Mast Cell and Monocyte Recruitment by S100A12 and Its Hinge Domain*

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S100A12 is expressed at sites of acute, chronic, and allergic inflammation. S100 proteins have regions of high sequence homology, but the “hinge” region between the conserved calcium binding domains is structurally and functionally divergent. Because the murine S100A8 hinge domain (mS100A882–93) is a monocyte chemoattractant whereas the human sequence (hS100A883–96) is inactive, we postulated that common hydrophobic amino acids within the S100A12 hinge sequence may be functional. The hinge domain, S100A1238–53, was chemotactic for human monocytes and murine mast cells in vitro. S100A1238–53 provoked an acute inflammatory response similar to that elicited by S100A12 in vivo and caused edema and leukocyte and mast cell recruitment. Circular dichroism studies showed that S100A1238–53 had increased helical structure in hydrophobic environments. Mutations in S100A1238–53 produced using an alanine scan confirmed that specific hydrophobic residues (I44A, I47A, and I53A) on the same face of the helix were critical for monocyte chemotaxis in vitro and generation of edema in vivo. In a hydrophobic environment such as the cell membrane, these critical residues would likely align on one face of an α-helix to facilitate receptor interaction. Interaction is unlikely to occur via the receptor for advanced glycation end products but, rather, via a G-protein-coupled mechanism.

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4The abbreviations used are: EN-RAGE, extracellular newly identified receptor for advanced glycation end products; SRAGE, soluble RAGE; MC, mast cells; BMMC, bone marrow-derived mast cells; PMC, peritoneal mast cells; CD, circular dichroism; TB, toluidine blue; β-hex, β-hexosaminidase; fMLP, formylmethionylleucylphenylalanine; ERK, extracellular signal-regulated kinase.

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vitro (21) and in vivo. (22) Although human S100A8, S100A9, and S100A8/A9 are chemotactic agents for neutrophils in vitro and in vivo (23), we found that human S100A8 (has 58% structural identity with murine S100A8) had little monocyte chemotactic activity, and the hinge domain (hS100A838–56) was inactive (21). Our structural modeling studies (7) predict that the chemotactic human homologue of the active hinge domain of murine S100A8 (mS100A842–55) may be S100A12 (S100A1238–53).

Here we show that S100A12 promotes migration of MC in vitro and in vivo and that S100A1238–53, a region containing the hinge domain, was chemotactic for murine bone marrow-derived mast cells (BMMC), rat peritoneal mast cells (PMC), and human monocytes in vitro. Like S100A12, S100A1238–53 provoked mild mast cell degranulation in vitro and leukocyte recruitment in vitro and in vivo and induced a Ca2+ influx in mouse BMMC. Amino acid residues important for activity were identified; those essential for MC activating activity and edema were hydrophobic (Leu40, Ile44, Ile47, and Ile53). Dose response curves indicated a single receptor on MC and high and low affinity receptors on THP-1 mononcytoid cells that were inhibited by pertussis toxin, suggesting involvement of G-protein-coupled receptors in signal transduction. Results strengthen our proposal for a role for S100A12 in allergic inflammation and may lead to rational design of S100A12 antagonists.

EXPERIMENTAL PROCEDURES

Human Subjects and Animals—Peripheral blood from normal donors was obtained with approval of the Human Care and Ethics Committee of the University of New South Wales with written informed consent. All animals were specific pathogen-free; BALB/c mice were used as a source of BMMC; Quackenbush Swiss mice were used to study responses to S100A12 in vivo, and Sprague-Dawley rats (10 weeks old) were the source of PMC. Experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales.

Protein and Peptides—Recombinant human S100A12 was expressed and purified as described (2). Chain assembly of S100A1238–53 (KELANTIKNIKAVI) and its alanine scan mutants (24) was performed manually as described (25). Endotoxin levels in all preparations were <10 pg/10 μg using the chromogenic limulus amebocyte lysate assay (Cape Cod Associates, Woods Hole, MA).

Electrospray-Ionization Mass Spectrometry—Electrospray-ionization mass spectrometry was performed on an LCT time-of-flight mass spectrometer (MicroMass, Manchester, UK). Samples were injected into a moving solvent (100 μl/min; 70% aqueous acetonitrile + 0.1% formic acid) and entered the analyzer through an interface plate. Full scan mass spectra were acquired over the mass range 500–2000 Da. Molecular masses were calculated from the observed m/z value using Mass Lynx software (MicroMass).

Circular Dichroism (CD) Spectroscopy—CD spectra were recorded on a Jasco J-730 spectropolarimeter. Samples (59 μM) were dissolved in 50 mM phosphate buffer, pH 7.2, and data between 190 and 260 nm were recorded.

Cells—The monocytoid cell line (THP-1; American Type Culture Collection, Manassas, VA) was maintained at 37 °C in 5% CO2 in air in RPMI 1640 (Invitrogen) supplemented with 10% heat-damaged (56 °C, 30 min) bovine calf serum (HyClone Laboratories, Logan, UT). Media was sterilized by filtration through Zetapore 0.2-μm membrane (Cuno, Meriden, CT) to remove contaminating traces of endotoxin.

Peripheral blood mononuclear cells from buffy coats of citrated blood of normal donors were isolated using Ficoll-Paque™ Plus (Amersham Biosciences), washed 3 times with Ca2+, Mg2+-free Dulbecco’s phosphate-buffered saline (Invitrogen), and resuspended in 0.2% bovine calf serum/RPMI. BMMC were differentiated from murine bone marrow stem cells cultured in conditioned media consisting of supernatants from WEHI-3B (source of interleukin-3) and NIH-3T3 fibroblasts (source of stem cell factor) and 10% bovine calf serum in RPMI 1640 as described (26). Cells were passaged weekly and differentiated until >98% MC (>3 weeks), assessed by metachromatic staining of granules with toluidine blue (TB).

PMC harvested from rats by peritoneal lavage were isolated as described (27). Purity by TB staining was ~99% MC, and viability was >98%. Cells were suspended in 0.2% bovine serum albumin (Sigma)/RPMI.

Chemotaxis and Inhibition Studies—Chemotaxis of calcein (Molecular Probes Inc., Eugene, OR)-loaded cells was performed as described (2) using 96-well chambers separated by a polycarbonate membrane (NeuroProbe Inc., Bethesda, MD) with pores of 5 μm for peripheral blood mononuclear cells, 8 μm for BMMC, and 10 μm for THP-1, with six replicates per sample. C5a (Sigma; 10−8 M) was employed as a positive control. Chambers were incubated at 37 °C in 5% CO2 in air for 1.5 h (peripheral blood mononuclear cells), 2 h (THP-1), or 4 h (BMMC).

To determine involvement of RAGE, soluble RAGE peptide (sRAGE42–59) or the control peptide with the reverse sequence (rvRAGE) was preincubated with S100A12 at 10:1 molar excess overnight at 4 °C before migration assay. Alternatively, because RAGE signaling involves downstream mitogen-activated protein kinase activation via extracellular signal-regulated protein kinase (ERK1/2), THP-1 cells were incubated with PD98059 (Calbiochem; 10 μM) for 2 h before assay. To determine involvement of G-protein-coupled receptors, THP-1 cells were preincubated with pertussis toxin (Sigma, 0.1 μg/ml) for 2 h before testing. Results are expressed as numbers of migrated cells as described (2) or as migration index for pertussis toxin and PD98059-treated cells.

F-actin Polymerization and Cytosolic Ca2+ Flux—Cells (0.5 × 105/ml) in Hanks’ balanced salt solution and 0.2% bovine serum albumin were prewarmed at 37 °C in 5% CO2 before the addition of S100A12 or S100A1238–53 diluted in the same buffer at 37 °C (2). Cells (2.5 × 105) in 96-well plates cells were mixed with 50 μl of stimulants to yield final concentrations between 10−9 and 10−12 M. Cells were permeabilized and stained for F-actin for 60 min with a mixture of 0.05% (w/v) digitonin (Calbiochem) and 0.5 μM fluorescein isothiocyanate-phalloidin (Sigma). fMLP (10−9 M) was used as positive control.

Changes in [Ca2+]i were monitored using the fluorescent probe Fluo-3 (Molecular Probes) (2). Fluorescence emission...
was assayed immediately and for 10–20 cycles (3 s/cycle) at 
\( \lambda_{ex} = 480 \text{ nm} \) and \( \lambda_{em} = 530 \text{ nm} \). C5a (10\(^{-7} \text{ M} \)) was used as positive control.

**Mediator Production**—MC degranulation was assessed by measuring \( \beta \)-hexosaminidase (\( \beta \)-hex) or histamine release. Supernatants and cell pellets (lysed with 100 \( \mu \text{L} \) 1% Triton X-100) from stimulated BMMC or PMC were assayed for \( \beta \)-hex as described; (28) histamine was measured by enzyme-linked immunosorbent assay (ImmuBio-Logical Laboratories, Hamburg, Germany). Data are expressed as percentage (\% release calculated as (\( \beta \)-hex or histamine in supernatant)/\( \beta \)-hex or histamine in supernatant + lysate) \( \times 100 \).

**In Vivo Studies**—To assess inflammatory responses in vivo, mice were injected with 10 \( \mu \text{g} \) of S100A12 or S100A12\(^{38–53} \) in 1 ml of Hanks’ balanced salt solution intraperitoneally. Peritoneal cavities were lavaged (5 \times 5 ml of citrated Hanks’ balanced salt solution). 8 and 24 h later cells were harvested by centrifugation, and total MC numbers were determined using cytospin preparations after TB staining. Alternatively, S100A12, S100A12\(^{38–53} \), or mutant peptides (2 \( \mu \text{g}/30 \mu \text{L} \) of Hanks’ balanced salt solution/site) were injected into the footpad. Footpad thickness was measured with an electronic caliper (Müntoyo, Japan) before and 20 and 30 min and 1 and 2 h after injections by two blinded investigators. Edema was defined as positive if the difference in footpad thickness 60 min post-injection was 0.1 mm greater than the pre-injection reading. To determine changes in vascular permeability, Evan’s blue (1% in saline; 0.05 ml/mouse) was injected intravenously 30 min before footpad injection. The assessment was expressed as a clinical score: 0, no blue, no edema; 1, edema, no blue; 2, edema, light blue; 3, edema, blue; 4, edema, dark blue. For some samples footpads were harvested 2 and 8 h post-injection, and leukocyte infiltration was assessed on formalin-fixed sections stained with hematoxylin and eosin or TB to detect MC. Macrophages were identified with anti-Mac-3 mAb (BD Biosciences Pharmingen), and reactivity was detected with biotin-stained with hematoxylin and eosin or TB to detect MC.

**Statistical Analysis**—Data are expressed as the means \( \pm \) S.D. Data from two groups were assessed using the Student’s \( t \) test and multiple groups by analysis of variance followed by the Bonferroni test, and \( p \) values < 0.05 were considered significant.

**RESULTS**

Human A12 Is a Mast Cell Chemoattractant in Vitro and in Vivo—Because S100A12 activates MC, and as MC numbers increase in asthmatic tissue, their chemoattractant response was tested. S100A12 stimulated BMMC migration in a dose-dependent manner that was optimal at 10\(^{-11} \text{ M} \) (Fig. 1; 2.74 \( \pm \) 0.19 \( \times \) 10\(^4 \) cells migrated compared with control, 2.02 \( \pm \) 0.17 \( \times \) 10\(^4 \) cells, \( p < 0.05 \)). Directional, rather than random migration, was confirmed when the gradient was negated by equivalent amounts of S100A12 in the upper and lower chambers (Fig. 1; \( p < 0.01 \) relative to maximal chemotaxis at 10\(^{-11} \text{ M} \) S100A12 in the lower chamber only). Human cord blood MC migration was also significantly greater than control values with 10\(^{-9} \sim 10^{-10} \text{ M} \) S100A12 (\( p = 0.02 \) compared with control; not shown).

Responses of both types of MC to S100A12 were equivalent or greater than those provoked by a pre-optimized concentration of C5a. Because BMMC are responsive to S100A12 (Ref. 4 and Fig. 1), these were routinely used as they are more rapidly differentiated in vitro at significantly less cost.

**Chemotactic Activity of S100A12 \(^{38–53} \)**—To determine whether the hinge domain of S100A12 could provoke a chemoattractant response, migration of THP-1 monocyticoid cells, peripheral blood mononuclear cells (S100A12 is only chemotactic for monocytes) (2), and mouse BMMC were tested. S100A12 consistently provoked THP-1 cell migration (Fig. 2A) in a biphasic manner with two prominent peaks at 10\(^{-9} \) and 10\(^{-12} \text{ M} \) (both \( p < 0.001 \) compared with negative control). S100A12\(^{38–53} \) was also chemotactic at these concentrations (\( p < 0.05 \) compared with negative control), although the peptide was significantly less potent than the full-length protein. When equivalent levels of S100A12\(^{38–53} \) were included in the upper and lower chambers, basal migration did not significantly increase (Fig. 2A), confirming directional migration. Similar dose response optima were obtained for migration of blood monocytes in response to S100A12, although the peptide was not active at 10\(^{-9} \text{ M} \) (Fig. 2B). Chemotactants cause polymerization of actin, a major component of microfilaments important for cell migration. S100A12 (10\(^{-11} \text{ M} \)) increased F-actin content in THP-1 cells within 20 s, which plateaued then increased and was sustained over 2 min. fMLP caused similar increases but was less sustained. S100A12\(^{38–53} \) provoked a somewhat slower response with a second more intense rise in F-actin content apparent after 60 s, which returned to base line within 120 s (Fig. 2C).

sRAGE, a RAGE antagonist, reduced migration of THP-1 cells in response to 10\(^{-9} \text{ M} \) S100A12 (\( p < 0.01 \)), whereas migration at 10\(^{-12} \text{ M} \) S100A12 was unaffected; the control, RvRAGE peptide, did affect migration to S100A12 at either concentration (Fig. 3A). ERK1/2 may play a role in RAGE signaling through direct interaction with RAGE (29); however, PD98059, a selective ERK1/2 inhibitor, did not affect S100A12-provoked migration.
THP-1 cell migration at either concentration or affect migration to C5a (Fig. 3B). In contrast, S100A12 induced THP-1 migration at both concentrations, and C5a were significantly reduced by pertussis toxin (p < 0.05; Fig. 3C). 

S100A1238–53-induced migration of BMMC was optimal at 10⁻¹¹ M (p < 0.01) (Fig. 4A). Maximal responses of native or hinge S100A12 preparations were consistently more potent than those of S100A1238–53. Data were expressed as the mean ± S.D. of cells in each of six wells from three experiments. p < 0.05 (*), p < 0.001 (**) compared with negative control; †, p < 0.05 compared with S100A12 at the same concentration; ‡, p < 0.01 (#) compared with upper chambers containing S100A12 or S100A1238–53.

FIGURE 2. Monocyte chemotactic activity of S100A12 and S100A1238–53. A, biphasic response of THP-1 cells induced by 10⁻⁹ and 10⁻¹² M S100A12 (■) and S100A1238–53 (▲). Directional migration was confirmed with equivalent concentrations of stimulants in both chambers (S100A1238–53 (– – –), C5a 10⁻⁸ M (●), and basal migration (□)). Data are expressed as the mean ± S.D. of cells in each of six wells from three experiments. p < 0.05 (*) and p < 0.01 (**) compared with negative control; †, p < 0.05 compared with S100A12 at the same concentration; ‡, p < 0.05 (δ) and p < 0.01 (#) compared with upper chambers containing S100A12 or S100A1238–53. B, S100A12 (■) or S100A1238–53 (▲)-provoked migration of mononuclear cells (monocytes). Data are expressed as the mean ± S.D. of cells in each of six wells from four experiments. p < 0.05 (*) and p < 0.01 (**) compared with negative control.

FIGURE 3. Mechanism of S100A12-induced THP-1 chemotaxis. A, sRAGE inhibited migration of THP-1 cells in response to 10⁻⁹ M but not 10⁻¹² M S100A12. Control peptide (RvRAGE) did not affect S100A12-induced migration. Data are expressed as net cell numbers ± S.D.; n = 3 (*, p < 0.01 compared with S100A12 alone). B, chemotactic activities induced by S100A12 at either concentration were not altered in THP-1 cells pretreated with the ERK1/2 inhibitor PD98059 (10 μM; white bars) and were not significantly different to untreated cells (black bars). Data expressed as migration index (numbers of migrated cells per well/numbers of unchallenged control cells/well; n = 4). C, pertussis toxin (PTX, 0.1 μg/ml) attenuated migration induced by S100A12 at 10⁻⁹ M (dark gray bars) and 10⁻¹² M (light gray bars) and by C5a (10⁻⁸ M) (black bars). Data are expressed as the migration index; n = 10.
than the predetermined optimal dose of 10^{-8} M C5a. Chemotactants generally generate a Ca^{2+} influx, and S100A12 activates this response in monocyteid cells (2). Resting [Ca^{2+}] in BMMC increased within 3 s (9–12 s, Fig. 4B) in response to S100A12 and S100A12^{38–53} and was maintained over 10–12 s then declined in a manner similar to the C5a-provoked response (Fig. 4B). Ca^{2+} influx occurred with between 10^{-11} and 10^{-6} M S100A12 or S100A12^{38–53} (not shown).

**MC Degranulation**—Because S100A12 provokes MC degranulation (4), S100A12^{38–53} was tested. Both the peptide and the protein induced significantly more β-hex release than unstimulated BMMC (basal release, 4.4%; Fig. 5A). Levels of β-hex were similar with S100A12 and the peptide, with increases of 12 and 7% with S100A12 and S100A12^{38–53} (10 μM) and 7 and 6% with 1 μM, respectively (p < 0.001 at 10 μM; p < 0.05 at 1 μM). The capacity for degranulation was confirmed using PMC (Fig. 5B). With 10 μM S100A12 or S100A12^{38–53}, β-hex increased 2.3- and 2.4-fold and histamine 1.8- and 1.9-fold, respectively (Fig. 5C), representing ~30% release of total cell histamine. Although 1 μM S100A12 or S100A12^{38–53} provoked some degranulation (Fig. 5A), lower concentrations (<0.1 μM) were ineffective (data not shown).

**Leukocyte Recruitment in Vivo**—S100A12 generates inflammatory changes in the microcirculation and induces a localized MC-dependent edema and leukocyte recruitment after 8 h (4). Footpad swelling increased 60 min post-injection of S100A12 or S100A12^{38–53} and was sustained for up to 2 h (Fig. 6A; p < 0.05, S100A12; p < 0.01, S100A12^{38–53} 1 or 2 h post-injection compared with vehicle control). Blood vessel permeability peaked 60 min post-injection and was similar for both preparations (see Table 1; the native peptide is represented by A41A and A51A). Histological analysis confirmed leukocyte accumulation 2 h post-injection into footpads (Fig. 6B, b and c). Marked MC degranulation was evident with S100A12 (Fig. 5B, e) and with S100A12^{38–53} (Fig. 6B, f) compared with vehicle (Fig. 6B, d). Total leukocyte numbers increased some 3-fold 8 h post-injection (Table 1; p < 0.01). Macrophage numbers increased ~5-fold (p < 0.01) and MC some ~2-fold (p < 0.05) with both...
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Inflammatory cells in footpads of mice 8 h post-injection of S100A12 or S100A1238–53.

Leukocyte recruitment after S100A12 injection

TABLE 1

Leukocyte recruitment after S100A12 injection

S100A12 preparations. Neutrophils comprised the remainder of the leukocytes. S100A1238–53 consistently recruited more MC when injected into the skin than S100A12 (p < 0.05).

Eleven-fold more MC were evident 8 h post-intraperitoneal injection of S100A12 (Fig. 6C, 11.4 ± 1.36 × 10^4 compared with 0.9 ± 0.27 × 10^4 in control mice; p < 0.001), and at 24 h numbers (4.4 ± 0.38 × 10^4) were still significantly higher (7-fold, p < 0.001) than in vehicle-injected mice (0.6 ± 0.20 × 10^3). In contrast to the skin, mucosal MC numbers recruited by S100A1238–53 were ~50% less than those elicited by S100A12, and numbers reached basal levels within 24 h, suggesting more rapid dispersal of the peptide or differences in responses between mucosal and skin MC.

Functional Amino Acids in S100A1238–53—Mapping of functional epitopes was performed using Ala scan mutagenesis. The 16 mutants (A41A and A51A were the same as S100A1238–53) were screened for their ability to induce footpad edema and chemotactic activity for THP-1 cells. Selected mutants were also tested for their ability to provoke MC degranulation (Fig. 5, B and C). L40A, N46A, I47A, and I53A mutants did not provoke significant edema (Table 2). K38A and K48A had somewhat less activity for THP-1 cells. Selected mutants were also tested for their ability to provoke MC degranulation (Fig. 5, B and C). L40A, N46A, I47A, and I53A mutants did not provoke significant edema (Table 2). K38A and K48A had somewhat reduced activity with clinical scores around 2.0. Because the isoleucine mutants did not cause edema, we predicted that these might be required for MC activation. I44A, I47A, and I53A did not induce β-hex or histamine release from PMC, whereas K50A, which had no effect on edema but was essential for monocyte chemotaxis at 10^{-9} M, generated similar levels of β-hex and histamine as S100A12 or S100A1238–53 (Fig. 5, B and C).

S100A1238–53 was chemotactic for THP-1 cells at 10^{-9} and 10^{-12} M (Fig. 2A), and mutants tested at these concentrations had different effects (Table 2). Peptides containing K38A, L40A, N46A, I47A, D49A, K50A, and I53A were significantly less active when tested at 10^{-9} M, whereas residues Asn-42 and Ile-44 were essential for chemotaxis at 10^{-12} M (Table 2). Although activities of mutants did not coordinate in vivo and in vitro, taken together K38A, N46A, D49A, and K50A were required for chemotaxis provoked by 10^{-9} M pep-
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Mast cells are important effector cells in allergic inflammation, infection, and in some chronic inflammatory conditions and are widely distributed in vascularized tissues and certain epithelia. Their localized accumulation may be due to redistribution of neighboring MC in response to factors such as stem cell factor, transforming growth factor-β, C5a, CXC chemokines (30), and interleukin-15 (31). Our recent studies showed S100A12 to be a potent activator of MC, and its expression in macrophages in the vicinity of tryptase-positive MC, in eosinophils infiltrating the airways of patients with asthma, and elevated levels in the sputum of patients with eosinophilic asthma implicate it in pathogenesis (4). S100A12-activated MC produce chemokines that could contribute to the high numbers of infiltrating monocytes found in the airways of patients with allergic asthma (32). However, S100A12 is also a potent chemotactant for monocytes, (2), and here we demonstrate its chemotactic activity for MC, with optimal activity in the low nanomolar range and potency greater than C5a. These properties strongly support a role for this protein in allergic inflammation.

The hinge region of murine S100A8 (S100A842–55), but not of human S100A8 (hS100A843–56), is chemotactic for murine and human monocytes even though murine S100A8 has the highest overall sequence similarity (58%) with human S100A8. The low sequence identity (21%) within the hinge domains of these homologues suggested functional differences, and electrostatic maps showed the hinge regions of murine S100A8 and S100A12 to be structurally more similar (7) even though their overall identity (32%) is low (Fig. 7F). Like murine S100A8, (21) S100A12 is chemotactic for monocytes, (2) with concentration optima of 10–9 and 10–12 M. S100A1228–53 was less potent than the native protein; THP-1 cells exhibited a similar biphasic dose response, whereas monocytes were unresponsive to higher concentrations (10–9 M). Both agonists rapidly increased F-actin polymerization in THP-1 cells. Some CXC chemokines (33), fMLP (34), and leukotriene B4 (35) also have high and low affinity receptors that may facilitate leukocyte recruitment to broader concentrations of chemoattractants (35).

In contrast to monocytes, BMMC responded to native S100A12 and to S100A1228–53 with equal magnitude, with a bell-shaped dose response over a narrow optimal concentration range around 10–11 M, and both induced a Ca2+ influx of a magnitude comparable with that generated by C5a. Prominent MC degranulation was evident after footpad injection of S100A1228–53, and vascular permeability and leukocyte numbers, including macrophages and MC, increased. The hinge region peptide consistently generated edema and leukocyte recruitment after footpad injection into mice.

The C-terminal and hinge domains of S100 proteins can have distinct functions. Studies with S100B confirmed that at least two sites interact with effector proteins and suggest that the C-terminal domain may be common to interactions with a number of effector proteins, whereas that containing the hinge domain may be target-specific (36). Comparisons of the crystal structure of human S100A9, which has high sequence homology with S100A12, indicate that the hinge region is a target-binding site in these proteins (37), a proposal supported by the crystal structure of S100A12 (38). CD spectroscopy confirmed that the S100A12 hinge domain adopted a random structure in aqueous buffer, with increasing hydrophobicity resulting in a higher propensity to form an α-helical conformation. Helical wheel analysis suggests that in a hydrophobic environment such as a cell membrane, critical residues in this region would align on one face of the helix to interact with a receptor. The hydrophobic residues Asn-42, Ile-44, Ile-47, and Ile-53 were essential for monocyte chemotaxis, MC activation, and edema in vivo; Asn-46 was also critical for chemotaxis. Similarly, the hinge region of murine S100A8 has considerable secondary structure in a hydrophobic environment (39). Upon Ca2+ bind-

### TABLE 2

Activity of hinge mutants in vivo and in vitro

| Peptide mutant | Footpad injection clinical score | Chemotactic activity |
|---------------|---------------------------------|----------------------|
|               | 10–9                            | 10–12                |
| K38A          | 2.0 ± 1.0                       | 6.3 ± 1.2            | 9.6 ± 0.9          |
| E39A          | 2.6 ± 0.5                       | 9.1 ± 1.4            | 9.8 ± 1.7          |
| L40A          | 1.3 ± 0.5                       | 7.7 ± 1.2            | 8.3 ± 1.9          |
| A41A          | 3.5 ± 0.8                       | 10.3 ± 1.2           | 10.7 ± 0.4         |
| N42A          | 2.8 ± 0.8                       | 10.2 ± 0.6           | 7.7 ± 1.6          |
| T43A          | 2.6 ± 0.8                       | 9.1 ± 1.9            | 9.2 ± 1.7          |
| I47A          | 0.2 ± 0.4                       | 8.9 ± 0.9            | 7.9 ± 0.6          |
| K45A          | 2.7 ± 0.4                       | 10.5 ± 1.0           | 10.9 ± 0.7         |
| A51A          | 2.7 ± 0.9                       | 7.1 ± 1.2            | 10.1 ± 1.7         |
| A41A          | 0.0 ± 0.0                       | 7.1 ± 1.2            | 9.3 ± 1.3          |
| K48A          | 2.0 ± 0.8                       | 10.3 ± 1.2           | 8.2 ± 0.3          |
| D49A          | 2.3 ± 1.6                       | 7.7 ± 1.2            | 8.9 ± 1.2          |
| K50A          | 3.2 ± 0.8                       | 6.8 ± 0.5            | 9.3 ± 0.5          |
| A51A          | 3.2 ± 0.8                       | 10.2 ± 1.2           | 10.7 ± 0.4         |
| V52A          | 2.2 ± 0.6                       | 9.1 ± 0.8            | 10.5 ± 1.2         |
| I53A          | 0.4 ± 0.4                       | 7.9 ± 0.7            | 8.5 ± 1.1          |
| S100A12       | 1.2 ± 0.8                       | 11.9 ± 1.6           | 10.6 ± 1.1         |

*p < 0.05 compared to native hinge.

*p < 0.05 compared to clinical scores of the native peptide (A41A and A51A, bold italics) 60 min post-injection.

*p < .01 compared to clinical scores of the native peptide (A41A and A51A, bold italics) 60 min post-injection.

tide, and L40A, I44A, I47A, and I53A were essential for edema and optimal chemotactic responses.

### Structural Analysis

The secondary structure of S100A1228–53 was examined by CD spectroscopy in aqueous and hydrophobic environments (Fig. 7, A–D). It adopted a random structure in aqueous buffer and had a higher propensity to form an α-helical conformation, as indicated by characteristic minima at A208 and A222 nm, with increasing solvent hydrophobicity. Each of the inactive mutants had CD spectra identical to native S100A1228–53 (not shown).

Helical wheel analysis showed that residues important for chemotactic activity of the hinge domain occur on one side of the helix (Fig. 7E). These include Asn-42, Ile-44, Asn046, Ile-47, and Ile-53. Residues essential for edema were also hydrophobic (Leu-40, Ile-44, Ile-47, and Ile-53) Alignments of S100A12, mS100A8, and hS100A8 are given (Fig. 7F); identical amino acids are indicated in red. Asn-46 and Ile-47 were critical amino acids for S100A12 chemotaxis, and these are also located in the hinge domain of mS100A8 but not in hS100A8.

### DISCUSSION

Mast cells are important effector cells in allergic inflammation, infection, and in some chronic inflammatory conditions and are widely distributed in vascularized tissues and certain epithelia. Their localized accumulation may be due to redistribution of neighboring MC in response to factors such as stem cell factor, transforming growth factor-β, C5a, CXC chemokines (30), and interleukin-15 (31). Our recent studies showed S100A12 to be a potent activator of MC, and its expression in macrophages in the vicinity of tryptase-positive MC, in eosinophils infiltrating the airways of patients with asthma, and elevated levels in the sputum of patients with eosinophilic asthma were critical amino acids in this region, a proposal supported by the crystal structure of S100A12 (38). CD spectroscopy confirmed that the S100A12 hinge domain adopted a random structure in aqueous buffer, with increasing hydrophobicity resulting in a higher propensity to form an α-helical conformation. Helical wheel analysis suggests that in a hydrophobic environment such as a cell membrane, critical residues in this region would align on one face of the helix to interact with a receptor. The hydrophobic residues Asn-42, Ile-44, Ile-47, and Ile-53 were essential for monocyte chemotaxis, MC activation, and edema in vivo; Asn-46 was also critical for chemotaxis. Similarly, the hinge region of murine S100A8 has considerable secondary structure in a hydrophobic environment (39). Upon Ca2+ bind-
Dose response curves indicated high and low affinity receptors for S100A12 on THP-1 monocytoid cells, and the Ala scan supported this notion as Asn-42 and Ile-44 were essential for the chemotactic response provoked by 10^{-12} M S100A12^{38–53}, whereas mutation of Lys-38, Leu-40, Asn-46, Ile-47, Asp-49, Lys-50, and Ile-53 all significantly reduced migration provoked by 10^{-9} M S100A12^{38–53}, suggesting involvement of additional charged interactions. Identification of S100 receptors has been elusive. RAGE is proposed as a pan S100 receptor, although there are clearly additional/alternate receptors (4, 42). S100A12 was the first of this class of protein found to bind RAGE (1). However, we recently showed that although human MC respond to S100A12, RAGE mRNA/protein expression was not found in these cells. Studies using RAGE-null mice show that RAGE plays little role in adaptive immune responses (43), and although a soluble RAGE antagonist (sRAGE) reduced delayed-type hypersensitivity responses proposed to be mediated by S100A12 (1), this was similarly suppressive in RAGE-null mice, and undefined effects other than simply blocking cell-surface RAGE function are proposed (43). sRAGE inhibited the ability of S100A12 to promote migration of THP-1 cells at 10^{-9} M, but this was not affected by inhibiting ERK1/2 even though RAGE is expressed by these cells (4). Attempts to neutralize RAGE with appropriate antibodies were not successful (not shown). Furthermore, as for murine S100A8 (20), both peaks of S100A12-induced migration of THP-1 cells were pertussis toxin-sensitive, suggesting a G-protein-coupled mechanism. Although these parameters were not tested using MC, we showed that human cord blood-derived MC do not express RAGE (4), and it is possible that MC and THP-1 cells share a common, as yet unidentified receptor. Because S100A12 binds the two C-type domains of RAGE (44), we hypothesized that an unidentified receptor may have sequence similarity to this region. However, database searches (BLASTP) yielded no homology matches to G-protein-coupled receptors when we searched with both C-type domains together or separately. We are currently investigating the nature of S100A12 interactions with MC.

This study indicates that S00A12 may contribute to monocyte and MC migration and MC activation and identifies the
hinge region as a functional domain. It reinforces a potential role for S100A12 in allergic inflammation and forms the basis for the design of antagonists that may have useful therapeutic applications. Delineation of the structural motifs that mediate S100A12-target interactions and their relevance in vivo are required to fully understand its role in the pathogenesis of inflammatory diseases.

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