Specific Citrullination Causes Assembly of a Globular S100A3 Homotetramer

A PUTATIVE Ca$^{2+}$ MODULATOR MATURES HUMAN HAIR CUTICLE*[1]

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S100A3 is a unique member of the Ca$^{2+}$-binding S100 protein family with the highest cysteine content and affinity for Zn$^{2+}$. This protein is highly expressed in the differentiating cuticular cells within the hair follicle and organized into mature hair cuticles. Previous studies suggest a close association of S100A3 with epithelial differentiation, leading to hair shaft formation, but its molecular function is still unknown. By two-dimensional PAGE-Western blot analyses using a modified citrulline antibody, we discovered that more than half of the arginine residues of native S100A3 are progressively converted to citrullines by Ca$^{2+}$-dependent peptidylarginine deiminases. Confocal immunofluorescent microscopy showed that the cytoplasmic S100A3 within the cuticular layer is mostly co-localized with the type III isoform of peptidylarginine deiminase (PAD3) but not with PAD1. Recombinant PAD1 and PAD2 are capable of converting all 4 arginines in recombinant S100A3, whereas PAD3 specifically converts only Arg-51 into citrulline. Gel filtration analyses showed that either enzymatic conversion of Arg-51 in S100A3 to citrulline or its mutational substitution with alanine (R51A) promotes a homotetramer assembly. Fluorescent titration of R51A suggested that its potential Ca$^{2+}$ binding property increased during tetramerization. A prototype structural model of the globular Ca$^{2+}$-bound S100A3 tetramer with citrulline residues is presented. High concentrations of S100A3 homotetramer might provide the millimolar level of Ca$^{2+}$ required for hair cuticular barrier formation.

Several EF-hand type Ca$^{2+}$-binding proteins are involved in a multitude of Ca$^{2+}$-dependent cellular processes. The S100 protein family is the largest subgroup of more than 20 members characterized by two highly conserved Ca$^{2+}$-binding domains: a classical C-terminal EF-hand with a canonical Ca$^{2+}$-binding loop and a S100 specific N-terminal EF-hand motif (1, 2). Extensive studies have revealed diverse functional roles of several S100 proteins in variety of cellular processes, such as cell growth and differentiation, cell cycle regulation, transcription, and signal transduction receptor activities. They are also associated with human diseases, including inflammation, brain disorders, cancer, diabetes, heart failure, and pathological conditions of the skin and hair follicle. The basic structural and functional unit of most S100 proteins was previously thought to be a noncovalently associated antiparallel dimer; however, there is increasing evidence that some members assemble into higher order oligomers, thereby conferring their biological function (3–7).

In terrestrial animals, Ca$^{2+}$ is an essential divalent cation in the formation of the epithelial protective barrier by superficial tissues that are exposed to the external environment (8). A specialized protein structure termed the cornified cell envelope (CE) encapsulates the corneocytes of mammalian skin epidermis and hair fiber cuticle. A number of the genes encoding the CE precursor proteins are clustered in the epidermal differentiation complex on human chromosome 1q21 (9) with genes for EF-hand type Ca$^{2+}$-binding proteins, including both 16 S100 proteins (2) and more than four larger peptides with an S100-like domain at the N terminus (e.g. profilaggrin and trichohyalin) (10). Most of these genes were reported to be coordinately expressed during epithelial terminal differentiation, and the translation products were incorporated to the CE (8); however, the functional role of S100 proteins and S100-like domains responsible for regulating the intracellular Ca$^{2+}$ ion in the epithelial cornifying processes is poorly understood.

Two Ca$^{2+}$-dependent protein-modifying enzymes, peptidylarginine deiminase (PAD; EC 3.5.3.15), which converts peptidylarginine residues to citrulline (11), and transglutaminase (EC 2.3.2.13), which introduces N$\gamma$-(γ-glutamyl)lysine isopeptide cross-links (12), are involved in the epithelial barrier formation. Although this biochemical process is believed to be precisely regulated by the intracellular Ca$^{2+}$ concentration, there remains controversy regarding the mechanism by which PAD and transglutaminase could be activated in vivo, since both require a nearly millimolar level of Ca$^{2+}$ to exhibit their full activities in vitro (12, 18). Several epithelial protein barrier

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1 The on-line version of this article (available at http://www.ibc.org) contains supplemental method and Fig. S1.

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The abbreviations used are: CE, cornified envelope; PAD, peptidylarginine deiminase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; DTT, dithiothreitol; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholinolinesulphonic acid; HPLC, high pressure liquid chromatography; Cit, citrulline.

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components containing Ca\(^{2+}\)-binding domains have been reported to be natural substrates of PAD (the mature filaggrin subunit, proteolytically processed from profilaggrin (13)) or of transglutaminase (S100A7, S100A10, and S100A11 (14, 15)) in skin epidermis or of both these enzymes (trichohyalin (16, 17)) in hair follicles. It has been proposed that the Ca\(^{2+}\) ions bound to these proteins are presented to the Ca\(^{2+}\)-dependent protein-modifying enzymes (8, 11); however, more direct evidence is necessary to support this notion.

We previously identified S00A3, which is unique in its high cysteine content (10 of 101 amino acids) (19) and Zn\(^{2+}\)-binding property (K\(_d\) = 1.5–11 \(\mu\)M) (20, 21), as a predominant protein present in human hair cuticles (22). The flattened cuticular layers composed of the mechanically tough but dead cells confer the physical and chemical barrier function (23); however, little is known about the terminal differentiation processes of this specialized epithelium equipped with a thick protein envelope (8). Although our previous investigation suggested that S100A3 is closely associated with the cuticular differentiation within the hair follicle (24–26), the very low affinity of S100A3 for Ca\(^{2+}\) (8). Although our previous investigation suggested that S100A3 is known about the terminal differentiation processes of this

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**EXPERIMENTAL PROCEDURES**

**Extraction and Purification of S100A3 from Hair Follicles and Cuticles—**About 200 beard follicles were plucked from a Japanese male using hair tweezers. In a mortar, nonkeratinized parts of follicles were crushed in 2 ml of 0.2 M Tris–HCl buffer (pH 7.6) containing 0.1 M DTT, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. After centrifugation at 14,000 g, supernatants were stored at \(-70^\circ\)C. The combined follicle extract of 20 preparations (i.e., 4,000 follicles) and the extract of hair cuticles (200 mg) using 10 ml of the above extraction buffer at 37 °C for 16 h were dialyzed against 20 mM Tris, 1 mM EDTA buffer (pH 7.6) containing 10 mM DTT and then loaded onto a Macro-Prep® column (1 ml). Bound S100A3 was eluted by a linear gradient from 0 to 0.6 M NaCl. Fractions containing S100A3 were combined, and (NH\(_4\))\(_2\)SO\(_4\) was added to 1.5 M and then loaded onto a Q-Sepharose\textsuperscript{TM} column (1 ml) preequilibrated with the same buffer. Bound S100A3 was eluted by a linear gradient from 1.5 to 0 M (NH\(_4\))\(_2\)SO\(_4\). Resultant crude S100A3 fractions were centrifugally desalted and condensed to 100 \(\mu\)l using Microcon\textsuperscript{R} YM-3 filters (Millipore Corp., Bedford, MA). Samples preheated under reducing conditions in NuPAGE\textsuperscript{R} LDS sample buffer (Invitrogen) were loaded into 4 of 10 wells of 4–12% Bis-Tris gel (7.8 \(\times\) 6.3 \(\times\) 0.15 cm; Invitrogen) and then electrophoresed using MES-SDS buffer at 200 V for 35 min. After staining with Rapidstain\textsuperscript{TM} reagent (Calbiochem), each S100A3 band was excised and transferred to an AE6100 electrophoretic apparatus (Atto, Tokyo, Japan). S100A3 was eluted from a gel slice at 100 V for 45 min, followed by 1 min of reversed electric current. Purified S100A3 aliquots were combined and condensed after supplementation of DTT to 5 mM and then subjected to electrospray ionization-mass spectrometric analyses (see supplemental method).

**Citrullination Contents in S100A3—**Peptidylcitrullinyl residues in S100A3 were detected by Western blot analyses of two-dimensional polyacrylamide gels using an antibody to citrulline residues (34). Samples were loaded onto immobilized pH gradient strips with pH range 3–5 (Sigma) by in-gel rehydration. Isoelectric focusing was performed at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min. The second dimension was performed with precast NuPAGE\textsuperscript{R} 4–12% Bis-Tris Zoom gel (7.8 \(\times\) 6.3 \(\times\) 0.1 cm; Invitrogen) using MES-SDS buffer at 200 V for 35 min. Citrulline residues were chemically modified to form a ureido group adduct in 0.0125% FeCl\(_3\), 2.3 M H\(_2\)SO\(_4\), 1.5 M H\(_3\)PO\(_4\), 0.25% 2,3-butanedione monoxime, 0.125% antipyrine, and 0.25% acetic acid on the protein-transferred polyvinylidene difluoride membrane prefixed with 1% glutaraldehyde at 37 °C for 16 h. An anti-citrulline (modified) detection kit (Upstate Biotechnology, Inc., Lake Placid, NY) was employed according to the supplier’s manual.

The pl of each S100A3 spot reflects the exact number of peptidylcitrullinyl residues. Relative intensities of silver-stained S100A3 spots were measured using image-analyzing software (Scion). The background of a nonspot area was subtracted from each signal intensity (I) value. The conversion rate was calculated via the equation for the acetylated native form (conversion (%)) = (N\(_0\) \(\times\) I\(_{pt\ 4.3}\)/I\(_{total}\) + N\(_1\) \(\times\) I\(_{pt\ 4.3}\)/I\(_{total}\) + N\(_2\) \(\times\) I\(_{pt\ 4.1}\)/I\(_{total}\) + N\(_3\) \(\times\) I\(_{pt\ 3.9}\)/I\(_{total}\) + N\(_4\) \(\times\) I\(_{pt\ 3.8}\)/I\(_{total}\))/4 \(\times\) 100 (where N\(_0\), N\(_1\), N\(_2\), N\(_3\), and N\(_4\) represent the forms with 0, 1, 2, 3, and 4 converted arginines, respectively) and for the recombinant modified by PAD enzymes (conversion (%)) = (N\(_0\) \(\times\) I\(_{pt\ 4.5}\)/I\(_{total}\) + N\(_1\) \(\times\) I\(_{pt\ 4.3}\)/I\(_{total}\) + N\(_2\) \(\times\) I\(_{pt\ 4.3}\)/I\(_{total}\) + N\(_3\) \(\times\) I\(_{pt\ 4.1}\)/I\(_{total}\) + N\(_4\) \(\times\) I\(_{pt\ 4.0}\)/I\(_{total}\))/4 \(\times\) 100.

**Confocal Immunofluorescent Microscopy—**Plucked beard follicles were fixed in 4% paraformaldehyde in phosphate-buffered saline for 16 h and embedded in paraffin. Sections with 6-\(\mu\)m thickness were deparaffinized and rehydrated, and then antigens were retrieved by heating to 95 °C in a modified citrate buffer (S1700; Dakocytomation, Carpenteria, CA) for 30 min. Polyclonal rabbit antibodies raised by us against the multiple antigen peptides of PAD3 (a partial sequence, D\(_{233}\)DKVSYEVPRLHGD\(_{234}\)) and a commercially available antibody to PAD1 from Covalab (Lyon, France), both purified by their own antigen-bound affinity columns, were employed. Commercially available rabbit antibody immunized with recombinant PAD2 from Shima Laboratory (Tokyo, Japan) was used after preabsorption with recombinant PAD1 and PAD3.
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The pretreated sections were reacted with diluted primary rabbit antibody to human PAD1 (5 μg/ml), PAD2 (15 μg/ml), or PAD3 (5 μg/ml). All reaction steps were performed for 45 min at room temperature, followed by three rinses with phosphate-buffered saline. Sections were then incubated with 10 μg/ml of Alexa Fluor® 488-labeled goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR). Subsequently, biotinylated antibody to human S100A3 (5 μg/ml), prepared using an EZ-link® NHS-PEO solid phase biotinylation kit (Pierce) (26), was applied to detect the second target antigen. Finally, sections were incubated with 10 μg/ml Alexa Fluor® 594-streptavidin conjugate (Molecular Probes). Images were acquired and processed using an LSM510 fluorescent confocal microscope and its accompanying software (Carl Zeiss AG, Göttingen, Germany).

In Vitro Modification of S100A3—Active recombinant human PAD1, PAD2, and PAD3 enzymes were prepared according to the previously reported procedures (18, 35). Conventionally, 1 μg of recombinant S100A3 was reacted with 25 milliunits of PAD enzymes in 20 μl of 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM CaCl₂ and 5 mM DTT at 37 °C. One unit was defined as the amount of PAD able to citrullinate 1 μmol of benzoyl-L-arginine ethyl ester in the same reaction buffer for 1 h at 55 °C (35).

Peptide Mapping—After purification on a Superdex 75 column to remove the enzymes, recombinant S100A3 modified by three recombinant PAD enzymes (25 milliunits/μg S100A3) at 37 °C for 24 h and native S100A3 purified from the hair follicle were reduced and alkylated with a half-volume of 1 x iodoacetamide dissolved in 3 x Tris-HCl (pH 8.4) for 15 min. After blocking unreacted reagent with DTT, the reaction mixture was passed through a NAP™ 5 column, equilibrated with 5 mM Tris-HCl (pH 8.0). Concentrated modified S100A3 was digested with endoproteinase Lys-C (Roche Applied Science) in 25 mM Tris-HCl containing 1 mM EDTA (pH 8.0) at 37 °C overnight.

6 μl of the Lys-C digest were loaded on a reversed-phase capillary HPLC column (PepMap C18, 0.3 x 150 mm; LC Packings). The peptides were separated with the following HPLC program: (i) isocratic flow at 5% B (v/v) for 5 min; (ii) linear gradient from 5 to 20% B in 5 min; (iii) linear gradient from 20 to 60% B in 20 min; (iv) linear gradient from 60 to 100% B in 5 min; (v) isocratic flow at 100% B for 10 min. Solvents A and B were 0.1% trifluoroacetic acid and 80% CH₃CN in 0.07% trifluoroacetic acid, respectively. The flow rate was 4 μl/min, and peptides were detected at 220 nm. Peptides eluting from the HPLC column were collected onto a 600-μm AnchorChip™ target (Bruker Daltonics, Leipzig, Germany), using a Probit microfraction collector (LC Packings). Fractions were collected in 20-s intervals. 1.3 μl of matrix solution was added to each fraction. A 1:5 dilution of saturated α-cyano-4-hydroxyquinidine acid in 33% CH₃CN, 0.1% trifluoroacetic acid in ethanol/acetone (2:1) was used as matrix solution. Mass mapping was performed with an Autoflex® MALDI-TOF mass spectrometer (Bruker Daltonics). Peptides were analyzed in the positive ion mode with delayed extraction (70 ns) and the following voltages: source, 19 kV; extraction, 16.55 kV; lens, 8.35 kV; reflector, 20 kV.

Mutated S100A3 Production—The point mutation for Arg/Ala substitution in human S100A3 was introduced by PCR using the QuikChange® II mutagenesis system (Stratagene) with pMal-c2-S100A3 (20) as a template according to the instruction manual. Briefly, the mutagenic primer sets used were as listed below, with modified bases shown underlined: for R3A, 5’-GAGTGGAGATGGCCGCCCTCTTGAGAC-AGG-3’ and 5’-CCTGCTCCAGGGCCGCACATCTC-CACTC-3’; for R22A, 5’-CGAGGATACGCCAGGGCCCTGTGGGGAACATAC-3’ and 5’-GTATTGTCGCCACAGGCCCCCTGCGTATTCCTGG-3’; and for R77A, 5’-CTTTGTGGAGATGGCTGCCCCACATTGC- TTGCTTGCTCCTC-3’ and 5’-GAGCCAGGAATGACTGAGG-CACCATACTCCACAAAAG-3’.

The resulting mutated plasmids containing staggered nicks were processed for transformation after the template plasmid digestion by DpnI. S100A3 and its mutated proteins were expressed as maltose-binding fusion protein in Escherichia coli and purified under anaerobic conditions, as previously described (21).

Apparent Molecular Mass Analyses—The native apparent molecular mass of S100A3 in solution was determined by size exclusion chromatography using a Superdex™ 75 10/300 GL column (GE Healthcare) in 50 mM Tris buffer containing 150 mM KCl, 1 mM DTT, and various concentrations of CaCl₂ or EGTA (pH 7.5) at a flow rate of 0.5 ml/min. The column was standardized with the low molecular mass calibration proteins (GE Healthcare).

Titration of Ca²⁺-induced Conformational Change—Fluorescent titration of the single Trp residue of recombinant S100A3 and its mutated proteins (2 μM) was carried out in 50 mM Tris buffer containing 150 mM KCl and 1 mM DTT (pH 7.5), as previously described (20, 21). Emission fluorescent intensities at 340 nm (5-nm slit) excited at 295 nm (10-nm slit) after cumulative additions of CaCl₂ were recorded using an RF5000 spectrofluorophotometer (Shimazu, Kyoto, Japan).

RESULTS

Citrullinated S100A3 Derived from Hair Follicles and Cuticles—We purified the native form of S100A3 protein derived from beard follicles and matured hair cuticles (Fig. 1 and Table 1). It was difficult to estimate S100A3 content in the matured cuticles, since only a small percentage of the cuticular proteins were extracted. Total soluble protein in the nonkeratinized cells of plucked follicles was estimated to be 10% of the wet mass, and S100A3 content was found to be about 3% of this. This tissue expression level is highest among known abundant brain proteins. Given that S100A3 is found almost only in differentiating cuticular cells, which account for less than 10% of the follicular tissue, this indicates that in the condensed (dehydrated) cuticular cells, it could reach high, even millimolar, concentrations.

Different S100A3 elution profiles from both anion exchange and hydrophobic interaction chromatography (Fig. 1B) suggested that its molecular characteristics are altered during the...
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FIGURE 1. S100A3 protein purification from immature and mature cuticles. A, the hair shaft consists of an outermost layer of cuticle cells, an inner fibrous cortex, and a central vacuolated medulla that is surrounded by inner root sheath. These keratinized tissues originate from the epithelial matrix cells within the hair follicle bulb. S100A3 was isolated from the immature cuticular cells contained in the plucked beard follicle (blue dots) and the mature cuticles separated from the hair shaft (red dots). B, anion exchange and hydrophobic interaction chromatography of native S100A3. Protein extracts from beard follicles (9.8 mg; open circle) and hair cuticles (1.4 mg; closed circle) were loaded on a Q-Sepharose column (1 ml) and eluted by an increasing NaCl linear gradient (0–0.6 M). S100A3 derived from cuticles (closed bar) was eluted later than the follicle derivatives (open bar). After adding ammonium sulfate to 1.5 M, S100A3-containing fractions were loaded on a Macro-prep butyl resin column (1 ml). Although most S100A3 derived from follicles was collected in the pass-through fractions (open bar), a decreasing ammonium sulfate linear gradient (1.5–0.0 M) is required for elution of the cuticle derivatives (closed bar). Fraction numberings were aligned with the linear gradient. C, purification steps of the beard follicle and hair cuticle derivatives, as shown by a silver-stained NuPAGE gel loaded with crude extracts (1/8000 volume), Q-Sepharose column fraction (1/4000 volume), butyl resin column fraction (1/2000 volume), electrical elution effluent (1/500 volume), and recombinant S100A3 (50 ng) under reducing condition.

| Purification step | From 4000 beard follicles | From 200 mg of hair cuticles |
|-------------------|---------------------------|-----------------------------|
|                   | Amount*                   | Yield                       | Amount*               | Yield                       |
| Crude extract     | 307 g                     | 3%                          | 294 g                | 3%                          |
| Anion exchange    | 224 g                     | 73%                         | 173 g                | 59%                         |
| chromatography    | 168 g                     | 54%                         | 106 g                | 36%                         |
| Hydrophobic       | 66 g                      | 21%                         | 23 g                 | 8%                          |
| interaction       |                           |                             |                      |                             |
| chromatography    |                           |                             |                      |                             |
| Electric elution  |                           |                             |                      |                             |

A. Measured densitometrically with recombinant S100A3 as a standard. B. Separated from about 20 g of hair fiber.

cuticular differentiation. Two-dimensional PAGE-Western blots probed by anti-S100A3 antibody showed that the proportions of the isoelectric variants were different for follicular and cuticular S100A3 but did not change during purification steps (Fig. 2A). Although several spots are found in follicle and cuticle derivatives, electrospray ionization-mass spectrometry revealed a single peak with mass 11,624 Da for the S100A3 purified from hair follicle and two peaks (11,624 and 11,640 Da) for the mature cuticle derivative, consistent with the calculated mass of the native form (N-terminal methionine replaced by an acetyl group) (36) and its singly oxidized form (Fig. 2B). The acidic shifts of S100A3 cannot be explained by multiple oxidation of Cys residues, as may occur in artificially oxidized hair (37).

In this study, we found that Ca2+-dependent PAD enzymes converted more than half of the arginine residues in S100A3 to citrullines within the hair cuticular cells. Using an immunodetection method for the chemically modified citrulline (34), we observed S100A3 protein spots containing citrullines besides the acetylated form (Fig. 2A). S100A3 contains 4 arginine residues, and the beard follicle derivative consists of noncitrullinated and singly citrullinated forms (pI 4.5 and 4.3), whereas various arginine residues in the mature S100A3 were converted to citrullines, resulting in their lower pI values (pI 4.3, 4.1, 3.9, and 3.8). A doublet spot observed at pI 4.3 in the follicle derivative probably consists of monocitrullinated forms, where different arginines were converted. Calculated conversion rates of S100A3 were 19% for nonkeratinized beard follicle derivatives and 59% for the matured cuticle derivatives. Naturally occurring S100A3 appears to be citrullinated during cuticular differentiation.

PAD Isozymes Modified S100A3 in Hair Follicle—In the mammalian cutaneous epithelium, protein-citrullination is reported to be catalyzed by PAD1, PAD2, and PAD3 (38) among the five known isozymes (39). In hair follicles, PAD3 is located in the cuticular cells expressing S100A3 (24–26) as well as in the inner root sheaths and the medulla, where it co-localizes with trichohyalin (35, 40). We determined how PAD isozymes co-localize with S100A3 in order to elucidate the role of PAD isozymes in S100A3 citrullination.

Immunofluorescent laser-scanning confocal microscopy of beard follicles showed that S100A3 is primarily localized in the cuticular cells but also in some cortical cells just under the cuticle (Fig. 3). Changes in PAD distribution with cuticular differentiation were detected using specific anti-PAD antibodies (supplemental Fig. S1). All confocal fluorescent signals of PAD enzymes appeared prior to the S100A3 signal as granular patches, but subsequently the localization of isozymes is cytoplasmic. PAD1 was found in the differentiating hair cuticular and cortical cells and, to a lesser extent, in the inner root sheath.
The granules with PAD1 immunoreactivity were clearly discriminated from the nuclear and cytoplasmic distribution of S100A3. PAD1 in the differentiating cortical cells appeared to be associated with hair keratin fiber, as has been described for the epidermis (18, 38). PAD2 was observed as granular patches from the early differentiation stages of all hair epithelial matrix daughter cells; however, as each cellular differentiation advanced, PAD2 appeared in the cytoplasm and partially co-localized with S100A3 in the cuticular and cortical cells. Anti-PAD3 antibody intensely labeled the differentiating cells within the inner root sheath and cuticular layers and medulla, but a PAD3 signal was absent from the matrix in the lower follicle bulb and hair cortex, as previously reported (35). Cytoplasmic distribution of PAD3 was seen at an earlier cuticular differentiation stage than for PAD2 and almost completely overlaps the S100A3 reaction. cu, cuticle; co, cortex; m, medulla; irs, inner root sheath. Scale bar, 50 μm.

Differential Citrullination of S100A3 Depending on Isoype of PADs—Recombinant human S100A3 protein was incubated with the three recombinant PAD isoforms in vitro, and the extent of the arginine conversions catalyzed by active enzymes was then examined. The number of peptidylcitrulline residues in S100A3 varied depending on the isozyme used, as shown by two-dimensional PAGE patterns (Fig. 4A). PAD1 and PAD2 are
capable of converting 70–80% of arginine residues in S100A3, whereas the maximum conversion rate by PAD3 was below 30% (Fig. 4B). PAD3 exhibited the highest selectivity for one specific residue in S100A3. All three PAD isozymes were able to catalyze arginine citrullination at lower Ca2+/H11001 levels in S100A3 (Fig. 4C) than needed for synthetic substrates and recombinant filagrin subunit (18). PAD2 and PAD3 enzymes that conduct S100A3 citrullination in vivo required higher Ca2+/H11001 concentrations for half-maximal activation (0.35 mM for PAD2 and 0.53 mM for PAD3) than needed by PAD1 enzyme (0.18 mM). These results suggest that millimolar Ca2+/H11001 concentrations are necessary to activate PAD2 and PAD3 in cuticular cells.

The conversion rates of each arginine residue in S100A3 were deduced from a HPLC profile of the peptide fragments (Fig. 5). S100A3 released all arginine-containing peptides (1MARPLEQAVAAIVCTFQEYAGRCGDK26 (from recombinant), Ac-2ARPLEQAVAAIVCTFQEYAGRCGDK26 (from native form), 41ELATWTPTEFRECDYNK57, and 67DCEVFYEYVRSCLCILYCHEYFK91) by endoproteinase Lys-C digestion. Digestion of citrullinated S100A3 revealed additional
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citrulline containing peptides at slightly different retention times due to their increased hydrophobicity (41). MALDI-TOF mass spectrometry of these peptides confirmed the contents of citrulline; the molecular mass of the peptides increases by +1 Da/number of converted arginine residues. The peak area ratio of the modified peptide revealed that both PAD1 and PAD2 converted Arg-3, Arg-22, and Arg-51 completely and Arg-77 partially. In contrast, with PAD3, conversion was complete for Arg-51 and partial for Arg-3 or Arg-22 but did not affect Arg-77 at all. Similarly, specific conversion of Arg-51 (74%) in native S100A3 derived from hair follicle is the principal action of PAD3 in vivo, although we were unable to determine the conversion rates of each site in the mature cuticle derivative.

Specific Citrullination-mediated Ca$^{2+}$-dependent S100A3 Homotetramerization—Protein citrullination by Ca$^{2+}$-dependent PAD enzymes is known to confer large structural and mechanical effects on the target proteins by altering inter- and intramolecular ionic or hydrophobic interactions (11). In this study, we found that naturally occurring S100A3 derived from the cuticles and that from the beard follicle and recombinant S100A3 modified by PAD enzymes in vitro have higher apparent molecular masses in the presence of Ca$^{2+}$ (Fig. 6). A size exclusion chromatography calibrated using globular protein standards showed apparent molecular masses (40–42 kDa) consistent with the calculated mass for S100A3 tetramer (46.5 kDa). Nonmodified S100A3 mainly forms a dimer either in the absence or presence of Ca$^{2+}$. Its hemispheric structure resulted in a slightly larger apparent molecular mass (28 kDa) than the calculated mass (23.4 kDa). Since the tetramer dissociated to the dimer at Ca$^{2+}$ concentrations below 1 mM (data not shown) or upon the addition of EGTA in the elution buffer, it is apparent that S100A3 tetramerized in a Ca$^{2+}$-dependent manner similarly to the S100A8/S100A9 heterotetramer (5) and S100A12 hexamer (6).

In order to elucidate the masking effect of each positive charge of 4 arginine residues in S100A3 on its Ca$^{2+}$-binding property and tetramerization, we substituted them with Ala by site-directed mutagenesis. Titration of the single tryptophan fluorescence in S100A3 (20, 21) showed that the conformation of R51A and R77A apparently changed at lower Ca$^{2+}$ concentrations than in the wild-type protein, but the Ca$^{2+}$ concentrations needed for the conformational change of R3A and R22A were not altered (Fig. 7A). Estimated Ca$^{2+}$-binding properties of R51A ($K_D = 3.4$ mM) and R77A ($K_D = 4.8$ mM) showed 3.9- and 2.7-fold increases in affinity compared with the wild type protein ($K_D = 13$ mM). Gel filtration analyses in the presence of 5 mM Ca$^{2+}$ showed that wild type, R3A, and R22A all form a dimer only, whereas R51A (mainly) and R77A (to a small extent) form a tetramer (Fig. 7B). Since the recombinant S100A3 modified by PADs in vitro could only partially assemble into tetramers after previous disassociation to dimers by Ca$^{2+}$ exclusion from the reaction mixture, it was difficult to precisely determine their Ca$^{2+}$-binding properties. However, taking account the lower Ca$^{2+}$ concentration required for the tetramerization of citrullinated S100A3 proteins compared with R51A (1 mM versus 5 mM), their $K_D$ values for Ca$^{2+}$ binding are presumed to be around the 1 mM level.

S100A3 self-association enhanced its own Ca$^{2+}$-binding property under in vivo conditions where there is preferential citrullination of Arg-51. Considering that incomplete Arg-51 conversion resulted in partial tetramerization (e.g. follicle...
S100A3; Fig. 6), citrullination of Arg-51 in all S100A3 elements appears to be necessary for its efficient tetramerization. Masking of Arg-77 caused a conformational change upon Ca\(^{2+}\) binding, but tetramerization proceeded very slowly (Fig. 7). Since Arg-77 is normally preserved in vivo, the R77A tetramer is probably assembled in an unusual manner in vitro. In recombinant S100A3 modified by PAD enzymes, Arg-51 was completely converted to Cit-51 by all three PAD isozymes. In the presence of Ca\(^{2+}\), they similarly tetramerized regardless of the different conversion rates of Arg-3 and Arg-22 (Figs. 5 and 6). Single substitution of these residues with alanine did not affect either Ca\(^{2+}\)-induced conformational change or tetramerization (Fig. 7). Thus, we conclude that citrulline residues in S100A3 converted in vivo other than Cit-51 are irrelevant to S100A3 tetramerization.

**DISCUSSION**

Studies on the biological function of several S100 proteins capable of organizing into larger assemblies than dimers have recently increased in number, but the physiological condition promoting this has not been elucidated for any S100 oligomers. Although S100A3 tends to assemble as homotetramers or higher oligomers at supraphysiological Ca\(^{2+}\) concentrations (20), it is not known if there are circumstances that allow S100A3 tetramer formation in vivo. In this study, we show for the first time that an irreversible post-translational modification enables S100A3 tetramerization during human hair cuticular differentiation.

PAD enzymes have been reported to catalyze the conversion of arginine residues in keratins and keratin matrix proteins into citrulline residues in the presence of Ca\(^{2+}\) (11). Previous investigation has shown that this irreversible modification unfolds the protein structure of natural substrates, such as filaggrin in the skin epidermis and trichohyalin in the hair follicle (13), resulting in different outcomes. Citrullinated filaggrin disassociates from keratin filaments and is hydrolyzed into amino acids (13), whereas citrullinated trichohyalin becomes soluble and susceptible to cross-linking by another Ca\(^{2+}\)-dependent enzyme, transglutaminase (16, 17). In contrast, citrullinated S100A3 dimer assembled into a homotetramer. This irreversible modification of S100A3 appears to be a crucial step prior to its organization into the amorphous composites of the mature cuticles (25).

Although neither the functional significance of each converted residue nor the catalytic order of the responsible PAD isozymes has yet been defined with any natural substrates, we reveal a definite functional role of a specific conversion of Arg-51 in S100A3 protein, principally conducted by PAD3, which promotes S100A3 tetramerization. Since Arg-51 within the hinge region is not conserved in the amino acid sequences represents the mean value of three independent experiments. B, gel filtration analyses of wild type and mutated S100A3 proteins (10 \(\mu\)g each) premixed in the elution buffer containing 5 mM CaCl\(_2\) were loaded onto a Superdex 75 column. Wild type, R3A, and R22A migrated as a dimer (d), whereas R51A migrated mainly as a tetramer (t). R77A migrated only partially in the tetrameric form, but its portion increased after prolonged incubation. The arrows indicate the elution time of the standard proteins.
of any other human S100 protein, this posttranslational regulation is probably specific to S100A3.

No general type of assembly has been reported with S100 oligomers (7); however, determination of the citrullination site of S100A3 essential for its tetramerization offered an insight on its manner of assembly. Based on the known crystal structure of apo-S100A3 dimer (Protein Data Bank code 1KSO) (27, 28), we propose a prototype model of the S100A3 tetramer (Fig. 8). Since Arg-51 is not involved in any intramolecular function in the dimer (1KSO), its conversion per se does not directly affect the Ca\(^{2+}\)-binding property of S100A3. Increased intracellular Ca\(^{2+}\) level to nearly millimolar range is still insufficient for dimeric S100A3 binding to Ca\(^{2+}\). In the S100A3 dimer, a symmetric pair of Cit-51 occurs on the planar surface formed by helices III and IV. Hence, we hypothesize that the apo-S100A3 dimer initially associates with another dimer rotated around an axis connecting Cit-51 residues. Such interaction cannot occur between the nonmodified dimers because of the electric repulsion between the positive charges of Arg-51.

Most dimeric S100 proteins have been reported to reorient helix III and expose a broad hydrophobic cleft upon Ca\(^{2+}\) binding (1). Our fluorescent analyses showed that a similar conformational change could be induced with R51A at the same Ca\(^{2+}\) concentration that promotes its tetrameric association (Fig. 7). This suggests that the potential Ca\(^{2+}\)-binding property of S100A3 is elicited during its tetrameric association. Since structural modification is supposed to enable target protein recognition by S100A3, it is intriguing that elements of S100A3 undergo a similar conformational change within the tetramer. The Ca\(^{2+}\) affinity of S100 proteins is generally low at the intracellular Ca\(^{2+}\) level but can be increased once they associate with their target protein (42). Our results show for the first time that the potential Ca\(^{2+}\)-dependent role of S100A3 in vivo could be acquired by its self-association.

As a result of the S100A3 tetramerization following Arg-51 conversion, Arg-77 is buried within its docking interface. Although Arg-3 and Arg-22 located on the tetramer surface are susceptible to citrullination by PAD2, Arg-77 is normally preserved due to the structural constraint. Interestingly, masking of this positive charge (R77A) enabled a conformational change similar to that following R51A conversion but failed to cause assembly of the S100A3 tetramer. On the helix IV, which constitutes a tetramerization plane of the apo-S100A3 dimer, the positive charge of Arg-77 might draw a neighboring negative charge of Glu-74 within the C-terminal canonical EF-hand motif. In the crystal structure of the S100A3 dimer (1KSO), the two monomers are similar but not identical and are termed chains A and B. Interaction between Arg-77 and Glu-74 is seen only in chain B. Preserved Arg-77 in vivo might hamper the bidentate coordination of the carboxyl group (Glu-74) with the Ca\(^{2+}\) ion, which is invariant in most S100 proteins, and reduce the potential Ca\(^{2+}\)-binding property of dimeric S100A3.

S100A3 tetramer and bound Ca\(^{2+}\) ions (presumed 8 ions/tetramer) in the differentiating cuticular cells are estimated to be present at submolar and low millimolar levels, respectively. Our results showed that the nearly millimolar range of Ca\(^{2+}\) concentration is needed for S100A3 modification by the PAD enzymes in vitro (Fig. 4C). In order to achieve this condition, it is necessary that the bulk of Ca\(^{2+}\) stored in the S100A3 tetramer be utilized efficiently.

The presence of 10 cysteine residues in S100A3 suggests the formation of covalently linked polymeric complex structures in the cornifying cuticular cells in nonreducing conditions. Although the presence of intermolecular disulfide bridges between them has not been demonstrated, an intramolecular disulfide bridge between Cys-23 and Cys-30 easily forms under aerobic conditions and is postulated to distort the S100-specific N-terminal EF-hand, decreasing its affinity to Ca\(^{2+}\) (21). Ca\(^{2+}\) stored within citrullinated S100A3 tetramer might be released into the local environment as it becomes less reductive.

The most important question this study raises is why the enhancement of the Ca\(^{2+}\)-binding property of S100A3 during tetramerization is under the regulation of Ca\(^{2+}\)-dependent PAD activity. One possible explanation is that S100A3 tetramers take up and release Ca\(^{2+}\) ions within the restricted zone (Fig. 8). Amassed Ca\(^{2+}\) ions could activate Ca\(^{2+}\)-dependent...
protein-modifying enzymes. Such a microenvironmental Ca\(^{2+}\) transient might extend within the cuticular cellular boundary to construct its thick protein barrier structure. More work is needed to reveal the precise functional role of S100A3 tetramers in the epithelial Ca\(^{2+}\)-cycling system.

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