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ORIGINAL ARTICLE

Active Secretion of Dimerized S100A11 Induced by the Peroxisome in Mesothelioma Cells

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Abstract S100A11, a small Ca^{2+} binding protein, acts extracellularly as a mediator of cancer progression. That raises the question of how a protein that lacks the classical secretory signal is able to be secreted outside cells without being damaged. Some insights into this question have been obtained, and there has been accumulating evidence indicating a pivotal role of a non-classical vesicle-mediated pathway using lysosomes or peroxisomes for the protein secretion. To obtain a more precise insight into the secretory mechanism of S100A11, we first screened representative cancer cells exhibiting significantly active secretion of S100A11. From the results of profiling, we turned our attention to aggressive cancer mesothelioma cells. In mesothelioma cells, we found that abundant dimeric S100A11 was produced selectively in the peroxisome after transportation of monomeric S100A11 through an interaction with PEX14, a peroxisome membrane protein, resulting in peroxisomal secretion of dimerized S100A11. In an extracellular environment in vitro, dimerized S100A11 promoted mesothelial cell invasion indirectly with the help of fibroblast cells. Overall, the results indicate that the peroxisome functions as an essential vesicle for the production of dimerized S100A11 and the subsequent secretion of the protein from mesothelioma cells and that peroxisome-mediated secretion of dimerized S100A11 might play a critical role in mesothelioma progression in a tumor microenvironment.

Keywords S100A11 · Mesothelioma · Peroxisome · Lysosome · Tumor microenvironment · Metastasis

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Abbreviations

Ca	Calcium
FBS	Fetal bovine serum
DTT	Dithiothreitol
RAGE	Receptor for advanced glycation endproducts
HMGB1	High mobility group box 1
HSP70	Heat shock protein 70
IL-1β	Interleukin-1β
ER	Endoplasmic reticulum
HE	Hematoxylin-eosin
ANXAI	Annexin I
$cPLA_2$	cytosolic phospholipase A_2
EGFR	Epidermal growth factor receptor

Introduction

S100 proteins constitute a family of 20 different members (S100A1 to S100A16, S100β, S100G, S100P, and S100Z) in humans, each of which is able to bind calcium by their common EF-hand motifs. S100 proteins are named after their solubility at 100 % saturation with ammonium sulfate. The proteins are small with a molecular weight ranging from 9 to 14 kDa and they have acidic properties. Interestingly the proteins act as monomeric, homo and heterodimeric, or multimeric forms in intracellular as well as extracellular spaces [1]. Among the proteins, one of our focuses is on S100A11 since we have been studying this protein for a long time to pursue its interesting biological significance [2–12].

S100A11 is expressed in ubiquitous tissues at different levels, high levels in the skin and placenta, intermediate levels in the heart, spleen, kidney, liver, and lung, low levels in the skeletal muscle, colon, and thymus, and very low level in the brain [3]. S100A11 has been proposed to play pleiotropic cellular functions in various biological contexts, such as contact inhibition [2], epidermal differentiation [4, 6], senescence [3], apoptosis [5], inflammation [13, 14], and cancer progression [10, 12, 15–17].

A confluent condition of normal human fibroblast cells results in cell growth arrest, and the same phenomenon is observed for human epidermal keratinocytes after being stimulated with TGF-β or high Ca^{2+}. Interestingly, both phenomena are linked to nuclear accumulation of S100A11 [2–8]. Namely, in both cases, intracellular S100A11 is phosphorylated by PKCα at 10Thr and transferred to the nucleus, resulting in induction of p21/WAF1, a strong cyclin-dependent kinase inhibitor, through activation of Sp1 transcription factor.

In normal human keratinocytes, S100A11 in the cytoplasmic compartment also plays a role as a growth inhibitor when the cells are treated with high Ca^{2+}. Ca^{2+}-dependent binding of S100A11 to annexin I (ANXAI) facilitates the binding to cytosolic phospholipase A_2 ($cPLA_2$), resulting in inhibition of the arachidonic acid cascade that induces down-regulation of cell proliferation [9].

On the other hand, S100A11 exerts an extracellular autocrine effect when the protein is secreted from normal keratinocytes, epithelial cancer cells [10] and also chondrocytes [13, 14]. In the extracellular space, S100A11, as in the case of other members of the S100 protein family, can form a homodimer in solution through a disulfide bridge between cysteine residues in a Ca^{2+}-independent manner [10, 18] or by an enzymatic modification via transglutaminase [14]. Unlike the monomeric form of S100A11, the dimerized form readily engages a cell surface receptor, receptor for advanced glycation endproducts (RAGE) [10]. The binding of S100A11 to RAGE induces growth stimulation through Akt-mediated upregulation of EGF in not only normal keratinocytes but also various epithelial cancer cells [10]. Cecil et al. reported that the binding of S100A11 to RAGE regulates chondrocyte differentiation, resulting in hypertrophy through a p38 MAPK-mediated increase in the size of cells and expression of type X collagen [13, 14]. These findings indicate that the S100A11-RAGE signal is multiple and contributes to diverse cellular processes in different contexts of pathogenesis.

S100A11 is hence an interesting molecule with dual opposing functions in intracellular and extracellular environments. However, there are still unknown mechanisms for the extracellular S100A11. That raises the question of how the protein, which lacks the classical secretory signal through an endoplasmic reticulum (ER)/Golgi-pathway, is secreted outside the cells.

In this study, we first showed that S100A11 was highly upregulated for secretion in various cancer cells, especially in mesothelioma cells, aggressive cancer cells. Analysis of a non-classical vesicle-mediated secretory pathway revealed the contribution of two different cellular vesicles, lysosome and peroxisome. Interestingly, S100A11 in a dimeric form was specifically formed in the peroxisome through binding with PEX14, a peroxisomal membrane protein. We also found that the peroxisomal extract has a critical role in the disulfide bridge-mediated dimer formation of S100A11. Finally, we found that dimeric S100A11 promoted mesothelial cell invasion indirectly with the help of fibroblast cells. These novel findings provide insights into the extracellular role of dimerized S100A11 in the mesothelioma cell microenvironment linked to cancer progression.

Materials and Methods

Cell Lines

The following human cancer cell lines were used: HEK293T (embryonic kidney cell line stably expressing the SV40 large T antigen; RIKEN BioResource Center, Tsukuba, Japan), SK-BR-3 (breast cancer cell line; ATCC, Rockville, MD), NCI-

2170 (lung cancer cell line; ATCC), T24 (bladder cancer cell line; ATCC), PC-3 (prostate adenocarcinoma cell line; ATCC), LNCaP (prostate carcinoma cell line; ATCC), DU145 (prostate carcinoma cell line; ATCC), HeLa (cervix adenocarcinoma cell line; ATCC), A431 (skin epidermoid carcinoma cell line; ATCC), BSCC-93 (RIKEN BioResource Center), DJM-1 (JCRB Cell Bank, Tokyo, Japan), HSC-5 (RIKEN BioResource Center), HepG2 (hepatocellular carcinoma cell line; ATCC), Hep3B (hepatocellular carcinoma cell line; ATCC), Huh-7 (hepatocellular carcinoma cell line; JCRB Cell Bank), PK-8 (pancreatic carcinoma cell line; RIKEN BioResource Center), PL45 (pancreatic carcinoma cell line; ATCC), NCI-H2052 (mesothelioma cell line; ATCC), NCI-H2452 (mesothelioma cell line; ATCC), Met-5A (Immortalized mesothelial cell line; ATCC) and LP-9 (normal mesothelial cell line; Coriell Cell Repository, Camden, NJ). LP-9 cells were cultured using Ham's F12 medium/Medium 199 (1:1 mixture) with 10 % fetal bovine serum, 2 mM L-glutamine, 1.7 nM epidermal growth factor, and 1100 nM hydrocortisone [19]. The other cell lines were all cultivated in D/F medium (Invitrogen, Carlsbad, CA) supplemented with 10 % FBS.

Recombinant Proteins

Human S100A11 cDNAs (wt and mut ΔSH3, which lacks dimerization) were cloned into the pGEX-6P1 vector (GE Healthcare, Piscataway, NJ) for expression in *E. coli*. Purification of dimerized S100A11 (wt) and monomeric S100A11 (ΔSH3) was performed under conditions reported previously [10]. Biotinylated monomeric S100A11 (wt) was also prepared as described previously [10]. The extracellular domain of receptor for RAGE fused with Fc region of IgG1 (exRAGE-Fc chimera) was obtained from a commercial source (R&D Systems, Minneapolis, MN).

Mammalian Gene Expression Plasmids

All of the mammalian gene expression constructs used in this study were made using the pIDT-SMART (C-TSC) vector [20] as the backbone to express cargo genes. A series of vesicle-targeting S100A11 (Wt: wild, LTS: lysosome-targeting signal, PTS: peroxisome-targeting signal) expression constructs were made to express ectopic S100A11s as C-terminal Myc-6His-tagged forms. In the constructs, KFERQ sequence as a representative LTS [21], which is located behind the C-terminal epitope, was used to efficiently localize S100A11 in the lysosome. Two representative PTSs, SKL [22] and KANL [23] sequences, which are both located at the C-terminal site behind the epitope, induce S100A11 accumulation in the peroxisome. Although the function of KFERQ sequence is not restricted to the specific protein site, the functions of SKL and KANL sequences are restricted to the protein C-terminal end. S100A11 lacking Ca-binding ability (mut ΔCa [4, 12]) and cysteine (Cys)-replaced variants of S100A11 (ΔSH1: Cys13Ser, ΔSH2: Cys91Ser, ΔSH3: Cys13Ser + Cys91Ser) were also made to be expressed as C-terminal 3Myc-6His-tagged forms. Human cDNAs encoding PEX5, PEX7 and PEX14 were designed to be expressed as C-terminal 3Flag-6His-tagged forms. Human cDNA encoding LAMP1 was tagged with C-terminal 3Myc-6His epitope. Transient transfection of the above-described plasmids into cultured cells was performed using FuGENE-HD (Promega BioSciences, San Luis Obispo, CA).

Western Blot Analysis and Co-Immunoprecipitation

Western blot analysis was performed under conventional conditions. The antibodies used were as follows: rabbit anti-S100A11 antibody that we made [2–10], mouse anti-HA tag antibody (Cell Signaling Technology, Beverly, MA), mouse anti-Myc antibody (Cell Signaling Technology), mouse anti-Flag antibody (Sigma-Aldrich, St Louis, MO), rabbit anti-human RAGE antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human PEX14 antibody (Novus Biologicals, Littleton, CO), and mouse anti-human tubulin antibody (Sigma-Aldrich). The second antibody was horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling Technology). Positive signals were detected by a chemiluminescence system (ECL plus, GE Healthcare Bio-Sciences, Piscataway, NJ).

Agarose beads conjugated with monoclonal anti-DYKDDDDK tag antibody (the Flag tag being captured by the antibody beads, WAKO, Tokyo, Japan), monoclonal anti-Myc tag antibody (MBL, Nagoya, Japan) and monoclonal anti-HA tag antibody (Sigma-Aldrich) were used for co-immunoprecipitation experiments.

siRNA

Human PEX14 siRNA (siPEX14: No.1: ID# s10324, Lot# ASO22891; No.2: ID# s10325, Lot# ASO22893; No.3: ID# s10326, Lot# ASO22892) and Control siRNA (siCont: Silenser® Negative Control siRNA #1) were purchased from Ambion/Thermo Fisher Scientific (Waltham, MA). The siRNAs (20 nM) were transfected using Lipofectamin RNAiMAX reagent (Invitrogen/Thermo Fisher Scientific).

Quantitative RT-PCR

Cultured cells were washed with phosphate-buffered saline and total RNA was extracted using ISOGEN II Isolation Reagent (Nippon Gene, Tokyo, Japan), and then reverse-transcription was performed using ReverTraAce qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-time PCR was performed using FastStart SYBR Green Master (Roche, Tokyo,

Japan) with specific primers (forward primer: tctccaagacagagttcctaagc; reverse primer: tcatgcggtcaaggacac) for detection of human S100A11 on a LightCycler 480 system II (Roche).

Immunocytochemistry

To simultaneously visualize endogenous S100A11 and the peroxisome or lysosome, fixed cells on coverslips were treated with rabbit anti-human S100A11 antibody [2–10] and mouse anti-human Catalase antibody (Abnova, Taipei, Taiwan) to label the peroxisome or mouse anti-human LAMP1 antibody (Novus Biologicals) to label the lysosome at RT for 1 h and further treated with Alexa 594–conjugated goat anti-rabbit IgG antibody (Molecular Probes/Thermo Fisher Scientific) and with Alexa 488–conjugated goat anti-mouse IgG antibody (Molecular Probes/Thermo Fisher Scientific) under the same conditions as those reported previously [2, 3]. Coverslips were finally mounted using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA).

Immunohistochemistry

Human mesothelioma tissues were fixed in 10 % buffered formalin solution. Paraffin-embedded sections were deparaffinized and antigen retrieval was performed by conventional trypsin treatment, and then endogenous peroxide activity was then quenched by incubation in 3 % hydrogen peroxide (Kanto Chemical Co., Inc, Hokkaido, Japan) for 10 min at RT. Slides were incubated with rabbit anti-S100A11 antibody (at a dilution 1:100) as a primary antibody overnight at 4 °C. After the slides had been washed with 0.05 % Tween-20 in PBS (−), they were incubated with AEC (red color) reagent (HISTOFINE simple stain AEC solution, Nichirei Biosciences, Tokyo, Japan). The study using the tissue specimens was approved by the Research Ethics Committee in Niigata University Medical and Dental Hospital. Informed consent was obtained from each patient for use of these materials.

Cell Invasion Assay

Cell invasion was assayed using a Boyden chamber method with filter inserts (pore size, 8 μm) pre-coated with Matrigel in 24-well plates (BD Biosciences, Franklin Lakes, NJ). To avoid any function of S100A11 secreted from cells, Met-5A cells were selected since the cells show no secretion of their own S100A11 (Fig. 1d). Met-5A cells (5×10^4 cells/insert) were seeded on the top chamber. The top and bottom chambers were both filled with the same 0.5 % FBS-low serum D/F medium. The recombinant S100A11 (Wt and ΔSH3) was then set in the bottom chamber at a final concentration of 100 ng/ml. After incubation for 48 h, cells that appeared on the lower surface through the filter were counted after staining with hematoxylin-eosin (HE) dye. The cell invasiveness was then quantified by cell counting in five non-overlapping fields at a magnification of × 100 and was represented as the average of four independent experiments.

To study the contribution of another cells, we seeded OUMS-24 normal human fibroblasts (8×10^4cells/well) on the bottom chamber and filled the chamber with 0.5 % FBS-low serum medium. Before seeding Met-5A cells on the top chamber, OUMS-24 cells on the bottom chamber were treated or not treated with recombinant S100A11 (Wt and mut ΔSH3, each at 100 ng/ml) for 24 h. After setting Met-5A cells (5×10^4 cells/insert) on the upper chamber, an additional 48-h incubation was done to assess the invasiveness of Met-5A cells.

Statistical Analysis

Data are expressed as means ± SD. We used simple pair-wise comparison with Student's *t* test (two-tailed distribution with two-sample equal variance). $P < 0.05$ was considered significant.

Results

Active Secretion of S100A11 in Mesothelioma Cells

We have shown that active secretion of S100A11 is often coupled with higher expression levels of S100A11 in skin squamous cell carcinomas [10]. To know S100A11 secretion levels more broadly in a variety of cancers, we first performed Northern dot blot analysis to detect *S100A11* gene expression in tumor specimens. By this approach, we found that *S100A11* mRNA levels are increased in many cancer tissues (T) compared to the levels of their normal counterpart tissues (N), especially in the colon, lung, bladder, uterus, cervix, thyroid gland, skin, small intestine, and pancreas (Fig. 1a). Cell lines partially matched to this tissue exhibition were then analyzed for S100A11 secretion. Immortalized HEK293T cells were used as a negative control of S100A11 secretion, and NCI-2052 mesothelioma cells were additionally included as a subject of our interest. In parallel with the expression profile shown in Fig. 1a, we found that the secretion of S100A11 was particularly up-regulated in bladder T24 cells, cervix Hela cells, vulva A-431 cells, skin-derived BSCC-93, DJM-1 and HSC-5 cells, pancreas-derived PK-8 and PL45 cells, and NCI-H2052 mesothelioma cells. PL-45 pancreatic and NCI-H2052 mesothelioma cells showed extraordinarily higher levels of secretion (Fig. 1b).

Since results of previous studies have shown very high expression levels of S100A11 in pancreatic cancer cells [24, 25], we turned our attention to mesothelioma cells. To investigate more

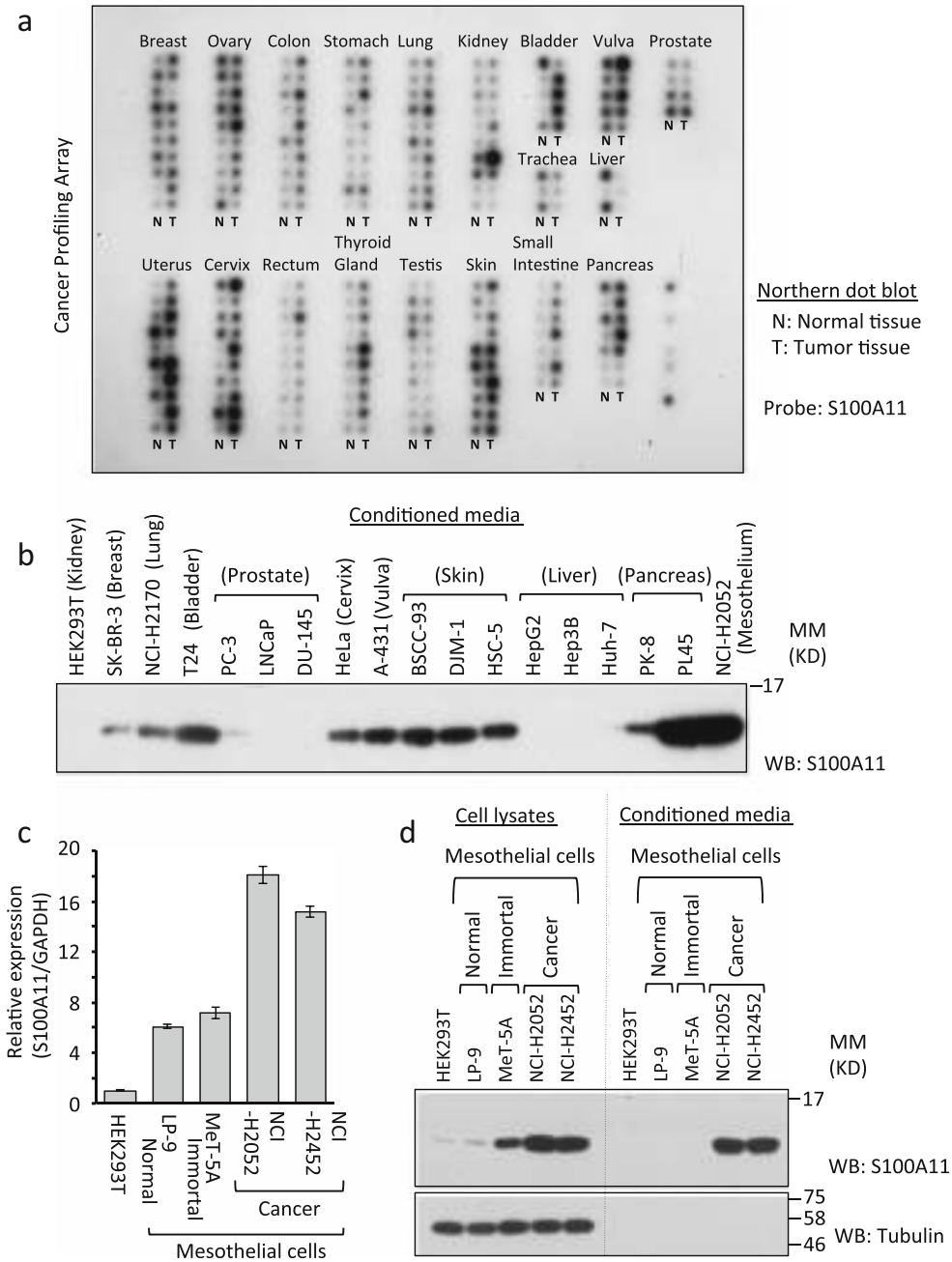

Fig. 1 Expression and secretion profiles of S100A11 in human cancers. **a** Cancer profiling Array II (BD Biosciences) was used to evaluate *S100A11* gene expression in different cancer species compared to their normal counterparts from human tissue specimens. **b** Protein levels of extracellular S100A11 in conditioned culture media were analyzed for various cell lines by Western blot analysis. The prepared protein samples were all in a reduced condition. **c** Total RNAs prepared from various mesothelial cell lines were analyzed for expression of S100A11 by quantitative real-time PCR. GAPDH was used as a control for calibration of the assessment. **d** Protein specimens (cell extracts and their corresponding conditioned culture media) from the indicated cell lines were analyzed for S100A11 by Western blotting. The prepared protein samples were all in a reduced condition. Tubulin was used as a control for loaded amounts of cell extracts and as a successful preparation of secreted proteins without any contamination of cellular proteins

precisely the increase in S100A11 expression in mesothelioma cells, we performed quantitative real-time PCR analysis using additional mesothelioma NCI-H2452 cells, normal LP-9 mesothelial cells and immortalized Met-5A mesothelial cells. Both cancer cell lines showed approximately two-fold higher expression levels of S100A11 than those in normal and immortalized cells (Fig. 1c). Interestingly, the secretion levels of S100A11 protein by the two cancer cell lines were markedly increased, while there was no secretion by either normal LP-9 cells or immortalized Met-5A cells (Fig. 1d).

Based on these findings, we investigated the expression levels of S100A11 in tumor samples from patients with epithelioid and sarcomatoid mesotheliomas. As shown in Fig. 2, S100A11 was abundantly present in the both types of tumor cells. These results together with the results of histological examination suggest that active secretion of S100A11 by mesothelioma cells occurs not only in vitro but also in vivo.

Non-Classical Secretory Route(s) of S100A11

Since S100A11 protein lacks the classical secretory leader signal heading to and passing through an ER/Golgi-pathway, we examined other potential secretory routes of S100A11. Non-classical vesicle-mediated secretory pathways have been

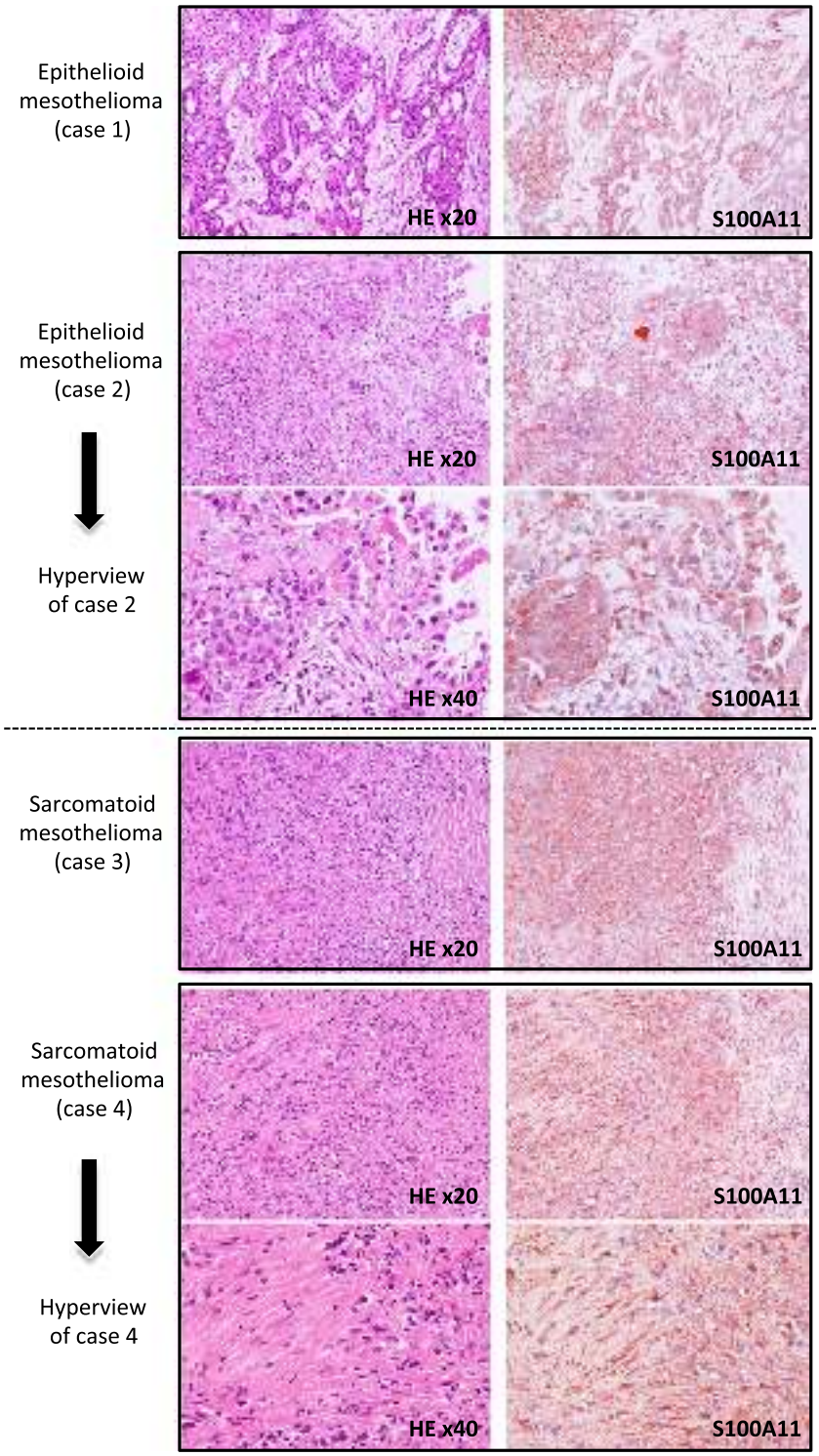

Fig. 2 Immunohistchemical analysis of S100A11 in mesothelioma tissues. S100A11 was immunostained (right images) in epithelioid mesothelioma (*upper*) and sarcomatoid mesothelioma (*lower*). The pictures on the left show hematoxylin-eosin (HE)-stained images corresponding to the S100A11-stained images on the right side

proposed for several leaderless proteins including high mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), interleukin (IL)-1β and catalase, which use specific vesicles, lysosomes [26, 27] or peroxisomes [28, 29]. We then attempted to specify a candidate pathway involved in mediating S100A11 secretion in mesothelioma cells. Fluorescence microscopic observation at low magnification showed that intracellular S100A11 was distributed broadly in the cytoplasm and partially in the nuclei in some cells (Fig. 3a-left), probably due to different stages of the cell cycle [2–8]. By stretching a selected cell image, we were able to determine that S100A11 is distributed as a dot-like pattern in the cytoplasm (Fig. 3a-right). To identify the dots, we further performed double staining of S100A11 with two types of vesicle

marker proteins, LAMP1 for the lysosome or catalase for the peroxisome. We found that S100A11 partially co-localized with both lysosomes and peroxisomes in NCI-H2452 mesothelioma cells (Fig. 3b). The partial co-localization pattern of S100A11 with the peroxisome was also confirmed similarly in a different pancreatic cancer cell line, PK-8 (Fig. 3c).

In order to further explore a functionally important secretory route of S100A11, we made gene expression

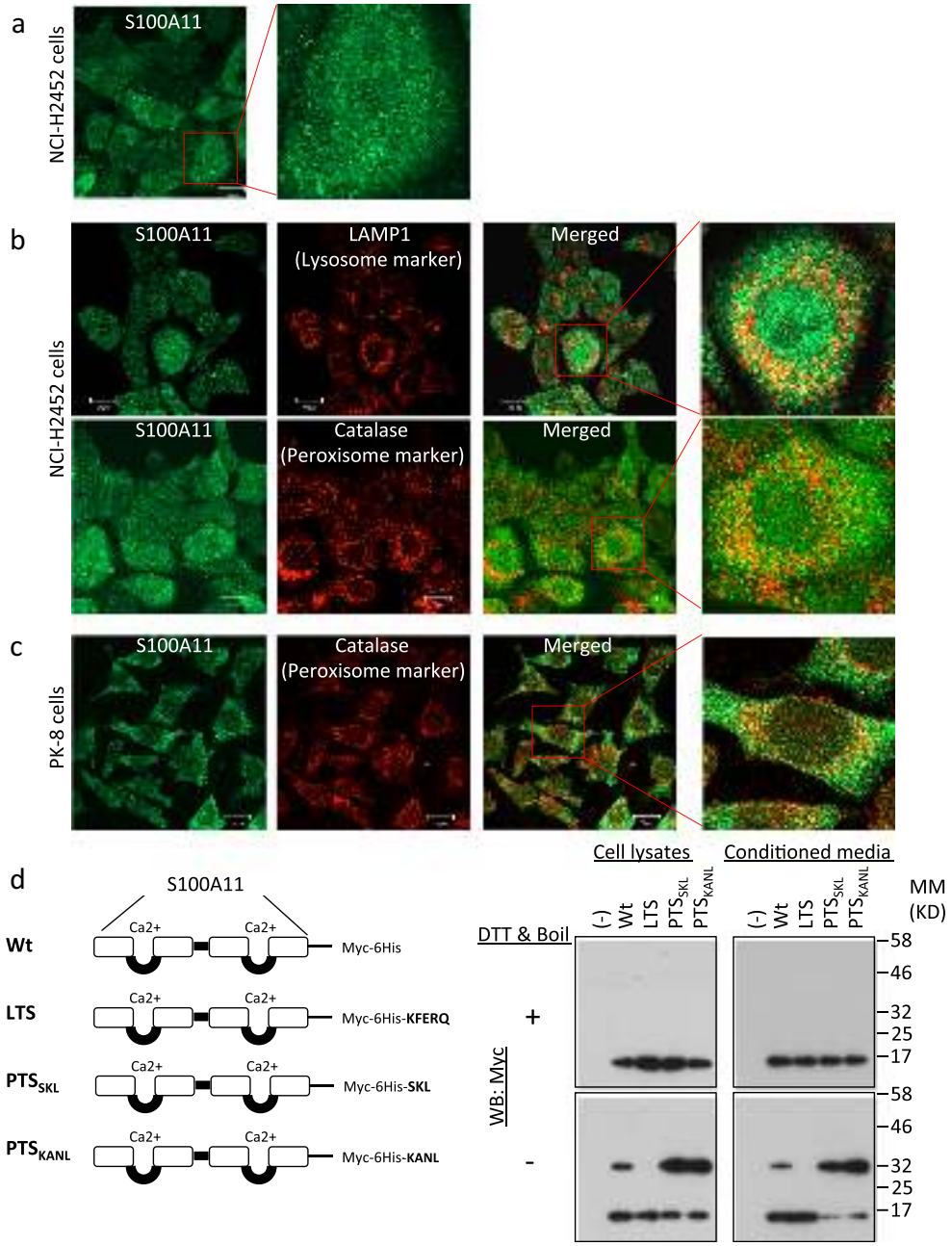

Fig. 3 Non-classical secretory route(s) of S100A11. **a** Immunofluorescence staining was performed to observe localization of S100A11 (*green*). **b** The fixed NCI-H2452 cells were further stained to simultaneously visualize S100A11 (*green*) with the lysosome (*red*) or with the peroxisome (*red*). **c** Immunofluorescence staining of S100A11 (*green*) with the peroxisome (*red*) was also performed in another pancreatic cancer cell line, PK-8. Scale bars in **a**, **b**, and **c** are all 20 μm in length. **d** Schema of selective vesicle-targeting S100A11 is shown on the left side. We gave the wild type (Wt) S100A11 the KFERQ sequence (workable at any location), a representative lysosomal targeting signal (LTS), to force accumulation of ectopic S100A11 (termed LTS) in the lysosome. Two peroxisomal targeting signals (PTSs), SKL and KANL sequences (workable at the C-terminal site), were given to S100A11 to abundantly express S100A11 (termed PTS_{SKL} and PTS_{KANL}, respectively) in the peroxisome. HEK293T cells were transiently transfected with these plasmids expressing modified S100A11s. After preparation of cell extracts and their paired culture media from the indicated transfectants, they were analyzed for S100A11 by Western blotting under a reduced condition (DTT and boil +) and non-reduced condition (DTT and boil -). Tubulin was used as a control for loaded amounts of cell extracts and as a successful preparation of secreted proteins without any contamination of cellular proteins (*right*)

constructs for S100A11 variants that deliver selective vesicle-targeting S100A11, LTS for lysosomal transportation and PTS_{SKL} and PTS_{KANL} for peroxisomal transportation (see Materials and Methods, Fig. 3d-left). In this experimental setting, we analyzed S100A11 under reducing or non-reducing conditions (dithiothreitol (DTT) & Boil + or –) because it has been reported that S100A11 is readily fashioned as disulfide bond-mediated dimeric form in a physiologically functional mode in both intracellular and extracellular spaces [10, 18]. From analysis under the reduced condition (DTT & Boil +), we found that secretion levels of ectopic S100A11 were not altered regardless of the selective localization in different vesicles. However, under the non-reduced condition (DTT & Boil -), dimerization of S100A11 was accelerated with peroxisomal localization (PTS_{SKL} and PTS_{KANL}) but not with lysosomal localization (LTS) and was secreted to the culture medium (Fig. 3b-right). These results indicate that the peroxisome plays a critical role in disulfide bond-mediated dimerization of S100A11 and in secretion of dimerized S100A11.

Effects of Ca^{2+} Binding and Disulfide-Bond Formation on S100A11 Secretion

We next examined the structural requirements of S100A11 for its secretion process, and we focused first on Ca^{2+} binding, which induces a structural arrangement of S100A11 [30, 31], and then on the disulfide bond that mediates the dimerization of S100A11. By using expression constructs of mutant S100A11s (ΔCa, ΔSH1, ΔSH2, and ΔSH3, see Materials and Methods, Fig. 4a-left and b-left), we found that all of them were secreted without any difference in their protein contents in culture media compared to Wt (Fig. 4a-right and b-right). Importantly, Fig. 4b-right showed that the N-terminal 13Cys is required for dimer formation of S100A11, being consistent with a previous report [18].

Interaction of S100A11 with PEX14 in the Process of Peroxisomal Secretion of S100A11

To determine the mechanism underlying peroxisomal secretion of S100A11, we screened potential interactions between S100A11 and PEX proteins, PEX5, PEX7 and PEX14, that play a significant role in selective

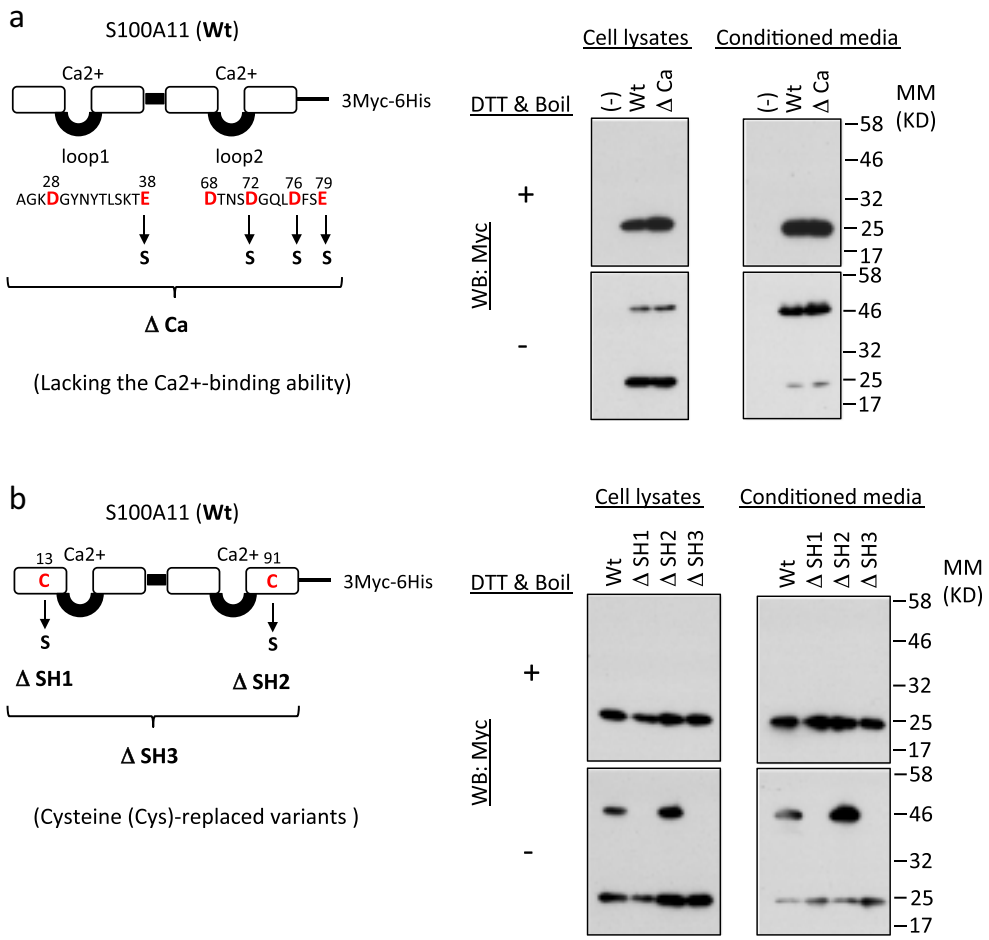

Fig. 4 Effects of Ca^{2+}-binding and disulfide-bond formation on S100A11 secretion. **a** Schema of a mutant S100A11 that lacks Ca^{2+}-binding ability (termed ΔCa) is shown on the left side. **b** Schema of a mutant S100A11 in which cysteine residues were replaced by serine (termed ΔSH1: Cys13Ser, ΔSH2: Cys91Ser, ΔSH3: Cys13Ser + Cys91Ser, respectively) is shown on the left side. HEK293T cells were transiently transfected with these plasmids expressing mut S100A11s (ΔCa, ΔSH1, ΔSH2, ΔSH3). Analysis of expressed S100A11 was performed in the same way as that in the experiment for which results are shown in Fig. 3d (*right*)

transportation of peroxisomal proteins [32, 33]. Among the PEX proteins, only PEX14 bound to S100A11 in a monomeric state (Fig. 5a). The interaction was not altered in the case of S100A11 mutants (ΔCa, ΔSH1, ΔSH2, and ΔSH3) (Fig. 5b). The results might be reasonable since all variants retained secretion abilities (Fig. 4), suggesting a significant function of PEX14 in the secretion process. To address the essential role of PEX14 in the dimerization and following secretion of S100A11, PEX14 siRNAs were used. Application of PEX14 siRNAs efficiently suppressed the expression of endogenous PEX14 (Fig. 5c-left). Notably, the validated siRNAs induced cessation of both dimerization and secretion of S100A11 (Fig. 5c-right). Thus, the notion that cytoplasmic S100A11 in a monomeric state first binds to PEX14 and is then taken up into the peroxisome and is dimerized in a peroxisomal oxidative condition, and is finally secreted appears plausible.

Role of Peroxisome in Disulfide Bond-Mediated Dimer Formation of S100A11

To determine the role of the inner environment of the peroxisome for inducing disulfide bond mediated-dimerization of S100A11, we incubated biotin-conjugated monomeric recombinant S100A11 (see Materials and Methods, [10]) in extracts prepared from isolated peroxisome or lysosome vesicles (Fig. 6a). The mixed monomeric S100A11 changed into a dimeric form in a time-dependent manner when the protein was in reaction with the peroxisomal extract but not the lysosomal extract (Fig. 6b). These results indicate that the peroxisome plays a pivotal role in the dimer formation of S100A11.

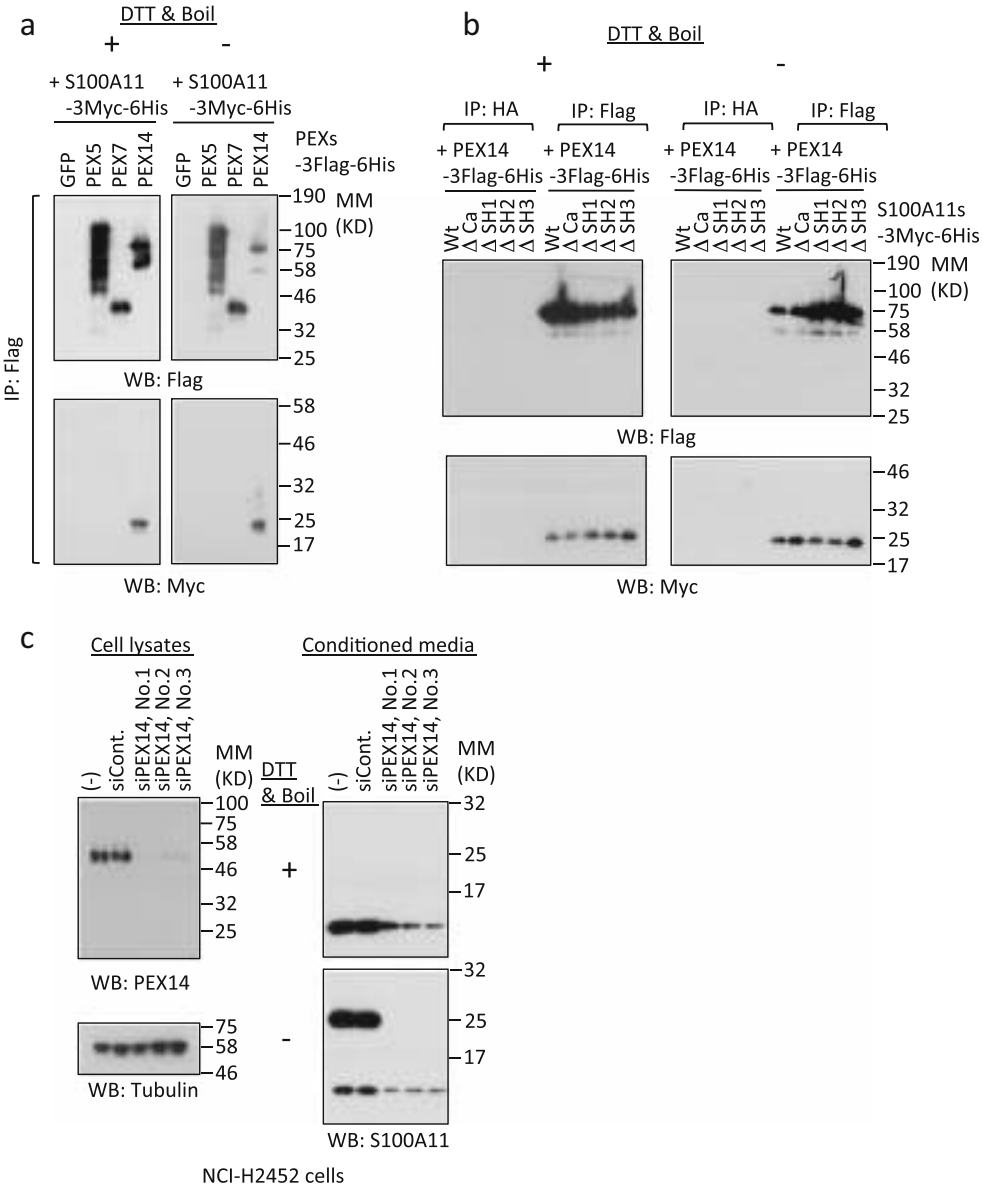

Fig. 5 Interaction of S100A11 with PEX14 in the process of peroxisomal secretion of S100A11. **a** HEK293T cells were co-transfected with S100A11 tagged with 3Myc-6His and candidates (PEX5, PEX7, or PEX14) tagged with 3Flag-6His. Cell extracts were then prepared and analyzed by Western blotting with immunoprecipitation (IP) for the expressed PEX proteins. **b** The same experiment as that in **a** was performed except for the use of mut S100A11s (ΔCa, ΔSH1, ΔSH2, ΔSH3). **c** PEX14 siRNAs (siPEX14 No.1 ~ 3, 20 nM)-induced down-regulation of PEX14 (*left*) was assessed for the dimerization and secretion states of endogenous S100A11 in NCI-H2452 mesothelioma cells (*right*)

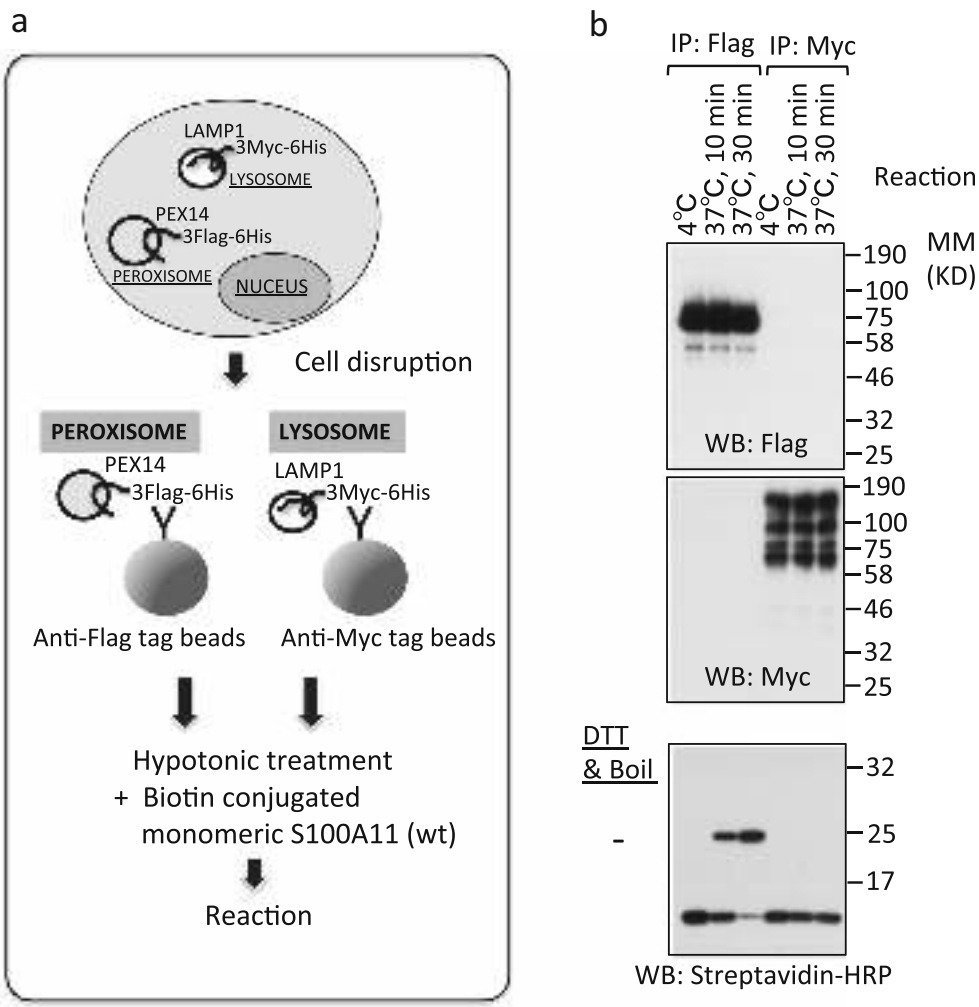

Fig. 6 Role of peroxisome in disulfide bond-mediated dimer formation of S100A11. **a** Schema of the preparation for isolated lysosomes and peroxisomes and their extracts is shown on the left side. HEK293T cells were temporarily co-transfected with PEX14-3Flag-6His and LAMP1-3Myc-6His. Cell extracts prepared without the use of any detergent were used to isolate two vesicles, the peroxisome and the lysosome, by using the indicated beads. After washing the vesicles that had bound to the beads, the precipitates were treated with a hypotonic buffer to disrupt the vesicle membrane and to collect the extracts. **b** To avoid detection of endogenous S100A11 from HEK293T cells, biotin-conjugated recombinant S100A11 kept in a monomeric form was used. After incubation of the recombinant S100A11 in a reaction mixture with the vesicle extracts that was prepared beforehand, the mixed S100A11 was evaluated by Western blotting using HRP-conjugated streptavidin under a non-reduced condition (DTT & boil -) (*lower*)

Biological Role of Dimerized S100A11 in the Tumor Microenvironment In Vitro

To determine the physiological significance of secreted S100A11 in the dimerized form, we prepared highly purified recombinant S100A11 (Wt and ΔSH3: lacking dimerization ability) (see Materials and Methods, Fig. 7a). S100A11 is known as a functional ligand of RAGE [10, 11, 13, 14], so in this context, we first checked the binding capacity of dimerized S100A11 to RAGE compared to that of monomeric ΔSH3 and we found that the dimerized form shows much higher affinity to RAGE (Fig. 7b). This prompted us to examine further the role of dimerized S100A11 in cancer processes. Mesothelioma cells show a metastatic feature that should be regulated in part by the tumor microenvironment containing abundant dimerized S100A11. According to this hypothesis, we tried to determine the biological function of dimerized S100A11 in mesothelioma invasiveness. In this assessment, we selected immortalized Met-5A cells to avoid an autocrine effect of their own secreted S100A11 and we performed a conventional in vitro cell-based invasion assay using a Boyden chamber. We unexpectedly found that S100A11 proteins even in the dimerized form were not able to induce any positive effect on cell invasion (Fig. 7c-left). The tumor microenvironment contains not only tumor cells but also various other cells such as fibroblast cells, mesothelial cells, vascular cells, and immune cells. To express such a microenvironmental feature in the Boyden chamber, we combined fibroblast cells in the system and newly found that dimeric S100A11, but not monomeric type, indirectly promoted invasion of Met-5A cells through the fibroblast stimulation (Fig. 7c-right). These results suggest that extracellular dimerized S100A11, which is formed through the peroxisomal secretory pathway in mesothelioma cells, may play a critical role in mesothelioma progression linked to triggering metastasis in a tumor microenvironment.

Discussion

Increasing evidence has shown that there is a group of secretory proteins without signal peptides, defined as unconventional

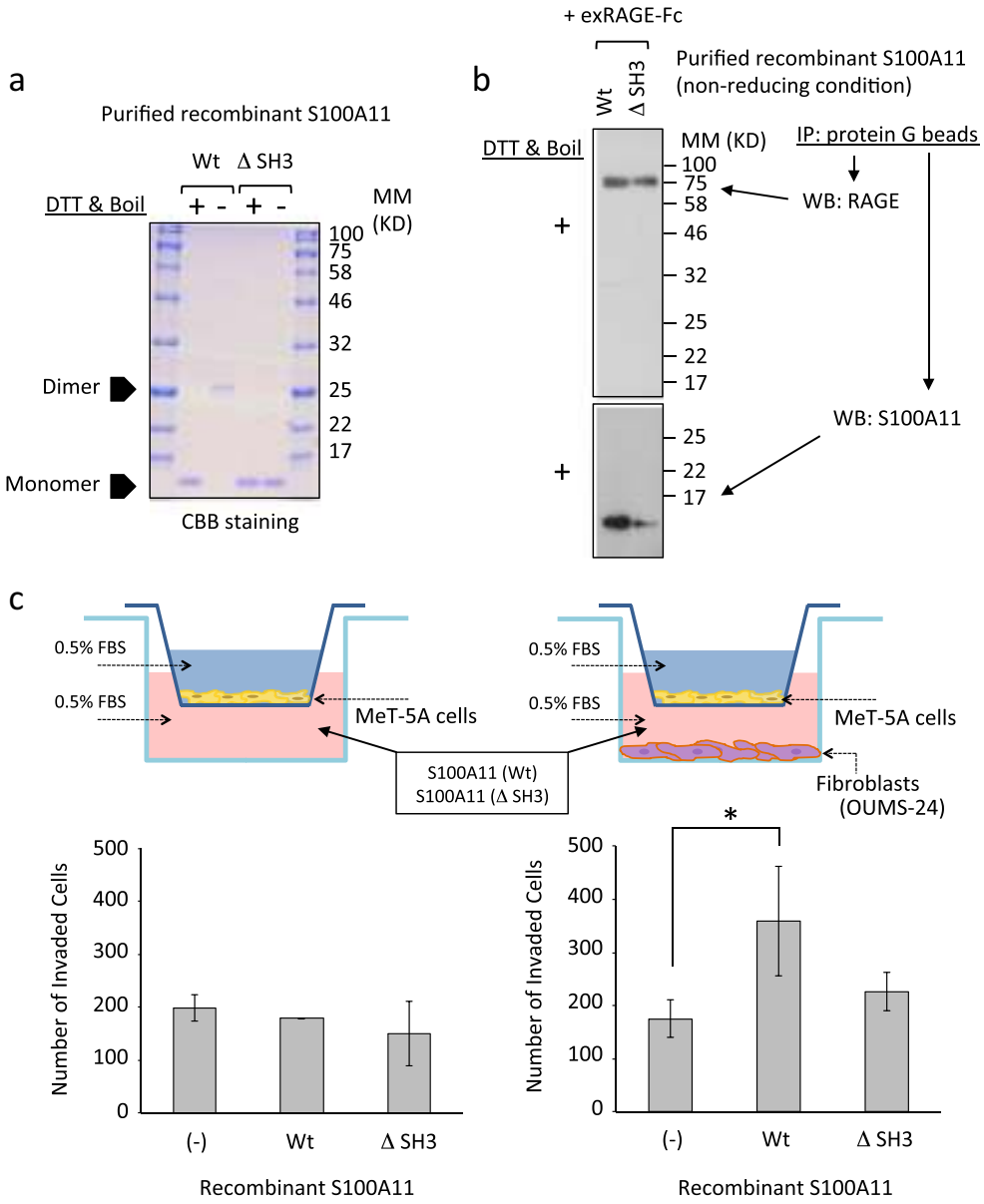

Fig. 7 Biological role of dimerized S100A11 in the tumor microenvironment in vitro. **a** Recombinant human S100A11 (Wt and ΔSH3) was purified from an *E. Coli* expression system. The purified protein was subjected to SDS-PAGE and detected by Coomassie Brilliant Blue (CBB) staining. Wt showed the dimerized form, while ΔSH3 showed a constitutively monomeric form under a non-reduced condition. **b** In vitro binding analysis was performed to assess interaction affinity for each of the purified S100A11s (Wt and ΔSH3) to exRAGE-Fc protein prepared from a commercial source. The exRAGE-Fc was incubated with purified S100A11 proteins, precipitated by protein G conjugated beads, and analyzed by Western blotting for the precipitates. **c** Invasion of immortalized Met-5A mesothelial cells was assessed by a Boyden chamber method in the absence (*left*) or presence (*right*) of OUMS-24 fibroblast culture. The Met-5A cells (*left and right*) and fibroblasts (*right alone*) were placed at the top insert and at the bottom well, respectively, and the purified recombinant S100A11s (Wt or ΔSH3) were also set at the bottom well (*left and right*). The experimental schematics and results are displayed at the upper side and lower side, respectively. Data are means ± SD, $*P < 0.05$

secretory proteins, that are exported via an ER/Golgi-independent pathway to perform extracellular functions. This pathway has been termed unconventional or non-classical secretion [34]. S100A11 also lacks a classical signal peptide sequence for secretion. Secretion of S100A11 was first reported in chondrocytes by Cecil et al. [13]. We also showed that S100A11 was actively secreted from epithelial cancer cells [10]. Although several possible mechanisms have been proposed and partly verified for unconventional secretion routes, the mechanisms by which S100A11 and other S100 family members are secreted remain to be clarified. One clue comes from secretable proteins lacking signal peptide such as HMGB1, HSP70, and IL-1β, which have been proposed to use a lysosome for their secretion [26, 27]. In this respect, it is noteworthy that abundant accumulation of S100A11 in the lysosome appeared in MCF-7 breast cancer cells when the cells were changed to highly metastatic cells by forced expression with the active form of ErbB2 oncogene [35], and this might be linked to active secretion of S100A11 via the lysosomal pathway. In fact, we also found that S100A11 is secreted via the lysosome (Fig. 3), but the lysosome was not able to contribute to the secretion of dimerized S100A11 (Figs. 3d and 6). In this point of view, we fortuitously succeeded in identifying another important pathway through a peroxisome, which plays a pivotal role in the production of disulfide bond-mediated dimerized S100A11 (Fig. 6) and its following secretion (Figs. 3d and 5c).

Peroxisomes are about the same size as lysosomes and, like lysosomes, are enclosed by a single membrane. Owing to the oxidative function of the organelle, it is reasonable that the peroxisomal inner environment is suitable for disulfide-bond formation of S100A11. This is supported by our experimental results using an in vitro reaction system with monomeric S100A11 and extracts from isolated peroxisomes or isolated

lysosomes. In this experimental setting, only the peroxisome fraction induced dimerization of S100A11 (Fig. 6). The peroxisomal secretory phenomenon raises the question of how S100A11 moves to and accumulates in the peroxisome. To obtain an answer, we investigated a potential interaction between S100A11 and PEX proteins, which are in charge of selective transport machinery for peroxisomal proteins [32, 33]. PEX5 and PEX7 play a role in recognition of the peroxisomal targeting signal (PTS), which is delivered to a PEX14 receptor that takes the PTS proteins into the peroxisome. By an interaction study, we found that monomeric S100A11 bound to the PEX14 receptor but not those of the other PEXs (PEX5 and PEX7) (Fig. 5a and b). In addition, down-regulation of PEX14 caused cessation of the coupled process of S100A11 dimerization and secretion of the dimerized form (Fig. 5c). These results indicate that the monomeric form of cytosolic S100A11 is incorporated into the peroxisome via binding with PEX14, resulting in dimer formation, and linking to peroxisomal secretion.

We would like to also touch on the matter of how the secretion of S100A11 is highly up-regulated in mesothelioma cells. We previously reported that S100A11 was actively secreted from epithelial cancer cells through the activation of epidermal growth factor receptor (EGFR) [10]. We hence consider that active secretion of S100A11 by mesothelioma cells occurred in a way similar to that of the EGF-EGFR pathway, since endogenous expression of EGFR in mesothelioma cells used in this experiment was highly up-regulated (data not shown). However, no significant up-regulation of any *PEX* genes including PEX14, even with EGF treatment, was observed in mesothelioma cells when we investigated their expression levels in mesothelioma cells (NCI-H2052 and NCI-H2452) compared with the expression levels in normal LP-9 cells and immortalized Met-5A cells using quantitative real-time PCR analysis (data not shown). Contemporary studies focused on post-translational modification of proteins for their activation, decomposition, and localization. Due to presence of many kinds of modifications including phosphorylation, acetylation and O-GlcNAcylation, further studies will be required to fully elucidate mechanisms involved in targeting S100A11 towards PEX14 and its regulation in the process of peroxisomal secretion.

To evaluate the biological significance of dimerized S100A11 in mesothelioma cells, we took a conventional approach to monitor cell invasion. By an in vitro cell-based assay, we newly found that dimeric S100A11, but not monomeric type, enhanced mesotherial cell invasion indirectly with the help of fibroblast cells (Fig. 7c-right). Considering the very weak invasive ability as a non-cancerous immortalization feature in Met-5A cells, we speculated that dimerized S100A11 could not positively work directly to the Met-5A cells in the case of invasion (Fig. 7c-left) even with RAGE-positive expression (data not shown). We have shown that dimerized S100A11 as well as S100A8/A9 heterodimer protein functions as a cytokine to induce further production of growth factors, cytokines, and chemokines when normal human keratinocytes were treated with these recombinant proteins [10, 13, 14, 36, 37]. That gave us an idea that various cytokines might also be induced from OUMS-24 fibroblasts by treatment with dimerized recombinant S100A11 that might cause an increase in the invasiveness of Met-5A cells. These novel findings are useful for understanding the extracellular role of dimerized S100A11 in the mesothelioma cell microenvironment linked to cancer progression.

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Compliance with Ethical Standards Immunohistochemical studies using human tissue specimens were approved by the Research Ethics Committee in Niigata University Medical and Dental Hospital, and only samples in Niigata University Graduate School of Medicine and Dental Sciences were used. Informed consent was obtained from each patient for use of these materials.

Conflict of Interests The authors declare that they have no conflicts of interest.

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