Interaction between S100A8/A9 and Annexin A6 Is Involved in the Calcium-induced Cell Surface Exposition of S100A8/A9\(^*\)

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The calcium binding S100A8/A9 complex (MRP8/14; calgranulin) is considered as an important proinflammatory mediator in acute and chronic inflammation and has recently gained attention as a molecular marker up-regulated in various human cancers. Here, we report that S100A8/A9 is expressed in breast cancer cell lines and is up-regulated by interleukin-\(1\)\(\beta\) and tumor necrosis factor-\(\alpha\) in SKBR3 and MCF-7 cells. We identified the phospholipid-binding protein annexin A6 as a potential S100A8/A9 binding protein by affinity chromatography. This finding was verified by Southwestern overlay experiments and by coimmunoprecipitation with the S100A8/A9-specific monoclonal antibody 27E10. Immunocytochemical experiments demonstrated that S100A8/A9 and annexin A6 colocalize in SKBR3 breast cancer cells predominantly in membranous structures. Upon calcium influx both S100A8/A9 and annexin A6 are exposed on the cell surface of SKBR3 cells. Subcellular fractionation studies suggested that after A23187 stimulation membrane association of S100A8/A9 is not enhanced. However, both S100A8/A9 and annexin A6 are exposed on the cell surface of SKBR3 cells upon calcium influx. Experiments with artificial liposomes indicated that S100A8/A9 is able to associate with membranes independently of both annexin A6 and independently of calcium. Finally, cell surface expression of S100A8/A9 could not be observed in A23187-treated A431 and HaCaT cells. Both cell lines are known to be devoid of annexin A6. Repression of annexin A6 expression by small interfering RNA in SKBR3 cells abolishes the cell surface exposition of S100A8/A9 upon calcium influx, suggesting that annexin A6 contributes to the calcium-dependent cell surface exposition of the membrane-associated S100A8/A9 complex.

S100A8/A9 (MRP8/14; calgranulin) was originally discovered as immunogenic protein expressed and specifically released from neutrophils. Subsequently, it has emerged as a proinflammatory marker protein in many acute and chronic inflammatory diseases. Additionally, increased S100A8 and S100A9 levels have also been detected in various human cancers as breast and prostate cancer (1, 2). Although a number of putative functions have been proposed for S100A8/A9, its biological role particularly in cancer cells remains to be defined. Its expression pattern in inflammation and in cancer suggests that S100A8/A9 play a prominent role in inflammation-associated cancer.

S100A8 and -A9 are constitutively expressed in myeloid cells. They are secreted by activated myeloid cells by a so far unidentified mechanism, and elevated sera levels have been found in patients suffering from several inflammatory disorders (3). In search for an extracellular function of the S100A8/A9 protein complex a number of interaction partners or receptors have been identified. Robinson et al. (2002) (4) have reported that extracellular secreted S100A8/A9 binds to heparan sulfate glycosaminoglycans present on endothelial surfaces. Kerkhoff et al. (5) identified the FAT/CD36 as a receptor facilitating the uptake of the arachidonic acid bound to S100A8/A9. Furthermore, S100A8 has been shown to interact with TLR4, amplifying phagocyte activation during sepsis (6). S100A8 and/or S100A9 reportedly increase Mac-1 affinity (7), mediate neutrophil adhesion to fibronectin (8), may induce neutrophil chemotaxis (9), and are able to mediate apoptosis (10, 11). The receptor for advanced glycation end products (RAGE) has been proposed to serve as cellular receptor of S100A8 and -A9, thereby mediating some of the activities described (12). However, direct evidence for a physical interaction of RAGE and S100A8/A9 has not been shown so far.

Intracellularly, the S100 proteins are considered as calcium sensors, changing their conformation in response to calcium influx and then mediating the calcium signal by binding to other intracellular proteins. In migrating monocytes the S100A8/A9 complex has been found to be associated with cytoskeletal tubulin and to modulate transendothelial migration (13). Recently, it has been found that S100A8/A9 promotes NADPH oxide activation by interaction with components of the phagocytic NADPH oxidase multienzyme complex (14–16).

Two reasons prompted us to search for additional intracellular molecular targets of S100A8/A9 in breast cancer cells. First, beside their expression in myeloid cells, S100A8 and S100A9 are expressed under cancerous conditions in glandular...
epithelial tissues, and they are overexpressed in poorly differ-
entiated adenocarcinoma (1, 2). Mammalian cancer cells, how-
ever, have neither the high migratory potential of monocytes
nor produce high amounts of reactive oxygen species as neu-
rophils, suggesting that the two S100 proteins may have a dif-
ferent intracellular function(s) in cancerous cells. Second,
although most of S100A8/A9 resides in the cytoplasm, part of
the proteins is associated with cellular membranes (17, 18). So
far nothing is known about interacting partners of the mem-
brane-associated S100A8/A9. As mentioned above, besides
myeloid cells, S100A8 and -A9 are expressed in cancerous cells
of secretory tissues as breast and prostate. Cells originating
from such glandular tissues are rich in membrane structures,
suggesting that membrane-associated molecular targets for the
S100A8/A9 proteins could be potentially found in these cells.
Therefore, we used breast cancer cells to search for further
molecular partners interacting with S100A8/A9.

Here, we report that the phospholipid-binding protein
annexin A6 is a potential molecular interacting partner of the
S100A8/A9 complex. The interaction with annexin A6 links
S100A8/A9 to membrane-related cellular events.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokines—Breast cancer SKBR 3, MCF-7,
and MDA-MB-468 cells as well as keratinocyte like A431 cells
and HaCaT cells were provided by the DSMZ (Deutsche
Sammlung für Mikroorganismen und Zellkultur). All cell lines
except MCF-7 were propagated in Dulbecco’s modified Eagle’s
medium according to standard procedures. MCF-7 cells were
propagated in RPMI1640 medium. IL-1β and tumor necrosis
factor-α were purchased from Tebu/Preprotech. A23187 was
obtained from Sigma.

Recombinant Proteins—Generation and purification of recombinant S100 proteins are described elsewhere (18). The
cDNA encoding annexin A6 was amplified from pC3AnxA6
(provided by V. Gerke) via PCR and subcloned into pQE32.
Expression of recombinant His-tagged annexin A6 in Esche-
richia coli carrying pQE32AnxA6 was induced with isopropyl
1-thio-β-d-galactopyranoside. The annexin A6 containing
E. coli lysate was used for Southwestern analysis. Purified recombinant annexin A6 was insoluble in physiological buffers
and was not used for further studies.

SDS-PAGE, Western Blot, and Southwestern Analysis—SDS-
PAGE and Western blots were performed according to stand-
ard procedures and developed using the ECL PlusTM Western
blotting detection reagents from Amersham Biosciences. Anti-
nexin antibodies were purchased from Santa Cruz Biotech-
nologies (annexin VI sc-1931; annexin II sc-30757).

For Southwestern overlay analysis, three blots were incu-
bated in Tris-buffered saline, 1% bovine serum albumin. Two
membranes were incubated with either S100A8/A9 or S100A9
protein (10 µg/ml in 30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2
mM CaCl2, 1 mM dithiothreitol) for 4 h at room temperature as

indicated. Membranes were washed (4 × 5 min) with Tris-
buffered saline in the presence of 0.2 mM CaCl2 and incubated
with primary antibody. Membranes were washed again (4 × 5
min) with Tris-buffered saline in the presence of 0.2 mM CaCl2
and incubated for 1 h with goat anti-(rabbit IgG)-horseradish
peroxidase conjugate. Finally, membranes were washed (4 × 5
min) with Tris-buffered saline in the presence of 0.2 mM CaCl2,
and immunoreactive bands were visualized by enhanced
chemiluminescence. A third blot was stained with the annexin
A6 antibody to verify the identity of annexin A6.

Quantitative Real-time Reverse Transcriptase-PCR—Total
RNA was isolated according the protocol of the manufactur-
er (Qiagen, Düsseldorf, Germany). For real-time RT-PCR, RNA
from every experiment was analyzed in duplicate. cDNA was
synthesized from 2.5 µg of total RNA using reverse tran-
scriptase (MBI Fermentas). Primers were designed using the
Primer Express software package (Applied Biosystems, Foster
City, CA) and obtained from MWG Biotech (Ebersberg,
Germany).

The procedures and conditions for real-time RT-PCR using
the QuantiTect SYBR Green PCR kit (Qiagen) were the same as
described elsewhere (19). Gene expression was normalized
with respect to the endogenous housekeeping control gene
glyceraldehyde 3-phosphate dehydrogenase.

Affinity Chromatography—Affinity purification was per-
fomed according to Koltzsch et al. (20). Briefly, an affinity
column was generated by immobilization of His-tagged S100A8/A9 protein on Ni-NTA-agarose. Five milligrams of purified,
His-tagged S100A8 and His-S100A9, respectively, were dissolved in buffer C (30 mM Tris, pH 7.2, 20 mM imidaz-
ole, pH 7.2, 300 mM NaCl, 2 mM MgCl2, 0.5 mM CaCl2, 10 mM β mercaptoethanol, Roche Applied Science protease inhibitor
mixture “Complete,” EDTA-free, 1.5 mM phenylmethylsulfonyl
fluoride) and added to 2 ml of Ni-NTA-agarose equilibrated in
the same buffer. The Ni-NTA-agarose/S100A protein slurrys
were then transferred to 10-ml polypropylene columns (Pierce)
and washed with 10 column volumes of buffer C to remove
unbound protein.

A SKBR3 lysate was prepared in lysis buffer (30 mM Tris, pH
7.2, 150 mM NaCl, 2 mM MgCl2, 1% Triton X-100, 1 mM dithi-
othreitol, Complete medium, EDTA-free, 1 mM EDTA, 1.5 mM
phenylmethylsulfonyl fluoride) and cleared by subsequent cen-
trifugation with 1,000 and 10,000 × g. The lysate was preincu-
bated with equilibrated Ni-NTA-agarose to eliminate unspe-
cifically binding proteins. Before loading and while gentle
stirring, CaCl2 was added carefully to the preincubated cell
lysate to a final concentration of 0.7 mM. The preincubated
SKBR3 lysate was then loaded onto the chromatography col-
umn. The column was washed with 10 column volumes of
buffer C and finally with 10 column volumes buffer C1 (same as
buffer C but with 0.7 mM EGTA instead of 0.7 mM CaCl2)
for 4 h at room temperature as

2 The abbreviations used are: IL, interleukin; RT, reverse transcriptase; SUV,
small unilamellar vesicles; Ni-NTA, nickel-nitriotriacetic acid; MALDI-TOF,
matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-
buffered saline; MES, 4-morpholineethanesulfonic acid.
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50% methanol, and washed in acetic acid/methanol/water (10/45/45, v/v/v) for 30 min and subsequently in water for 30 min. It was then shrunk in acetonitrile and dried. After trypsin digestion (Roche Applied Science) the peptide-containing supernatant was transferred to a clean Eppendorf tube, and peptides were extracted 3 times with 70 μl of acetonitrile/water/formic acid (50/45/5, v/v/v). The lyophilized extract was dissolved in 7 μl of water/acetonitrile (95/5, v/v) containing 0.1% trifluoroic acid (Merck) and purified using ZipTips (Millipore, Bedford, MA). Peptides were eluted with 8 μl of acetonitrile, 0.1% aqueous trifluoric acid (70/30 v/v). For matrix preparation, 10 μg of α-cyano-4-hydroxycinnamic acid (Sigma) were washed with acetone and dissolved in 1 ml of 49.5/49.5/1 (v/v/v) acetonitrile, ethanol, 0.1% aqueous trifluoric acid. 0.5 μl of this matrix preparation was spotted onto the target followed by the same amount of peptide sample, and both solutions were mixed directly on the target for MALDI peptide mapping on TofSpec 2E (Micromass Ltd., Manchester, UK). Digests were run in positive ion reflectron mode using a matrix suppression of 500. Masses were externally calibrated and internally corrected using the lock mass option of the instrument providing m/z values better than 50 ppm up to m/z 2500. Nanospray tandem mass spectrometry of tryptic peptides was performed using iontrap (Bruker Daltonics, Bremen, Germany) and quadrupole-TOF (Micromass). Data base searches were performed as described earlier (20) using available to the public via the internet.

Coimmunoprecipitation—The S100A8/A9 heterodimer-specific monoclonal antibody 27E10 was purified from hybridoma supernatants using protein G-coupled Sepharose as described by the manufacturer (Amersham Biosciences). The immunoprecipitation experiments were performed according to Kerkhoff et al. (21). Briefly, aliquots of the cell supernatants were subjected to preadsorption by incubation for 1 h after the addition of 100 μl/ml rabbit IgG (Calbiochem) followed by incubation for 1 h after the addition of 100 μl/ml protein G-Sepharose fast flow (Amersham Biosciences). After centrifugation for 10 min at 14,000 × g, 1 μg/ml nonspecific mouse IgG1 or 1 μg/ml monoclonal antibody 27E10 were added to the supernatants and incubated for 1 h. Protein G-Sepharose (30 μl/ml) was added, and samples were further incubated for 1 h. Sepharose was collected by centrifugation, and the supernatants were discarded. The pellets were resuspended in SDS loading buffer. Immunoprecipitation was performed in calcium containing buffer (0.5 mM CaCl₂).

Immunocytochemistry—Cells fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. After quenching in 50 mM NH₄Cl for 10 min cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated in PBS supplemented with 5% bovine serum albumin for 30 min. Cells were then incubated with a rabbit anti S100A9 and a goat anti-annexin A6 antibody (Santa-Cruz Biotechnology, catalog no. sc-1931) for 30 min and washed 3 times with PBS, 5% bovine serum albumin. Subsequently, primary antibodies against annexin A6 and S100A9 were stained with Cy5- and Cy2-conjugated secondary antibodies, respectively, or with Alexa594- and Alexa488-conjugated secondary antibodies (Molecular Probes), respectively. Finally, cells were washed 3 times with PBS, once with distilled H₂O, mounted in Mowiol, and analyzed using a confocal laser scanning microscope (Zeiss, Jena, Germany; LSM 510).

Subcellular Fractionation—SKBR3 cells were Dounce-homogenized in ice-cold MES buffer (25 mM MES, pH 6.5, 150 mM NaCl, complete protease inhibitors from Roche Applied Science). Subsequently total cell lysates were differentially centrifuged at 1,000, 10,000, and 100,000 × g at 4 °C. Pellets and the final supernatant were harvested after the centrifugation steps and analyzed via Western blotting.

Preparation of Endosomes—After cell lysis the homogenate was centrifuged, and the postnuclear supernatant served as a crude membrane extract. Early and late endosomes were prepared as described elsewhere (22). Briefly, post-nuclear supernatant was brought to a final 40.2% sucrose (w/v) concentration by adding 80% sucrose (3 mM imidazole, pH 7.4) and 35, 25, 16, and 10% sucrose. The gradient was centrifuged for 90 min at 35,000 rpm, 4 °C in a swing out Beckman SW40 rotor. After centrifugation, 1-ml fractions were collected from top to bottom, and the proteins were precipitated with trichloroacetic acid.

Lipid Raft Fractionation Using a Sucrose Gradient—10⁷ SKBR3 cells were incubated in MES buffer (25 mM MES, pH 6.5, 150 mM NaCl, protease inhibitors) containing 1% Triton X-100 at 4 °C for 20 min and then homogenized using a loose-fitting Dounce homogenizer (10 strokes). The homogenates (corresponding to 3 × 10⁷ cells) were then adjusted to 40% sucrose by the addition of an equal volume of an 80% sucrose solution prepared in the above buffer but lacking Triton X-100, placed in the bottom of ultracentrifuge tubes, and then overlaid with a discontinuous sucrose gradient of 4 ml of 30% (w/v) sucrose and 4 ml of 5% (w/v) sucrose, both prepared in MES buffer lacking Triton X-100. The samples were centrifuged at 35,000 rpm (200,000 × g) in an SW41 rotor (Beckman Instruments) for 16–20 h, fractionated into 1-ml fractions sequentially from the top of the gradient, and concentrated by precipitation with trichloroacetic acid.

The pellets were dissolved in denaturing Laemmlı loading buffer. Equal quantities of each fraction were loaded onto an SDS-PAGE. Western blots were performed according to standard procedures and developed using the ECL Plus™ Western blotting detection reagents from Amersham Biosciences.

Artificial Liposomes—Lipid vesicles were prepared by dissolving 30 mg of lipids of the liposome kit from Sigma (L4395) in chloroform and subsequent evaporation of the solvent under N₂. MES buffer (25 mM MES, pH 6.5, 150 mM NaCl, complete protease inhibitors from Roche Applied Science) were added to the dried lipids. Small unilamellar vesicles (SUVs) were prepared by sonification of the hydrated lipids (suspended in buffer) with a Branson sonicator for at least 8 min on ice or until the suspension became opaque.

Liposomes were subsequently incubated with a cell lysates or/and purified S100A8/A9 complex for 15 min at room temperature and were then adjusted to 40% sucrose by the addition of an equal volume of an 80% sucrose solution, placed in the bottom of ultracentrifuge tubes, and then overlaid with a discontinuous sucrose gradient (1 ml of 30% (w/v) sucrose, 1 ml of 20% sucrose, 1 ml of 10% sucrose, and 1 ml of 5% (w/v) sucrose...
prevented in MES buffer). After centrifugation at 140,000 × g for 16 h, the samples were fractionated into 1-ml fractions sequentially from the top of the gradient and concentrated by precipitation with trichloroacetic acid. The fractions were loaded onto a SDS-PAGE gel, and S100 proteins were detected by Western blotting.

Small Interfering RNA Transfection—SKBR3 cells were transfected with annexinA6 small interfering RNA (Santa Cruz; sc-29688) using Oligofectamine (Invitrogen) or Hyperfect (Qiagen) transfection reagents according to the manufacturer’s protocols. 48 h after transfection, cells were harvested. Part of the cells were lysed, an aliquot of the sample was loaded on a SDS-PAGE, and the remaining cells were used for further FACS analysis.

Flow Cytometry—For flow cytometry, cells (5 × 10⁵) were incubated in 150 μl of PBS, 1% fetal calf serum with the indicated antibodies (1 μg/ml) for 60 min at 4 °C. Cells were washed twice in 1 ml of PBS, 1% fetal calf serum. Then a 1 μg/ml concentration of secondary fluorescein isothiocyanate-conjugated antibodies was added to the cells and incubated for 30 min on ice. Finally, cells were washed twice, resuspended in 500 μl of PBS, 1% fetal calf serum, and analyzed using a FACSCalibur flow cytometer equipped with CellQuestPro software (BD Pharmingen). IgG1 was used as isotype control for the monoclonal antibody 27E10.

RESULTS

S100A8/A9 Are Expressed in Breast Cancer Cell Lines—First, we investigated the expression of S100A8 and S100A9 in various breast cancer cell lines by Western blots and quantitative RT-PCR. Using Western blot analysis in MCF-7 cells, both proteins were hardly detected; in SKBR3 cells a moderate level of expression was found, and in MDA-MB-468 both proteins were highly expressed in comparison to the other two cell lines. These results were verified by quantitative RT-PCR (Fig. 1B). However, the expression of both S100 proteins in breast cancer cells is at least 10-fold lower compared with that of granulocytes (Fig. 1A). Furthermore, we found that IL-1β (Figs. 1C and 5) and tumor necrosis factor-α (data not shown) up-regulate the expression of both S100 genes in MCF-7 as well as in SKBR3 cells but not in MDA-MB-468 cells.

Identification of Annexin A6 as a Potential S100A8/A9 Interacting Protein—For further experiments we decided to work with the moderately S100-expressing SKBR3 cells. Cleared lysates from SKBR3 cells were applied to S100A8/A9 affinity chromatography followed by SDS-PAGE. As shown in Fig. 2, a protein of ~70 kDa in size was eluted from the S100A8/A9 affinity column using a calcium-free elution buffer. The experiment was repeated three times, and this protein species was eluted in all of the experiments. Other proteins that have also been eluted from the affinity column could only be isolated in one or two of the set of three experiments conducted. The 70-kDa protein band was excised from the gel, digested with}

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**FIGURE 1. Expression of S100A8 and -A9 in breast cancer cells.** A, cell lysates of different breast cell lines (SKBR3, MCF-7, MDA-MB-468) and of the keratinocyte cell line HaCaT were subjected to SDS-PAGE as indicated. The amount of protein loaded on the PAGE corresponds to 5 × 10⁵ cells. S100A9 and annexin A6 were detected via Western blotting using the corresponding antibodies. The amount of proteins of the granulocyte lysate corresponds to 5 × 10⁶ cells. B, total RNA was isolated using Qiagen kit, and the amount of S100A8 and -A9 mRNA was determined by quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control. The values are presented as 15-ΔΔCt values and are the mean values of three independent experiments. Mean ± S.D. is shown. C, Western blot indicating the up-regulation of S100A9 upon IL-1β (10 ng/ml) stimulation of SKBR3 cells on the protein level. Expression of annexin A6 is not affected by IL-1β.
trypsin, and subjected to MALDI-mass spectrometry for peptide mass fingerprint. The data obtained were analyzed using Swissprot and NCBI databases. The identified protein was annexin A6.

To confirm these data we performed co-immunoprecipitation experiments using the S100A8/A9 hetero-oligomer-specific monoclonal 27E10 antibody. A SKBR3 and MDA-MB-468 cell lysate was incubated with 27E10, and the immunoprecipitate was subjected to SDS-PAGE followed by Western blotting. A 70-kDa protein band was recognized by an anti annexin A6 antibody (Fig. 2B).

Annexin A6 is also present in neutrophilic granulocytes. However, because of the high expression of S100A8/A9 in granulocytes there is a massive molar excess of S100A8/A9 compared with the annexin A6 (Fig. 1). We asked whether S100A8/A9 is bound to annexin A6 in granulocytes. Using a granulocyte lysate, the 27E10 antibody precipitates a 55-kDa protein species that was recognized by anti-annexin A6 antibody (data not shown). As shown earlier (24), mild proteolytic digestion of annexin A6 in granulocyte lysate results in a 55-kDa band that is recognized by the annexin A6 antibody. Various protease inhibitors tested did not prevent the degradation in the granulocyte lysate. Thus, the 55-kDa protein might be regarded as a degradation product of the granulocytic annexin A6.

We then performed overlay/Southwestern experiments to further confirm the interaction of S100A8/A9 with annexin A6. As shown in Fig. 2C, the S100A8/A9 complex binds to the blotted recombinant annexin A6. Neither S100A8 (data not shown) nor S100A9 (Fig. 2C) alone was bound to annexin A6 under the conditions used, suggesting that solely the hetero-oligomeric S100A8/A9 complex specifically interacts with annexin A6. Taken together we conclude from these data that annexin A6 is an interaction partner of the S100A8/A9 complex.

S100A8/A9 and Annexin A6 Colocalize Particularly in Membranes of the SKBR3 Cells—Next, double fluorescence immunocytochemical analysis was used to examine the localization of S100A9 and annexin A6 in SKBR3 cells (Fig. 3). S100A9 expression is shown as green fluorescence in cytoplasm and membranes (Fig. 3, left panels). Annexin A6 expression is...
shown as red or blue fluorescence (Fig. 3, middle panels). The overlapping images (Fig. 3, right panels) revealed evidence for the colocalization of S100A9 and annexin A6 particularly in the cell membranes.

Annexins are well known to be associated with membrane-related events as endocytosis and exocytosis. Therefore, we prepared endosomes from SKBR3 cells and analyzed them. As shown in Fig. 4A, S100A8/A9 and annexin A6 are present in both early and late endosomal fractions, whereas annexin A2 is only present in early endosomes.

Then, lipid rafts were prepared from SKBR3 cells and analyzed for annexin A6 and S100A9. Western blotting revealed that annexin A6 is present in lipid rafts, whereas the S100 proteins are present in lipid rafts and other non-lipid raft fractions. However, in contrast to annexin A6, the majority of S100A8/A9 was found in the cytosolic high sucrose fraction.

We further analyzed the presence of annexin A6 and S100A8/A9 in distinct subcellular compartments by differential centrifugation (Fig. 4B). As expected, S100A8/A9 was predominantly present in the cytosolic fraction. Annexin A6 was detected in the nuclear (1000 \( \times \) g), organelle (10,000 \( \times \) g), membrane (100,000 \( \times \) g), and cytosolic fraction. Interestingly, S100A9 and A6 were detected in all cellular fractions after their up-regulation by IL-1β (Fig. 5A).

To investigate whether the subcellular distribution of S100A8/A9 is dependent on the presence of annexin A6, we performed differential centrifugation with cell lysates of A431 cells, an epidermal cell line that is supposed to be devoid of annexin A6 (25). In these cells the subcellular distribution of S100A8/A9 was identical to that of annexin A6-expressing SKBR3 cells (Fig. 5B). However, in contrast to other studies, we observed that A431 cells expressed minute amounts of annexin A6 that appeared to be exclusively present in the cytosolic fraction. Taken together, both immunocytochemical and biochemical data suggest that specifically membrane-associated S100A8/A9 may interact with annexin A6.

**Lipid Association of S100A8/A9 Is Independent of Annexin A6**—It has been reported that S100A8/A9 translocates to cell membranes after elevated intracellular calcium levels. Thus, we speculated that this translocation may be guided by annexin A6.
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S100A8/A9 Is Exposed to the Cell Surface upon Calcium Influx in SKBR3 Cells but Not in Cells Devoid of Annexin A6—In monocytes, S100A8/A9 is exposed to the cell surface after A23187-induced calcium ion influx. Therefore, we stimulated breast cancer SKBR3 cells with calcium (500 μM) and A23187 followed by staining the live cells with S100A8/A9 hetero-oligomer specific monoclonal antibody 27E10. Whereas no cell surface staining was detected in non-stimulated SKBR3 cells, A23187-treated cells expose the S100A8/A9 complex on their surface (Fig. 7). To investigate whether annexin A6 is also exposed to the cell surface, we used anti-annexin A6 antibodies for FACS analysis. Indeed, similar to S100A8/A9, annexin A6 was also exposed on the cell surfaces upon A23187-induced calcium ion influx (Fig. 7). Annexin A6 is not present on the cell surface in untreated SKBR3 cells. We further analyzed cell surface expression of S100A8/A9 in A431 and HaCaT cells, both cell lines expressing S100A8 and -A9 but devoid of annexin A6 (Fig. 7). Here, calcium/A23187 induction of the cells did not result in exposing S100A8/A9 on the cell surface. Transfection of A431 cells with an annexin A6-expressing plasmid failed to facilitate the calcium induced S100A8/A9 exposition to the A431 cell surface (data not shown). However, repression of the annexin A6 expression by small interfering RNA transfection abrogates the calcium-induced exposition of S100A8/A9 in SKBR3 cells, suggesting that annexin A6 is involved in the cell surface exposition of S100A8/A9 at least in these cells.

A23187-induced exposition of S100A8/A9 to the cell surface of SKBR3 cells was not accompanied with an increase in membrane-associated S100A8/A9 as shown in cell fractionation experiments (Fig. 5A), suggesting that a translocation of the S100 complex from cytosol to the cell membrane has not occurred. Instead the membrane-associated S100A8/A9 appeared to translocate from the inner to the outer membrane leaflet.

DISCUSSION

By far the largest group of calcium sensors is the EF-hand calcium-binding protein family, of which more than 600 have been identified from the human genome. The S100 proteins are small acidic proteins (10–12 kDa) that functionally belong to the group of damage-associated molecular pattern proteins (26). Here, we demonstrate that S100A8 and S100A9 are expressed in breast cancer cell lines. This finding confirms earlier data of histological breast cancer specimens (27–29). The level of expression varies between the different breast cancer cell lines probably due to the different proportions of S100A8/A9-expressing cells within the cell lines (see the supplemental data). Unfortunately, we do not know so far which cellular phenotype relies upon this differential expression level, but breast cancer cells may be a good model to study. We further show that S100A9 expression was up-regulated by IL-1β. Recently, Li et al. (23) have reported that oncostatin induces S100A9 via a STAT3-dependent pathway in MCF-7 cells and that at least in MCF-7 cells oncostatin M-mediated growth inhibition is ablated by the repression of S100A9 expression. Stunningly, in contrast to oncostatin M IL-1β is regarded as a cytokine that promotes breast cancer proliferation (30).

Annexins are another class of Ca\(^{2+}\)-regulated proteins. They are characterized by the unique architecture of their Ca\(^{2+}\)-binding sites, which enables them to peripherally dock onto negatively charged membrane surfaces in their Ca\(^{2+}\)-bound conformation. This property links annexins to many membrane-related events, such as the regulated organization of membrane domains and/or membrane-cytoskeleton linkages, certain exocytic and endocytic transport steps, and the regulation of ion fluxes across membranes (31).

Our observation that members of the S100 protein family physically interact with a member of the annexin protein family does not stand alone. Various members of both families seem to have a certain affinity to each other. Interactions between S100A1 and the annexins A5 and A6, between S100A6 and the annexins A11, A2, A5, and A6, between S100A12 and annexin A5, between S100B and annexin A6, as well as between S100A11 and the annexins A1 and A6 have been reported (32–33). Cell surface expression is shown with the monoclonal antibody 27E10, which specifically binds to the heteromeric S100A8/A9 complex. Because our results suggest that annexin A6 and S100A8/A9 interact, they may form a heterotetrameric complex similar to the annexin A2-S100A10 heterotetramer.

Biochemical analysis indicates that a fraction of the cellular S100A8/A9 protein was bound to annexin A6. We observed that only a minor amount of S100A8/A9 was present in membrane-containing fractions, whereas annexin A6 was predominantly present in membrane-containing fractions. Co-localization and biochemical experiments suggest that interaction between both proteins may take place at membranes. This is strengthened by the correlation that both S100A8/A9 and annexin A6 were exposed on the cell surface upon calcium stimulation in SKBR3 cells but not in A431 or HaCaT cells, which are both devoid of annexin A6. Interestingly, the minute amount of annexin A6 that we detected after biochemical fractionation of A431 cells (Fig. 5B) was located in the cytosol and
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Not in membrane-containing fractions. The observed expression of minute amounts of annexin A6 may rely on clonal variations of the A431 cell line.

The association of S100A8/A9 with liposomes was independent of calcium ions (Fig. 7). This is in agreement with our observation that calcium influx did not increase the amount of S100A8/A9 in the cell membrane fractions (Fig. 6, top panel). Earlier reports (17, 34, 35) demonstrated the calcium-dependent translocation of S100A8/A9 in monocytes, neutrophils, and keratinocytes, but the authors also concede that part of the S100 complex is associated with membranes independent of calcium and of the S100A8/A9 phosphorylation status (17). Concerning the latter, it has been reported that p38 mitogen-activated protein kinase mediates phosphorylation of S100A8 and S100A9, which in turn favors the membrane association of S100A8/A9 (36, 37). Our data (Figs. 5A and 7) let us speculate that in SKBR3 cells, calcium influx induced a translocation of the membrane-associated S100 complex from the inner cell membrane leaflet to the outer one. In SKBR3 cells we could not observe that calcium influx induced a translocation from the cytosol to the membrane. It remains to be elucidated how membrane-associated S100 complex differs molecularly from the cytosolic S100 complex and what causes part of the complex to associate with membranes.

Annexin A6 is the largest member of the vertebrate family of ubiquitous calcium- and membrane-binding proteins with not yet clearly defined physiological functions. It occurs in two splice variants which may have different cellular functions (38). Because annexin A6 is down-regulated in lactating breast, it has been earlier considered as a member of the annexin family which inhibits secretion. Recently, however, annexin A6 has been associated with the regulation of secretory events in plasma cells (39) and pancreatic acinar cells (40). Thus, we are tempted to speculate that the association of S100A8/A9 with annexin A6 may play a role in the secretion of the S100A8/A9 proteins themselves. However, at least monocytes release S100A8/A9 via a non-classical, tubulin-dependent pathway (41), which excludes a role of annexin A6 in S100A8/A9 secretion. Annexin A6 regulates the plasma membrane localization of the GTPase-activating protein p120GAP, one of the most important inactivators of Ras (42). It could also be speculated that annexin A6 may guide the S100 complex to membranes. However, the fact that S100A8/A9 associates to liposomes in the absence of annexin A6 clearly contradicts this hypothesis.

In summary, we demonstrated that in breast cancer cells expression of S100A8 and -A9 is up-regulated by IL-1β. We present evidence that annexin A6 interacts with S100A8/A9 and that this interaction takes place in membranous structures. Finally, the presence of annexin A6 in the cell membrane might be necessary but not sufficient for the calcium-induced cell surface exposition of S100A8/A9.

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