S100A13 and S100A6 exhibit distinct translocation pathways in endothelial cells

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Summary

S100 proteins have attracted great interest in recent years because of their cell- and tissue-specific expression and association with various human pathologies. Most S100 proteins are small acidic proteins with calcium-binding domains – the EF hands. It is thought that this group of proteins carry out their cellular functions by interacting with specific target proteins, an interaction that is mainly dependent on exposure of hydrophobic patches, which result from calcium binding. S100A13, one of the most recently identified members of the S100 family, is expressed in various tissues. Interestingly, hydrophobic exposure was not observed upon calcium binding to S100A13 even though the dimeric form displays two high- and two low- affinity sites for calcium. Here, we followed the translocation of S100A13 in response to an increase in intracellular calcium levels, as protein translocation has been implicated in assembly of signaling complexes and signaling cascades, and several other S100 proteins are involved in such events. Translocation of S100A13 was observed in endothelial cells in response to angiotensin II, and the process was dependent on the classic Golgi-ER pathway. By contrast, S100A6 translocation was found to be distinct and dependent on actin-stress fibers. These experiments suggest that different S100 proteins utilize distinct translocation pathways, which might lead them to certain subcellular compartments in order to perform their physiological tasks in the same cellular environment.

Key words: S100A13, S100A6, Protein translocation, Calcium, Angiotensin II, ER-Golgi, Actin filament

Introduction

S100 proteins have attracted increased attention in recent years owing to their cell- and tissue-specific expression and their involvement in several human diseases such as cystic fibrosis, rheumatoid arthritis, acute inflammatory lesions and acute myocardial ischemia (Schafer and Heizmann, 1996; Heizmann and Cox, 1998; Donato, 2001; Heizmann et al., 2002). Twenty members have been identified so far, and altogether S100 proteins represent the largest subgroup in the EF-hand calcium-binding protein family. Another important aspect of the S100 family is that most of their genes are located in a gene cluster on human chromosome 1q21, which is structurally conserved in evolution (Ridinger et al., 1998). Within this chromosomal region, several gene rearrangements during tumor development have been described (Schafer et al., 1995). Indeed, some S100 proteins are involved in metastasis and cancer development (Nagy et al., 2001; Ninomiya et al., 2001).

Among the 20 members reported in the S100 calcium-binding protein family, S100A13 is the only member that exhibits a ubiquitous expression in a broad range of tissues (Ridinger et al., 2000). S100A13 is one of the latest member of the S100 family and was originally identified by screening the EST database (Wicki et al., 1996). It is similar to most other S100 proteins – homodimeric S100A13 possesses two high- and two low-affinity sites for calcium (Ridinger et al., 2000). However, it does not show a calcium-dependent exposure of the hydrophobic surface, which is essential for the interaction of S100 proteins with their target proteins. S100A13 is associated with the secretion of brain-derived fibroblast growth factor-1 (FGF-1) and p40-synaptotagmin in response to heat shock (Jackson et al., 1992; Landriscina et al., 2001). In addition, the anti-allergic and anti-inflammatory drug amlexanox which binds to S100A12 and S100A13, represses the secretion of FGF-1, S100A13 and p40-synaptotagmin 1 multi-aggregates (Carreira et al., 1998; Shishibori et al., 1999). Therefore, S100A13 was postulated to play an important role in the release of FGF-1, as the growth factor lacks a classical signal sequence for secretion.

Calcium plays an important role as a second messenger in various signaling pathways. A sudden increase in the intracellular calcium level alters physiological cellular functions such as cell cycle progression, differentiation and muscle contraction (Schäfer and Heizmann, 1996). One of the most commonly used factors to increase the intracellular calcium levels is angiotensin II. Angiotensin II is a potent smooth muscle constrictor, which increases cytosolic calcium levels by interacting with angiotensin receptors (Schiffrin, 1998; Watts et al., 1998; Purdy and Arendshorst, 1999; Takeuchi, 1999; Rossier and Capponi, 2000). Through a GTP-binding protein, angiotensin II activates phospholipase C, which generates inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and releases calcium from intracellular stores (Marks, 1992; Capponi, 1996).

Translocation involves protein relocation to different

intracellular compartments, and it has been implicated in association with signal transduction cascades. Protein translocation in response to intracellular calcium change has long been observed with protein kinase C (PKC) (Damron et al., 1998; Oancea and Meyer, 1998; Marsigliante et al., 2001) and recently with some S100 proteins (Goebeler et al., 1995; Guignard et al., 1996; van den Bos et al., 1996; Brett et al., 2001). S100A8 and S100A9 show calcium-dependent translocation in myelomonocytic cells and in epithelial cells (Goebeler et al., 1995; van den Bos et al., 1996). S100A2, S100A4 and S100A6 translocate in response to ionophore A23187, cyclic ADP-ribose and thapsigargin in some tumor cells (Mueller et al., 1999), and translocation of S100A11 and S100B has been described in human glioblastoma cell lines (Davey et al., 2000; Davey et al., 2001). Since S100 proteins lack the classical signal sequence for secretion, it is of great interest to investigate the translocation pathways of this group of proteins. Novel pathways such as tubulin-filament-dependent translocation have been reported in secretion and translocation of S100A8, S100A9 and S100A11 (Rammes et al., 1997; Davey et al., 2000).

In an attempt to study the cellular function of S100A13, we investigated translocation in response to intracellular calcium rise by stimulating ECV (a human umbilical vein-derived endothelial cell line) with angiotensin II and immunohistochemical staining. The translocation pathways of S100A13 and S100A6 were studied by using various inhibitors to block the distinct pathways. We report here a classic translocation pathway for S100A13 in contrast to the novel pathways utilized by other members of the S100 family. By contrast, translocation of S100A6 in response to angiotensin-II-stimulated calcium rise seems to be an actin-filament-dependent process in endothelial cells, suggesting that two S100 proteins utilize different translocation pathways in the same cellular environment.

Materials and Methods

Recombinant human S100A13 protein purification

For a high yield of inducible protein expression and ease of protein purification, human S100A13 cDNA was cloned into *Bam*HI and *Hin*dIII sites of the prokaryotic expression vector, pPROEXtm HTb (Invitrogen, Basel Switzerland). Cell lysis and protein purification by a Ni-NTA (nitrilo-tri-acetic acid) column (Invitrogen, Basel Switzerland) were performed according to the manufacturer's protocol. The S100A13 fusion protein was then cleaved by TEV (Tobacco Etch Virus) protease (Invitrogen, Basel Switzerland) at 30°C for 4 to 6 hours. Recombinant S100A13 was further purified by gel filtration to remove contaminants using Sephacryl S200 (Amersham Pharmacia Biotech, Uppsala, Sweden) in buffer containing 20 mM Tris, 150 mM KCl and 0.1 mM EDTA (ethylene diamine tetraacetate) at pH 7.5. The fractions from gel filtration were collected, and the presence of purified S100A13 was identified and confirmed by western blotting and mass spectrometry.

Calcium and magnesium binding

Calcium binding was measured at 25°C by the flow dialysis method in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM KCl). Protein concentrations were 20-30 μM. Treatment of the raw data and evaluation of the intrinsic metal-binding constants was performed as described previously (Cox, 1996). The data were analyzed with the equation of Adair for four binding sites:

$$\nu=\{K_1[Ca^{2+}] + 2K_1K_2[Ca^{2+}]^2 + 3K_1K_2K_3[Ca^{2+}]^3 + 4K_1K_2K_3K_4[Ca^{2+}]^4\}/\{1 + K_1[Ca^{2+}] + K_1K_2[Ca^{2+}]^2 + K_1K_2K_3[Ca^{2+}]^3 + K_1K_2K_3K_4[Ca^{2+}]^4\}$$

where K_1, K_2 etc. are the stoichiometric association constants for the binding of the first and second calcium to the protein. The intrinsic association constants, used in this study to present the data, are linked to stoichiometric ones by the statistical factors 4, 3/2, 2/3 and 1/4 (Cox, 1996). The antagonism between calcium and zinc was tested with the competition equation for each site:

$$K'_{Ca}/K'_{Ca.app}=1 + K'_{Zn.comp}\,[Mg^{2+}]$$

where K'_{Ca} and $K'_{Ca.app}$ are the intrinsic Ca^{2+}-binding constants for a given site in the absence and presence of Zn^{2+} and $K'_{Zn.comp}$ is the calculated Zn^{2+}-binding constant for this site.

SDS PAGE and western blotting

SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and western blotting were used throughout the purification of recombinant human S100A13 to monitor the presence of the protein in certain fractions. Either a pre-cast 4-20% Tris-glycine gradient (BioWhittaker Molecular Applications, Rockland, USA) or a 14% Tris SDS PAGE gel was used for both Coomassie G-250 (Pierco, Illinois, USA) staining and western blotting onto 0.45 μm nitrocellulose membrane (BIO-RAD, Hercules, USA). ECV cell extract was prepared by resuspending 5×10^6 cells in lysis buffer containing 50 mM Tris, 250 mM NaCl, 2 mM EDTA and 1% nonidet P40 at pH 8.0. Protein denaturation was carried out in loading buffer (16% SDS, 48% glycerin, 0.2 M Tris, pH 6.8, 2% β-mercaptoethanol and 0.01% bromophenol blue) at 95°C for 10 minutes. Primary antibodies against the human recombinant proteins (rabbit anti-S100A1, anti-S100A3, anti-S100A4, anti-S100A5, anti-S100A6, anti-S100A13 and anti-S100B) used for western blotting were diluted 1:1,000; and horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma, Steinheim, Germany) was used at a dilution of 1:10,000. The specificity and the crossreactivity of the antibodies were described previously (Ilg et al., 1996; Ridinger et al., 2000; Schafer et al., 2000). Visualization of the protein was achieved by ECL (enhanced chemiluminescence) (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cell culture

ECV, a kind gift from T. Maciag (ECV-304), was maintained in 10% FCS-M199 medium with antibiotics (Invitrogen, Basel, Switzerland) at 37°C and 5% CO_2. For immunofluorescence stainings, 5×10^4 cells were plated on the fibronectin-coated coverslips (2 μg/ml) (Sigma, Steinheim, Germany) one day prior to the experiment in a 24-well plate. Addition of various drugs was performed in M199 medium without FCS (fetal calf serum), the working concentrations and incubation times used are as follows: 45 minutes with 5 μg/ml brefeldin A (Calbiochem), 120 minutes with 1 μM amlexanox (Takeda Chemical Industries Ltd., Japan), 30 minutes with 1 μM demecolcine (Sigma, Steinheim, Germany), 180 minutes with 1 mM bafilomycin A1 (Sigma, Steinheim, Germany). Cells were then stimulated by 0.1 μM angiotensin II (Sigma, Steinheim, Germany) in stimulation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM glucose, 1 mM HEPES, and 1 mM $CaCl_2$ for 10 minutes before fixation.

Immunofluorescence staining

Cells were fixed after angiotensin II stimulation with 3.7% formaldehyde in M199 medium for 15 minutes at 37°C. Permeabilization was carried out using cold methanol at room temperature for 10 minutes, and cells were washed with 5% horse

serum M199. The primary antibodies [rabbit anti-S100 antibodies and mouse anti-tubulin antibody (Sigma, Steinheim, Germany)] were diluted 1:200 and incubated with the cells at 37°C for 1 hour. Mouse monoclonal anti β-COP antibody (Sigma, Steinheim, Germany) was used at a dilution of 1:80. Various S100 antibodies were pre-absorbed by mixing with 10 μg antigen overnight at 4°C followed by centrifugation at 16,060 ***g***. The supernatant was then used instead of the primary antibody as a negative control. Cells were washed twice with 5% horse serum M199 medium and incubated with goat Cy3-conjugated anti-rabbit IgG or goat Cy3-conjugated anti-mouse IgG secondary antibody (1:200) (Jackson Immuno-Research Laboratories, Inc.) for 1 hour at 37°C. For double staining, a goat anti-human S100A6 antibody and a rabbit anti-human S100A13, antibody were used. Phalloidin-TRITC (Sigma, Steinheim, Germany) was used to locate actin stress fibers in the cells, and the standard staining protocol provided by the manufacturer was followed using labeled phalloidin at 0.5 μM and incubation for 1 hour. To visualize the protein distribution, a Cy3-conjugated anti-goat IgG secondary antibody (1:200) and a Cy2-conjugated anti-rabbit IgG secondary antibody (1:200) were used in the double staining experiment. After incubation with the secondary antibody, the cells were again washed twice in 5% horse serum M199 medium followed by a PBS (pH 9.0) (phosphate-buffered saline) wash. Slide mounting was performed using Mowiol as mounting medium (Hoechst, Frankfurt, Germany), and the slides were stored at 4°C in the dark.

Microscopy and confocal laser-scanning microscopy

Immunostained cells were analyzed by a Zeiss Axioskop microscope using a 40× oil objective lens. Images were taken by an AxioCam and processed in AxioVision 2.05 and Adobe Photoshop 5.5. For confocal microscopy, a Zeiss Axioplan fluorescence microscope equipped with a confocal scanning unit MRC-600 (Biorad) was used. The slides were visualized using a 40× oil objective lens and an argon krypton laser with an excitation wavelength of 568 nm for Cy3 labeling. The images were enlarged by 2.5× and processed using Adobe Photoshop.

Results

Purification of human recombinant S100A13

In order to study the metal-binding properties of S100A13 and the interaction with the anti-allergic drug amlexanox, recombinant S100A13 was purified. A strong induction of protein expression was observed upon addition of IPTG (isopropyl-1-thio-b-D-galactopyranoside) (Fig. 1A, lane 2) compared with the cell lysate in lane 1 where no IPTG was added. The fusion protein was purified over a Ni-NTA column (Fig. 1A, lane 3), digested with TEV protease to remove the histidine repeats (Fig. 1B, lane 2) and further purified by gel filtration (Fig. 1B, lane 3). The molecular mass of the final product determined by mass spectrometry amounts to 11,743 kDa, which corresponds to the expected mass calculated according to the protein sequence with deletion of the first methionine and five extra amino acids (GAMGS) at the N-terminus. This verifies the correct synthesis of the protein and complete removal of the histidine tag. The same calcium-binding profile was observed on both newly purified recombinant S100A13 and the one purified previously by a different method (Ridinger et al., 2000), which indicates that these five amino acids do not influence the binding properties of S100A13.

Metal-binding studies of S100A13

Calcium binding to S100A13 was monitored by flow dialysis

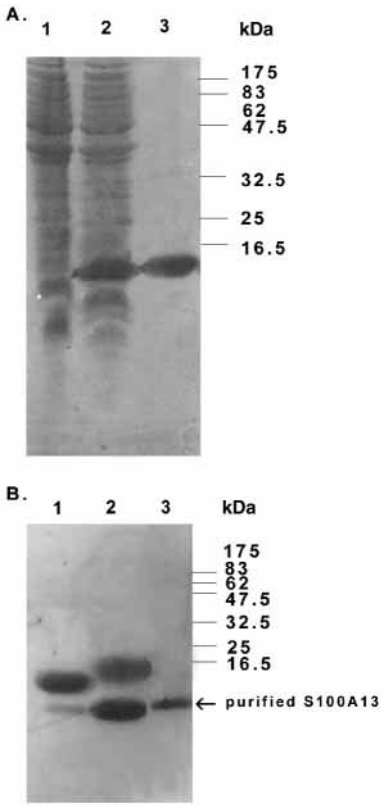

Fig. 1. Purification of recombinant S100A13. **(**A) Recombinant S100A13 was purified using the pPROEX HTb expression system and Ni-NTA affinity chromatography. 20 μl of protein extract from transformed DH5α cells was loaded before (lane 1) and 3 hours after (lane 2) IPTG induction. Lane 3 shows the purified fusion protein S100A13 from the Ni-NTA column. Protein extract was loaded on a 14% SDS Tricine-PAGE under reducing condition, followed by Coomassie G-250 staining. (B) Purified S100A13 from the Ni-NTA column is shown in lane 1. The fusion protein was then digested by TEV protease to remove the histidine tag as shown in lane 2, and S100A13 purified by gel filtration is shown in lane 3.

in the absence and presence of 100 μM zinc (Fig. 2, Table 1). In the absence of zinc, the isotherm strongly resembles the one reported previously (Ridinger et al., 2000), with two high- and two low-affinity sites ($[Ca^{2+}]_{0.5}$ of 10 μM and 500 μM, respectively) per dimer and positive cooperativity within each group of sites (n_H of 1.4 to 1.5). In the presence of zinc, the calcium-binding parameters of the high-affinity set were not changed, but a global 1.8-fold decrease in $[Ca^{2+}]_{0.5}$ was observed for the low-affinity sites. Close inspection reveals that zinc decreased merely the value of K'_4 and thus disturbed the positive cooperativity in the set of low calcium-affinity sites. With the competition equation, $[Zn^{2+}]_{0.5}$ of 150 μM was calculated for this pair of sites, although the zinc-calcium antagonism is a priori not caused by direct competition (Heizmann and Cox, 1998). A direct equilibrium gel filtration experiment at room temperature in buffer A containing 50 μM free zinc yielded 0.6 protein-bound zinc per dimer, thus confirming the distinct but weak interaction of zince with this protein. The binding of zinc induces only small conformational changes in the environment of Trp77, as shown by fluorescence and difference spectrophotometry (Ridinger et al., 2000).

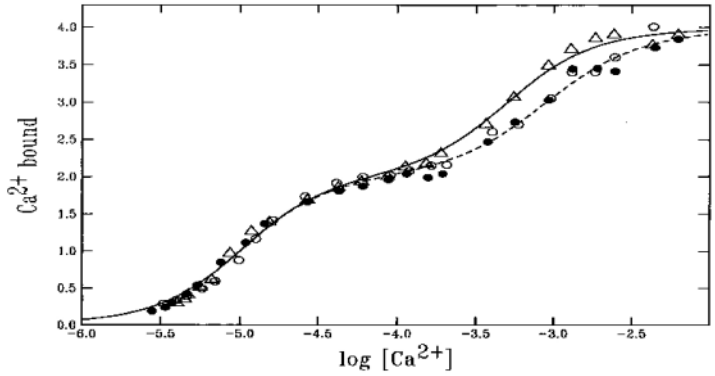

Fig. 2. Calcium binding to S100A13 and the effect of zinc. Flow dialysis at 25°C in 50 mM Tris HCl, pH 7.5, 150 mM KCl, with (circles; two different experiments) or without 100 μM zinc (triangles). Protein concentration is 25 μM. The lines connecting the symbols were generated using the Adair equation for four sites with the constants (translated to stoichiometric constants as described in the Materials and Methods) indicated in Table 1.

Metal-free S100A13, which has a molten globule characteristic, strongly enhances the fluorescence of the hydrophobic probe TNS (2-p-toluidinylnaphthalene-6-sulfonate) (Ridinger et al., 2000), and calcium binding to S100A13 strongly reduces the interaction and fluorescence of the hydrophobic probe TNS. However, addition of 0.15 mM zinc to the apoprotein has no effect at all, suggesting that hydrophobic properties are not influenced by zinc binding. We also measured the calcium-binding profiles in the presence of a five-fold excess (125 μM) of the anti-allergic drug amlexanox over S100A13. In these flow dialysis experiments the perfusion buffer contained also 1 μM of the drug, and the loss of drug in the protein-containing compartment was reduced to 10%. This calcium-binding profile (data not shown) was not significantly different from the one in the absence of zinc shown in Fig. 2. Thus we can conclude by virtue of the rule of linked functions (Weber, 1975) that if the drug binds directly to S100A13, the binding is independent of calcium.

Subcellular localization of S100 proteins in human endothelial cells

Several S100 proteins have different subcellular distribution patterns in various cell types in relation to their physiological functions (Mandinova et al., 1998; Mueller et al., 1999; Stradal and Gimona, 1999). In an attempt to understand the function of S100A13, we examined the subcellular localization of endogenous S100A13 in ECV cells by immunostaining using a rabbit polyclonal anti-human S100A13 antibody. As shown in Fig. 3A, S100A13 is located predominantly in the cell nucleus, which is different from its perinuclear localization in human umbilical vein endothelial cells (HUVECs) (Ridinger et al., 2000). To directly compare the subcellular localization of S100 proteins, we also identified S100A1, S100A4, S100A6 and S100B in ECV. Nuclear staining was observed with S100A4 (Fig. 3E) and S100A5 (Fig. 3F) antibodies; partial nuclear staining was observed with the S100A1 (Fig. 3C) antibody – the protein is located only in certain areas within the nucleus. S100A6 (Fig. 3B) and S100B (Fig. 3G) were mainly found in the cytoplasm and perinuclear area of the cells. Only background staining was observed with an S100A3 antibody, which implies that the hair-specific S100A3 is not expressed at significant levels in this particular cell line (Fig. 3D). Pre-absorbed S100 antibodies were used as negative controls, and only background staining was observed (Fig. 3H).

The presence of several S100 proteins in ECV cells was confirmed by western blotting using specific S100 antibodies as shown in Fig. 4. S100A3 is also absent in this blot, whereas other S100 proteins appeared as monomers and various polymers. Similar protein separation and intensity was observed in each lane by Ponceau staining (data not shown) prior to western blotting.

Translocation pathway of endogenous S100A13 in endothelial cells

We observed S100 protein translocation in response to an increase in intracellular calcium levels by angiotensin II stimulation. As shown in Fig. 5A, endogenous S100A13 is mainly distributed in the nuclei of ECV cells. Upon angiotensin II stimulation, S100A13 translocated within small vesicles in the cytoplasm (Fig. 5B). Since no vesicles were observed with unstimulated cells, the redistribution of the small S100 protein is not caused by the fixation process (Fig. 5A). Protein translocation was observed by stimulating cells with angiotensin II for 5 minutes, but the phenomenon was more pronounced after 10 minutes of stimulation. However, vesicles remained for up to 1 hour after the stimulation. To further investigate the mechanism of protein translocation, subcellular localization of endogenously expressed S100A13 was determined after treatment with inhibitors of various secretion pathways. Brefeldin A was used to impede protein transport from the ER to the Golgi complex (Misumi et al., 1986). The drug was added to the cells and incubated for 45 minutes before stimulation with angiotensin II. Significantly fewer cells with vesicles translocating in the cytoplasm were observed (Fig. 5C) compared with treatment with angiotensin II alone (Fig. 5B). This implies an ER-Golgi-dependent translocation pathway of S100A13 in ECV, which is unique for the S100 family since S100 proteins lack the classic signal sequence for secretion. Other secretion pathways such as the actin filament pathway, which can be blocked by amlexanox (Tarantini et al., 2001),

Table 1. Intrinsic calcium-binding affinity of S100A13 and the effect of zinc

	No Zn^{2+} (M^{-1})	+100 mM Zn^{2+} (M^{-1})
K′1	1.4×10^4	1.4×10^4
K′2	1.2×10^5	1.2×10^5
K′3	2.0×10^3	1.5×10^3
K′4	1.2×10^4	5.0×10^3

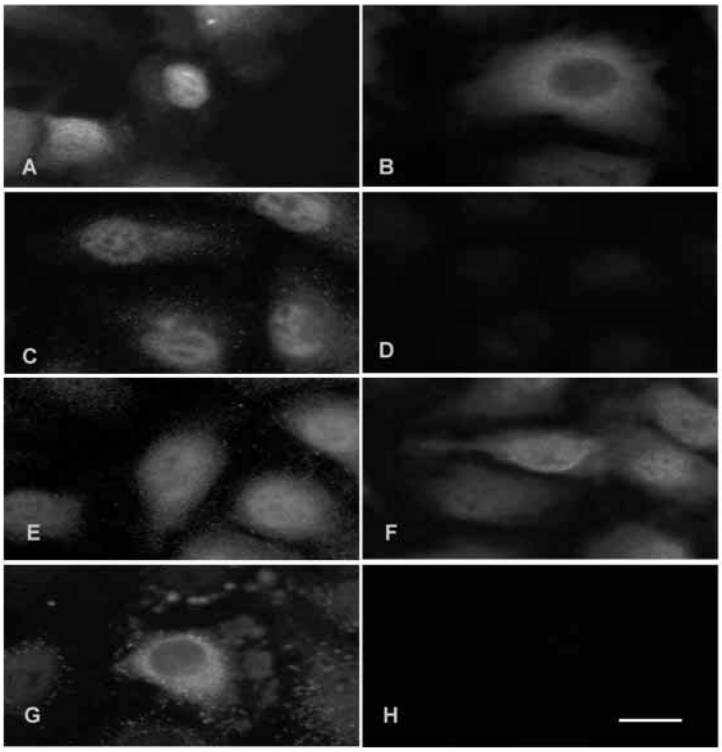

Fig. 3. Subcellular localization of S100 proteins in endothelial cells (ECV). Several S100 proteins were found in ECV, and different subcellular distributions of the proteins was observed by staining with the appropriate antibodies. Rabbit anti-S100 antibodies were used at a 1:1000 dilution, and a goat Cy3-conjugated anti-rabbit antibody was used for visualization under the 40× oil objective lens in the Zeiss light microscope with a filter of 450 to 490 nm. Nuclear localization was observed in cells stained with S100A13 (A), S100A1 (C), S100A4 (E) and S100A5 (F) antibodies; perinuclear stainings were observed with S100A6 (B) and S100B (G) antibodies. A very faint staining was seen with the S100A3 antibody (D). H shows one example of background staining with pre-absorbed S100A6 antibody, and no obvious staining was observed. The same observation was made with S100A13 and the other pre-absorbed S100 antibodies, indicating the specificity of the antibodies used. Bar, 15 μm.

and the tubulin-dependent translocation, which is inhibited by demecolcine (Davey et al., 2000), have therefore been investigated in this study. Translocation in response to angiotensin II was not disturbed by adding either amlexanox (Fig. 5D) or demecolcine (Fig. 5F), which indicates that the translocation of S100A13 is independent of the actin and tubulin filament pathways. In an attempt to identify the type of vesicles that translocate S100A13 in response to an intracellular calcium rise, bafilomycin A was used to inhibit the formation of late endosomes. Bafilomycin A inhibits vacuolar proton ATPase, which is known to generate the acidic lumernal environment of endosomes and lysosomes (Palokangas et al., 1998). Inhibition of acidification by bafilomycin A affects a number of other cellular translocation pathways such as protein recycling from the plasma membrane to the trans-Golgi and transport between the early and late endosomes (the endosomal carrier vesicles) (Clague et al., 1994; Reaves and Banting, 1994; van Weert et al., 1995; Gustafson et al., 2000). Indeed, fewer cells responding to angiotensin II stimulation were observed with bafilomycin A treatment (Fig. 5E). This set of data was also investigated quantitatively by counting the fraction of cells with vesicle appearance. Fig. 8A displays the fraction of cells with vesicular translocation of S100A13 in response to angiotensin II and various secretion inhibitors. Significantly fewer cells with translocating S100A13-containing vesicles were observed after treatment with either brefeldin A (approximately a 50% decrease compared with cells stimulated with angiotensin II) or bafilomycin A (approximately a 75% decrease compared with cells stimulated with angiotensin II alone) in response to angiotensin II; addition of amlexanox or demecolcine had no significant effect on the translocation of S100A13 elicited by angiotensin II.

Translocation pathway of endogenous S100A6 in endothelial cells

To examine whether the unique translocation pathway observed with S100A13 in ECV is an universal phenomenon in this particular cell line, we investigated the translocation pathway of S100A6 by immunohistochemistry (Fig. 6). Endogenous S100A6 is localized in the cytoplasm and perinuclear area of the cells (Fig. 6A) and transported in slightly larger vesicles than the ones observed with S100A13

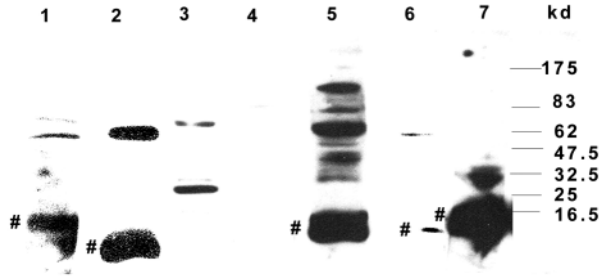

Fig. 4. Western blot analysis of S100 proteins expressed in endothelial cells (ECV). 35 μg of total protein extract was loaded in each lane of a pre-cast 4-20% Tris-glycine gradient SDS PAGE gel and blotted onto the nitrocellulose membrane. Various rabbit anti-S100 antibodies were used as primary antibodies, and signals were detected by horseradish-peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence. The following S100 proteins were identified in ECV – S100A13 (lane 1), S100A6 (lane 2), S100A1 (lane 3), S100A4 (lane 5), S100A5 (lane 6) and S100B (lane 7). S100A3 was not detected in this cell line as shown in lane 4. # indicates monomeric protein, and the dimeric and polymeric forms are seen above these.

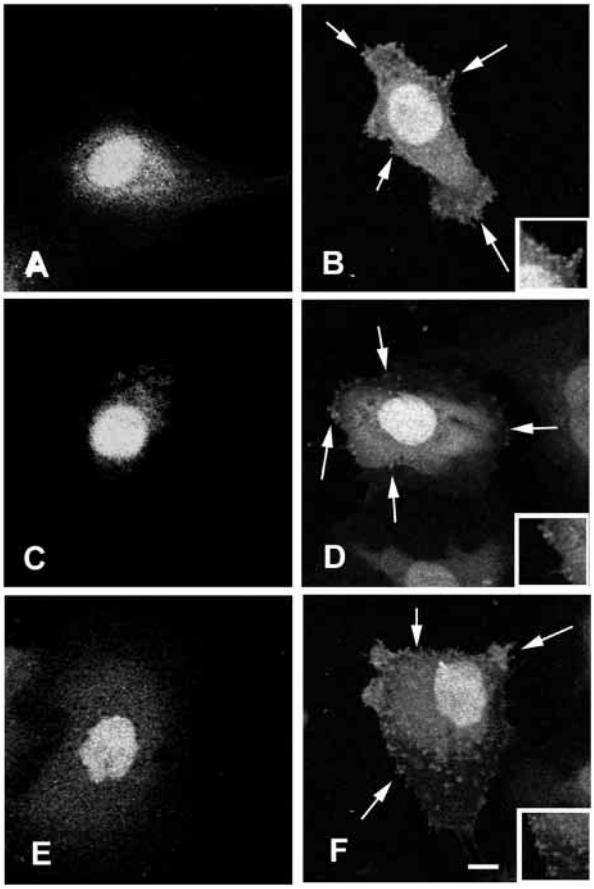

Fig. 5. Localization and translocation of endogenous S100A13 in endothelial cells (ECV). (A) S100A13 protein was mainly localized in the nucleus of the untreated cells. Similar nuclear localization of S100A13 was also observed in cells treated with brefeldin A (C) and bafilomycin A (E) before angiotensin II stimulation. By contrast, small vesicles at the cellular periphery (indicated by arrows) were evident after angiotensin II stimulation (B); and treatment with amlexanox (D) or demecolcine (F) before addition of angiotensin II had no effect on the translocation of the protein. Areas with a series of vesicles were enlarged by 20% and presented in the small squares at the right hand corners (B,D,F). The samples were analyzed by confocal microscopy. Bar, 15 μm (F).

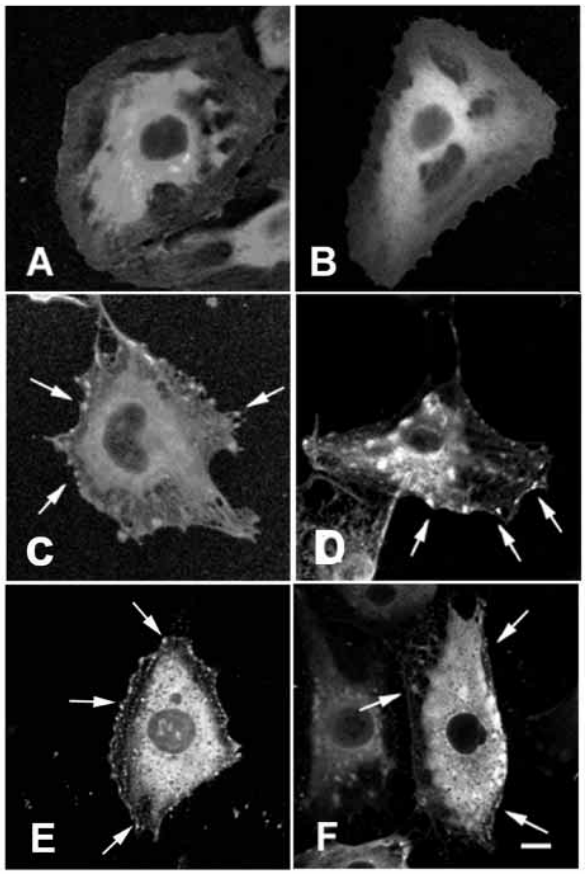

Fig. 6. Localization and translocation of endogenous S100A6. (A) S100A6 was mainly distributed around the cell nuclei and in the cytoplasm of ECV cells; S100A6 translocated in the form of vesicles (indicated by arrows) in cells stimulated by angiotensin II (C); (B) Fewer cells with vesicle formation were observed after amlexanox treatment. However, S100A6 translocation in angiotensin-II-stimulated cells was not affected by brefeldin A (D) nor by demecolcine (E) or bafilomycin A (F). All the samples were analyzed by confocal microscopy. Bar, 15 μm.

in response to angiotensin II stimulation (Fig. 6C). Addition of brefeldin A (Fig. 6D), demecolcine (Fig. 6E) or bafilomycin A (Fig. 6F) had no effect on the translocation of S100A6 in response to angiotensin II. However, treatment with amlexanox effectively inhibited the translocation process (Fig. 6B). Cell counting reveals a nearly 50% decrease in the number of cells producing vesicles with S100A6 when cells were treated with amlexanox before angiotensin II stimulation (Fig. 8B). This implies an actin-stress-fiber-dependent translocation pathway of S100A6 in contrast to the ER-Golgi-dependent S100A13 translocation in ECV.

Effects of the inhibitory chemicals

To confirm the effects of the chemicals we used to block various translocation pathways, we used anti-β-COP antibody to confirm the disruption of the ER-Golgi compartments induced by brefeldin A. Brefeldin A induces fusion of Golgi to ER and inhibits protein transport to the post Golgi compartment (Lippincott-Schwartz et al., 1990). The vesicle coat protein β-COP is a cis-Golgi membrane-resident protein and was found in a different location following brefeldin A treatment in HUVECs (Humphries et al., 1997). A scattered distribution of β-COP indicates dissociation of the protein from cis-Golgi membranes and fusion between Golgi and ER compartments (Fig. 7A,B). Similarly, labeled phalloidin, which binds to actin fibers, was used to monitor actin depolymerization in cells treated with amlexanox (Fig. 7C,D). The destruction of tubulin filaments in cells treated with demecolcine was visualized by using a monoclonal anti-tubulin antibody and a Cy3-conjugated secondary antibody (Fig. 7E,F).

Distinct S100A13 and S100A6 translocation

In order to confirm that the translocating vesicles containing either S100A13 or S100A6 go through distinct pathways, double staining with two antibodies obtained from different

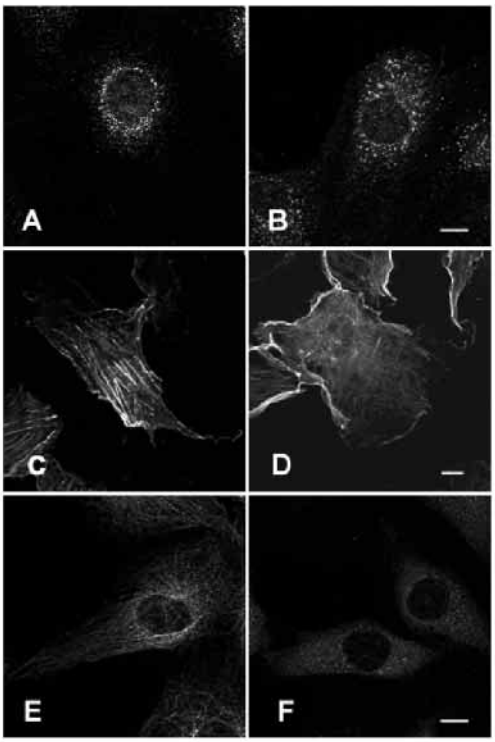

Fig. 7. Effects of the inhibitory drugs used to study the various translocation pathways. Cells were left untreated (A,C,E), incubated with brefeldin A (B), amlexanox (D) or demecolcin (F) and stained with mouse anti-β-COP (A,B) and mouse anti-tubulin (E,F) antibodies. Phalloidin-TRITC was used to localize actin stress fibers (C,D). The scale bar in B applies for both A and B; the scale bar in D applies for C and D; and the scale bar in F applies for E and F. All the scale bars are 15 μm.

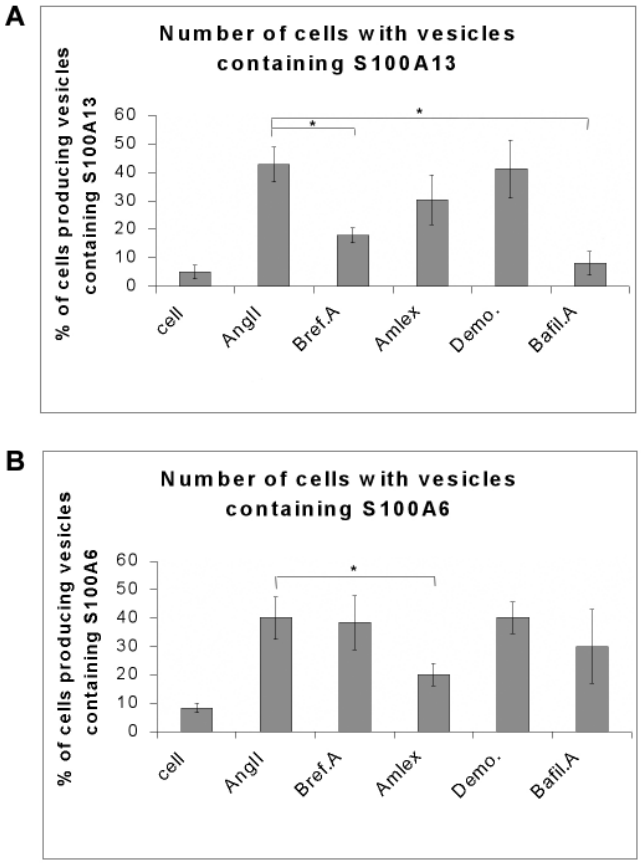

Fig. 8. Quantification of S100 protein translocation. Cells producing vesicles containing either S100A13 or S100A6 were counted and divided by the total cell counts (approximately 200 cells were counted for each experiment), and the columns represent the mean of three experiments. (A) Percentage of cells with S100A13 translocating in vesicles in response to angiotensin II alone or after treatment with the drugs indicated. * indicates a statistically significant difference between cells stimulated with angiotensin II alone and treated with brefeldin A or bafilomycin A ($P<0.05$). (B) Percentage of cells with S100A6 translocating in vesicles in response to angiotensin II and the drugs indicated. * indicates a statistically significant difference between cells treated with angiotensin II alone and with amlexanox ($P<0.1$). The P value was calculated by using Student-t-variant R. A. Fisher and F. Yates.

species was performed. Translocation of S100A13 (green) was observed by following Cy2-conjugated antibody after angiotensin II stimulation in Fig. 9A. As described earlier, the protein is mainly observed in the cell nucleus and in the vesicles within the cytoplasm after angiotensin II stimulation. With the same cell, S100A6 was also localized in vesicles and in the cytoplasm (Fig. 9B). However, a superimposed image of the two pictures (Fig. 9A,B) reveals that vesicles containing S100A13 (green) are distinct from the vesicles containing S100A6 (red) after angiotensin II stimulation (Fig. 9C). These data confirm that S100A6 and S100A13 are localized in distinct vesicles as implied by their use of different translocation pathways.

Discussion

The growing interest in S100 calcium-binding proteins has led to various investigations to understand their biochemical and physiological roles. To further investigate the biochemical properties of the most recent member of the family, S100A13, we improved the purification of the recombinant protein. The newly purified protein has the same calcium-affinity profile (Fig. 2) as the one purified previously (Ridinger et al., 2000), but with a better control and higher yield in protein expression. It is thought that the cellular function of S100 proteins relies on their ability to interact with target proteins by exposing a hydrophobic domain at the N-terminus as a result of calcium binding. However, the addition of calcium does not induce hydrophobic exposure in S100A13 (Ridinger et al., 2000), which leads us to speculate that the binding of other cations alone or in concert with calcium might create the hydrophobic domain. Since several reports have demonstrated the high binding affinity of S100 proteins to zinc (Harder and Gerke, 1993; Fritz et al., 1998; Davey et al., 2000; Schafer et al., 2000), we investigated the effect of zinc on the calcium affinity of S100A13. However, only a weak negative effect of zinc was observed on the set of low-affinity binding sites for calcium in flow dialysis (Fig. 2). Moreover, the binding of zinc to the metal-free protein shows only a weak affinity and does not induce any conformational changes (Ridinger et al., 2000). Under identical experimental conditions, zinc increases the calcium affinity of one pair of the binding sites in the dimeric S100A12 more than a 100-fold (C.W.H., unpublished). The

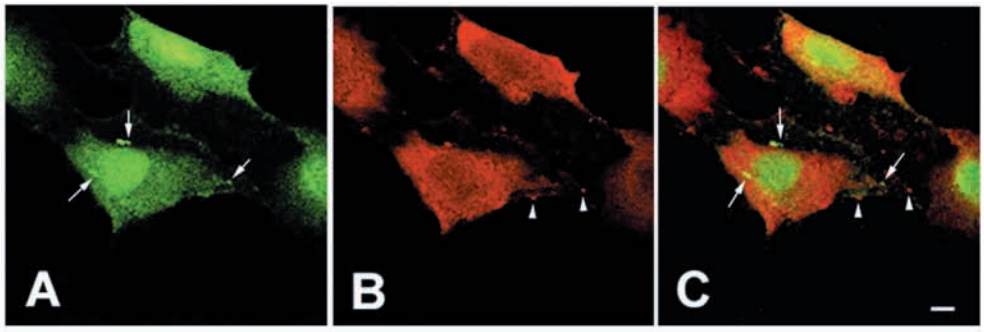

Fig. 9. S100A13 and S100A6 are contained in distinct vesicles. **(**A) S100A13 distribution in cells stimulated by angiotensin II and visualized by a Cy2-conjugated anti-goat antibody. (B) S100A6 distribution in cells stimulated by angiotensin II and visualized by Cy3-conjugated anti-rabbit antibody. (C) A superimposed image of A and B. The sample was analyzed by confocal microscopy. Bar, 15 μm. Arrows indicate vesicles containing S100A13 and arrowheads indicate vesicles with S100A6.

relative insensitivity of S100A13 for zinc was also observed in the case of S100A6 (Pedrocchi et al., 1994). In another set of flow dialysis experiments, we examined the calcium-binding affinity of S100A13 in the presence of the anti-allergic drug, amlexanox. However, the calcium affinity of S100A13 was not altered in the presence of amlexanox even though an interaction of S100A13 to the drug was observed in the amlexanox column (Shishibori et al., 1999). On the basis of our in situ biochemical data, we conclude that no hydrophobic exposure of S100A13 is triggered by binding of zinc ions, that amlexanox does not modify the calcium-binding characteristics, and thus, by virtue of the linked functions (Weber, 1975; Cox, 1996), amlexanox binding is independent of calcium ions.

Translocation of S100 proteins may play an important role in assembly of signaling complexes to activate specific signaling pathways (Mueller et al., 1999; Davey et al., 2000; Davey et al., 2001). We observed relocation of S100A13 in response to either angiotensin II or thapsigargin (data not shown), both of which are known to elevate calcium intracellularly. It is tempting to associate the translocation process of S100A13 with alternation of the intracellular calcium levels even though the biochemical data indicated that calcium has no effect on the hydrophobic interaction of the protein. Thus, we hypothesize that an increase in intracellular calcium levels might not have a direct effect on the translocation of S100A13. Instead, the translocation process might depend on the interaction with unknown target proteins, which are activated in the cells saturated with a high concentration of calcium. A similar observation was reported in the case of S100A10, which does not bind to calcium either, but forms a tight complex with another calcium-binding protein, annexin II, and is thought to be involved in endo- and exocytosis (Harder and Gerke, 1993). Alternatively, the homeostasis of other ions present in the cells might be influenced by the sudden increase of calcium and then participate in translocation of S100A13. One potential candidate is copper since it has been reported lately that it induces the assembly of S100A13, FGF-1 and synaptotagmin-1 multiprotein aggregate (Landriscina et al., 2001).

Interestingly, translocation after angiotensin II stimulation was also observed with S100A6. S100A6 is another unique member in the S100 calcium-binding family, as the interaction of S100A6 to its target proteins is calcium-dependent even though addition of calcium only induces very modest conformational changes of the protein (Sastry et al., 1998). Although no hydrophobic exposure for target protein interaction was observed in either S100A13 or S100A6 proteins in response to calcium binding, both proteins translocated in response to an increase of intracellular calcium levels in ECV cells. This implies that both S100A13 and S100A6 have different modes for transducing calcium signals and interacting with their target proteins from other members of the S100 family.

The so-called alternative secretion pathway, which is independent of the classic ER-Golgi route, seems to be the most favorable pathway for relocation of S100 proteins since S100 proteins lack the signal sequence to anchor the plasma membrane for secretion. Several S100 proteins such as S100B, S100A8 and S100A9 utilize the tubulin pathway for secretion (Rammes et al., 1997; Davey et al., 2000). However, the translocation of S100A13 in response to angiotensin II was inhibited by addition of brefeldin A, which impairs the protein transport to the post-Golgi compartment, and no inhibition of translocation was observed with addition of demecolcine and amlexanox. Our results (Fig. 5, Fig. 8A) imply that the translocation of S100A13 in ECV is associated with the classic ER-Golgi pathway, which is novel in the S100 protein family. By contrast, the translocation of S100A6 is independent of the ER-Golgi and the tubulin pathway, but actin stress fibers might play an important role instead (Fig. 6, Fig. 8B).

In order to identify the type of vesicle we observed in translocation of S100 proteins, we used the H^+-ATPase inhibitor bafilomycin A to inhibit acidification, which affects a variety of intracellular pathways, including transport from early to late endosomes and recycling from the plasma membrane to the trans-Golgi (Palokangas et al., 1998; Gustafson et al., 2000). Apart from inhibiting the H^+-ATPase activity in Golgi cisternae, bafilomycin A also disrupts Golgi stack morphology and might block the coat formation of the secreting vesicles (Gustafson et al., 2000). As we have observed that S100A13 translocates through the ER-Golgi pathway in small vesicles, the translocation process might be inhibited by bafilomycin A. Indeed, translocation of S100A13 but not of S100A6 was inhibited by bafilomycin A (Fig. 8). The translocating vesicles carrying S100A13 in response to angiotensin II stimulation seem to be different from the ones that translocate S100A6 as shown in Fig. 9C. This confirms the

distinct translocation routes of S100A13 and S100A6 in response to the increase of intracellular calcium by angiotensin II in the same cellular environment.

In contrast to other members of the S100 family, we report here a classic translocation pathway of S100A13 in ECV cells in response to intracellular calcium increase by angiotensin II. The vesicles responsible for S100A13 translocation are possibly early endosomes as the translocation process can be inhibited by bafilomycin A. We also identified actin-stress-fiber-dependent translocation of S100A6 in this study. The distinct translocation pathways of the two S100 proteins in the same cell imply the existence of different signal transduction mechanisms of S100 proteins in response to intracellular calcium changes. Co-staining with other Golgi-ER early endosomal markers and searching for the possible target proteins that interact with S100A13 will be our future goals to understand the physiological role of this unique protein in the S100 family.

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