Expression of S100A6 (Calcyclin), a member of the S100 family and of Zn\(^{2+}\)-binding proteins is elevated in a number of malignant tumors. In vitro the protein associates with several actin-binding proteins and annexins in a Ca\(^{2+}\)-dependent manner. We have now studied the subcellular localization of S100A6 using a new, specific monoclonal antibody. Immunofluorescence microscopy of unfixed, ultrathin, frozen sections demonstrated a dual localization of S100A6 at the nuclear envelope and the plasma membrane of porcine smooth muscle only in the presence of Ca\(^{2+}\). The same localization was found by immunofluorescence and immunogold electron microscopy as well as by confocal laser scanning microscopy with cultured, fixed, human CaKi-2 and porcine ST interphase cells. Upon cell division, however, S100A6 was found exclusively in the cytoplasm. Cell fractionation studies showed that S100A6 was present in the microsomal fraction in the presence of Ca\(^{2+}\) and was released from this fraction by the addition of EGTA/EDTA but not by Triton X-100. The data demonstrate that S100A6 is localized both at the plasma membrane and the nuclear envelope in vivo and suggest a Ca\(^{2+}\)-dependent interaction with annexins or other components of the nuclear envelope.

Because calcium plays a crucial role in the regulation of several nuclear functions (1, 2), a strong interest exists in identifying Ca\(^{2+}\)-binding proteins involved in the perception and transduction of signals involved in cell cycle control and cell death. The S100 family comprises 16 closely related members, 13 of which form a gene cluster in the epidermal differentiation complex of chromosome 1 in humans (3). S100 proteins consist of two Ca\(^{2+}\)-binding EF-hands and short individual N and C termini. The first EF-hand is conserved among the S100 family and the second EF-hand follows the canonical consensus. Almost all S100 proteins form stable homomeric complexes of chromosome 1 in humans (3). S100 proteins are present in a variety of malignant tumors. In vitro studies revealed Ca\(^{2+}\)-dependent interactions of the large S100 protein family has attracted substantial interest in the last decade due to their tissue- and cell type-specific expression patterns and their involvement in several diseases (4). The S100 gene products play a role in multiple proliferation- and differentiation-related events and appear to be involved in autoimmune diseases and cancer (5). S100B is predominantly expressed in brain and has been implicated in Alzheimer's disease, epilepsy, and neurodegenerative symptoms commonly associated with Down Syndrome (7). S100A7 is overexpressed in psoriatic lesions (8), and the S100A8/A9 (MRP8/MRP14) heterodimer plays a role in inflammation and cystic fibrosis (9) (for review see Ref. 6). In contrast to the above, S100A2 is down-regulated in breast cancer and the promotor is activated upon the interaction with wild-type p53 (10). Thus, S100A2 was suggested to be a tumor suppressor-related gene.

S100A6 (calcyclin) was first identified by molecular cloning of the growth factor-inducible gene 2A9 (11). Its expression is elevated in response to growth factor-stimulated proliferation of quiescent fibroblasts, indicating a role in cell cycle progression (12). Further studies showed that this elevation in expression is mediated by a growth factor-responsive element in the promotor region of S100A6 (13) and peaks in the transition from G\(_0\) to S-phase in serum-stimulated quiescent smooth muscle cells (14). S100A6 was also found to be overexpressed in several tumors like acute myeloid leukemia (11) and neuroblastoma (15), and also in a variety of melanoma cell lines (16). Overexpression of S100A6 correlates with the metastatic behavior in nude mice and an up-regulated expression has also been observed in wound healing after corneal injury (17). S100A6 was additionally identified and purified from murine Ehrlich ascites tumor cells (18) and Gou et al. (19) described a correlation between the expression of S100A6 and the metastatic behavior of Ras-transformed NIH 3T3 fibroblasts. In benign tissues, S100A6 is expressed predominantly in fibroblasts and epithelial cells (20) but is also present in neuronal and smooth muscle cells. To elucidate the in vivo functions of S100A6 recent efforts focused on the identification of cellular binding partners and the metal-binding characteristics (21). Several in vitro studies revealed Ca\(^{2+}\)-dependent interactions with actin binding proteins like caldesmon (22), tropomyosin (23), and calponin (24). More recently a novel protein of yet unknown function, termed p30 or CaCYBP, has been reported to bind to S100A6 in vitro (25). In addition, members of the annexin family were shown to interact with S100A6 in a Ca\(^{2+}\)-dependent manner. Thus, affinity chromatography using immobilized calcyclin revealed an interaction with annexin II and annexin VI (26). The interaction of S100A6 with annexin XI has been studied in more detail, and the binding sites on both molecules have been mapped (27). Notably, annexin XI has been reported to target to the nucleus and to display a distinct localization during mitosis (28).

The localization of S100A6 has been also studied in transformed (15, 29) or normal tissues (20, 30), and in cultured smooth muscle cells (31) using a polyclonal antibody. In this study we have reinvestigated the Ca\(^{2+}\)-dependent subcellular localization of S100A6 using a novel highly specific monoclonal antibody and show that the protein localizes to the plasma membrane and the nuclear envelope.
membrane and the nuclear envelope of smooth and nonmuscle cells.

**EXPERIMENTAL PROCEDURES**

All chemicals were from Merck with the exception of HEPES and Triton X-100 (Sigma), fetal bovine serum (HyClone), Dulbecco’s modified Eagle’s medium and minimal essential medium (Life Technologies).

**Antibodies—** Monoclonal anti-porcine calcyolin (clone CACY-100) was from Sigma. Ultradyn Frozen Sections—Fresh porcine stomach smooth muscle tissue was excised in 2 × 2 × 20 mm strips and submerged in buffer containing 20 mM imidazole, pH 7.0, 150 mM NaCl, 5 mM MgCl₂, 5% (v/v) glycerol, 1 mM NaN₃, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin-leupeptin (Roche), and 2 mM sucrose as cryoprotectant at 4 °C overnight. Muscle strips were then mounted onto aluminum cryostat and then frozen in liquid nitrogen. Cryosections of 5–8 μm were cut using a Reichert-Jung FC4E cryo-ultramicrometer. Sections were then transferred to silanized coverslips, dried for 1 h at room temperature, and subjected to immunofluorescence staining.

**Cell Culture and Immunofluorescence Microscopy—** Porcine testis (ST) and human CaKi-2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. For immunofluorescence microscopy, cells were grown on Falcon tissue culture dishes in complete Eagle’s medium and minimal essential medium (Life Technologies). Cells were washed with PBS and fixed for 10 min with 3.7% paraformaldehyde, 2.5% glutaraldehyde in HBS for 15 min. The blocks were dehydrated in ethanol and embedded in Araldite (TAAB). Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss EM 10A electron microscope.

**RESULTS AND DISCUSSION**

A monoclonal antibody specific for S100A6 was generated as described earlier (35) using porcine smooth muscle S100 proteins as the antigen. Clone CACY-100 specifically recognizes S100A6 but not S100A2 or S100b on Western blots of whole cell and tissue extracts (Fig. 1).

We first determined the subcellular localization of S100A6 in unfixed, frozen sections of porcine stomach smooth muscle. In the presence of Ca²⁺, S100A6 displayed a dual localization, on the plasma membrane and in the nucleus. Longitudinal sections revealed an association along thin thread-like structures across the plasma membrane and in the nucleus. Longitudinal sections revealed an association along thin thread-like structures across the plasma membrane and in the nucleus. These data suggested that S100A6 colocalizes with two membranous cellular compartments, namely the plasma membrane and the nuclear envelope of smooth muscle cells in a Ca²⁺-dependent manner. To support the results obtained with unfixed tissue sections, we next studied the distribution of S100A6 in cultured porcine ST cells fixed and labeled both in the presence or absence of divalent cations. In the presence of Ca²⁺, staining of the plasma membrane was observed in cells extracted with 0.1% Triton X-100 (Fig. 3A). When the Triton X-100 concentration was increased to 0.3%, localization at the plasma membrane was lost and a weak cytoplasmic distribution in addition to a strong concentration in the nucleus was observed. Notably, in the presence of Zn²⁺, S100A6 localization was indistinguishable from that seen in cells stained in the absence of both Ca²⁺ and Zn²⁺. We also examined the association of S100A6 with vesicular structures and organelles. In the absence of Ca²⁺, S100A6 was localized to intracellular organelles and vesicles (Fig. 3B). In the presence of horseradish peroxidase-conjugated secondary antibodies, the ECL chemiluminescence system (Amersham Pharmacia Biotech). Electron Microscopy—Nuclei of cultured pig ST cells were isolated as described by Kihlmark and Hallberg (34), resuspended in HBS containing phenylmethylsulfonyl fluoride, pepstatin-leupeptin, and 0.05% Triton X-100 on ice for 5 min by centrifugation. The pellet was resuspended in HBS containing 1 mg/ml bovine serum albumin and incubated with the monoclonal anti-S100A6 antibody for 1 h at room temperature. The nuclei were then washed 3 times in HBS and incubated with the monoclonal anti-S100A6 antibody for 1 h at room temperature. After washing the nuclei 3 times in HBS, they were incubated with a 10-nm gold-labeled secondary antibody (BioCell) for 45 min at room temperature, washed 3 times and fixed in 3.7% paraformaldehyde, 2.5% glutaraldehyde in HBS for 15 min. The blocks were dehydrated in ethanol and embedded in Araldite (TAAB). Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss EM 10A electron microscope.

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1 The abbreviation used is: HBS, HEPES-buffered saline.

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**FIG. 1. Specificity of monoclonal anti-S100A6 antibody.** Coomassie Blue-stained polyacrylamide gel electrophoresis gel (A) and Western blot (B) showing the reactivity of monoclonal antibody clone CACY-100. The antibody specifically recognizes recombinant (lane 1) and native porcine S100A6 (lane 9), detects S100A6 in whole cell extracts of porcine ST (lane 3), porcine LLC PK1 (lane 4), or human CaKi-2 cells (lane 5), and in tissue extracts of porcine stomach (lane 6) and porcine uterus (lane 7). The absence of S100A2 (lane 2) or S100b from bovine brain (lane 8). Note the reactivity with both monomer (M) and the stable dimer (D) in purified native S100b (lane 9). Position of molecular weight markers is indicated in panel A.
Confocal laser scanning microscopy was used to further confirm the dual localization of S100A6 at the plasma membrane and the nuclear envelope. Cells were extracted with 0.1% Triton X-100 in the presence of Ca\(^{2+}\), S100A6 was detected along the plasma membrane (A). In contrast, extraction with 0.3% Triton X-100 in the absence of Ca\(^{2+}\) revealed S100A6 localization also in the cytoplasm and more strongly at the nuclear envelope (B). Only the nuclear staining was retained in the presence of EGTA (C). Note the diffuse distribution of S100A6 in mitotic cells (stained with antibody CACY-100 in the presence of Ca\(^{2+}\)).

In contrast to the results obtained with unfixed tissue sections, S100A6 remained associated with the nuclear envelope of fixed cultured cells even in the presence of EGTA/EDTA. Together with the observed requirement for increased Triton X-100 concentrations, these data strongly suggest that S100A6 localizes at the inner face of the nuclear envelope and that this binding is mediated via a binding partner, which is sensitive to formaldehyde fixation. We therefore used immunogold electron microscopy on isolated nuclei to further test the hypothesis that S100A6 colocalized with inner structures of the nuclear envelope. The nuclear envelope consists of three compartments, namely the plasma membrane, the inner nuclear membrane, and the lamina. As expected, gold particles were detected exclusively in the space between the outer and the inner nuclear membrane, and in the vicinity of the nuclear lamina (Fig. 5).

Both fluorescence and electron microscopy suggested a membrane association of S100A6 requiring the presence Ca\(^{2+}\). To further support this conclusion, we carried out cell fractionations. As seen in Fig. 6, S100A6 was detected in the microsomal fraction of cultured porcine ST cells by Western blotting, and this association was strictly Ca\(^{2+}\)-dependent. S100A6 was released from the pellet upon addition of EGTA/EDTA. In contrast, the addition of 0.5% Triton X-100 to the extraction buffer failed to solubilize S100A6. However, significant amounts of S100A6 were also found in the soluble fraction, in agreement with the results obtained in the microscopic studies. These data also support previous studies by Leśniak and Filipiak (36) that demonstrated the Ca\(^{2+}\)-dependent interaction of S100A6 with microsomal membranes from murine Ehrlich ascites tumor cells.

Taken together, our data suggest that S100A6 is located in the cytoplasm and is targeted to two membranous compartments, namely the plasma membrane and the nuclear envelope upon the increase of cytoplasmic Ca\(^{2+}\), mediated by yet unidentified signals. The variable extractability of S100A6 associated with the plasma membrane versus the nuclear envelope...
points to an interaction of S100A6 with two different targets. Interaction with the plasma membrane was strongly dependent on the presence of Ca\textsuperscript{2+} but was lost upon the addition of Triton X-100 despite fixation with paraformaldehyde. The interaction with the Triton X-100 insoluble fraction, i.e. the nucleus envelope, likewise depended on the presence of Ca\textsuperscript{2+}, as shown in the cell fractionation experiments and in unfixed tissue sections, but S100A6 remained firmly attached to its putative target after fixation even in the presence of EGTA. In contrast, cytoplasmic S100A6 was extracted in the presence and absence of Ca\textsuperscript{2+} despite prior fixation with formaldehyde. Interestingly, several S100 proteins have been shown recently to interact directly with intermediate filament proteins (37). Thus, the association of S100A6 with the intermediate filaments of the nuclear lamina is plausible.

The potential binding partners for S100A6 that have thus far been identified can be divided into two groups, one comprising several actin-binding proteins, and the second embracing members of the annexin family. In this study we failed to detect any colocalization of S100A6 with actin-rich structures, although an interaction with components of the actin cytoskeleton cannot be ruled out completely. Annexin II and annexin VI were described to interact with S100A6 and have been shown to colocalize with the plasma membrane (38). Barwise and Walker (38) demonstrated that annexin II relocates to granular structures at the plasma membrane, whereas annexin VI adopts a more homogeneous distribution at the plasma membrane in cells treated with the calcium ionophore A23187. More importantly, this group also claimed that the intranuclear portion of annexins IV and V translocates to the nuclear membrane under the same conditions. Likewise, annexin XI displays partial nuclear localization and is one of the potential targets of S100A6. The binding sites of both molecules have been mapped within residues 4–7 in S100A6 (39) and residues 52–59 in annexin XI (27), respectively. Furthermore annexin XI has been reported to alter its localization during mitosis, forming an arc-like structure around the mitotic spindle (28). Our results indicate that S100A6 is localized at multiple discrete sites in the cell including the inner face of the nuclear envelope and that the protein undergoes specific translocations during mitosis. The colocalization of S100A6 with these membranous compartments is strictly dependent on the presence of Ca\textsuperscript{2+} and is likely to correspond to the sites of functionally active S100A6. From these data we therefore suggest an interaction of S100A6 in vivo with members of the annexin family, and in particular with nuclear annexin XI. Moreover, we have shown that refined methods are required to reveal the localization of S100A6 at multiple distinct compartments in the cell. Our further efforts are directed toward characterizing the interaction between S100A6 and annexin XI in vitro and in vivo.

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Ca$^{2+}$-dependent Association of S100A6 (Calcyclin) with the Plasma Membrane and the Nuclear Envelope
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