

## Ca<sup>2+</sup>-dependent interaction of S100A2 with muscle and nonmuscle tropomyosins

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### SUMMARY

Zero-length chemical crosslinking with 1-ethyl-3-[3-(dimethyl amino)propyl]carbodiimide (EDC) indicated an association of the Ca<sup>2+</sup>-binding protein S100A2 with tropomyosin (TM) *in vitro*. The mobility of the crosslinked product on SDS-PAGE gels indicated the formation of a 1:1 complex between S100A2 and TM and the interaction was Ca<sup>2+</sup> dependent. Monoclonal antibodies were raised against S100A2 and used to determine its cellular localization in the porcine epithelial cell line LLC PK1. It was found that the localization of S100A2 depended on the differentiation state of the cells, being absent from actin stress fibers in sparsely seeded cultures, but present in the actin-containing microvilli characteristic of differentiated cells. Immunoprecipitations of [<sup>35</sup>S]methionine-labeled extracts using S100A2 as well as TM-specific antibodies

failed to co-precipitate TM and S100A2, indicating a transient association between these two molecules in solution. Affinity chromatography of cell extracts on immobilized recombinant TMs, however, confirmed the Ca<sup>2+</sup>-dependent interaction between S100A2 and both muscle TMs as well as with high and low molecular mass nonmuscle TMs, suggesting that the binding site resides in one of the conserved regions of TM. Our data demonstrate the possible interaction of S100A2 with TM that is not bound to the microfilaments and indicate a differentiation-related function for S100A2 in LLC PK1 cells. The possible functional implications of this interaction are discussed.

Key words: Tropomyosin, Cross linking, Binding, Localization, S100A2

### INTRODUCTION

A large number of cellular processes including secretion, cell proliferation and muscle contraction are regulated by transient increases in intracellular Ca<sup>2+</sup> levels in response to extracellular signals (Kligman and Hilt, 1988; Berridge, 1995). These Ca<sup>2+</sup> signals are often characterized by the appearance of waves or spikes (Berridge, 1993; Fay, 1995; Nelson et al., 1995; see Bootman and Berridge, 1995, for review) and are in turn transduced by a variety of Ca<sup>2+</sup>-binding proteins. Most Ca<sup>2+</sup>-binding proteins identified thus far are expressed in a cell-type specific manner, where they may act as either Ca<sup>2+</sup> sensors or Ca<sup>2+</sup> buffers (Skelton et al., 1994; Ikura, 1996) and are involved in the regulation of multiple cellular processes (for reviews see Kligman and Hilt, 1988; Heizmann and Hunziker, 1991) such as cell cycle progression and cell growth (Calabretta et al., 1986), signal transduction events (Barger et al., 1992; Skelton et al., 1994), the activation of enzymes (reviewed by Donato, 1992) and the modulation of cytoskeletal interactions with the plasma membrane (Gerke and Weber, 1985a,b; Glenney and Tack, 1985). They have also been implicated in the regulation of smooth muscle contraction (Mani and Kay, 1993; Wills et al., 1993, 1994a,b; Kriajewska et al., 1994;

Fujii et al., 1990, 1994), the development of a number of human diseases (Allore et al., 1988; Marks and Allore, 1990; Van Eldik and Griffin, 1994; Renaud et al., 1994; Föhr et al., 1994; Sheng et al., 1994; Pedrocchi et al., 1994a,b; Tonini et al., 1991, 1995) as well as tumor cell metastasis (Takenaga et al., 1994a; Gibbs et al., 1994; Parker et al., 1994a,b).

S100 proteins belong to a family of small, acidic, EF-hand-containing, Ca<sup>2+</sup>-binding polypeptides that were originally identified in brain tissue (Moore, 1965). Over the past decade a large number of new S100-related proteins have been identified and to date this subfamily consists of 13 members, which occur as either monomers or dimers (for a complete review see Kawasaki and Kretsinger, 1994). Nine genes coding for different S100 members have been found to be clustered on chromosome 21 (Kawasaki and Kretsinger, 1994; Engelkamp et al., 1993; Schäfer and Heizmann, 1996). For most members of the S100 family the cellular effector protein has yet to be determined, but recent findings indicate that cytoskeletal elements are most likely involved. For example in smooth muscle, S100 proteins have been shown to bind directly to the actin-associated proteins calponin (CaP) (Wills et al., 1993; Fujii et al., 1994), caldesmon (CaD) (Mani and Kay, 1993; Fujii et al., 1990) and nonmuscle myosin heavy chain (Kria-

jewska et al., 1994). For nonmuscle cells a direct association of S100A4 (pEL 98) with nonmuscle tropomyosin, both in vitro and in vivo, has been reported (Takenaga et al., 1994c) and S100A4 has been found to be distributed along actin stress fibers (Gibbs et al., 1994; Takenaga et al., 1994b), further pointing towards elements associated with the actin cytoskeleton as possible targets for S100 proteins.

The protein tropomyosin (TM) is a ubiquitous component of the thin actin-containing filaments in muscle cells and is also found in many actin-assemblies of nonmuscle cells. While it appears to be an essential component of the thin filament-regulating complex in muscle its role in nonmuscle cells, in particular with respect to the regulation and assembly of actin filament subcomponents, is still poorly understood (Lees-Miller and Helfman, 1991). Moreover, the disruption of actin filament organization observed in virus-transformed cells and cells from malignant tissues, commonly lacking one or more TM isoforms (Leonardi et al., 1982; Cooper et al., 1987; Prasad et al., 1993; Takenaga et al., 1988, 1994d; Takenaga and Masuda, 1994), points to a pivotal role of TM in regulating actin cytoskeleton organization. Recent efforts have been directed at identifying components that are involved in the organization of the actin cytoskeleton and its interaction with other molecules. The actin- and TM-binding protein calponin (CaP) is predominantly expressed in smooth muscle cells (reviewed by Gimona and Small, 1996) and has been shown to interact with Ca<sup>2+</sup>-binding proteins of the S100 family (Mani and Kay, 1990; Wills et al., 1993, 1994a,b; Fujii et al., 1994). It appears likely from these findings that a complex containing CaP (and its homologues), TM and S100 family members modulate actin organization as well as the interaction of contractile and cytoskeletal elements in smooth muscle and nonmuscle cells. Clear evidence for such a role has, however, not yet been forthcoming.

In this study we describe the purification of potential Ca<sup>2+</sup>-sensory proteins from smooth muscle, based on their ability to bind to hydrophobic chromatography media due to the presence of one or more regulatory domains (Ikura, 1996). We show that S100A2 is present in smooth muscle tissue, that it binds directly to muscle and nonmuscle TM in vitro in a 1:1 fashion and that this interaction is Ca<sup>2+</sup> dependent. Using a monoclonal antibody specific for S100A2 we further demonstrate that the protein is colocalized with actin in microvilli in a differentiation-dependent fashion in LLC PK1 epithelial cells. The results presented support the hypothesis that S100 proteins are directly involved in the regulation of cytoskeletal organization.

## MATERIALS AND METHODS

### Purification of S100A2 from porcine and avian smooth muscle

Fresh muscle tissue was minced at 4°C and then homogenized in a solution containing 40 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM Imidazole, 10 mM bis-Tris, 50 mg/ml streptomycin, pH 6.6. Solid ammonium sulfate was added to 40% saturation and after centrifugation (Sorvall GSA, 12,000 rpm for 35 minutes) the floating residue was discarded by filtration through glass wool. The filtered supernatant was further fractionated with ammonium sulfate between 60 and 75% saturation and the pellet, containing S100 proteins S100A2 and S100A6 (calcyclin) as well as calmodulin, was collected by further centrifu-

gation at 15,000 g. Pellets were stored at -20°C and pooled from several preparations (totalling around 4 kg wet muscle). The pellets were dissolved in 500 ml AA buffer (20 mM Imidazole, pH 6.9, 60 mM KCl, 0.1 mM dithioerithrol (DTE)) supplemented with 0.5 mM EGTA and dialyzed overnight against the same solution with at least three solution changes. To the final dialysate 1 mM CaCl<sub>2</sub> and 25% solid ammonium sulfate were added and the preparation was clarified by centrifugation (Sorvall SS 34, 18,000 rpm, 20 minutes). The supernatant was applied to a phenyl Sepharose CL 6B column (2.2 cm × 15 cm) equilibrated in AA containing 25% ammonium sulfate and 0.5 mM CaCl<sub>2</sub>. The column was developed in an EGTA gradient (0.5-2.5 mM EGTA). S100 protein- and calmodulin-containing fractions were identified by urea-glycerol gel electrophoresis as described (Sobieszek and Jertschin, 1986) and the proteins collected by precipitation with 60% ammonium sulfate with the pH adjusted to 4.2. After dissolving the pellets in a minimal volume of AA buffer and desalting over an AcA 54 gel filtration column in the same buffer, the protein-containing fractions were applied onto a DEAE ion exchange column (2.2 cm × 20 cm). The column was washed and bound proteins were eluted with a NaCl gradient (2 cm × 500 ml AA, 60-400 mM NaCl). S100 proteins eluted at around 80 mM NaCl and were well separated from calmodulin, which eluted at 280 mM NaCl. The preparations were concentrated by ammonium sulfate, dissolved and dialyzed against AA containing 0.5 mM CaCl<sub>2</sub> and stored at -70°C until further use.

### Immunoprecipitations

Immunoprecipitations were performed essentially as described earlier (Gimona et al., 1995), with modifications as indicated. LLC PK1 cells, grown to 75% confluence, were washed three times with ice-cold PBS, pH 7.4, containing 2 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub>. Proteins for immunoprecipitation were extracted subsequently in 200 µl/60 mm dish of immunoprecipitation (IP) buffer (20 mM Imidazole, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM PMSF, 1 mM NaN<sub>3</sub>, pH 7.0) for 60 minutes on ice. The extract was collected and cellular residues were removed by centrifugation at 14,000 rpm in an Eppendorf Microfuge. The extracts were precleared for 60 minutes with Protein A-Sepharose (Pharmacia) in IP buffer and the supernatant was transferred to a fresh tube (washed twice for 5 minutes in IP buffer to reduce nonspecific binding). 5 µg of anti-S100A2 antibody was added and the suspension was incubated on ice for 60 minutes. Following the addition of 30 µl of Protein A-Sepharose, incubation was continued for another 60 minutes. The beads were washed three times in IP buffer and once in PBS and subsequently prepared for gel electrophoresis. Precipitated proteins were released from the beads by boiling in SDS sample mix for SDS-PAGE and western blotting analysis or with 9 M urea for 2-D analysis as previously described (Gimona et al., 1995).

### Cell culture and immunofluorescence

LLC PK1 cells (ATCC) were grown in high glucose DMEM supplemented with 10% FBS (HYCLONE), penicillin/streptomycin (Gibco) at 37°C and 5% CO<sub>2</sub>. For immunofluorescence microscopy cells were cultured on 12 mm glass coverslips. Cells were washed three times in Cytoskeleton buffer (10 mM MES, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 5 mM glucose, 5% (v/v) glycerol, 1 mM PMSF, 1 mM NaN<sub>3</sub>, pH 6.1). The cells were subsequently extracted in IP buffer containing 2 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub> for 5 minutes and fixed in 3% PFA for 30 minutes. Incubations with antibodies were performed in TBS (20 mM Tris-HCl, 154 mM NaCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1 mM NaN<sub>3</sub>, pH 7.5). Fluorescent images were recorded on a Zeiss Axiophot using a ×63 oil immersion lens and Kodak P3200 Tmax film.

### Electrophoresis and western blotting

Analytical SDS-gel electrophoresis on 10-20% gradient polyacrylamide mini-slab gels and western blotting onto nitrocellulose

(Hybond, Amersham) was performed as described elsewhere (Gimona et al., 1990). Transferred proteins were visualized using horseradish-peroxidase-coupled secondary antibodies and the ECL chemiluminescence detection system (Du Pont).

### Antibodies

Monoclonal anti-TM antibody (clone 311) was from Sigma, FITC and rhodamine-labelled phalloidin and secondary antibodies were from Molecular Probes. Monoclonal anti-porcine S100A2 (SH-L1) and anti-porcine Calcyclin (clone CACY-100) were produced in mice using purified porcine smooth muscle S100 proteins as antigens, essentially as described (Gimona et al., 1994). Briefly, the immunogen was dissolved in PBS and Balb/c mice were immunized with 20 µg of protein emulsified in Freund's complete adjuvant. Booster injections were given at 3-week intervals and fusion between spleen cells of immunized mice and NS-1 mouse myeloma cells was performed 3 days after the third booster injection. Hybridization was induced by exposure of the mixed cells to polyethylene glycol (molecular mass 1500 Da). After fusion, mixed cell cultures were grown in DMEM supplemented with 10% heat-inactivated horse serum containing  $10^{-4}$  M hypoxanthine,  $4 \times 10^{-7}$  M aminopterin and  $1.6 \times 10^{-5}$  M thymidine (HAT medium). Cultures were re-fed with DMEM-HAT medium every 2-3 days. After 10-14 days culture, supernatants were screened for specific antibodies by ELISA and immunoblotting using the immunogen as probe. Positive cells were isolated and cells were cloned by the limiting dilution method. The mouse immunoglobulin isotype (both IgG1) was determined using the Sigma Isotype Kit (ISO-1). The antibodies are now commercially available from Sigma Chemical Company.

### Column binding assays

Recombinant TMs were expressed and purified according to Pittenger et al. (1992, 1995). 2 ml of a 5 mg/ml solution were bound to 1 ml *N*-hydroxysuccinimide (NHS)-activated Sepharose columns (Pharmacia) according to the manufacturer's instructions and blocked with 200 mM glycine. LLC PK1 cell extracts were passed through the columns in IP buffer containing either 2 mM CaCl<sub>2</sub> or 2 mM EGTA/2 mM EDTA, and washed in the same buffer. The specificity of the binding was probed by washing the column in the respective buffers containing 500 mM KCl, and the S100A2 retained on the column was finally eluted by the addition of 2 mM EGTA/2 mM EDTA and collected in 1 ml fractions.

### Metabolic labelling of LLC PK1 cells

Cells grown to 70% confluence in 100 mm Petri dishes were incubated for 16 hours in methionine-free DMEM (Gibco) in the presence of 100 µCi [<sup>35</sup>S]Met-translabel (New England Nuclear). Extracts for immunoprecipitations were made as described above.

### Two-dimensional gel electrophoresis

[<sup>35</sup>S]Methionine-labelled cell extracts and immunoprecipitated sub-fractions were solubilized in 9 M urea (United States Biochemicals) and the proteins analyzed on IEF gels (pH range 3-10) in the first dimension and on 15% slab SDS-PAGE gels in the second dimension as described by Garrels (1983), with the modifications given by Patterson and Latter (1993). Gels were either dried and exposed to X-ray films for 7-14 days or subsequently blotted onto nitrocellulose for western blotting analysis (see above).

### Chemical crosslinking by EDC/NHS

Proteins were crosslinked in solution (20 mM Imidazole, pH 7.0, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>) at 1:1 molar ratios using 5 mM NHS and 5 mM EDC (Sigma) as described (Leszyk et al., 1990). Crosslinking reactions were stopped and prepared for SDS-PAGE by the addition of an equal volume of SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTE, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and subsequent boiling for 2 minutes.

### Microsequencing

S100 proteins (about 40 µg) were separated by reversed phase HPLC (Hewlett-Packard 1090) on a Vydac C4 column (2.1 mm × 250 mm, 5 µm, 300 Å). Proteins were eluted with an acetonitrile gradient and monitored by UV absorbance at 214 and 280 nm, and each fraction was tested with S100A2 and S100A6 antibodies on western blots. Fractions used for sequencing were lyophilized and dissolved in 200 µl 0.1 M Tris-HCl, pH 9.0, and digested with 300 ng of *Achromobacter* protease I for 20 hours at 30°C. The digests were further separated on a Vydac C18 reversed phase HPLC column (1 mm × 250 mm, 10 µm, 300 Å) and the peaks subjected to peptide sequencing in an automated protein sequencer (Applied Biosystems, model 470A, 473A or 477A).

## RESULTS

### Isolation and microsequencing of S100A2

S100 proteins were purified from porcine and avian smooth muscle tissue on the basis of their interaction with a hydrophobic chromatography matrix in the presence of Ca<sup>2+</sup>. Purified porcine stomach S100 proteins separated into two fractions after reversed phase HPLC (on C4 column), the first consisting of S100A6 (not shown) and the second containing S100A2 (Fig. 1A). Peak fractions of both proteins were digested and sequenced (see Materials and Methods). From several overlapping fragments a continuous stretch of 72 amino acids was generated (Fig. 1B, lowest sequence) and sequence comparisons revealed 100% identity to the sequence of bovine S100L (or S100A2, according to the new nomenclature).

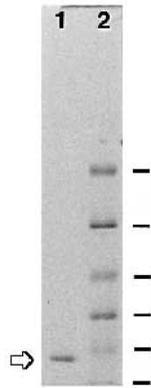
### Specificity of monoclonal anti-S100A2 antibody

The monoclonal S100A2 antibody (clone SH-L1) recognized a single band on western blots of extracts of pig stomach and chicken gizzard smooth muscle and porcine LLC PK1 cells (Fig. 2, lanes 2-4). No reactions, however, were obtained in preparations of mouse uterus smooth muscle and cultured rat embryo fibroblast cells (Fig 2, lanes 5 and 6), nor with a variety of other rodent and human cell lines (data not shown), indicating either species specificity of the antibody or the absence of the protein in the cell lines and tissues tested. The S100A2 antibody did not cross-react with purified smooth muscle caltropin or bovine S100b (kindly provided by Dr C. M. Kay), but specifically recognized a single spot on two-dimensional gels of whole cell extracts corresponding to that of S100A2 (M. Gimona, unpublished).

### Column binding assays

Recombinant TM 1 was immobilized on NHS-activated Sepharose columns (see Materials and Methods). Extracts of LLC PK1 cells prepared in the presence of 2 mM CaCl<sub>2</sub> or 2 mM EGTA/EDTA were passed through the columns in the presence or absence of 2 mM CaCl<sub>2</sub>. Western blots demonstrated that S100A2 bound to the immobilized TM in a Ca<sup>2+</sup>-dependent manner (Fig. 3), although only about 20% of the soluble S100A2 present in the cell extracts was retained on the column. Bound S100A2 remained associated with immobilized TM even at high ionic strength (up to 500 mM KCl) in the presence of 2 mM CaCl<sub>2</sub> (Fig. 3B), but was readily eluted from the column by the addition of 2 mM EGTA/EDTA (Fig. 3C). Binding was completely abolished in extracts prepared and applied onto the column in the presence of 2 mM EGTA/EDTA. Similar binding was also observed with recom-

A



**Fig. 1.** Purification and sequencing of S100A2 from porcine stomach smooth muscle. (A) Coomassie blue-stained 10-20% SDS gel of purified S100A2. Lane 1, purified S100A2 (open arrow); lane 2, molecular mass markers (from top to bottom: 68, 46, 30, 21.5, 14 and 6.5 kDa, respectively). (B) Purified S100A2 proteins were separated by reversed phase HPLC, peak fractions digested in situ and individual fragments repurified on reversed phase HPLC (see Materials and Methods). The combined amino acid sequences obtained (lowest sequence) are aligned with those of other S100 family proteins identifying the sequenced protein as the porcine S100A2 homolog. po., porcine; hu., human; bo., bovine; \* indicates identical amino acid. Sequences were obtained from Kawasaki and Kretsinger (1994).

B

	1	10	20	30	40
hu. S100b	- S E L E K A M V A L I D V F H Q Y S G R E G D K H K L K K S E L K E L I N N E L S H F L E				
po. S100b	- S E L E K A V V A L I D V F H Q Y S G R E G D K H K L K K S E L K E L I N S E L S H F L E				
hu. CAPL	A C P L E K A L D V M V S T F H K Y S G K E G D K F K L N K S E L K E L L T R E L P S F L G				
bo. S100A2	S S P L E Q A L A V M V A T F H K Y S G Q E G D K F K L S K G E M K E L L H K E L P S F V G				
	*****				
po. S100 A2	S P L E Q A L A V M V A T F H K Y S G Q E G D K F K L S K G E M K E L L H K E L P S F V G				
	50	60	70	80	90
	E I K E Q E V V D K V M E T L D N D G D G E C D F Q E F M A F V A M V T T A C H E F F E H E				
	E I K E Q E V V D K V M E T L D S D G D G E C D F Q E F M A F V A M V T T A C H E F F E H E				
	K R T D E A A F Q K L M S N L D S N R D N E V D F Q E Y C V F L S C I A M M C N E F F E G F				
	E K V D E E G L K K L M G D L D E N S D Q Q V D F Q E Y A V F L A L I T I M C N D F F Q G S				
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binant TM 2, TM 3, TM 5a, TM  $\alpha$ -smooth and TM  $\beta$ -skeletal (not shown), suggesting that the binding site(s) may reside in region(s) common to all TM isoforms.

Binding of S100A2 to immobilized TMs was competed by preincubation of the cell extracts with 1 mg/ml free recombinant TM 1 or TM 5a prior to application onto the column. Although the amount of S100A2 retained on the column was reduced by about 80% as compared to the uncompleted extracts, binding was not completely abolished (data not shown).

### Chemical zero-length crosslinking

Purified recombinant TMs (TM1, TM 2, TM3, TM5a, TM  $\alpha$ -smooth and TM  $\beta$ -skeletal), as well as two C-terminal truncations ('cla') lacking the carboxy-terminal 27 amino acids of skeletal  $\alpha$ -TM and skeletal  $\beta$ -TM (see Pittenger et al., 1995), were chemically crosslinked with 5 mM NHS and 5 mM EDC. As shown in Fig. 4, for TM 1, dimer and tetramer species were formed and this result was independent of the presence of CaCl<sub>2</sub>. Identical results were obtained with all the recombinant TMs tested (not shown). In contrast, purified S100A2 could not be crosslinked into higher molecular mass aggregates by EDC even in the presence of 2 mM CaCl<sub>2</sub> (see Fig. 5).

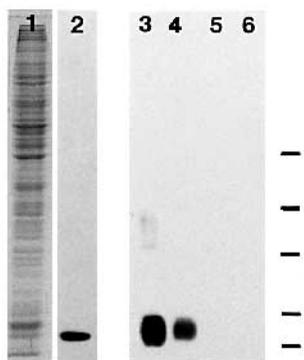
However, when TMs and S100A2 were incubated together in the presence of 2 mM CaCl<sub>2</sub> and crosslinked with EDC/NHS, a novel band, migrating about 10 kDa higher than the respective TM monomer band, appeared on Coomassie blue-stained SDS-PAGE gels (Fig. 5). Fig. 6 shows the Ca<sup>2+</sup>-dependent formation of this band for three TM isoforms, in

combination with S100A2. Western blot analysis showed that the additional band contained TM, as indicated by the cross reaction with an anti-TM antibody (clone TM 311) (Fig. 6B). When such western blots were probed sequentially with the anti-S100A2 antibody (Fig. 7A and C) followed by reaction with anti-TM antibody (Fig. 7B and D), the additional band was seen to contain both S100A2 and TM. The generation of this TM:S100A2 hybrid band was observed with all TM isoforms tested. The new band corresponded in its mobility on SDS-PAGE gels to a 1:1 complex between TM and S100A2.

### Immunoprecipitation using extracts of LLC PK1 cells

The S100A2 antibody selectively precipitated S100A2 from extracts of LLC PK1 cells, but only in the presence of Ca<sup>2+</sup> (Fig. 8, lane 2). Extracts of LLC PK1 cells radioactively labelled with [<sup>35</sup>S]methionine were used to detect proteins that co-immunoprecipitated with S100A2. The same dependence on the presence of Ca<sup>2+</sup> in the precipitation mixture that was shown in the column binding assays was seen for the immunoprecipitation. Autoradiographs from 10-20% SDS gels revealed the presence of an additional band of slightly higher molecular mass in the S100A2 precipitates (Fig. 9A, lane 1, arrow). No bands in this area could be detected in control precipitations using nonspecific mouse antisera (lane 2).

Two-dimensional gels of whole LLC PK1 cell extracts and of immunoprecipitated material were electroblotted onto nitrocellulose and probed with either anti-S100A2 or anti-calcylin

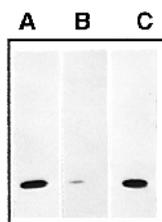


**Fig. 2.** Characterization of S100A2 antibody. Lane 1, Coomassie blue-stained gel of LLC PK1 whole cell extract; lanes 2-6, western blots probed with anti-S100A2 antibody. Cell and tissue extracts were electrophoresed on 10-20% SDS-PAGE gels and electroblotted onto nitrocellulose. The antibody reacts with a single band of apparent molecular mass of 10 kDa in extracts of LLC PK1 cells (lane 2), pig stomach smooth muscle (lane 3) and chicken gizzard smooth muscle (lane 4), but not with extracts from rat embryo fibroblasts (lane 5) and mouse uterus smooth muscle (lane 6). Molecular mass markers from top to bottom: 46, 30, 21.5, 14 and 6.5 kDa, respectively.

(S100A6) antibody to determine the position of the respective proteins. The observed positions for these two proteins, in the pI range of 4.1-4.3, are in agreement for their reported pI values. Identical gels were prepared for autoradiography and exposed for 2 to 3 weeks. The gels revealed several low molecular mass polypeptides co-precipitating with S100A2. S100A6, which runs close to S100A2 (Fig. 9B,C) was not present in the precipitate, as confirmed by western blotting using a monoclonal antibody specific for S100A6 (not shown).

### Cellular localization

LLC PK1 cells grown in culture show at least two morphologically distinct phenotypes, one fibroblast-like and one epithelial-like. Sparsely seeded cells grow as single cells or in small clusters and exhibit a fibroblastic phenotype containing several prominent stress fibers (Fig. 10A). At the periphery of cell clusters these actin bundles are often found to be organized

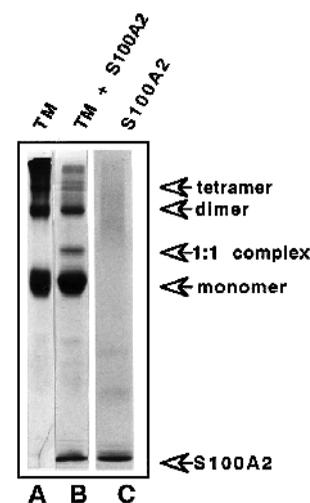


**Fig. 3.**  $\text{Ca}^{2+}$ -dependent binding of S100A2 to immobilized tropomyosin. Western blot probed with anti-S100A2 antibody. Whole cell extracts from a confluent 9 cm cell culture dish of LLC PK1 cells prepared in IP buffer were applied onto a 1 ml HiTrap column (Pharmacia) carrying immobilized recombinant TM 1 (see Materials and Methods) and washed with 10 column volumes of the same buffer. Bound S100A2 remained on the column at high ionic strength (IP buffer containing 500 mM KCl, lane B) in the presence of 2 mM  $\text{CaCl}_2$  and was eluted by the addition of 2 mM EGTA to the wash buffer (lane C).

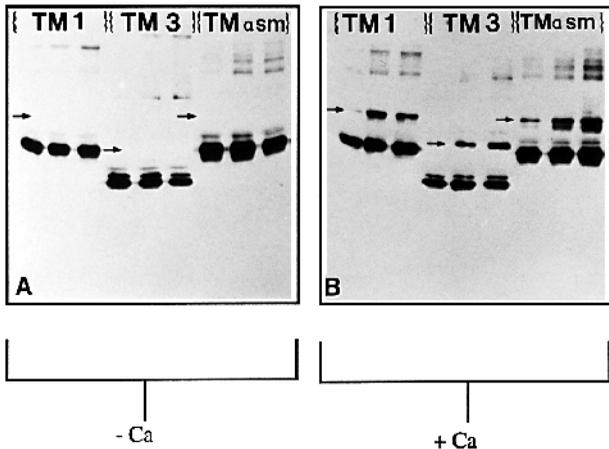


**Fig. 4.** EDC crosslinking of recombinant TM 1. Western blot of TM 1 crosslinked in the presence of 2 mM  $\text{CaCl}_2$  and probed with monoclonal TM antibody (clone TM 311). Proteins were incubated for 15 minutes at 25°C in 20 mM Imidazole, 2 mM  $\text{MgCl}_2$ , 1 mM  $\text{NaN}_3$ , pH 7.0, in the presence of either 2 mM  $\text{CaCl}_2$  or 2 mM EGTA, prior to the addition of NHS and EDC and reactions were stopped by the addition of  $\beta$ -mercaptoethanol followed by subsequent boiling and preparation for SDS-PAGE analysis. Samples were removed from the protein/EDC mixtures at several incubation times and were analyzed on SDS-PAGE gels. Upon continuing incubation the crosslinking of the TM monomer subunit into the dimeric and tetrameric species was observed. Times of incubation in minutes are indicated.

into thick, cable-like structures (Fig. 10C). When such cells were stained for S100A2 no distinct localization for this protein could be observed (Fig. 10B and D). S100A2 was found to be distributed diffusely throughout the cytoplasm and no colocalization with actin or TM was observed. Tropomyosin was found, as expected, to colocalize with actin stress fibers (Fig. 11A,A') but could not be detected in microvilli (Fig. 11B,B').

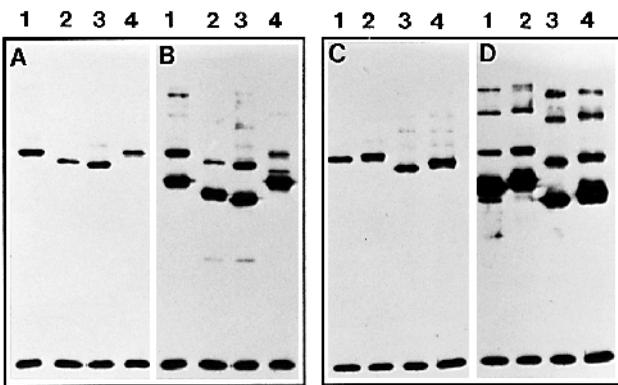


**Fig. 5.** EDC crosslinking TM 1 and S100A2. Coomassie blue-stained 10-20% SDS-PAGE gel. Proteins were incubated as in Fig. 4 and crosslinked for 15 minutes at 25°C with 5 mM EDC, 5 mM NHS. When S100A2 and TM 1 were combined at an equimolar ratio prior to crosslinking, a new band of about 46 kDa was generated (B), which corresponds to a 1:1 complex of TM 1 and S100A2. This band is not seen with either TM 1 (A) or S100A2 (C) alone. S100A2 shows no EDC-induced aggregation under the assay conditions. Note the reduction in dimer and tetramer stabilization in the presence of S100A2.

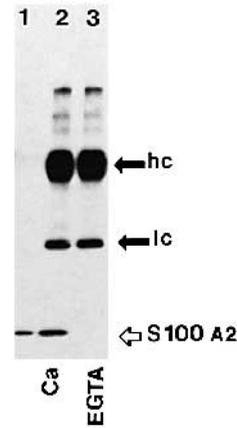


**Fig. 6.** The association of S100A2 with TM is Ca<sup>2+</sup>-sensitive. Western blots of proteins crosslinked in the absence (A) or presence (B) of 2 mM CaCl<sub>2</sub> probed with antibody TM 311. Arrows indicate the position of the new band observed in Coomassie blue-stained gels. Samples were taken after 1, 5 and 15 minutes of incubation with 5 mM EDC, 5 mM NHS. Results are shown for three TM isoforms: TM1, TM3 and TM α-smooth.

When LLC PK1 cells were grown to a higher density, the cell shape changed to a more compact and often dome-shaped morphology, characteristic for epithelial cells. In many cases the cells spontaneously differentiated into epithelia and extended a large number of microvilli at the apical surface of the cells (Fig. 12A). The few stress fibers still present in the differentiated cells remained at the basal surface. When such dense cultures were double stained with anti-S100A2 antibody and Phalloidin it was evident that S100A2 undergoes a significant cellular redistribution. Whereas S100A2 remained distributed diffusely throughout the cytoplasm in cells which had not formed microvilli on their apical surface, differentiated



**Fig. 7.** The additional crosslinked product contains both TM and S100A2. Western blots of 10-20% SDS-PAGE gels sequentially probed with S100A2 (A and C) and TM 311 antibodies (B and D). The band at 46-48 kDa (depending on the TM isoform used) corresponding to that observed in the Coomassie blue-stained gel is recognized by both antibodies, indicating the presence of both S100A2 and TM in this complex. Samples were taken after 15 minutes of incubation with 5 mM EDC, 5 mM NHS. (A and B) Lane 1, TM 1; lane 2, TM 2; lane 3, TM 3; lane 4, TM α-smooth. (C and D) Lane 1, TM α-skeletal; lane 2, TM β-skeletal; lane 3, TM α-skeletal/cla; lane 4, TM β-skeletal/cla (see Results).

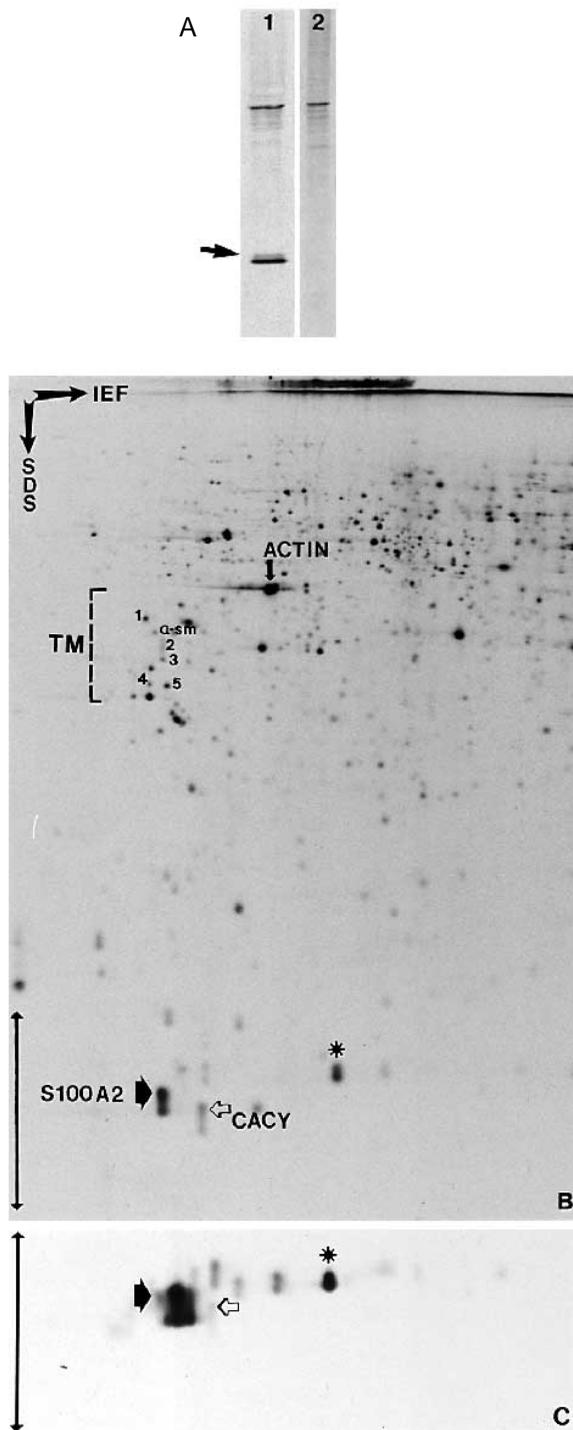


**Fig. 8.** Immunoprecipitation of S100A2 is Ca<sup>2+</sup> sensitive. Western blot of a 10-20% SDS-PAGE gel probed with the anti-S100A2 antibody. Extracts of LLC PK1 cells were immunoprecipitated with anti-S100A2 antibody in the presence of 2 mM CaCl<sub>2</sub> (lane 2) or 2 mM EGTA (lane 3). The S100A2 band (open arrow) is recognized by the antibody in the precipitate containing CaCl<sub>2</sub> (lane 2) and the extract (lane 1), but not in the EGTA extract. Arrows indicate the positions of the heavy (hc) and light (lc) chains of the antibody.

cells showed a restricted colocalization of S100A2 with actin in the micovilli (Fig. 12).

## DISCUSSION

Further knowledge about the possible targets of EF hand-containing Ca<sup>2+</sup>-binding proteins is required to gain more insight into their *in vivo* functions. Our interest has focused on the identification of novel smooth muscle proteins involved in the regulation of the cytoskeleton *in vivo*. We show that S100A2 interacts with recombinant TM in a Ca<sup>2+</sup>-dependent fashion. We have recently demonstrated that another Ca<sup>2+</sup>-binding protein, caltropin, binds directly to smooth muscle calponin (CaP), a potential modulator of the contraction/relaxation events in this cell type (Wills et al., 1994a,b). In the present study we have purified another member of the S100 family of Ca<sup>2+</sup>-binding proteins from porcine and avian smooth muscle tissue, namely S100A2, and demonstrate that porcine S100A2 shares a 100% sequence identity over the N-terminal 72 amino acids to the bovine variant (S100L) first described by Glenney et al. (1989). S100L was found to be localized diffusely in the cytoplasm and the nucleus of bovine epithelial cells (MDBK), and no association with structures of the actin cytoskeleton or a direct interaction with any other cytoskeletal or cytoplasmic protein could be determined (Glenney et al., 1989). We have raised a monoclonal antibody against porcine S100A2, which recognizes its antigen on western blots of SDS gels in both the presence and absence of Ca<sup>2+</sup>. Since the antibody, however, fails to complex with its antigen in the presence of EGTA (or EDTA) in immunoprecipitation experiments, we conclude that the epitope is in the vicinity of the Ca<sup>2+</sup>-binding loops of S100A2. The binding of Ca<sup>2+</sup> has been shown to induce conformational changes in S100 proteins (Baudier and Gerard, 1986; Becker et al., 1992; Mani and Kay, 1987), which may render the epitope inaccessible to the antibody in the Ca<sup>2+</sup>-free



**Fig. 9.** One- (A) and two-dimensional (B and C) gel electrophoresis of immunoprecipitated proteins. (A) 10-20% SDS-PAGE. Autoradiograph of  $^{35}\text{S}$ -labelled LLC PK1 extracts immunoprecipitated with S100A2 antibody (lane 1) or nonspecific serum (lane 2) in the presence of 2 mM  $\text{CaCl}_2$ . Note the additional band (arrow) migrating above the S100A2 band. (B and C) Autoradiographs of 15% SDS-PAGE gels. (B) Whole LLC PK1 extract. (C) Immunoprecipitation with S100A2 antibody in the presence of 2 mM  $\text{CaCl}_2$ . The positions of actin, tropomyosins (TM), S100A2 (solid arrow) and calyculin (open arrow) are indicated. Note the co-precipitation of an additional low molecular mass polypeptide (asterisk).

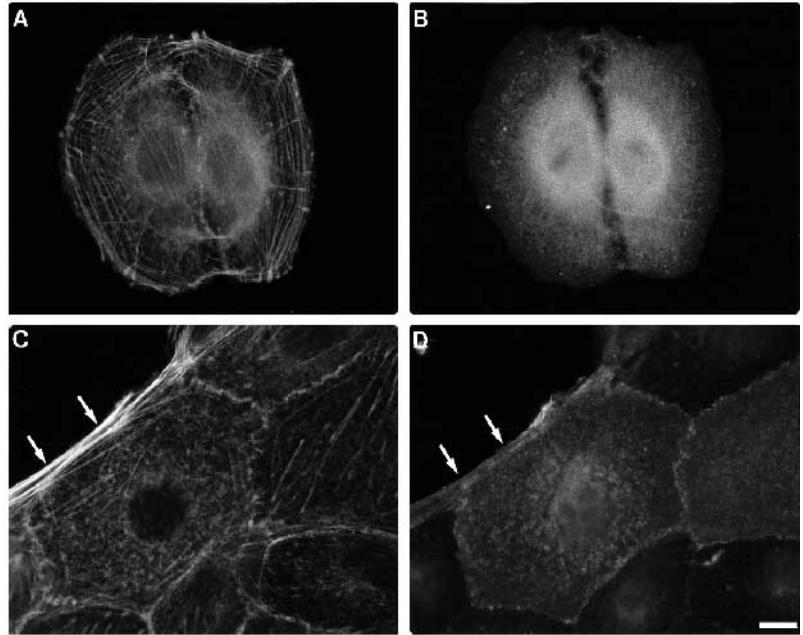
form whereas it may be exposed when the molecule is in the  $\text{Ca}^{2+}$ -bound state.

Since there is the possibility that the binding of the SH-L1 antibody to S100A2 blocks the binding site used in the interaction with TM (which in turn would prevent the co-precipitation of the two polypeptides), we have performed the reverse experiment, using a monoclonal TM antibody (clone TM 311). This antibody precipitated TMs only, and no S100A2, supporting the previous suggestion that the interaction between S100A2 and TM in solution is only transient (M. Gimona and D. Helfman, unpublished).

### In vitro association of S100A2 with TMs

Recently, Takenaga et al. (1994a,b,c), using truncated recombinant TM 2 and TM 5, have demonstrated that another  $\text{Ca}^{2+}$ -binding protein, S100A4 (also known as pEL 98, *mts* 1, CAPL), which shares about 60% sequence homology with S100A2, can interact with TM in vitro and that this association is also dependent on the presence of  $\text{Ca}^{2+}$ . In our column-binding and crosslinking experiments we demonstrate that S100A2 also binds directly to TMs in a  $\text{Ca}^{2+}$ -dependent fashion. This association is seen with both muscle and nonmuscle TMs, as well as with C-terminally truncated TM molecules, indicating that the binding site may reside in one of the conserved regions of the molecule encoded by exons 3-5 and 7-8 (Lees-Miller and Helfman, 1991). Chemical crosslinking of recombinant TMs with purified S100A2 results in the formation of an additional band migrating above the TM monomer subunit on SDS gels. This new band contains both TM and S100A2, as demonstrated by differential immunostaining of western blots, and represents a 1:1 complex between TM and S100A2 as predicted from its electrophoretic mobility (molecular mass between 42-48 kDa). There are two possible explanations for the generation of this complex. TM in solution is in an equilibrium state between monomers and dimers, although the dimer represents the thermodynamically favorable conformation. If S100A2 associates with the monomeric fraction of TM, the 1:1 complexes should be the predominant species generated by zero-length crosslinking. If, however, the interaction between S100A2 and TM occurs while TM is in its dimeric state, the stable crosslinking of a 1:1 complex infers that this interaction possibly influences the ability of TM to dimerize for the following reasons. Crosslinking of one (or two) molecule(s) of S100A2 to the existing TM dimer (Fig. 13A) would result in the generation of a band migrating above the crosslinked TM dimer band (at around 85 kDa in the case of a  $2 \times \text{TM} + 1 \times \text{S100A2}$ , or 95 kDa for  $2 \times \text{TM} + 2 \times \text{S100A2}$ ), assuming that the TM dimer is equally accessible to EDC and is crosslinked at least at the same rate as the S100A2:TM complex. A logical explanation for the observed crosslinking products is, therefore, that the binding of S100A2 to TM interferes with the coiled-coil of TM dimers in such a way that zero-length crosslinking is prevented due to an increased distance between the TM subunits (Fig. 13C). This increase in distance could be achieved by partially unfolding the coiled-coil TM molecule as a result of steric interference. Upon preparation for SDS-gel electrophoresis, these non-crosslinked TM subunits fall apart but carry the crosslinked S100A2 molecule with them, resulting in the generation of the new crosslinking product (Fig. 13D).

In agreement with our findings for S100A2, Golitsina et al.

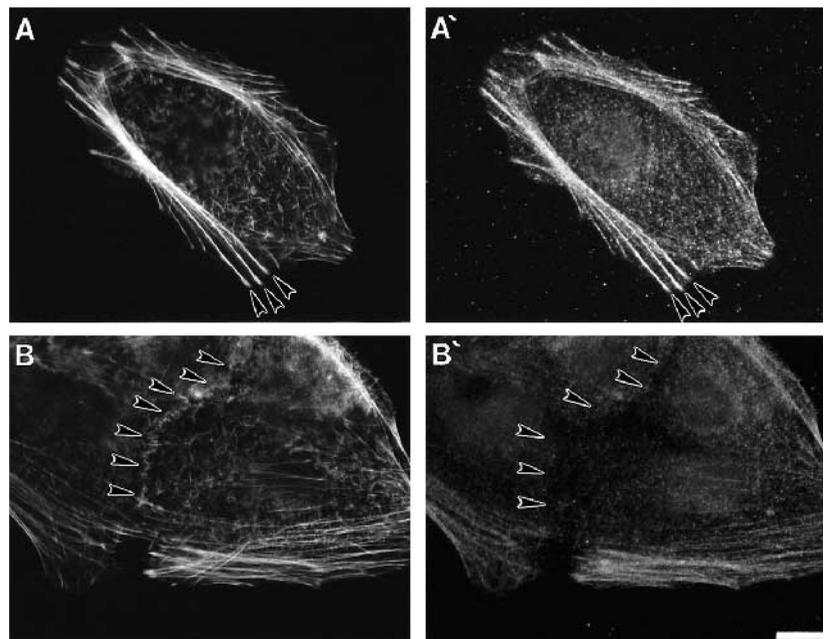


**Fig. 10.** S100A2 is not associated with actin-containing stress fibers. Immunofluorescence staining showing the diffuse cytoplasmic and perinuclear localization of S100A2 in LLC PK1 cells in fibroblastic growth phase. (A,C) Phalloidin-stained cells. (B,D) The same cells stained for S100A2. Sparsely seeded cells exhibit a well-defined actin stress fiber network. Note thick actin bundles at the boundaries of cells (arrows in C). S100A2 is absent from both structures. Bar, 20  $\mu$ m.

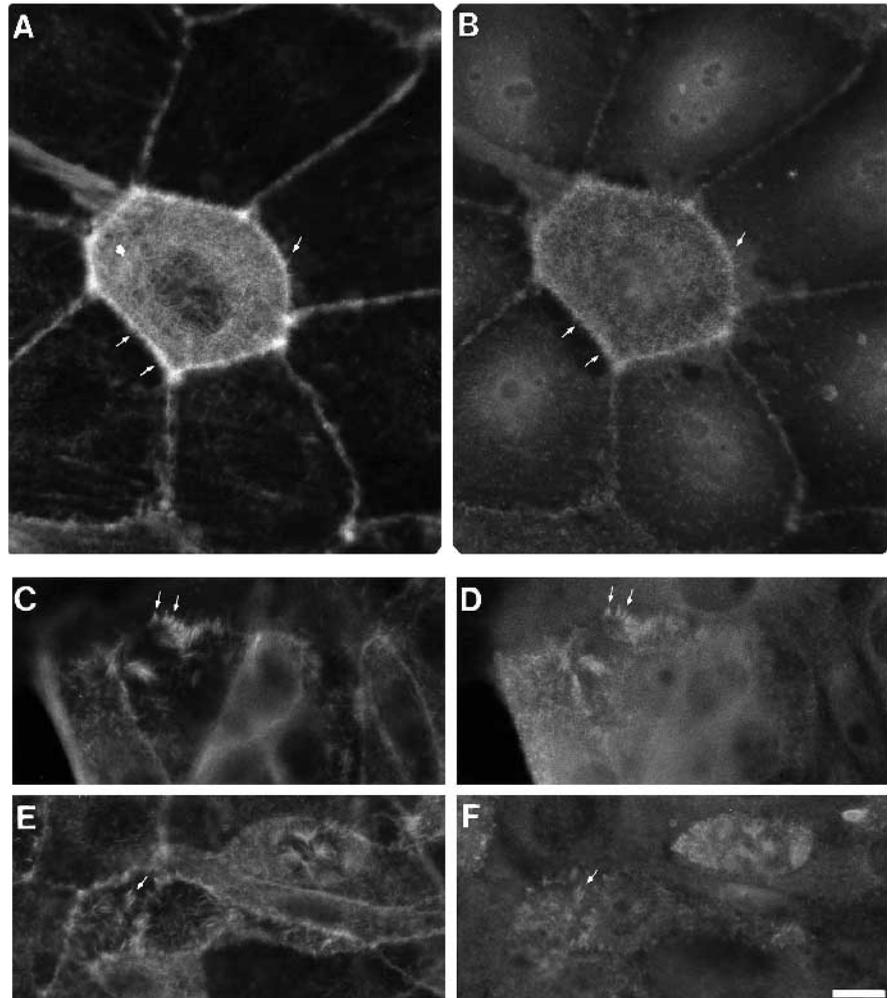
(1996) demonstrated the association of calyculin (S100A6) with muscle TMs, forming UV crosslinked complexes between S100A6 monomers and TM. This association, however, was seen not to cause the dissociation of actin-bound TM from the filament, indicating functional differences between the two S100 proteins.

In our crosslinking experiments we failed to generate EDC crosslinked S100A2 homodimers. It was suggested earlier that S100A2 in solution exists as a dimer, based on gel filtration studies (Glenney et al., 1989), and homo- or heterodimers represent the native functional conformation found in a number of S100 proteins (Barger et al., 1992; Donato, 1991; Gerke and Weber, 1985a; Pedrocchi et al., 1994b). Golitsina et al. (1996) have further demonstrated, that S100A6 can be dimerized in

solution by oxidation on cysteine residues, resulting in the formation of interchain SH- bridges. The crosslinking procedure employed in this study stabilizes molecular interactions predominantly on lysine residues. S100A2 contains nine such residues at positions 17, 25, 27, 30, 34, 39, 48, 55 and 56, but only one cysteine residue at position 85. The use of only one attachment site between two monomers of S100A2 situated close to the C-terminal end of the molecule could allow for rotational freedom in solution, resulting in distances between the two polypeptide chains big enough to prevent the EDC crosslinking, which depends on a minimal distance of the crosslinkable side chains. We are currently investigating the possibility of interchain SH- bridges in S100A2. Alternatively, the co-immunoprecipitation of another low molecular mass



**Fig. 11.** Tropomyosin localizes in actin stress fibers but is absent in microvilli. Whereas tropomyosin is associated with F-actin along the entire length of actin stress fibers (arrowheads in A and A'), no immunostaining is obtained in the microvilli (arrowheads in B and B'). (A and B) Phalloidin; (A' and B') anti-TM. Bar, 20  $\mu$ m.



**Fig. 12.** S100A2 colocalizes with F-actin in microvilli of LLC PK1 cells. Immunofluorescence staining demonstrating the colocalization of F-actin and S100A2 in microvilli (arrows). Cells grown at high density for 24 hours in the presence of 10% FBS show spontaneous differentiation into brush border-type cells and develop microvilli at their apical surface. S100A2 is found concentrated in these structures. (A,C,E): phalloidin; (B,D,F): SH-L1. Bar, 20  $\mu\text{m}$ .

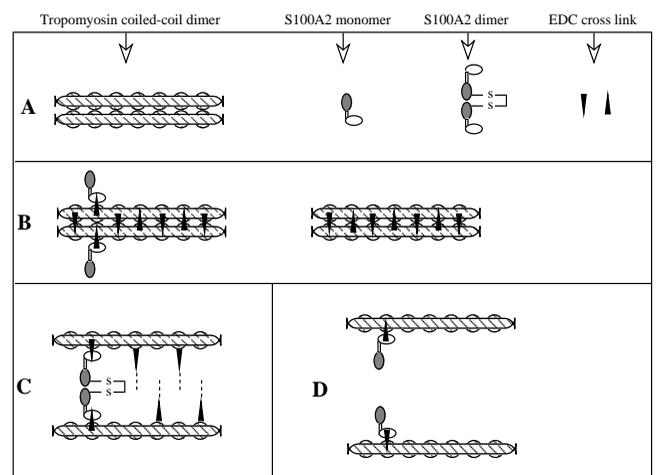
protein in a divalent cation-dependent fashion suggests the formation of a heterodimeric complex of S100A2 *in vivo*.

### Cellular localization in LLC PK1 cells

Whereas the distribution of S100A2 in subconfluent LLC PK1 cultures exhibits the same diffuse pattern as observed by Glenney and coworkers (Glenney et al., 1989) in MDBK cells, the ability of the former cell line to undergo spontaneous differentiation into microvilli-containing epithelial-like cells (Amieva and Furthmayr, 1995) reveals a differentiation-dependent localization, namely in the microvilli formed on the

apical surface. This observation is in agreement with earlier findings for S100 $\beta$  and CaM, which were also found to exhibit differential localization patterns upon changes in the cellular organization (Zimmer and Van Eldik, 1989). The expression of many S100 proteins has been found to be linked to differentiation or cell cycle-related events, suggesting the possibility that temporal and spatial regulation is necessary for the con-

**Fig. 13.** Schematic diagram of S100A2-TM crosslinking products. In solution, the coiled-coil dimer is the predominant form of TM, whereas S100A2 may exist as monomers or disulfide-crosslinked dimers (A). Upon addition of the crosslinking agent EDC (arrowheads in A), both TM:TM and TM:S100A2 complexes become irreversibly stabilized (B). In addition to the TM dimers, the formation of a 2:2 complex between TM and S100A2 could be formed. The 1:1 complex observed in the crosslinking experiments can be generated by preventing crosslinking of the two TM subunits by increasing the distance of the two TM chains (C). Upon preparation for electrophoresis the S-S bridges in S100A2 are being reduced, thereby liberating the irreversibly crosslinked 1:1 complex consisting of one TM subunit and one molecule of S100A2 (D).



trolled interaction with cytoskeletal components. The expression levels of several S100 proteins have been demonstrated to be elevated during tumorigenesis and metastasis and it has been suggested that abnormal expression of these proteins may contribute to the development of a metastatic phenotype. In contrast to other members of the S100 family, the expression of S100A2 has been reported to be downregulated in tumor cells (Lee et al., 1992), further supporting the hypothesis that S100A2 is involved in the organization of the cytoskeleton.

The localization data appear to contradict the results obtained from the in vitro binding and crosslinking experiments. S100A2 and TM exhibit a mutually exclusive localization pattern in LLC PK1 cells, with S100A2 being absent from the TM-coated actin stress fibers whereas it is present in the microvilli, which contain tightly bundled actin devoid of TM. This discrepancy may, however, point towards a possible physiological role for S100A2 in vivo. The formation of a coiled-coil dimer is a requirement for correct TM function and F-actin binding. The generation of a 1:1 complex between S100A2 and TM in solution will shift the monomer-dimer equilibrium of TM to the left and a high concentration of S100A2 in the microvilli would therefore be capable of preventing TM from binding to F-actin bundles in these structures. The observations made earlier (Burgess et al., 1987) that TM inhibits the bundling action of villin in vitro lends support to this hypothesis and is further in agreement with the recent findings by Takenaga et al. (1994c), who suggested that a possible function for pEL98 (S100A4) could be to prevent the interaction of TM with actin. Alternatively, the Ca<sup>2+</sup>-dependent unfolding of the TM dimer by S100A2 might expose the single TM subunits to proteolytic degradation, further preventing TM from competing for binding sites on the F-actin filament.

We therefore propose a direct modulatory role for S100A2 in the organization of the actin cytoskeleton via a Ca<sup>2+</sup>-sensitive interaction with TM. Further studies using recombinant S100A2 will have to address the identification of the binding site(s) for TM in S100A2 and the nature of the polypeptides identified in the co-immunoprecipitations, as well as the localization and functional role of S100A2 in smooth muscle.

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