

# Isolation of a New Member of the S100 Protein Family: Amino Acid Sequence, Tissue, and Subcellular Distribution

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**Abstract.** A low molecular mass protein which we term S100L was isolated from bovine lung. S100L possesses many of the properties of brain S100 such as self association, Ca<sup>++</sup>-binding (2 sites per subunit) with moderate affinity, and exposure of a hydrophobic site upon Ca<sup>++</sup>-saturation. Antibodies to brain S100 proteins, however, do not cross react with S100L. Tryptic peptides derived from S100L were sequenced revealing similarity to other members of the S100 family. Oligonucleotide probes based on these sequences were used to screen a cDNA library derived from a bovine kidney cell line (MDBK). A 562-nucleotide cDNA was sequenced and found to contain the complete coding region of S100L. The predicted amino acid sequence displays striking similarity, yet is clearly

distinct from other members of the S100 protein family. Polyclonal and monoclonal antibodies were raised against S100L and used to determine the tissue and subcellular distribution of this molecule. The S100L protein is expressed at high levels in bovine kidney and lung tissue, low levels in brain and intestine, with intermediate levels in muscle. The MDBK cell line was found to contain both S100L and the calpactin light chain, another member of this protein family. S100L was not found associated with a higher molecular mass subunit in MDBK cells while the calpactin light chain was tightly bound to the calpactin heavy chain. Double label immunofluorescence microscopy confirmed the observation that the calpactin light chain and S100L have a different distribution in these cells.

**T**HE S100 proteins were first identified as low molecular mass, acidic Ca<sup>++</sup>-binding proteins from brain tissue (42). S100 purified from brain is composed of two distinct polypeptides,  $\alpha$  and  $\beta$  (26, 27), which are the same size and share 58% amino acid sequence identity (25). Originally thought to be exclusively in brain, more recent evidence has shown that both the  $\alpha$  and  $\beta$  subunits of S100 also exist in other tissues (32, 40, 41, 51). Further sequence analysis has revealed that S100 is a member of a larger protein family that includes the cystic fibrosis antigen (6) also termed mrp-8 (44), the light chain of the cytoskeletal protein calpactin (8, 12, 18, 23), and predicted proteins derived from RNA abundantly expressed in fibroblasts (termed pEL98 [21], 18A2 [28], 42A [37], or p9kA [1]), and growth factor-treated cells (termed 2A9 or calcyclin [4, 9]).

The most extensively studied proteins of this family are brain S100 and the calpactin light chain. Brain S100 proteins are isolated as homo or heterodimers of  $\alpha$  and  $\beta$  subunits (26, 27). Similarly the calpactin I light chain exists as a homodimer when the heavy chain is removed (13). The function of S100 is uncertain. Kligman and Marshak (33) purified a neurite extension factor that was identical by amino acid

sequence to the  $\beta$  subunit of S100. By contrast, Molin et al. have suggested that S100  $\alpha$  may be involved in pH, electrolyte, and water regulation, from the observation that it is found in salivary and sweat glands (40), as well as certain cells of the kidney (41). Other studies have suggested that S100 may regulate the phosphorylation of certain proteins (2, 22). Both S100  $\alpha$  and  $\beta$  subunits are known to bind Ca<sup>++</sup>-ions (24, 35, 36), a property not shared with the calpactin I light chain (13, 15).

Calpactins are members of a class of Ca<sup>++</sup>-binding proteins that interact with membrane lipids in vitro (10, 11, 14-16, 48). Two related but distinct calpactins (I and II) are also substrates of the tyrosine-specific protein kinases (7, 16, 46) and bind to actin filaments (11, 16). Calpactin I is isolated as a tetramer of heavy (38 kD) and light (11 kD) chain subunits, or as a 38-kD monomer, whereas other members of this family are found only as monomeric proteins (8, 11, 16). The calpactin I light chain binds to the amino terminal tail of the heavy chain (19, 30, 31), a region of the molecule that contains the sites of phosphorylation (18), and has been suggested to regulate the association of calpactin with the cytoskeleton (53, 45). Thus the calpactin light chain is thought to be a regulatory element in calpactin I. The amino acid sequence of the calpactin I light chain has revealed a 50% sequence identity with the  $\alpha$  subunit of S100 (12, 18, 23).

During the course of investigations of the calpactins we isolated a low molecular mass protein from lung which had

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many of the properties of brain S100. Further analysis has shown that this protein represents a new member of the S100 family, and accordingly we refer to it as S100L. Antibodies raised to S100L reveal that it is expressed at high levels in the Madin-Darby bovine kidney (MDBK)<sup>1</sup> cell line from which the cDNA encoding S100L was cloned. In addition to S100L, MDBK cells express the light chain of calpactin, however these two related proteins appear to be targeted differently.

## Materials and Methods

### Proteins and Antibodies

S100L was isolated from bovine lung as follows: Frozen lung (1 kg) was homogenized in 2 vol of 20 mM Tris (pH 8.0), 5 mM EDTA for 2 min in a waring blender. After centrifugation (15,000 g for 30 min at 4°C) the supernatant was adjusted to 60% ammonium sulfate, stirred for 30 min, and centrifuged as above. The supernatant was adjusted to 85% ammonium sulfate (pH 4.7), stirred for 30 min and centrifuged for 1 h at 15,000 g. The pellet was dissolved in 4 vol (~400 ml) 10 mM Tris, and CaCl<sub>2</sub> was added (from a 1 M stock) to 1 mM free Ca<sup>++</sup> while maintaining the pH at 7.5. After stirring for 1 h at 4°C, the solution was centrifuged at 100,000 g for 1 h and the supernatant fluid was applied to a 25-ml phenyl-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) column at room temperature. The column was washed with 20 vol of buffer I (100 mM NaCl, 10 mM imidazole, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, pH 7.3) followed by buffer I with 5 mM EGTA substituted for CaCl<sub>2</sub>. The effluent was monitored at 280 nm and the protein fraction that eluted with EGTA was collected and applied directly to a Mono-Q FPLC (Pharmacia Fine Chemicals) ion exchange column. This column was developed with a 100–500 mM NaCl gradient and fractions were analyzed by SDS-PAGE. The S100 peak was dialyzed against buffer I and applied to a second (1 ml) phenyl-Sepharose column, eluting as above. S100L was dialyzed against H<sub>2</sub>O and stored frozen at -80°C. The same procedure was used to isolate S100 from bovine brain and cardiac muscle. Antibodies were elicited in rabbits to cardiac and lung S100 proteins. Rabbits were immunized initially with 200–400 µg protein in 50% Freund's complete adjuvant followed by two immunizations of 200 µg S100 in 50% Freund's incomplete adjuvant at 6-wk intervals. 10 d after the last immunization rabbits were bled out by cardiac puncture while under general anesthesia. Balb/c mice were immunized as above but the final immunization was with 200 µg protein injected intravenously in PBS. Monoclonal antibodies were generated from these mice as in reference 53.

The complex of calpactin I heavy and light chains was purified as described previously (15). Polyclonal and monoclonal antibodies to the calpactin subunits were as in references 16 and 53. Rabbit antibodies to S100 α and β (51) were generously provided by Linda Van Eldik (Vanderbilt University). Rabbit antibodies to unfractionated brain S100 proteins were purchased from Dako Corp. (Santa Barbara, CA) and used at a dilution of 1:100.

### Peptide and DNA Sequence Determination

Intact S100L was subjected to sequence determination after electrophoresis, transfer to immobilon (millipore), and staining with Coomassie Blue, and was generously performed by Paul Matsudaira's laboratory (Whitehead Institution) as described in reference 38. Since no sequence was observed for 500 pmol S100L, we conclude that the amino terminus was blocked. The protein was digested with cyanogen bromide (25), or with trypsin (50 µg/ml) at 37°C overnight. Peptides were fractionated by reversed phase HPLC (C-8 column), eluting with a 20–80% acetonitrile gradient. Absorbance was monitored at 209 nm and selected peptides were analyzed by amino acid sequence analysis using a gas phase sequencer (Applied Biosystems, Inc., Foster City, CA) operated by the University of California at San Diego Chemistry Department.

The peptide sequence was aligned with the other members of the S100 family and degenerate oligonucleotide probes were synthesized based on the

reverse translation of the S100L peptides, taking into account codon usage in the same positions of the other S100 family members. The oligonucleotide mixtures were as follows:

probe A 3'-CC(G,C)GT(T,C)CTCCC(T,A)CTGTTCAA(A,G)TTC-5'  
probe B 3'-TT(T,C)CTCGAC(G,A)A(G,C)GTGTTCTCGA(G,A)GG-5'

The probes were labeled with <sup>32</sup>P-ATP and T<sub>4</sub> oligonucleotide kinase (Promega Biotech, Madison, WI) and used to screen the Okyama-Berg plasmid-based cDNA library derived from MDBK cells (34). Bacterial colonies that were positive with both probes (five of 30,000 screened) were replated and rescreened with the "A" probe. The insert size was determined on plasmid DNA cut with Bam HI. All inserts were 500–700 bp in length and the longest (S100-I-1) was selected for sequence determination.

S100-I-1 was subcloned into the pibi 76 vector and the orientation was determined on DNA cut with Pst I. Single-stranded DNA was prepared and sequenced by the dideoxy method using the Sequenase kit (United States Biochemicals, Cleveland, OH) and <sup>35</sup>S-dATP. Nucleotide sequence was analyzed on 6–8% denaturing polyacrylamide gels.

### Other Methods

Immunoprecipitations were performed as follows. MDBK cells, obtained from the American Type Culture Collection (Rockville, MD) were grown in DME containing 20% FBS. Cells were incubated overnight in DME (without methionine) containing 10% dialyzed FBS and 50 µCi <sup>35</sup>S-trans label (ICN Radiochemicals, Irvine, CA). Cells were then lysed 5 min at 4°C in 400 µl of either buffer A (20 mM Tris, 100 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, 0.1% SDS, 0.1% deoxycholate) or buffer B (20 mM Tris, 1 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.1% Triton X-100). Lysates were clarified by centrifugation at 100,000 g, 30 min, divided into four equal aliquots, and incubated with 5 µg monoclonal anti-calpactin light chain (a), 5 µg rabbit anti-calpactin I heavy chain (b), 5 µg rabbit anti-S100L (c), or without antibody (d). After 3 h 15 µl of goat anti-mouse IgG antibody was added to samples a and d and further incubated for 30 min. Washed pansorbin (Calbiochem-Behring Corp., San Diego, CA) was then added (25 µl of a 10% solution) and rotated end-over-end for an additional 30 min. The pansorbin was collected by centrifugation and washed with the appropriate lysis buffer three times. Immunoprecipitated protein was eluted with SDS sample buffer, run on a 12% acrylamide SDS gel, treated with enlightening (New England Nuclear, Boston, MA), dried, and exposed to refreshed x-ray film overnight.

Immunofluorescence microscopy was performed as described (53) with the following modifications. MDBK cells grown on glass coverslips were fixed in PBS containing 4% formaldehyde for 1.5 h followed by further fixation and permeabilization in methanol/acetone (1:1) at -20°C for 5 min. Coverslips were air dried, rehydrated in PBS containing 2% BSA, and incubated in rabbit anti-S100L (10 µg/ml) together with mouse anti-calpactin light chain (2 µg/ml). The second antibody incubation was fluorescein-conjugated anti-mouse and rhodamine-conjugated anti-rabbit IgG. Cells were examined with a Nikon Optiphot microscope equipped with epielimination using the appropriate filter combinations. Controls, omitting one of the first antibodies, were always included, and were photographed and printed exactly as the double antibody experiments.

ELISA were performed as in reference 20. For the quantitation of S100L in tissue samples, dilutions of the soluble extracts were preincubated with antibody (1:50,000 dilution of ascites fluid) for 1 h at 37°C in PBS containing 5% powdered milk. The solutions were then added to an ELISA plate and processed by our standard procedure (20). A standard curve of known amounts of soluble S100L as inhibitor was always run in parallel. All assays were performed in duplicate and the entire assay was performed twice using fresh reagents with essentially the same results. The results presented (Table III) represents one of the two assays. Half-maximal inhibition was observed at 2–4 nM S100L.

Detergent extractions were performed (53) with 1 mM Ca<sup>2+</sup>. Ca<sup>++</sup>-binding was performed as described previously (16) with the specified Ca<sup>++</sup> level using 100–200 µM S100L (assuming a molecular mass of 10,000 D). Protein was determined with the Pierce BCA assay using BSA standards. Amino acid composition of S100L was determined by OCS Laboratories (Denton, TX) for 6 N HCl hydrolyzed protein and after performic acid oxidation. Analytical gel filtration was performed using a FPLC superose 12 (Pharmacia Fine Chemicals) equilibrated in 100 mM KCl, 10 mM imidazole, 2 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>PO<sub>4</sub>. The column was calibrated with the protein standards BSA (66,000 D), ovalbumin (43,000 D), soybean trypsin inhibitor (21,500 D), and cytochrome C (14,300 D). The K<sub>av</sub> was defined as the  $V_e - V_o / V_i - V_o$ , where V<sub>o</sub> was determined with blue dextran and modifications in reference 53 necessary to retain S100 on the filter.

1. Abbreviation used in this paper: MDBK, Madin-Darby bovine kidney.

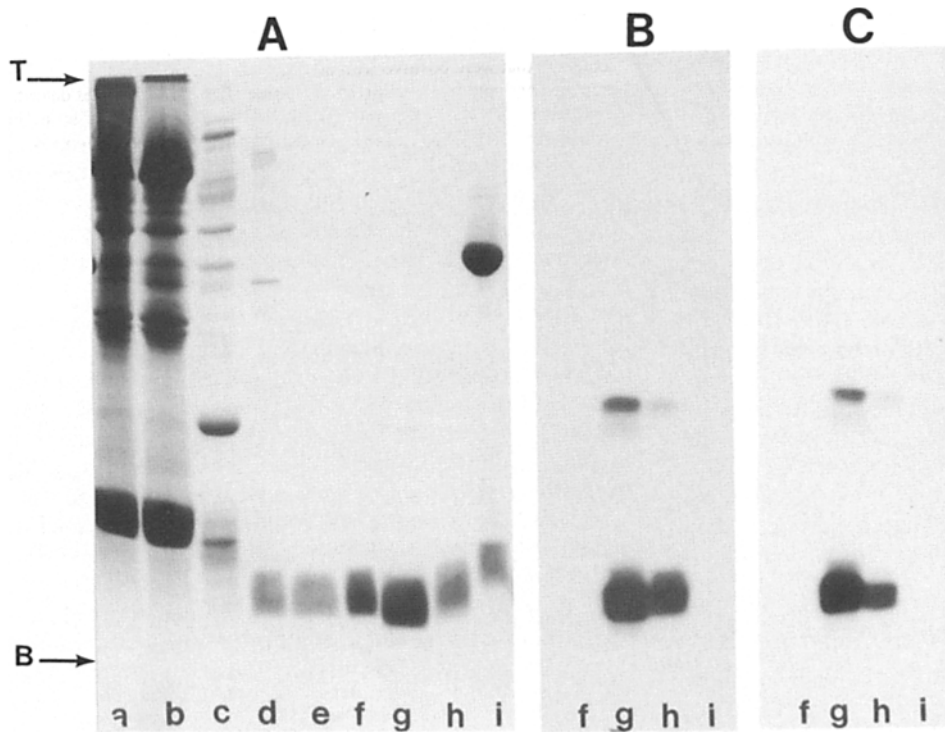


Figure 1. Isolation of S100 from bovine lung. The soluble proteins of lung (lane *a*) were precipitated with ammonium sulfate. The protein resolubilized in buffer containing  $\text{Ca}^{++}$  (lane *b*) was then applied to a column of phenyl-Sephrose and S100 containing protein was eluted with EGTA (lane *c*). The S100 was further purified by ion-exchange chromatography on an FPLC mono-Q column eluting with a linear salt gradient (lane *d*). The S100 fraction was then applied to a second phenyl-Sephrose column and eluted with EGTA as before (lanes *e* and *f*). For comparison the same procedure was used for S100 from brain (lanes *g*) and cardiac muscle (lanes *h*). Calpactin I complex of heavy and light chain subunits (lanes *i*) was purified from lung tissue by standard procedures. The gel was stained with Coomassie Blue (A) and the top and bottom of the gel are indicated on the side. The S100 samples (lanes *f-i*) were also run on adjacent lanes of the gel, transferred to immobilon, and tested for reactivity with anti-S100 $\alpha$  (B) or anti-S100 $\beta$  (C). Reactivity was assessed with  $^{125}\text{I}$ -protein A and autoradiography.

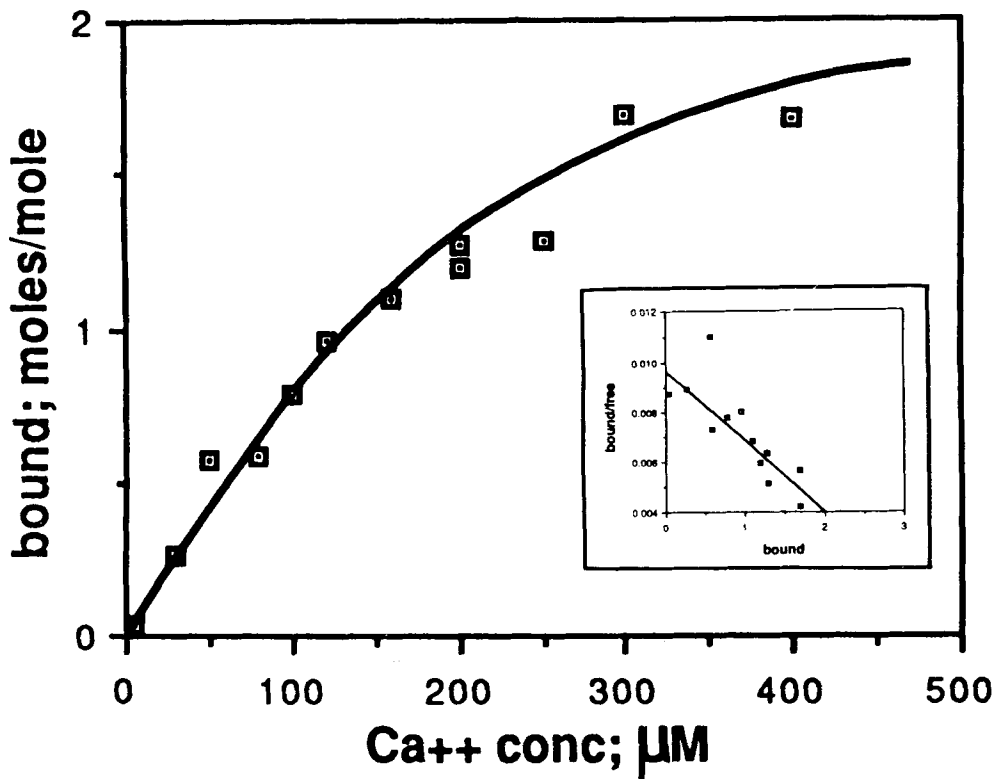


Figure 2.  $\text{Ca}^{++}$ -binding by S100 isolated from lung. Purified S100L (see Fig. 1, lane *f*) was tested for the ability to bind  $\text{Ca}^{++}$  by equilibrium dialysis using trace levels of  $^{45}\text{Ca}^{++}$  and the indicated level of total  $\text{CaCl}_2$ . The amount of  $\text{Ca}^{++}$  bound was calculated for an S100L monomer. (Inset) Scatchard analysis of the binding data showing that two  $\text{Ca}^{++}$  sites were titrated with a  $K_d$  of  $\sim 100 \mu\text{M}$ .

## Results

### Isolation of a $Ca^{++}$ -binding Protein from Lung

A low molecular mass protein was purified from bovine lung using methods developed for brain S100 proteins (3, 24, 49). This involved the extraction from tissues in EDTA buffers, precipitation with 85% ammonium sulfate at pH 4.7,  $Ca^{++}$ -dependent hydrophobic interaction chromatography on a phenyl-Sepharose column and ion exchange chromatography. A limited number of components bound to the phenyl-Sepharose column and were eluted with EGTA, including a predominant band migrating at the position of calmodulin and polypeptides with apparent molecular masses slightly lower than the 11-kD light chain of calpactin (Fig. 1). Ion exchange chromatography of this extract on an FPLC Mono Q column resulted in the isolation of one of these components in pure form. Since this protein comigrated on SDS-PAGE with S100 isolated by the same procedure from brain and cardiac muscle, we tested rabbit antibodies directed to the  $\alpha$  and  $\beta$  subunits of S100 for reactivity with the lung protein by Western blots (17). As shown in Fig. 1, anti-S100 reacted with the proteins isolated from brain and heart but not with the protein from lung or the light chain of calpactin. This indicated that the lung protein, although having similar properties to brain S100, was distinct from  $\alpha$  or  $\beta$  subunits. For this reason we referred to this protein as S100L.

### $Ca^{++}$ Binding and Self Association of the S100L Protein

Since S100 proteins are known to be  $Ca^{++}$ -binding proteins, we tested the ability of S100L to bind  $Ca^{++}$  ions, using the technique of equilibrium dialysis with  $^{45}Ca^{++}$ . As shown in Fig. 2, S100L binds stoichiometric amounts of  $Ca^{++}$  with a relatively low affinity. At saturation 2  $Ca^{++}$  ions were bound to each subunit with a  $K_d$  of  $\sim 100 \mu M$  (Fig. 2). The dialysis buffer contained 2 mM  $MgCl_2$  to insure that we were not detecting  $Ca^{++}/Mg^{++}$  sites.

S100 proteins are known to self associate into homo- or heterodimers. When we subjected S100L to analytical gel filtration on a calibrated superose 12 column (Fig. 3) it eluted much earlier than would be expected for a 10-kD monomer. We always observed S100L eluting as a single symmetrical peak just after the position of ovalbumen. This suggests that

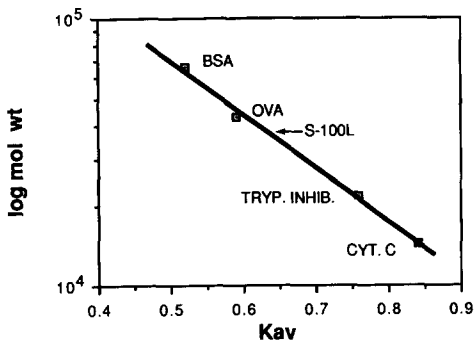


Figure 3. Gel filtration of S100L on a calibrated superose 12 column under nondenaturing conditions. The molecular mass standards BSA (BSA, 68 kD), ovalbumen (OVA, 43 kD), soybean trypsin inhibitor (TRYP. INHIB., 21.5 kD), and cytochrome c (CYT. C, 14 kD) were used to calibrate the column.

under these conditions it exists as a self associated complex (dimer, trimer, or tetramer).

### The Sequence of S100L

Since S100 subunits have been defined by amino acid sequence we decided to determine the primary sequence of S100L by the sequencing of peptides and cloning the cDNA encoding the protein. Intact S100L was found to have a blocked amino terminus, so we fragmented the protein with either CNBr or trypsin, and peptides were isolated by reversed-phase HPLC. Two peptide sequences were obtained as indicated in Fig. 4 and aligned with the other members of the S100 family. Degenerate oligonucleotide probes were then used to screen an Okyama and Berg cDNA library derived from the MDBK cell line (34), a rich source of this protein (see below). Five colonies (of 30,000 tested) were positive with both probes and the one with the longest DNA insert (S100-1-1) was sequenced. Fig. 4 shows the nucleotide sequence of the S100-1-1 clone. It contains 562 nucleotides with an open reading frame that encodes a 96-amino acid ( $\sim 10$  kD) protein. The two peptide sequences derived from S100L (see above) matched the predicted protein sequence from this DNA exactly. This indicates that the S100-1-1 cDNA encodes the entire S100L protein. To further confirm that this cDNA encodes S100L, the amino acid composition of S100L was compared to the deduced amino acid composition (Table I). Excellent agreement was found between these two values including single arginine and cysteine residues.

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A GAT GGA GGT ATG GCT TCT GGC CAG GGA GCC ACA GGC TGA GGC
GGA GAG CCA GCC TGC CTG CCC ATT CTA AGA GGG ACT TGG AGA GGC
AGA TTG GGG CAC AAG GGC AGC AGA CGA TCC TGC AGG CTG GGC TGA
GGC ATC ACC CTC CCT TGC CAC CCC TGT AGC CTC AGA TCC AAG ATG
Met
TCC AGT CCC CTG GAG CAG GCG CTG GCT GTG ATG GTC GCC ACC TTC
Ser Ser Pro Leu Glu Gln Ala Leu Ala Val Met Val Ala Thr Phe
CAC AAG TAC TCT GGC CAA GAG GGC GAC AAG TTC AAG CTG AGT AAG
His Lys Tyr Ser Gly Gln Glu Gly Asp Lys Phe Lys Leu Ser Lys
GGG GAG ATG AAG GAA CTT CTG CAC AAG GAG CTG CCC AGC TTT GTG
Gly Glu Met Lys Glu Leu Leu His Lys Glu Leu Pro Ser Phe Val
GGG GAG AAG GTG GAT GAG GAG GGC CTG AAG AAG CTG ATG GGT GAT
Gly Glu Lys Val Asp Glu Glu Gly Leu Lys Lys Leu Met Gly Asp
CTG GAT GAG AAC AGT GAC CAG CAG GTG GAC TTC CAG GAG TAC GCC
Leu Asp Glu Asn Ser Asp Gln Gln Val Asp Phe Gln Glu Tyr Ala
GTC TTC CTG GCC CTC ATC ACG ATC ATG TGC AAT GAT TTC TTC CAG
Val Phe Leu Ala Leu Ile Thr Ile Met Cys Asn Asp Phe Phe Gln
GGC TCC CCA GCA CGG TCC TGA TGC AGA GCT TGT GGC TCC CTG CTA
Gly Ser Pro Ala Arg Ser End
TGG GTC TCT TCG GCC CAG GAG GAC TCT CTA TCT TTT TTA GTT TTA
CTC AAT AAA CTT TTT TTG CTG GTT

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Figure 4. Nucleotide and deduced protein sequence of the insert from the clone S100-1-1, selected from a library derived from the MDBK cell line. An open reading frame, beginning with the ATG (underlined) encodes a 96-amino acid protein which includes the exact sequence of the two peptides (underlined) that were derived from S100L. Also underlined is the polyadenylation signal.

**Table I. Amino Acid Composition of S100L and Comparison to that Predicted from the cDNA Sequence**

Amino acid	Residues/96AAs	Interpretation	Predicted
asp + asn	9.9	10	9
thr	3.0	3	2
ser	7.7	8	8
glu + gln	16.1	16	16
pro	2.6	3	3
gly	9.4	9	7
ala	6.2	6	6
cys	1.3	1	1
val	5.5	6	6
met	2.8	3	4
ile	1.9	2	2
leu	9.7	10	11
tyr	1.9	2	2
phe	5.7	6	7
lys	8.7	9	9
his	2.2	2	2
trp	0	0	0
arg	1.4	1	1

The amino acid sequence of S100L was compared to other members of this protein family (Fig. 5, Table II), revealing a striking homology to all of the other known S100 related proteins. The highest degree of similarity (60%) was found between S100L and predicted proteins termed pEL98 (21), p9Ka (1), 42c (37), or 18a2 (28). Since S100L is a Ca<sup>++</sup>-binding protein and at least one other member of this family has lost this property, we focused on the sequences surrounding the Ca<sup>++</sup>-binding pockets. As shown in Table II, S100L displays a more marked sequence similarity in this region compared to the other known Ca<sup>++</sup>-binding proteins such as S100  $\alpha$  and  $\beta$ .

**Antibodies to S100L**

S100L, purified from bovine lung was used to elicit antibodies in rabbits (polyclonal) and mice (monoclonal). Both antibody preparations reacted with S100L displayed on Western blots (Fig. 6, left) with the highest level found in the bovine kidney cell line (MDBK). We used a sensitive ELISA to quantitate S100L in the soluble fraction of various bovine tissues. As shown in Table III, S100L is abundantly expressed in lung and kidney and undetectable in brain and intestine with other tissues containing intermediate levels.

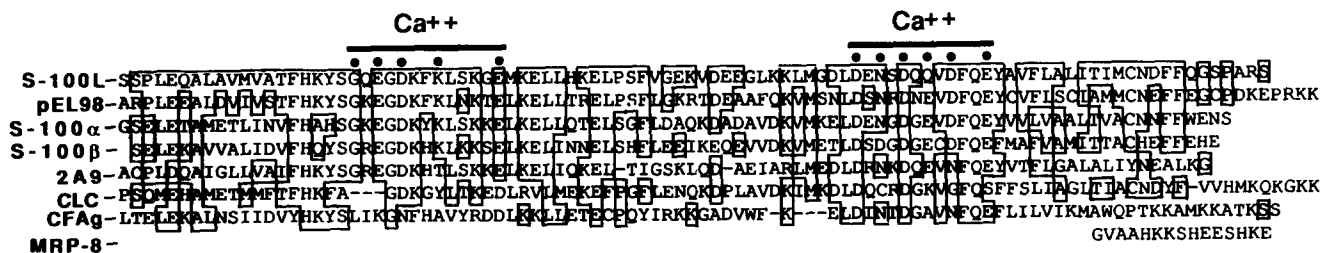
**Table II. Comparison of the Percent Amino Acid Sequence Identity between S100L and the Other Members of This Family**

Species	Percent amino acid sequence identity		
	Overall	1st Ca <sup>++</sup> site	2nd Ca <sup>++</sup> site
pEL98	60	80	70
S100 $\alpha$	47	80	80
S100 $\beta$	43	70	60
2A9	45	70	75
CLC	36	40	40
CFAg	25	10	60

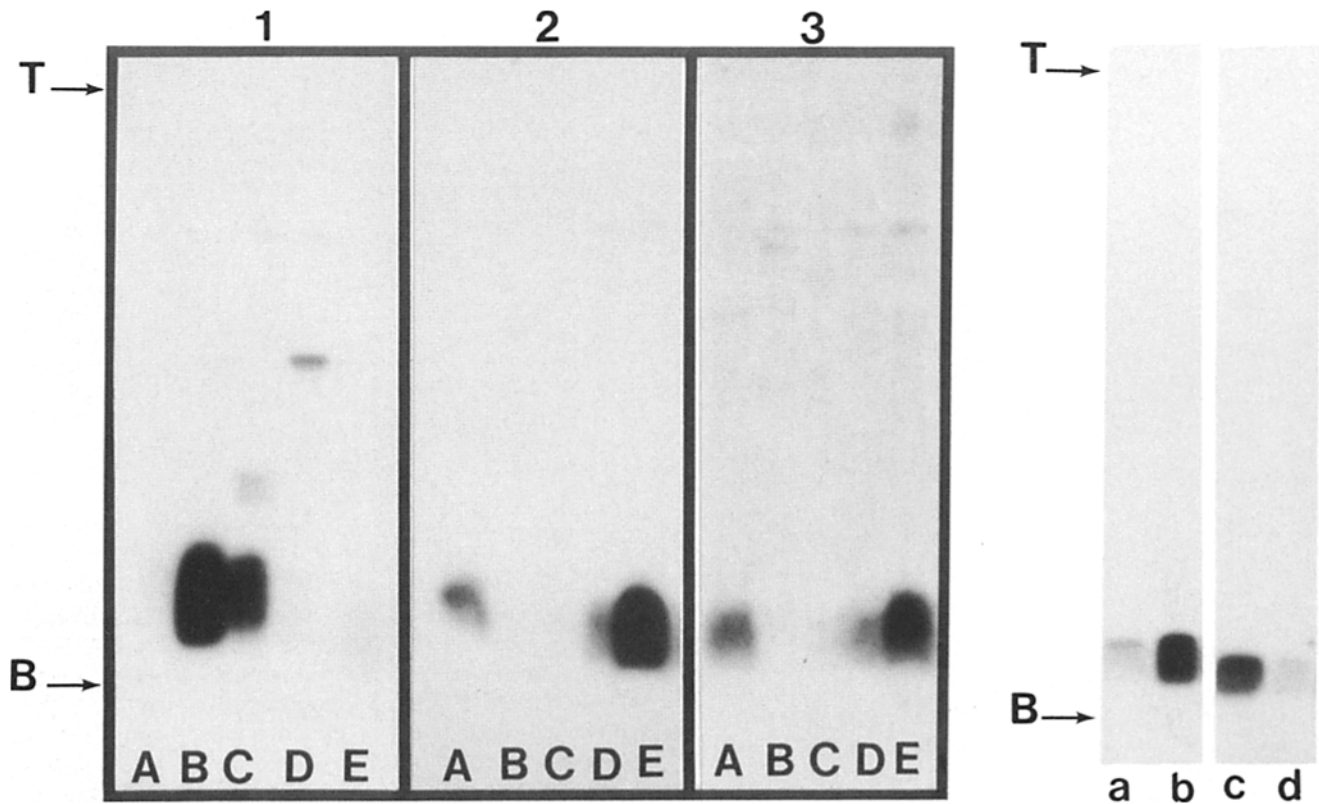
The sequences were aligned as in Fig. 5 and the overall identity was determined as well as in the regions of the Ca<sup>++</sup>-binding sites (see Fig. 5). CFA, cystic fibrosis antigen; CLC, calpactin light chain.

Since the MDBK cell line is known to express both the cytoskeleton-associated calpactin light chain (53) and S100L (see above) we explored the distribution of these two molecules in some detail. Detergent extracts of MDBK cells were separated into soluble and cytoskeletal fractions at high Ca<sup>++</sup> (1 mM) and probed with antibodies to S100L or the calpactin light chain. As shown in Fig. 6, right, under these conditions the calpactin light chain fractionated with the detergent-insoluble cytoskeleton while S100L was in the soluble fraction. This suggested that the two proteins are in distinct subcellular locations in MDBK cells.

S100L and the calpactin light chain were immunoprecipitated from <sup>35</sup>S-labeled MDBK cells. When the immunoprecipitation was performed in a standard buffer containing 0.1% SDS and 0.1% deoxycholate, the antibodies precipitated only a single subunit (either heavy chain or light chain; Fig. 7). When the SDS and deoxycholate were omitted, two bands were detectable in the anti-calpactin I precipitates. Antibodies to the calpactin light chain precipitated a polypeptide at the position of the calpactin heavy chain and antibodies to the calpactin heavy chain caused the precipitation of the calpactin light chain (Fig. 7). Antibodies to S100 precipitated a single component migrating slightly faster than the calpactin light chain under both buffer conditions. A higher molecular mass band was also observed in these precipitates, but appeared to be nonspecifically precipitated by the rabbit antibodies. Thus, there was no hint of association of a 39-kD subunit with S100 under these conditions. In addition, this



**Figure 5.** Comparison of the amino acid sequence of S100L with the other members of this family (see text for references). The predicted Ca<sup>++</sup> binding sites and amino acid residues thought to be involved are indicated above. Identically placed amino acids between S100L and the other family members are enclosed. The calpactin light chain is abbreviated CLC.



**Figure 6.** (Left) Detection of S100L in total cell lysates with polyclonal and monoclonal antibodies. Bovine lung (A), brain (B), cardiac muscle (C), kidney (D), or MDBK (E) cells were homogenized directly in SDS sample buffer and run on a 13% acrylamide SDS gel. Protein was transferred to immobilon and probed with antibodies to  $\alpha$ ,  $\beta$  S100 (1), rabbit anti-S100L polyclonal antibodies (2), or a mouse monoclonal antibody to S100L (3). Detection was with  $^{125}\text{I}$ -protein A (1 and 2) or  $^{125}\text{I}$ -anti-mouse IgG (3) and autoradiography. (Right) Detergent extractability of S100-related proteins in MDBK cells. Cells were lysed in a buffer containing 0.5% Triton X-100 and separated into detergent-soluble (a and c) and cytoskeletal (b and d) fractions by centrifugation. Equal volumes of the fractions were run on a 12% acrylamide SDS gel; the protein was transferred to immobilon and probed with antibodies to the calpactin light chain (a and b) or S100 (c and d). Reactivity was detected with  $^{125}\text{I}$ -labeled anti-mouse IgG for the mouse antibody (a and b) and  $^{125}\text{I}$ -protein A for the rabbit antibody (c and d) followed by autoradiography.

**Table III. Quantitation of S100L in the Soluble Fraction of Various Tissues**

Tissue	ng S100L/mg total protein
Lung	210
Skeletal muscle	20
Kidney	380
Brain	<1
Adrenal gland	2
Cardiac muscle	70
Intestine	<2
Liver	100

Tissues were homogenized in 5 mM EDTA and clarified by centrifugation. Dilutions of the tissue extract were tested for the S100 content using the anti-S100L monoclonal antibody as described in Materials and Methods.

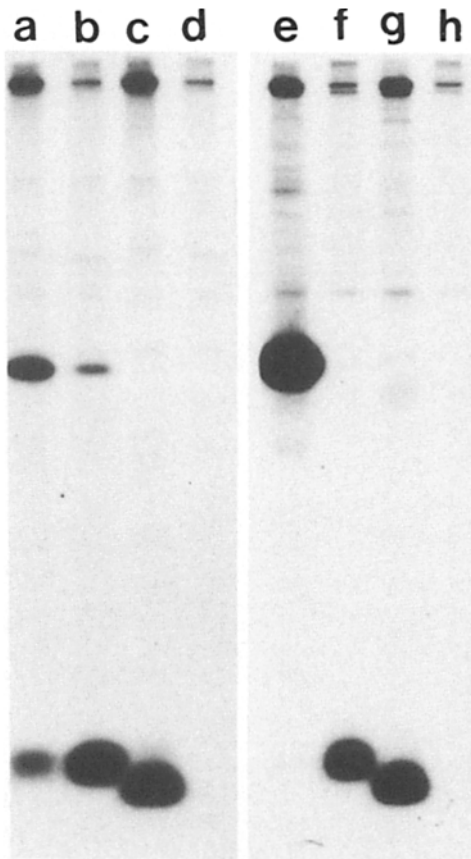
gel system resolved S100 from the calpactin light chain. If heterodimers exist in MDBK cells, one might expect to detect S100 in precipitates with anti-calpactin light chain and vice versa, but no evidence for this was found.

Antibodies to calpactin and S100L were used in double la-

bel immunofluorescence microscopy on MDBK cells grown on coverslips. A double fixation method using formaldehyde and organic solvents was needed for adequate fixation of S100L in these experiments. In MDBK cells, S100L was found throughout the cell, whereas in these same cells the calpactin light chain was strictly cytoplasmic and was excluded from the nucleus (Fig. 8). The level of resolution was not high enough to tell whether the calpactin light chain was present only under the apical membrane, but we can conclude that it is not concentrated at regions of cell-cell contact under these conditions (Fig. 8).

### Discussion

With the large amount of amino acid sequence information currently being generated, one of the most common means of gaining insight into the function of a newly identified protein is by comparison to the sequences of proteins that have been studied in more detail. The protein reported here, S100L, is clearly related by amino acid sequence to a number of other proteins of the S100 family. The first two members of the S100 protein family identified were those isolated from



**Figure 7.** Immunoprecipitation of calpactin I and S100L from MDBK cells labeled with [<sup>35</sup>S]methionine. Cells were labeled overnight and extracted in buffer B, without SDS or deoxycholate (lanes *a-d*) or in buffer A, containing 0.5% Triton, 0.1% SDS, and 0.1% deoxycholate (lanes *e-h*). The lysates were subjected to immunoprecipitation with rabbit antibodies to the calpactin I heavy chain (lanes *a* and *e*), S100L (lanes *c* and *g*), or monoclonal antibody II48 to the calpactin I light chain (lanes *b* and *f*) or a control (lanes *d* and *h*) omitting the first antibody. Immunoprecipitated proteins were run on a 12% acrylamide SDS gel and processed for fluorography. The high molecular mass protein was nonspecifically precipitated with normal rabbit IgG.

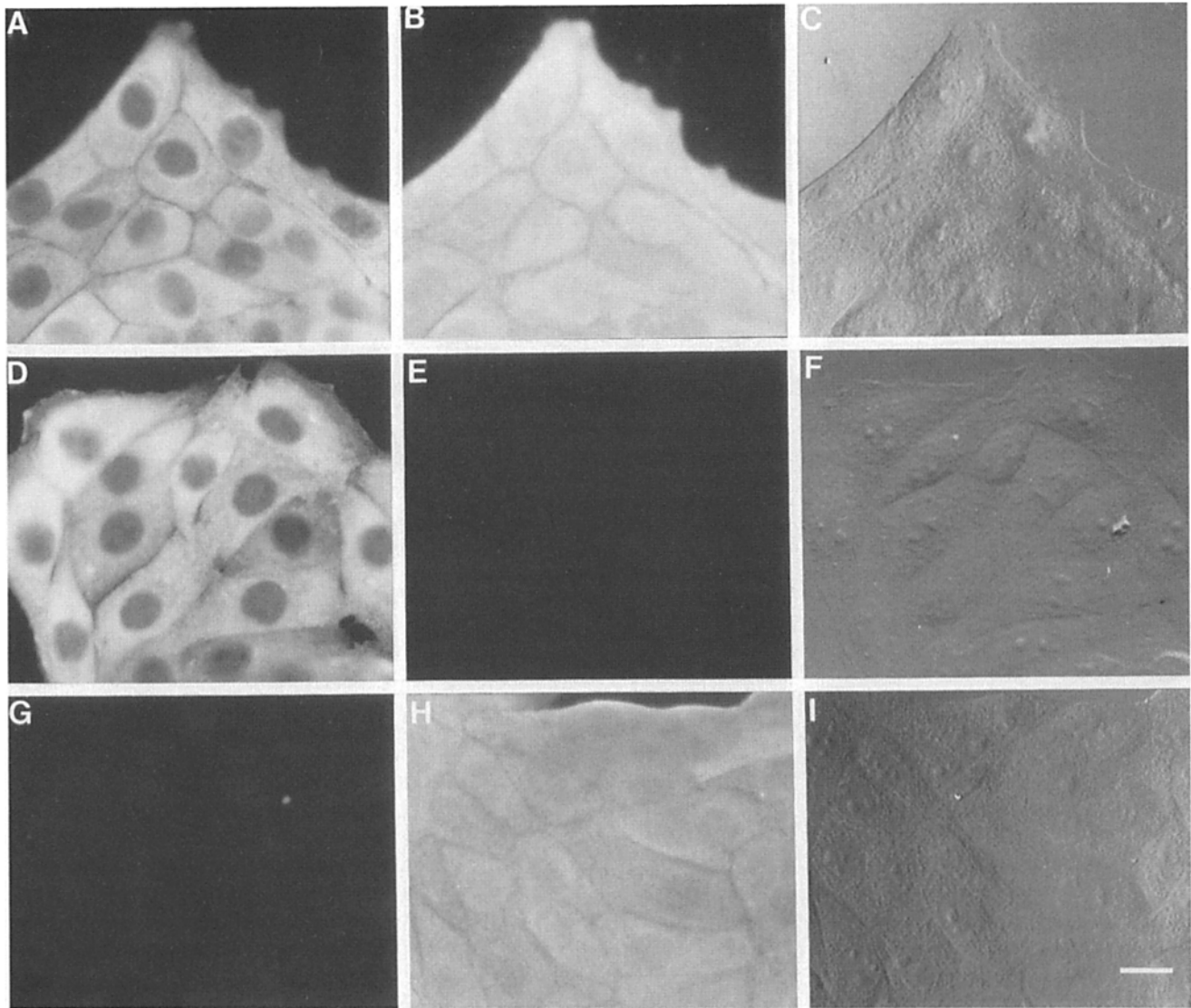
brain. Although found in 1965 as an acidic protein present at high levels in brain tissue (42), it was not until 1977 that two distinct subunits ( $\alpha$  and  $\beta$ ) were resolved (26). Amino acid sequence analysis demonstrated that  $\alpha$  and  $\beta$  S100 evolved from a common ancestor, and have retained 58% sequence identity. The third member of the family was identified in 1985 when we (18), and Gerke and Weber (12) found that the p10 light chain of the cytoskeletal protein calpactin is related to the S100 proteins (50% sequence identity with S100 $\alpha$ ). The cDNA encoding p11 has now been cloned (47) as well as the cDNA encoding a highly related RNA induced when PC12 cells are induced with nerve growth factor (NGF) (37). The fourth member of this family was then provided by Calabretta et al. (4) and Ferrari et al. (9) with the cloning of a cDNA (2A9) which codes for a protein (calcyclin) with a high degree of sequence homology to both the calpactin light chain and S100. The mRNA is induced when quiescent fibroblasts are stimulated to proliferate, however, the protein has

yet to be identified. The fifth member of the S100 family is a serum protein of the same size and having a sequence related to S100, present at elevated levels in patients with cystic fibrosis (6). The function of this cystic fibrosis antigen is unknown but, like S100, it apparently binds Ca<sup>++</sup>-ions (6). Odink et al. recently reported the sequence of a Ca<sup>++</sup>-binding protein (MRP-8) apparently identical to the cystic fibrosis antigen (44). The last 15 amino acids of MRP-8 differ from the cystic fibrosis antigens due to a single base change in the coding region in the cDNA, possibly due to a sequencing error in the initial report. The sixth S100 related protein is encoded by an RNA expressed at high levels in an established cell line (3T3 cells), but absent in primary cultures of mouse embryo fibroblasts (21). This protein is also known to be induced in cells converted from a cuboidal stem cell to myoepithelial-like cells (1), in PC12 cells induced with NGF (37), and in 3T3 cells stimulated with serum (28). The putative proteins are related by sequence to S100 (49% identity) and are approximately the same size (99 amino acids). S100L, reported here, becomes the seventh member of this family. S100L is most closely related to pEL98/18A2/42A/p9Ka, sharing 60% amino acid sequence identity (see Table II). It is unlikely that these represent the same protein from two species (S100L is a bovine protein whereas the others are rodent). The other members of this family are more highly conserved between species. Bovine and human S100 $\beta$ , for instance, are >90% identical at the amino acid sequence level (29), as are the bovine and murine equivalents of the calpactin light chain (47) and the rabbit and human calcyclins (43). Clearly a novel member of this family is indicated. There is no reason to believe that these are the only members of this family.

S100 proteins were initially thought to reside exclusively in glial cells in the brain (39). More recent work has shown that not only are the  $\alpha$  and  $\beta$  "brain" proteins found elsewhere but the other related proteins are widespread. The S100L protein, for instance, has a distinct tissue distribution, including the complete absence from brain. It is possible that all cells have a requirement for an S100 protein that can be filled by one of the seven known members of this family (or by an as yet unidentified member). Future studies on this protein family must address the question of both common and unique features of various members.

The calpactin light chain is known to associate with the 39-kD heavy chain both in vitro and in vivo. One purpose of the present study was to test whether other S100 proteins bind to the calpactin heavy chain in vitro and to find a cell type that coexpresses S100 and the calpactin light chain to analyze the associations occurring in vivo. The MDBK cell line was found to express high levels of proteins reactive with antibodies to S100L and to calpactin heavy chains. Previous studies have noted the presence of S100 $\alpha$  in certain cells of the distal nephron of the kidney (41). The identification of a kidney cell line that overexpresses S100L along with the availability of polyclonal and monoclonal antibodies to S100L should help in the elucidation of the function of this protein.

The light chain of calpactin is known to bind to a region of the heavy chain (the amino terminal tail; 19, 31) which also contains the site of phosphorylation by pp60src (18). Previous studies have shown that the expression of the light chain is coupled to the heavy chain in every tissue examined (45, 53). Whereas in some cells in culture, the calpactin heavy



**Figure 8.** Double-label immunofluorescence microscopy of S100L and the calpactin light chain in MDBK cells. Cells were grown on coverslips, fixed, permeabilized, and treated with either mouse monoclonal antibody I148 to the calpactin light chain (*D–F*), rabbit polyclonal antibodies to S100 (*G–I*), or a mixture of the two (*A–C*). After incubation with the first antibodies, coverslips were washed and further treated with fluorescein-conjugated anti-mouse (*A, D, and G*) together with rhodamine anti-rabbit (*B, E, and H*), and viewed with the appropriate filters. Nomarski images of the same fields are shown in *C, F, and I*. Bar, 10  $\mu$ M.

chain is present at higher levels than the light chain (16, 53), we have never observed the reverse situation (i.e., free light chain). Unlike the calpactin light chain, other S100 proteins have not been shown to be tightly associated with a 36-kD subunit, and it is always isolated as a free homo- or heterodimer. This is not to suggest that S100 may not associate with other proteins in the cell. Indeed, other studies have shown that S100 can bind to and activate the enzyme aldolase (50) and that a population of S100 is associated with membranes (5). What we show here is that the high affinity interaction with the calpactin heavy chain and hence the cytoskeleton is not found with S100L. In a recent study, Zimmer and Van Eldik found that the binding proteins for S100  $\beta$  and another calcium binding protein, calmodulin, change when c6

glioma cells are induced to differentiate (52). It may be that the signal transduction mediated by calcium ions is more complex than imagined.

In MDBK cells the calpactin light chain and S100L have distinct distributions (Fig. 7). S100L appears to be present throughout the nucleus and cytoplasm in MDBK cells (Fig. 7) a distribution that is found for S100  $\alpha$  in adipocytes (24). The calpactin light chain, by contrast, is only found in the cytoplasm in MDBK cells presumably in association with elements of the cytoskeleton (Fig. 5). Heterodimers of S100 and the calpactin light chain were not precipitated with either antibody and antibodies to S100 did not coprecipitate a 30–40-kD polypeptide as did antibodies to the calpactin light chain. In these cells the calpactin light chain could be



demonstrated in the cytoskeleton, whereas S100 was strictly detergent soluble under these conditions. It is possible that there is yet another biological activity shared by the S100 family of proteins which has been overlooked thus far. It may be that this activity is coupled to the cytoskeleton by calpactin and is present in a soluble pool with S100. Distinct roles for S100 and the calpactin light chain are suggested by this study.

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