

# Binding of pEL98 Protein, an S100-related Calcium-binding Protein, to Nonmuscle Tropomyosin

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**Abstract.** The cDNA coding for mouse fibroblast tropomyosin isoform 2 (TM2) was placed into a bacterial expression vector to produce a fusion protein containing glutathione-S-transferase (GST) and TM2 (GST/TM2). Glutathione-Sepharose beads bearing GST/TM2 were incubated with [<sup>35</sup>S]methionine-labeled NIH 3T3 cell extracts and the materials bound to the fusion proteins were analyzed to identify proteins that interact with TM2. A protein of 10 kD was found to bind to GST/TM2, but not to GST. The binding of the 10-kD protein to GST/TM2 was dependent on the presence of Ca<sup>2+</sup> and inhibited by molar excess of free TM2 in a competition assay. The 10-kD protein-binding site was mapped to the region spanning residues 39-107 on TM2 by using several COOH-terminal and NH<sub>2</sub>-terminal truncation mutants of TM2. The 10-kD protein was isolated from an extract of NIH 3T3 cells transformed by *v-Ha-ras* by affinity chromatography

on a GST/TM2 truncation mutant followed by SDS-PAGE and electroelution. Partial amino acid sequence analysis of the purified 10-kD protein, two-dimensional polyacrylamide gel analysis and a binding experiment revealed that the 10-kD protein was identical to a calcium-binding protein derived from mRNA named pEL98 or 18A2 that is homologous to S100 protein. Immunoblot analysis of the distribution of the 10-kD protein in Triton-soluble and -insoluble fractions of NIH 3T3 cells revealed that some of the 10-kD protein was associated with the Triton-insoluble cytoskeletal residue in a Ca<sup>2+</sup>-dependent manner. Furthermore, immunofluorescent staining of NIH 3T3 cells showed that some of the 10-kD protein colocalized with nonmuscle TMs in microfilament bundles. These results suggest that some of the pEL98 protein interacts with microfilament-associated nonmuscle TMs in NIH 3T3 cells.

**T**ROPOMYOSINS (TMs)<sup>1</sup> are ubiquitous actin-binding proteins found in muscle and nonmuscle cells (25, 38, 41, 44, 57). Nonmuscle cells express multiple TM isoforms with a broad range of molecular weight. TMs isolated from rat fibroblasts can be grouped into high (termed TM1, TM2, and TM3) and low molecular weight TM isoforms (termed TM4 and TM5) (42). These nonmuscle TM isoforms are associated with actin in microfilaments. Although their functions in microfilaments are poorly understood, they are proposed to play a regulatory role in defining actin filament assembly and organization during cell motility, cell division, changes in cell shape, and differentiation. This is based on several observations. TM inhibits the actions of vil-

lin (9), gelsolin (15), and actin-depolymerizing proteins (6) toward actin filaments. In transformed and more malignant cells, the synthesis of at least one of the high molecular weight TMs is reduced (7, 10, 25, 26, 37, 38, 41, 54, 55), which is thought to be responsible, in part, for the disorganization of actin filament bundles and subsequent changes in characteristics of transformed cells (36). Furthermore, disruption of the single TM gene in budding yeast leads to disappearance of cytoplasmic actin cables and reduced cell growth (39). Thus, interactions between TM and actin are important in a wide variety of cellular processes. However, the components and mechanisms responsible for regulating TM-actin interactions in nonmuscle cells remain uncertain.

In skeletal and cardiac muscle, each TM molecule is associated with a troponin complex consisting of troponin I, troponin T, and troponin C (the calcium-binding component) along the actin filaments. In vitro studies demonstrate that binding of troponin promotes the head-to-tail association of TM and also enhances the binding of TM to actin (for reviews see references 35, 48, 64). The troponin complex

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1. *Abbreviations used in this paper:* MAP, microtubule-associated protein; TM, tropomyosin.

functions to confer calcium sensitivity on regulation by TM of actomyosin ATPase activity (35, 48, 64). In smooth muscle, caldesmon, a calmodulin- and F-actin-binding protein, is associated with TM along the actin filaments (for review see reference 49). It inhibits actomyosin  $Mg^{2+}$ ATPase activity and the inhibition is reversed by  $Ca^{2+}$ -calmodulin (49). In addition, calponin, a recently discovered troponin-like molecule, binds actin and TM and inhibits actomyosin  $Mg^{2+}$ ATPase (1, 51, 52).

Since nonmuscle cells do not contain a troponin complex, much effort has been directed at identifying cellular proteins that are directly involved in the mechanisms of regulation of TM-actin interactions in nonmuscle cells. To date, several cellular proteins that regulate TM-actin interaction have been identified and they can be classified into two groups. One group includes proteins such as villin (9) and  $M_r$  55,000 bundling protein (43) that can prevent the interaction of TM with actin and/or dissociate TM from actin by binding to actin and competing for the same binding sites on actin filaments. The other group includes proteins such as tropomodulin and nonmuscle caldesmon that can bind directly to TM and affect TM-actin interactions. Tropomodulin binds to (one of) the ends of the erythrocyte TM molecules and weakens TM-actin interactions by blocking head-to-tail association of TM molecules along the actin filament (16, 17, 50). Results from immunofluorescence labeling of isolated myofibrils at resting and stretched lengths using anti-erythrocyte tropomodulin antibodies indicate that tropomodulin is localized at or near the free (pointed) ends of the thin filaments (18). In addition, antibody to tropomodulin cross-reacts with 43 kD polypeptides in muscle, brain, lens, neutrophils, and endothelial cells (17), suggesting that tropomodulin-like molecules exist in non-erythroid cells. Like smooth muscle caldesmon, nonmuscle caldesmon inhibits actomyosin  $Mg^{2+}$ ATPase activity and the inhibition is reversed by  $Ca^{2+}$ -calmodulin. It is associated with TM along the actin filaments (49) and potentiates the binding of low molecular weight TMs to F-actin (62) and the TM inhibition of the severing of actin filaments by gelsolin (29). In Rous sarcoma virus-transformed cells, the amount of caldesmon decreases and the distribution of caldesmon changes to a diffuse state (47). The changes in nonmuscle caldesmon are thought to be a cause of a loss of  $Ca^{2+}$  regulation and the redistribution of actin stress fibers to a less-ordered state in transformed cells. Thus, regulation of TM-actin association involves coordinate interactions of many regulatory proteins.

The fact that TM-binding protein(s) homologous to muscle troponin T or I have not been isolated from nonmuscle cells, together with the speculation that regulation of TM-actin associations may involve many regulatory proteins led us to search for additional proteins that bind to TM directly and regulate TM-actin interactions. We show here that the pEL98 protein, which is an S100-related calcium-binding protein and the expression of which is reported to be related to "immortalization" of cells (21), binds directly to nonmuscle TMs in a  $Ca^{2+}$ -dependent manner both in vitro and in vivo. To date, many S100-related proteins, all of which have EF hand  $Ca^{2+}$ -binding motif, have been isolated and they are implicated to play a role in a wide variety of biological events such as cell immortalization, cell growth, differentiation, and tumor cell metastasis (for reviews see references 13, 22, 27). Although their functions and effector proteins,

if any, are poorly understood, several studies have shown that S100 protein and S100-related proteins may participate in regulating cytoskeletal organization. For example, S100 protein inhibits the binding of caldesmon to F-actin, and reverses the inhibitory action of caldesmon on skeletal muscle actomyosin ATPase activity (19). S100 protein also inhibits a tubulin-dependent ATPase activity found in microtubule-associated proteins (MAPs) in a  $Ca^{2+}$ -dependent manner (2). Furthermore, it has been reported that p11 associates with components of the cytoskeleton in fibroblasts (20) and that p9Ka is localized along actin stress fibers (12). The results presented here indicate that nonmuscle TMs are one of the effector proteins of the pEL98 protein and that the pEL98 protein may be involved in regulating cytoskeletal organization.

## Materials and Methods

### Cell Culture Methods

NIH 3T3 and NIH 3T3 cells transformed by v-Ha-ras (pH 1-3) were grown in MEM supplemented with 10% heat-inactivated ( $56^{\circ}C$ , 30 min) calf serum, 100  $\mu$ g/ml of streptomycin and 100 U/ml of penicillin. They were cultured in a humidified atmosphere of 5%  $CO_2$  in air at  $37^{\circ}C$ .

### cDNA Clones and Expression Constructs

The cDNA clones for mouse TM2, TM5, and  $\beta$ -actin have been described: the GenBank entry names being MUSTRO2IS, MUSTROP5, and MUSACTBR, respectively (53, 56, 58). The sequence of pEL98 cDNA has been described elsewhere (21). The coding sequence of TM2 cDNA was excised from pGEM-3Z (Promega Biotec, Madison, WI) using the TM NcoI site and the DraI site in the 3'-untranslated region. The fragments were treated with T4 DNA polymerase and the synthetic oligonucleotide 5'-GATCCGAATTCGGATC-3' was added to create the BamHI and EcoRI sites in the 5' and 3' ends, respectively. After digestion with BamHI and EcoRI the fragment was ligated into a BamHI/EcoRI cut pGEX-2T expression vector (Pharmacia LKB Biotechnology, Uppsala, Sweden) to form a plasmid pGEX/TM2. Expression plasmids pGEX/TM5, pGEX/ $\beta$ -actin, and pGEX/pEL98 were prepared by the same procedure except that the coding sequence of TM5 cDNA,  $\beta$ -actin cDNA or pEL98 cDNA was excised by the TM5 NcoI site and the SspI site, by the  $\beta$ -actin NcoI site and the DraI site, or by the pEL98 NcoI site and the Nsp(7524)I site, respectively.

3' and 5' deletion mutants of TM2 were constructed by using various restriction enzyme recognition sequences in the TM2 cDNA sequence. Briefly, pGEX/TM2 was cleaved by restriction enzymes, and the fragments containing the BamHI site and the 5' region of TM2 cDNA or the EcoRI site and the 3' region were isolated. They were blunt ended with T4 DNA polymerase, ligated to the appropriate-length EcoRI or BamHI linker, respectively, required to maintain an open reading frame, and cleaved with BamHI and EcoRI. The deleted fragments were then cloned into a BamHI/EcoRI cut pGEX-2T. The restriction enzyme recognition sites used to produce 3' and 5' truncations were PvuII at 168 bp of the GenBank entry MUSTRO2IS; MaeII, 305; FokI, 313; AspHI, 360; Ball, 373; BglII, 406; BanII, 456; SacI, 608; and AvaI, 764.

Competent *Escherichia coli* JM109 cells were transformed with each of the pGEX/TM2 clones containing the various deletions, pGEX/TM5, pGEX/ $\beta$ -actin, or pGEX/pEL98 and recombinant clones were screened by restriction enzyme analysis and SDS-PAGE analysis (34) of overproduced fusion proteins.

### Purification of Fusion Proteins

Expression of fusion proteins was induced by 100  $\mu$ M isopropylthio- $\beta$ -D-galactoside for 2-3 h. After induction, the bacteria were pelleted and washed once with cold DPBS containing 1 mM PMSF. Bacteria were resuspended in 25 mM Hepes, pH 7.6, 300 mM KCl, 1 mM EGTA, 12.5 mM  $MgCl_2$ , 0.5% Triton X-100, 10% glycerol, 1 mM DTT, and 1  $\mu$ g/ml each of aprotinin, leupeptin and pepstatin A, and lysozyme was added to 500  $\mu$ g/ml. Samples were incubated on ice for 20 min, sonicated for a total of 5 min at 1-min intervals, and then spun in a type 42.1 rotor (Beckman

Instruments, Inc., Palo Alto, CA) at 30,000 *g* for 30 min at 4°C. The supernatant was removed and loaded onto a column of glutathione-Sepharose 4B (Pharmacia LKB Biotechnology). Fusion proteins were eluted by 10 mM glutathione in 50 mM Tris-HCl, pH 9.5, dialyzed overnight against DPBS, and then concentrated by ultrafiltration. In some cases, fusion proteins were further purified by DEAE-cellulose chromatography. The purity of the fusion proteins was checked by SDS-PAGE.

### Cleavage of Fusion Proteins with Thrombin

Cleavage of GST/TM2 or GST/pEL98 (pEL98 protein containing GST fusion protein) with thrombin was performed as described (3) with some modifications. Briefly, purified GST/TM2 or GST/pEL98 in 2.5 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl was incubated with 3% (wt/wt) thrombin (activity, ~1,000 NIH U/mg protein; Sigma Immunochemicals, St. Louis, MO) for 18 h at 37°C. The resulting solution was applied to a column of glutathione-Sepharose three consecutive times to remove GST and undigested fusion proteins. The flow through containing released TM2 or pEL98 protein was collected, dialyzed extensively against 50 mM Tris-HCl, pH 7.5, and applied to a column of DE52 (Whatman, Kent, UK) preequilibrated with the same buffer. The column was eluted with a linear NaCl gradient (0–0.5 M) and the fractions containing TM2 or pEL98 protein were pooled. The purified TM2 and pEL98 protein were used for a competition assay described in the text and generation of polyclonal anti-pEL98 protein antibodies, respectively.

### Metabolic Labeling and Preparation of Cell Extract

Subconfluent NIH 3T3 cells in 60-mm plastic culture dishes were labeled with 50  $\mu$ Ci/ml of Tran<sup>35</sup>S-label (sp act >1,000 Ci/mmol; ICN Radiochemicals, Irvine, CA) for 18 h in methionine-free DME containing 0.1% dialyzed calf serum. After rinsing the cells twice with cold DPBS, they were lysed with 200  $\mu$ l of cold extraction buffer consisting of 1% (vol/vol) Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF and 10  $\mu$ g/ml each of aprotinin, leupeptin, and pepstatin A for 10 min on ice. In some experiments, the cells were lysed with DPBS containing 1% (vol/vol) Triton X-100 and 1 mM PMSF or similar buffer containing 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>, additionally. The resulting cell extract containing soluble cellular proteins was centrifuged at 10,000 *g* for 10 min and the supernatant was used immediately for a binding assay or stored frozen at –80°C.

### Binding Assay

Cell extracts were precleared by incubation with a slurry of glutathione-Sepharose beads bearing ~20  $\mu$ g of GST and Sepharose 4B beads. This preadsorption step was proved to be effective in reducing nonspecific binding. The extracts were diluted 10 times with the lysis buffer in which Triton X-100 was omitted, and incubated with glutathione-Sepharose beads bearing 10  $\mu$ g of fusion proteins in the presence of CaCl<sub>2</sub> or EGTA for up to 2 h at room temperature. The effect of Ca<sup>2+</sup> on the binding was assayed under the same conditions as above but using calcium/EGTA mixtures (4 mM EGTA plus increasing concentrations of CaCl<sub>2</sub>) to vary the free Ca<sup>2+</sup>. The free Ca<sup>2+</sup> concentration was calculated as reported (24). The beads were washed five times with DPBS containing 0.1% (vol/vol) Triton X-100, 1 mM PMSF and 1 mM CaCl<sub>2</sub> or 1 mM free Ca<sup>2+</sup>, and then boiled for 3 min in SDS gel sample buffer (34). The released materials were subjected to SDS-PAGE. The gels were fixed and treated with ENLIGHTNING (New England Nuclear, Boston, MA) before they were dried and exposed to Fuji RX medical X-ray film with an intensifying screen at –80°C.

### Binding Assay for In Vitro Translation Product of the pEL98 Transcript

In vitro translation of the pEL98 transcript was performed by the method described previously (53). Briefly, the pEL98 cDNA in pUC18 was excised by PstI and KpnI and the fragment was subcloned into the PstI/KpnI site of pGEM-3Z (Promega Corp.). In vitro transcription was carried out by using SP6 RNA polymerase in the presence of m<sup>7</sup>G(S')ppp(S')G as a cap analogue. In vitro translation was done in a reticulocyte lysate cell-free system (Amersham International, Amersham, UK) using [<sup>35</sup>S]methionine as a tracer amino acid. The reticulocyte lysate was diluted into 0.1% (vol/vol) Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 10  $\mu$ g/ml each of aprotinin, leupeptin, and pepstatin A. A binding assay was performed as described above.

### Purification of the 10-kD Protein

pHI-3 cells were lysed with the lysis buffer described above for 30 min at 4°C with gentle stirring. Extracts clarified by centrifugation at 30,000 *g* in a Beckman type 42.1 rotor for 30 min at 4°C were first applied to a column of plain Sepharose 4B (15 ml) (Pharmacia LKB Biotechnology). The extracts (~160 mg total protein) were then applied to a GST/TM2(1-107) (see text) affinity matrix. The affinity matrix was prepared by coupling 2 mg/ml of purified GST/TM2(1-107) to CNBr-activated Sepharose (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. The resulting matrix contained approximately 5 mg of the fusion protein per ml of Sepharose. The column was washed with 0.1% (vol/vol) Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM CaCl<sub>2</sub>, and 1 mM PMSF until no protein was eluted. Elution was achieved using similar buffer in which the cations were replaced by 20 mM EGTA and Triton X-100 was omitted. 10-kD fractions detected by SDS-PAGE were pooled and concentrated by ultrafiltration. The concentrated materials were next applied to preparative SDS-PAGE. After the gel was stained briefly with Coomassie brilliant blue R-250, the 10-kD band was excised and the protein was eluted from the minced gel electrophoretically by the method described elsewhere (4). Typically, the yield was 3–5  $\mu$ g of the 10-kD protein from ~160 mg of total protein.

### Enzymatic Digestion and Amino Acid Sequence Analysis

Purified 10-kD protein was digested with lysylendopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 50 mM Tris-HCl, pH 9.0 and 1 mM EDTA for 18 h at 30°C, the enzyme to protein ratio being 1:100 (wt/wt). The resulting peptides were separated by reverse-phase HPLC on a C<sub>18</sub> column (TSKgel ODS-80T<sub>M</sub> 4.6  $\times$  150 mm, TOSOH Inc., Tokyo, Japan) preequilibrated with 0.08% trifluoroacetic acid in 5% acetonitrile. Separation of the peptides was done by generation of a 40-min linear gradient (5–65% acetonitrile in 0.08% trifluoroacetic acid) at a flow rate of 1 ml/min, and the separated peptides were stored at –80°C for subsequent sequence analysis.

Amino acid sequence analysis was performed on a protein sequencer (model 6600; Milligen/Bioresearch, Burlington, MA) equipped with an on-line analyzer for the phenylthiohydrantoin derivatives.

### Antibody Production

Purified pEL98 protein (400  $\mu$ g in 0.5 ml DPBS) prepared by digestion of GST/pEL98 with thrombin as described above was mixed with an equal volume of Freund's complete adjuvant, emulsified, and used to immunize a New Zealand White female rabbit. Booster injections of the protein (200  $\mu$ g) emulsified in Freund's incomplete adjuvant were made and the titer of the serum was monitored by ELISA (14). Affinity-purified anti-pEL98 antibody was prepared according to a previously published method (11).

### Western Blot Analysis

Samples were electrophoresed on 15% polyacrylamide gels under reducing conditions as described (34). The resolved proteins were electrophoretically transferred to nitrocellulose membrane (59) and the 10-kD (pEL98) protein was detected using anti-pEL98 antibodies and an ECL Western blotting detection kit (Amersham International).

### Subcellular Fractionation

Monolayers of NIH 3T3 cells in 35-mm plastic dishes were washed three times with ice-cold DPBS and then overlaid with 200  $\mu$ l of the extraction buffer containing 0.2 mM EGTA, 1 mM or 2  $\mu$ M free Ca<sup>2+</sup> (see above). After 2 min at room temperature, the supernatant containing extracted soluble cellular proteins was removed. The soluble proteins were precipitated by addition of 5 vol ethanol. After standing at –80°C for 20 min, the precipitate was recovered by centrifugation and dried under vacuum. The residual monolayer was gently washed three times with the wash buffer containing 0.2 mM EGTA or 1 mM Ca<sup>2+</sup>, depending on the buffer with which cells were extracted. The residues were scraped off from the culture dishes by a rubber policeman and collected by centrifugation.

### Indirect Immunofluorescence Microscopy

NIH 3T3 cells on coverslips were washed three times with DPBS and fixed

for 30 min with 4% formaldehyde and 5% sucrose in DPBS. After washing, the cells were permeabilized in 0.5% Triton X-100 in DPBS for 4 min followed by washing three times with DPBS. In some experiments, prior to fixation cells were extracted for 2 min at room temperature with extraction buffer (0.1 M Pipes, pH 6.9, 5 mM MgCl<sub>2</sub>, 0.05% Triton X-100, 4 M glycerol) containing 0.2 mM EGTA (44) or 1 mM or 2 μM free Ca<sup>2+</sup>, followed by washing the residues three times with wash buffer (0.1 M Pipes, pH 6.9, 5 mM MgCl<sub>2</sub>) containing 0.2 mM EGTA or 1 mM Ca<sup>2+</sup>, respectively. The cells or the cytoskeletal residues were then treated with 3% BSA in DPBS containing 0.1% glycine for 1 h to block nonspecific binding sites. After extensive washing, the cells or the cytoskeletal residues were incubated for 1 h with affinity-purified anti-pEL98 antibodies and/or mouse anti-TM monoclonal antibody (TM311; Sigma Immunochemicals) in DPBS containing 1 mg/ml BSA at room temperature. After washing with DPBS, the first antibodies were localized with the appropriate secondary antibodies. The secondary antibodies used were: TRITC-goat anti-rabbit IgG, FITC-goat anti-rabbit IgG, and TRITC-goat anti-mouse IgG. For double-label studies, cells were simultaneously stained with a mixture of the primary antibodies, rinsed, and simultaneously stained with a mixture of the fluorescent secondary antibodies. After rinsing, the coverslips were mounted in 50% glycerol in DPBS containing 1 mg/ml *p*-phenylenediamine to inhibit photobleaching.

### Other Procedures

Two-dimensional gel electrophoresis was carried out by the method of O'Farrell (46).

Protein concentration was determined by the method of Bradford (8) using bovine serum albumin as a standard.

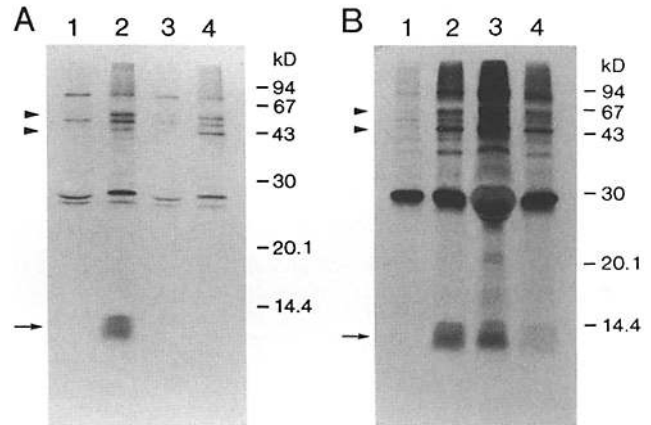
## Results

### Detection of Cellular Proteins That Bind to the GST/TM2 Fusion Protein

To detect cellular proteins that bind to GST/TM2 but not to GST, glutathione-Sepharose beads bearing GST or GST/TM2 were incubated with <sup>35</sup>S-labeled NIH 3T3 cell extracts. Initially, we performed binding assays in DPBS or DPBS containing 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>. After washing the beads extensively, the beads were boiled in SDS gel sample buffer and the materials released were analyzed by SDS-PAGE. The results are shown in Fig. 1 A. When the binding assay was performed in DPBS, two cellular proteins with 43 and 55 kD that bound to GST/TM2, but not to GST, were detected (Fig. 1 A, lanes 3 and 4). These proteins were also detected when the binding assay was performed in DPBS containing divalent cations (Fig. 1 A, lanes 1 and 2). Strikingly, an additional cellular protein of 10 kD could be detected only when DPBS containing divalent cations was used (Fig. 1 A, lane 2), suggesting that the binding was dependent on the presence of Ca<sup>2+</sup> or Mg<sup>2+</sup>.

To ensure further that binding of the 10-kD protein to the beads bearing GST/TM2 requires divalent cations, the complex was treated with DPBS containing 20 mM EDTA and the materials released were analyzed separately from that remaining with the beads. The results showed that the 10-kD protein was eluted by EDTA from the complex, while it was not eluted when EDTA was omitted (data not shown).

We next performed a competition assay in which molar excess of free GST or TM2 was included to determine whether bindings of these cellular proteins to GST/TM2 are specific. As shown in Fig. 1 B, the binding of the 10-kD protein was significantly inhibited by 14-fold molar excess of TM2 (Fig. 1 B, lanes 2 and 4), but not by 19-fold molar excess of GST (lanes 2 and 3). On the other hand, the bindings of the 55- and the 43-kD proteins were not noticeably inhibited by free

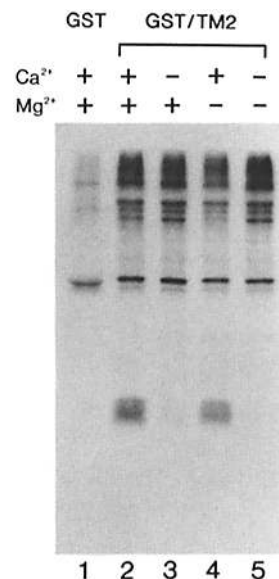


**Figure 1.** Detection of the 10-kD protein that binds to GST/TM2. (A) SDS-PAGE analysis of cellular proteins that bind to glutathione-Sepharose beads bearing GST or GST/TM2. Beads bearing 10 μg of GST (lanes 1 and 3) or GST/TM2 (lanes 2 and 4) were incubated with <sup>35</sup>S-labeled cell lysates of NIH 3T3 cells for 1 h in DPBS containing 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> (lanes 1 and 2) or DPBS (lanes 3 and 4). (B) Competition assay for the binding of the 10-kD protein to GST/TM2. Beads bearing 10 μg of GST (lanes 1) or GST/TM2 (lanes 2–4) were incubated with <sup>35</sup>S-labeled cell lysates of NIH 3T3 cells for 1 h in DPBS containing Ca<sup>2+</sup> and Mg<sup>2+</sup>. For a competition assay, 14-fold molar excess of GST (lanes 3) or 19-fold molar excess of TM2 (lane 4) was included in the incubation mixture. After washing the beads extensively, the materials bound were released in SDS sample buffer and loaded onto a 15% gel. Arrow indicates the position of the 10-kD protein. Arrowheads indicate the locations of 55- and 43-kD proteins. Molecular weight standards (*kD*) are indicated at right.

TM2. From these results, we concluded that the binding of the 10-kD protein to TM2 was specific and thereafter focused on the 10-kD protein.

### Ca<sup>2+</sup>-dependent Binding of the 10-kD Protein to the GST/TM2 Fusion Protein

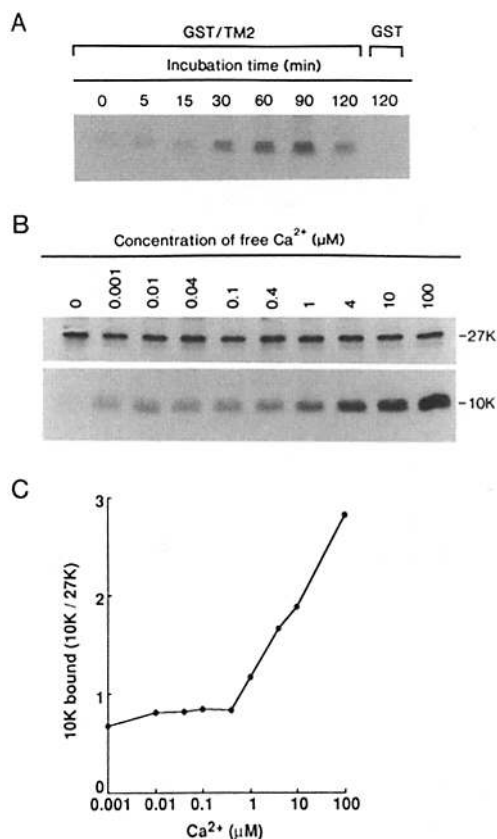
To determine which divalent cation is responsible for the



**Figure 2.** Ca<sup>2+</sup>-dependent binding of the 10-kD protein to GST/TM2. Glutathione-Sepharose beads bearing 10 μg of GST (lane 1) or GST/TM2 (lanes 2–5) were incubated with <sup>35</sup>S-labeled cell lysates of NIH 3T3 cells for 1 h in Tris-buffered saline containing 1 mM CaCl<sub>2</sub> or 0.5 mM MgCl<sub>2</sub> or both. After the incubation, the beads were washed and the materials bound were released in SDS sample buffer and loaded onto a 15% gel. Arrow indicates the position of the 10-kD protein. Note that the binding of the 10-kD protein to GST/TM2 occurs only when Ca<sup>2+</sup> is present in the incubation mixture.

binding of the 10-kD protein to GST/TM2, we carried out binding assays in Tris-buffered saline containing  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  or both. In the following experiments, we used Tris-buffered saline free of KCl instead of DPBS because the binding of  $\text{Ca}^{2+}$  by some calcium-binding proteins is antagonized by the presence of potassium ions (5). The results shown in Fig. 2 clearly indicate that the binding was apparently dependent on the presence of  $\text{Ca}^{2+}$  (lanes 2 and 4), but not of  $\text{Mg}^{2+}$  (lanes 2 and 3).

Fig. 3 shows the kinetics of the binding of the 10-kD protein to GST/TM2. In these experiments, calcium/EGTA buffers were used to vary the free  $\text{Ca}^{2+}$ . The binding was

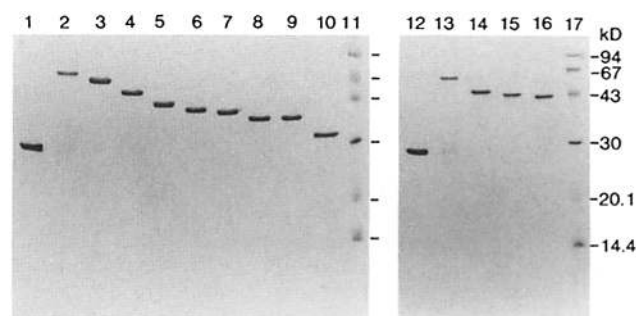


**Figure 3.** Kinetics of the binding of the 10-kD protein to GST/TM2. (A) Time course of the binding. Glutathione-Sepharose beads bearing 10  $\mu\text{g}$  of GST or GST/TM2 were incubated with  $^{35}\text{S}$ -labeled cell lysates of NIH 3T3 cells for various times in Tris-buffered saline containing 2  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . Only the region of interest is shown. (B) Effects of the concentrations of  $\text{Ca}^{2+}$  on the binding. Glutathione-Sepharose beads bearing 10  $\mu\text{g}$  of GST/TM2 were incubated with  $^{35}\text{S}$ -labeled cell lysates of NIH 3T3 cells for 1 h in Tris-buffered saline containing 1 mM EGTA (the concentration of  $\text{Ca}^{2+}$  is indicated as 0) or various concentrations of free  $\text{Ca}^{2+}$ . After the incubation, the beads were washed and the materials bound were released in SDS sample buffer and loaded onto a 15% gel. (Top) 27-kD protein that bound to the beads nonspecifically. (Bottom) 10-kD protein. Only the regions of interest are shown. (C) Effects of the concentrations of  $\text{Ca}^{2+}$  on the binding. The lanes in B were scanned with a densitometer and the amount of 10-kD protein bound was normalized to the amount of 27-kD protein. The values (10K/27K) at various  $\text{Ca}^{2+}$  concentrations were plotted after subtracting the value which represents the amount of 10-kD protein bound in the presence of 1 mM EGTA.

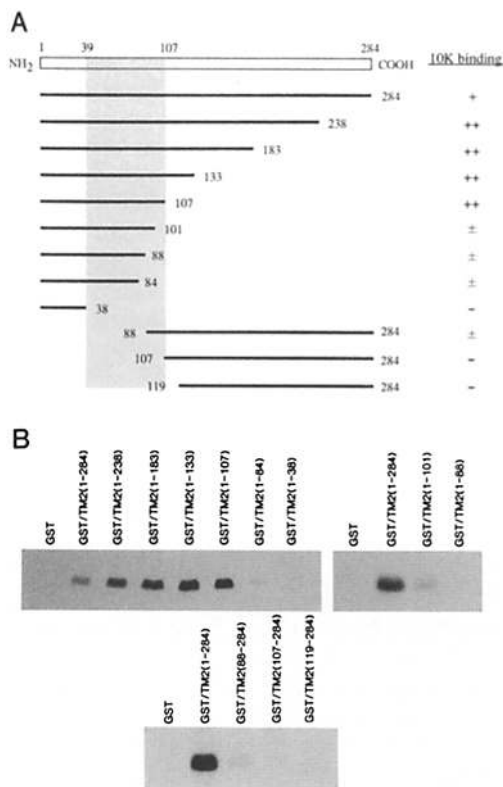
detectable as early as 5 min after the incubation started in the presence of 2  $\mu\text{M}$   $\text{Ca}^{2+}$  and the amount of the 10-kD protein bound increased thereafter up to 90 min (Fig. 3 A). The binding was dependent on the concentration of  $\text{Ca}^{2+}$  (Fig. 3 B). The lanes were scanned with a densitometer and the ratios of the amount of the 10-kD protein (bottom) to that of the 27-kD protein (top) at various  $\text{Ca}^{2+}$  concentrations were plotted (Fig. 3 C). The binding of the 27-kD protein (probably GST derived from cell extracts) was nonspecific and the amount was unchanged irrespective of  $\text{Ca}^{2+}$  concentrations. Then, we used it as a control. A significant amount of the 10-kD protein was already seen at 0.001  $\mu\text{M}$   $\text{Ca}^{2+}$  and the amount was constant up to 0.4  $\mu\text{M}$   $\text{Ca}^{2+}$  and then a sharp increase in the amount was observed at the concentrations of  $\text{Ca}^{2+}$  between 1 and 100  $\mu\text{M}$ . The amount of the 10-kD protein bound was nearly saturable above 100  $\mu\text{M}$   $\text{Ca}^{2+}$  (data not shown).

#### Localization of the 10-kD Protein Binding Site on TM2

To locate the 10-kD protein-binding sites on TM2, a series of eight deletions from the 3' end and three deletions from the 5' end of TM2 cDNA was constructed by the use of restriction enzyme recognition sites as described in Materials and Methods and the corresponding fusion proteins expressed in *Escherichia coli* were purified (Fig. 4). Fig. 5 summarizes the truncation mutants. Then, their abilities to interact with the 10-kD protein in the presence of  $\text{Ca}^{2+}$  were assessed and the results are shown in Fig. 5 (A and B). The 10-kD protein interacted with GST/TM2 (1-238), GST/TM2 (1-183), GST/TM2 (1-133), and GST/TM2(1-107) fusion proteins; the interaction being comparable to, or more efficient than that observed between the 10-kD protein and GST/TM2 on a molar basis. The 10-kD protein interacted with GST/TM2(1-101), GST/TM2(1-88), GST/TM2(1-84), and GST/TM2(88-284) to a much lesser extent, and it did not interact



**Figure 4.** SDS-PAGE analysis of purified GST/TM2 fusion protein and various truncation mutants. Bacterially expressed fusion proteins containing GST were purified by chromatography on glutathione-Sepharose as described in Materials and Methods. Some fusion proteins were further purified by anion exchange column chromatography. 2  $\mu\text{g}$  of purified fusion protein were loaded on a gel. After electrophoresis, the gel was stained with Coomassie blue and destained. Lane 1 and 12, GST; lane 2 and 13, GST/TM2; lane 3, GST/TM2 (1-238); lane 4, GST/TM2(1-183); lane 5, GST/TM2(1-133); lane 6, GST/TM2(1-107); lane 7, GST/TM2(1-101); lane 8, GST/TM2(1-88); lane 9, GST/TM2(1-84); lane 10, GST/TM2(1-38); lane 14, GST/TM2(88-284); lane 15, GST/TM2(107-284); lane 16, GST/TM2(119-284); and lanes 11 and 17, molecular weight standards.

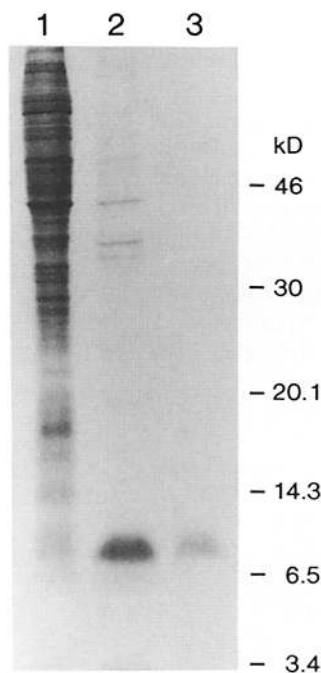


**Figure 5.** Binding of the 10-kD protein to various truncation mutants of GST/TM2. (A) Schematic drawing of the truncation mutants of GST/TM2 and their binding activity to the 10-kD protein. (++) Strong 10-kD protein-binding activity of the truncation mutants; (+) moderate binding activity; (±) very weak binding activity; and (-) indicates no detectable binding activity. The region primarily responsible for the binding of the 10-kD protein is mapped to residues between 39-107 on TM2 (shadowed box). (B) Binding activity of various truncation mutants to the 10-kD protein. Glutathione-Sepharose beads bearing 10  $\mu$ g of each truncation mutant were incubated with  $^{35}$ S-labeled cell lysates of NIH 3T3 cells for 1 h in Tris-buffered saline containing 1 mM  $\text{CaCl}_2$ . After the incubation, the beads were washed and the materials bound were released in SDS sample buffer and loaded onto a 15% gel. Only the regions of interest are shown.

with GST/TM2(1-38), GST/TM2(107-284), and GST/TM2(119-284). These results suggest that the integrity of the region between residues 39-107 of TM2 is required for the 10-kD protein binding.

### Isolation and Identification of the 10-kD Protein

Preliminary experiments showed that the 10-kD protein was more abundant in v-Ha-ras-transformed NIH 3T3 cells (pH 1-3) than in NIH 3T3 cells, as assessed by a binding assay (data not shown; see also Fig. 10). Therefore, we tried to purify the 10-kD protein from an extract of pH 1-3 cells. The GST/TM2(1-107) fusion protein was coupled to Sepharose and used as an affinity matrix to select the 10-kD protein. We chose this truncated form of fusion protein because the yield of it was greater than that of GST/TM2 when purified from an extract of *E. coli* (data not shown), probably due to its solubility, and the 10-kD protein bound well to the truncated fusion protein (Fig. 5). An extract of pH 1-3 cells (Fig.



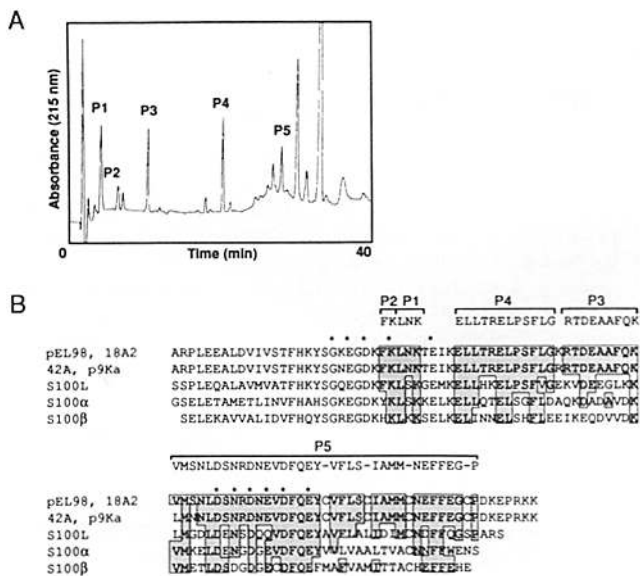
**Figure 6.** Purification of the 10-kD protein from an extract of pH 1-3 cells. Lane 1, Triton X-100-soluble fraction (30  $\mu$ g protein); lane 2, materials eluted from the affinity column using cation-free buffer containing 20 mM EGTA (10  $\mu$ g protein); lane 3, purified 10-kD protein prepared by electroelution (1  $\mu$ g protein). Protein molecular weight standards (in kD) are shown at right. After electrophoresis, the gel was stained with Coomassie blue and destained.

6, lane 1) was applied to the affinity matrix in the presence of  $\text{Ca}^{2+}$ . Elution with an EGTA-containing buffer released a protein that had an apparent molecular mass of 10 kD with some minor contaminants which were revealed by SDS-PAGE (Fig. 6, lane 2). The results indicate that the 10-kD protein interacts with the affinity matrix in a  $\text{Ca}^{2+}$ -dependent manner, supporting the *in vitro* binding experiments described above. To further purify the 10-kD protein, the 10-kD band was excised from the gels, and the protein was electroeluted (Fig. 6, lane 3). The purified protein was then subjected to amino acid sequence analysis.

Initial attempts to sequence intact 10-kD protein were unsuccessful, indicating that the  $\text{NH}_2$  terminus of the 10-kD protein might be blocked. Then, lysylendopeptidase was utilized to digest the purified 10-kD protein. The resulting peptides were separated on a  $\text{C}_{18}$  column. Fig. 7 A shows HPLC peptide maps of enzymatic digests of the 10-kD protein. Among the separated peptides, amino acid sequences of five peptides could be analyzed (P1, P2, P3, P4, and P5), as shown in Fig. 7 B. A computer homology search of the Swiss-Prot data base revealed a complete match with S100-related calcium-binding proteins (pEL98, 18A2) (21, 31) whose amino acid sequences were deduced from the corresponding cDNAs (Fig. 7 B). The partial amino acid sequence of the 10-kD protein also showed a high homology (96.8%) with proteins (42A, p9Ka) and with other S100-related proteins.

To further confirm if the 10-kD protein is identical to the pEL98 or 18A2 protein, we performed two separate experiments. First, *in vitro* translation product of the pEL98 RNA transcript and the 10-kD protein, both of which were labeled with [ $^{35}$ S]methionine, were mixed and then co-electrophoresed on a two-dimensional polyacrylamide gel. As previously reported (21), the *in vitro* translation product of the pEL98 RNA transcript showed a major radioactive spot with a pI value of  $\sim 5.4$  and minor spots with different pI values



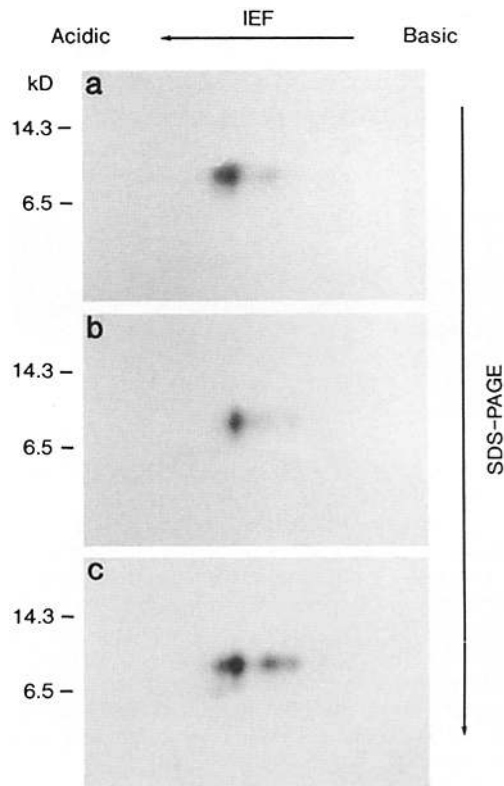


**Figure 7.** Determination of the amino acid sequences of peptides prepared by digestion of the purified 10-kD protein with lysylendopeptidase. (A) Analytical HPLC peptide maps of the 10-kD protein. A preparation of the 10-kD protein was digested with lysylendopeptidase in 50 mM Tris-HCl, pH 9.0 and 1 mM EDTA for 18 h at 30°C, the enzyme to protein ratio being 1:100 (wt/wt). The cleavage products were separated by reverse-phase HPLC on a C<sub>18</sub> column. The peptides (P1-P5) were used to determine the sequence of the 10-kD protein. (B) Comparison of the amino acid sequences among the 10-kD protein and S100-related proteins. The amino acid sequence of the peptides (P1-P5) are shown in single letter code at the top. The horizontal bars indicate unidentified amino acid residues. Methionine residues corresponding to the initiation codon are not represented. Identical amino acids to those of the 10-kD protein are shaded. Asterisks indicate the possible amino acid residues involved in the interaction with calcium ion. The sequences of pEL98, 18A2, 42A, p9Ka S100a, S100b, and S100L are from references 21, 30, 31, and 40, respectively.

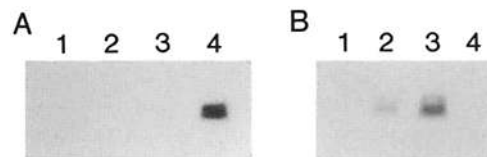
(Fig. 8 b). The 10-kD protein also displayed a major and several minor radioactive spots (Fig. 8 a). When the two were mixed and co-electrophoresed, they co-migrated (Fig. 8 c). Second, we examined whether the in vitro translation product of the pEL98 RNA transcript is able to bind to GST/TM2. The in vitro translation product was incubated with the beads bearing GST or GST/TM2 in the presence of 1 mM CaCl<sub>2</sub> or 1 mM EGTA. As shown in Fig. 9 A, the in vitro translation product bound to GST/TM2 (lane 4), but not to GST (lane 3), in the presence of Ca<sup>2+</sup>, while it did not bind to GST/TM2 in the presence of EGTA (lane 2). From these observations, we concluded that the 10-kD protein is identical to the pEL98 protein.

#### Binding of the pEL98 Protein to the GST/TM5 or the GST/β-actin Fusion Proteins

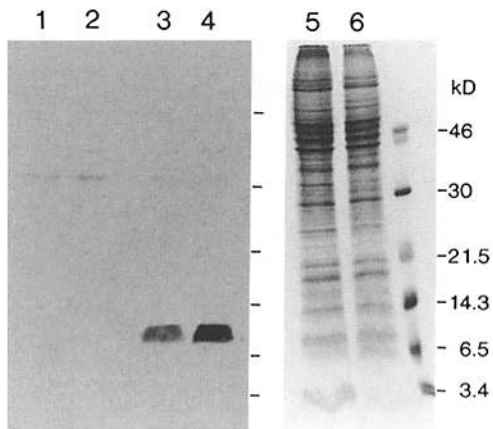
To examine whether the in vitro translation product of the pEL98 RNA transcript can also bind to the low molecular weight TMs, GST/TM5 (tropomyosin isoform 5 containing GST fusion protein) was generated and used. As shown in Fig. 9 B, the product was found to bind to GST/TM5 more efficiently than to GST/TM2 (lanes 2 and 3). This result was



**Figure 8.** Two-dimensional gel analysis of the 10-kD protein and the in vitro translation product of the pEL98 transcript. (a) [<sup>35</sup>S]Methionine-labeled 10-kD protein. Glutathione-Sepharose beads bearing 20 μg of GST/TM2 were incubated with <sup>35</sup>S-labeled cell lysates of NIH 3T3 cells for 2 h in Tris-buffered saline containing 1 mM CaCl<sub>2</sub>. The materials bound to GST/TM2 were analyzed. (b) In vitro translation product of the pEL98 transcript. The insert of pEL98 cDNA was subcloned into pGEM-3Z and in vitro transcription was carried out by using SP6 RNA polymerase. In vitro translation was performed in a reticulocyte lysate cell-free system using [<sup>35</sup>S]methionine as a tracer amino acid. (c) A mixture of samples a and b. Only the regions of interest (pI 5-6) are shown. Molecular weight standards (kD) are shown at left.



**Figure 9.** Binding of the in vitro translation product of the pEL98 transcript to GST, GST/TM2, GST/TM5, or GST/β-actin. (A) Glutathione-Sepharose beads bearing 10 μg of GST (lanes 1 and 3) or GST/TM2 (lanes 2 and 4) were incubated with the in vitro translation product of the pEL98 transcript for 2 h in Tris-buffered saline containing 1 mM EGTA (lanes 1 and 2) or 1 mM CaCl<sub>2</sub> (lanes 3 and 4). The translation product was prepared as described in the legend of Fig. 8. After the incubation, the beads were washed and the materials bound were released in SDS sample buffer and loaded onto a 15% gel. Note that the translation product binds to GST/TM2 only in the presence of Ca<sup>2+</sup>. (B) Glutathione-Sepharose beads bearing 10 μg of GST (lane 1), GST/TM2 (lane 2), GST/TM5 (lane 3), or GST/β-actin (lane 4) were incubated with the in vitro translation product of the pEL98 transcript for 2 h in Tris-buffered saline containing 1 mM CaCl<sub>2</sub>. Note that the product binds to GST/TM2 and GST/TM5 but not to GST/β-actin. Only the regions of interest are shown.



**Figure 10.** Western blot analysis of the specificity of the antiserum directed against the 10-kD (pEL98) protein. Triton X-100-soluble cell extracts (20  $\mu$ g of protein) of NIH 3T3 cells (lanes 1, 3, and 5) and pH 1-3 cells (lanes 2, 4, and 6) were electrophoresed on 15% polyacrylamide gels under reducing conditions. The resolved proteins were electrophoretically transferred to nitrocellulose membrane and the 10-kD (pEL98) protein was detected using polyclonal antibodies and an ECL Western blotting detection kit (Amersham International). (Lanes 1 and 2) Preimmune serum; (lanes 3 and 4) antiserum against the 10-kD (pEL98) protein; (lanes 5 and 6) Coomassie blue staining of the gel. Molecular weight standards (kD) are indicated at right.

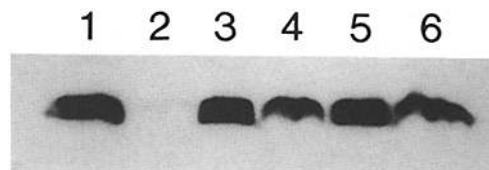
confirmed in multiple experiments, and since the beads were bearing the same amount (10  $\mu$ g) of GST/TM2 and GST/TM5, the difference was significant even on a molar basis. On the other hand, the product did not bind to GST/ $\beta$ -actin ( $\beta$ -actin containing GST fusion protein) (Fig. 9 B, lane 4), demonstrating the specificity of the binding of the 10-kD (pEL98) protein to TMs.

#### **Western Blot Analysis of the Specificity of Antibodies Directed against the 10-kD (pEL98) Protein**

We produced a rabbit antiserum directed against recombinant pEL98 protein, and the specificity of the antiserum was confirmed by Western blot analysis. As shown in Fig. 10, the antiserum recognized a protein with a molecular mass corresponding to the 10-kD protein (lanes 3 and 4), whereas the preimmune serum did not (lanes 1 and 2). We also compared the amount of the 10-kD protein in NIH 3T3 and pH 1-3 cells and found that the amount of the 10-kD protein in pH 1-3 cells was increased over that of NIH 3T3 cells (Fig. 10, lanes 3 and 4).

#### **Western Blot Analysis of the Distribution of the 10-kD (pEL98) Protein**

To examine the distribution of the 10-kD (pEL98) protein in Triton-soluble and -insoluble cytoskeletal residues of NIH 3T3 cells, we extracted the cells with Triton X-100 in the presence of 1 mM or 2  $\mu$ M free  $\text{Ca}^{2+}$  or 0.2 mM EGTA. Proteins in the soluble fraction and the insoluble residues were resolved by SDS-PAGE and the 10-kD (pEL98) protein was detected by Western blot analysis. As shown in Fig. 11, when the cells were extracted in the presence of EGTA, the 10-kD (pEL98) protein was scarcely detected in the Triton-



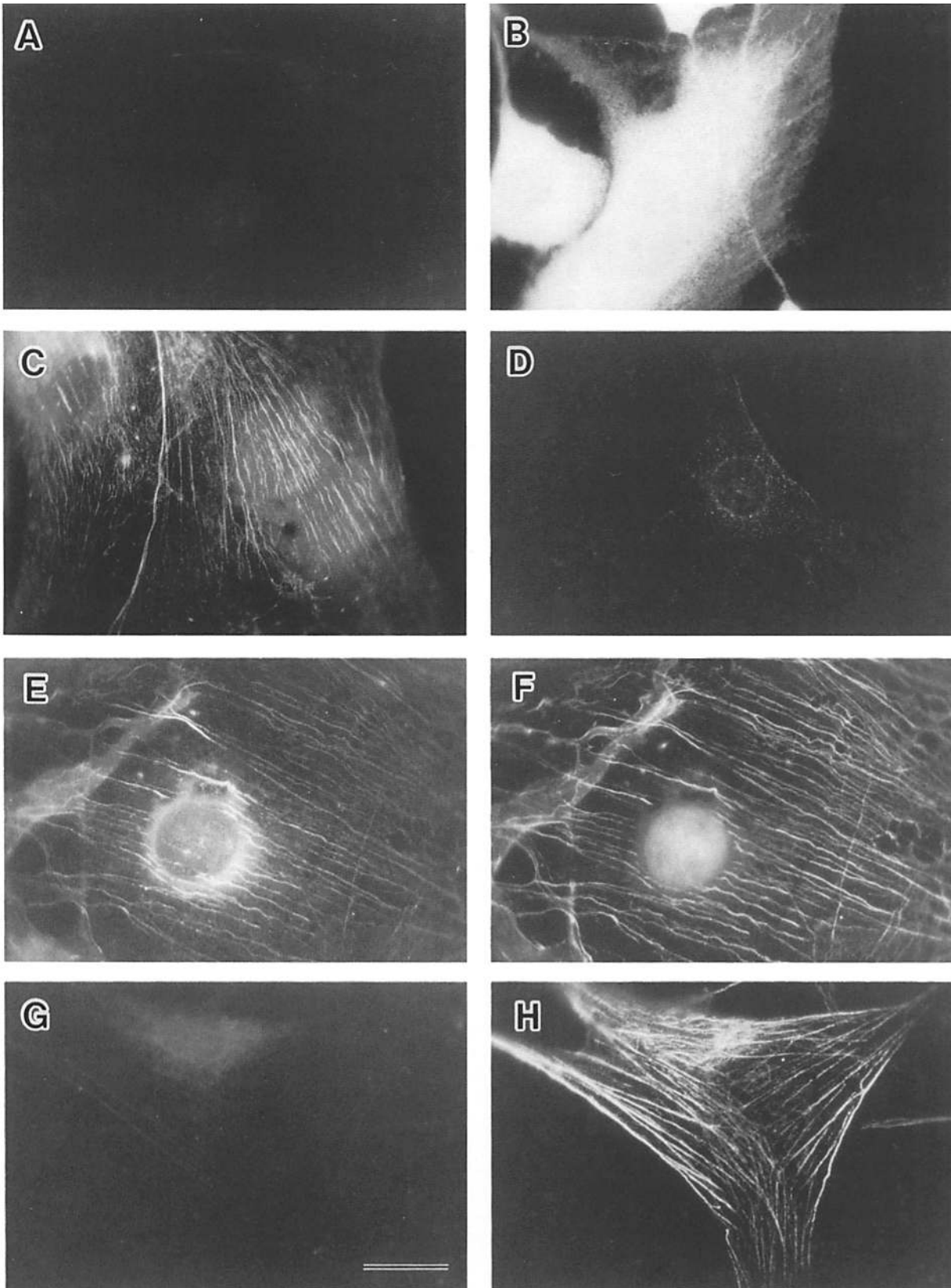
**Figure 11.** Immunoblot of NIH 3T3 cell subfractions. NIH 3T3 cells were extracted for 2 min with 0.05% Triton X-100 in the presence of 0.2 mM EGTA (lanes 1 and 2), 1 mM  $\text{Ca}^{2+}$  (lanes 3 and 4) or 2  $\mu$ M  $\text{Ca}^{2+}$  (lanes 5 and 6). Proteins in the soluble fraction (lanes 1, 3, and 5) and the insoluble fraction (lanes 2, 4, and 6) were applied to SDS-PAGE, and transferred to nitrocellulose membrane. The 10-kD (pEL98) protein was detected as described in the legend of Fig. 10. Only the region of interest is shown.

insoluble residues (lane 2) and almost all of the protein was recovered in the Triton X-100-soluble fraction (lane 1). On the other hand, some of the 10-kD (pEL98) protein could be detected in the Triton X-100-insoluble residues when the cells were extracted in the presence of 1 mM  $\text{Ca}^{2+}$  (Fig. 11, lane 4). The 10-kD (pEL98) protein was also detected in the Triton-insoluble residues even when the cells were extracted in the presence of 2  $\mu$ M  $\text{Ca}^{2+}$  (Fig. 11, lane 6). These results suggest that a part of the 10-kD (pEL98) protein indeed is associated with the cytoskeletal residues in a  $\text{Ca}^{2+}$ -dependent manner.

#### **Cellular Localization of the 10-kD (pEL98) Protein**

To determine the cellular localization of the 10-kD (pEL98) protein, NIH 3T3 cells or their cytoskeletal residues were examined by indirect immunofluorescence microscopy by using the affinity-purified anti-pEL98 antiserum and fluorochrome-conjugated secondary antibodies. When the cells permeabilized with detergent were stained with the affinity-purified antiserum, bright, diffuse fluorescent staining was observed in the cytoplasm of a cell (Fig. 12 B), while only faint staining was apparent when the preimmune serum was used (Fig. 12 A). In some cases, fibrillar streaks were seen in the cytoplasm of a well-spread cell (Fig. 12 B). On the other hand, when cytoskeletal residues prepared by extraction with solution containing 1 mM free  $\text{Ca}^{2+}$  were stained with the antiserum, fibrillar streaks were clearly observed (Fig. 12 C), while only background level of staining was seen when cytoskeletal residues were prepared by extraction with solution containing EGTA (Fig. 12 D). Fibrillar streaks were also observed when cytoskeletal residues were prepared with extraction solution containing 2  $\mu$ M  $\text{Ca}^{2+}$ , although the fluorescence was slightly less intense than that seen in Fig. 12 C (data not shown). Double-label staining with the antiserum and rhodamine-phalloidin revealed that the fibrillar streaks distributed along the actin microfilament bundles (data not shown). The distribution of the 10-kD (pEL98) protein relative to that of TM was examined by double-label immunofluorescence microscopy. The cytoskeletal residues prepared by extraction with solution containing 1 mM  $\text{Ca}^{2+}$  were stained with both a mouse monoclonal anti-TM antibody and the affinity-purified antiserum. The results showed that the 10-kD (pEL98) protein colocalized with TM (Fig. 12, E and F). Again, essentially no fibril-





**Figure 12.** Localization of the 10-kD (pEL98) protein in NIH 3T3 cells. (*A* and *B*) Cells were permeabilized with 0.5% Triton X-100 for 4 min after fixation. (*C*, *E*, and *F*) Cytoskeletal residues prepared by treating the cells with 0.05% Triton X-100 in the presence of 1 mM  $\text{Ca}^{2+}$  for 2 min before fixation. (*D*, *G*, and *H*) Cytoskeletal residues prepared by treating the cells with 0.05% Triton X-100 in the presence of 0.2 mM EGTA for 2 min before fixation. Cells were stained with preimmune serum (*A*) or affinity-purified anti-10-kD (pEL98) protein antibodies (*B*) followed by TRITC-conjugated goat anti-rabbit IgG antibodies. Cytoskeletal residues were stained with affinity-purified anti-10-kD (pEL98) protein antibodies (*C* and *D*) followed by TRITC-conjugated goat anti-rabbit IgG antibodies. Cytoskeletal residues were double-stained with affinity-purified anti-10-kD (pEL98) protein (*E* and *G*) and mouse TM311 antibodies (*F* and *H*) followed by FITC-conjugated goat anti-rabbit IgG antibodies and TRITC-conjugated anti-mouse IgG antibodies, respectively. TM311 antibody recognizes TM1, TM2, and TM3. Bar, 20  $\mu\text{m}$ .

lar distribution of the 10-kD (pEL98) protein was observed when the cytoskeletal residues were prepared by extraction with solution containing EGTA (Fig. 12 G), while TM could be seen (Fig. 12 H). These results suggest that some of the 10-kD (pEL98) protein associates with TMs in a Ca<sup>2+</sup>-dependent manner in microfilament bundles, while a majority of the protein exists diffusely in the cytoplasm of a cell.

## Discussion

We report here the detection of a protein (10-kD protein) that binds directly to the nonmuscle tropomyosin isoform TM2 in a Ca<sup>2+</sup>-dependent manner *in vitro*. This conclusion is supported by the following evidence. First, the 10-kD protein bound to GST/TM2, but not to GST, in a Ca<sup>2+</sup>-dependent manner in an *in vitro* binding assay. Second, the binding was inhibited by molar excess of free TM2, but not by GST, in a competition assay. Third, the protein bound to an affinity matrix made from GST/TM2(1-107) coupled to Sepharose and was eluted with EGTA. Next, we identified the 10-kD protein as the translation product of pEL98 mRNA. This conclusion is supported by the observations that: (a) partial amino acid sequence of the 10-kD protein was identical with that of the pEL98 (or 18A2) protein that was deduced from the cDNA (21, 31); (b) the 10-kD protein co-migrated with the *in vitro* translation product of the pEL98 transcript on a two-dimensional polyacrylamide gel; and (c) the *in vitro* translation product of the pEL98 transcript bound to GST/TM2, but not to GST, in a Ca<sup>2+</sup>-dependent manner. In addition, the specificity of the binding of the 10-kD (pEL98) protein to TM was confirmed by the fact that it did not bind to GST/ $\beta$ -actin fusion proteins.

The concentrations of Ca<sup>2+</sup> required for binding of the 10-kD (pEL98) protein to GST/TM2 were revealed to be in the range of 1–100  $\mu$ M in a binding experiment (Fig. 3, B and C). Although binding of the 10-kD (pEL98) protein could be observed to some extent at the concentrations of Ca<sup>2+</sup> between 0.001 and 0.1  $\mu$ M, this binding is probably due to the Ca<sup>2+</sup>-bound form of the 10-kD (pEL98) protein that is already present in the cell lysates. Alternatively, although it is quite unlikely, the 10-kD (pEL98) protein might have high-affinity sites for Ca<sup>2+</sup>. Since intracellular calcium concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) do not rise above 1  $\mu$ M, it is possible to argue that the binding between the 10-kD (pEL98) protein and TM observed in this study should not be of physiological significance. However, several *in vitro* studies have shown that a number of cytoplasmic proteins accepted as being calcium-sensitive exhibit their calcium sensitivity in the 1–100  $\mu$ M range. For instance, calmodulin is reported to require 1–10  $\mu$ M Ca<sup>2+</sup> to activate phosphodiesterase activity (32). Ca<sup>2+</sup>-calmodulin-dependent phosphorylation in synaptosomal cytosol occurs in the 1–50  $\mu$ M range (45). Protein kinase C requires 1–100  $\mu$ M Ca<sup>2+</sup> for its kinase activity (33) and Ca<sup>2+</sup>-dependent cysteine proteases, calpain I and calpain II, requires 1–10  $\mu$ M Ca<sup>2+</sup> and 0.1–1 mM Ca<sup>2+</sup> for their activity, respectively (63). In addition, the ability of S100 protein to relieve caldesmon-induced ATPase inhibition was observed at 10–100  $\mu$ M Ca<sup>2+</sup> (19). Therefore, on the basis of these works, the binding between the 10-kD (pEL98) protein and TM seems to be physiologically relevant. Supporting this notion, a part of the 10-kD (pEL98) protein was visualized *in situ* as fibrillar streaks in the cytoplasm of a cell

by immunofluorescent staining studies (see Fig. 12 B) and was detected in Triton-insoluble cytoskeletal residues prepared in the presence of micromolar concentrations of Ca<sup>2+</sup> (Fig. 11). Furthermore, some of the 10-kD (pEL98) protein could be detected in the cytoskeletal residues prepared by extraction with solution containing Ca<sup>2+</sup> (Fig. 12, C and E) and colocalized with TMs (Fig. 12, E and F), but not in the residues prepared by extraction with solution containing EGTA (Fig. 12, D and G). Thus, these results suggest that the 10-kD (pEL98) protein indeed binds to TMs *in vivo* in a Ca<sup>2+</sup>-dependent manner.

By using several COOH-terminal and NH<sub>2</sub>-terminal truncation mutants of TM2, the region containing residues between 39–107 of TM2 appeared to be primarily responsible for binding to the 10-kD (pEL98) protein (Fig. 5). Amino acid residues between 81–107 are within a conserved region of TM isoforms and almost identical between TM2 and TM5, while NH<sub>2</sub>-terminal region of TM2 is different from that of TM5 (53, 56). The fact that the *in vitro* translation product of the pEL98 transcript also bound to GST/TM5 suggests that the region containing residues 81–107 are necessary for binding of the 10-kD (pEL98) protein; yet the pEL98 protein appears to bind more efficiently to GST/TM5 (Fig. 9 B). Thus, the variable NH<sub>2</sub>-terminal region may also be necessary for binding of the 10-kD protein. It is possible that sequences between residues 81–107 could be necessary for folding and/or dimerization of the NH<sub>2</sub>-terminal portion of TM and that the actual binding site is in the variable NH<sub>2</sub>-terminal region of TMs (residues 39–80 in the case of TM2). Further study is needed to clarify this point.

So far, proteins that bind directly to TM in nonmuscle cells are limited. Tropomodulin, a 43-kD TM-binding protein, is one of them. In our binding assays, we failed to detect tropomodulin-like polypeptides. Although a 43-kD protein was repeatedly detected in the binding assays, we concluded that it was nonspecific because molar excess of free TM2 did not inhibit the binding in a competition assay (Fig. 1 B). The failure may be mainly due to the localization of tropomodulin since it locates in the Triton X-100-insoluble membrane cytoskeleton fraction, but not in the cytosol, of erythrocytes (16, 17). It is unknown whether this is the case for nonerythroid cells, but Triton X-100-soluble fraction of NIH 3T3 cells that we used in our binding assays may not contain tropomodulin-like polypeptides. Similarly, we could not clearly detect nonmuscle caldesmon of 70–80 kD in our binding assays. This may be due to either the lack of nonmuscle caldesmon in Triton X-100-soluble fraction of NIH 3T3 cells because it localizes within the subset of actin filaments in normal cells (49) or the weak *in vitro* binding of caldesmon to TM under physiological ionic conditions and/or the absence of F-actin which enhances caldesmon-TM interaction (23, 28) under our binding conditions.

The function(s) of the 10-kD (pEL98) protein in microfilaments is unknown, but several possibilities might be speculated. (a) The 10-kD (pEL98) protein interferes with the functions of tropomodulin or nonmuscle caldesmon directly. This seems to be unlikely, because the 10-kD (pEL98) protein-binding site is located between residues 39–107 of TM, whereas tropomodulin binds to (one of) the ends of the TM molecules (17) and the major caldesmon-binding site is located between residues 142–227 of TM (61). Therefore, the 10-kD (pEL98) protein may not compete with these TM-

binding proteins by simply competing for their binding sites on TM. However, we can not exclude the possibility that the binding of the 10-kD protein to TM affects the functions of these TM-binding proteins indirectly. (b) The 10-kD (pEL98) protein enhances the binding of nonmuscle TMs to F-actin and stabilizes actin filament organization. This seems to coincide with the immunofluorescent staining results in which a part of the 10-kD (pEL98) protein localized in well-organized microfilament bundles. If so, however, we can not account for the increase in amount of the pEL98 mRNA in many transformed cell lines which show less-ordered actin microfilament organization compared to their normal counterparts and the previous observation that the amount of pEL98 mRNA is more abundant in "immortalized" BALB/c3T3 cells than in "primary" cells (21), both of which show well-organized actin microfilaments. Considering that a rise in  $[Ca^{2+}]_i$  in a cell often results in dynamic cellular changes such as cell division, cell migration and changes in cell shape which need dynamic changes in cytoarchitecture, this possibility seems to be unlikely. (c) The 10-kD (pEL98) protein prevents the interaction of TMs with actin and/or dissociate TMs from actin. At least, the increase in the amount of pEL98 mRNA in many transformed cells might be able to be explained partly by this possibility. However, this contrasts with the immunofluorescent staining results. (d) The 10-kD (pEL98) protein functions to confer  $Ca^{2+}$  sensitivity to TM regulation of actomyosin ATPase activity as a troponin complex does so. This possibility is plausible and valuable to be tested in *in vitro* reconstitution experiments using purified proteins such as TM, actin and myosin light chain kinase.

The pEL98 (18A2, p9Ka, or 42A) protein is the putative calcium-binding protein that is highly homologous to S100 protein (22, 27). Like calmodulin, S100 protein interacts with other proteins and modulates their biological functions in the presence of  $Ca^{2+}$  (13). S100-related proteins are thought to have similar functions (22, 27). However, the exact nature of effector proteins of S100-related proteins are poorly understood. The present study showed that one of the effector proteins of the pEL98 protein is TMs. However, the presence of a large amount of the 10-kD (pEL98) protein in the cytoplasm of a cell implies that the protein is also involved in other fundamental cell activities. If so, 10-kD (pEL98) protein might interact with proteins other than TMs after  $Ca^{2+}$  binding. Interestingly, our preliminary experiments showed that several cellular proteins were co-immunoprecipitated with the 10-kD (pEL98) protein in the presence of  $Ca^{2+}$ , but not in the presence of EGTA, in immunoprecipitation assays (data not shown). Analyses of these co-immunoprecipitated proteins may facilitate identification of the effector proteins of the 10-kD (pEL98) protein.

Recently, a protein named calvasculin which shows a high homology with the pEL98 (18A2, 42A, or p9Ka) protein is reported to be synthesized constitutively and secreted by bovine aortic smooth muscle cells (60). It shows a cytoplasmic granular distribution that was typical of a secreted protein in BASM cells and 3Y1 cells and exhibits  $Ca^{2+}$ -dependent binding to 36-kD microfibril-associated glycoprotein (60). We examined the distribution of the 10-kD (pEL98) protein in 3Y1 cells and also in normal rat kidney cells and observed essentially the same distributions of the protein in these cells as in NIH 3T3 cells and never observed a cytoplasmic gran-

ular distribution of the protein (data not shown). Whether the 10-kD (pEL98) protein is related to calvasculin and whether the protein displays different functions in different cell types need to be examined.

pEL98 and 18A2 were identified as a mRNA related to cell immortalization (21) and a serum-inducible mRNA in BALB/c3T3 cells (31), respectively. The p9Ka mRNA was reported to be induced during differentiation of rat mammary epithelial stem cells to myoepithelial-like cells and has recently been shown to be involved in regulation of metastatic phenotype in rat mammary epithelial cells (12). 42A was identified as a mRNA regulated by nerve growth factor in a rat pheochromocytoma cell line (40). Thus, these S100-related proteins are thought to play a role in a wide variety of biological events. These proteins are identical or alike each other and therefore probably involved in the mechanism(s) by which TM-actin interactions are regulated in nonmuscle cells. However, we are unable to explain all of these biological phenomena by this mechanism. Further studies are needed to evaluate the function(s) of these S100-related proteins.

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