Interaction of Chicken Liver Tropomyosin with Glutamate Dehydrogenase

Authors: Akutsu, Satonari, Tobita-Teramoto, Takayuki, Xie, Lin, Hirabayashi, Tamio, and Miyazaki, Jun-Ichi

Source: Zoological Science, 17(7) : 871-879

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.871

ZOOLOGICAL SCIENCE **17**: 871–879 (2000)

Interaction of Chicken Liver Tropomyosin with Glutamate Dehydrogenase

Satonari Akutsu, Takayuki Tobita-Teramoto[†], Lin Xie, Tamio Hirabayashi, and Jun-Ichi Miyazaki*

Institute of Biological Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan.

ABSTRACT—Tropomyosin (TM) is one of the actin-regulatory proteins and stabilizes the filamentous structure of F-actin. Although it has been suggested that multiple TM isoforms play important roles in nonmuscle cells and several TM-binding proteins have been identified and characterized, the functional properties of nonmuscle TM isoforms are not well understood. In order to investigate the roles of TM in nonmuscle cells, we searched for novel nonmuscle TM-binding proteins from the chicken liver total acetone powder extract by TM-affinity column chromatography. As a consequence, a protein of 57 kDa was mainly eluted from the column and its partial amino acid sequence of the protease-digested fragment was determined. Homology search using SWISS-PLOT showed that the 57 kDa protein was nearly identical to chicken glutamate dehydrogenase (GDH: EC 1.4.1.3). Purified GDH had the ability to bind to the TM-affinity column, indicating that GDH associated directly with liver TM. The binding of GDH to TM was abolished in the presence of 0.1 mM ATP, that is known to decrease the rate of the GDH enzymatic activity at pH 7.5. Thus, we have demonstrated the interaction between nonmuscle TM and GDH for the first time.

INTRODUCTION

Tropomyosin (TM), an α-helical coiled-coil protein, is one of the constituents of thin filaments in myofibrillar cells and of microfilaments in nonmuscle cells (Pittenger *et al*., 1994; Lin *et al*., 1997). The dimeric form of TM polymerizes in a head-to-tail manner and associates with F-actin in the two grooves of filaments to stabilize the filamentous structure. Especially in higher vertebrates, a number of TM isoforms is generated from four genes by using their alternative promoters and alternative exons. They are generally categorized into two types of high molecular weight (high M_r) and low molecular weight (low M_r) isoforms. It is well recognized that both high and low M_r TM isoforms are distributed in nonmuscular tissues as well as in embryonic muscles. As is the case of actin, isoform transition occurs from mainly low M_r isoforms to the muscle-specific high M_r ones during muscle maturation (Hosoya *et al*., 1989; Lin *et al*., 1997).

Numerous biochemical, immunocytochemical, and genetic studies have been devoted to elucidate the functional significance of nonmuscle TM isoforms and documented their involvement in cell shape stabilization, cytokinesis during cell division, intracellular granule movement, and organelle distribution (Pittenger *et al*., 1994; Lin *et al*., 1997). Thus, it is strongly suggested that TM isoforms play significant functions in nonmuscle cells. Nevertheless, those roles can not be ascribed exclusively to nonmuscle TM isoforms. TM-binding proteins are certainly involved in the regulation of these physiological events through inducing conformational changes of TM and utilizing it as a scaffold.

It is well known that TM regulates an actomyosin interaction in myofibrillar cells in association with the troponin complex in a Ca^{2+}-dependent fashion (Ebashi *et al*., 1968). In nonmuscle cells, the troponin complex is absent, but several TM-binding proteins were identified. Caldesmon and calponin, that were originally isolated from smooth muscle, bind to both TM and F-actin in a Ca^{2+}-calmodulin dependent manner (Sobue *et al*., 1981; Takahashi *et al*., 1986). Later, low M_r caldesmon and acidic calponin were detected from nonmuscular sources and characterized (Matsumura and Yamashiro, 1993; Appligate *et al*., 1994; Trabelsi-Terzidis *et al*., 1995). It is noteworthy that nonmuscle caldesmon increases the ability of low M_r TM isoforms to associate with F-actin (Yamashiro-Matsumura and Matsumura, 1988), and that it also enhances the affinity of rat nonmuscle high M_r TM-1 to F-actin, but not that of another nonmuscle high M_r, TM-2 (Pittenger *et al*., 1995). Although acidic calponin exhibits extensive homology in the primary structure to the smooth muscle basic calponin except for the C-terminal domain (Appligate *et al*., 1994), it

* Corresponding author: Tel. +81-298-53-4665;
FAX. +81-298-53-6614.
E-mail: junichi@sakura.cc.tsukuba.ac.jp
[†] Present address: Department of Biology, Keio University, Hiyoshi 4-1-1, Kohoku-ku, Yokohama, Kanagawa 223-0061, Japan.

has not been demonstrated yet whether it can bind to TM. Tropomodulin, that was firstly identified in human erythrocytes as a TM-binding protein and also found in the striated muscle, associates with the pointed-end of microfilaments (Fowler, 1996). The pointed-end capping activity of tropomodulin is greater in the presence of TM than in its absence. Other proteins that interact with nonmuscle TM, including human serum albumin (Hitchcock-DeGregori *et al*., 1985), myelin basic protein (Dobrowolski *et al*., 1986), plasma gelsolin (Koepf and Burtnick, 1992), S100 proteins (Takenaga *et al*., 1994; Gimona *et al*., 1997), were reported. Despite the existence of several proteins that can associate with TM, the physiological roles of these proteins are not fully understood.

Liver is the largest nonmuscular organ and plays central roles in diverse metabolic pathways, such as storage of glycogen, detoxification, bile acid formation and lipid turnover. Several studies have given evidences that the actin cytoskeleton is involved in the contraction of bile canaliculi in parenchymal cells and of fenestrae in nonparenchymal sinusoidal endothelial cells (Tsukada *et al*., 1995; Steffan *et al*., 1987; Gatmaitan *et al*., 1996). However, detailed physiological properties of microfilaments and their associated proteins in the liver parenchymal and nonparenchymal cells such as Küpffer cells and fat-storing cells are scarcely investigated and elucidated.

In this study, to obtain further information on the physiological properties of nonmuscle TM isoforms, we searched for novel nonmuscle TM-binding proteins from the chicken liver total acetone powder extract by TM-affinity column chromatography. A protein of 57 kDa, which was eluted from the TM-affinity column, was identified as an enzyme, glutamate dehydrogenase (GDH: EC 1.4.1.3). Purified GDH was also retained on the TM-affinity column in the absence of ATP, but not in its presence, indicating that GDH directly associated with chicken liver TM in an ATP-dependent manner.

MATERIALS AND METHODS

Preparation of proteins

All procedures were performed in a cold room unless otherwise mentioned. Chicken liver TM was prepared according to Tobita *et al.* (1996) with some modifications. Briefly, proteins were extracted from the chicken liver acetone powder with 20 volumes (v/w) of the extraction buffer containing 1 M KCl, 0.1 M KH_2PO_4 and 0.1% (v/v) 2-mercaptoethanol, pH 7.0 for overnight. After boiling the supernatant, isoelectric precipitation (pH 4.5) and ammonium sulfate fractionation in 40–70% saturation were carried out. The resultant precipitate was dissolved in 1 ml of 7 M urea and 0.1% 2-mercaptoethanol and dialyzed against the same solution for overnight. The dialysate was subjected to agarose-IEF according to Hirabayashi (1981). After IEF, the bands corresponding to TM were excised from the agarose gels and dialyzed against the extraction buffer for overnight. Finally, the dialysate was ultracentrifuged at 195,000 x g for 30 min using a Beckman TLA 100.3 rotor. The resulting supernatant was recovered and stored at -80°C until use.

Chicken liver glutamate dehydrogenase (GDH) was prepared as follows. The chicken liver was dissected, washed with cold PBS to remove blood and minced in 4 volumes (v/w) of the extraction buffer consisting of 1% (v/v) Triton X-100, 0.05% (w/v) cetyltrimethylammonium bromide (CTAB), 10 mM K-PO_4, 1 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT, pH 7.5 using a Sorvall Mixer on ice for about 1 min. The mixture was extracted for 1 hr with stirring and centrifuged at 37,000 x g for 20 min using a Beckman JA-20 rotor. The supernatant was pooled and the resulting pellet was re-extracted in the same way as described above. These two extracts were combined and ammonium sulfate fractionation was performed in 0-30 and 30–70% saturation. The precipitate from ammonium sulfate fractionation in 30–70% saturation was dissolved in buffer I containing 10 mM K-PO_4, 1 mM EDTA, 0.1 mM PMSF and 0.1 mM DTT, pH 7.5 and dialyzed against the same buffer for overnight. The dialysate was applied onto a DEAE-TOYOPEARL S650 column (TOSOH: 2.2x20 cm) equilibrated with buffer I using a FPLC System (amersham pharmacia biotech). After washing with the equilibration buffer to remove unabsorbed proteins, bound proteins were eluted with a linear concentration gradient of KCl from 0 to 0.5 M. GDH-active fractions were collected and ammonium sulfate fractionation in 0–70% saturation was done. The pellet was dissolved in 1 M ammonium sulfate in buffer I, pH 7.5 and applied onto a Butyl-TOYOPEARL S650 column (TOSOH: 2.2x20 cm) equilibrated with the same buffer. After washing with the starting buffer, adsorbed proteins were eluted with a decreasing linear concentration gradient of ammonium sulfate from 1 to 0 M. GDH was eluted at the concentration between 0.25 and 0 M ammonium sulfate. These fractions were subjected to ammonium sulfate fractionation. The centrifugal pellet was suspended, dialyzed against buffer I, and applied to a Hydroxylapatite column (BIO-RAD: 1.6x15 cm) equilibrated with buffer I. After washing the column, GDH-containing fractions were eluted with a linear concentration gradient of K-PO_4 from 10 to 500 mM. GDH-containing fractions were desalted, dialyzed, and loaded onto a HiTrap Blue affinity column (amersham pharmacia biotech) equilibrated with buffer I. After washing, bound proteins were eluted at 3 steps with different concentrations of KCl, 0–0.9 M, 0.9–2 M and 3 M in buffer I. GDH was eluted at a concentration of KCl more than 0.9 M and these fractions were concentrated and desalted with 0.15 M KCl in buffer I, pH 7.5 using Centriprep-10 (Amicon). The GDH-enriched fraction was applied onto a HiLoad Superdex 200 pg column (amersham pharmacia biotech: 1.6x60 cm) pre-equilibrated with 0.15 M KCl in buffer I, pH 7.5 and eluted with the same buffer.

Actin was prepared from a rabbit skeletal muscle according to Spudich and Watt (1971).

Determination of protein concentration

Concentrations of chicken liver TM and GDH were determined using A_{280}=0.24 for 1 mg/ml of platelet TM (Côte and Smillie, 1981) and A_{280}=0.95 for 1 mg/ml of chicken liver GDH (Frieden, 1962), respectively. Those of other proteins including crude fractions of TM or GDH were calculated using A_{280}=0.63 for 1 mg/ml of BSA as a standard.

Electrophoresis and immunoblotting

SDS-PAGE was performed by the method of Laemmli (1970). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out according to Hirabayashi (1981). After electrophoresis, gels were stained generally with CBB-R or with CBB-G (Neuhoff *et al*., 1988), if necessary. Immunoblotting was performed according to Towbin *et al.* (1979). After blocking with 2% BSA in TBS for 1 hr, the membrane was incubated with the rabbit primary antibody against chicken small intestinal mucosa TM (Xie *et al*., 1992) and subsequently with the biotinylated goat-anti rabbit IgG antibody. The antigen-antibody reaction was amplified with the ABC reagent (VECTASTAIN ABC kit, Vector Laboratory Inc.) and the immunoreactive proteins were detected using a Konica Immunostain Kit (Konica).

Co-sedimentation assay

Polymerized rabbit skeletal muscle actin (1 mg/ml) and chicken liver TM (0.25 mg/ml) were mixed well in a solution consisting of 0.1 M KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF

and 0.1 mM DTT, pH 7.5 and incubated at 25°C for 2 hr. F-actin or TM alone was also incubated in the same way as a control. Then, the samples were ultracentrifuged at 250,000 x g for 20 min using a Beckman TLA 100.3 rotor. The resultant supernatants and the precipitates were analyzed by 2D-PAGE.

TM-affinity column chromatography

Purified chicken liver TM (2 mg) was coupled to 1 ml of HiTrap NHS-activated Sepharose 4B column (amersham pharmacia biotech) according to the manufacturer's protocol. The BSA-affinity column was prepared in the same way to detect nonspecifically bound proteins to the TM-affinity column. The liver proteins were extracted from the liver tissue or acetone powder with 20 volumes of the buffer consisting of 10% sucrose, 1 M KCl, 50 mM Tris-HCl, 20 mM EDTA, 0.1 mM PMSF and 0.1 mM DTT, pH 8.0 at 4°C for overnight. After centrifugation, the resultant supernatant was subjected to ammonium sulfate fractionation in 0–60% saturation. Centrifugal precipitates were dialyzed against the equilibration buffer containing 50 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF and 0.1 mM DTT, pH 7.5. Then, the proteins from the chicken liver tissue or acetone powder were applied onto the column. Purified GDH was also loaded on the TM- or the BSA-affinity column to ensure the association with TM. After the column was washed with 10 or 20 column volumes of the equilibration buffer, bound proteins were eluted with 15 column volumes of a linear concentration gradient of KCl from 50 mM to 1 M. Eluted fractions of 1.5 ml in each were collected and analyzed by SDS-PAGE.

Partial amino acid sequencing of the TM-binding protein

Effluents from the TM-affinity column were concentrated using Centricon-10 (Amicon) and subjected to 2D-PAGE. After staining the gel with CBB-R, the spot of the TM-binding protein was cut out and dialyzed against 62.5 mM Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol and 1% SDS, pH 6.8 at 4°C for overnight. Then, the dialysate was treated with *Staphylococcus aureus* V8 protease as described previously (Cleveland *et al.*, 1977). Protease-digested fragments were separated on SDS-PAGE and the gel was transferred to the Immobillon-P membrane. The CBB-R-stainable fragment was excised and the N-terminal sequence was determined with a 477A protein sequencer (Applied Biosystems). The obtained amino acid sequence was analyzed by homology search with SWISS-PLOT.

GDH enzymatic assay

Enzymatic activities of GDH were measured spectrophotometrically at 340 nm using a Beckman DU-530 spectrophotometer in the direction of reductive amination of 2-oxoglutarate into L-glutamate. The reaction solution was composed of 0.1 M ammonium acetate, 50 mM triethanolamine, 7 mM 2-oxoglutarate, 2.5 mM EDTA, 1 mM ADP and 0.2 mM NADH (Schmidt, 1974). One unit of enzyme was defined as the amount of enzyme required oxidizing 1 μM NADH per min at 25°C.

RESULTS

Purification of chicken liver TM

For detection of novel TM-binding proteins from nonmuscular sources by biochemical approaches, it is indispensable to obtain highly pure nonmuscle TM because of the possibility that misleading results might be caused by minor contaminants in preparations. Recently, we succeeded to purify the sea urchin egg TM of remarkably high purity by extracting TM from the agarose-IEF gels at the final purification step (Tobita *et al.*, 1996). Considering that the amount of TM is far less in nonmuscular tissues than in muscular ones, we isolated nonmuscle TM from the chicken liver according to the purification procedure. As a results, we could prepare highly purified liver TM (Fig. 1), that was cross-reacted with the anti-TM antiserum (Fig. 1A, lane W). The 2D-PAGE pattern showed that the chicken liver TM was composed of multiple high and low M_r isoforms ranging from 32 to 45 kDa (Fig. 1B). About 2 mg of TM was obtained from 10 g of the chicken liver acetone powder. Since Graceffa and Lehrer revealed that the head-to-tail polymerization of TM is irreversibly lost in contact with 8 M urea for a long time (Graceffa and Lehrer, 1986), we car-

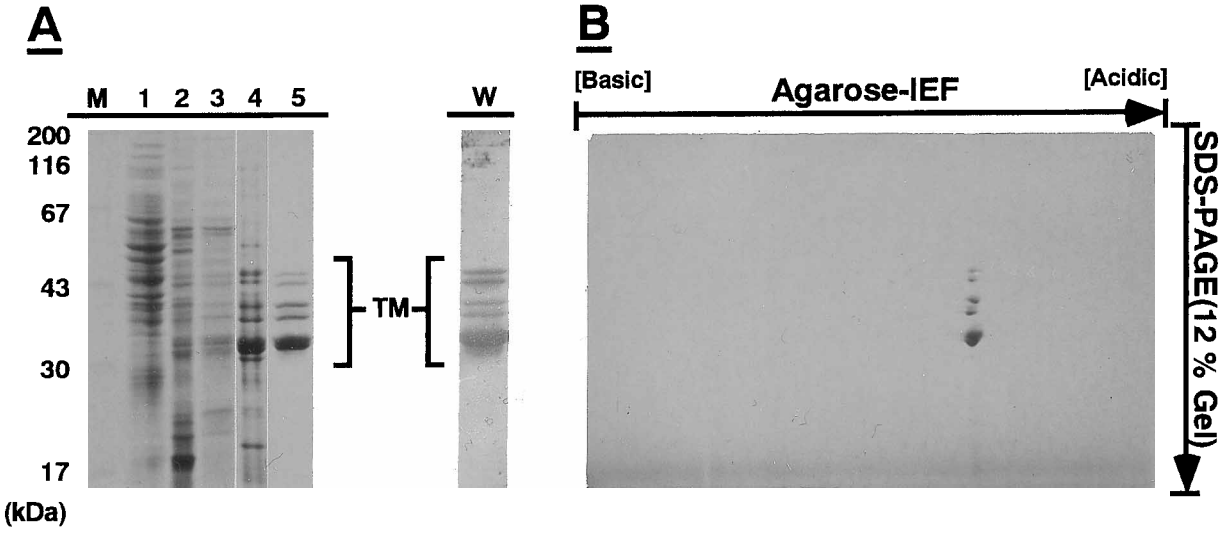

Fig. 1. Purification of TM from the chicken liver. **A**, Chicken liver TM was prepared as described in Materials and Methods. Fractions obtained through the course of purification steps were analyzed by SDS-PAGE. Lane **1**, extract from the chicken liver acetone powder; **2**, supernatant after heat treatment; **3**, precipitate from isoelectric precipitation at pH 4.5; **4**, precipitate from ammonium sulfate fractionation in 40–70% saturation; **5**, purified chicken liver TM after extraction from agarose-IEF gels. **M** denotes the molecular weight marker and the apparent molecular masses are shown on the left side of the gel. **W** represents the immunoblot with an anti-TM antiserum. **B**, Purified chicken liver TM was also subjected to 2D-PAGE. Multiple high and low Mr TM isoforms were observed.

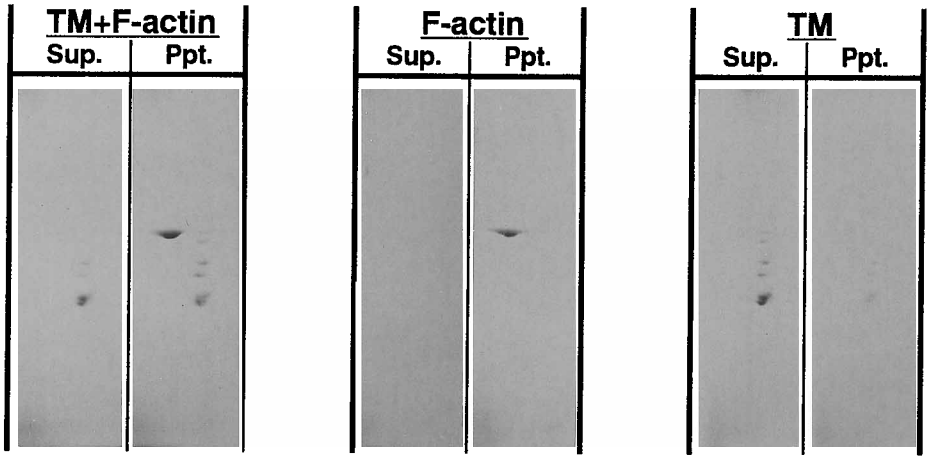

Fig. 2. Co-sedimentation of chicken liver TM isoforms with F-actin. In order to examine the ability of the purified TM to bind to F-actin, rabbit skeletal muscle F-actin (1 mg/ml), TM (0.25 mg/ml) and their mixture were incubated at 25°C for 2 hr. After ultracentrifugation, the resultant supernatants and precipitates were analyzed by 2D-PAGE.

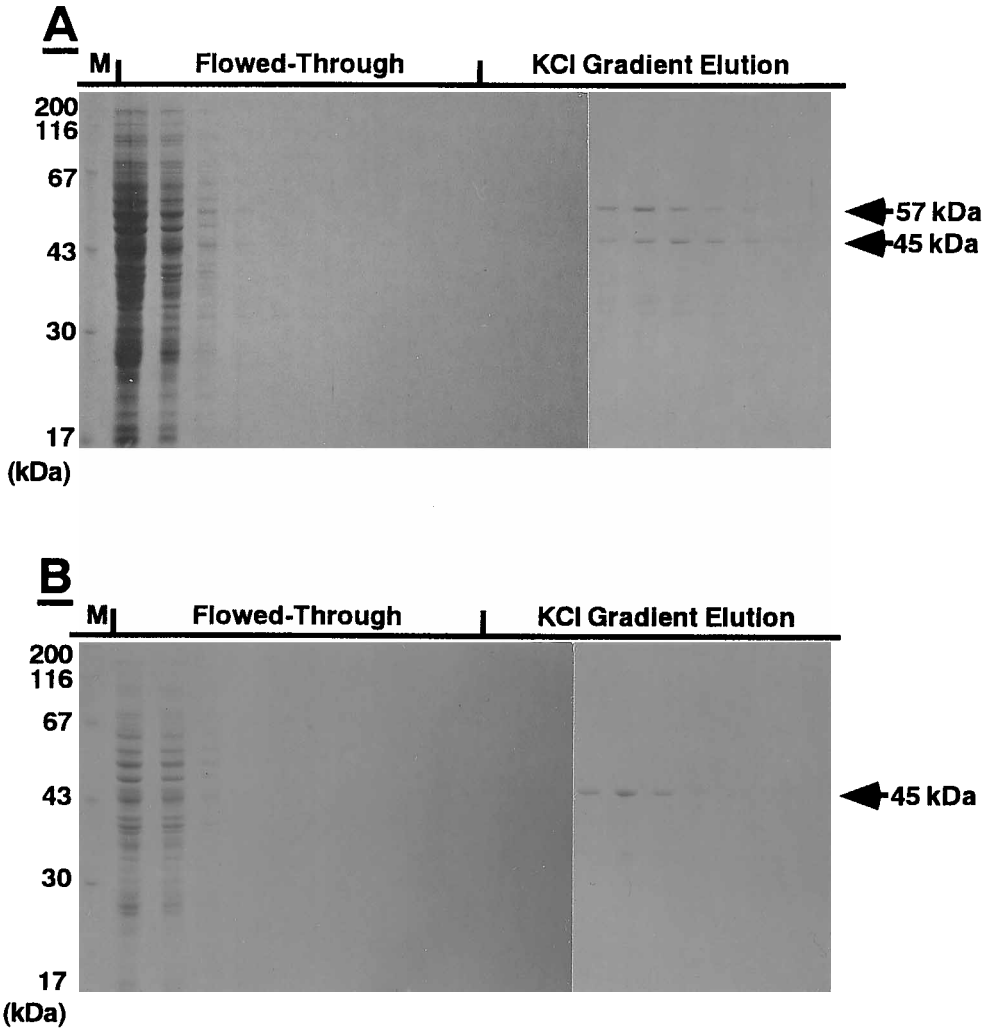

Fig. 3. Affinity column chromatography. Two mg of chicken liver TM (**A**) or BSA (**B**) was coupled to the HiTrap NHS-activated Sepharose 4B column and column chromatography was carried out. The total extract from the chicken liver acetone powder was loaded onto the affinity columns. After washing with 10 column volumes of the equilibration buffer, bound proteins were eluted with an increasing concentration of KCl from 50 mM to 1 M. Flowed-through fractions and the eluted fractions were loaded onto 12% SDS-polyacrylamide gels. The proteins of 57 kDa and 45 kDa were eluted from the TM-affinity column (arrows in **A**). The 45 kDa protein was also eluted from the BSA-affinity column (arrow in **B**).

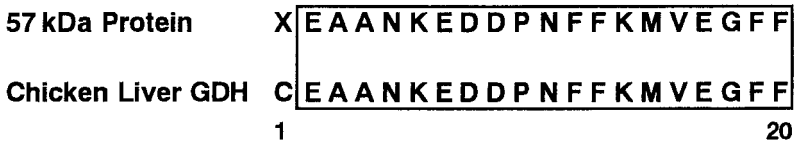

Fig. 4. Sequence comparison between the 57 kDa protein and chicken liver GDH. The N-terminal amino acid sequence of the V8 protease-digested 57 kDa protein was aligned with its homologous sequence of chicken liver GDH. Identical amino acids are boxed. X represents the amino acid residue that was not determined.

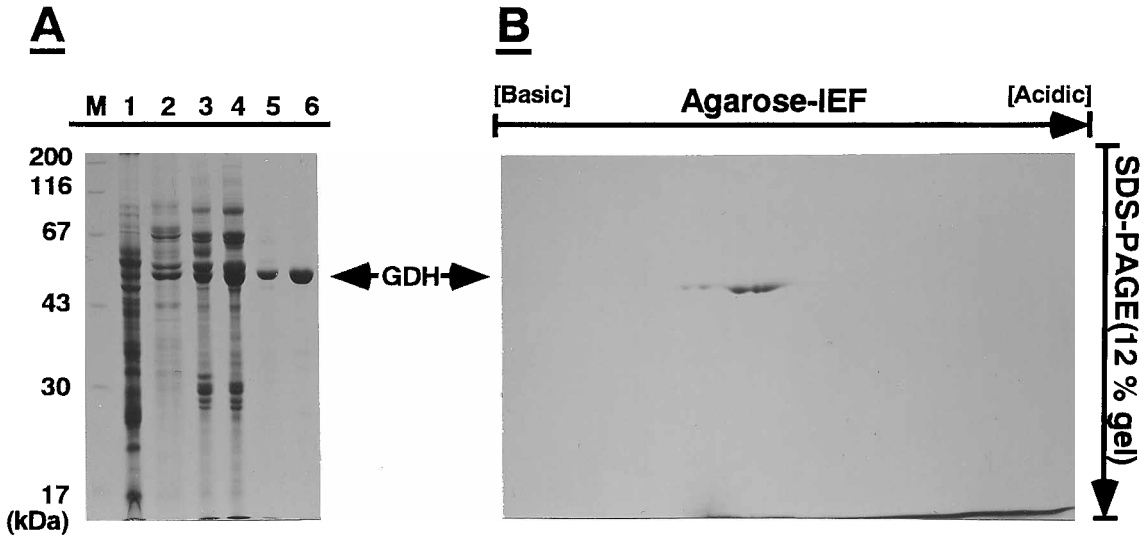

Fig. 5. Preparation of GDH from the chicken liver. **A**, Chicken liver GDH was prepared as described in Materials and Methods. Fractions obtained during the purification steps were loaded onto 12% SDS-polyacrylamide gel. Lane **1**, extract from the chicken liver tissue; **2**, GDH-containing fraction from a DEAE-TOYOPEARL S650 column; **3**, GDH-containing fraction from a Butyl-TOYOPEARL S650 column; **4**, GDH-containing fraction from a Hydroxylapatite column; **5**, GDH-containing fraction from a HiTrap Blue-affinity column; **6**, purified GDH eluted from a HiLoad Superdex 200 pg column. **M** indicates the molecular weight marker. **B**, The purified GDH was also analyzed by 2D-PAGE. Several spots of GDH were observed.

ried out co-sedimentation assays of TM with purified rabbit skeletal muscle F-actin to examine whether the purified TM had its authentic nature (Fig. 2). While TM isoforms alone were scarcely precipitated, they were pelleted in the presence of F-actin, indicating that the purified proteins were genuine TM.

Identification of the nonmuscle TM-binding protein in the chicken liver

In order to find out novel nonmuscle TM-binding proteins from the chicken liver, we prepared the TM-affinity column according to the manufacturer's protocol and carried out column chromatography. We also made the BSA-affinity column to distinguish proteins nonspecifically bound to the TM-affinity column from specific ones. The total extract from the chicken liver acetone powder was loaded onto the columns (Fig. 3). After washing with 10 column volumes of the equilibration buffer, bound proteins were eluted with an increasing concentration of KCl. Two proteins of 57 and 45 kDa were mainly detected by SDS-PAGE in the effluents from the TM-affinity column (Fig. 3A). Although almost the same amounts of the 45 kDa protein were eluted from both columns (Fig. 3B), the 57 kDa protein was eluted exclusively from the TM-affinity column and scarcely from the BSA-affinity column. When the total extract from the fresh tissue was applied onto the TM-affinity column, the protein of 57 kDa was also eluted, although its amount was considerably smaller (data not shown).

Table 1. Enzymatic activities during the purification steps of GDH.

Step	Specific Activity	Purification
Triton/CTAB Extract	0.32	1
DEAE-TOYOPEARL	2.29	7.16
Butyl-TOYOPEARL	8.38	26.19
Hydroxylapatite	16.99	53.09
HiTrap Blue	54.14	169.19
HiLoad Superdex 200 pg	143.67	448.97

Fractions obtained during its purification steps were measured spectrophotometrically at 340 nm in the direction of amination of 2-oxoglutarate into glutamate. An activator of GDH, 1 mM ADP, was included in the reaction mixture. Specific activities (units/mg/min.) of these fractions were aligned at the left column and their purification folds at the right column.

To examine whether the 57 kDa protein is a novel TM-binding protein, the protease-digested fragment of the 57 kDa protein was sequenced and the resultant N-terminal amino acid sequence was analyzed by homology search with SWISS-PLOT. The result indicated that the obtained partial amino

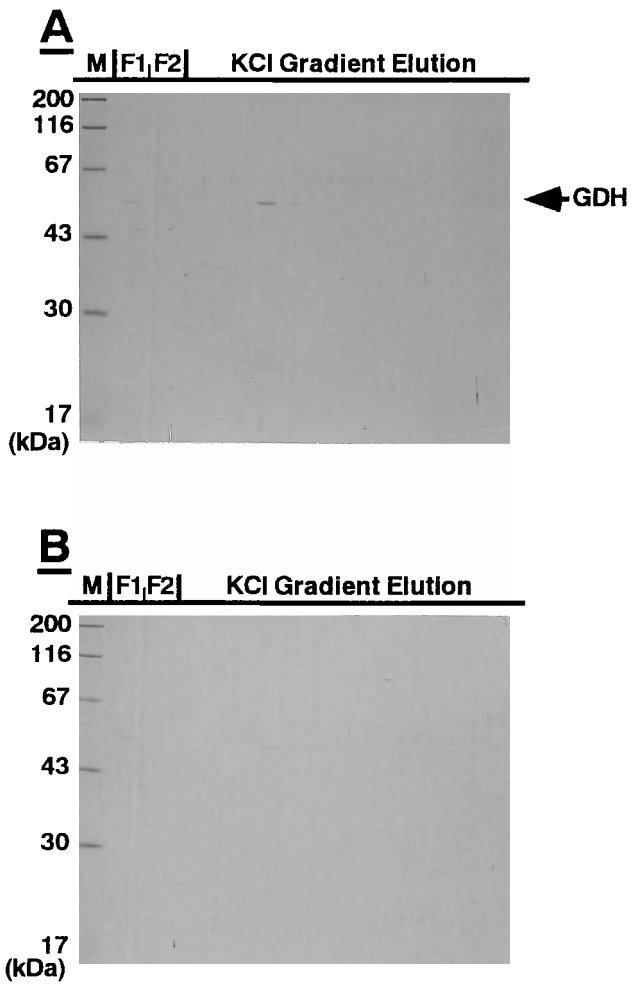

Fig. 6. Binding of purified GDH onto the TM-affinity column. Purified GDH (0.05 mg) was applied onto both TM- (**A**) and BSA- (**B**) affinity columns. After washing with 20 column volumes of the equilibration buffer, the bound protein was eluted with an increasing concentration gradient of KCl. Flowed-through fractions (**F1**, **F2**) and eluted fractions were analyzed by SDS-PAGE and the gels were stained with CBB-G. GDH eluted from the TM-affinity column was represented by an arrow (**A**), while it was scarcely eluted from the BSA-affinity column (**B**).

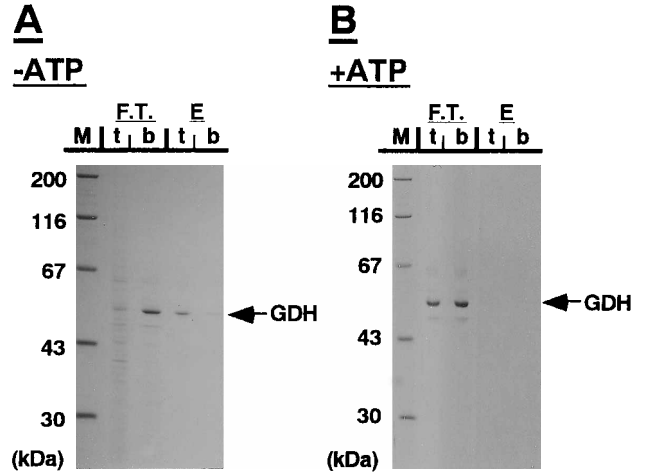

Fig. 7. Effect of ATP on the association of TM with GDH. In order to assess whether the interaction between TM and GDH is influenced by nucleotides, affinity column chromatography was performed in the absence (**A**) and the presence (**B**) of 0.1 mM ATP. Other conditions were the same as described in Fig. 6. In this experiment, flowed-through fractions (**F.T.**) and eluted fractions (**E**) from the TM- (lane **t**) and the BSA- (lane **b**) affinity columns were concentrated using Centricon-10 and were loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, the gels were stained with CBB-G. Note that GDH scarcely bound to the BSA-affinity column irrespective of the absence or the presence of ATP (lane **b** in **E**).

acid sequence was nearly identical to the N-terminal region of chicken liver glutamate dehydrogenase (GDH) (Moon *et al.*, 1973), that has not been reported to bind to TM so far (Fig. 4).

GDH directly associates with liver TM

To ensure the interaction between TM and GDH, we isolated GDH from the chicken liver through several column chromatographic steps as described in Materials and Methods. Fractions obtained during the purification steps were run on SDS-PAGE (Fig. 5A) and their enzymatic activities were represented in Table I. We could purify chicken liver GDH more than 400-fold in the relative enzymatic activity and the result of gel filtration column chromatography at the final purification step revealed its molecular mass of approximately 330 kDa, presumably showing a formation of a native hexamer (data not shown). The purified GDH was also subjected to 2D-PAGE (Fig. 5B) and shown to have several isoforms with approximately the same molecular weight of 57 kDa and different isoelectric points, suggesting that the GDH isoforms were derived from the several distinct genes or the post-translational modifications. All the protein spots were detected by immunoblotting with the anti-GDH antibody (Rockland, Inc.) (data not shown).

In order to determine whether GDH indeed associates with TM, the purified GDH was applied onto the TM-affinity column and column chromatography was performed in the same way as in Fig. 3 with the exception that the column was washed with 20 column volumes of the equilibration buffer after sample loading. GDH was retained on the TM-affinity column (Fig. 6A), while scarcely on the BSA-affinity column (Fig. 6B), as judged by SDS-PAGE. These results indicated that GDH directly associated with nonmuscle TM.

It is known that GDH is involved in the pathway of glutamate metabolism. Previous biochemical analyses revealed that the enzymatic activity is influenced by nucleotides (Smith *et al.*, 1975). In an attempt to address whether the interaction between TM and GDH is altered by nucleotides, we carried out affinity column chromatography in the presence of 0.1 mM ATP (Fig. 7B). As a consequence, the amount of GDH that bound to the TM-affinity column was greatly reduced in the presence of 0.1 mM ATP (Fig. 7B, lane t in E) relative to that in the absence of ATP (Fig. 7A, lane t in E), suggesting that the association of TM with GDH was regulated by ATP.

DISCUSSION

In the present study, we have demonstrated for the first time that chicken liver TM bound to GDH by affinity column chromatography and that the association of these two proteins was inhibited by ATP. Nevertheless, we found that not all GDH was retained on the TM-affinity column and the discernible amount of GDH was flowed through by washing with the equilibration buffer. It is conceivable that the affinity of TM to GDH might not be so high or that only the specific TM isoform(s) can bind to GDH, because purified chicken liver TM is composed of multiple high and low Mr isoforms. These possibilities remain to be investigated in the future study.

We have tried to confirm the association of TM with GDH by other procedures. Co-sedimentation assays of TM and GDH with F-actin were carried out, because it is strongly suggested that TM-binding protein can interact with TM in association with actin filaments, and because a small amount of actin was also eluted from the TM-affinity column (data not shown). However, a considerable amount of GDH was precipitated even in the absence of TM or F-actin, presumably owing to its tendency to aggregate (Smith *et al.*, 1975), making it difficult to judge whether GDH bound to TM on the actin filaments or not. The gel overlay method using biotinylated TM could not give the reliable results either, because it was almost impossible to eliminate nonspecific signals from presumably endogenous biotinylated proteins in the chicken liver, although various conditions of blocking and washing were tested. Therefore, we concluded that the affinity column chromatography is the best way to show clearly the interactions between TM and other proteins in the liver.

GDH is a ubiquitous protein that is widely expressed in organisms from prokaryotes to eukaryotes and is involved in the pathways of glutamate metabolism, insulin release, ureagenesis, and so on. In vertebrates, it is generally accepted that GDH is localized in mitochondria (Smith *et al.*, 1975). However, recent several investigations have indicated that GDH also exists in nuclei (Di Prisco and Garofano, 1975; McDaniel, 1995), lysosomes and/or vesicles (Rajas and Rousset, 1993; Rajas *et al.*, 1996), and endoplasmic reticulum (Kawajiri *et al.*, 1977; Lee *et al.*, 1999). In addition, two novel GDH isoforms have been identified in the rat brain. There are some differences between them in the primary structure, heat stability, and kinetic parameter (Cho *et al.*, 1995). Therefore, it is possible to say that the GDH isoforms from nonmitochondrial sources can be structurally and functionally distinguished from the mitochondrial one, although their physiological properties have not been understood in detail.

It has been reported that some enzymes interact with cytoskeletal proteins. For instance, the glycolytic enzyme, aldolase, is known to associate with the filamentous actin at low ionic strength (Arnold and Pette, 1968) and its binding site on actin has also been determined (O'Reilly and Clarke, 1993). Since colocalization of aldolase and F-actin has been observed *in vivo* (Pagliaro and Taylor, 1988), it is strongly suggested that the interaction between these two proteins is physiologically relevant. In addition, binding of aldolase with F-actin is inhibited in the presence of its substrate, fructose 1,6-bisphosphate, and affects the enzyme activity (Walsh *et al.*, 1977). Thus, F-actin can work not only as a scaffold of the glycolytic enzyme but also as a regulator of the enzymatic activity. Our preliminary study showed that the enzyme activity of GDH was not affected significantly in the presence of TM (data not shown), but the more detailed study to see if TM influences on the GDH activity is underway.

A novel 58 kDa microtubule-associated protein has been found to be localized in the membrane of Golgi apparatus and Golgi-derived vesicles (Bloom and Brashear, 1989). Recently, it has been shown that the protein is identified as formiminotransferase cyclodeaminase that is involved in histidine metabolism (Bashour and Bloom, 1998; Hennig *et al.*, 1998). It has been reported that the GDH isoform located at the lysosomal and/or vesicular membrane has also the capability to bind to microtubules in an ATP-dependent manner (Rajas and Rousset, 1993; Rajas *et al.*, 1996). Although the physiological properties of these two enzymes in association with microtubules have not been elucidated, the activities of these emzymes might be somehow regulated by microtubules as in the case of aldolase by F-actin. Alternatively, these enzymes might function as a ligand for linking cytoskeletal structures to organelles. Considering that TM can be related to organelle and/or vesicle transport (Hegmann *et al.*, 1989; Perham *et al.*, 1996), the previous studies encourage us to propose that the interaction of TM with GDH may play significant roles in intracellular metabolisms.

We have reported for the first time that GDH is associated with TM by affinity column chromatography. Therefore, it seems interesting to examine more precisely the intracellular localization of GDH, because it is well known that TM is distributed in microfilaments and ruffling membranes (Lin *et al.*, 1997), but not in mitochondria. In addition, our present study has shown that ATP influences the association of TM with GDH. Since the GDH activity is prevented in the presence of ATP at pH 7.5 and activated by ADP (Smith *et al.*, 1975), it is suggested that ATP can regulate its binding of TM and the enzymatic activity. Future biochemical and immunocytochemical studies and molecular cloning can give further insights into the physiological role of the interaction between TM and GDH.

ACKNOWLEDGMENTS

We would like to express our thanks to Dr. Osamu Numata (Institute of Biological Sciences, University of Tsukuba, Tsukuba, Japan) for generously allowing us to use a FPLC System (amersham pharmacia biotech) and to Mrs. Hiroko Kondou (Gene Experiment Center, University of Tsukuba, Tsukuba, Japan) for partial amino acid sequencing.

REFERENCES

Applegate D, Feng W, Green RS, Taubman MB (1994) Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. J Biol Chem 269: 10683–10690

Arnold H, Pette D (1968) Binding of glycolytic enzymes to structure proteins of the muscle. Eur J Biochem 6: 163–171
Bashour A-M, Bloom GS (1998) 58K, a microtubule-binding Golgi protein, is a formiminotransferase cyclodeaminase. J Biol Chem 273: 19612–19617
Bloom GS, Brashear TA (1989) A novel 58-kDa protein associates with the Golgi apparatus and microtubules. J Biol Chem 264: 10683–10692
Cho S-W, Lee J, Choi SY (1995) Two soluble forms of glutamate dehydrogenase isoproteins from bovine brain. Eur J Biochem 233: 340–346
Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252: 1102–1106
Côte GP, Smillie LB (1981) Preparation and some properties of equine platelet tropomyosin. J Biol Chem 256: 11004–11010
Di Prisco G, Garofano F (1975) Crystallization and partial characterization of glutamate dehydrogenase from ox liver nuclei. Biochemistry 14: 4673–4679
Dobrowolski Z, Barylko B, Drabikowski W (1986) Interaction of tropomyosin with myelin basic protein and its effect on the ATPase activity of actomyosin. Eur J Cell Biol 41: 65–71
Ebashi S, Kodama A, Ebashi F (1968) Troponin. I. Preparation and physiological function. J Biochem 64: 465–477
Fowler VM (1996) Regulation of actin filament length in erythrocytes and striated muscle. Curr Opin Cell Biol 8: 86–96
Frieden C (1962) The molecular weight of chicken-liver glutamate dehydrogenase. Biochim Biophys Acta 62: 421–423
Gatmaitan Z, Varticovski L, Ling L, Mikkelsen R, Steffen A-M, Arias IM (1996) Studies on fenestral contraction in rat liver endothelial cells in culture. Am J Pathol 148: 2027–2041
Gimona M, Lando Z, Dolginov Y, Vandekerckhove J, Kobayashi R, Sobieszek A, Helfman DM (1997) Ca^{2+}-dependent interaction of S100A2 with muscle and nonmuscle tropomyosins. J Cell Sci 110: 611–621
Graceffa P, Lehrer SS (1986) Tropomyosin's end-to-end polymerization is irreversibly lost on exposure to urea or cyanate. Biochem Biophys Res Commun 134: 64–70
Hegmann TE, Lin JL-C, Lin JJ-C (1989) Probing the role of nonmuscle tropomyosin isoforms in intracellular granule movement by microinjection of monoclonal antibodies. J Cell Biol 109: 1141–1152
Hennig D, Scales SJ, Moreau A, Murley LL, De Mey J, Kreis TE (1998) A formiminotransferase cyclodeaminase isoform is localized to the Golgi complex and can mediate interaction of trans-Golgi network-derived vesicles with microtubules. J Biol Chem 273: 19602–19611
Hirabayashi T (1981) Two-dimensional gel electrophoresis of chicken skeletal muscle proteins with agarose gels in the first dimension. Anal Biochem 117: 443–451
Hitchcock-DeGregori SE, Gerhard MD, Brown WE (1985) Platelet tropomyosin binding protein is identical with serum albumin. J Biol Chem 260: 3228–3231
Hosoya M, Miyazaki J-I, Hirabayashi T (1989) Tropomyosin isoforms in developing chicken gizzard smooth muscle. J Biochem 105: 712–717
Kawajiri K, Harano T, Omura T (1977) Biogenesis of the mitochondrial matrix enzyme, glutamate dehydrogenase, in rat liver cells. J Biochem 82: 1403–1416
Koepf EK, Burtnick LD (1992) Interaction of plasma gelsolin with tropomyosin. FEBS Lett 309: 56–58
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
Lee W-k, Shin S, Cho SS, Park J-s (1999) Purification and characterization of glutamate dehydrogenase as another isoprotein binding to the membrane of rough endoplasmic reticulum. J Cell Biochem 76: 244–253
Lin JJ-C, Warren KS, Wamboldt DD, Wang T, Lin JL-C (1997) Tropomyosin isoforms in nonmuscle cells. Int Rev Cytol 170: 1–38
McDaniel HG (1995) Comparison of the primary structure of nuclear and mitochondrial glutamate dehydrogenase from bovine liver. Arch Biochem Biophys 319: 316–321
Matsumura F, Yamashiro S (1993) Caldesmon. Curr Opin Cell Biol 5: 70–76
Moon K, Piszkiewicz D, Smith EL (1973) Amino acid sequence of chicken liver glutamate dehydrogenase. J Biol Chem 248: 3093–3107
Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255–262
O'Reilly G, Clarke F (1993) Identification of an actin binding region in aldolase. FEBS Lett 321: 69–72
Pagliaro L, Taylor DL (1988) Aldolase exists in both the fluid and solid phases of cytoplasm. J Cell Biol 107: 981–991
Pelham RJ, Jr., Lin JJ-C, Wang Y-L (1996) A high molecular mass non-muscle tropomyosin isoform stimulates retrograde organelle transport. J Cell Sci 109: 981–989
Pittenger MF, Kazzaz JA, Helfman DM (1994) Functional properties of non-muscle tropomyosin isoforms. Curr Opin Cell Biol 6: 96–104
Pittenger MF, Kistler A, D.M. Helfman DM (1995) Alternatively spliced exons of the β-tropomyosin gene exhibit different affinities for F-actin and effects with nonmuscle caldesmon. J Cell Sci 108: 3253–3265
Rajas F, Rousset B (1993) A membrane-bound form of glutamate dehydrogenase possesses an ATP-dependent high-affinity microtubule-binding activity. Biochem J 295: 447–455
Rajas F, Gire V, Rousset B (1996) Involvement of a membrane-bound form of glutamate dehydrogenase in the association of lysosomes to microtubules. J Biol Chem 271: 29882–29890
Schmidt E (1974) Glutamate Dehydrogenase. In "Methods of Enzymatic Analysis Vol 2" (2nd Ed.) Ed by UH Bergmeyer, Academic Press, New York, pp 650–659
Smith EL, Austen BM, Blumenthal KM, Nyc JF (1975) Glutamate Dehydrogenase. In "The Enzymes Vol XI" (3rd Ed.) Ed by PD Boyer, Academic Press, New York, pp 293–367
Sobue K, Muramoto Y, Fujita M, Kakiuchi S (1981) Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. Proc Natl Acad Sci USA 78: 5652–5655
Spudich JA, Watt S (1971) The regulation of rabbit muscle contraction. J Biol Chem 246: 4866–4871
Steffan A-M, Gendrault J-L, Kirn A (1987) Increase in the number of fenestrae in mouse endothelial liver cells by altering the cytoskeleton with cytochalasin B. Hepatology 7: 1230–1238
Takahashi K, Hiwada K, Kokubu T (1986) Isolation and characterization of a 34,000-dalton calmodulin- and F-actin-binding protein from chicken gizzard smooth muscle. Biochem Biophys Res Commun 141: 20–26
Takenaga K, Nakamura Y, Sakiyama S, Hasegawa Y, Sato K, Endo H (1994) Binding of pEL98 protein, an S100-related calcium-binding protein, to nonmuscle tropomyosin. J Cell Biol 124: 757–768
Tobita T, Hiraide F, Miyazaki J-I, Ishimoda-Takagi T (1996) Muscle-type tropomyosin of sea urchin egg increases the actin-binding of nonmuscle-type tropomyosin. J Biochem 120: 922–928
Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354
Trabelsi-Terzidis H, Fattoum A, Represa A, Dessi F, Ben-Ari Y, der Terrossian E (1995) Expression of an acidic isoform of calponin in rat brain: Western blots on one- or two-dimensional gels and immunolocalization in cultured cells. Biochem J 306: 211–215
Tsukada N, Ackerley CA, Phillips JM (1995) The structure and orga-

nization of the bile canalicular cytoskeleton with special reference to actin and actin-binding proteins. Hepatology 21: 1106–1113

Walsh TP, Clarke FM, Masters CJ (1977) Modification of the kinetic parameters of aldolase on binding to the actin-containing filaments of skeletal muscle. Biochem J 165: 165–167

Xie L, Hirabayashi T, Miyazaki J-I (1992) Histological distribution and developmental changes of tropomyosin isoforms in three chicken digestive organs. Cell Tissue Res 269: 391–401

Yamashiro-Matsumura S, Matsumura F (1988) Characterization of 83-kilodalton nonmuscle caldesmon from cultured rat cells: Stimulation of actin binding of nonmuscle tropomyosin and periodic localization along microfilaments like tropomyosin. J Cell Biol 106: 1973–1983

(Received February 16, 2000 / Accepted March 29, 2000)