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Biochemical and Immunohistochemical Studies on Tropomyosin and Glutamate Dehydrogenase in the Chicken Liver

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ABSTRACT—Recently, we have reported a novel tropomyosin (TM) -binding protein, glutamate dehydrogenase (GDH) and demonstrated by affinity column chromatography that chicken liver TM interacts with GDH in an ATP-dependent manner. To elucidate the physiological roles of the interaction between TM and GDH, we performed co-sedimentation assays of TM and GDH with F-actin, because it is known that TM exerts its physiological functions by associating with actin filaments. The results showed that TM and GDH co-pelleted with F-actin. GDH alone also co-precipitated with F-actin, but the amount of GDH sedimenting with F-actin was increased in the presence of chicken liver TM, suggesting that GDH is involved in the regulation of the actin cytoskeleton. We also prepared crude GDH from the nuclear and mitochondrial fractions obtained by subcellular fractionation of the chicken liver cells. Semi-nondenaturing 2D-PAGE revealed that partially purified GDH from the nuclear fraction was associated with TM, but not GDH from the mitochondrial fraction, suggesting preferential binding of TM to GDH. We determined the nucleotide sequence of chicken GDH cDNA and showed that the GDH transcript was widely expressed in the chicken organs. We examined the localization of TM and GDH by immunohistochemistry and revealed that they were distributed in the cytoplasm of the adult chicken liver. From these results, we propose two hypotheses on the physiological roles of the interaction between TM and GDH in nonmuscle cells.

Key Words: actin cytoskeleton, protein-protein interaction, intracellular localization, bifunctional protein, organelle movement

INTRODUCTION

It is well known that the actin-based cytoskeleton plays important roles in the intracellular physiological events such as muscle contraction, cell migration, cytokinesis, secretion, and so on. These multiple functions of the actin cytoskeleton are regulated by actin-binding proteins (Alberts *et al*., 1994). Tropomyosin (TM) is one of those proteins and binds to actin filaments along with their groove structures. Multiple TM isoforms, derived from four distinct genes by alternative splicing of their transcripts, are expressed especially in vertebrate nonmuscle cells and it is suggested that each TM has distinct function (Lees-Miller and Helfman, 1991; Pittenger *et al*., 1994; Lin *et al*., 1997).

It is revealed that TM plays important roles in the regulation of the actin cytoskeleton in nonmuscle cells. Disruption of the TM gene makes fission yeasts unable to divide into two daughter cells (Balasubramanian *et al*., 1992). Location of TM at the contractile ring, that mainly consisits of actin and actin-binding proteins during cytokinesis, is shown by the study using cultured cells (Warren *et al*., 1995). Interestingly, overexpression of human TM3, a high-M_r TM isoform, in Ptk-2 cells induces perinuclear localization of organella, but not TM5a, a low-M_r TM isoform (Pelham *et al*., 1996). By injecting the TM-antibody and/or the caldesmon antibody into cultured cells, intracellular granule movement is greatly inhibited (Hegmann *et al*., 1989; 1991). Differential localization of TM isoforms is observed in cultured fibroblasts (Lin *et al*., 1988), LLC-PK1 cells (Temm-Grove *et al*., 1998), and synchronized NIH3T3 cells (Percival *et al*., 2000). Despite these evidences, the functional properties of TM in vertebrate nonmuscle cells are not elucidated well.

We have focused on TM-binding proteins, because the actin cytoskeleton is also modulated by the proteins such as caldesmon (Matsumura and Yamashiro, 1993), calponin (Winder and Walsh, 1997), and tropomodulin (Fowler, 1996). It is significant to reveal the precise functional properties of TM and its associated proteins in order to elucidate the regulation of the actin cytoskeleton. We searched for novel nonmuscle TM-binding proteins from the chicken liver by affinity column chromatography and demonstrated that glutamate dehydrogenase (GDH), that plays a central roles in gluta-

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mate metabolism, bound to the TM in an ATP-dependent manner (Akutsu *et al*., 2000), like caldesmon and calponin which associate with TM and F-actin in a Ca^{2+}-dependent fashion. GDH interacted with TM in the absence of ATP, but the association was inhibited by 0.1 mM ATP. ATP is one of the allosteric inhibitors for the enzymatic activity of GDH (Smith, 1975), and thus the GDH activity is also regulated in an ATP-dependent manner.

GDH is ubiquitously expressed in various organisms from bacteria to mammals (Smith *et al*., 1975). In vertebrates, it is widely accepted that GDH locates in the mitochondrial matrix. However, recent studies suggest that GDH also localizes at the nucleus (DiPrisco and Garofano, 1975; McDaniel, 1995), rough endoplasmic reticulum (Lee *et al*., 1999), and lysosome (Rajas and Rousset, 1993; Rajas *et al*., 1996). The GDH isoform at the lysosomal membrane posseses microtubule-binding ability in an ATP-dependent manner, suggesting the involvement of GDH in lysosomal movement and distribution in mammalian cells (Rajas *et al*., 1996).

In this study, we investigate the interaction of chicken liver TM and GDH to elucidate its physiological significance. Co-sedimentation assays of TM and GDH with F-actin suggests that GDH is involved in the regulation of the actin cytoskeleton. Semi-nondenaturing 2D-PAGE shows that GDH obtained from the chicken liver nuclear fraction interacts with TM. We determine the nucleotide sequence of chicken GDH cDNA and demonstrate that only a single transcript is expressed in the liver. We also show by immunohistochemistry that TM and GDH are distributed in the cytoplasm of hepatocytes. From these results, we propose two hypotheses on physiological roles of the interaction between TM and GDH in nonmuscle cells.

MATERIALS AND METHODS

Preparation of chicken liver nuclear and mitochondrial fractions

The liver was excised from the adult chicken, cut into small pieces with scissors, and then homogenized with Potter-Elvejem homogenizer at low speed (less than 1,000 rpm) in 4 volumes (V/W) of 0.25 M sucrose. The homogenate was centrifuged at 2,500×g, 2°C for 15 min using BECKMAN JA-20 rotor and the resultant supernatant and precipitate were pooled. For preparing the mitochondrial fraction, the supernatant was centrifuged at 5,000×g, 2°C for 10 min. The pellet was suspended in 0.25 M sucrose and centrifuged at 24,000×g, 2°C for 10 min. The resultant precipitate was dissolved in 0.25 M sucrose and centrifuged again. The pellet was used as the chicken liver mitochondrial fraction. For preparing the nuclear fraction, the pellet obtained by centrifugation of the homogenate was suspended in 2.4 M sucrose and 2 mM $MgCl_2$ and subjected to ultracentrifugation at 100,000×g, 2°C for 1 hr using BECKMAN 50.2Ti rotor. The resultant precipitate was used as the chicken liver nuclear fraction. These nuclear and mitochondrial fractions were dissolved in 0.25 M sucrose and treated with cold acetone for several times. The resultant sediments were dried up thoroughly and stored at –20°C until use.

Preparation of proteins

All procedures were performed at 4°C unless otherwise mentioned. Chicken liver TM was prepared as described previously (Akutsu *et al*., 2000). Chicken GDH was partially purified from the nuclear and mitochondrial fractions obtained by subcellular fractionation of the chicken liver. Briefly, proteins were extracted from both the nuclear and the mitochondrial fractions with 1% Triton X-100, 0.05% cetyltrimethylammonium bromide, 10 mM K-PO_4, 1 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT, pH 7.5 and subsequently subjected to saturated ammonium sulfate fractionation. The resultant precipitates were dialyzed against Buffer I containing 10 mM K-PO_4, 1 mM EDTA, 0.1 mM PMSF, and 0.1 mM DTT, pH 7.5 for overnight. Dialyzed samples were loaded on HiTrap Blue affinity column (Amersham/Pharmacia Biotech) and bound proteins were eluted with graded KCl from 0 to 0.9 M and from 0.9 to 3 M and subsequently eluted with 3 M KCl. GDH-containing fractions were collected, concentrated, and then applied to HiLoad Superdex 200 prep grade gel filtration column (Amersham/Pharmacia Biotech). GDH-rich fractions were collected and used for the experiments described below. Chicken liver actin was prepared essentially according to Weir and Frederiksen (1980).

Electrophoresis and Western blotting

SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Proteins on the gels were stained either with CBB-R250 or CBB-G250 (Neuhoff *et al*., 1988) or transferred to the nitrocellulose membrane according to Towbin *et al*. (1979). Western blotting was performed using the GDH-antibody (1:100, Rockland Inc.) as the primary antibody and goat TRITC-conjugated anti-rabbit IgG (H+L) (1:100, Jackson ImmunoResearch) as the second antibody. Two-dimensional gel electrophoresis (2D-PAGE) was performed by the Hirabayashi's procedure (Hirabayashi, 1981). For semi-nondenaturing 2D-PAGE, 2D-PAGE was performed with the decreased urea concentration (2 M instead of 5 M) for preparing samples and agarose-IEF gels in the first dimension (Hirabayashi, 1981).

Affinity column chromatography

TM-affinity column chromatography was performed as reported previously (Akutsu *et al*., 2000). Purified chicken liver GDH was loaded on the TM-affinity column. Fractions eluted from the column were concentrated using Centricon-10 (Amicon) and subjected to 2D-PAGE. Proteins were stained with CBB-R or transferred to the nitrocellulose membrane (Towbin *et al*., 1979) for immunological detection with the GDH-antibody (1:100, Rockland, Inc.).

Co-sedimentation assay

Purified chicken liver TM, GDH, and F-actin were mixed well, incubated at room temperature for 2 hr in the buffer consisting of 0.1 M KCl, 6 mM $MgCl_2$, 20 mM Tris-HCl, pH7.5, 0.1 mM PMSF, and 0.1 mM DTT, and then ultracentrifuged at 270,000×g at 2°C for 20 min. Control experiments were also done in the same way using each or two of the three proteins. The supernatants and precipitates were loaded on the 12% SDS-polyacrylamide gel and proteins were stained with CBB-R.

Nucleotide sequencing of chicken GDH cDNA

Total RNA was extracted from the adult chicken liver using ISOGEN (NIPPON GENE) according to manufacturer's protocol and synthesis of first strand cDNA was done using the oligo $dT_{12–18}$ (Amersham/Pharmacia Biotech) primer and SuperScript reverse transcriptase (GIBCO BRL). RT-PCR was performed using specific primers designed from nucleotide sequences of mammalian GDH, S1 (5'-CGACCCCAACTTCTTCAAGATGG-3') and A1 (5'-TRAYG-TAGGCAGCTGTTCTCAGG-3'), and the primers designed from the partial nucleotide sequence of the GDH cDNA fragment amplified by RT-PCR with S1 and A1 primers, S2 (5'-CATTGGTCCTGGA-GTTGATGTGC-3') and A2 (5'-GCTTTGACCTTGTGAGCATTGGC-3').

KOD Dash DNA polymerase (TOYOBO) was used for amplification of specific fragments. Step-down PCR was performed, by denaturing at 94°C for 1 min, annealing for 1 min, and extension at 74°C for 2 min. The annealing temperature was lowered in each 3 cycles by 4°C from 74 to 58°C and at 54°C for last 20 cycles. RT-PCR products were subcloned into pBluescript II KS+ phagemid at Eco RV sites. Phagemids were isolated from transformed JM109 or DH5α competent cells by the alkaline-SDS method (Sambrook *et al*., 1989). GDH cDNA was subjected to cycle sequencing using ABI PRISM™ Big-Dye Terminator (Perkin Elmer) according to the manufacturer's protocol on ABI PRISM™ 377 Sequencer (Perkin Elmer).

Preparation of chicken liver tissue sections

The adult chicken liver was cut into small pieces (5–10 mm) and fixed with 10% formalin in PBS for 3 hr. Subsequently, they were washed with water for overnight, dehydrated with graded ethanol followed by buthanol, and finally embedded in paraffin. Sections were prepared using a microtome in 5 μm-width and placed on poly-L-lysine-coated slide glasses. Sections were deparaffinized with xylene, rehydrated with graded ethanol, and washed with water. They were used for immunohistochemical staining.

Immunohistochemical staining of chicken liver tissue sections

Chicken liver tissue sections were blocked with 5% BSA in PBS at room temperature for 1 hr and incubated with the preimmune serum (1:100), the TM-antiserum (1:100), and the GDH antibody (1:100, Rockland Inc.) for 2 hr. Washing with PBS for 5 min 3 times, they were stained with goat TRITC-conjugated anti-rabbit IgG (H+L) (1:100, Jackson ImmunoResearch) for 2 hr. Subsequently, to eliminate background staining, sections were stained with 0.1% amidoblack and 0.7% acetic acid for 20 min. After washing with water, sections were enclosed with 90% glycerol in PBS. Fluorescence images were photographed with a combination of Nikon OPTIPHOT XF-Ph microscope and Nikon FX-35WA camera.

RESULTS

Interaction of chicken liver TM and GDH

As demonstrated in the previous study (Akutsu *et al*., 2000), chicken liver TM bound to GDH, an allosteric enzyme that catalizes transition of L-glutamate into 2-oxoglutarate (Smith *et al*., 1975). 2D-PAGE of eluates obtained by TM-affinity column chromatography showed that purified chicken liver GDH directly associated with TM (Fig. 1A). Western blotting using the GDH-antibody revealed that the protein eluted from the TM-affinity column cross-reacted with the antibody (Fig. 1B), confirming that the protein was GDH. GDH appeared as some spots on the 2D-PAGE gel.

Association of TM with GDH under the semi-nondenaturing condition

Semi-nondenaturing 2D-PAGE was performed in order to confirm the interaction of TM and GDH (Fig. 2). Since it is reported that GDH is located at the mitochondrial matrix (Smith *et al*., 1975) and also at non-mitochondrial organella such as the nucleus (DiPrisco and Garofano, 1975) and the rough endoplasmic reticulum (Lee *et al*., 1999), GDH was partially purified both from chicken liver nuclear and mitochondrial fractions and subjected to the semi-nondenaturing 2D-PAGE. When TM and GDH form the protein complex,

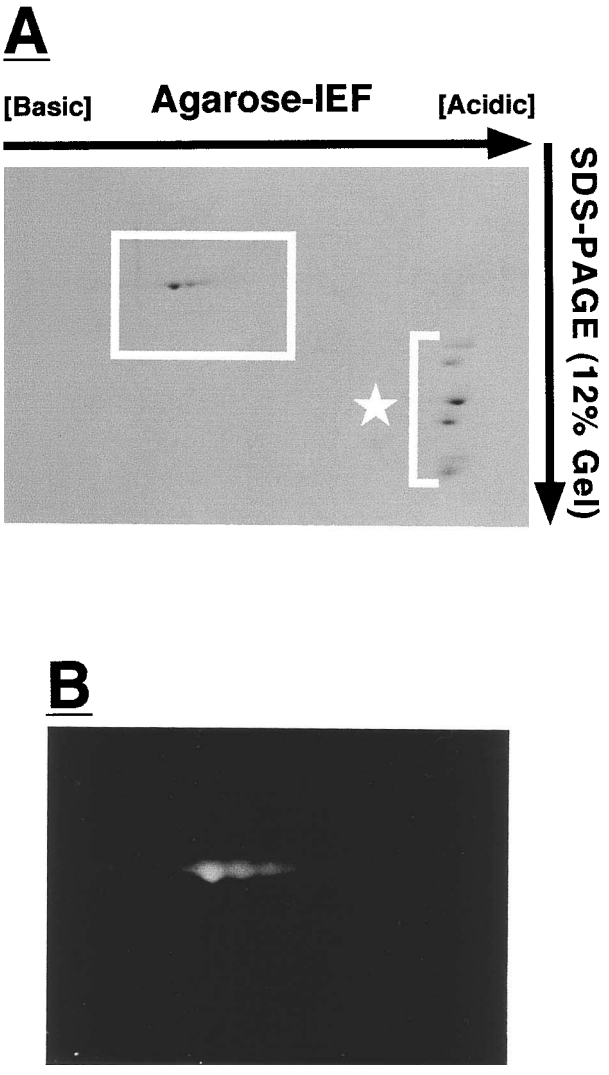

Fig. 1. Two-dimensional gel electrophoresis of eluates from the TM-affinity column. Purified GDH was loaded onto the TM-affinity column. After washing the column, bound protein was eluted with a linear concentration gradient of KCl from 0.05 to 1 M. The eluted fractions were concentrated and subjected to 2D-PAGE. Proteins were stained with CBB-R250 (A). The region including eluted proteins (A, boxed) was transferred to the nitrocellulose membrane and subsequently Western blotting was performed using the GDH-antibody (B). An asterisk in A indicates chicken liver TM dissociated from the affinity column.

they co-migrate on the semi-nondenaturing gel in the first dimension and align vertically on the SDS-polyacrylamide gel in the second dimension. When TM alone was subjected to semi-nondenaturing 2D-PAGE, faster-migrating minor components and slower-migrating major components were seen on the gel (Fig. 2A). The former was possibly composed of TM monomers and the latter of TM homodimers and/or heterodimers which were formed under the low concentration of urea in the first dimension. GDH was stuck on the top of the first dimension gels (Fig. 2B to E, arrowheads) and only a small portion of nuclear GDH entered the gel (Fig. 2B, double-arrowhead). However, almost all nuclear

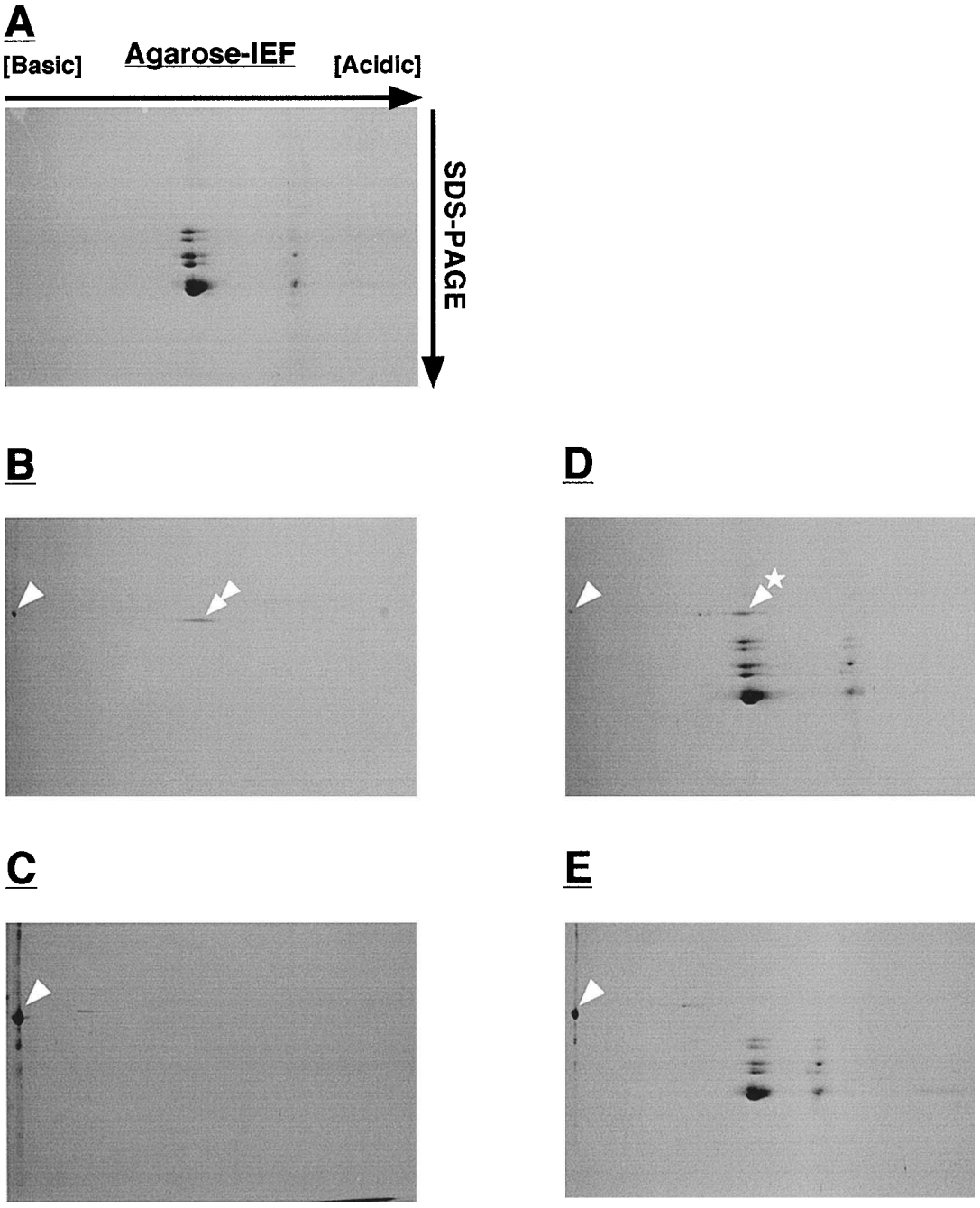

Fig. 2. Semi-nondenaturing 2D-PAGE with low concentration of urea in the first dimension. Semi-nondenaturing 2D-PAGE was performed using agarose gels containing 2 M urea instead of 5 M in the first dimension with chicken liver proteins of the following combinations, A, TM; B, nuclear GDH; C, mitochondrial GDH; D, TM+nuclear GDH; E, TM+mitochondrial GDH. Proteins were stained with CBB-G250. GDH hardly entered the first dimension gels (B to E, arrowheads), but a small amount of GDH prepared from the nuclear fraction did (B, double-arrowhead). In the presence of TM, nuclear GDH and TM co-migrated in the first dimension gel and aligned vertically on the second dimension gel (D, arrowhead accompanied with an asterisk), but mitochondrial GDH and TM did not (E).

GDH entered the gel in the presence of TM and co-migrated with TM (Fig. 2D, arrowhead accompanied with an asterisk). Mitochondrial GDH did not enter the gel even in the presence of TM (Fig. 2C and E). These results suggested that TM preferentially associated with the nuclear GDH.

Co-sedimentation of chicken liver TM and GDH with F-actin

Since it is widely accepted that TM plays its physiological roles by binding to actin filaments, we performed co-sedimentation assays of TM and GDH with F-actin. The resultant supernatants and precipitates were run on the 12% SDS-polyacrylamide gel (Fig. 3). While TM and GDH in the

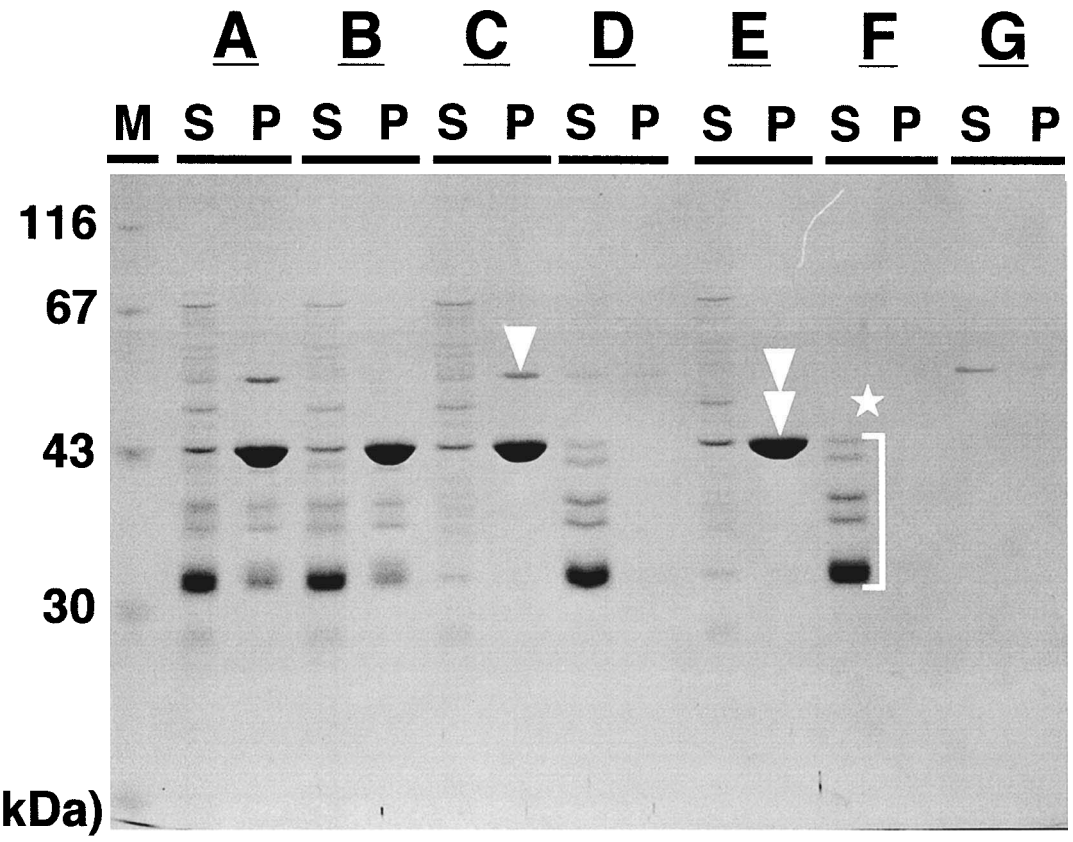

Fig. 3. Co-sedimentation assays of chicken liver TM and GDH in the presence of chicken liver F-actin. Co-sedimentation assays were performed in the following combinations of chicken liver proteins, A, chicken liver TM, GDH, and F-actin; B, TM and F-actin; C, GDH and F-actin; D, TM and GDH; E, F-actin alone; F, TM alone; G, GDH alone. After ultracentrifugation, resultant supernatants (S) and precipitates (P) were electrophoresed on the 12% SDS-polyacrylamide gel. Proteins were stained with CBB-R250. M, molecular weight marker; asterisk, chicken liver TM; arrowhead, chicken liver GDH; double-arrowhead, chicken liver F-actin.

absence of F-actin were in the supernatant after ultracentrifugation (Fig. 3D, F, and G), they co-pelleted with F-actin (Fig. 3A). Although TM or GDH alone co-sedimented with F-actin (Fig. 3B and C), the amount of GDH that co-precipitated with F-actin was slightly increased in the presence of chicken liver TM (Fig. 3A), suggesting that GDH is involved in the regulation of the actin cytoskeleton.

Expression of GDH mRNA and its nucleotide sequence

Recent studies have suggested the existence of two distinct GDH transcripts in human (Mavrothalassitis *et al*., 1988; Shashidharan *et al*., 1994). In order to see if several GDH transcripts are also expressed in the chicken, RT-PCR was performed using a template prepared from the adult chicken liver (Fig. 4A). A single fragment of approximately 1.4 kbp was specifically amplified with the S1 and A1 primer pair designed from the mammalian GDH cDNA sequences (Fig. 4A, SS, S1/A1) with no amplified product when either primer was used (Fig. 4A, SS, S1 and A1) or when reverse transcriptase was not added on first strand synthesis (Fig. 4A, No). We newly designed S2 and A2 primers from the partial nucleotide sequence of the 1.4 kbp fragment and performed RT-PCR using S1, S2, A1, and A2 primers. There is no information of genomic and cDNA sequences of GDH in avians. The fragments of 1 kbp were specifically amplified with the S1/A2 and S2/A1 primer pairs and the fragment of 0.5 kbp with the S2/A2 primer pair (Fig. 4B). Note that no amplified product was obtained when one of the primers was used (Fig. 4B, S1, S2, A1, and A2) or when reverse transcriptase was not added (Fig. 4B, No). The 1.4 kbp fragment was also amplified by RT-PCR using templates prepared from the adult chicken spleen (Sp), brain (Br), skeletal muscle (Sk), and heart (He), thus suggesting that the GDH mRNA was widely expressed in the chicken organs (Fig. 4C).

Amplified products with S1/A2, S2/A2, and S2/A1 primer pairs were cloned and sequenced. The nucleotide sequence (1,436 bp) and its deduced amino acid sequence are shown in Fig. 5. It contained most of the open reading frame, but residual 5'- and 3'- sequences of the open reading frame and non-coding sequences were not determined in this study. Sequence identities of the chicken GDH to mammalian GDH including human GDH1 (Mavrothalassitis *et al*., 1988), GDH2 (Shashidharan *et al*., 1994), rat liver GDH (Amuro *et al*., 1989), and mouse brain GDH (Tzimagiorgis and Moschonas, 1991) were from 82 to 85% in nucle-

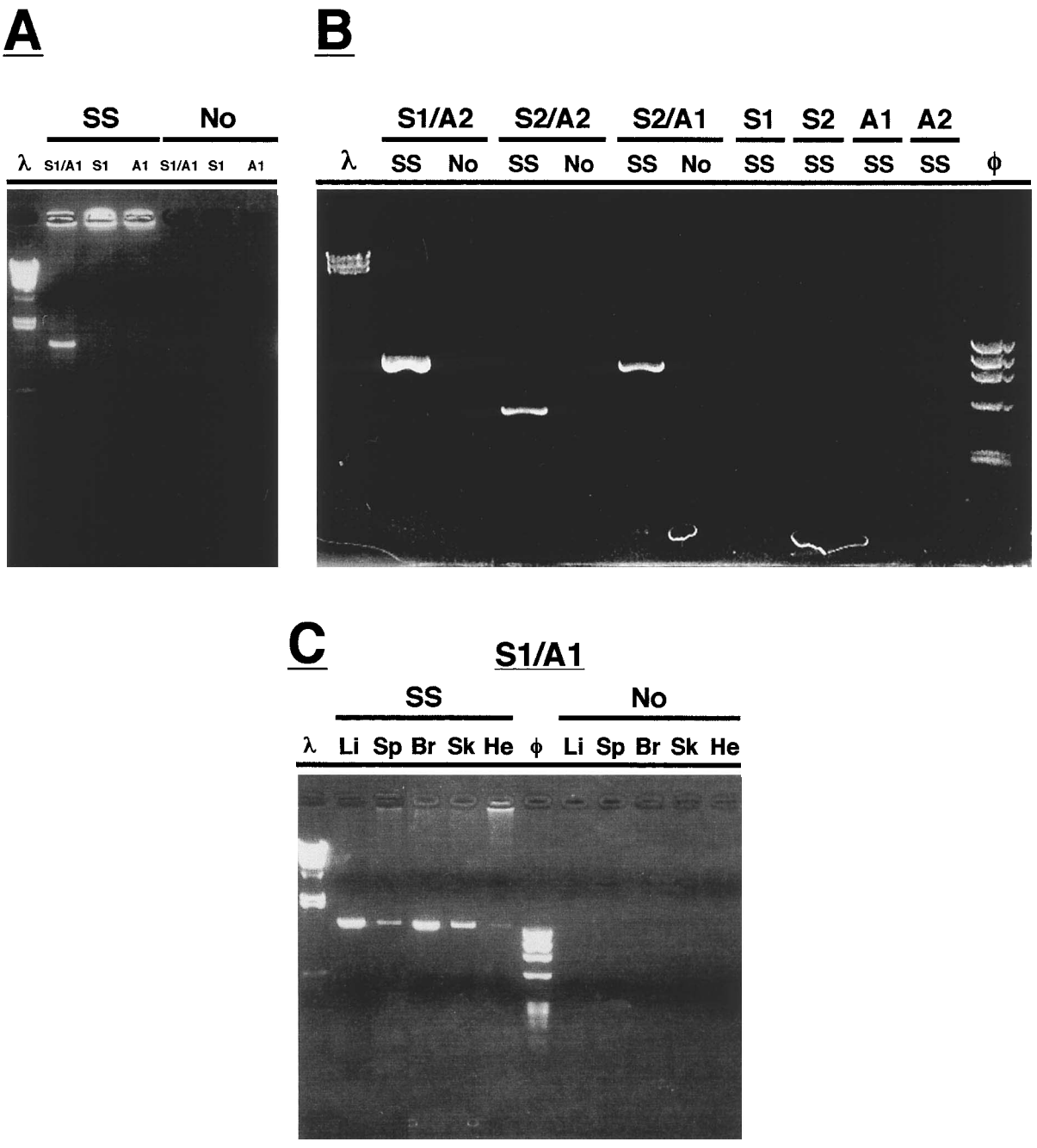

Fig. 4. RT-PCR for amplification of chicken GDH cDNA. A, RT-PCR was performed using the chicken liver total RNA as a template and S1 and A1 primers (S1/A1) which were designed from nucleotide sequences of mammalian GDH. RT-PCR was also carried out with either primer (S1 or A1) for negative control. Reverse transcriptase was added (SS) or not added (No) in the first strand synthesis. Amplified products were run on the 1% agarose gel. The fragment of approximately 1.4 kbp was specifically amplified. λ, λ DNA Hind III digest size marker. B, S2 and A2 primers were designed based on the partially determined nucleotide sequence of chicken GDH cDNA. Using S1, S2, A1, and A2 primers, RT-PCR was performed and the resultant products were subjected to agarose gel electrophoresis (S1/A2, S2/A2, S2/A1, S1, S2, A1, and A2, respectively). The fragments of 1 kbp (S1/A2 and S2/A1) and of 0.5 kbp (S2/A2) were specifically amplified. ϕ, ϕχ174 DNA Hae III digest size marker. C, RT-PCR was performed with S1 and A1 primers (S1/A1) and total RNA from the adult chicken organs as templates. Amplified products were subjected to agarose gel electrophoresis. Li, liver; Sp, spleen; Br, brain; Sk, skeletal muscle; He, heart. The fragment of approximately 1.4 kbp was amplified in all the organs.

otides, and more than 90% in amino acids. Thus, GDH was highly conserved between chicken and mammals.

The α-helical (Fig. 5, underlined) domains were deduced from the secondary structure of bovine liver GDH (Peterson and Smith, 1999). It is likely that GDH can associate with TM via their α-helical domains, because it is suggested that troponin T, one of TM-binding proteins, interacts with TM through their α-helical regions (Pearlstone *et al.*,

1976). Using the software of NetPhos 2.0, it was indicated that 16 serine, 6 threonine, and 5 tyrosine residues on the dedeced amino acid sequence of chicken liver GDH (Fig. 5, boxed letters) were candidates for phosphorylation (Blom *et al.*, 1999). Several spots of GDH seen on the 2D-PAGE gel (Fig. 1A) may be produced by phosphorylation, although the precise phosphorylation site(s) of chicken liver GDH has not been determined.

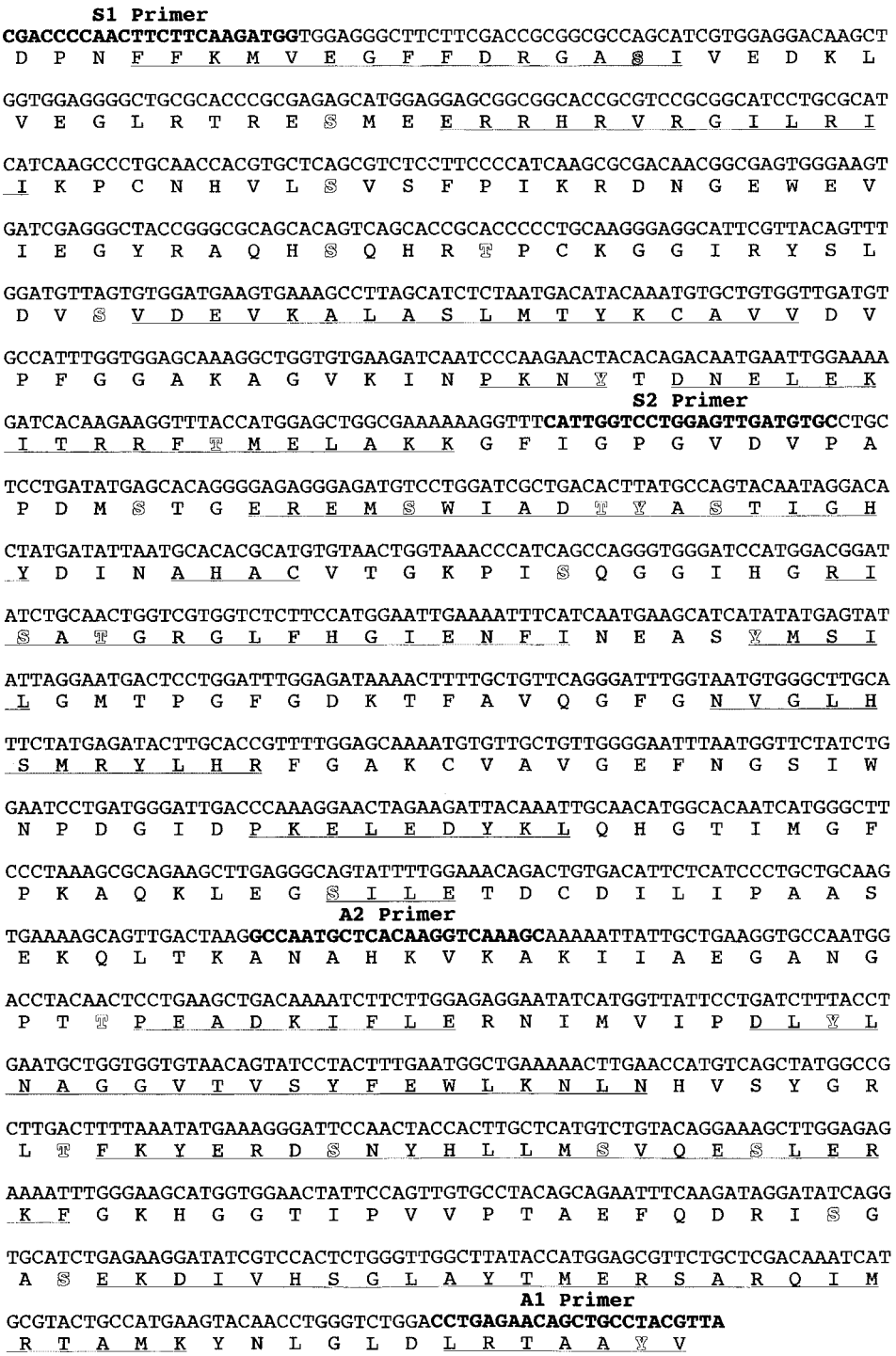

Fig. 5. Nucleotide sequencing of chicken GDH cDNA. Specifically amplified fragments with S1/A1, S1/A2, S2/A2, and S2/A1 primer pairs by RT-PCR were cloned and sequenced. The nucleotide sequence (1,436 bp) and the deduced amino acid sequence were aligned. Primers (S1, S2, A1, and A2) used in this experiment (bold letters) are denoted in the nucleotide sequence. The putative α-helical domains (underlined) and the predicted phosphorylation sites (boxed letters) are indicated in the amino acid sequence.

Immunohistochemical localization of TM and GDH in the chicken liver

To address localization of TM and GDH, paraffin-embedded adult chicken liver tissue sections were immunostained with the preimmune serum, the TM-antiserum, and the GDH-antibody, respectively (Fig. 6, ×100). Immunostaining results are shown with different regions of the tissue sections (Figs. 6 and 7). Although weak background staining

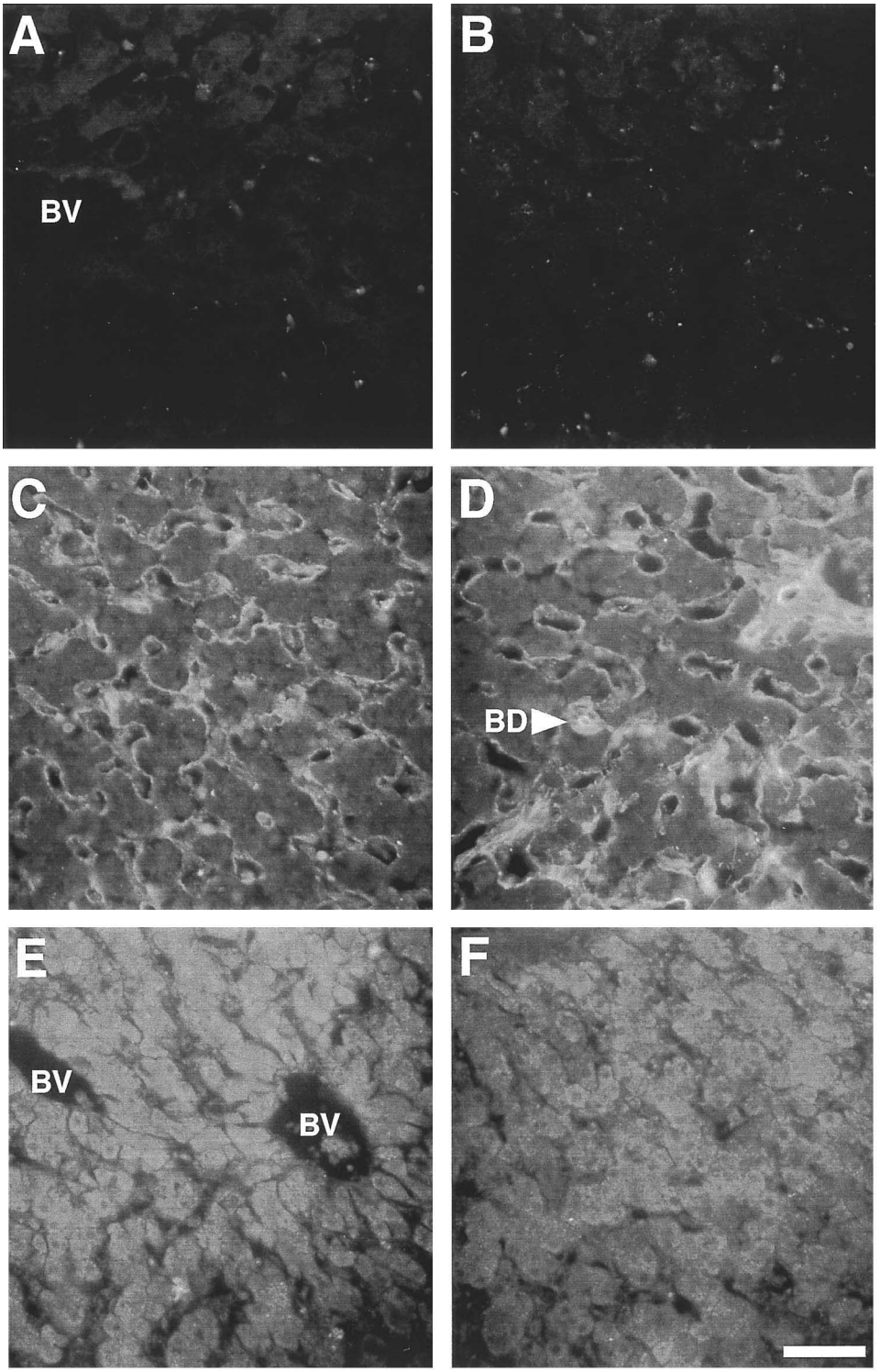

Fig. 6. Immunohistochemical observation of TM and GDH in the adult chicken liver. Formalin-fixed, paraffin-embedded adult chicken liver tissue sections were stained with the preimmune serum (1:100, A and B), the TM-antiserum (1:100, C and D), and the GDH-antibody (1:100, E and F), respectively. Goat TRITC-conjugated anti-rabbit IgG (H+L) antibody (1:100, Jackson ImmunoResearch) was used as the secondary antibody. BV and BD indicate the blood vessel and the bile duct, respectively. Bar, 50 μm; magnification, ×100.

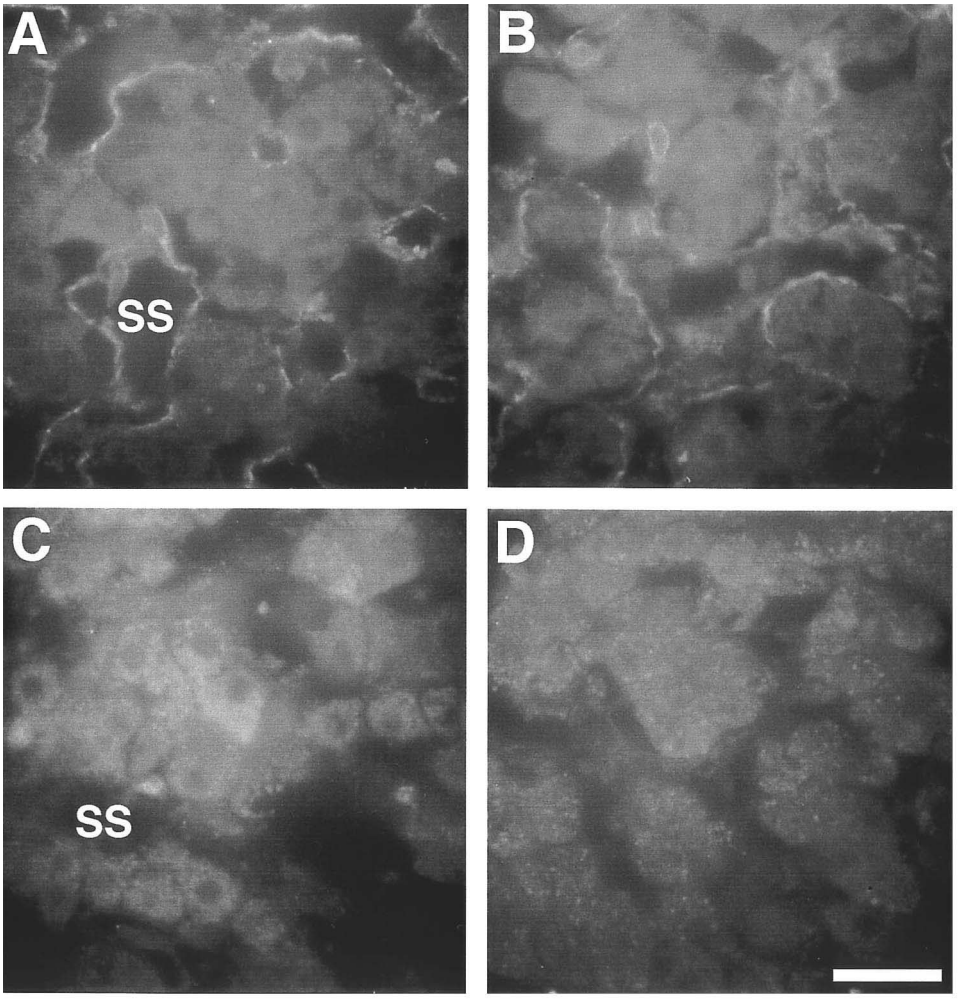

Fig. 7. Immunohistochemical observation of TM and GDH at the higher magnification. Formalin-fixed, paraffin-embedded chicken liver tissue sections were immunostained with the TM-antiserum (1:100, A and B) and the GDH-antibody (1:100, C and D). SS indicates the sinusoid. Bar, 20 μm; magnification, ×250.

was seen in the sections that were stained with the preimmune serum (Fig. 6A and B), immunostaining with the TM-antiserum and the GDH-antibody was distinctive from that with the preimmune serum. TM was localized in the peripheral regions of the sinusoids and bile ducts (BD) (Fig. 6C and D). Note that TM was also located at blood vessels (data not shown). GDH was distributed in the cytoplasm of hepatocytes, but not at the peripheries of sinusoids, bile ducts, and blood vessels (BV) (Fig. 6E and F). In order to demonstrate precise location of TM and GDH in the chicken liver, immunostained sections were observed at the higher (×250) magnification (Fig. 7). TM was localized weakly in the cytoplasm of hepatocytes and the peripheral regions of the sinusoids (SS) (Fig. 7A and B). GDH was distributed in the cytoplasm of hepatocytes with dotted structures (Fig. 7C and D).

DISCUSSION

In this study, we showed by co-sedimentation assays that GDH co-precipitated with F-actin and the amount of GDH sedimenting with F-actin was slightly increased by TM. It suggests that GDH is involved in the regulation of the actin cytoskeleton by binding to TM. It is not surprising that GDH co-sedimented with F-actin in the absence of TM, because several known TM-binding proteins, such as caldesmon (Matsumura and Yamashiro-Matsumura, 1993), calponin (Winder and Walsh, 1997), and tropomodulin (Fowler, 1996), bind both to TM and F-actin. It is known that caldesmon increases the association of TM with actin filaments, stabilizing the actin cytoskeleton (Yamashiro-Matsumura and Matsumura, 1988; Pittenger *et al*., 1995) and that the F-actin pointed end-capping activity of tropomodulin is enhanced in the presence of TM (Fowler, 1996). Thus, it is possible that the association of GDH with TM stabilizes the actin cytoskeleton. Alternatively, it is also possible that GDH utilizes the actin cytoskeleton via TM as a scaffold by exerting its enzymatic activity at paticular regions of the cytoplasm in nonmuscle cells.

Semi-nondenaturing 2D-PAGE showed that TM associ-

ated with nuclear GDH, but not with the mitochondrial GDH, suggesting preferential binding of TM to GDH. It has been widely accepted that GDH is localized in the mitochondrial inner space and also shown by recent biochemical and immunocytochemical studies that non-mitochondrial GDH isoforms are expressed in mammalian tissues. However, cloning and sequencing of chicken liver GDH cDNA in this study revealed that a single transcript was specifically amplified by RT-PCR. It is possible that other GDH transcripts could not be detected due to their small quantities in the adult chicken liver or that they have substantially different nucleotide sequences and thus could not be amplified by RT-PCR with primers used in this study. Alternatively, differences among transcripts may be restricted to the their 5'- and/or 3'-coding regions, sequences of which were not determined in this study, as rat liver microsomal aldehyde dehydrogenase cDNA has no mitochondrial signal sequence at the 5'-terminal region compared with the mitochondrial aldehyde dehydrogenase cDNA, but has an additional sequence at the 3'-terminal region encoding a hydrophobic segment, that is required for the precise location of the protein at the endoplasmic reticulum (Miyauchi *et al*., 1991; Masaki *et al*., 1994). Structural analyses of the chicken GDH gene(s) are useful to examine whether multiple transcripts can be produced or not. Human GDH isoforms, designated as GDH1 (Mavrothalassitis *et al*., 1988) and GDH2 (Shashidharan *et al*., 1994), are encoded by distinct genes. Our studies showed that several TM isoforms were present in the chicken liver. Therefore, it is interesting to examine specific interactions between TM isoforms and possible GDH isoforms.

Immunohistochemical staining of the chicken liver suggests that TM and GDH were localized in the cytoplasm of hepatocytes, but their precise localization could not be revealed in this study. It seems that the dotted structures detected with the GDH-antibody may be mitochondria. It has not been reported that TM is distributed in the mitochondrial inner space, and such dotted structures were not detected with the TM-antiserum. Several biochemical and immunocytochemical studies have indicated that GDH is localized at the lysosomal membrane (Rajas and Rousset, 1993; Rajas *et al*., 1996), nucleus (DiPrisco and Garofano, 1975; McDaniel, 1995), and rough endoplasmic reticulum (Lee *et al*., 1999). It is conceivable that a membrane-bound form of GDH can bind to the actin cytoskeleton via TM. It has been revealed that TM is involved in intracellular granule movement in cultured cells (Hegmann *et al*., 1989) and budding yeasts (Liu and Brescher, 1992), and that human TM3 isoform is related to organelle movement and distribution (Pelham *et al*., 1996). Thus, it is possible that an organelle movement is regulated by the interaction between TM and GDH. It is intriguing to examine by double staining of chicken liver tissue sections and cultured cells using the GDH-antibody and a specific antibody against an organelle membrane-bound protein in order to reveal GDH indeed locates at the organelle membrane.

We showed in the previous study (Akutsu *et al*., 2000) that GDH interacted with TM in the absence of ATP, an allosteric inhibitor of GDH enzymatic activity, but the interaction was inhibited by 0.1 mM ATP. From our previous and present results, we propose two hypotheses on physiological roles of the interaction between TM and GDH in nonmuscle cells (Fig. 8). First, GDH associates with TM on actin filaments in the absence of ATP. In the absence of the allosteric inhibitor, GDH stabilizes its filamentous structure together with TM, or exerts its enzyme activity on the actin cytoskeleton at particular regions of the cytoplasm because of the absence of the allosteric inhibitor. On the contrary, GDH dissociates from the actin filaments and its enzymatic activity is prevented in the presence of ATP (Fig. 8A). Sec-

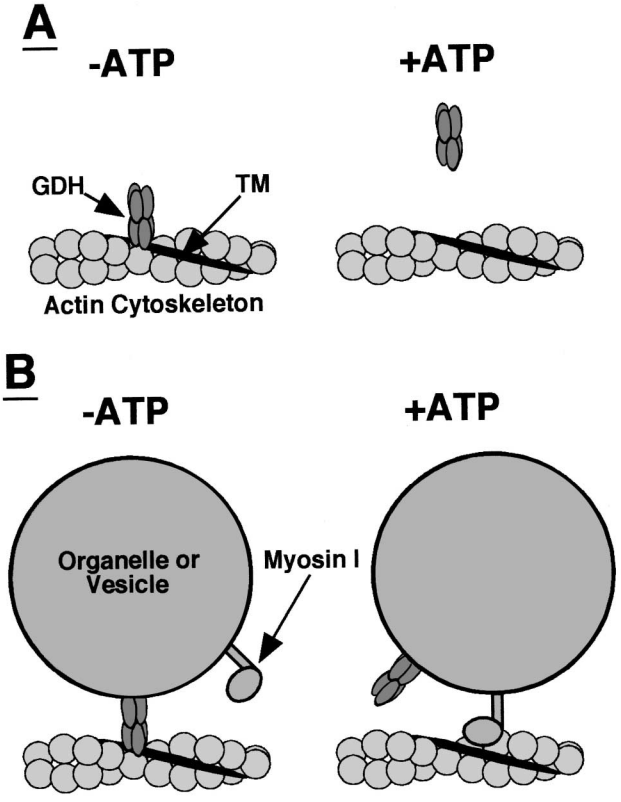

Fig. 8. Schematic presentation of hypotheses on physiological roles of the interaction between TM and GDH. A, GDH interacts with actin filaments via TM in the absence of ATP. GDH stabilizes the actin cytoskeleton together with TM, or exerts its enzymatic activity at particular portions of the cytoplasm in nonmuscle cells. In the presence of ATP, the association of TM with GDH is prevented. GDH dissociates from actin filaments and loses its enzymatic activity, because ATP is known as an inhibitor for the GDH enzymatic activity. B, The organelle membrane-bound GDH binds to actin filaments via TM in the absence of ATP. The organelle is anchored at limited positions of the cytoplasm in nonmuscle cells. In the presence of ATP, the association of TM with GDH is inhibited. Instead, an actin-based motor protein such as myosin I, which is known to locate at the organelle membrane, can bind to actin filaments and promote its motor activity utilizing ATP as energy. Thus, the organelle moves along the actin cytoskeleton to particular regions of the cytoplasm in nonmuscle cells.

ond, the organelle membrane-bound GDH interacts with actin filaments via TM in the absence of ATP, and thus the organelle is anchored at limited regions of the cytoplasm in nonmuscle cells. In the presence of ATP, the association of GDH with the actin filaments is prevented. Instead, an actin-based motor protein such as myosin I, which is known to locate at the organelle membrane (Mermall *et al*., 1998), can interact with actin filaments utilizing ATP as an energy source, resulting in movement of the organelle along the actin cytoskeleton to particular portions of the cytoplasm in nonmuscle cells (Fig. 8B).

The present results invoke bifunctional proteins. Translation elongation factor-1α catalyzes the GTP-dependent binding of aminoacyl tRNA to the ribosome acceptor site in the peptide elongation phase of protein synthesis and it also participates in the regulation of microtubules and actin filaments (Condeelis, 1995). Aldolase is a glycolytic enzyme and also recognized as an actin-binding protein (Arnold *et al*., 1968). Its enzymatic activity is modulated by interacting with actin filaments, and the interaction is prevented in the presence of its substrate, fructose 1, 6-bisphosphate (Walsh *et al*., 1977). Crystallins are known as major components of the lens. αB-crystallins are ubiquitously expressed in vertebrate tissues and exert chaperone activities (Graw, 1997). GDH catalizes transition of L-glutamate into 2-oxoglutarate and participates in the regulation of actin filaments and microtubules (Rajas and Rousset, 1993; Rajas *et al*., 1996). Therefore, it is suggested that GDH is adjoined to members of bifunctional proteins.

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