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Analyses of Expression and Localization of Two Mammalian-Type Transglutaminases in *Physarum polycephalum*, an Acellular Slime Mold

Fumitaka Wada[1], Atsuko Ogawa[1], Yuko Hanai[1], Akio Nakamura[2], Masatoshi Maki[1] and Kiyotaka Hitomi[1,*]

[1]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-8601; and [2]Department of Molecular and Cellular Pharmacology, Faculty of Medicine, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8510

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Transglutaminase (TGase) is an enzyme that modifies proteins by crosslinking or polyamination. *Physarum polycephalum*, an acellular slime mold, is the evolutionally lowest organism that has a mammalian-type transglutaminase. We have cloned a cDNA for *Physarum polycephalum* TGase (PpTGB), homologous to a previously identified TGase (PpTGA), whose sequence is similar to that of mammalian TGases. PpTGB encodes a primary sequence identical to that of PpTGA except for 11 amino acid residues at the N-terminus. Reverse transcription–PCR and Western blotting analyses showed that both PpTGA and PpTGB are expressed in microplasmodia and macroplasmodia during their life cycle, except for in sporangia. For biochemical characterization, we carried out the ectopical expressions of PpTGA and PpTGB in *Dictyostelium discoideum*. Subcellular fractionation of these *Dictyostelium* cells showed that the expressed PpTGA, but not PpTGB, localizes to the membrane fraction. Furthermore, in *Physarum*, subcellular fractionation and immunostaining indicated specific localization at the plasma membrane in macroplasmodia, while the localization was entirely cytoplasmic in microplasmodia.

Key words: calcium, crosslinking, *Dictyostelium discoideum*, *Physarum polycephalum*, transglutaminase.

Abbreviations: CBB, Coomassie Brilliant Blue R250; MoAb, monoclonal antibody; Myo II, myosin II; PBS, phosphate-buffered saline; PoAb, polyclonal antibody; PpTG(s), transglutaminase(s) from *Physarum polycephalum*; RT-PCR, reverse transcription polymerase chain reaction; TCA, trichloroacetic acid; TGase, transglutaminase.

Transglutaminases (TGases; EC 2.3.2.13) comprise a family of enzymes that catalyze the Ca^{2+}-dependent crosslinking reaction between the γ-carboxyamide group of glutamine residues and the ε-amino group of lysine residues or primary amine. The reaction leads to the formation of an isopeptide bond between two proteins and the covalent incorporation of polyamine into proteins (*1*, *2*). In mammals, crosslinking by TGases is essential for blood coagulation, skin-barrier formation, and apoptosis. In humans, nine isozymes have been identified, and each has a unique tissue distribution and physiological roles (*3*). TGase 2, a ubiquitously expressed isozyme, also plays critical roles in cell adhesion and signaling due to a non-crosslinking reaction (*4–8*).

Although the involvement of TGases in various biological processes has been reported, the physiological significance remains unclarified. Recent studies on mice lacking TGase 2, however, have shown no developmental abnormalities and only minor apparent phenotypic changes (*9–11*). In this case, other TGases could compensate for the loss of enzymatic activity and consequently, it would be difficult to find apparent phenotypes in gene-deficient animals.

To gain insight into the fundamental functions of TGases, investigations using lower organisms as model systems are profitable (*12*, *13*). Although the isopeptide crosslinking reaction is observed in various organisms from animals to microorganisms, their primary structures are diverse (*14*, *15*). TGases with papain-like characteristics, Ca^{2+}-dependency and an active-center Cys residue have been identified in vertebrates and arthropods, such as grasshoppers and horseshoe crabs (*16*, *17*). In bacteria, yeast, and lower invertebrates such as nematodes, no gene with a structure similar to the structures of mammalian TGases has been found (*18–21*). Recently, we found that Physarum polycephalum, an acellular slime mold, has a TGase whose primary structure is similar to that of mammalian TGases (*22*).

Physarum polycephalum has been used mainly in studies on development, inheritance of mitochondrial DNA and cell motility (*23*, *24*). This is one of the lowest eukaryotes with a unique life cycle which is characterized by spores, amoebae, macroplasmodia, and microplasmodia. The macroplasmodia are giant, multinucleated cells showing vigorous cytoplasmic streaming, a veined structure and no internal cell walls. Starvation of macroplasmodia together with exposure to light causes differentiation into sporangia, which undergo meiosis to form haploid spores. Germinating spores form amoebae, which can fuse to produce diploid macroplasmodia.

*To whom correspondence should be addressed. Tel: +81-52-789-5541, Fax: +81-52-789-5542, E-mail: hitomi@agr.nagoya-u.ac.jp

A special growing type of the plasmodia is formed in liquid culture, in which the plasmodia are reduced to small protoplasmic fragments called "microplasmodia" (*25*). Because of their small size and convenience of culture, microplasmodia have proved to be suitable for *in vivo* experiments.

In order to advance studies on the physiological significance of crosslinking reactions using *Physarum polycephalum*, we further characterized TGase (PpTG) in this evolutionally lower model organism. The expression levels of two identified PpTGs at each stages of development were analyzed at the mRNA and protein levels. For the biochemical characterization of PpTGs, expression of each recombinant protein in *Dictyostelium discoideum*, a cellular slime mold, was carried out and the subcellular localization of the PpTGs was determined. Moreover, localization in macroplasmodia and microplasmodia was analyzed by immunostaining using a monoclonal antibody (MoAb).

MATERIALS AND METHODS

Cell Culture—Macroplasmodia of *Physarum polycephalum* (strain Ng-1) were grown on Quaker Oatmeal (Quaker Oats Company, Chicago, IL) in the dark (*24*). The migrating sheets of plasmodia were collected and used for experiments. Microplasmodia of *Physarum polycephalum* (strain Ng-1) were cultured in the semi-defined liquid medium (SDM) of Daniel and Baldwin (*25*). All cultures were grown in complete darkness. The growth temperature was 25°C for plasmodia.

Dictyostelium discoideum strain Ax-2 was cultured axenically in HL5 medium using Proteose Peptone No. 3 (Difco Laboratories, MI, USA) at 22°C as described previously (*26*, *27*). The culture medium was supplemented with penicillin G (6 units/ml) and streptomycin sulfate (6 mg/ml).

cDNA Cloning of PpTGA and PpTGB—Total RNA from plasmodia was obtained by the AGPC (acid guanidium/phenol/chloroform) method. The first strand cDNA was synthesized using 1 μg of total RNA in a reaction mixture of 0.5 mM dNTPs, 40 U RNasin, 4 U AMV reverse transcriptase, and an antisense primer containing a stop codon, 5′-GTAGAAAGAATATTTTAAACG-3′, which was determined previously by cDNA cloning of PpTG (*22*). PCR was performed with sense primer, 5′-CTGGATCCATGACTACCGTATTCTTTAATGAAATTC-3′ and antisense primer, 5′-AGTCTAGATTAAACGACAATAACTTGGGCTTGG-3′. The sense primer has a *Bam*HI site upstream of the initiation codon and the antisense primer includes an *Xba*I site after the stop codon. Amplification conditions were as follows: 30 cycles at 94°C for 0.5 min, 60°C for 1 min, and 72°C for 1 min. The PCR product was cloned into TA-cloning vector pCR-TOPO (Invitrogen, CA) according to the manufacturer's instructions. The nucleotide sequences of the isolated clones were determined with an automated fluorescent sequencer, ABI PRISM 310, using a BigdyeTM terminator cycle sequencing ready reaction kit (PE Applied Biosystems).

RT-PCR Analysis—Total RNA from each development stage of *Physarum polycephalum* macroplasmodia developing on paper sheets was extracted at 4-h intervals after irradiation of light. The total RNA (1 μg) was reverse-transcribed in a reaction mixture of 0.5 mM dNTPs, 40 U RNasin in the supplied buffer, 4 U of AMV–reverse transcriptase and 1 μM oligo dT primer. The resulting cDNAs were subjected to PCR with primers 5′-TGGGTGGGGCACATTGTATG-3′ (sense, 205–224) and 5′-AACTGTATGGAAGTCCCAAT-3′ (antisense, 395–414), and 5′-TGGGTGGGGTACATTATACA-3′ (sense, 205–224) and 5′-AACTGAATAGAGGTCCCACA-3′ (antisense, 395–414) to specifically amplify PpTGA and PpTGB cDNAs, respectively. *Dictyostelium discoideum* pTX-FLAG expression plasmid vectors with PpTGA or PpTGB cDNA (0.1 pg/μl) insertions were used as positive controls. Amplification conditions were as follows: 30 cycles at 94°C for 0.5 min, 63°C for 0.5 min, and 72°C for 0.5 min.

Establishment of Hybridomas Producing MoAb against PpTGs—Recombinant protein for immunizing mice was produced from *E. coli* using full-length cDNA of PpTG (PpTGA) as described previously (*22*). BALB/c female mice were immunized three times and used for the preparation of splenocytes. Hybridomas were established using myeloma SP2 by standard methods. Among the established Sp2-hybridomas, positive hybridomas were selected for further studies based on growth rate and immunoreactivity in the enzyme-immunosorbent assay. Cloned hybridomas were transplanted intraperitoneally into balb/c mice. The IgG fraction contained in the ascitic fluid was purified by ammonium sulfate precipitation and DEAE-cellulose column chromatography.

Preparation of Total Cellular Lysates and Western Blotting—Total cellular extracts were prepared from microplasmodia and macroplasmodia. In the latter case, total extracts were prepared at 4 h intervals after irradiation of light from the slime mold that were developing on paper sheets. For analysis of the expression of PpTGs, harvested cells were lysed directly in SDS sample buffer (63 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, pH 6.8) containing 5 mM EDTA. The sample was heated for 3 min and sonicatied. Then, the sample was analyzed in 7.5% SDS–polyacrylamide gels (PAGE) followed by Western blotting using an anti-PpTG MoAb by standard methods. Immuno-signals were detected by the chemiluminescent method using Super Signal West Pico Chemiluminescent Substrate (Pierce).

Expressions of Recombinant PpTGs in Dictyostelium discoideum and Detection of TGase Activity—Both full length cDNA fragments that were inserted into pCR-TOPO vector were excised with *Bam*H I and *Xba*I and then ligated into the multiple cloning sites of the pTX-FLAG expression vector for *Dictyostelium discoideum*, which has a FLAG epitope upstream of multiple cloning sites (*28*). The resulting plasmids, pTX-FLAG-PpTGA and pTX-FLAG-PpTGB, were transformed into the cell line Ax-2 by electroporation. For both plasmids, several clonal cell lines were selected with G418 (10 μg/ml) in HL-5 medium.

The total lysate of *Dictyostelium* cells was directly dissolved with SDS sample buffer after washing with PBS. Samples were subjected to 7.5% SDS-PAGE followed by CBB (Coomassie Brilliant Blue) staining and Western blotting.

TGase activity was assayed by fluorometric measurement of monodansylcadaverine (MDC) (Sigma) conjugation to *N*,*N*-dimethylcasein. The standard assay mixture

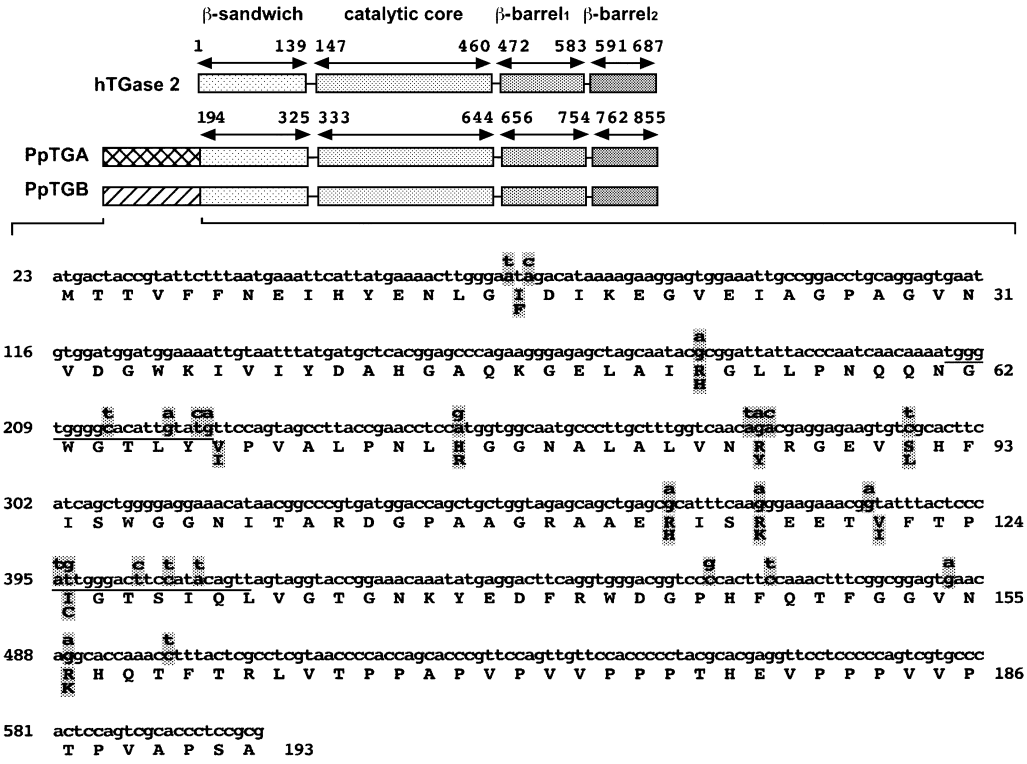

Fig. 1. **cDNA and deduced amino acid sequences of PpTGA and PpTGB in the N-terminal region.** The additional cDNA and amino acid sequences at the amino terminus of PpTGs compared with those of human TGase 2 (hTGase 2) are shown. The four structural domains are indicated by arrows with amino acid positions (top). The nucleotide and amino acid residue numbers are shown on the left and right sides, respectively. The gray background indicates places where the cDNA and deduced amino acid sequences of PpTGB differed from those of PpTGA. The single lines indicate the primer sequences used for RT-PCR (Fig. 2A).

contained 20 mM Tris-HCl (pH 7.5), 0.4 mM $CaCl_2$, 8 mM dithiothreitol, 0.2 mg/ml dimethyl casein, 0.4 mM MDC, and the cellular extract in 30 μl. Reactions were performed at 37°C for 1 h. Products were analyzed by SDS-PAGE in 12.5% gels. A fluorograph of the gel was obtained on a UV lightbox (302 nm) to detect the MDC-incorporated *N*,*N*-dimethylcasein.

Subcellular Fractionation Analysis—Dictyostelium cells expressing either PpTGA or PpTGB were harvested and washed twice with PBS and suspended in lysis buffer (10 mM Tris-HCl, 3 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, pH 7.5) containing protease inhibitors (1 mM PMSF, 25 ng/ml leupeptin, 1 μM pepstatin). Then the cells were lysed with a homogenizer and passed through a membrane filter (5.0 μm, MILLIPORE). Alternatively, cells were lysed by the addition of Triton X-100 at a final concentration of 1%, and then gently mixed for 15 min at 4°C. Cell lysates were centrifuged at 1,000 × *g* for 10 min at 4°C (pellet, P_1). The supernatants were centrifuged at 10,000 × *g* for 10 min at 4°C (pellet, P_2), and the obtained supernatants were further centrifuged at 100,000 × *g* for 30 min at 4°C (pellet, P_3; supernatant, S).

The samples were analyzed by 7.5% SDS-PAGE followed by Western blotting using anti-FLAG MoAb.

For subcellular fractionation of *Physarum* microplasmodia and macroplasmodia, the same procedures were used except for the size of the membrane filter (8 μm) and the antibody (anti-PpTG MoAb) used to detect the endogenous PpTGs.

Immunofluorescence Microscopy—Microplasmodia washed three times with TM buffer (20 mM Tris-HCl, 10 mM NaCl, 40 mM KCl, 7 mM $MgCl_2$, pH 7.5), or macroplasmodia grown on MEA medium consisting of 0.165% mycological pepton (Oxoid), 1% malt extract (Oxoid), 5 μg/ml hemin (ICN Biomedicals Inc.), 1.5% agar were fixed at 4°C for 15 min in a solution of 10% TCA (trichloroacetic acid) (*29*). The fixed cells were then washed three times with PBS and permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature. After washing in PBS, non-specific binding was blocked with PBS containing 1% BSA. Then the fixed cells were incubated for 1 h at room temperature in the presence of anti-PpTG MoAb and anti-myosin II (Myo II) polyclonal antibody (PoAb) in PBS containing 1% BSA. Subsequently, cells were incubated for 1 hr at room temperature with Alexa 488 Fluor goat anti–mouse IgG (Molecular Probe) and Cy3-conjugated goat anti–rabbit IgG (Molecular Probe) in PBS containing 1% BSA. For nuclear staining, samples were incubated with Hoechst 33342 (Dojindo, Japan) in PBS for 10 min at room temperature. Samples were mounted on a glass slide with antifading solution [25 mM Tris-HCl, 10% polyvinyl alcohol, 5% glycerol, 2.5% 1,4-diazobicyclo-(2,2,2)-octane, pH 8.7] and covered with coverslips. Immunofluorescence was analyzed under an inverted fluorescence apotome microscope, Axiovert 200M (Zeiss).

RESULTS

Identification and Cloning of PpTGA and PpTGB—Previously, we identified mammalian type TGase (PpTG) in *Physarum polycephalum* macroplasmodia by purification and cDNA cloning. During further analyses of the cDNA, another mRNA species with an identical sequence except for the 5′ region was found. We here designate the newly found PpTG and the previously identified one as PpTGB and PpTGA, respectively.

Initially, we attempted to obtain MoAbs able to distinguish PpTGA from PpTGB. Although suitable MoAbs

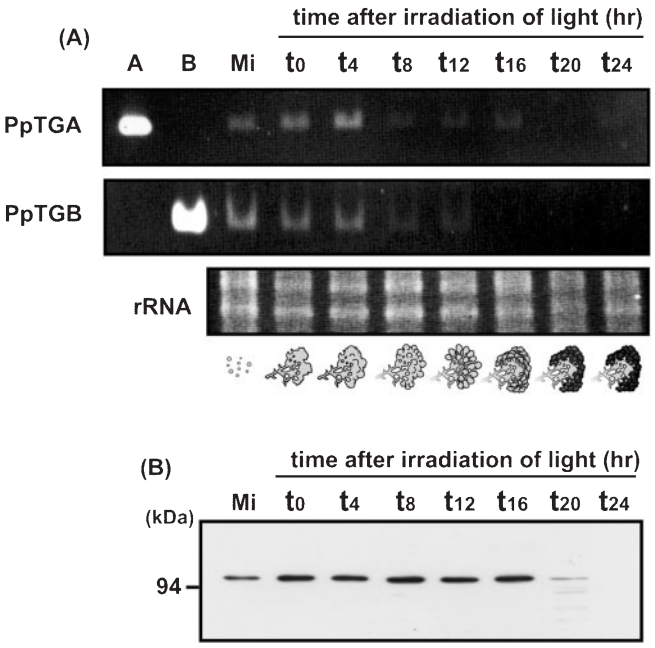

Fig. 2. **Stage-specific detection of PpTGs.** (A) Total RNA from microplasmodia (Mi) and each stage of macroplasmodia at the indicated times after light irradiation (t0–t24) was obtained by the AGPC method. The RNA was then subjected to RT-PCR analyses for PpTGA (top panel) and PpTGB (middle panel) mRNAs using specific primers. As a control, pTX-FLAG plasmid vectors inserted with either PpTGA or PpTGB were used as a template for PCR (A, B). Ethidium bromide staining is shown after agarose gel electrophoresis of total RNA where rRNAs are the major constituents (bottom panel). Illustrations show the process of sporulation. Plasmodium dissociates into cytoplasmic nodules (t12–t16), which culminate to form a fruiting body (t20–t24). (B) Total cell lysates of microplasmodia and each stage of macroplasmodia were prepared as described in "Materials and Methods." The proteins were subjected to 7.5% SDS-PAGE followed by Western blotting using anti-PpTG MoAb.

could not be obtained, the most specific MoAb reacting with both PpTGs was used for biochemical analyses during the course of this study.

As shown in Fig. 1, TGase 2, which is the most ubiquitously expressed isozyme among TGases in higher organisms, consists of four domains (β-sandwich, catalytic core, β-barrel 1, and β-barrel 2) (*30*). When compared with the primary sequence of human TGase 2, an additional extended region at the N-terminus was found in PpTGs. In this N-terminal region, 25 nucleotides and 11 amino acid residues were different between PpTGA and PpTGB. Except for this region, no differences in nucleotide sequence, including the catalytic domain, were found between the two PpTGs.

Expression of PpTGA and PpTGB during Development of Physarum plasmodia—The stage-specific expression of PpTGs was investigated at the mRNA (Fig. 2A) and protein levels (Fig. 2B). RT-PCR analyses capable of distinguishing the expression level of each transcript were carried out using specific primers for PpTGA and PpTGB. Expression of mRNA for both PpTGs was observed in microplasmodia (Mi), macroplasmodia and macroplasmodia developing into sporangia (t0–t24). The amount of PpTGA transcript in microplasmodia was smaller than that in macroplasmodia (t0). This result is in contrast to the level of PpTGB transcript. Both transcripts for PpTGA and PpTGB reached a peak by 4 h and then abruptly decreased at 8–16 h after light irradiation. Western blotting analyses revealed that the protein levels of PpTGs were higher in macroplasmodia than in microplasmodia, a pattern similar to that of the PpTGA transcript. During differentiation, the protein levels of PpTGs remained unchanged and then decreased at around 20 h upon sporulation.

Subcellular Localization of Recombinant PpTGs in Dictyostelium discoideum—As shown in Fig. 1, the N-terminal regions of PpTGA and PpTGB differ in sequence. In some TGases, cellular localization is affected by the N-terminal sequence: attachment of human TGase 1 to the plasma membrane by myristoylation and localization of starfish TGase in the nucleus (*31*, *32*). Since there are no distinct structural differences between the two PpTGs, analysis using *Physarum polycephalum* is limited. Therefore, in order to examine the differences in the cellular localization of PpTGs, we investigated each PpTG expressed separately in *Dictyostelium discoideum,* a species closely-related to *Physarum polycephalum* (*33*). The PpTGA or PpTGB cDNA was introduced into the *Dictyostelium*-expression vector (pTX-FLAG) and transfected

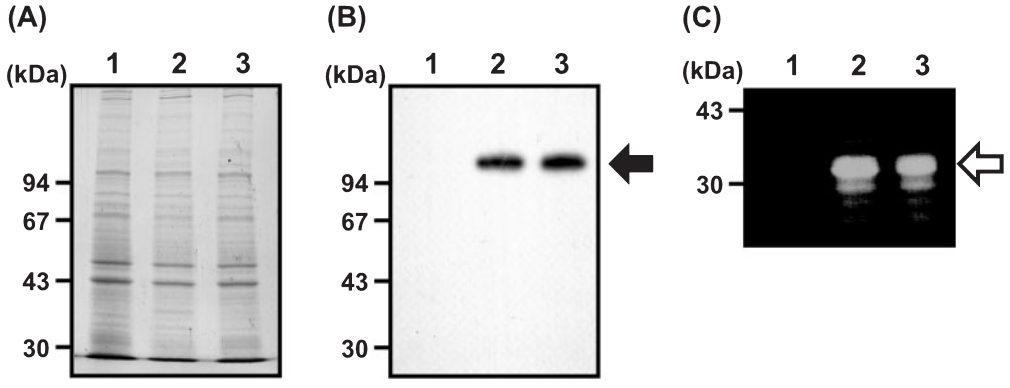

Fig. 3. **Expression of recombinant PpTGs in *Dictyostelium discoideum*.** The expressions of recombinant PpTGA and PpTGB were analyzed by 7.5% SDS-PAGE following by CBB staining (A) and Western blotting using anti-PpTG MoAb (B). Lane 1, total cellular protein of wild type *Dictyostelium discoideum* amoebae cells; lane 2 and lane 3, cellular protein from cells expressing PpTGA and PpTGB, respectively. The black arrow indicates the positions of PpTGs. (C) MDC was incorporated into *N*,*N*-dimethylcasein by incubation with a cellular extract of wild type amoebae cells (lane 1), and cells expressing PpTGA (lane 2) or PpTGB (lane 3). The reaction products were analyzed by 12.5% SDS-PAGE and a fluorograph was obtained on a UV lightbox. The white arrow indicates the position of *N*,*N*-dimethylcasein.

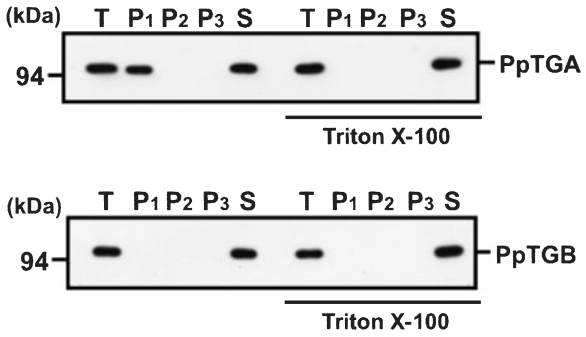

Fig. 4. **Subcellular fractionation of PpTGA and PpTGB in Dictyostelium.** *Dictyostelium* cells that ectopically expressed either PpTGA or PpTGB were suspended in lysis buffer and lysed by either homogenation (–Triton X-100) or by gentle mixing in the presence of 1% Triton X-100 as described in "MATERIALS AND METHODS." Subcellular fractionation was performed by sequential centrifugations. PpTGA and PpTGB proteins were detected by Western blotting using the anti–FLAG MoAb: T, total lysate; P_1, 1,000 × *g* pellet; P_2, 10,000 × *g* pellet; P_3, 100,000 × *g* pellet; S, supernatant.

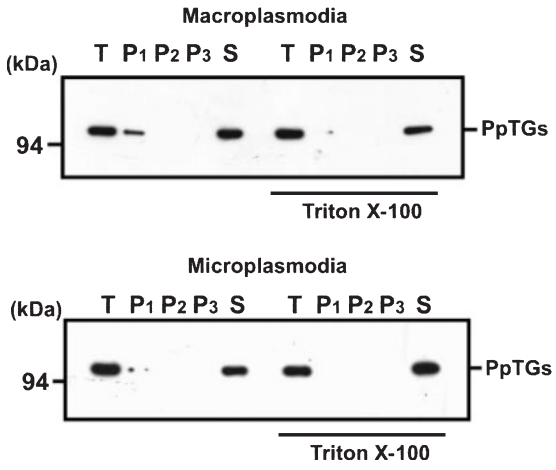

Fig. 5. **Subcellular fractionation of PpTGs in macroplasmodia and microplasmodia.** Subcellular fractionations of PpTGs in macroplasmodia and microplasmodia cells were performed as described to the legend to Fig. 4. PpTGs protein was detected by Western blotting using anti-PpTG MoAb.: T, total lysate; P_1, 1,000 × *g* pellet; P_2, 10,000 × *g* pellet; P_3, 100,000 × *g* pellet; S, supernatant.

into growing *Dictyostelium* amoebae. In cellular extracts of the established cell lines, recombinant PpTGA and PpTGB were expressed at a size similar to that of the native enzyme (Fig. 3, A and B).

To confirm that the recombinant PpTGs are enzymatically active, the cellular lysate was analyzed using dimethylcasein and fluorescent-labeled primary amine. Extracts of *Dictyostelium* amoebae expressing either PpTGA or PpTGB showed apparent enzymatic activity (Fig. 3C) when compared with the reaction products of the wild type. These results indicate that both PpTGs proteins are expressed with a structure similar to that of the native enzyme.

The subcellular localization of each PpTGA or PpTGB expressed in *Dictyostelium* was investigated by the differential centrifugation method followed by Western blotting using anti-FLAG MoAb (Fig. 4). In the absence of the non-ionic detergent Triton X-100, PpTGB was recovered only in the cytosolic fraction (S), whereas PpTGA was detected in both the 1,000 × *g* pellet (P_1) and cytosolic fraction (S). In the presence of Triton X-100 at a final concentration of 1% in the lysis buffer, however, PpTGA was recovered entirely in the cytosolic fraction (S). These findings indicate that PpTGA localizes preferentially to the cellular membrane as compared with PpTGB.

Subcellular Localization and Immunostaining of PpTGs in Physarum polycephalum—To obtain more information about the significance of each PpTG in *Physarum polycephalum*, we examined their cellular localization in microplasmodia and macroplasmodia by subcellular fractionation and immunostaining.

Subcellular fractionations of native PpTGs in microplasmodia and macroplasmodia were performed by the same procedure as used for *Dictyostelium*-expressing PpTGs using anti-PpTG MoAb (Fig. 5). In macroplasmodia, specific reactions were observed in both the 1,000 × *g* pellets (P_1) and cytosolic fraction (S). In the presence of Triton X-100, no band was found in the P_1 fraction. This result coincides with the distribution of PpTGA expressed in *Dictyostelium* (Fig. 4), suggesting that PpTGA preferentially localizes to the cellular membrane in macroplasmodia. In microplasmodia, both PpTGs were harvested mostly in the cytosolic fraction (S) and slightly in the P1 fraction. Since the mRNAs of both PpTGA and PpTGB are expressed in microplasmodia, PpTGs exist in the cytoplasm in this life stage.

To investigate further the cellular distribution of PpTGs in macroplasmodia and microplasmodia, immunolocalization in both phases was analyzed (Fig. 6). Immunostaining of Myo II, which is a cytoskeleton-associated protein used as a marker for cytoplasm protein, resulted in diffuse staining in both types (Fig. 6, B and H). From the merged image, PpTGs in macroplasmodia were apparently localized to the plasma membrane (Fig. 6, C and D). In contrast, diffuse cytoplasmic staining of PpTGs was observed in microplasmodia (Fig. 6, I and J). These observations are consistent with the results of subcellular fractionation.

By simultaneous staining with Hoechst 33342, a number of nuclei were observed in both plasmodia. The merged images of the Hoechst 33342–stained cells with anti-PpTG MoAb–immunostained cells suggest that PpTGs are not localized to the nucleus (Fig. 6, F and L).

DISCUSSION

We have reported here the cloning and biochemical characterization of two different TGases in *Physarum polycephalum*. By RT-PCR and Western blotting analyses, we confirmed that both PpTGs are expressed in most stages of macroplasmodia as well as in microplasmodia. It is possible that alternative splicing produces two variants of mRNAs, although single transcript was detected by Northern blotting (*22*).

In macroplasmodia, the amounts of both PpTGs at the protein level remained unaltered until 8–12 h after the onset of differentiation, a slightly longer period than that of mRNA expression (Fig. 2). When macroplasmodia changed to sporangia, which produce spores on their surface, the expression levels of PpTGs decreased. Although

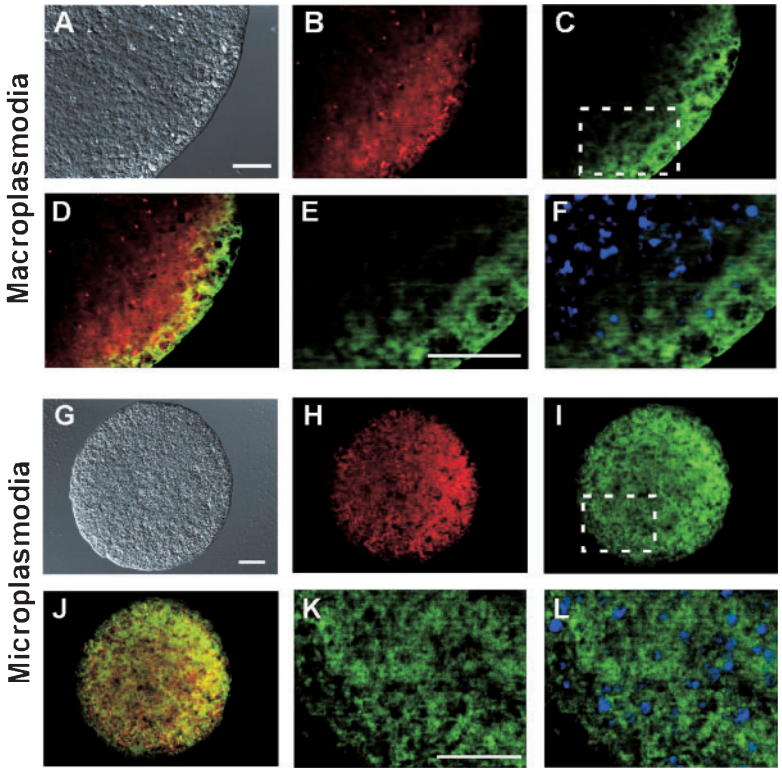

Fig. 6. **Immunolocalization of PpTGs and myosin II in macroplasmodia and microplasmodia.** Macroplasmodia (upper) and microplasmodia (lower) cells were stained with anti–Myo II PoAb (B and H) and anti–PpTG MoAb (C and I). A and G, differential interference contrast images; D and J, merged images of either B and C or H and I; E and K, higher magnifications images shown in C and I, respectively; F and L, merged images of Hoechst 33342 staining with E and K, respectively. Scale bars represent 100 μm.

the substrates of PpTGs in differentiating macroplasmodia have not been clarified, crosslinking might be involved in spore formation at an early stage. In sporulating cells of *Bacillus subtilis*, observations of both TGase activity and ε-(γ-Glu)Lys crosslinking products in the spore coat fraction have been reported (*34*).

Based on the results of Western blotting, the expression levels of PpTGs in macroplasmodia were higher than those in microplasmodia (Fig. 2B). This result correlates well with that of the PpTGA transcript and indicates that PpTGA is dominantly expressed in *Physarum*.

Macroplasmodia, growing on agar plates with sufficient nutrients, show rapid growth with active shuttle streaming in the cytoplasm. On the other hand, microplasmodia growing as cultured cells in liquid media proliferate more slowly and the elongation rate of the cell membrane is less than that of macroplasmodia. It is speculated that a higher activity level of PpTGs is necessary for vigorous cellular growth and elongation of the body.

Since PpTGA and PpTGB cannot be distinguished in *Physarum* by immunological detection, the ectopic expression of each cDNA was carried out for localization analyses. *Dictyostelium discoideum* was used as a host because the expression vector for this organism has already been established (*28*). Additionally, no gene homologous to PpTG was expressed in the slime mold. Since both recombinant proteins were enzymatically active (Fig. 3C), PpTGs in this slime mold could be used for biochemical analyses.

Subcellular analysis of the localization of PpTGs in the *Dictyostelium* cell lines, revealed distinct patterns. PpTGA, which mainly exists as an insoluble protein (P_1 fraction), was harvested in the soluble fraction following treatment with Triton X-100. This result indicates that the amino terminal sequence provides PpTGA with the ability to associate with the cellular membrane. Although the P_1 fraction contains the nucleus, the results of immuno- and Hoechst 33342 co-staining exclude the possibility of localization in the nucleus (Fig. 6).

In *Physarum*, in both macroplasmodia and microplasmodia, the subcellular localization of PpTGs showed a pattern similar to that of PpTGA expressed in *Dictyostelium*. This result further indicates that the PpTGA is dominantly expressed TGase in *Physarum*, particularly in the rapidly growing macroplasmodia.

Immunostaining revealed that PpTGs preferentially localize beneath the plasma membrane in macroplasmodia. In contrast, a positive signal was observed entirely in the cytoplasm of microplasmodia (Fig. 6). By simultaneous staining with Hoechst 33342, PpTGs appeared not to co-localize to the nucleus of microplasmodia and macroplasmodia. These observations correlate with the results of the subcellular localization analysis.

The mechanism by which the different amino acid residues in the N-terminal regions of PpTGA and PpTGB influence cellular localization remains unknown. TGases in lower eukaryotes commonly have longer additional N-terminal regions than those in mammals (*17*, *32*, *35*). In the starfish TGase, the N-terminal region is crucial for nuclear transport. In lower eukaryote TGase, it is possible that this region is involved in the regulation of localization, stability, and/or interaction with other proteins.

The physiological function of PpTGs remains unclarified. Enhancement of TGase activity has been observed following cellular injury, probably resulting in coagulation of the damaged area (*36*). In the same report, LAV1-2, which is a calcium-binding protein, appeared as a major substrate upon cellular damage. LAV1-2 has also

been characterized as CBP40, which reversibly forms large aggregates in a Ca^{2+}-dependent manner (*37*).

For elongation of the membrane during cellular growth and also in the case of repairing a damaged area, Ca^{2+} influx through the plasma membrane occurs. Therefore, it is possible that cross-linked CBP40 products formed by PpTGs, mainly by PpTGA beneath the cell surface, might be involved in these events. In order to clarify the significance of crosslinking by PpTGs, investigations to identify substrates other than CBP40 are in progress.

The nucleotide sequences of *Physarum polycephalum* TGases in this paper are available in the DDBJ/EMBL/GenBank database. The accession numbers of PpTGA and PpTGB are AB076663 and AB183248, respectively. This work was supported by a Grant-in-Aid for Scientific Research (C) No. 14560063 (to K.H.) and also by the TAKEDA Science Foundation (to K.H.). We thank Dr. H. Shibata in our laboratory for valuable suggestions.

REFERENCES

1. Griffin, M., Casadio, R., and Bergamini, C.M. (2002) Transglutaminases: *Nature*'s biological glues. *Biochem. J.* **368**, 377–396
2. Lorand, L. and Graham, R.M. (2003) Transglutminases: crosslinking enzymes with pleiotropic functions. *Nat. Rev. Mol. Cell. Biol.* **4**, 140–156
3. Aeschlimann, D., Koeller, M.K., Allen-Hoffmann, B.L., and Mosher, D.F. (1998) Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. Detection and identification of transglutaminase gene products based on reverse transcription-polymerase chain reaction with degenerated primers. *J. Biol. Chem.* **273**, 3452–3460
4. Fesus, L. and Piacentini, M. (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends Biochem. Sci.* **27**, 534–539
5. Akimov, S.S., Krylov, D., Fleichman, L.F., and Belkin, A.M. (2000) Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *J. Cell Biol.* **148**, 825–838
6. Takahashi, H., Isobe, T., Horibe, S., Takagi, J., Yokosaki, Y., Sheppard, D., and Saito, Y. (2000) Tissue transglutaminase, coagulation factor XIII, and the pro-polypeptide of von Willebrand factor are all ligands for the integrins alpha 9beta 1 and alpha 4beta 1. *J. Biol. Chem.* **275**, 23589–23595
7. Nakaoka, H., Perez, D.M., Baek, K.J., Das, T., Husain, A., Misono, K., Im, M.J., and Graham, R.M. (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* **264**, 1593–1596
8. Chen, J.S.K. and Metha, K. (1999) Tissue transglutaminase: an enzyme with a split personality. *Int. J. Biochem. Cell Biol.* **31**, 817–836
9. De Laurenzi, V. and Melino, G. (2001) Gene disruption of tissue Transglutaminase. *Mol. Cell. Biol.* **21**, 148–155
10. Nanda, N., Iismaa, S.E., Owens, W.A., Husain, A., Mackay, F., and Graham, R.M. (2001) Targeted inactivation of Gh/tissue transglutaminase II. *J. Biol. Chem.* **276**, 20673–20678
11. Bernassola, F., Federici, M., Corazzari, M., Terrinoni, A., Hribal, M.L., De Laurenzi, V., Ranalli, M., Massa, O., Sesti, G., McLean, W.H., Citro, G., Barbetti, F., and Melino, G. (2002) Role of transglutaminase 2 in glucose tolerance: knockout mice studies and a putative mutation in a MODY patient. *FASEB J.* **16**, 1371–1378
12. Osaki, T., Okino, N., Tokunaga, F., Iwanaga, S., and Kawabata, S. (2002) Proline-rich cell surface antigens of horseshoe crab hemocytes are substrates for protein cross-linking with a clotting protein coagulin. *J. Biol. Chem.* **277**, 40084–40090
13. Eschenlauer, S.C. and Page, A.P. (2003) The *Caenorhabditis elegans* ERp60 homolog protein disulfide isomerase-3 has disulfide isomerase and transglutaminase-like cross-linking activity and is involved in the maintenance of body morphology. *J. Biol. Chem.* **278**, 4227–4237
14. Makarova, K.S., Aravind, L., and Koonin, E.V. (1999) A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. *Protein Sci.* **8**, 1714–1719
15. Hitomi, K. (2003) Molecular evolution of transglutaminase: from bacteria to animal in *Recent Research Developmemts in Biophysics and Biochemistry* (Pandalai, S.G., ed.) Vol. **3**, pp. 223–234, Research Signpost, Kelara
16. Singer, M.A., Hortsch, M., Goodman, C.S., and Bentley, D. (1992) Annulin, a protein expressed at limb segment boundaries in the grasshopper embryo, is homologous to protein cross-linking transglutaminases. *Dev. Biol.* **154**, 143–159
17. Tokunaga, F., Muta, T., Iwanaga, S., Ichinose, A., Davie, E., Kuma, K., and Miyata, T. (1993) Limulus hemocyte transglutaminase. cDNA cloning, amino acid sequence, and tissue localization. *J. Biol. Chem.* **268**, 262–268
18. Kanaji, T., Ozaki, H., Takano, T., Kawajiri, H., Ide, H., Motoki, M., and Shimonoshi, Y. (1993) Primary structure of microbial transglutaminase from *Streptverticillium sp*. strain s-8112. *J. Biol. Chem.* **268**, 11565–11572
19. Ichikawa, S., Hatanaka, H., Yuuki, T., Iwamoto, N., Kojima, S., Nishiyama, C., Ogura, K., Okumura, Y., and Inagaki, F. (1998) Solution structure of Der f **2**, the major mite allergen for atopic diseases. *J. Biol. Chem.* **273**, 356–360
20. Chandrashekar, R., Tsuji, N., Morales, T., Ozols, V., and Metha, K. (1998) An ERp60-like protein from filarial parasite *Dirofilaria immitis* has both transglutaminase and protein disulfide isomerase activity. *Proc. Natl Acad. Sci. USA* **95**, 531–536
21. Natsuka, S., Takubo, R., Seki, R., and Ikura, K. (2001) Molecular cloning and expression of *Caenorhabditis elegans* ERp57-homologue with transglutaminase activity. *J. Biochem.* **130**, 731–735
22. Wada, F., Nakamura, A., Masutani, T., Ikura, K., Maki, M., and Hitomi, K. (2002) Identification of mammalian-type transglutaminase in *Physarum polycephalum*. *Eur. J. Biochem.* **269**, 3451–3460
23. Bailey, J. (1995) Plasmodium development in the myxomycete *Physarum polycephalum*: genetic control and cellular events. *Microbiology* **141**, 2355–2365
24. Kohama, K., Ishikawa, R., and Ishigami, M. (1998) Large-scale culture of *Physarum*: A simple way of growing plasmodia to purify actomyosin and myosin in *Cell Biology: a Laboratory Handbook*, 2nd ed., vol. **1**, pp. 466–471, Academic Press, New York
25. Daniel, J.W. and Baldwin, H.H. (1964) Methods of culture for plasmodial myxomycetes. *Methods Cell Physiol.* **1**, 9–41
26. Adachi, H., Takahashi, Y., Hasebe, T., Shirouzu, M., Yokoyama, S., and Sutoh, K. (1997) *Dictyostelium* IQGAP-related protein specifically involved in the completion of cytokinesis. *J. Cell Biol.* **137**, 891–898
27. Ohkouchi, S., Nishio K., Maeda, M., Hitomi, K., Adachi, H., and Maki, M. (2001) Identification and characterization of two penta-EF-hand Ca^{2+}-binding proteins in *Dictyostelium discoideum*. *J. Biochem.* **130**, 207–215
28. Levi, S., Polyakov, M., and Egelhoff, T.T. (2000) Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid* **44**, 231–238
29. Hayashi, K., Yonemura, S., Matsui T., and Tsukita, S. (1999) Immunofluorescence detection of ezrin/radxin/moesin (ERM) proteins with their carboxyl-terminal threonine phosphorylated in cultured cells and tissues. *J. Cell Sci.* **112**, 1149–1158
30. Casadio, R., Polverini, E., Mariani, P., Spinozzi, F., Carsughi, F., Fontana, A., Laureto, P.P., Matteucci, G., and Bergamini, C.M. (1999) The structural basis for the regulation of tissue transglutaminase by calcium ions. *Eur. J. Biochem.* **262**, 672–679
31. Steinert, P.M., Kim, S.-Y., Chung, S.-I., and Marekov, L.N. (1996) The transglutaminase 1 enzyme is variably acylated by

myristate and palmitate during differentiation in epidermal keratinocytes. *J. Biol. Chem.* **271**, 26242–26250
32. Sugino, H., Terakawa Y., Yamasaki, A., Nakamura, K., Higuchi, Y., Matsubara, J., Kuniyoshi, H., and Ikegami, S. (2002) Molecular characterization of a novel nuclear transglutaminase that is expressed during starfish embryogenesis. *Eur. J. Biochem.* **269**, 1957–1967
33. Baldauf, S.L. and Doolittle, W.F. (1997) Origin and evolution of the slime molds (Mycetozoa) *Proc. Natl Acad. Sci. USA* **94**, 12007–12012
34. Kobayashi, K., Kumazawa, Y., Miwa, K., and Yamanaka, S. (1996) ε-(γ-Glutamy)lysine cross-links of spore coat proteins and transglutaminase activity in *Bacillus subtilis*. *FEMS Microbiol. Lett.* **144**, 157–160
35. Cariello, L., Ristoratore, F., and Zanetti, L. (1997) A new transglutaminase-like from the ascidian *Ciona intestinalis*. *FEBS Lett.* **408**, 171–176
36. Mottahedeh, J. and Marsh, R. (1998) Characterization of 101-kDa transglutaminase from *Physarum polycepharum* and identification of LAV 1-2 as substrate. *J. Biol. Chem.* **273**, 29888–29895
37. Nakamura, A., Okagaki, T., Takagi, T., Nakashima, K., Yazawa, M., and Kohama, K. (2000) Calcium binding properties of recombinant calcium binding protein of lower eukaryote *Physarum polycephalum*. *Biochemistry* **39**, 3827–3834