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Glycogen phosphorylase in glycogen-rich cells is involved in the energy supply for ion regulation in fish gill epithelia

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Tseng Y-C, Huang C-J, Chang JC, Teng W-Y, Baba O, Fann M-J, Hwang P-P. Glycogen phosphorylase in glycogen-rich cells is involved in the energy supply for ion regulation in fish gill epithelia. *Am J Physiol Regul Integr Comp Physiol* 293: R482–R491, 2007. First published March 15, 2007; doi:10.1152/ajpregu.00681.2006.—The molecular and cellular mechanisms behind glycogen metabolism and the energy metabolite translocation between mammal neurons and astrocytes have been well studied. A similar mechanism is proposed for rapid mobilization of local energy stores to support energy-dependent transepithelial ion transport in gills of the Mozambique tilapia (*Oreochromis mossambicus*). A novel gill glycogen phosphorylase isoform (tGPGG), which catalyzes the initial degradation of glycogen, was identified in branchial epithelial cells of *O. mossambicus*. Double in situ hybridization and immunocytochemistry demonstrated that tGPGG mRNA and glycogen were colocalized in glycogen-rich cells (GRCs), which surround ionocytes (labeled with a Na⁺-K⁺-ATPase antiserum) in gill epithelia. Concanavalin-A (a marker for the apical membrane) labeling indicated that GRCs and mitochondria-rich cells share the same apical opening. Quantitative real-time PCR analyses showed that tGPGG mRNA expression levels specifically responded to environmental salinity changes. Indeed, the glycogen content, glycogen phosphorylase (GP) protein level and total activity, and the density of tGPGG-expressing cells (i.e., GRCs) in fish acclimated to seawater (SW) were significantly higher than those in freshwater controls. Short-term acclimation to SW caused an evident depletion in the glycogen content of GRCs. Taken altogether, tGPGG expression in GRCs is stimulated by hyperosmotic challenge, and this may catalyze initial glycogen degradation to provide the adjacent ionocytes with energy to carry out iono- and osmoregulatory functions.

osmoregulation; Na⁺-K⁺-ATPase; salinity; ionocytes

GLYCOGEN, A RAPIDLY AVAILABLE energy storage form, comprises the main readily mobilized energy resource deposit and is distributed in high-energy-consuming organs (10, 46). It has been well documented that glycogen serves as an emergency fuel supply for highly energy-dependent organs, such as the liver, muscles, and brain (5, 6, 35). Indeed, the rate of degradation or synthesis of glycogen is a function of the relative activities of glycogen phosphorylase (GP; EC 2.4.1.1) and glycogen synthase (EC 2.4.1.11). In the process of glycogen metabolism, GP is crucial for the utilization of glycogen, the storage form of glucose in all animal cells. GP itself is the rate-limiting enzyme and contains about 850 residues of 97

kDa, occurring in 2 forms: the more active glycogen phosphorylase a (GP_a) and the less-active glycogen phosphorylase b (GP_b). The homodimeric enzyme exists as three isoforms named according to the tissues in which they predominate, GPLL (liver), GPMM (skeletal muscle), and GPBB (brain), all of which are organs with great energy demands. The different GP isoforms vary in their responses to activation by phosphorylation and allosteric control, and thus, play different functional roles and possess different modes of regulation of glycogen metabolism in different cell types. Accordingly, these isoforms with distinct regulatory properties are able to meet the energy requirements of different tissues and cells (24).

Hepatic glucose production is central to glucose homeostasis, which is critical for the energy supply in the physiological operation of other organs (4). Glycogenolysis and the translocation of the subsequent metabolites during an emergency situation have been investigated in detail only in the mammalian central nervous system and retinal tissues (1, 7, 50). In the rat brain, GP is mainly expressed in astrocytes, astroglial cells, and ependymal cells of the ventricles but never in neurons (39). Astrocytes have been demonstrated to provide energy fuel, such as lactate, for neuron cells via the breakdown of astrocytic glycogen (38, 44). Highly active energy metabolism also occurs in mammalian retinal tissue. Glycogen and GP are present mainly in Müller glial cells, indicating that these cells provide energy for the functional operations of optic neurons (40). It would be interesting and challenging to see whether these cellular and physiological relationships in energy metabolism between astrocytes (or glial cells) and neurons exist in other highly energy-dependent physiological processes, like transepithelial transport.

Gills, one of the most important experimental models for studying transepithelial transport physiology (9, 14), are the major organ responsible for fish ionoregulatory and osmoregulatory mechanisms, and mitochondria-rich (MR) cells have been suggested to be the major ionocytes actively conducting ion transport in this epithelium (18, 19, 26, 33). In euryhaline fishes, acclimation to fluctuating salinities induces their gills to sufficiently and timely modulate and/or activate the operations of various ion transporters and enzymes, which are highly energy consuming (20, 29, 31, 32). The modulation and stimulation of ion transporters in gill epithelial MR cells in response to salinity challenges have been suggested to require

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prompt and extra energy supplies (33). In the very early literature, glycogen granules were reported in the cytoplasm of gill MR cells based on ultrastructural analyses (15, 37, 41), although no convincing cytochemical evidence has since been offered to support those data. Increments in the glycolytic potential such as the activities of GP, hexokinase, and pyruvate kinase were observed in gills, in parallel with increased environmental salinity (47). These findings imply the possibility of the involvement of glycogenolysis in gill epithelia during salinity challenges; so far, all of this existing knowledge requires convincing molecular and cellular evidence.

Using the benefits of advanced techniques in molecular and cellular biology, we aimed to elucidate the role of GP in the prompt energy supply for fish gill ionocytes during environmental stress. Using suppressive subtractive hybridization, we found that a clone of GP, which is a novel isoform (tGPGG), was differentially expressed in gills of freshwater (FW)- and seawater (SW)-acclimated tilapia (*Oreochromis mossambicus*, a euryhaline teleost). In addition, preliminary experiments showed that the novel tGPGG was specifically expressed in a group of cells other than MR cells. We proposed that the emergency energy supply for MR cells may be provided by neighboring non-MR cells, similar to the cellular and physiological relationships between mammalian brain astrocytes and neurons. In the present study, isoforms of GP were cloned and sequenced from tilapia; the effects of environmental salinity on the mRNA and protein expressions, and activity of GP, as well as the glycogen content in tilapia gill epithelial cells were examined; localization of GP and glycogen was carried out in these epithelial cells.

MATERIALS AND METHODS

Animals. Tilapia (*O. mossambicus*), 8–12 cm in total length and weighing 35–50 g, were obtained from stocks of the Institute of Cellular and Organismic Biology of Academia Sinica, and were reared in a tank with a FW circulating system at 25–28°C with a 12:12-h light-dark photoperiod. SW with different salinities was prepared by adding artificial sea salt (Taikong, Taipei, Taiwan) to the FW. For long-term acclimation experiments, FW tilapia were initially transferred to 15 ppt SW and then to 35 ppt SW for at least 2 wk to become SW tilapia. Our preliminary experiments indicated that acute exposure to 25 ppt SW for 1–6 h caused peaks in the changes of GP expression and activity. Therefore, in the short-term experiments, FW tilapia were acutely transferred to 25 ppt SW for 3 h. During the acclimation experiments, neither the FW control nor the SW experimental fish were fed. The experimental protocols were approved by

the Academia Sinica Institutional Animal Care and Utilization Committee (approval no. RFI-ZOOHP2006083).

Suppressive subtractive hybridization between SW and FW tilapia gills. The cDNAs obtained from FW- and SW-acclimated tilapia gill were subjected to suppressive subtractive hybridization with the PCR-Select cDNA Subtraction Kit (Clontech BD, Palo Alto, CA), as described by the manufacturer (17), and then randomly selected for PCR amplification. The SW gill-specific library consists of 350 clones, among which, two clones of GP were identified.

Isolation of epithelial cells from tilapia gills. Gill tissues have plenty of blood cells and muscle cells that contain a large amount of glycogen and express a high level of GP (Chang JC, Wu SM, Tseng YC, Lee YC, Baba O, and Hwang PP, unpublished observations); therefore, gill epithelial cells have to be isolated by a previous method (30) to exclude contamination by blood and muscle cells. Tilapia were anesthetized on ice, killed by spinal transection, and the gills were excised and immediately transferred to PBS (0.09% NaCl in 0.1 M phosphate buffer) to remove most of the blood cells. The epithelial tissues were scraped from the gill filaments in dissociation buffer (0.5 M EDTA and 500 μ l Percoll in PBS), agitated on ice, and then gently passed through a nylon mesh (with a mesh size of 100 μ m) to remove the larger tissue fragments. The cell suspension remnant was poured into Percoll (Sigma, St. Louis, MO) in PBS at a volumetric ratio of 3 (cell suspension): 2 (Percoll): 2 (PBS), and centrifuged for 10 min at 2,000 g and 4°C. The cell suspension was isolated by aspiration with a dropper, washed with PBS, and centrifuged for 10 min at 1,000 g and 4°C. Isolated cells were homogenized (see *Western blot analysis*), and 50 μ g of protein was checked for muscle cell contamination by Western blot analysis with an anti-chicken tropomyosin (a muscle-specific protein) monoclonal antibody (Sigma). The isolated cell suspension was stored at –80°C for the subsequent experiments.

Purification of mRNA. The total RNA of the isolated gill epithelial cells or other tissues (including the brain, liver, muscle, intestine, heart, spleen, and kidneys of FW tilapia) were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Promega, Madison, WI) to remove genomic DNA contamination. The mRNA for the RT-PCR was obtained with a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia, Piscataway, NJ). The mRNA quality was determined at 260 and 280 nm with a spectrophotometer (Hitachi U-2000, Tokyo, Japan) and stored at –20°C.

RT-PCR. For cDNA synthesis, mRNA was reverse-transcribed with the Superscript reverse transcriptase (Invitrogen), followed by heating to 70°C for 15 min to inactivate the reactions. *Escherichia coli* RNase H (Invitrogen) was added to remove the remnant RNA. For PCR amplification, 1 μ g cDNA was used as a template with 2.5 units ExTaq polymerase (Takara, Shiga, Japan), and 2.5 μ M of each primer. The degenerate primer sets for GP cloning were forward 5'-YGARCTRGARCCSWABAAGTT-3' and reverse 5'-CCKNG-GCACVAWDRHYTYTKYNGGV-3'. The primer sets for gene ex-

Table 1. RT-PCR primers designed for gene expressions in various tissues

Gene Name	Amplicon Size, bp	Sequence
GP (conserved)	758	Forward 5'-AGTGAAAATCAACCCAGCCT-3' Reverse 5'-CAGGGATGTTCTTGATCACC-3'
GPGG	171	Forward 5'-ACCAACCTCAAAATCCCACCA-3' Reverse 5'-GAACTCCAAGTCTCAACAG-3'
tGPBB	129	Forward 5'-AAGGCTTAAACCAACAGGAACG-3' Reverse 5'-CCAACCTCAAAATCCGCCAC-3'
tGPMM	321	Forward 5'-GATGCTTTTCAAACATGACCG-3' Reverse 5'-AACCAACAGGAACGGTAAAT-3'
tGPLL	108	Forward 5'-GGATGCTTTTCAAACATGA-3' Reverse 5'-TCGTCCACTCCACTGGATT-3'
β -Actin	1,960	Forward 5'-CACACTCACAGCTTGTGCGGGATATCATTT-3' Reverse 5'-CGGAACACATGTGCACCTTATTGGGATCGT-3'

pressions in various tissues are shown in Table 1. These amplicons were sequenced to confirm the PCR products. The amplicons were sequenced with a PE Applied Biosystems 377 DNA sequencer (DNA Sequencing Facility of Institute of Cellular and Organismic Biology, Academia Sinica) to confirm the PCR products.

Rapid amplification of cDNA ends, DNA cloning, sequencing, and bioinformatics analysis. To obtain the full-length cDNAs of GPs, first-strand cDNA synthesis and 5'- and 3'-RACE were conducted using the RNA Transcript (SMART) cDNA Amplification Kit (Clontech, Palo Alto, CA). The 5'-RACE was performed with the universal primer mix (UPM) A specific for the adaptor and a specific antisense primer for the GP gene (SRGP1, 5'-GGAGTGATGCCATTGGTTT-TGTTC-3'), whereas the second round of PCR was carried out with the nested universal primer (NUP) A, and a nested specific antisense primer (SRGP2: 5'-CAGGCGTCCAAGGCCTCCATT-3'). For the 3'-RACE, cDNA was first amplified with UPM A and a specific sense primer for GP (5'-AACCTGGACTG GCGGAGCTTATAG-3') and then reamplified with NUP A and a nested specific-sense primer (3RGP2: 5'-CCAAAAATCCAGAGCTGTTCAA-3'). The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced. DNA sequence analysis was performed as previously described. The entire amino acid sequence deduced with the CLUSTAL program (12, 13) was used for the multiple sequence alignments and phylogenetic analysis. The data set was treated by distance analysis using the Neighbor-joining (NJ) analytical method, and a 1,000-bootstrap replicate analysis was carried out using the MEGA program ver. 3.1.

Quantitative real-time PCR. Quantitative real-time PCR (qPCR) was carried out by use of a SYBR Green dye (Qiagen, Hilden, Germany)-based assay with an ABI Prism 7000 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Wellesley, MA) according to the manufacturer's instructions. Primers targeting the GP and the endogenous control gene, β -actin, were designed using the Primer Express 2.0 software (Applied Biosystems). In each assay, 25 ng cDNA was amplified in a 20- μ l reaction containing 2 \times SYBR Green Master mix, 300 nM of the forward and reverse primers, and nuclease-free water. Table 2 shows the primers designed for the specific GP isoforms. β -actin was used as an internal control to construct the standard curves.

Western blot analysis. Isolated gill epithelial cells were disrupted in homogenization buffer (100 mM imidazole, 5 mM EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate; pH 7.6), and then centrifuged at 4°C and 10,000 rpm for 10 min. The supernatant (a volume equivalent to 50 μ g protein) was supplemented with electrophoresis sample buffer (250 mM Tris base, 2 mM Na₂ EDTA, 2% SDS, and 5% dithiothreitol), and then incubated at 95°C for 10 min. The denatured samples were subjected to 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking in 5% nonfat milk, the blots were incubated with a mouse anti-human GPBB monoclonal antibody (Biotrend Chemikalien, Cologne, Germany) at a 1:3,000 dilution and then reacted with alkaline phosphatase (AP)-conjugated goat anti-mouse immunoglobulin G (diluted 1:1,000; Pierce, Rockford, IL). The immunoreactive proteins were visualized

with a BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) Substrate Kit for AP (Zymed Laboratories, San Francisco, CA). Immunoblots were scanned and exported to JPEG files, and the differences between the band intensities of FW and SW were compared using a commercial software package (Image-Pro Plus 4.0, Media Cybernetics, Silver Spring, MD).

Gill GP activity assay. Measurement of gill GP activity followed the procedure of Milligan (35) with some modifications. The isolated gill epithelial cells were homogenized with ice-cold homogenization buffer (100 mM imidazole, 100 mM KF, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) for the enzyme activity assay. Total GP (GPa + GPb) activity was measured by incubating the samples at 25°C in the presence of 1.6 mM 5'AMP, 45 mM potassium-phosphate buffer (pH 7.0), 0.2 mg/ml glycogen (dialyzed bovine muscle), 0.34 mM NADP, 4 μ M glucose-1,6-bisphosphate, 0.1 mM EDTA, 15 mM MgCl₂, 1.6 U/ml phosphoglucomutase, and 12 U/ml glucose-6-phosphate dehydrogenase. The changes in absorbance between the reactions with and without glycogen (the substrate) were measured at 340 nm with a spectrophotometer (Hitachi U-2000). Each sample was assayed in triplicate.

Gill Na⁺-K⁺-ATPase activity assay. Na⁺-K⁺-ATPase activity in isolated gill epithelial cells was determined as described by Hwang et al. (22). Na⁺-K⁺-ATPase activity was assayed by adding the supernatant of the homogenates to the reaction mixture containing 100 mM imidazole-HCl buffer (pH 7.6), 125 mM NaCl, 75 mM KCl, 7.5 mM MgCl₂, and 5 mM Na₂ATP. The reaction was run at 37°C for 30 min and then stopped by adding 200 μ l of ice-cold 30% trichloroacetic acid. Na⁺-K⁺-ATPase-specific activity (mmole Pi/mg protein/h) was defined as the difference between the inorganic phosphate liberated in the presence and absence of 0.5 mM ouabain in the reaction mixture. Each sample was assayed in triplicate.

In situ hybridization. RNA probe synthesis followed previously published procedures (25) with some modifications. Fragments of tGPGG obtained by PCR were inserted into a pGEM-T easy vector (Promega). DIG-labeled RNA probes were synthesized by *in vitro* transcription with T7 and SP6 RNA polymerase (Roche, Penzberg, Germany), the quality was examined using RNA gels, and the concentrations were determined by a dot-blot assay with standard DIG-labeled RNA (100 ng/ μ l) (Roche).

Tilapia gills were fixed in 4% paraformaldehyde at 4°C for 3 h, immersed in PBS containing 30% sucrose overnight, and then embedded in optimal cutting temperature compound embedding medium (Sakura, Tokyo, Japan) at -20°C. Cryosections at 10 μ m were made with a cryostat (CM 1900, Leica, Heidelberg, Germany) and were attached to poly-L-lysine-coated slides (Erie, Hooksett, NH). After washing with PBS-Tween, slides were prehybridized with hybridization buffer (HyB) containing 60% formamide, 5 \times SSC, and 0.1% Tween-20 in Diethylpyrocarbonate dH₂O for 5 min at 65°C. Then, hybridization was conducted with a DIG-labeled RNA probe for tilapia tGPGG in HyB⁺ (HyB plus 500 ng/ml yeast tRNA and 50 μ g/ml heparin) at 65°C overnight. Hybridized sections were washed with a series of HyB/SSC and SSC/PBST buffers at room temperature. After washing, the slides were blocked in blocking solution (5% sheep serum and 2 mg/ml BSA in PBST) and then treated with a

Table 2. Specific primer sets for quantitative real-time PCR

Gene Name	Amplicon Size, bp	Sequence
GP (conserved)	178	Forward 5' - ACCTCGATAAAATTCGAGCTCTCT - 3' Reverse 5' - GCTTTGTGAACCCAGAAATAC - 3'
tGPGG	105	Forward 5' - CGAGCCCAGGAAGCCATCGAA - 3' Reverse 5' - TGAAGGCTTAAACCAAACAGGAA - 3'
tGPBB	243	Forward 5' - AACATCGCTGCAACGGGAAAG - 3' Reverse 5' - CAAGTCCTCAAACAGTTCACTGA - 3'
β -Actin	135	Forward 5' - CGGAATCCACGAAACACCTA - 3' Reverse 5' - ATCTCCTTCTGCATCCTGTCA - 3'

1:2,000-diluted anti-DIG-AP antibody (Roche) for another 2 h at room temperature. Finally, staining reactions were conducted with NBT and BCIP.

The GP-expressing cell density (cells/unit surface area) in tilapia gill was counted. Images (3 to 4 images for each individual) were acquired with an Olympus (Tokyo, Japan) BX60 microscope equipped with Normarski lenses at about 0.306 mm² ($\times 400$ magnification) in the afferent side of the gill filaments, and cell numbers were calculated using a commercial software package (Image-Pro Plus 4.0).

Fluorescence immunocytochemistry. For simultaneous localizations of tGPGG mRNA and the Na⁺-K⁺-ATPase protein, sections were hybridized with the tGPGG RNA probe as described above and then were subjected to fluorescence immunocytochemistry. After blocking with 3% BSA, sections were incubated with the 1:300-diluted monoclonal glycogen antibody followed by incubation with the goat anti-mouse IgG conjugated with FITC (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). For Na⁺-K⁺-ATPase (a marker for MR cells) (21) identification, tGPGG-hybridized slides were incubated with the Na⁺-K⁺-ATPase $\alpha 5$ antibody conjugated with Texas red (Hybridoma Bank, University of Iowa, Ames, IA) at 4°C overnight. Some sections were double-labeled with the tGPGG RNA probe or Texas-Red conjugated concanavalin-A (a marker for the apical opening of MR cells) (0.5 mg/ml; Molecular Probes, Eugene, OR) and an anti-glycogen monoclonal antibody (1:300 dilution) (2), followed by a secondary antibody conjugated with FITC. After washing, image acquisition was conducted with an Olympus BX60 microscope equipped with Normarski lenses and appropriate filter sets for the simultaneous monitoring of AP, FITC, and Texas red.

Statistical analyses. Values are presented as the mean \pm SD. The level of significance was set to $P < 0.05$ in a two-tailed test. Student's *t*-test was used to compare tGP mRNA expression levels, relative protein abundances, total enzyme activities, and glycogen contents of FW gill epithelial cells with those of tilapia in SW.

RESULTS

Isolation of gill epithelial cells. As shown by the Western blot for tropomyosin (Fig. 1), tilapia gill cells not subjected to the isolation process showed a slight signal in muscle tissues; however, the isolated epithelial cells were totally free from muscle contamination. Accordingly, isolated gill epithelia cells were used in all subsequent experiments, to ensure that all GP data were free from muscle cell contamination.

Molecular cloning, sequencing, and phylogenetic analysis of tilapia GP. Using RT-PCR and rapid amplification of cDNA ends, the full-length cDNA of GP was cloned and sequenced

from tilapia gills. The gill GP cDNA consists of 2,565 bp (GenBank accession no. DQ010415) with an open reading frame encoding an 855-amino acid protein that contains several highly conserved catalytic regions (Fig. 2). The length of the deduced tilapia gill GP (tGPGG) protein was similar to that of other GP isoforms from mammals (842–863 amino acids) and birds (857 amino acids). For comparison, the partial cDNA sequences of the other GP isoforms were also cloned and sequenced from tilapia brain, liver, and muscle. The tGPGG showed the highest amino acid sequence identity of 81% with the chicken GP liver form (NP_989723). In addition, the tGPGG also showed 79%–81% identities to the GPLL forms of human (AAC17450), rat (NP_071604), mouse (NP_573461), and tilapia (DQ010416); 76–77% identities to the GPBB forms of human (NP_002853), rat (XP_342543), chicken (CAG31099), zebrafish (NP_997974), *Xenopus laevis* (AAH47245), and tilapia (DQ081728); and 75–77% identities to the GPMM forms of human (A27335), mouse, (NP_035354), rat (S34624), tetraodon (CAG00533), and tilapia (DQ010417). As shown in Fig. 3, a phylogenetic tree was generated using the NJ analysis to clarify the precise denominations and relationships among these GP isoforms from different species. The analysis was applied to the 3'-end amino acid sequences of GP isoforms of different species with *Drosophila* GP (NP_722762) used as the outgroup. According to the analysis, the tGPGG clustered with the GPLL but was indeed apart from the other members.

GP expression in various tilapia tissues. Expressions of GP mRNA were evaluated by 30 cycles of RT-PCR using total RNA extracted from different tilapia tissues. As shown in Fig. 4A, primers designed from the GP conserved region were used to examine the PCR product of a 796-bp fragment (nucleic acids 1358–2154) of the GP isoforms. The mRNA expression of all GP isoforms was ubiquitous among the tissues examined, including the brain, gills, heart, muscles, kidneys, intestines, and liver, but not the spleen. The highest expression level was in the liver, while the intestines showed the lowest expression. Furthermore, isoform-specific primers were designed from the 3'-end untranslated region to examine the differential expressions of the tGPGG, tGPBB, tGPLL, and tGPMM in various tissues of tilapia. The four GP isoforms showed distinct expression patterns in the tissues examined (Fig. 4B). The tGPBB was mainly expressed in the brain and heart, with a little in the intestine (Fig. 4B), while tGPLL was in the liver and kidneys and also some in other tissues, including the brain, muscles, intestine, and heart (Fig. 4B). The tGPMM was mainly expressed in the muscles and kidneys, and a little in the brain (Fig. 4B). However, the tGPGG was predominantly expressed in gills, and only a very minute expression was found in other tissues. Moreover, tilapia gills mainly expressed the tGPGG and only little tGPBB (Fig. 4B).

Localization of tGPGG mRNA and glycogen in tilapia gills. To identify the cell types that specifically express tGPGG mRNA, an in vitro synthesized RNA probe was used to detect the tGPGG mRNA in cryosections of tilapia gills (Fig. 5A), and subsequent double immunocytochemical labeling with an anti-Na⁺-K⁺-ATPase α subunit (a marker for MR cells) antibody was carried out (Fig. 5B). As shown in Fig. 5C (merged images of Fig. 5, A and B), tGPGG mRNA was clearly found in a few groups of gill epithelial cells but was never colocalized with the Na⁺-K⁺-ATPase protein, a marker for MR cells, which

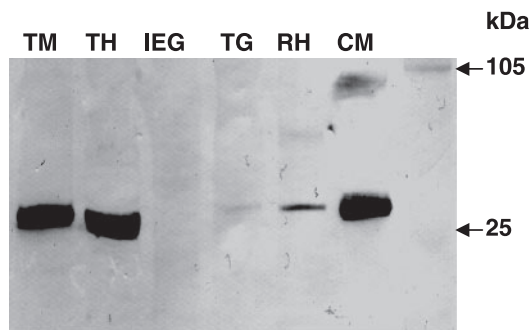


Fig. 1. Immunoblot of the muscle-specific protein, tropomyosin, in different cells preparations/populations. Isolated epithelial cells (IEG) from tilapia gills were free from contamination by muscle cells. CM, chicken muscle; RH, rat heart; TG, tilapia gill cells not subjected to the isolation process; TH, tilapia heart; TM, tilapia muscle.

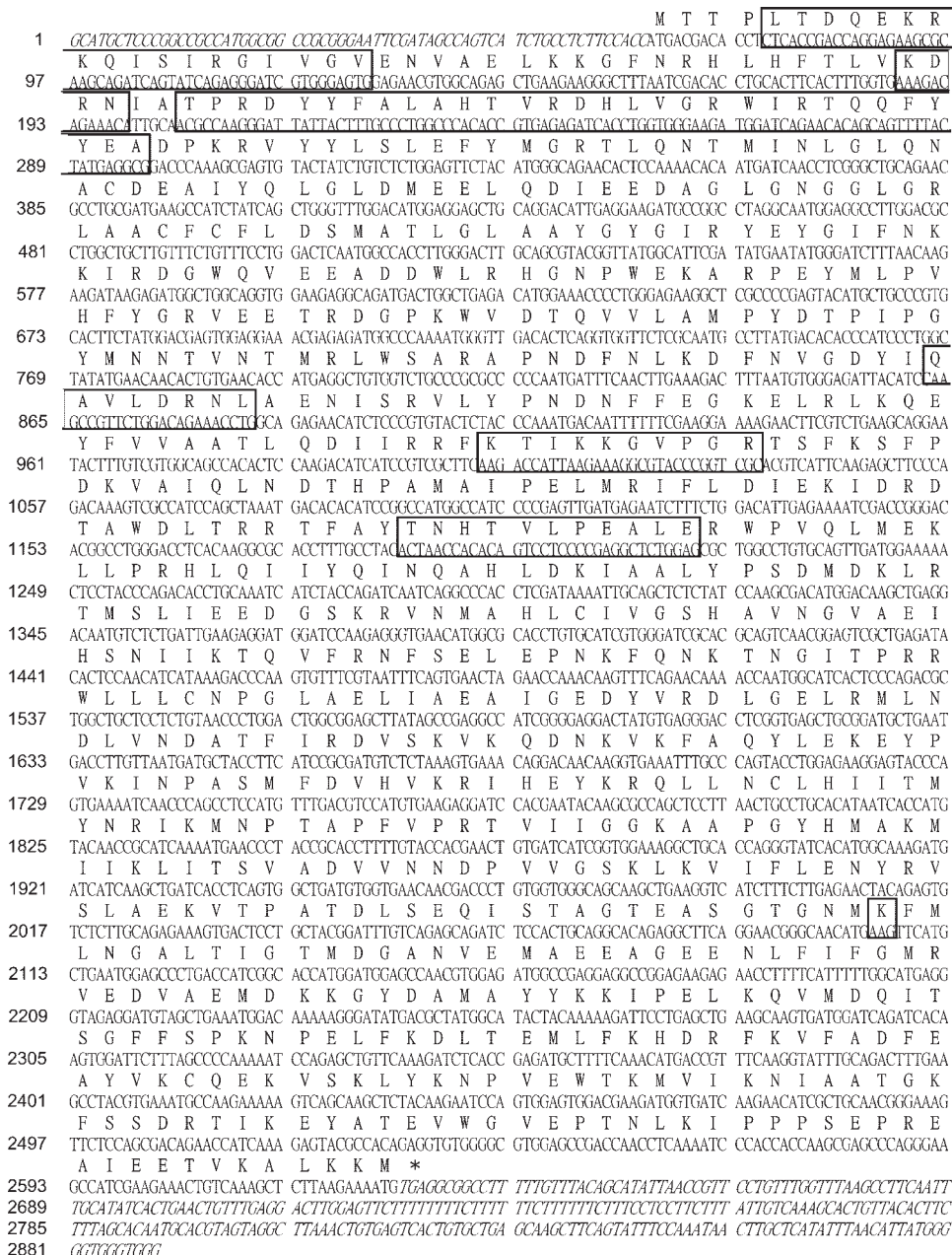


Fig. 2. Nucleotide and deduced amino acid sequences of glycogen phosphorylase (GP) cDNA from tilapia gill epithelial cells. *Translational stop codon. Regions enclosed in boxes are GP regulation domains postulated from mammalian counterparts. Accession no., DQ010415.

indicates that tGPGG mRNA was predominantly expressed in a group of gill cells that were adjacent to MR cells.

An immunocytochemical approach was also applied to localize the deposits of glycogen in tilapia gill cells. The specificity of the antiglycogen antibody that was used in the present study was confirmed by incubating the antibody with glycogen before the immunolabeling. As shown in Fig. 5, D–F, the immunoreactions of glycogen in tilapia gill cells were abolished in a concentration-dependent pattern by preabsorption with 0.25 (Fig. 5E) and 25 mg/ml (Fig. 5F) glycogen. In further double-labeling with the tGPGG RNA probe and the antiglycogen antibody (Fig. 5, G–I), both tGPGG mRNA and glycogen were found to be colocalized in the same group of gill cells, named glycogen-rich cells (GRCs) (Fig. 5A–C). Subse-

quent double-labeling with the glycogen antibody and Con-A (a marker for the apical opening of MR cells) indicated that all GRCs showed Con-A-positive apical openings (Fig. 6B). On the basis of confocal microscopic observations of serial sections, GRC and MR cells appeared to share the same apical openings, forming multicellular complexes, as previously reported (Fig. 6, A and B) (19).

Effects of environmental salinity on GP expression and function, and glycogen contents in tilapia gills. GP gene expression levels between FW- and SW-acclimated tilapia gills were compared using qPCR. Using the primers for the consensus of all GP isoforms, the mRNA expression levels of all GP isoforms in gill epithelial cells from SW tilapia were significantly higher by about twofold than those of the FW control

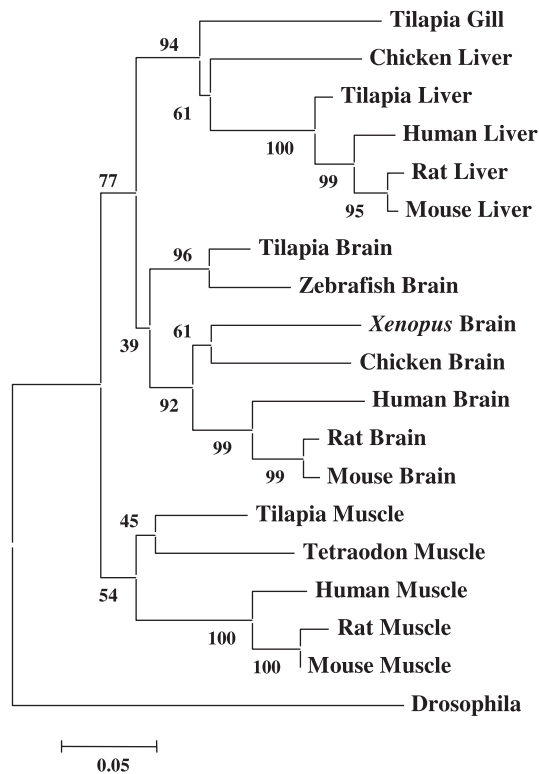


Fig. 3. Phylogenetic tree constructed with the complete amino acid sequences of GPs. Analysis was conducted with the neighbor-joining method using a 1,000-bootstrap analysis. Numbers indicate the bootstrap values. The GenBank accession numbers of the sequences used are as follows: the GP gill form of tilapia, DQ010415; the GP liver forms of chicken, NP_989723; tilapia, DQ010416; human, AAC17450; rat, NP_071604; and mouse, NP_573461; the GP brain forms of tilapia, DQ081728; zebrafish, NP_997974; *Xenopus laevis*, AAH47245; chicken, CAG31099; human, NP_002853; rat, XP_342543; and mouse, NP_722476; and the GP muscle forms of tilapia, DQ010417; tetraodon, CAG00533; human, A27335; rat, S34624; mouse, NP_035354; and *Drosophila melanogaster*, NP_722762.

group (Fig. 7). On the basis of the subsequent analysis with isoform-specific primers, this difference was ascribed mainly to the tGPGG, whose expression was specifically stimulated by environmental salinity (Fig. 7). No product was found in the qPCR for tGPMM and tGPLL (data not shown). In terms of normalized mRNA levels as shown in Fig. 7, GP in gill GRCs was composed of about 80% tGPGG and 20% tGPBB, and only the tGPGG was significantly stimulated by SW challenge. The tGPGG mRNA level in SW was 2.1-fold higher than in cells of FW gills.

The differential expressions of GP mRNA reflect the profiles of protein expression and enzyme activity in gill GRCs. Western blot analysis using an anti-human GPBB monoclonal antibody detected a protein band corresponding to ~97 kDa from tilapia gill epithelial cells, liver, muscles, and brain (a positive control) (Fig. 8B). Preincubation of the commercial GPBB antibody with the recombinant protein expressed from a GP fragment (amino acids 434–569; with a predicted protein size of ~15 kDa) decreased the intensity of the immunoreaction of the blots (Fig. 8A), thus indicating antibody specificity to tilapia GP. Supporting the data of GP mRNA expression, the GP protein level in gill GRCs in both short- and long-term SW-acclimated tilapia was higher than that in the FW group (Table 3). Similarly, the specific glycogenolytic enzyme total

GP activity (GP_a + GP_b) in gill GRCs of the SW groups was also significantly higher (Table 3). The enhancement of protein levels and enzyme activities by SW challenge may be a consequence of tGPGG stimulation, since this isoform is the major component of the GPs in tilapia gill GRCs, and its mRNA specifically responded to the salinity change as described above (Fig. 7, Table 3). Supporting these GP qPCR, Western blot analysis, and activity data, the densities of tGPGG-expressing cells (i.e., GRCs) were much higher (about 3 times) in SW tilapia gills than in FW ones (Table 3, Fig. 9).

The glycogen content of tilapia gill GRCs after acute exposure to 25 ppt SW for 3 h decreased by about 40% compared with that of the FW control group. On the other hand, the glycogen content of gill GRCs from tilapia acclimated to 35 ppt SW for the long term was about 3.5-fold higher than that from the FW control group (Table 3), indicating that 2 wk of SW acclimation resulted in an evident increase of the glycogen deposits in gill GRCs, even as GP activity was simultaneously being stimulated (Table 3).

DISCUSSION

The major findings of the present study are as follows. A novel tilapia gill GP isoform, tGPGG, which differs from the other three isoforms (GPBB, GPLL, and GPMM) was identified, and the tGPGG was mainly found to be expressed in gill

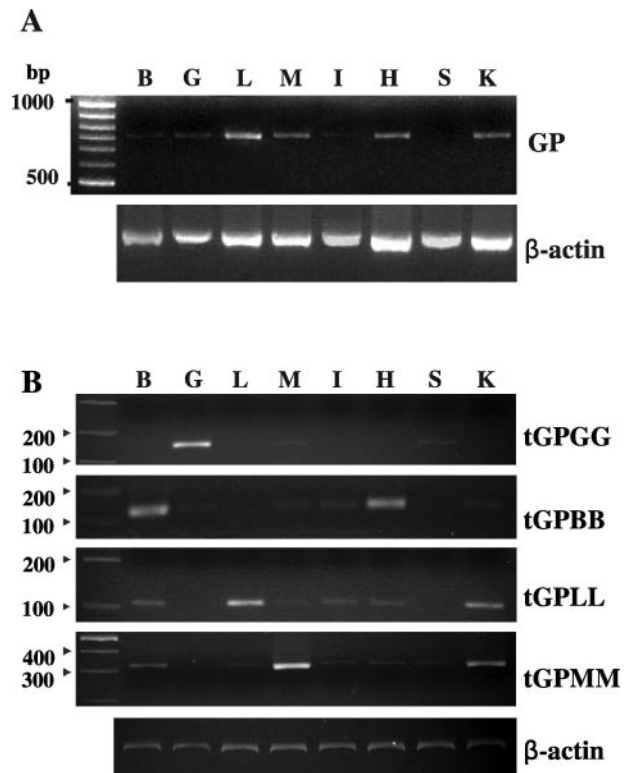


Fig. 4. RT-PCR analysis of GPs in various tissues of tilapia. Primers were designed for the consensus of all GP isoforms (A) and specifically for the gill (tGPGG), brain (tGPBB), liver (tGPLL), and muscle (tGPMM) forms, respectively (B). β-actin was used as an internal control. Gill epithelial cells mainly expressed tGPGG and a very small amount of tGPBB. B, brain; G, gill epithelial cells; L, liver; M, muscle; I, intestine; H, heart; S, spleen; K, kidney. Accession nos.: tGPGG, DQ010415; tGPBB, DQ081728; tGPLL, DQ010416; and tGPMM, DQ010417.

epithelial cells. A change in the environmental salinity effectively stimulated tilapia gill GPGG mRNA expression, which was accompanied by upregulation of the protein level and total enzyme activity. In situ hybridization and immunocytochemical results indicated that the tGPGG and glycogen deposits were colocalized in a specific group of cells, GRCs, which surround MR cells in tilapia gills. Con-A labeling indicated that GRCs and MR cells share the same apical opening and form multicellular complexes. Acute exposure to 25 ppt SW for 3 h caused an evident depletion of the glycogen contents of gill GRCs, while a 2-wk acclimation to 35 ppt SW increased glycogen deposits.

The full-length cDNA of 1 GPGG and the partial cDNAs of three other identified GPs were cloned and sequenced from tilapia. Alignment results indicated that these GP isoforms have higher than 88% identities with the reported vertebrate GPs at the amino acid sequence level. The deduced amino acid sequences of these GP isoforms possess all the major structural

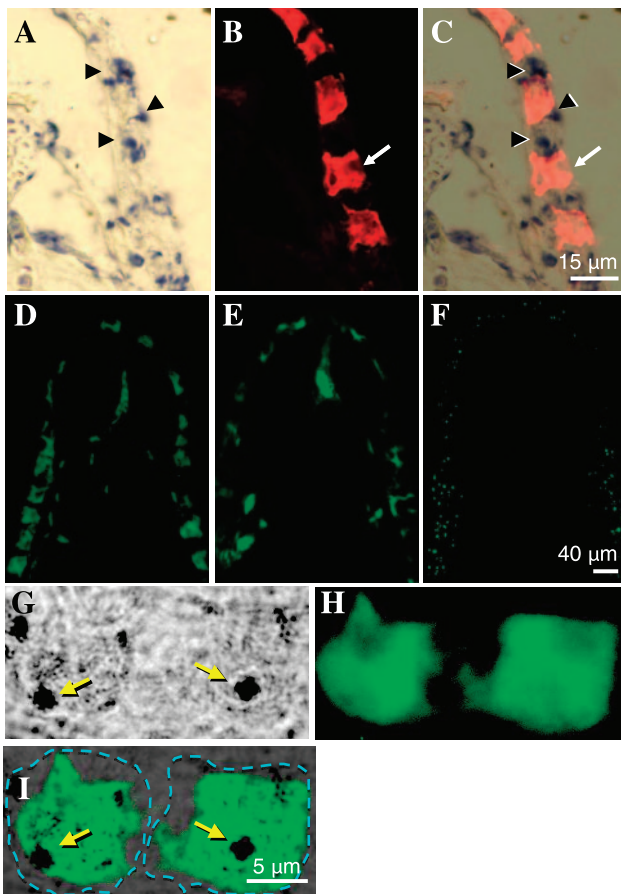


Fig. 5. In situ hybridization and immunocytochemistry of gill epithelial cryosections. *A*, *B*, and *C*: Double labeling for tilapia gill glycogen phosphorylase isoform (tGPGG) RNA mRNA (*A*) and $\text{Na}^+\text{-K}^+\text{-ATPase}$ α subunit [a mitochondrial-rich (MR) cell marker] (*B*) in the same section. Merged image (*C*) indicating that the tGPGG (arrowheads) was not colocalized with $\text{Na}^+\text{-K}^+\text{-ATPase}$ (white arrows). (*D*–*F*) Immunocytochemistry with a glycogen-specific antibody preabsorbed with glycogen at 0 (*D*), 0.25 (*E*), and 25 mg/ml (*F*). Effects of the preabsorption confirmed the specific reaction of glycogen in gill epithelial cells (*G*, *H*, and *I*). Double-labeling for tGPGG mRNA (*G*) and glycogen (*H*) in the same section. Merged image (*I*) indicating that the tGPGG (yellow arrows) was colocalized with glycogen in the glycogen-rich cells (GRCs) (outlined with a dashed line).

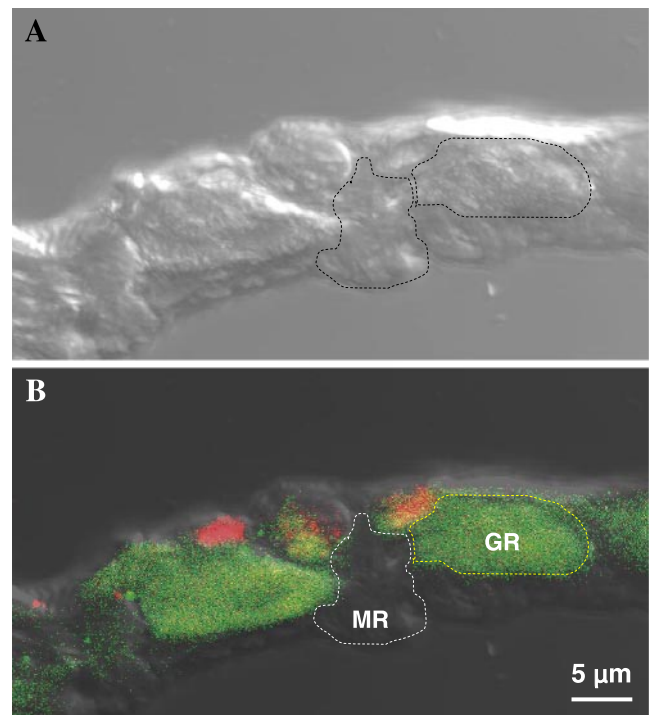


Fig. 6. Double-labeling with the glycogen antibody and concanavalin-A (Con-A). *A*: differential interference contrast (DIC) image of a gill epithelial cryosection. *B*: merged images of Con-A (red) and glycogen (green) signals. The image was reconstructed with three serial sections (at a total thickness of $0.45\ \mu\text{m}$) by confocal microscopy. A mitochondrial-rich (MR) cell and a glycogen-rich (GR) cell are outlined with a dashed line, and they shared the same apical opening (red signal of Con-A).

features and sequence motif characteristics of the glycogen metabolic functional domains: the phosphorylation residue, Ser-14; 2 AMP binding sites, amino acids 42–45 and 48–78; the tower helix, amino acids 267–274; the adenine loop, 315–324; the pyridoxal phosphate attachment site, Lys-680; and the glucose analog binding site, 376–386 amino acids (45).

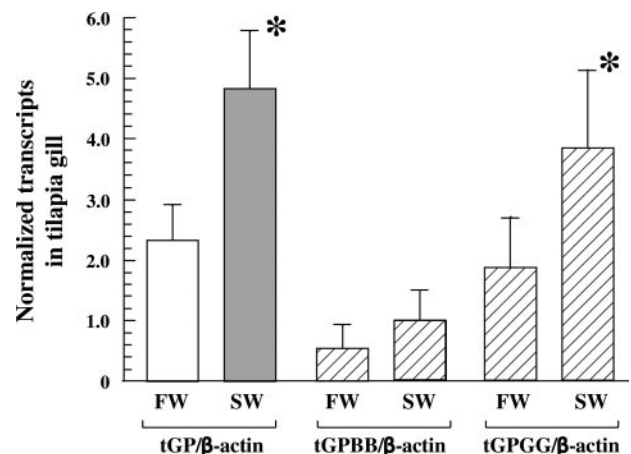


Fig. 7. Quantified real-time PCR (qPCR) comparisons of tilapia glycogen phosphorylase (tGP) mRNA expressions in gill epithelial cells between freshwater- (FW) and seawater (SW)-acclimated tilapia. SW stimulated the expression of tGP, and it mainly resulted from the specific response of the tilapia gill GP isoform (tGPGG) to SW with β -actin as an internal control. Values are presented as the means \pm SD ($n = 6$). *Significant difference between the FW and SW groups ($P < 0.05$, Student's *t*-test).

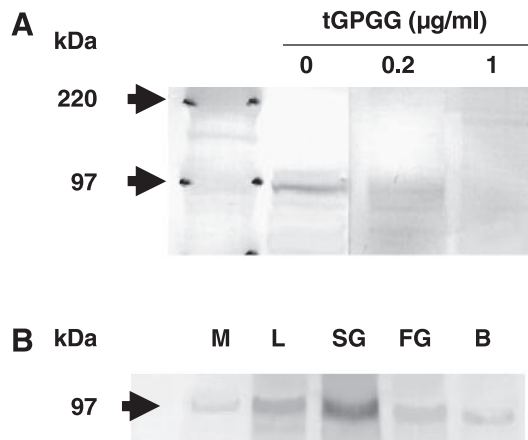


Fig. 8. Western blot analysis for glycogen phosphorylase in gill cells with preincubation of the tilapia gill GP isoform (tGPGG) recombinant protein (A) and in tilapia tissues (B). Preincubation with the tGPGG recombinant protein (amino acids 434–569; with a predicted protein size of ~15 kDa) negated the immunoreactions of the antibody in a concentration-dependent pattern (A). The GP protein level was higher in SW-acclimated gill epithelial cells than in FW-acclimated ones (B). FG, FW gill cells; SG, SW gill cells.

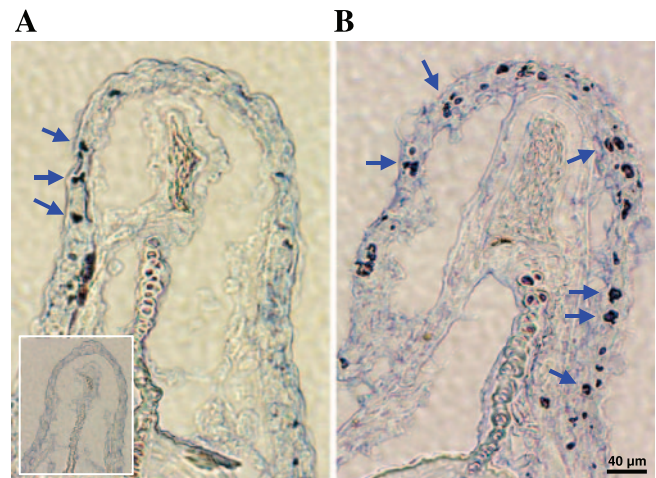


Fig. 9. In situ hybridization of the tGPGG mRNA expressed in gill epithelial cells from FW (A) and SW-acclimated (B) tilapia. Much greater and stronger tGPGG mRNA signals (arrows) were found in SW cells (B) than in FW ones (A). Insertion, hybridization with a sense mRNA probe as the negative control. Bar = 40 µm.

The phylogenetic analysis of the deduced c-terminal amino acid sequences of GPs showed that tilapia have LL, MM, and BB forms of GP, which are grouped with their vertebrate counterparts. Notably, an additional GP isoform, GG, which is ascribed to the group of the liver form, is probably a new GP isoform, based on the phylogenetic analysis and the expression profile (discussed below). In the GPLL group, the tGPGG, however, showed the highest divergence from the other members.

Supporting the results of the phylogenetic analysis, mRNA expression profiles also provided evidence for the presence of a novel GP isoform, the tGPGG, in tilapia gills. Tilapia GPGG was mainly and predominantly expressed in gills. In addition, only the tGPGG specifically showed a response to the environmental salinity challenge. A much lower amount of the tGPBB was also expressed in tilapia gills, but its expression did not show a significant correlation with environmental salinity, suggesting that the tGPGG plays a major role, while the tGPBB plays a minor part in glycogenolysis of fish gills. Taking all of these molecular and physiological data into account, we concluded that the tGPGG is a novel isoform in the GP family. We searched the genome database of pufferfish (*Tetraodon nigroviridis*) and found a gill GP isoform, which is

a homolog of the tGPGG (data not shown). Hence, it is evolutionally interesting and important to determine whether the gill GP isoform exists only in aquatic animals that have gills.

Glycogen granules have for a long time been reported by ultrastructural observations to be localized in the cytosol of MR cells, the major ionocytes in fish gills (15, 37, 41); however, this has never been certified by a convincing molecular or biochemical approach until the present study. A specific glycogen antibody was used to localize plenty of glycogen deposits in the as yet unidentified group of gill cells that specifically expresses tGPGG mRNA. These GRCs showed the absence of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (a marker for the gill MR cells) and were located surrounding gill MR cells. A more sensitive approach, like immunoelectromicroscopy, should be used to precisely examine the presence of glycogen deposits in gill MR cells. Another notable finding of the present study is to indicate that GRCs and MR cells form multicellular complexes, raising a possibility that GRCs may be a population of the previous reported accessory cells (9, 19). Previously, accessory cells were suggested to be another type of gill cells or immature MR cells, and their physiological roles are still being debated (9, 16, 18, 28, 42, 43). The present study may provide new insights

Table 3. Effects of short- and long-term acclimation to SW on GP expression and activity, the number of GP-expressing cells, and the glycogen contents of tilapia gill epithelial cells compared with FW controls

	Short-Term Acclimation		Long-Term Acclimation	
	FW Control	25-ppt SW for 3 h	FW Control	35-ppt SW for 2 wk
Relative expression of GP mRNA (arbitrary value)	0.99 ± 0.21 (n = 5)	1.82 ± 0.36* (n = 5)	0.801 ± 0.06 (n = 5)	1.79 ± 0.06* (n = 5)
Relative abundance of the GP protein (arbitrary value)	0.91 ± 0.11 (n = 4)	2.09 ± 0.10* (n = 4)	0.97 ± 0.40 (n = 4)	6.92 ± 2.27* (n = 4)
GP total activity (GP _a + GP _b), IU/g protein	6.46 ± 0.77 (n = 4)	15.10 ± 1.46* (n = 4)	6.64 ± 1.17 (n = 8)	11.89 ± 1.34* (n = 8)
Density of GP-expressing cells, cell/mm ²	†	†	127 ± 61 (n = 4)	411 ± 83* (n = 4)
Glycogen content, mg/g wet weight	0.41 ± 0.04 (n = 4)	0.26 ± 0.03* (n = 4)	0.33 ± 0.18 (n = 10)	1.16 ± 0.33* (n = 10)

Values are presented as the means (SD); n = number in parentheses. *Significant difference from the respective FW control (Student's *t*-test, *P* < 0.05). †No determination could be made. SW, softwater; GP, glycogen phosphorylase; FW, freshwater.

to possible functions of accessory cells; however, more sensitive analysis, like immunoelectron microscopy, is necessary to further confirm GRCs to be accessory cells. The present immunocytochemistry and in situ hybridization data raise the possibility that the relationship in energy translocation between mammal astrocytes and neurons may also occur between GRCs (i.e., accessory cells) and MR cells in fish gills. MR cells, which contain obviously higher densities of mitochondria and $\text{Na}^+\text{-K}^+\text{-ATPase}$ (26), reveal a much higher demand for energy fuel. The major emergency energy source for MR cells may reasonably be proposed to come from the surrounding GRCs, because these GRCs express GP, which can degrade glycogen deposits and release energy metabolites. Our subsequent molecular physiological experiments further demonstrated that this spatial combination of GRCs and MR cells is of similar functional significance for energy metabolism and translocation as that of mammalian astrocytes and neurons.

Upon encountering environmental stress, GP is stimulated to increase glucose or glucose-6-phosphate levels for additional energy fuel to meet specific physiological demands. In mammals, hypoglycemic stress is known to increase GP expression in specific tissues to enhance glucose uptake to both facilitate the metabolic energy supply (5) and protect cells from hypoxic injury (40). A similar mechanism also seems to be exhibited by fish gills upon environmental salinity challenge. It has been well documented in fish that more energy is required to support the activation of gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ expression and activity during acclimation to different salinities (20, 23, 27, 29). The present study examines for the first time the cellular and molecular mechanisms of emergent energy metabolism for the ionoregulation and osmoregulation processes in fish gills. Acclimation to SW stimulated mRNA and protein expressions, as well as the activity of GP and the cell density of GRCs in tilapia gills. Supporting these molecular and cellular events, an evident depletion of the glycogen deposits in tilapia gill GRCs was found after acute exposure to 25 ppt SW for 3 h. Notably, the glycogen content in tilapia acclimated to 35 ppt SW for a long term was much higher than that in FW control fish, and even gill GP expression and activity were stimulated under the same conditions. These results possibly imply that deposition of a higher amount of glycogen is necessary for the maintenance of higher $\text{Na}^+\text{-K}^+\text{-ATPase}$ in tilapia gills after a long-term acclimation to SW, as our subsequent experiments indicated that the expression of gill glycogen synthase was about 1.5 times upregulated after long-term acclimation to SW (Chang JC, Wu SM, Tseng YC, Lee YC, Baba O, and Hwang PP, unpublished data). In a previous study in euryhaline sea bream (*Sparus auratus*), acclimation to high salinity also caused parallel enhancements in the total GP activity and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in gills but had no significant effect on the gill glycogen content (48). This controversy may be due to species differences or differences in the experimental designs and the measurement methods. Isolated gill epithelial cells were used in the present study, while in the case of sea bream, the gill glycogen content was directly measured without isolating contaminants (other cell types). According to our preliminary experiments, nonepithelial cells contained over 65% of the glycogen content of the entire gill. Measurement of glycogen content in the entire gills (48) may have obscure changes in the glycogen content in gill GRCs.

Cellular and physiological relationships in energy metabolism between mammal astrocytes and neurons may also exist in other organs, including fish gills, although much remains to be studied to elucidate the entire outline of glycogen metabolism for ionoregulation and osmoregulation in fish gills. In mammalian, glucose transporters and/or monocarboxylate transporters transport glucose, lactate, and/or pyruvate between astrocytes and neurons. Further studies are required to examine which transporters are involved in energy translocation between GRCs and MR cells in fish gills. For instance, both epinephrine and norepinephrine are known to stimulate brain and liver glycogenolysis via activation of the PKA/GPK pathway (11, 36). Modulation of neuroendocrine control of the ionoregulation and osmoregulation in fish gills upon environmental stress has been well studied (49); thus, it will be interesting and challenging to examine how the neuroendocrine system controls GP expression and function in fish gills.

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