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

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1Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei; 2Institute of Zoology, National Taiwan University, Taipei; 3Institute of Biological Chemistry, Academia Sinica, Nankang; 4Department of Hard Tissue Engineering, Tokyo Medical and Dental University, Tokyo, Japan; and 5Department of Life Sciences, National Yang Ming University, Taipei, Taiwan, Republic of China

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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. The molecular and cellular mechanisms behind glycogen metabolism and the energy metabolite translocation between mammalian 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 (MR) cells have been well documented that glycogen serves as an emergency 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 (GPα) and the less-active glycogen phosphorylase b (GPβ). 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, 30). 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
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 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. RFZOOH2006083).

Suppressive subtractive hybridization between SW and FW tilapia gills. The cDNAs obtained from FW- and SW-acclimated tilapia gills 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 μM EGTA) 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 μg 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 remaining RNA. For PCR amplification, 1 μg cDNA was used as a template with 2.5 units ExTaq polymerase (Takara, Shiga, Japan), and 2.5 μM of each primer. The degenerate primer sets for GP cloning were forward 5’-YGARCTRGRCCSWABAAGTT-3’ and reverse 5’-CKNG-GCACVAVDRHYYTVKNGGV-3’. The primer sets for gene ex-

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

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Amplicon Size, bp</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP (conserved)</td>
<td>758</td>
<td>Forward 5’-AGTGAAATCCAAACCCACGGCT-3’</td>
</tr>
<tr>
<td>GPGG</td>
<td>171</td>
<td>Reverse 5’-CAGGCAATGTTCCTGGATGACC-3’</td>
</tr>
<tr>
<td>tGPBB</td>
<td>129</td>
<td>Forward 5’-ACCAACCTCAAAATCCCCACCA-3’</td>
</tr>
<tr>
<td>tGPMM</td>
<td>321</td>
<td>Reverse 5’-GAACTCGAAGTCTGGAGACACG-3’</td>
</tr>
<tr>
<td>tGPLL</td>
<td>108</td>
<td>Forward 5’-GGCATGCTGGTTTTGACCACACCC-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>1,960</td>
<td>Reverse 5’-ACCAACCTCAAAATCCCCACCA-3’</td>
</tr>
</tbody>
</table>
Table 2. Specific primer sets for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Amplicon Size, bp</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP (conserved)</td>
<td>178</td>
<td>Forward 5′-ACCTCGATATTTGACGAGCTCTTCT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 3′-GCTTTGACTCAGGAGTTTATAGT-5′</td>
</tr>
<tr>
<td>tGPGG</td>
<td>105</td>
<td>Forward 5′-CCCAAAAATCCCAAGCCTTTCCAT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 3′-GAAAGGTTTAAACCAAACAGGAA-5′</td>
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<tr>
<td>tGPBB</td>
<td>243</td>
<td>Forward 5′-ACCTCGTGGGATTTGACGAGCCTT-3′</td>
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<tr>
<td></td>
<td></td>
<td>Reverse 3′-TGAAGGCTTAAACCAAACAGGAA-5′</td>
</tr>
<tr>
<td>β-Actin</td>
<td>135</td>
<td>Forward 5′-ACCTCGAATTTGACGAGCTCTTCT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 3′-GCTTTGACTCAGGAGTTTATAGT-5′</td>
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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 (×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 α5 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 ± SD. The level of significance was set to $P < 0.05$ in a two-tailed test. Student’s t-test was used to compare gp 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 GPMM 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](image-url) 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.
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). Subsequent 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. 6A 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 consensuses 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.
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. 8B), 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 (GPa + GPa) 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 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.

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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 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).
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 isomorphism, 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 isofrom 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 Na\(^+\)-K\(^+\)-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 into the functions of these cells.

### 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

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

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, seawater; 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 Na\(^{+}\)K\(^{-}\)-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 glycogenolysis and release energy metabolites. Our recent 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.

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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Regulation of glycogen metabolism in gills and liver of the euryhaline tilapia (*Oreochromis mossambicus*) during acclimation to seawater

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Summary

Glucose, which plays a central role in providing energy for metabolism, is primarily stored as glycogen. The synthesis and degradation of glycogen are mainly initialized by glycogen synthase (GS) and glycogen phosphorylase (GP), respectively. The present study aimed to examine the glycogen metabolism in fish liver and gills during acute exposure to seawater. In tilapia (*Oreochromis mossambicus*) gill, GP, GS and glycogen were immunocytochemically colocalized in a specific group of glycogen-rich (GR) cells, which are adjacent to the gill’s main ionocytes, mitochondrion-rich (MR) cells. Na+/K+-ATPase activity in the gills, protein expression and/or activity of GP and GS and the glycogen content of the gills and liver were examined in tilapia after their acute transfer from freshwater (FW) to 25‰ seawater (SW). Gill Na+/K+-ATPase activity rapidly increased immediately after SW transfer. Glycogen content in both the gills and liver were significantly depleted after SW transfer, but the depletion occurred earlier in gills than in the liver. Gill GP activity and protein expression were upregulated 1–3 h post-transfer and eventually recovered to the normal level as determined in the control group. At the same time, GS protein expression was downregulated. Similar changes in liver GP and GS protein expression were also observed but they occurred later at 6–12 h post-transfer. In conclusion, GR cells are initially stimulated to provide prompt energy for neighboring MR cells that trigger ion-secretion mechanisms. Several hours later, the liver begins to degrade its glycogen stores for the subsequent energy supply.

Key words: glycogen phosphorylase, glycogen synthase, Na+/K+-ATPase, osmoregulation, mitochondrion-rich cells, salinity.

Introduction

Glucose plays a central role in providing energy for metabolism. It is primarily stored in animal tissues as a long-branching high-molecular-mass polysaccharide called glycogen (Roach et al., 1998). Glycogen metabolism is the principal energy source in both vertebrates and invertebrates, especially during environmental fluctuations (Karlsson, 1979; Hoffman and Katz, 1998; Oliveira et al., 2004; Bacca et al., 2005). The synthesis and degradation of glycogen are mainly initiated by glycogen synthase (GS; EC 2.4.1.11) and glycogen phosphorylase (GP; EC 2.4.1.1), respectively. GS, which constitutes an additional mechanism of control over glycogen metabolism, plays a major role in glycogen storage and is also the rate-limiting step in the glycogen synthesis pathway (Fernandez-Novell et al., 1992; Garcia-Rocha et al., 2001). On the other hand, GP is a rate-limiting enzyme that maintains energy support for various tissues. It is composed of two identical subunits that control the breakdown of glycogen to glucose 1-phosphate (Newgard et al., 1989; Frolow and Milligan, 2004; Greenberg et al., 2006).

Because gills are directly exposed to the outer aquatic environment, they have multiple functions including gas exchange, acid–base balance and ionic/osmotic regulation (Evans et al., 2005). In terms of the mechanisms of ionic/osmotic regulation, mitochondrion-rich (MR) cells in gill epithelia are the main sites responsible for active ion transport functions, which are conducted by the operations of various ion transporters and enzymes. These operations are highly energy-consuming processes (Hirose et al., 2003; Evans et al., 2005).

It has been well documented that acclimation to hypertonic seawater (SW) in euryhaline teleosts involves a timely and sufficient activation of an ion excretion system. This includes the morphological modifications of gill MR cells and stimulation of both the expression and activity of ion transporters (Hwang, 1987; Hwang et al., 1989; Marshall et al., 1999; Lee et al., 2003). These processes require an additional energy supply, reflecting changes in oxygen consumption in fish upon encountering fluctuations in environmental salinity (Boeuf and Payan, 2001). However, monitoring oxygen consumption, which most studies have emphasized, is not a direct approach for determining energy metabolism in a gill cell that conducts ion regulation (Morgan and Iwama, 1991; Morgan and Iwama,
Glycogen metabolism during seawater acclimation

Several studies have addressed the major role that carbohydrate plays in energy metabolism for osmoregulation. Energy may be mainly supplied by the oxidation of glucose and lactate obtained from the circulation as a result of carbohydrate metabolism (Perry and Walsh, 1989; Morgan et al., 1997). Indeed, consumption of the glycogen content of the liver has been reported in rainbow trout (Oncorhynchus mykiss) after transfer to SW (Soengas et al., 1991). Hepatic metabolism was found to be related to salinity acclimation in euryhaline fishes (Nakano et al., 1998; Sangiao-Alvarelos et al., 2003).

In earlier studies on teleost gills, glycogen granules/particles were found in MR cells by electron microscopic observations (Philpott and Copeland, 1963); however, this was not further confirmed by molecular evidence. Recently, an antibody against glycogen was used to identify glycogen deposits localized in a group of cells, glycogen-rich (GR) cells, in tilapia gills, and these GR cells express a gill form of GP (Tseng et al., 2007). Moreover, the glycogen content and expression/activity of GP in these cells were affected by environmental salinity (Tseng et al., 2007). These results imply that glycogenesis and glycolysis in fish gills may be involved in energy metabolism during acclimation to salinity changes; however, the detailed mechanism is still unclear. No study has clarified the partitioning of energy supplements between the liver and gills in gill energy requirement during acclimation to salinity changes.

In the present study, we attempted to examine glycogen metabolism in fish liver and gills during acute exposure to SW. Mozambique tilapia (Oreochromis mossambicus), a euryhaline cichlid, was selected for study due to its ability to adapt to acute fluctuations in salinity. The following experiments were performed: (1) GS was cloned and sequenced from tilapia, and the expression of GS in various tissues, including the gills, was examined; (2) immunocytochemistry was used to localize GP, GS and glycogen in tilapia gill cells; (3) time-course changes in glycogen content in tilapia liver and gills after exposure to SW were examined; (4) time-course changes in GP protein and/or activity levels in tilapia liver and gills after exposure to SW were examined and (5) time-course changes in GS protein level in tilapia liver and gills after exposure to SW were examined.

Materials and methods

Experimental animals

Tilapia (Oreochromis mossambicus Peters), 40–60 g in body mass and 10–14 cm in total length, were obtained from stocks of the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. The fish were kept in local freshwater (FW) circulating in a tank at 27–28°C under a 14 h:10 h light:dark photoperiod and were fed daily with artificial feed pellets (Fu-So, Taipei, Taiwan). The experimental protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no. RFIZOOP2006083).

Acclimation experiments

Seawater (SW) was prepared by adding appropriate amounts of artificial sea salt (Taikong, Taipei, Taiwan) into the FW. In exposure experiments, FW tilapia were directly transferred to 25‰ SW and sampled at 1, 3, 6, 12, 24, 48 and 168 h after the transfer. The fish in the acute exposure experiment were not fed. In total, eight fish were sacrificed at each sampling point: four fish for the experimental treatment (FW to SW) and another four fish for the control (FW transferred to FW). All samples were processed at the same time: 09.30–11.30 h in order to normalize the effects of circadian rhythms on physiological metabolism. Sampled gills and liver were immediately placed in ice-cold 1% phosphate-buffered saline (PBS) and were subsequently subjected to the treatments described below.

Isolation of epithelial cells from tilapia gills

Preliminary experiments indicated that blood cells and muscle cells contain considerable amounts of glycogen, GP and GS. Isolation of epithelial cells from gill tissue is necessary in order to exclude the effects of these non-epithelial cells. The isolation method followed a previous paper (Tseng et al., 2007). Gills were carefully rinsed with PBS and then tissue was scraped from the gill filaments and immediately kept in dissociation buffer (0.5 mol l−1 EDTA, 500 µl Percoll in PBS) on ice. After rigorous agitation with a stirring bar on ice for 30 min, gill cells were isolated from the tissue by gently filtering the solution through a 100-µm nylon mesh to remove the larger tissue fragments. The filtered cell suspension was poured into a Percoll (Sigma, St Louis, MO, USA) solution (2∶1∶1 cell suspension∶Percoll∶PBS) and centrifuged for 10 min at 2000 g at 4°C. The epithelial cell fraction was collected, washed with PBS and centrifuged. Afterwards, the isolated cells were subjected to western blotting with an anti-tropomyosin (a muscle-specific protein) monoclonal antibody (mAb) (Sigma) to confirm that there was no contamination of muscle cells (data not shown). The isolated epithelial cells were stored at −80°C.

Preparation of mRNA

In the process of preparing mRNA, 200–300 mg of gill tissues was homogenized in 3 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated according to the manufacturer’s protocols. The amount and quality of total RNA were determined by measuring the absorbance at 260 and 280 nm with a spectrophotometer (Hitachi U-2000, Tokyo, Japan) and analysis using RNA-denatured gels. The total amount of RNA was subsequently extracted with a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia, Piscataway, NJ, USA). Finally, the pellets of mRNA were precipitated with 0.1 mg glycogen, 1/10 volume of 3 mol l−1 NaOAc and 95% ethanol and stored at −20°C.

cDNA synthesis from mRNA

For cDNA synthesis, 0.36 µg of mRNA was reverse-transcribed in a final volume of 20 µl containing 0.5 mmol l−1 dNTPs, 2.5 µmol l−1 oligo (dT)18, 5 mmol l−1 dithiothreitol and 200 units of PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA) for 1.5 h at 42°C, followed by a 15-min incubation period at 70°C. The cDNA samples were finally stored at −20°C.

Cloning of tilapia GS cDNA from the gills

Degenerate oligonucleotide primers for PCR were designed based on the conserved sequences of GS from different vertebrates. The primer set for cloning GS consisted of forward (5’-AATGTAAGAGTAGTCTCAGC-3’) and reverse (5’-
GCAAAGATGGCCYCTCCTCAT-3’ sequences. For PCR amplification, 2 µl cDNA was used as a template in a 50 µl final reaction volume containing 0.25 mmol l⁻¹ dNTP, 2.5 units EXTaq polymerase (Takara, Shiga, Japan) and 0.2 µmol l⁻¹ of each primer. The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and the nucleotide sequence was determined with an ABI 377 DNA Sequencer (ABI, Warrington, UK). Sequences were analyzed with the BLASTx program (NCBI).

The 5' and 3' RACE (rapid amplification of cDNA ends) cDNAs were cloned with a commercial kit (SMART RACE cDNA Amplification Kit; Clontech) following the manufacturer’s protocols. The specific primers of GS for 5' and 3' RACE were 5'-CGGGACACAGTGAGGATGGG-3' and 5'-ACAGCAGCCCACTCCTCAA-3', respectively. The RACE PCR products were also subcloned into the pGEM-T Easy vector and sequenced. The entire amino acid sequence deduced by the CLUSTAL program (Higgins and Sharp, 1988; Higgins et al., 1996) was used for multiple sequence alignments and analysis of the phylogenetic tree. The dataset was subjected to a distance analysis using the maximum parsimony (MP analysis) method, and 1000 bootstrap replicates of the analysis were carried out with the MEGA program version 2.1.

**GS mRNA expression in different tissues**

Total RNA samples were extracted from the brain, gills, liver, muscle, intestines, heart, spleen and kidneys in FW tilapia. The samples were subjected to RT-PCR analysis, and β-actin gene expression was utilized as an internal control. The primer set for GS consisted of the forward (5’-TGGGATCAGTCAGCT-GTGTA-3’) and reverse sequences (5’-TGTCCCTCCAGCATG-TTGTGAGT-3’) (a 187-bp fragment), and that for the control β-actin consisted of the forward (5’-CGGAATCCACGAAAC-CACCTA-3’) and reverse sequences (5’-ATCTCCTGCTCATG-TGTCCTCCAGCATG-TTGTGAGT-3’) (a 135-bp fragment). Denaturation was performed for 3 min at 95°C, and thereafter for 30 s at 95°C. The annealing time was 30 s at 57°C, and the elongation time was 30 s at 72°C. The reaction was run for 30 cycles. All amplitcons were sequenced to confirm that the PCR products were the desired gene fragments.

**Western blotting**

Both mouse and tilapia tissues (isolated gill epithelial cells and liver tissues) were homogenized with the homogenization solution (100 mmol l⁻¹ imidazole-HCl, 5 mmol l⁻¹ Na₂EDTA, 200 mmol l⁻¹ sucrose and 0.1% sodium deoxycholate; pH 7.6) at 600 revs min⁻¹ on ice. After centrifugation at 4°C and 9800 g for 30 min, the total protein concentration of the supernatant was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) at 110 V for 2 h. After being transferred to polyvinylidene difluoride membranes, the blots were incubated in 5% nonfat milk for 3 h at room temperature and then washed twice with PBST buffer (0.01 mol l⁻¹ phosphate, 0.09% NaCl, pH 7.5 and 0.05% Tween 20). The blotted membranes were incubated overnight with a rabbit anti-human GS polyclonal antibody (Rockland, Gilbertsville, PA, USA; diluted 1:750) at 4°C. After being washed twice with PBST buffer, the blotted membranes were reacted for another 2 h with an alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, Cambridgeshire, UK, diluted 1:1000). After washing with PBST buffer, immunoreactive proteins were visualized with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in staining buffer. Immunoblots were scanned and exported as JPEG files, and the differences between the band intensities of FW and SW samples were compared using a commercial software package (Image-Pro Plus 4.0; Media Cybernetics, Silver Spring, MD, USA).

**Na⁺/K⁺-ATPase activity assay**

Na⁺/K⁺-ATPase activity was determined as described by Hwang et al. (Hwang et al., 1989). Isolated gill epithelial cells were briefly homogenized in the homogenization solution [100 mmol l⁻¹ imidazole-HCl buffer (pH 7.6), 5 mmol l⁻¹ Na₂EDTA, 200 mmol l⁻¹ sucrose and 0.1% sodium deoxycholate]. The Na⁺/K⁺-ATPase activity was assayed in the reaction mixture containing 100 mmol l⁻¹ imidazole-HCl buffer (pH 7.6), 125 mmol l⁻¹ NaCl, 75 mmol l⁻¹ KCl, 7.5 mmol l⁻¹ MgCl₂ and 5 mmol l⁻¹ Na₂ATP. The reaction was run at 37°C for 30 min and then stopped by adding 200 ml of ice-cold 30% trichloroacetic acid. The enzyme activity was defined as the difference between the inorganic phosphates liberated in the presence and absence of 3.75 mmol l⁻¹ ouabain in the reaction mixture. Each sample was assayed in triplicate.

**GP activity assay**

Measurement of gill glycogen phosphorylase activity followed a procedure described by Milligan (Milligan, 2003), with some modifications. Isolated gill epithelial cells were homogenized in ice-cold homogenization solution (100 mmol l⁻¹ imidazole, 100 mmol l⁻¹ KF, 5 mmol l⁻¹ EDTA and 1 mmol l⁻¹ phenylmethylsulfonylfluoride). Total GP (GPₐ+GPₐβ) activity was measured by incubating the samples at 25°C in the presence of 1.6 mmol l⁻¹ 5’AMP, 45 mmol l⁻¹ potassium-phosphate buffer (pH 7.0), 0.2 mg ml⁻¹ glycogen, 0.34 mmol l⁻¹ NADP, 4 µmol l⁻¹ glucose-1,6-bisphosphate, 0.1 mmol l⁻¹ EDTA, 15 mmol l⁻¹ MgCl₂, 1.6 µmol l⁻¹ phosphoglucomutase and 12 µmol l⁻¹ glucose-6-phosphate dehydrogenase. The absorbance changes between the reactions with and without glycogen (the substrate) were measured at 340 nm with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Each sample was assayed in triplicate.

**Glycogen content**

Isolated gill epithelial cells and liver tissues were homogenized in 30% KOH and heated to 100°C for 30 min. The samples were supplemented with two volumes of 100% ethanol and incubated overnight. Glycogen was precipitated by centrifugation after the addition of 2–3 drops of Na₂SO₄. The glycogen pellets were washed with 66% ethanol and then completely dried. The glycogen content was analyzed in 0.2% anthrone reagent dissolved in H₂SO₄ using a Hitachi spectrophotometer.
Immunocytochemistry

Fresh gills were fixed with 4% paraformaldehyde at 4°C for 3 h and then gradually immersed in PBS containing 5%, 10% and 20% concentrations of sucrose for 15 min, with each step at room temperature. Finally, they were soaked in a mixed PBS solution (OCT compound:20% sucrose, 1:2) overnight and then embedded in OCT compound embedding medium (Sakura, Tokyo, Japan) at –20°C. Cryosections (5 µm thick) were made with a cryostat (CM 1900; Leica, Heidelberg, Germany) and placed onto poly-L-lysine-coated slides (Erie, Hooksett, NH, USA). Prepared slides were then rinsed in PBS and blocked with 3% bovine serum albumin (BSA) for 30 min. Afterwards, the slides were incubated with the Na⁺/K⁺-ATPase α5 mAb (Hydromba Bank, University of Iowa, Ames, IA, USA; diluted 1:200), mouse anti-human GP brain form mAb (Biorentend Chemikalien, Cologne, Germany; diluted 1:200) and/or glycogen mAb (Baba, 1993) (diluted 1:200) overnight at 4°C, respectively. The slides were then washed twice with PBS and incubated with anti-mouse IgG conjugated with Texas Red (Jackson ImmunoResearch Lab., West Grove, PA, USA; diluted 1:200) for 2 h at room temperature. After being washed with PBS twice, the slides were incubated again with the anti-human GS polyclonal antibody (diluted 1:200) overnight at 4°C and then washed again before being incubated with anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) for 2 h at room temperature. The images were acquired with a Leica TCS-NT confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Observations were made in the trailing edge of gills where most of the MR cells locate.

Statistical analysis

Values are presented as means ± s.d. (N=6). All time-course data were statistically analyzed by two-way analysis of variance (ANOVA), and pairwise multiple comparisons were made by Tukey’s test (P<0.05).

Results

Tilapia glycogen synthase (tGS) cloning and sequencing

Full-length GS cDNA was cloned and sequenced from tilapia gills (Fig. 1) (GenBank Accession No. EF565371). The complete sequence obtained was 2660 nucleotides (nt) long and contained a 2133-nt open reading frame, a 359-nt 5′-untranslated region and a 168-nt 3′-untranslated region with a poly (A) tail. The deduced amino acid sequence encodes a protein of 711 amino acids. Using commercial software (Vector NTI; Invitrogen, San Diego, CA, USA) to analyze the amino acid sequence, we discovered that the GS from tilapia gills (tGS) is about 70% identical to the zebrafish GS muscle form, 65% to the mouse GS brain form, 55% to the mouse liver form and 62.5% to the human GS muscle form (Fig. 1). The complete sequence obtained was 2660 nucleotides (nt) long and contained a 2133-nt open reading frame, a 359-nt 5′-untranslated region and a 168-nt 3′-untranslated region with a poly (A) tail. The deduced amino acid sequence encodes a protein of 711 amino acids. Using commercial software (Vector NTI; Invitrogen, San Diego, CA, USA) to analyze the amino acid sequence, we discovered that the GS from tilapia gills (tGS) is about 70% identical to the zebrafish GS muscle form, 65% to the mouse GS brain form, 55% to the mouse liver form and 62.5% to the human GS muscle form (Fig. 1).

Phylogenetic analysis of the tGS amino acid sequence

In Fig. 2, a phylogenetic tree was generated based on the MP analysis of the full-length amino acid sequences of the GS isoforms (muscle and liver) from eight species (mouse, rat, rabbit, human, monkey, zebrafish, tilapia and Drosophila). The tGS cloned from the gill had the highest homology to the muscle form.

tGS gene expressions in different tissues

tGS mRNA expressions in different tissues of tilapia were examined by RT-PCR with β-actin as the internal control. Fig. 3 clearly indicates that the PCR products of the 187-bp fragment of tGS were present. The tGS gene was ubiquitously expressed among various tissues, with higher expression levels in the brain, heart and muscle.

Western blotting of GS

Tilapia liver and isolated gill cells, and mouse muscle and kidney cells (as a positive control, following the Product Specification Sheet of Rockland) were subjected to western blotting with an anti-human GS polyclonal antibody. The antibody was raised against a synthetic peptide, A-Q-G-Y-R-Y-P-R-P-A-pS-V-P, which was derived from the 631–643 amino acid sequence of the human muscle GS. The 631–643 amino acid sequence of the tGS and mouse muscle GS showed 77% and 92% identities, respectively, to that of the human muscle GS. As shown in Fig. 4, immunoreactive bands with a similar size were found in all of the tissues examined. The size was about 80.9 kDa in mouse kidney, tilapia liver and gill cells, and 83.9 kDa in mouse muscle. Negative experiments with the pre-immune serum showed no bands (data not shown). These results indicate that the antibody recognizes different isoforms of the tGS and, thus, can be used to detect the tGS protein in gills and liver.

Localization of GS, Na⁺/K⁺-ATPase, GP and glycogen in tilapia gills

The specificities of anti-GP, anti-glycogen and anti-Na⁺/K⁺-ATPase antibodies have been confirmed in previous studies (Tseng et al., 2007; Hwang et al., 1999). Double immunohistochemical labeling was conducted to localize GS, GP, glycogen and MR cells. Differential interference contrast (DIC) observations showed distinct images of the outline of the afferent edge of a Gill filament (Fig. 5A,D,H). FITC signals for GS, and Texas Red signals for Na⁺/K⁺-ATPase, were both found in gill epithelial cells, but they were not colocalized in the same cells (Fig. 5B,C), clearly indicating that GS was localized in a specific type of cells, GR cells (Tseng et al., 2007) (also see below). Based on confocal-microscopic observations from the serial sections in more than 10 individuals, all these GS-expressing GR cells were adjacent to MR cells (Fig. 5B,C). Moreover, GS and GP were colocalized in the same GR cells (Fig. 5E–G), and GS and glycogen were also found in the same type of cells (Fig. 5I–K). As a result, GS, GP and glycogen were all colocalized in the same group of cells, GR cells, which were recently identified because of their abundant deposits of glycogen (Tseng et al., 2007).

Time-course changes in gill Na⁺/K⁺-ATPase activity after transfer to 25‰ SW

FW tilapia were transferred to 25‰ SW and FW (as a
control and then the activity of gill Na⁺/K⁺-ATPase was measured at different time points after the transfer. Gill Na⁺/K⁺-ATPase activity of the FW control group was maintained at 4–6 μmol Pi h⁻¹ mg⁻¹ protein during the entire experiment (Fig. 6). However, the activity in the 25‰ SW group showed dramatic changes, with two peaks (Fig. 6). The activity exhibited a significant increase (of about 1.9-fold) from the first hour immediately after transfer to 25‰ SW, and then remained at a low level, and finally recovered to the level of the control (Fig. 7A, B). Glycogen deposits in gill GR cells were depleted by about 50% in the first hour immediately after transfer from FW to 25‰ SW, then remained at a low level, and finally recovered to the level of the control (Fig. 7A). On the other hand, it was not until the sixth hour that the glycogen content began to be depleted in the liver, and the content of glycogen in the liver was maintained at a lower level, at ~40–60% of that of the control FW group, until the end of the experiment (Fig. 7B).

**Time-course changes in glycogen content in isolated gill and liver cells after transfer to 25‰ SW**

Similar to the above transfer experiment, glycogen content in isolated gill and liver cells was measured at different time points after the transfer. Glycogen content in isolated gill (mainly in GR cells) and liver cells was affected by environmental salinity (Fig. 7A, B). Glycogen deposits in Gill GR cells were depleted by about 50% in the first hour immediately after transfer from FW to 25‰ SW, then remained at a low level, and finally recovered to the level of the control. On the other hand, it was not until the sixth hour that the glycogen content began to be depleted in the liver, and the content of glycogen in the liver was maintained at a lower level, at ~40–60% of that of the control FW group, until the end of the experiment (Fig. 7B).
Fig. 2. Phylogenic analysis constructed with complete amino acid sequences of glycogen synthase (GS) by maximum parsimony methods with 1000 replications. The GenBank Accession Nos of the sequences used are as follows: GS muscle form of the mouse, NP_109603; human, NP_002094; rat, XP_341859; rabbit, P13834; monkey, AF529178; zebrafish, NP_957474. GS liver form of the mouse, NP_663547; rat, NP_037221; human, NP_068776; zebrafish, NP_001018199. GS isoform C from Drosophila, NP_731968.

The notable findings of the present study were as follows: (1) GS was cloned and sequenced from fish gills for the first time; (2) GS, GP and glycogen were co-localized in a specific group of gill cells, i.e. GR cells, which are adjacent to MR cells (the major ionocytes); (3) glycogen content in both gill and liver was significantly depleted after acute transfer from FW to 25‰ SW, but the depletion occurred earlier in gills than in the liver; and (4) gill Na⁺/K⁺-ATPase activity rapidly increased immediately after SW transfer. Following this, gill GP activity and protein expression were upregulated 1–3 h post-transfer and eventually recovered, while at the same time GS protein expression was downregulated. Similar changes in liver GP and GS protein expressions were observed but they occurred more slowly, at 6–12 h post-transfer to 25‰ seawater.

The amino acid sequence of the tilapia GS (tGS) from the gills showed the highest identity of 70% with the zebrafish GS muscle form and 55–65% with the mammalian counterparts. Phosphorylation of the C-terminal serine residues of mammalian GS, a key regulator for controlling enzyme activity (Cohen, 1982; Roach, 1990), is conserved in the tGS. Moreover, the tGS also has two Lys residues, Lys38 and Lys300, which have been identified as binding sites for UDP-glucose in the mammalian enzyme (Tagaya et al., 1985; Mahrenholz et al., 1988). Based on these results, the tGS may be a homologue of the mammalian GS muscle form.

The mechanisms of glycogen synthesis and degradation are generally known in mammal tissues, including the liver, muscle and other organs (Smythe and Cohen, 1991; Bollen et al., 1998), and this mechanism seems to be similar in fish gills, an energy-consuming organ. Glycogen was reported to exist in the cytoplasm of fish branchial MR cells according to electron microscopic observations (Philpott and Copeland, 1963; Nakao, 1974). This traditional inference seems unlikely because of the...
present and previous (Tseng et al., 2007) convincing molecular and cellular evidence of the colocalization of GP, GS and glycogen in a specific group of cells (GR cells) but not in MR cells in fish gills. As addressed by Tseng et al., GR cells and MR cells share the same apical opening to form a multicellular complex (Tseng et al., 2007). More sensitive analysis, like immuno-electron microscopy, is necessary to further confirm whether GR cells are actually previously reported accessory cells or other transporting cells (Tseng et al., 2007). However, the present result raises the possibility that the relationship in energy translocation between mammal astrocytes and neurons may also occur between GR cells and MR cells in fish gills. In the rat brain, GP and glycogen mainly exist in astrocytes, astroglial cells and ependymal cells of ventricles but never in neurons (Pfeiffer-Guglielmi et al., 2003). During energy deprivation in the central nervous system, glycogen is degraded to lactate, which is shuttled from astrocytes to high-energy-requiring neurons (Ransom and Fern, 1997; Brown et al., 2003; Pfeiffer-Guglielmi et al., 2007).

Acclimation to SW in fish was suggested to be involved in activation of ion secretion pathways in gill MR cells (Evans et al., 2005), and this requires additional energy support (Morgan et al., 1997). Immediately after an acute challenge with SW, gill Na⁺/K⁺-ATPase activity showed a rapid and drastic increase from the first hour in order to recover the impaired internal ion levels and osmolalities in tilapia (Hwang et al., 1989), indicating that a prompt energy supply for stimulating ion-secretion mechanisms is critically needed. Indeed, Morgan et al. (Morgan et al., 1997) examined the oxygen consumption in tilapia after transfer to SW and found a significant increase after 4 days of

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**Fig. 5.** Immunohistochemical images of freshwater tilapia gill frozen sections. (A–C) Double labeling for glycogen synthase (GS) and Na⁺/K⁺-ATPase (NaK); (D–G) double labeling (arrows) for GS and glycogen phosphorylase (GP); (H–K) double labeling (arrows) for GS and glycogen. A, D and H are DIC images. GS, GP and glycogen were co-localized in a specific group of glycogen-rich cells (GRC) that are adjacent to mitochondrion-rich cells (MRC; labeled with Na⁺/K⁺-ATPase). White dots indicate the outline of the cells. Scale bar: 10 μm (A–C) and 20 μm (D–K).
Data are presented as means ± s.d. (N in tilapia transferred from freshwater (FW) to 25‰ seawater (SW)). Other studies, however, reported a reduction in oxygen consumption in tilapia after 2 acclimation. Fig. 6. Time-course changes in gill Na⁺/K⁺-ATPase activities in tilapia transferred from freshwater (FW) to 25‰ seawater (SW). Data are presented as means ± s.d. (N=6). *Indicates a significant difference from the respective control in FW (P<0.05). Different letters indicate significant differences (P<0.05) among sampling times in fish transferred to SW.

Glycogen metabolism during seawater acclimation suggested to be an essential energy source for fish osmoregulation during acclimation to different salinities; hence, changes in glycogen content are another suitable indicator for monitoring energetic metabolism in a specific organ or cell (Assem and Hanke, 1979; Nakano et al., 1998; Sangiao-Alvarellos et al., 2005). Effects of environmental salinities on glycogen content in the liver, gills and/or other organs have been examined in several studies. Nakano et al. found no significant changes in liver glycogen contents in tilapia (O. mossambicus) after transfer from FW to 23‰ SW for 0.5–96 h (Nakano et al., 1998). Likewise, Assem and Hanke found no significant difference in glycogen content of the liver in the same species 6–168 h after transfer from FW to 25‰ SW (Assem and Hanke, 1979). The controversy between these previous data and the present results in tilapia may be partially due to differences in the fish size, the conditions (e.g. temperature) in which the fish were kept, the method of sampling, etc. On the other hand, Sangiao-Alvarellos et al. examined metabolic changes in the liver and gills in gilthead sea bream (Sparus auratus) during acclimation between 38‰ and 55‰ SW and found that glycogen was depleted in the liver on the first day while that in the gills accumulated (Sangiao-Alvarellos et al., 2005). These results imply that mobilization of liver glycogen may provide...
endogenous carbohydrate fuel to the gills (Sangiao-Alvarellos et al., 2005); however, no further investigations have been done within the first day after transfer, which is a very critical period for a fish exposed to a salinity stress as we addressed above. The present study examines the differential roles of the gills and liver and their spatial and temporal relationships to the energy supply for osmoregulation during acute exposure to a high-salinity environment. The present data of changes in glycogen content indicate that in gills in may provide prompt energy from the first hour after SW transfer; subsequently, from 6 h post-transfer, liver glycogen may become the major carbohydrate reserve supporting the operation of ion-secretion mechanisms in tilapia gills. Furthermore, the glycogen content in the liver is much greater, about 250–300-fold, than that in the gills, and this also reasonably reflects the different partitions of the two organs in energy supply. SW acclimation stimulates the activities of Na⁺/K⁺-ATPase and other transporters or enzymes in gills, intestine and other osmoregulatory organs (Kelly et al., 1999; Ando et al., 2003; Evans et al., 2005). Liver glycogen may provide energy for all the processes in these organs. It is impossible to distinguish the partitions of liver glycogen to different organs or different transporters; therefore, it is not surprising to find that the profile of liver glycogen (started to deplete at 6 h) was not totally in concert with that of gill Na⁺/K⁺-ATPase (second increase from 24 h).

In both the gills and liver of gilthead sea bream, GP activities and glycogen content changed in parallel immediately after transfer from 38‰ to 55‰ SW, but neither GS activity nor protein expression was studied (Sangiao-Alvarellos et al., 2005). Obviously, these previous findings might not correctly reflect the functional regulation of glycogenesis and glycogenolysis during hyperosmotic acclimation. On the contrary, in the present study, tilapia gill GP and GS protein expressions and/or activities showed opposite changes, increasing (upregulation of glycogenolysis) and decreasing (downregulation of glycogenesis), respectively, from the first to the third hours after transfer to 25‰ SW, and these profiles reasonably reflect depletion of gill glycogen. Similar results were also found in tilapia liver, but stimulation of glycogenolysis in the liver began later, from the sixth to the 12th hour after transfer. In previous studies on rainbow trout (O. mykiss), acclimation to 12‰ or 28‰ SW also stimulated liver glycogenolysis, which was associated with an increase in GP activity, a decrease in GS activity and a decline in glycogen levels (Soengas et al., 1991; Soengas et al., 1993). Based on our data, stimulation of glycogenolysis in the gills and thereafter in the liver probably provides a sequential energy supply for the operation of ion-secretion mechanisms, including Na⁺/K⁺-ATPase and other transporters, in tilapia gills during acute exposure to SW. Glycogen is stored in the liver as a reserve of glucose for extrahepatic tissues, and the liver, acting as a sensor of blood glucose stores, mobilizes glycogen according to peripheral needs (Bollen et al., 1998). Therefore, it is not surprising to find in the present study and other previous work that mobilization of liver glycogen may provide carbohydrate reserves to fuel glycolysis in gills for operation of ion-regulation mechanisms during acclimation to salinity changes (Assem and Hanke, 1979; Soengas et al., 1991; Soengas et al., 1993; Sangiao-Alvarellos et al., 2005). The notable new finding from the present study is that the gills accumulate glycogen as a local carbohydrate reserve, and upon acute salinity stress, the glycogen in the gills is initially degraded to provide a prompt emergency energy supply for activation of salt secretion mechanisms before glycogenolysis in the liver is stimulated. However, direct evidence to demonstrate the energy shuttle between gill cells is still lacking and requires further studies. The role of the gills themselves in the emergency energy supply for osmoregulation seems to have been overlooked in previous studies that examined metabolic changes in gills of the gilthead sea bream during acclimation to different salinities (Sangiao-Alvarellos et al., 2003; Sangiao-Alvarellos et al., 2005). There are some possible reasons for this: first, those studies did not examine short-term changes (in terms of hours) as discussed above; second, perhaps there are differences in the methodologies for measuring glycogen content and GP expression and activity. Our preliminary experiments showed that blood in gill tissues comprised as much as >60% and >80%, respectively, of the glycogen content and GP protein amount in the whole gill (data not shown). Thus, the present study using isolated gill epithelial cells to examine metabolic changes in gill cells avoided the contaminating effects from blood and other cells. The actual changes in gill cells might have been obscured in previous studies by Sangiao-Alvarellos et al. (Sangiao-Alvarellos et al., 2003; Sangiao-Alvarellos et al., 2005), since they used whole gill tissues without isolating the gill cells.

Taking all this into account, we propose a model for carbohydrate metabolism for osmoregulation in tilapia gills during acute exposure to salinity stress: GR cells accumulate...
glycogen as a local carbohydrate reserve in gills, glucogenolysis in gill GR cells is initially stimulated to provide prompt energy for neighboring MR cells in order to trigger ion-secretion mechanisms, and several hours later, the liver begins to degrade its glycogen for the subsequent energy supply. This local and systematic partitioning of glycogen metabolism for emergency energy requirements is similar to what has been well documented in mammal brains (neurons and astrocytes) and liver.

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Glycogen metabolism during seawater acclimation  3503

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