RESEARCH ARTICLE

Dynamic Regulation of Aquaglyceroporin Expression in Erythrocyte Cultures From Cold- and Warm-Acclimated Cope's Gray Treefrog, *Hyla chrysoscelis*

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ABSTRACT

Cope's gray treefrog, *Hyla chrysoscelis*,is a freeze-tolerant anuran which accumulates and distributes glycerol as a cryoprotectant before freezing. We hypothesize that HC-3, an aquaglyceroporin member of the MIP family of water pores, may play an important role in the process of freeze tolerance by mediating transmembrane passage of glycerol and water during cold-acclimation. The objectives of this study were two-fold: to examine HC-3 protein abundance and cellular localization in erythrocytes from cold- and warm-acclimated frogs and to develop and characterize an erythrocyte cell culture system for examining HC-3 gene regulation. Compared with warm-acclimated frogs, erythrocytes from cold-acclimated frogs had higher HC-3 protein expression and enhanced plasma membrane localization. Furthermore, erythrocytes from cold- and warm-acclimated frogs maintained in culture at 4 and 20°C exhibited time- and temperature-dependent regulation of HC-3 expression and an increase in the abundance of high molecular weight immunoreactive species within 24 hr of culture at 20°C. Deglycosylation of erythrocyte proteins resulted in the disappearance of the high molecular weight species, indicating that HC-3 is post-translationally modified by *N*-linked glycosylation. Erythrocytes cultured in media containing glycerol also showed an increased abundance of the high molecular weight bands and enhanced plasma membrane localization of HC-3, suggesting a role for glycerol in regulating HC-3 subcellular trafficking. Thus, the development of this erythrocyte cell culture system from *H. chrysoscelis* opened an opportunity to study the properties of cells with changing expression of an aquaglyceroporin, HC-3, and to explore the factors regulating that expression. *J. Exp. Zool. 315:424–437, 2011.* © 2011 Wiley-Liss, Inc.

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Hyla chrysoscelis, Cope's gray treefrog, undergoes a process of thermal acclimation that prepares the animals for the possibility of freezing and subsequent freeze recovery (Costanzo et al., '92; Irwin and Lee, 2003; Layne and Stapleton, 2009). The physiological processes that help freeze-tolerant gray treefrogs

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(including *H. chrysoscelis* and its sister species *H. versicolor*) accomplish this feat result in the accumulation of high concentrations of extracellular glycerol (Schmid, '82; Storey and Storey, '85; Costanzo et al., '92; Layne and Jones, 2001; Irwin and Lee, 2003; Zimmerman et al., 2007). During cold-acclimation, the glycerol that is accumulated crosses cell membranes and is present in both the intracellular and extracellular fluid, where it serves as a colligative cryoprotectant to moderate the osmotic shifts in water that develop during ice crystal formation (Storey, '97). In addition, glycerol as well as other carbohydrate cryoprotectants including glucose which accumulates in other species of freeze-tolerant anurans are thought to stabilize the structures of biomolecules during the process of freezing (Storey, '90).

One route for transmembrane transport of glycerol is via aquaporin/aquaglyceroporin (AQP/GLP) members of the MIP family of transmembrane proteins (Thomas et al., 2002). Several amphibian orthologs of mammalian aquaporins have been identified, including members of both the AQP and GLP functional classes (Ma et al., '96; Tanii et al., 2002; Virkki et al., 2002; Kubota et al., 2006; Akabane et al., 2007; Ogushi et al., 2007; Zimmerman et al., 2007; Mochida et al., 2008; Suzuki and Tanaka, 2009). In addition, two anuran-specific aquaporin types appear to have evolved to support physiologic responses to changes in the external environment, including those that occur during metamorphosis and the transition from an aquatic to a terrestrial environment (Suzuki et al., 2007; Suzuki and Tanaka, 2009; Ogushi et al., 2010; Suzuki and Tanaka, 2010).

To date, three AQP/GLPs (HC-1, HC-2, and HC-3) have been isolated and characterized from *H. chrysoscelis*. HC-1 and HC-2 are classical water-permeable aquaporins, whereas HC-3, an ortholog of mammalian AQP3, functions as an aquaglyceroporin (Zimmerman et al., 2007). We have previously shown that HC-3 mRNA and/or protein is expressed in several tissues, some of which show dynamic regulation of HC-3 expression, depending on thermal acclimation state of the animal (Zimmerman et al., 2007; Pandey et al., 2010; Goldstein et al., 2010). HC-3 protein is also highly expressed in erythrocytes from *H. chrysoscelis*, where it is more abundant in erythrocytes from cold-acclimated treefrogs compared with warm-acclimated treefrogs (Goldstein et al., 2010). In addition, erythrocytes expressing HC-3 exhibit high glycerol permeability, which is abolished by $HgCl_2$, a known AQP/GLP inhibitor, suggesting that glycerol permeability is rendered through GLPs (Goldstein et al., 2010). In view of these observations, we hypothesized that AQP/GLPs may also function in the physiology of freeze tolerance in Cope's gray treefrog (Zimmerman et al., 2007). In order to more clearly define the gene regulatory mechanisms that influence HC-3 expression and its functional role in glycerol transport, we have characterized HC-3 protein abundance and cellular localization in erythrocytes from cold- and warm-acclimated frogs and have developed an erythrocyte cell culture system in which HC-3 protein expression can be dynamically regulated. The development of this in vitro model presents an opportunity to explore the functional properties of cells with changing expression of the aquaglyceroporin, HC-3, and to explore the factors regulating that expression.

MATERIALS AND METHODS

Animals

Male gray treefrogs of the freeze-tolerant anuran species, *Hyla chrysoscelis*, were identified and collected as previously described (Zimmerman et al., 2007; Goldstein et al., 2010; Pandey et al., 2010). Animals were housed in small groups at ambient temperature with natural seasonal light cycles throughout the summer months and were fed crickets three times per week. Concurrent with natural autumnal temperature and light cycle changes, animals were moved to climate and light controlled rooms. Warm-acclimated animals were housed in pairs, maintained at 21°C with a 12:12-hr light cycle, and fed crickets three times per week. Cold-acclimated animals were progressively acclimated to 5°C over a period of 2 months, with a concomitant reduction in light exposure to 8:16 hr light cycle (Zimmerman et al., 2007; Goldstein et al., 2010). Cold-acclimated animals were maintained at 5°C with 8:16 hr light cycle for a minimum of 4 weeks before blood collection. For all conditions, water was available in cages ad libitum. The methods of collection, housing procedures, and experimental protocols for the care and use of *H. chrysoscelis* were approved by the Institutional Animal Care and Use Committee (IACUC) at Wright State University, where the frogs were housed. All animals subjected to nonrecovery blood draw procedures were euthanized by decapitation under anesthesia, as approved by the IACUC.

Establishment of *H. chrysoscelis* Erythrocyte Cultures

Blood was drawn from the trunk or brachial artery of *H. chrysoscelis* and was collected in heparinized capillary tubes. To attain sufficient number of erythrocytes for each experiment, and to control for the interindividual variation that naturally exists between noninbred individual gray treefrogs collected from the wild, blood was pooled from three to four frogs. Blood was transferred to 15-mL conical tubes containing 5 mL of complete cell culture media (CCCM (250 mOsM): RPMI 1640 medium supplemented with L-glutamine (Invitrogen, Carlsbad, CA), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Invitrogen), 5% fetal bovine serum (Fisher Scientific, Hanover Park, IL). Blood samples were centrifuged at 1,000 × *g* for 10 min. Approximately 5×10^6 cells were resuspended in either CCCM or in CCCM containing 0.156 M glycerol (400 mOsm) in a volume of 5 mL and placed in 25-cm^2 Corning tissue culture flasks. This glycerol concentration was chosen for two reasons: (1) the plasma glycerol concentration in cold-acclimated gray treefrogs reaches > 100 mM, which further

increases to >400 mM upon freezing (Storey and Storey, '85; Layne and Jones, 2001); and (2) the addition of 0.156 M glycerol increases the osmolarity of the media to ~400 mOsM which approximates plasma osmolarity in cold-acclimated treefrogs. For the majority of experiments, suspension cultures were maintained at 20 or 4°C in flasks positioned upright at a 45° angle on a plate shaker with constant rotation (190 rpm). Media were replenished every 24 hr to provide optimal cell viability. In the cell culture optimization experiments, the media was not replaced every 24 hr in a subset of cultures, and other cultures were maintained without shaking. Trypan blue dye exclusion method was used to determine cellular viability at time 0, 24, 48, 72, and 96 hr ($n = 3$ for each time point) as per the manufacturer's instruction (Invitrogen).

Western Blot Analysis

HC-3 protein expression was analyzed by Western blotting using established methods (Krane et al., '99). Approximately 1×10^6 erythrocytes were collected from cultures at selected time points and centrifuged at $1{,}000 \times g$ for 10 min. Pelleted cells were resuspended in 40 µl of ice-cold membrane isolation solution (5 mM tri-ethanolamine, 125 mM sucrose), containing a 1:1,000 dilution of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 2 mg/mL of phenylmethylsulfonyl fluoride (PMSF), and lysed by three consecutive freeze–thaw cycles (1 min on dry ice:1 min at 37°C). Total protein concentration in each sample was quantified using the Pierce BCA Protein Assay Reagent Kit (ThermoScientific, Waltham, MA) according to the manufacturer's protocol. Twenty-five micrograms of protein was size fractionated by SDS-PAGE (MiniProtean II apparatus; Bio-Rad, Hercules, CA) on a 12% denaturing polyacrylamide gel and electro-transferred to polyvinylidene difluoride membranes (SequiBlot; Bio-Rad). Western hybridization was carried out overnight at 4°C using a peptide-derived, monospecific rabbit polyclonal antibody raised against 16 C-terminal amino acids of HC-3 (0. 44 µg/mL; Goldstein et al., 2010), or mouse-anti-β-Actin antibody (diluted 1:5,000; Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000; Santa Cruz Biotech, CA) or goat anti-mouse secondary antibody (1:1,000; Santa Cruz Biotech). Immunoreactive signal was detected using enhanced chemiluminescence substrate (West Pico SuperSignal, Pierce, Rockford, IL) and visualized on X-ray film (Kodak Film, Rochester, NY) with multiple exposures. Relative band intensities were determined by densitometry using Vision works software on BioSpectrum® Imaging System (UVP, Upland, CA). A preabsorption control was performed by preincubating the HC-3 primary antibody with a 200-fold molar excess of the immunizing peptide for 1 hr at room temperature before the application to the membrane. Western blotting with preimmune serum showed no visible immunoreactive bands (not shown).

Deglycosylation

Total cellular proteins were isolated from cultured erythrocytes in membrane isolation solution (without PMSF) in the presence of the protease inhibitor cocktail (Sigma-Aldrich). Twenty micrograms of protein was preincubated in denaturing buffer (0.5% SDS, 0.04 M DTT) at 100°C for 10 min, then incubated in reaction buffer (50 mM sodium phosphate buffer (pH 7.5) with 1% Nonidet P-40) in the presence or absence of 500 U of Peptide-*N*-glycosidase F (PNGase F; New England BioLabs, Ipswich, MA) for 1 hr at 37°C as per the manufacturer's instruction. Control and enzyme-treated proteins were size fractionated by using SDS-PAGE and immunoblotted as described above.

Immunocytochemistry

For freshly isolated erythrocytes, roughly 15 µl of blood collected from the brachial artery was placed on a gelatin-coated slide, smeared with a 1 × 1 inch coverslip, and allowed to dry at room temperature. For cells in culture, 20 µl of the cell suspension was applied to a gelatin-coated slide, smeared, and allowed to dry at room temperature. Immunocytochemistry was performed essentially as described (Pandey et al., 2010). Slides were submerged in a periodate-lysine-paraformaldehyde solution (4% paraformaldehyde, 75 mM lysine, 37.5 mM sodium periodate, and 10 mM Na_2HPO_4, pH 7.2) and fixated for 20 min at room temperature. Cells were permeabilized by exposure to 0.2% Triton X-100 and subsequently submerged in 1% glycine −0.1% sodium borohydride (pH 8.0) to reduce any free paraformaldehyde remaining on the samples. Slides were blocked with a 10% blocking serum (10% donkey serum, 4% bovine serum albumin, and 0.05% Tween-20) for 1 hr at room temperature and incubated with a 1:1,000 dilution of HC-3 primary antibody (0.44 µg/mL) in 1% blocking serum overnight at 4°C. Slides were washed and labeled with goat anti-rabbit fluorescein-conjugated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1,000 in 1% blocking serum. Cells were treated with RNase (4 mg/mL; Promega, Madison, WI) for 5 min and stained with propidium iodide as per the manufacturer's instruction (Sigma-Aldrich). Immunofluorescence was analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope. A preabsorption control was performed by preincubating primary antibody with a 200-fold molar excess of the immunizing peptide for 1 hr at room temperature before application to the slide.

Statistics

The abundance of HC-3 protein expression (normalized to β-actin) in freshly isolated erythrocytes from cold-acclimated treefrogs is represented as a percentage of normalized HC-3 expression from warm-acclimated treefrogs (set at 100%). The statistical analysis of HC-3 and β-actin density ratios for protein expression in freshly isolated erythrocytes from cold- and warm-acclimated frogs was performed using an unpaired Student's *t* test with equal variance ($n = 3$ for each condition). For the other comparisons ($n = 3$ observations per time point, or condition),

repeated measures analyses of variance with time and treatment as factors were employed. For cell viability studies, the percentage of live cells at each timepoint was represented relative to time zero (100%; $n = 3$ per timepoint per condition). For the erythrocyte culture experiments, HC-3 protein abundance (native or glycosylated) was normalized to β-actin expression and represented as a percentage of normalized HC-3 expression at time zero (set at 100%). For the erythrocyte culture experiments, the coefficient of variation was calculated to determine the variation of normalized HC-3 expression in freshly isolated erythrocytes at time zero ($n = 3$ per acclimation state were used as controls for each experimental condition). Post hoc multiple pairwise comparisons were carried out using the Bonferroni corrections method for simultaneous comparisons. A P-value below 0.05 was considered statistically significant.

RESULTS

HC-3 Protein Abundance and Subcellular Localization in Freshly Isolated Erythrocytes

Previous experiments have shown differential regulation of HC-3 expression in multiple tissues depending upon the thermal acclimation state of *H. chrysoscelis* (Zimmerman et al., 2007; Goldstein et al., 2010; Pandey et al., 2010). In this study, Western blotting of erythrocyte proteins freshly isolated from cold- vs. warm-acclimated treefrogs showed that native HC-3 protein abundance is 2.3-fold higher in erythrocytes from cold-acclimated frogs as compared with warm-acclimated frogs (Fig. 1A and B). Western blots from erythrocyte proteins obtained from cold-acclimated frogs also showed high molecular weight immunoreactive species which appear as a smear ranging from >65 to 120 kDa on the Western blot (Fig. 1A). It is likely that the high molecular weight immunoreactive species represents glycosylated HC-3 (Pandey et al., 2010). Immunocytochemistry of erythrocytes from cold-acclimated treefrogs showed robust plasma membrane-associated fluorescence, indicating that HC-3 protein is primarily localized to the plasma membrane (Fig. 2A). In contrast, HC-3 expression appears as punctuate fluorescence scattered throughout the cytoplasm of freshly isolated erythrocytes from warm-acclimated treefrogs, where it is likely associated with internal membranous structures (i.e. vesicles) of the cell (Fig. 2B and C). Preincubating the primary antibody with immunizing peptide abolished all fluorescent signal, indicating that the fluorescence observed is HC-3 specific (Fig. 2D). Taken together, these data indicate that HC-3 protein expression is markedly upregulated in erythrocytes from cold-acclimated frogs vs. warm-acclimated frogs, and is predominantly localized to the plasma membrane in erythrocytes from cold-acclimated treefrogs.

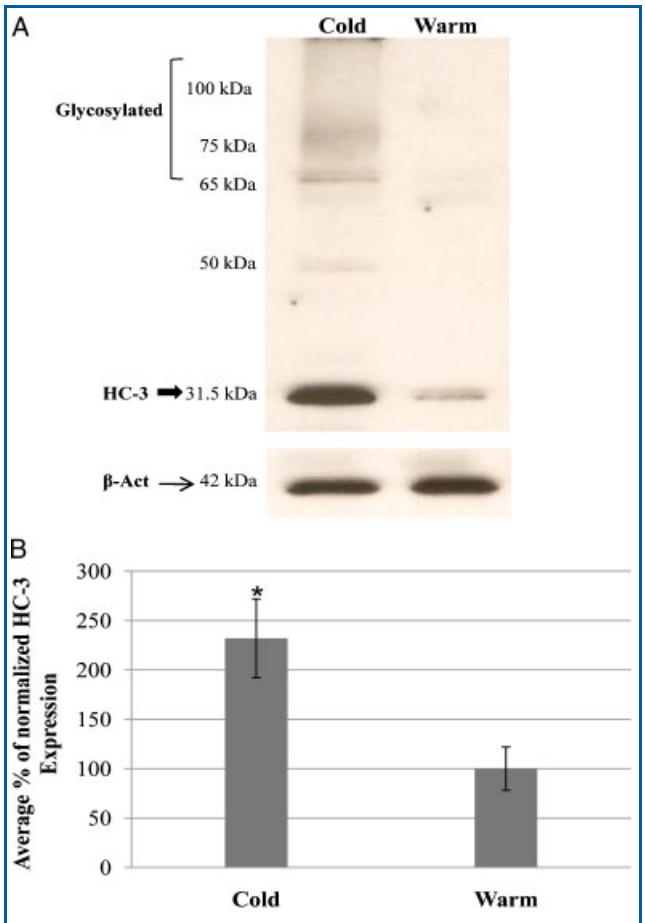

Figure 1. Protein expression and subcellular localization of HC-3 protein in freshly isolated erythrocytes from cold- and warm-acclimated frogs. (A) Native HC-3 protein expression (31.5 kDa) in erythrocytes from cold- and warm-acclimated frogs was detected using Western blotting. Immunoblotting also detected high molecular weight bands between >65 and 120 kDa which correspond to glycosylated HC-3. β-actin expression (42 kDa) is shown as a control for the equivalency of protein loading. (B) Densitometry was used to quantify the levels of native HC-3 expression in freshly isolated erythrocytes from cold- vs. warm-acclimated frogs. HC-3 expression is 2.3-fold more abundant in erythrocytes from cold-acclimated frogs as compared with expression in warm-acclimated frog erythrocytes ($P<0.05$). Densitometric results are represented as an averaged percentage of normalized HC-3 expression ± SD ($n = 3$) relative to levels in erythrocytes from warm-acclimated frogs (100%).

Establishing an Erythrocyte Cell Culture Model

In order to assess the potential cellular mechanisms responsible for differential regulation of HC-3 protein expression observed in vivo, we sought to develop an in vitro cell culture system using erythrocytes from *H. chrysoscelis*. Erythrocytes from *H. chrysoscelis* are nucleated, metabolically active, and can be repetitively harvested as a highly homogenous cell population. At first, we optimized the conditions required for maintaining

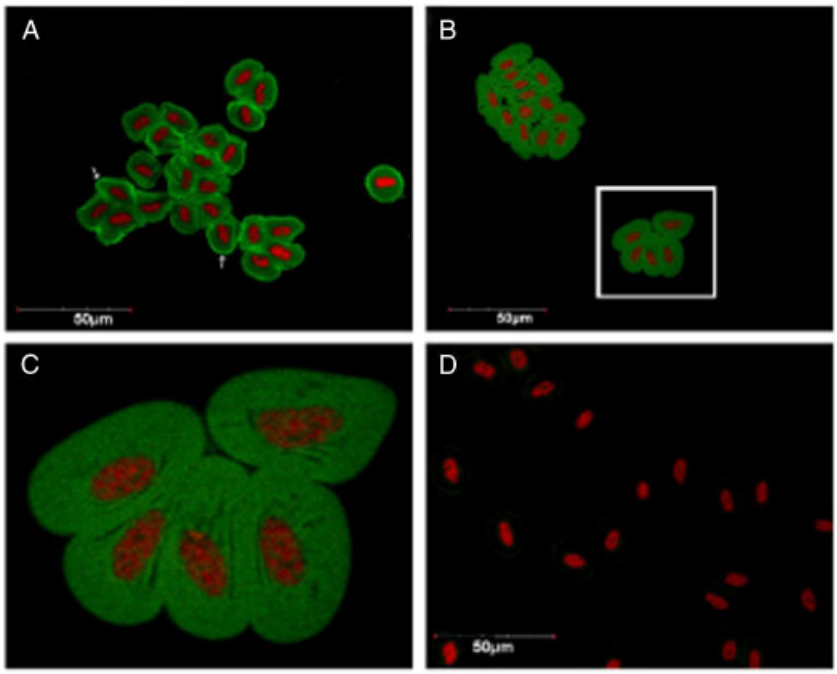

Figure 2. Immunofluorescence of HC-3 protein expression in erythrocytes freshly isolated from cold- and warm-acclimated frogs. (A) Immunocytochemistry showed enriched HC-3 protein (green) localization to the plasma membrane of erythrocytes freshly isolated from cold-acclimated frogs (60 ×). (B) Immunocytochemistry of HC-3 expression in freshly isolated erythrocytes from warm-acclimated treefrogs appeared to be predominantly in the cytoplasm of erythrocytes (60 ×). Propidium iodide (red) was used as a counterstain. (C) Enlarged image of panel (B); HC-3 immunocytochemistry in freshly isolated erythrocytes reveals a punctuate staining pattern throughout the cell that likely represents HC-3 expression in cytoplasmic vesicles. (D) No immunofluorescent signal is visible in the peptide-preabsorption control. Scale bars represent 50 μm. Arrows indicate HC-3 membrane expression.

erythrocytes in culture by exploring cell viability in response to the frequency of media replacement, shaking vs. nonshaking culture conditions, and alterations in media composition. Cell viability was determined by Trypan blue dye exclusion method. In the first experiment, we examined the effect of media replacement on cell viability in continuously shaken cultures. A repeated measures ANOVA showed that there was a significant two-way interaction between time in culture and media replacement on cell viability. There was no significant difference in cell viability in cultures with media replacement compared with those without media replacement at the 24 or 48 hr time points. However, by 72 hr, cell viability in cultures receiving daily media replacement was significantly different from the viability of cells in culture without media replacement (Fig. 3A; Table 1).

Next we compared cell viability in shaken vs. static cultures, when media were replaced daily. Repeated measures ANOVA showed that there was a significant two-way interaction between time in culture and treatment condition (shaking vs. static cultures). There were statistically significant differences in cell viability in shaken vs. static cultures at 24, 48, 72, and 96 hr (Fig. 3B; Table 1). At each of the time points, cell viability in shaken cultures was significantly higher than those grown in static cultures. Thus, it was determined from these studies that conditions optimal for *H. chrysoscelis* erythrocyte survival in culture required daily media replacement and continuous shaking.

Since glycerol naturally accumulates in *H. chrysoscelis* during cold-acclimation, we also sought to determine whether the addition of glycerol to the media would affect erythrocyte viability. On average, 95% of erythrocytes remained viable after 96 hr in glycerol-containing media (250 mOsm CCCM+150 mM glycerol, 400 mOsm total), not statistically different from 96% viability in CCCM (250 mOsm) (Fig. 3C).

Temperature-Dependent Regulation of HC-3 Expression in Erythrocyte Cultures

Using the optimized in vitro cell culture system described above, we examined HC-3 expression in cultured erythrocytes under various culture conditions. To determine whether changes in in vitro culture temperature affect HC-3 expression, erythrocytes from cold- and warm-acclimated frogs were isolated and cultured at 4 and 20°C for 12, 24, 48, and 72 hr. A repeated measures ANOVA indicated that there was no significant interaction

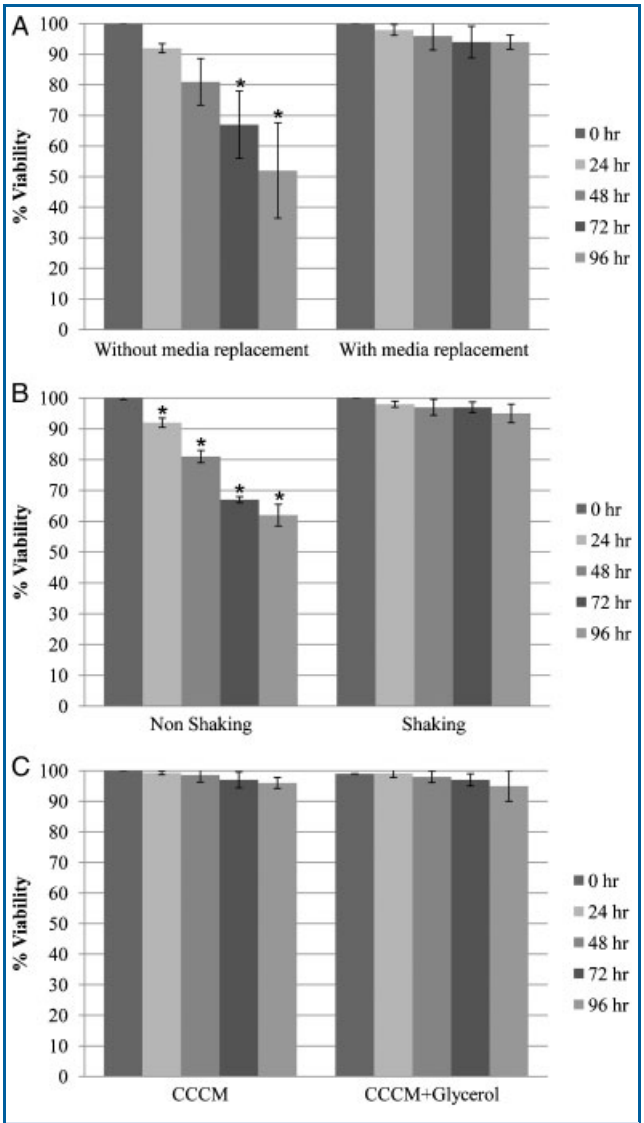

Figure 3. Determination of erythrocyte viability in optimized cell culture conditions. Erythrocytes were cultured in complete cell culture media (CCCM; 250 mOsM) (**A**) without or with media replacement every 24 hr; (**B**) without or with shaking (190 rpm); (**C**) without or with 0.156 M glycerol (400 mOsM media). Cells were removed from each culture at 24, 48, 72, and 96 hr, and cell viability was determined using Trypan blue exclusion staining. Viability at time zero represents the percentage of freshly harvested live erythrocytes. Results are shown as an average percentage of live cells in culture ± SD as compared with time zero ($n = 3$). A P-value < 0.05 was considered statistically significant (*).

between time and temperature when comparing native HC-3 protein expression in erythrocytes cultured at 4 vs. 20°C (Fig. 4A and C). Thus, native HC-3 expression remained constant throughout the 72-hr time course, regardless of culture temperature.

Similarly, the abundance of glycosylated HC-3 expression (>65–120 kDa) was not significantly different between time points when cultured at 4°C. However, there was a significant interaction between time and temperature: the abundance of glycosylated HC-3 in erythrocytes cultured at 20°C was statistically significantly higher at 24 and 48 hr when compared with the abundance of glycosylated HC-3 protein in cells cultured at 4°C for the same amount of time (Fig. 4A and E). There was a >10-fold increase in glycosylated HC-3 expression in erythrocytes cultured for 24 and 48 hr at 20°C as compared to expression in freshly isolated erythrocytes (time zero). The appearance of an uneven immunoreactive streak between 31.5 and 65 kDa observed at 20°C at the 24 and 48 hr time points in Figure 4A is likely due to retarded electrophoretic mobility of increased amounts of native HC-3 protein and was not interpreted as glycosylated HC-3 protein. Though expression of native HC-3 protein is not statistically significantly different from time zero ($P = 0.06$), there appears to be a moderate increase in native abundance.

In a similar fashion, cultured erythrocytes from warm-acclimated animals also demonstrated time- and temperature-dependent differential expression of glycosylated HC-3, whereas native HC-3 expression was not affected by time or culture temperature. There were no statistically significant differences in expression of native HC-3 protein between erythrocytes cultured at 4 or 20°C at any time point (Fig. 4B and D). But the expression levels of the high molecular weight bands (between >65 and 120 kDa) was significantly higher in erythrocytes cultured at 20°C at 24, 48, and 72 hr compared with the cells cultured at 4°C. Glycosylated HC-3 abundance was increased by 3- to 5-fold at 24, 48, and 72 hr in erythrocytes cultured at 20°C as compared with expression in freshly isolated erythrocytes (Fig. 4B and F). Thus cells cultured at 20°C regardless of the original source and acclimation state of the animal showed significantly higher expression levels of the high molecular weight immunoreactive bands (>65–100 kDa) relative to the 4°C cultures (Fig. 4A, B, E, and F). These data suggest that HC-3 expression in cultured erythrocytes is subject to post-translational modification via glycosylation that is regulated in part, by an interaction between factors related to the time in culture and culture temperature.

Deglycosylation of HC-3 in Cultured Erythrocytes

We suspected that the high molecular weight smear observed in our Western blots represented glycosylated forms of HC-3. Treatment of protein lysates with PNGase F, an enzyme that catalyses the release of *N*-linked glycan moieties from glycoproteins, resulted in collapse of the high molecular weight "smear" into discrete immunospecific bands at 23 kDa, 31.5 kDa (native HC-3), and 35 kDa (Fig. 5). The prominent 23-kDa immunoreactive band migrates at a molecular mass lower than that of native HC-3 (31.5 kDa). The reason for this is not clear. However, these results

Table 1. Percentage of cell viability of cultured erythrocytes from *Hyla chrysoscelis*.

Culture condition		Time in culture				
Media replacement	Shaking	0 hr	24 hr	48 hr	72 hr	96 hr
+	+	100	98 ± 1.1	96 ± 4.6	94 ± 5.0	95 ± 2.0
−	+	100	92 ± 1.5	81 ± 7.6	67 ± 11*	52 ± 5.6*
+	+	100	98 ± 1.0	97 ± 2.6	97 ± 1.7	95 ± 3.0
+	−	100	92 ± 1.5**	81 ± 2.0**	67 ± 1.0**	62 ± 3.4**

Values are represented as percent cell viability ± SD as compared with time zero (100%) ($n = 3$ for each time point for each condition).
*Pairwise comparison of media replacement vs. no media replacement with constant shaking, $P < 0.05$.
**Pairwise comparison of shaken cultures vs. static cultures with media replacement, $P < 0.05$.

are consistent with those previously published for PNGase F treatment of protein lysates from treefrog kidney in which the deglycosylated HC-3 protein was observed at ~25 kDa (Pandey et al., 2010). Results from similar experiments conducted on AQP3, the mammalian ortholog of HC-3 also show deglycosylated AQP3 protein with increased electrophoretic mobility on SDS-PAGE, resulting in immunoreactivity at a molecular mass lower than predicted for the monomeric form (~25 kDa vs. native 31.5 kDa; Roudier et al., 2002). Preabsorption of the HC-3 primary antibody with immunizing peptide resulted in the disappearance of these bands. Nonspecific bands were observed at 65 kDa in the presence and absence of the enzyme, whereas an additional nonspecific band of 55 kDa appeared in the presence of the enzyme (Fig. 5, arrowheads). These findings suggest that HC-3 is subject to post-translational modification by *N*-linked glycosylation, most prominently so in cells cultured at 20°C.

Evidence for Glycerol-Dependent Post-Translational Modification of HC-3 Expression in Erythrocyte Cultures

As stated above, *H. chrysoscelis* accumulates intracellular and extracellular glycerol as part of its freeze tolerance strategy. Because the distribution of this glycerol into and out of cells is likely to depend on transport via aquaglyceroporins, we tested whether the presence of glycerol in the media could induce changes in HC-3 abundance and/or localization. A repeated measures ANOVA indicated that there was no significant interaction between time and media composition when comparing native HC-3 protein expression in erythrocytes from cold-acclimated treefrogs (cultured at 4°C), or in erythrocytes from warm-acclimated treefrogs, (cultured at 20°C) in the presence or absence of glycerol (Fig. 6A–D). Native HC-3 abundance was not significantly different between erythrocytes cultured in the presence of glycerol vs. those cultured in the absence of glycerol at any timepoint within the 72-hr time course. This is true regardless of whether the erythrocytes were isolated from cold-acclimated frogs (Fig. 6A and C) or warm-acclimated frogs (Fig. 6B and D).

However, a repeated measures ANOVA indicated that there was a significant interaction between time and media composition when comparing glycosylated HC-3 expression. For erythrocytes isolated from cold-acclimated treefrogs and cultured at 4°C, the abundance of the high molecular weight bands (between >65 and 120 kDa) was significantly different at 24 and 48 hr in erythrocytes cultured in glycerol-containing media as compared with cells in media lacking glycerol. The expression of the high molecular weight moieties increased by 1.7-fold and 1.2-fold at 24 and 48 hr, respectively in glycerol-containing media as compared with expression in control media (Fig. 6A and E).

Similar results were obtained for erythrocytes originating from warm-acclimated treefrogs. The abundance of the high molecular weight glycosylated HC-3 protein was significantly different in erythrocytes cultured for 48 hr in glycerol-containing media as compared with expression in those cultured for 48 hr in control media (Fig. 6B and F). The level of glycosylated HC-3 protein increased by 2.2-fold at 48 hr in glycerol-containing media as compared with control media (Fig. 6B and F). These data indicate that the abundance of glycosylated HC-3 is regulated, in part, by the interaction between factors related to the time in culture and the presence of glycerol in the media.

Unlike the results presented above (Fig. 4B and F), erythrocytes harvested from warm-acclimated treefrogs and cultured under the same set of conditions in this set of experiments (i.e. 20°C in CCCM) did not respond similarly by increasing the abundance of glycosylated HC-3; rather, the amount of glycosylated HC-3 remained unchanged throughout the time course (Fig. 6B and F). It is possible that the differences in the gene regulatory responses seen between these two groups of experiments can be attributed at least in part, to the interindividual variation that exists in the physiology and genetics of wild-caught frogs.

Membrane Localization of HC-3 in the Presence of Glycerol

Consistent with the immunocytochemistry results from freshly isolated erythrocytes (Fig. 2A and B), erythrocytes from cold-acclimated treefrogs cultured at 4°C displayed prominent membrane expression. Immunofluorescence revealed a pronounced

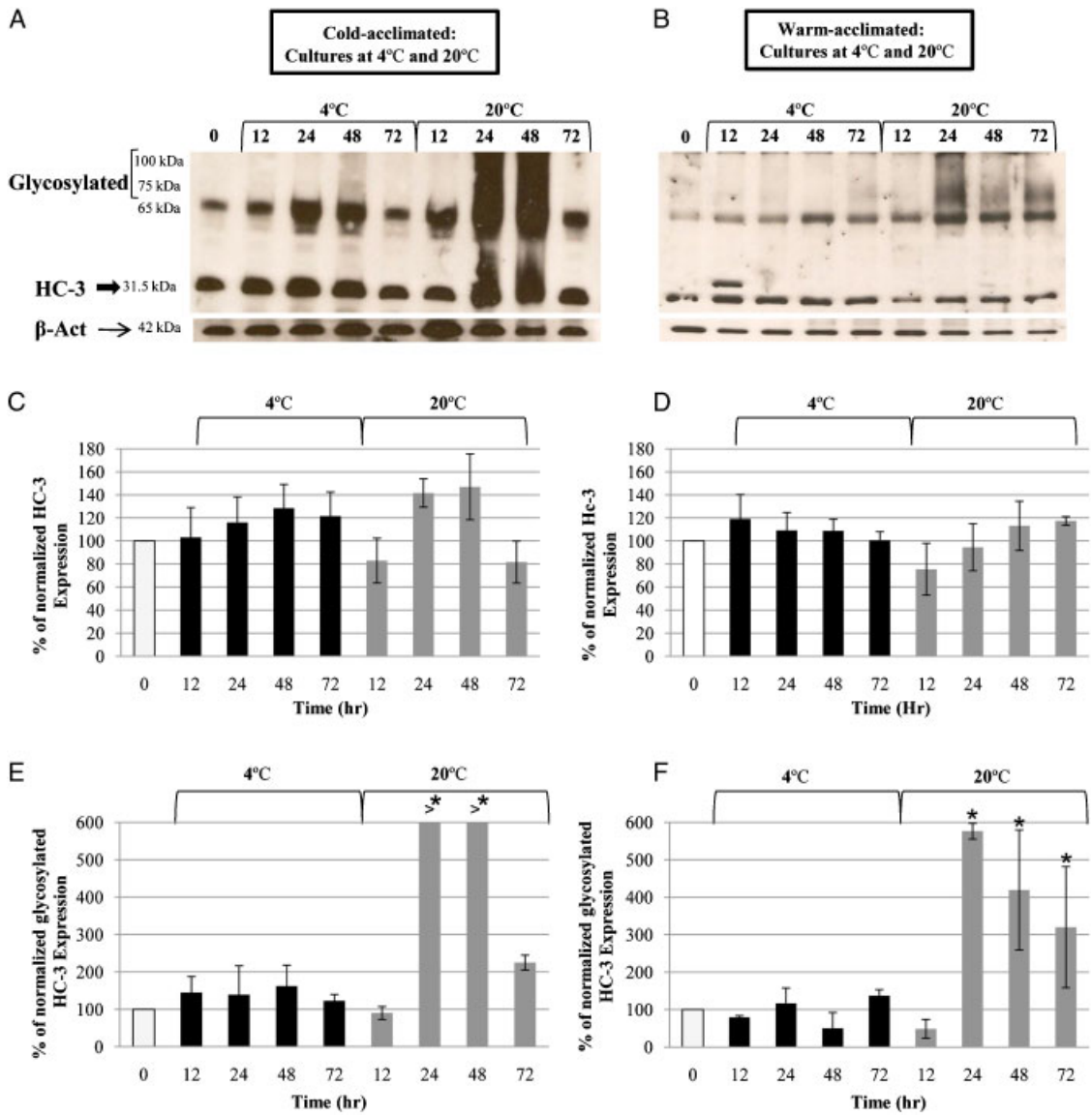

Figure 4. HC-3 protein expression in erythrocytes cultured at 4 and 20°C. Erythrocytes from cold- and warm-acclimated frogs were isolated and cultured at 4 or 20°C. The relative abundance of native (31.5 kDa) and glycosylated (between >65 and 120 kDa) HC-3 protein expression was detected by Western blotting of proteins isolated at 12, 24, 48, and 72 hr from erythrocytes, originating from (**A**) cold-acclimated frogs or (**B**) warm-acclimated frogs, and cultured at 4 and 20°C. β-actin expression (42 kDa) is shown as a control for the equivalency of protein loading. Densitometric analysis of native HC-3 expression (31.5 kDa) was used to determine relative HC-3 expression in erythrocytes from (**C**) cold-acclimated and (**D**) warm-acclimated frogs, cultured at 4 or 20°C ($n = 3$ observations for each condition). Densitometric analysis of HC-3 expression (>65–120 kDa) was used to determine relative glycosylated HC-3 expression in erythrocytes from (**E**) cold-acclimated and (**F**) warm- acclimated frogs cultured at 4 or 20°C ($n = 3$ observations for each condition). Results are represented as percentage of HC-3 expression (normalized to β-actin) relative to normalized HC-3 protein abundance at time zero from freshly isolated erythrocytes from (C, E) cold-acclimated and (D, F) warm-acclimated frogs (100%). A P-value < 0.05 was considered statistically significant (*). Percentages that exceed the maximum on the scale are indicated (>). The coefficient of variation (CV) of native HC-3 protein for freshly isolated erythrocytes from cold- and warm-acclimated frogs was determined to be 0.6 and 0.38, respectively, and for glycosylated HC-3 protein for freshly isolated erythrocytes from cold- and warm-acclimated frogs was determined to be 0.16 and 0.1, respectively.

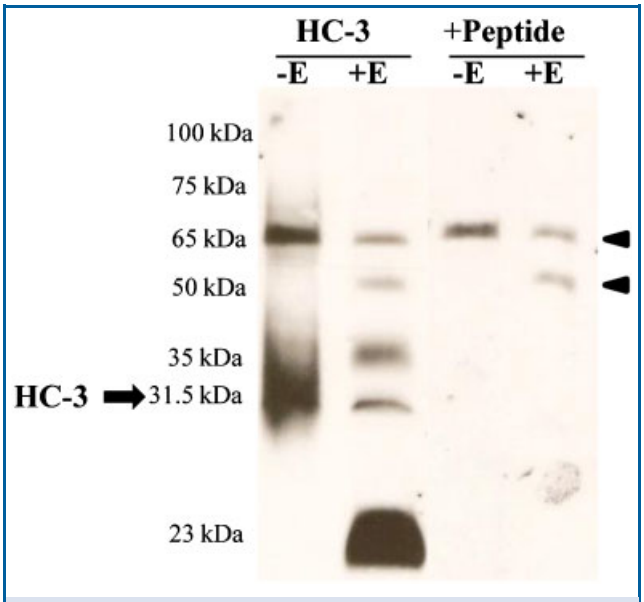

Figure 5. Deglycosylation of HC-3 protein. Protein lysates from erythrocytes cultured in CCCM at 20°C for 24 hr were digested with peptide-N-glycosidase F. HC-3 protein expression was assessed in control (−E) and deglycosylated (+E) protein lysates by Western blotting. HC-3 specific immunoreactivity was observed at 23, 31.5, and 35 kDa in the protein lysates subjected to deglycosylation. Preincubation of the HC-3 antibody with antigenic peptide blocked the 23-, 31.5-, and 35-kDa bands. The arrow indicates native HC-3 protein expression at 31.5 kDa. Arrowheads show the presence of nonspecific bands at 55 and 65 kDa.

increase in fluorescence intensity in the membrane when cells were cultured in media containing glycerol (Fig. 7). The shift in HC-3 localization to the membrane is concomitant with a reduction in the expression of green fluorescence in the internal membranous structures of the cytoplasm in both cold- and warm-acclimated cells (Fig. 7). It is not possible to discern from this assay whether the HC-3 protein that migrates to the plasma membrane is glycosylated or unglycosylated.

Interestingly, cells cultured in glycerol-containing media formed multicellular clusters and exhibited what appeared to be polarized expression of HC-3 along the membrane surface when cells abutted each other (Fig. 7B, D, F, and H). This effect was especially prominent in erythrocytes harvested from warm-acclimated treefrogs, cultured for 24 hr in glycerol-containing media (Fig. 7F) and was absent or much reduced in other conditions (e.g., without glycerol; Fig. 7A, C, E, and G), indicating that the polarized expression is unlikely to be a technical artifact from overlapping cells. A subset of erythrocytes cultured for 48 hr in the presence of glycerol also showed the opposite trend in HC-3 membrane polarization, where HC-3 expression appeared to polarize at the membrane surface not adjacent to a nearby cell (Fig. 7H). The reason for the observed polarization is not currently known, but the effect was reproducible and consistently occurred in glycerol-containing cultures. Thus, it appears that erythrocytes cultured in the presence of glycerol appear to regulate membrane trafficking of HC-3 from the cytosol to the plasma membrane in a manner consistent with the presence of enhanced membrane expression in erythrocytes from cold-acclimated frogs which naturally accumulate glycerol in vivo during the process of thermal acclimation in preparation for freezing.

DISCUSSION

The existence of the aquaglyceroporins and their ability to facilitate transmembrane flux of small solutes has been known for several years. Yet, an understanding of the functional role for this aquaporin subtype in animal physiology has only recently begun to emerge, with much of the characterization to date occurring in mammalian models. Erythrocytes from mammals express both the aquaporin AQP1 (Agre et al., '93) and at least one aquaglyceroporin. AQP3 is expressed in human and rat (Roudier et al., 2002) and AQP9 in murine erythrocytes (Liu et al., 2007). Functionally, human AQP3 and murine AQP9 have been shown to contribute to erythrocyte glycerol permeability, and have also been implicated in the process of intra-erythrocytic malarial infection (Liu et al., 2007; Bietz et al., 2009). It is clear that aquaglyceroporins function in mammals in numerous tissues to support normal glycerol metabolism, and may play other roles in the etiology of related disease pathologies.

Less is known about the potential physiological role for GLPs in anurans. We hypothesize that AQP/GLPs may function in the physiology of freeze tolerance in Cope's gray treefrog (Zimmerman et al., 2007). During the weeks or months of cold-acclimation, glycerol likely accrues from hepatic synthesis pathways concomitant with a possible contribution from triglyceride degradation in adipose tissue, and the solute is retained through diminished renal function and/or excretion (Schmid, '82; Storey and Storey, '85; Costanzo et al., '92; Kuriyama et al., '97; Layne and Jones, 2001; Irwin and Lee, 2003; Zimmerman et al., 2007; Layne and Stapleton, 2009). Gray treefrogs supercool by a few degrees before actual ice crystallization. Under those circumstances, ice formation is likely rapid once initiated, and the need for rapid exit of water from cells may necessitate aquaporin-based pathways. Functional studies of transmembrane transport of water and glycerol in other systems provide corroborative evidence for the probable involvement of AQP/GLPs in the process of freeze tolerance; aquaporins may confer the capacity for rapid water fluxes necessary during freezing (Muldrew and McGann, '94; Tanghe et al., 2004, 2005, 2006), whereas glyceroporins may enhance both exit of water and entry of cryoprotectant (Hagedorn et al., 2002; Yamaji et al., 2006; Izumi et al., 2006, 2007; Edashige et al., 2007; Philip et al., 2008). If freeze-induced damage does occur, aquaporins may be

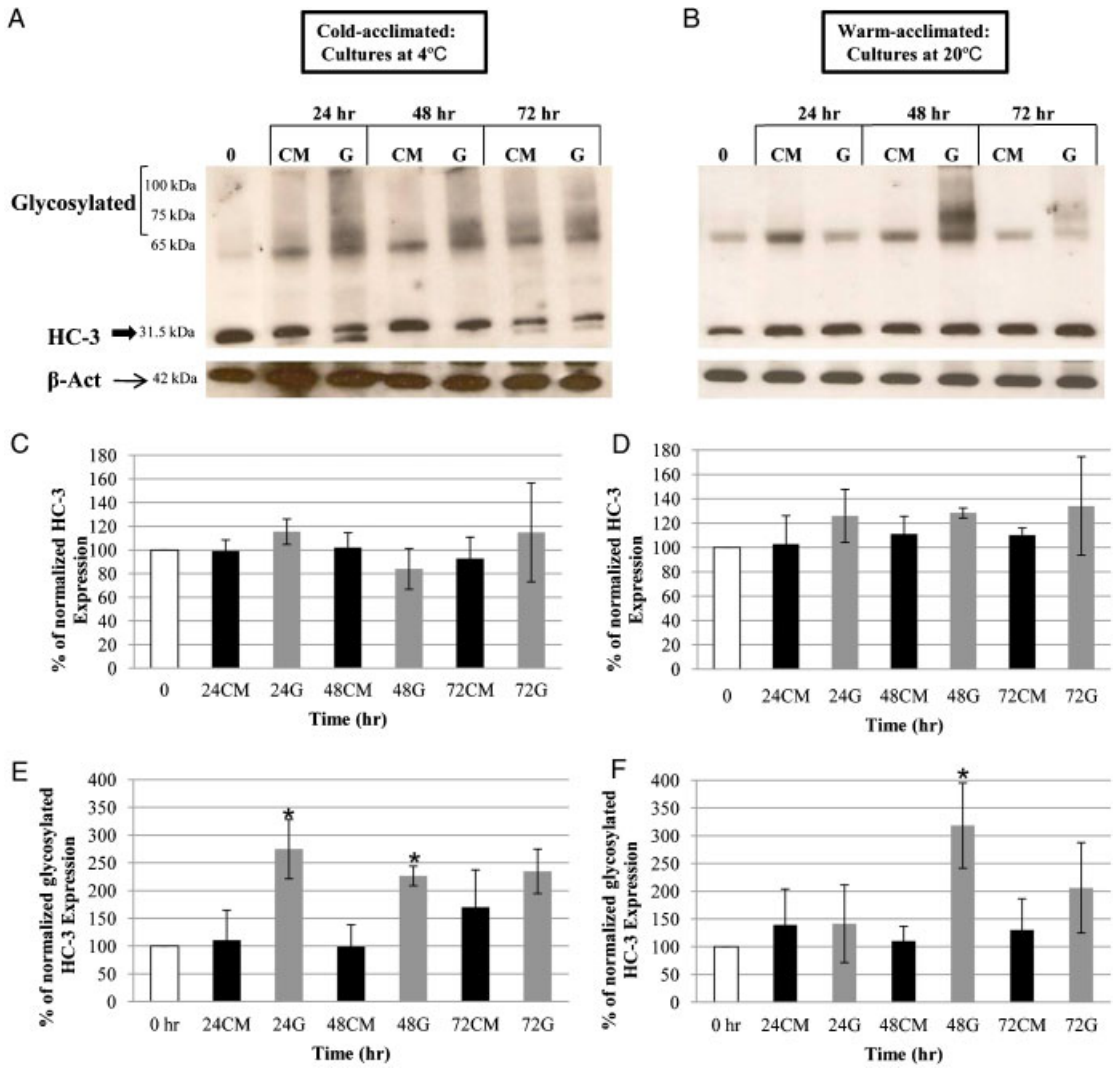

Figure 6. HC-3 protein expression in erythrocytes cultured in the absence (CM) or presence (G) of 0.156 M glycerol. HC-3 protein abundance was determined by Western blotting of proteins from cultured erythrocytes from (A) cold-acclimated or (B) warm-acclimated frogs were cultured at 4 or 20°C, respectively, in the absence (CM) or presence (G) of 0.156 M glycerol. Proteins were isolated from each erythrocyte culture at 24, 48, and 72 hr. HC-3 abundance (native HC-3 at 31.5 kDa and glycosylated HC-3 between >65 and 120 kDa) was determined by Western blotting. β-actin (42 kDa) is shown as a gel loading control. Densitometric analysis of native HC-3 expression (31.5 kDa) was used to determine relative HC-3 expression in (C) erythrocytes from cold-acclimated frogs cultured at 4°C in the absence or presence of glycerol, and (D) erythrocytes from warm-acclimated frogs, cultured at 20°C in the absence or presence of glycerol ($n = 3$ observations for each condition). Densitometric analysis of HC-3 expression (>65–120 kDa) was used to determine relative glycosylated HC-3 expression in (E) erythrocytes from cold-acclimated frogs cultured at 4°C in the absence or presence of glycerol, and (F) erythrocytes from warm-acclimated frogs, cultured at 20°C in the absence or presence of glycerol ($n = 3$ observations for each condition). Results are represented as percentage of HC-3 expression (normalized to β-actin) relative to normalized HC-3 protein abundance at time zero from freshly isolated erythrocytes from (C, E) cold-acclimated and (D, F) warm-acclimated frogs (100%). A P-value <0.05 was considered statistically significant (*). The CV of native HC-3 protein for freshly isolated erythrocytes from cold- and warm-acclimated frogs was determined to be 0.25 and 0.57 and of glycosylated form was found to be 0.075 and 0.073, respectively. CV, coefficient of variation.

important in cellular recovery and tissue healing (Costanzo et al., '93; Gallagher and Huang, '97; Hara-Chikuma and Verkman, 2008). Furthermore, overexpression of AQPs in *Saccharomyces cerevisiae* has been shown to improve freeze tolerance (Tanghe et al., 2002), whereas heterologous expression of AQP3 in fish embryos and artificial expression of AQP3 in mouse oocytes

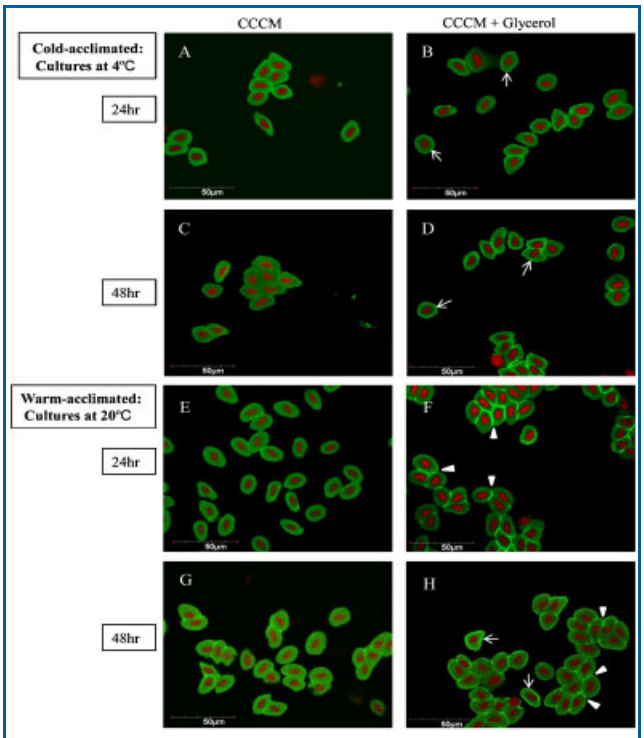

Figure 7. Immunofluorescence of HC-3 in erythrocytes cultured in the absence (CCCM) or presence of 0.156 M glycerol (CCCM+ Glycerol) in the media. Immunocytochemistry was used to determine the cellular/subcellular localization of 1. HC-3 in erythrocytes obtained from cold-acclimated frogs that were cultured for 24 hr (A) and 48 hr (C) at 4°C in the absence of glycerol or for 24 hr (B) and 48 hr (D) at 4°C in the presence of glycerol and 2. HC-3 in erythrocytes obtained from warm-acclimated frogs that were cultured for 24 hr (E) and 48 hr (G) at 20°C in the absence of glycerol, or for 24 hr (F) and 48 hr (H) at 20°C in the presence of glycerol. A fluroescein labeled secondary antibody was used to detect HC-3 immunoreactivity (green). Nuclei (red) are counter-stained with propidium iodide. Scale bars represent 50 µm. Arrows indicate HC-3 membrane expression. Arrowheads indicate areas of apparent polarized expression.

improves viability following cryopreservation (Hagedorn et al., 2002; Edashige et al., 2003).

We have previously shown that erythrocytes from *H. chrysoscelis* express the GLP HC-3, and they have high glycerol permeability that is inhibited by $HgCl_2$ (Goldstein et al., 2010). In this study, we further characterize HC-3 expression in erythrocytes from cold- and warm-acclimated treefrogs and describe the development of an in vitro erythrocyte culture system in which we can document dynamic regulation of HC-3.

Erythrocytes harvested from cold-acclimated frogs showed an increased abundance (2.3 fold increase) of native HC-3 protein expression, enhanced membrane localization, and upregulated post-translation modification via glycosylation as compared with expression in warm-acclimated treefrogs (Figs. 1 and 2). These results are consistent with the hypothesis that HC-3 expression is dynamically regulated in erythrocytes as part of the thermal acclimation process, and that its function in the membrane is important in preparing erythrocytes for tolerating freezing.

Based on this in vivo evidence for HC-3 regulation, we sought to establish and optimize an in vitro cell culture system that would support dynamic regulation of HC-3 expression in response to environmental cues using erythrocytes. We found that cultured erythrocytes remained viable for $\geq$96 hr with continuous shaking in culture media, and in media containing glycerol, when the media was replaced every 24 hrs (Fig. 3). Cultured erythrocytes maintained robust native HC-3 expression which did not significantly differ from expression in freshly isolated erythrocytes (Figs. 4 and 6). Perhaps the most informative for the analysis of HC-3-regulated expression are the observed HC-3 expression changes that consistently occur within and between culture conditions. Erythrocytes, regardless of the original acclimation state, showed a significant increase in the abundance of glycosylated HC-3 protein at 48 and 72 hr when cultured at 20°C as compared with 4°C indicating that HC-3 expression is subjected to time and temperature-dependant regulation in vitro. Similarly, erythrocytes cultured in glycerol-containing media consistently expressed significantly more glycosylated HC-3 at 48 hr than did erythrocytes cultured in CCCM. The effect of glycerol was evaluated by comparing cells exposed to CCCM (250 mOsM) or CCCM plus 150 mM glycerol (400 mOsM). Because the osmolarity of the media was different, it is not possible to be certain whether the observed effects are specific to glycerol or are the result of general cellular responses to hyperosmolarity. In the future, it will be possible to further address these mechanistic questions using the cell culture model system described here.

Furthermore, we showed that treatment of erythrocyte protein extracts with PNGase F resulted in the shift of immunoreactive bands toward the native conformer, indicating that a substantial fraction of HC-3 is subject to *N*-linked glycosylation (Fig. 5). *N*-linked glycosylation is a common post-translational modification in other aquaporins, including amphibian aquaporins. Western blots of AQP3 from human erythrocytes show a similar pattern of high molecular weight bands that correspond to the glycosylated form of human AQP3 (Roudier et al., 2002). Furthermore, AQP1 exists in both glycosylated and nonglycosylated functional monomeric forms, where approximately 50% of native red blood cell AQP1 is glycosylated (van Hoek et al., '95). Initial reports suggested that glycosylation of AQP1 was not required for either water transport of or for assembly. However, more recent reports have detailed that variations in the

proportion of glycosylated: nonglycosylated AQP1 is correlated to clinical diagnoses of disease and abnormal cell function (Ticozzi-Valerio et al., 2007). Approximately about 15–25% of AQP2 is glycosylated (Buck et al., 2004; Hendricks et al., 2004), and studies of mutant and wildtype AQP2 reveal that *N*-linked glycosylation facilitates protein transport from Golgi to the cell surface (Hendricks et al., 2004) and steady state stability of AQP2 proteins (Buck et al., 2004). However, the potential connections between internal glycosylation processes, membrane presentation, and cell surface exposure of oligosaccharides have not been thoroughly examined for any aquaporin. And even for AQP2, only a fraction of the protein present in the plasma membrane was glycosylated. Little has been published on how glycosylation may affect function and expression in different tissues under varying physiological conditions.

We also document that treefrog erythrocytes cultured in the presence of glycerol show evidence for trafficking of HC-3 to the plasma membrane, even in cells from warm-acclimated treefrogs (Fig. 7). As stated above, the interpretation of these data are somewhat confounded by the fact that the media was made hyperosmotic by the addition of glycerol. As such, the mechanistic link between glycerol presence and aquaporin expression remains unresolved. However, one possible contributory mechanism is that glycerol has been identified as a "chemical chaperone," which, in a subset of mutant AQP2 proteins, functions to correct defects in protein folding and trafficking (Tamarappoo and Verkman, '98; Marr et al., 2001). The mechanism(s) by which glycerol corrects these defects is (are) not currently known, though several explanations have been proposed (Tamarappoo and Verkman, '98; Molinari, 2007). It has been suggested that chemical chaperones, including glycerol, may act as osmolytes to modify the ER environment, or bind directly to nascent proteins to stabilize, promote appropriate folding, and prevent aggregation (reviewed in Molinari, 2007). Stability in the ER may also improve *N*-linked glycosylation, thereby resulting in a more stable protein structure (Molinari, 2007). Thus, if, in addition to its osmoregulatory role, glycerol acts in gray treefrogs as a chemical chaperone to stabilize proteins and cellular membranes during and after freezing, then HC-3 may be needed ubiquitously in the animal to support the cellular availability of that solute. Thus, HC-3, by facilitating glycerol influx, may regulate its own expression through post-translational glycosylation and enhanced membrane trafficking. If that is the case, then transmembrane proteins in addition to HC-3 may show similar membrane trafficking patterns as a result of HC-3 mediated glycerol accumulation. Further experiments are required to test this hypothesis.

In conclusion, the results of this study support the hypothesis that HC-3 is dynamically regulated in erythrocytes in vivo as a result of thermal acclimation, and that the patterns of regulated expression can be substantially recapitulated in vitro, in an optimized erythrocyte culture system. The erythrocyte culture system developed and characterized in this study can be used to further elucidate potential mechanisms of HC-3 regulation and function in erythrocytes, including the relation between post-translational glycosylation, cellular localization, and functional role of this aquaglyceroporin.

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