AQUAGLYCEROPORIN EXPRESSION AND REGULATION IN ERYTHROCYTES FROM FREEZE TOLERANT COPE’S GRAY TREEFROG, *HYLA CHRYSSOSCELIS*

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AQUAGLYCEROPORIN EXPRESSION AND REGULATION IN ERYTHROCYTES FROM FREEZE TOLERANT COPE’S GRAY TREEFROG, *HYLA CHRYSOSCELIS*.

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ABSTRACT

AQUAGLYCEROPORIN EXPRESSION AND REGULATION IN ERYTHROCYTES FROM FREEZE TOLERANT COPE’S GRAY TREEFROG, HYLA CHRYSOSCELIS.

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Cope’s gray treefrog, Hyla chrysoscelis is a freeze tolerant anuran that accumulates glycerol as a cryoprotectant during cold acclimation. Aquaporins, members of the major intrinsic protein (MIP) family of transmembrane water pores, may play an important role in the mechanism of freeze tolerance by mediating glycerol and water transport across cell membranes. Thus, we hypothesize that HC-3, an ortholog of mammalian aquaglyceroporin AQP3, enhances membrane permeability to glycerol, facilitating the cellular response to osmotic gradients formed when extracellular water freezes. To address the hypothesis, erythrocytes from H. chrysoscelis were used as an in vitro cell culture model to study the regulation of HC-3 protein expression. Compared with warm-acclimated frogs, erythrocytes from cold-acclimated frogs showed abundant HC-3 protein and enhanced plasma membrane localization of HC-3. Erythrocytes, regardless of the original acclimation state, exhibited time and temperature-dependent regulation of HC-3 expression and an increase in the abundance of high molecular weight immunoreactive proteins.
species within 24 hr of culture at 20°C. Likewise, erythrocytes cultured in glycerol-containing media consistently expressed relatively more glycosylated HC-3 than erythrocytes cultured in normal cell culture media. Thus, part of the regulation of HC-3 expression that occurs naturally during cold-acclimation is cell-based. Erythrocyte protein, when subjected to deglycosylation resulted in downward shift of high molecular weight HC-3 protein, demonstrating that HC-3 is post-translationally modified by N-linked glycosylation. In the absence of genomic knockout tools, a novel method of antisense HC-3 morpholino delivered in to cultured suspension erythrocytes via a peptide mediated Endo-Porter was developed where HC-3 protein expression was reduced by 94% in morpholino targeted cells (as assessed by Western blotting) as compared to controls. In addition, immunocytochemistry revealed a substantial decrease in HC-3 membrane expression in >65% of erythrocytes, with an additional 30% of erythrocytes showing no HC-3 expression, indicating that the method efficiently knocked down expression in >95% of cells. Furthermore, erythrocytes cultured for 48 hrs in media made hyperosmotic (400 mOsM) through the addition of 150 mM glycerol or urea showed enhanced membrane localization of HC-3 compared with those cultured in control media or media made hypertonic by addition of 150 mM sorbitol or 75 mM NaCl. The degree of hypotonic- induced cell shape changes were less for erythrocytes cultured in glycerol containing media compared to erythrocytes cultured in control media. In addition cell lysis data indicate that 60 % of erythrocytes cultured in glycerol or urea were still intact after 15 minutes as compared to controls, which were completely lysed within 15 minutes of challenge. Taken together, these studies strongly implicate a role for HC-3 in freeze tolerance in H. chrysoscelis.
Dedicated to Krishna and my guru Sadhguru Jaggi Vasudev
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LIST OF ABBREVIATIONS

AFPs……………….. Antifreeze proteins

AQPs……………….. Aquaporins

AQP0-AQP12……… Aquaporin 0-Aquaporin 12

AVP……………….. Arginine vasopressin

AVT……………….. Arginine vasotocin

ar/R………………. aromatic/Arginine

a.u………………….. arbitrary units

cAMP………………. Cyclic adenosine monophosphate

CCCM…………….. Complete cell culture media

CHIP28……………. Channel-like integral protein of 28 kDa

CM………………….. Control media

Co…………………. Colton null individuals

DMSO…………….. Dimethyl sulfoxide
ECF……………….Extracellular fluid

FA……………….Freeze avoidance

FT……………….Freeze tolerance

GFR…………….Glomerular filtration rate

GLPs…………….Aquaglyceroporins

HgCl₂………….Mercuric chloride

HIF -1α…………Hypoxia-inducible factor -1α

ICF…………….Intracellular fluid

IMCD………….....Inner medullary collecting duct

INPs…………….Ice-nucleating proteins

KO……………….Knockout

LA:SA…………..Long axis: Short axis ratio

MAPK………….Mitogen activated protein kinase

Min……………….Minutes

MIP………………Major intrinsic protein

NaCl…………….Sodium chloride

NDI……………...Nephrogenic diabetes insipidus
NPA………………..Asparagine, proline, alanine

$P_f$……………….Osmotic permeability coefficient

$P_{gly}$……………Glycerol permeability

PBS……………….Phosphate buffer saline

PMO………………Phophorodiamidate morpholino oligos

PMSF………………Phenylmethylsulfonyl fluoride

PNGaseF………………Peptide: N-glycosidase F

PVDF……………..Polyvinylidene difluoride

PKA………………Protein kinase A

RBCs………………Red blood cells

RT………………Room temperature

SCPs………………Supercooling points

SDS-PAGE…………Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec………………Seconds

TonE………………Tonicity responsive enhancer

Tmax………………Time taken to reach maximum LA: SA
CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Cold adaptations

In environments where organisms are exposed to subzero temperatures, animals survive by employing a combination of behavioral, physiological and biochemical strategies (Storey and Storey, 1988). Some animals avoid freezing (freeze avoidance; FA) by supercooling, whereby body water remains in a liquid state without freezing at temperatures of 0°C or below (Barnes, 1989). Most of the insect and arthropod species that survive at low temperatures fall under this category. The range of supercooling points (SCPs) varies from -10 to -30°C, although some arctic species supercool to below -60°C (Lee and Costanzo, 1998). The supercooing capacity in freeze avoidant species can be enhanced by three mechanisms; avoidance of ice-nucleating proteins (INPs), production of antifreeze proteins (AFPs) and accumulation of carbohydrate cryoprotectants (reviewed in Duman, 2001). Ice nucleating proteins lower the supercooling capacity by initiating the process of freezing on which ice crystals grow. Therefore, seasonal removal of INPs is a common adaptation among FA species (reviewed in Zachariassen, 1982). Many insects stop feeding and clear the gut prior to winter in order to avoid INPs (reviewed in Duman, 2001). AFPs adsorb onto the surface of ice crystals and prevent crystal growth by depressing the freezing point relative to the melting point using thermal hysteresis (reviewed in DeVries, 1983). AFPs, first
discovered in fish are also present in FA insects, plants, bacteria and fungi (reviewed in Clark and Worland, 2008). Further depression of SCP is achieved in part through the accumulation of high levels of membrane-permeant carbohydrate cryoprotectants such as glucose or glycerol. These cryoprotectants not only colligatively depress the freezing point, but also increase the supercooling capacity (reviewed in Costanzo, 2012). As opposed to FA strategies, freeze tolerant organisms withstand freezing of up to 50-65% of their total body water. It is thought that freeze tolerance (FT) evolved independently in diverse phylogeny in response to habitat, organismal physiology and temperature extremes (reviewed in Storey and Storey, 2005). A variety of species of microorganisms, plants, invertebrates and vertebrates employ FT as a part of overwintering strategy (reviewed in Storey and Storey, 1988).

**Mechanism of freeze tolerance**

The success or failure of FT is dependent upon the accumulation of carbohydrate cryoprotectants prior to freezing, which are essential for survival. In their absence, ice formation in the extracellular fluid leads to an increase in solute concentration as solutes are excluded from water, generating an osmotic gradient across the cell membrane (reviewed in Mazur, 1984). As a result of this osmotic imbalance, water efflux occurs, resulting in cell volume reduction and an increase in intracellular osmolarity leading to cellular dehydration (Figure 1). Moreover, ice crystals formed during freezing gradually increase in size, damaging the cell membranous structures (Storey, 2004). Thus cell death is due to both mechanical injury from ice crystals as well as hyperosmotic shrinkage of cells. Therefore FT organisms employ several adaptive strategies in order to prevent
injury from ice crystal damage and to maintain osmotic balance. Freeze tolerant insects
use endogenous INPs which help in preventing the formation of intracellular ice
(reviewed in Zachariassen, 1982). Additionally several FT insects and fish are known to
have AFPs whose fundamental function is to protect cell membranes from low
temperature damage (reviewed in Duman, 2001; Tomczak et al., 2002). However, most
FT organisms including FT anurans use cryoprotectant solutes such as glucose or
glycerol for protection against freeze-thaw induced cell damage (reviewed in Storey,
1990). Glucose and glycerol have been shown to be produced by the liver in response to
ice formation in some freeze tolerant anurans (Cai and Storey, 1997). These
cryoprotectants are low molecular weight carbohydrates that maintain cell homeostasis
by i) regulating the shifts in the fluid volume between the intra- and extracellular
compartments, ii) stabilizing membrane and protein structures and iii) preventing
intracellular ice formation (Storey et al., 1996). The same mechanism of cryoprotectant
production is also found in drought resistant plants which indicate that adaptations to cold
might have evolved from pre-existing strategies of desiccation resistance (Storey et al.,
1996). In anurans, the freezing process begins at the skin surface and propagates towards
internal structures. As freezing progresses, ice crystals are formed on skeletal muscle
fibers and the abdominal cavity is filled with a mass of ice (Storey and Storey, 1992). In
its fully frozen state, internal structures shrink, blood flow is completely shutdown,
cardiac activities are arrested and breathing is stopped. At this point approximately 65%
of total body water content turns into ice (Costanzo et al, 1995). During thawing, the first
vital sign detected is a renewed heart beat which allows circulation to be restored to the
rest of the organs (reviewed in Storey, 1990).
Freeze tolerance in frogs

Among amphibians, five species of North American anurans are known to have FT capabilities including wood frog (*Rana sylvatica*), gray treefrog (*Hyla versicolor*), Cope’s gray treefrog (*Hyla chrysoscelis*), spring peeper (*Hyla crucifer*) and western chorus frog (*Pseudacris triseriata*) (reviewed in Storey and Storey, 1988). Freeze tolerant anurans use glucose as a cryoprotectant except for those of the gray treefrogs (*H. versicolor* and *H. chrysoscelis*), which employ glycerol (Zimmerman et al., 2007; Layne and Stapleton, 2009). Upon contact with ice, *R. sylvatica* immediately triggers the breakdown of glycogen stored in the liver and glucose levels rise to 150-300 mM in central organs as compared to only 5 mM in unfrozen frogs (Costanzo et al., 1993). In contrast, *H. chrysoscelis* accumulates high concentrations of glycerol during cold acclimation (≥100 mM) which persists even after freezing (Layne and Jones, 2001; Irwin and Lee, 2003; Zimmerman et al., 2007). To maintain high levels of glycerol, gray treefrogs reduce glomerular filtration rate (GFR) and excretion rates during cold acclimation (Zimmerman et al., 2007). Thus the necessity for water and glycerol redistribution to different tissues is vital to many physiological processes required for survival of frogs during the events of freezing and thawing. The facilitated transmembrane transport of water and glycerol is thought to be mediated through the Major Intrinsic Protein (MIP) family of transmembrane channels, namely aquaporins (AQPs) and aquaglyceroporins (GLPs) (Zimmerman et al., 2007).
**Discovery of aquaporins**

Aquaporins (AQPs) are members of the MIP family of transmembrane pores that function to increase plasma membrane water permeability in response to osmotic gradients. The first water channel was isolated in 1991 by Dr. Peter Agre and colleagues while attempting to purify the 32 kDa Rh protein from red blood cells (Smith and Agre, 1991). The cRNA of this channel-like integral protein of 28 kDa (CHIP-28), when injected into oocytes of *Xenopus laevis* enhanced osmotic water permeability ($P_f$) and greatly increased the rates of hypotonic induced swelling. Merccuric chloride ($\text{HgCl}_2$), inhibited swelling of oocytes blocking the water permeability through the protein channel (Preston and Agre, 1991; Preston et al., 1992; Preston et al., 1993). With the discovery of more water channels, the common name of ‘aquaporins’ was introduced and CHIP-28 was renamed aquaporin 1 (AQP1) (Agre, 1997). For this breakthrough discovery, Dr. Peter Agre was a co-recipient of the 2003 Nobel Prize in Chemistry.

**Structure of aquaporins**

Hydropathy analysis of the primary amino acid sequence of AQP1 revealed six transmembrane $\alpha$ helices connected by three extracellular loops (A, C and E) and two intracellular loops (B and D) and cytosolic N and C termini (Jung et al., 1994) (Figure 2). Intracellular loop B and extracellular loop E contain highly conserved Asparagine-Proline-Alanine (NPA) motifs which fold back into the membrane forming an aqueous pore (Jung et al. 1994). The nucleotide sequence of the N and C terminal halves are closely related and thought to have evolved through an intragenic duplication event of the coding regions of the gene (Pao et al., 1991). The channel assembles as a homotetramer
though each monomer forms a functional water pore (Jung et al., 1994). Thirteen functionally and phylogenetically distinct AQPs, AQP0-AQP12 have been identified in mammals based on sequence homology to AQP1 (reviewed in Krane and Goldstein, 2007). AQP0, 1, 2, 4, 5, 6 and 8 are classical AQPs which are permeable to water alone whereas AQP 3, 7, 9, 10 are classified as GLPs that are permeable to water as well as select small organic solutes like glycerol and urea (Ishibashi et al., 2000; reviewed in Rojek et al., 2008). The third class of AQPs, AQP 11 and 12 are considered unorthodox AQPs sharing only 20% sequence homology with other MIP family members (reviewed in Rojek et al., 2008). A subset of aquaporin family members have also shown to transport arsinite (AQP7 and AQP9; Liu et al, 2002), anions (AQP6; Ikeda et al., 2002) and ammonia (AQP8; Saparov et al., 2007). The permeability property of AQP11 is still controversial. However studies by Yakata and colleagues show that AQP11 mediates a slow rate of osmotically-driven transmembrane water flux (Yakata et al., 2011).

Structural studies have significantly contributed to the understanding of the molecular basis of water/glycerol transport and selectivity through AQPs/GLPs. AQPs and GLPs differ from each other at five key amino acid positions (P1-P5) located in transmembrane domain 3 (P1), extracellular loop E (P2, P3) and transmembrane domain 6 (P4, P5) (reviewed in Krane and Goldstein, 2007, see also Figure 2). Replacement of amino acids (P1-P5) in AQPs by corresponding GLP-specific amino acid residues abolishes water transport, signifying the crucial role of these amino acids in aquaporin function (Heymann and Engel, 2000).

Large scale real-time molecular dynamics simulation studies have substantially contributed to the understanding of the mechanisms of water/glycerol permeation and
selectivity of AQPs/GLPs (Wang and Tajkhorshid, 2007). The rate of water transport and water selectivity is determined by the interaction of polar water molecules passing through the pore with amino acid side chains at two main positions within the pore. The first selection filter occurs at the ar/R (aromatic/Arginine) region in loop E, formed by Phe, His, Cys and Arg amino acid residues that create a restriction within the pore of maximum width of approximately 2.8\AA that is identical to the diameter of a single water molecule in AQPs (Sui et al., 2001). In GLPs, the ar/R region is wider (3.4 \AA) and exhibits a higher degree of hydrophobicity as compared to AQPs. As a result, GLPs can efficiently permeate small organic solutes like glycerol and urea. The fixed positive charges in the ar/R region prevents the passage of protons and other positive ions by disrupting the hydrogen bonds between water molecules and thus modify the orientation of water molecules flowing through the pore (Hub and de Groot, 2008). The second filter is a size exclusion filter formed by the two NPA motifs present in loops B and E that allows only small molecules to pass through the channel while excluding larger size particles. Thus the combination of charge and size restriction filters forms the basis for the permeability characteristics of AQPs and GLPs (de Groot and Grubmüller, 2001).

The binding of mercury to the Cys 189 residue present in ar/R region near the NPA motif in loop E blocks water permeation through most though not all AQPs. Mercury acts by disrupting the hydrogen bonds between the AQP/GLP protein and water molecules (Hirano et al., 2010). Nearly all AQPs/GLPs are mercury sensitive except for AQP4 and its orthologues, which are mercury insensitive due to lack of cysteine residue (Yukutake et al., 2008).
Expression of AQPs/GLPs

MIP family members are expressed in all living organisms ranging from bacteria and fungi to insects, plants and animals. Each of the thirteen known MIP family members identified in mammals has a unique tissue distribution (Zelenina et al., 2010) which is summarized in Table 1. In general, AQPs are expressed in cells of tissues responsible for secretory and absorptive processes (Table 1). Some AQPs are expressed in cells from a wide variety of tissues (i.e. AQP1) whereas other AQPs show high tissue and cell specific expression (i.e. AQP2; Table 1). The expression of aquaglyceroporins in mammalian tissues parallels the expression of several AQPs, especially in the kidney. However, GLPs have diverse yet specialized functions in select physiological and metabolic processes (reviewed in Krane and Goldstein, 2007). Erythrocytes from mammals express both AQP1 and a GLP (Agre et al., 1993). AQP3 is expressed in human and rat erythrocytes (Roudier et al., 2002) whereas AQP9 is expressed in murine erythrocytes (Liu et al., 2007). AQP3 and AQP9 are reported to contribute to erythrocyte glycerol permeability (Liu et al., 2007). AQP3 and AQP9 are expressed in rat, mouse and human, epidermal keratinocytes and function to facilitate transmembrane glycerol transport for proper skin hydration (Matsuzaki et al., 1999; Sougrat et al., 2002; Rojek et al., 2007). In mammalian kidney, AQP3 is expressed on the basolateral membrane of inner medullary collecting duct (IMCD) cells facilitates water efflux that enters through apically localized AQP2. AQP3 is important for urine concentration (Ecelbarger et al., 1995; Ishibashi et al., 1997; Coleman et al., 2000). AQP7 is expressed in mouse proximal straight tubules of the kidney participate in plasma glycerol reabsorption (reviewed in Sohara et al, 2009). Facilitated glycerol release through AQP7, expressed in mouse adipocytes is important
for fat metabolism (Skowronski et al., 2007). In humans and rodents, AQP9 is localized to the sinusoidal membrane of hepatocytes (reviewed in Rodriguez et al., 2011). Glycerol released into the bloodstream during fasting from adipocytes through AQP7 is transported into hepatocytes through AQP9 for gluconeogenesis (Maeda et al., 2009). The coordinated regulation of AQP7 and AQP9 expression in adipose and hepatic tissue respectively, is essential for the control of whole-body glucose homeostasis and lipid accumulation (reviewed in Rodriguez et al., 2011).

**Regulation of AQP/GLP gene expression**

The expression of functional aquaporin protein in the plasma membrane may be regulated at multiple levels, including transcription and translation, RNA or protein half life, post-translational modification, and membrane trafficking. Studies have shown that in the short term, regulation of AQP/GLP expression occurs rapidly in response to receptor-mediated cell signaling to support secretory or absorptive processes (reviewed in Zelinina, 2010). Transcriptional and translational control of AQP/GLP gene expression provides mechanisms for long-term regulation of gene expression in response to chronic physiological cues (reviewed in Zelinina, 2010). A number of studies have been published regarding the regulation of AQP2 expression in the principal cells of mammalian collecting duct (reviewed in Carbrey and Agre, 2009). AQP2 transcription and protein synthesis is upregulated in response to chronic dehydration (Hasler et al., 2002), whereas AQP2 membrane translocation is triggered by vasopressin (reviewed in Brown, 2003). Combined, these gene regulatory mechanisms are critical for kidney function and urinary concentrating mechanisms. (reviewed in Carbrey and Agre, 2009).
Glycosylation

The addition of sugar moieties (glycans) to a newly synthesized protein via covalent linkage, attached to either a nitrogen or oxygen of the amino acid side chains is referred to as N-linked glycosylation or O-linked glycosylation, respectively. It occurs in the endoplasmic reticulum (reviewed in Spiro, 2002). Glycosylated proteins are then transported to the golgi apparatus where N- or O-linked glycoproteins are further processed in the cis- and trans-golgi network by addition or removal of sugar residues producing a mature protein. The nascent glycoprotein exits the golgi, and in the case of aquaporins, resides within intracellular vesicles until exocytosis is triggered to translocate the pore to the plasma membrane (Zhou et al., 2005). Glycosylation appears to be important in protein folding, stability, and transmembrane insertion of many proteins including AQP1, -2, -4 as well as GLP, AQP3, -9 and -10 undergo N-linked glycosylation (Buck et al., 2004; Oberg et al., 2011). Fifty percent of AQP1 protein is glycosylated in red blood cells (van Hoek et al., 1995), whereas only 15-20% of AQP2 is glycosylated in collecting duct principal cells (Hendricks et al., 2004). Variations in the proportion of glycosylated: unglycosylated AQP1 have been associated with clinical diagnoses of disease and abnormal cell function (Ticozzi-Valerio et al., 2007). Using site-directed mutagenesis, the impact of N-linked glycans on the stability of the AQP2 glycoprotein was evaluated in X. laevis oocytes (Buck et al., 2004). Addition of N-linked glycans significantly improved the stability of mutant AQP2 protein, but did not have any effect on wildtype. Moreover glycosylated mutant AQP2 protein was more stable as compared to unglycosylated mutant AQP2 (Buck et al., 2004). Disruption of AQP2 glycosylation resulted in improper folding and
hence the proteins are not appropriately assembled. As a result, proteins were retained in the ER and targeted for degradation. Thus, one form of recessive nephrogenic diabetes insipidus (NDI) results from impaired intracellular transport of mutant AQP2 indicating the physiological significance of AQP2 glycosylation for AQP2 function (Deen et al., 1995; Marr et al., 2002).

**Vesicular trafficking and membrane insertion**

Vesicular trafficking of AQPs is a short term regulatory mechanism, happening within seconds leading to immediate changes in membrane permeability (reviewed in Zelinina, 2010). The regulation of membrane translocation is a common feature of several AQPs/GLPs, where it serves to provide acute, hormonal or nervous control of absorptive or secretory processes. Vesicular trafficking is best understood in AQP2 which is expressed in mammalian kidney principal cells of collecting duct. In response to dehydration, the antidiuretic hormone, arginine vasopressin (AVP) binds to the vasopressin V2-receptors in the basolateral membranes of the principal cells of the collecting duct initiating a cascade of events that lead to a rise in intracellular cAMP levels, protein kinase A (PKA) activation and AQP2 phosphorylation (Nielsen et al., 1995; van Balkom et al., 2002). Intracellular vesicles containing phosphorylated AQP2 are targeted and fuse to the apical membrane enhancing plasma membrane water permeability and water reabsorption (Kuwahara et al., 1995).

In addition, other AQP/GLPs undergo vesicular trafficking in response to a number of different physiological signals. For example, AQP5 translocates from intracellular vesicles to the apical membrane of rat parotid gland cells in response to
epinephrine to facilitate salivary secretion (Ishikawa et al., 2005). Vesicular trafficking and membrane insertion of AQP8 in rat hepatocytes is regulated by glucagon (Gradilone et al., 2003). Epinephrine triggers vesicular trafficking to the membrane in cultured Caco-2 cells to support glycerol absorption process in gastrointestinal tract (AQP3; Yasui et al., 2008). Under steady state conditions, AQP3 and AQP7 reside within intracellular vesicles of mouse adipocytes. Adipocytes on stimulation with isoproterenol and epinephrine respectively, promote translocation of AQP3 and AQP7 proteins to the membrane, promoting glycerol release (Kishida et al., 2000; reviewed in Rodriguez et al., 2011).

**Tonic control of AQP/GLP gene expression**

Several AQPs have been shown to be regulated by increased osmotic stress. Hypertonicity upregulates AQP1 mRNA expression in cultured mouse IMCD cells via a hypertonicity response element found in the AQP1 promoter (Jenq et al., 1998; Umenishi and Schrier, 2002). Hypertonicity increases AQP5 transcription in murine lung epithelial cells through a mitogen activated protein kinase (MAPK) signaling cascade (Hoffert et al., 2000). Hypertonicity also activates hypoxia-inducible factor-1α (HIF1 α) to bind to the HIF1 α binding site in the AQP5 promoter to induce AQP5 transcription (Zhou et al., 2007). AQP9 mRNA and protein expression is also upregulated through hypertonicity-induced MAPK in cultured rat astrocytes (Arima et al., 2003). Similarly hypertonicity-induced transcription factor binding of the tonicity responsive enhancer (TonE) present within the AQP9 promoter upregulates AQP9 mRNA expression (Bell et al., 2009). Phosphorylation and membrane trafficking of AQPs is also regulated by hyper- and/or hypotonicity. Hypotonicity results in a decrease in AQP2 Ser 256 phosphorylation and
AQP2 removal from the membrane in cultured renal CD8 cells (Tamma et al., 2007). In contrast, hypertonicity enhances AQP2 membrane localization in rat collecting duct cells (Hasler et al., 2008). These results indicate that the osmotic stress influences the expression of GLPs.

**Pathophysiology of AQPs/GLPs**

In humans, genetic mutations in select AQP/GLP genes results in clinical disease. For example, a genetic mutation in AQP0 cause congenital cataracts, suggesting that normal AQP0 function is crucial in maintaining transparency in the eye (reviewed in Chepelinsky, 2009). Humans lacking AQP1 protein were identified as Colton (Co) null individuals who lack the Colton blood group antigen (Preston et al., 1994). Interestingly, Colton-null individuals do not show any clinical symptoms under normal physiological conditions; however, they display a mild urinary concentrating defect under controlled clinical tests of water deprivation stress (King et al., 2001). Genetic forms of NDI are caused by mutations in AQP2. Autosomal dominant NDI is caused by AQP2 mutations that affect protein processing, folding, and membrane translocation, whereas recessive NDI results from missense, deletion, and frameshift mutations that truncate the protein, or render it non-functional in the membrane (Marr et al., 2002). In addition, it has been reported that downregulation of AQP5 in the salivary glands of Sjogren’s syndrome correlates with the phenotype, suggesting a role for AQP5 dysregulation in this disease (Steinfeld et al., 2001).

Knockout (KO) mouse models further provide considerable insights into the role of AQPs and GLPs in whole animal physiology. Table 2 summarizes the phenotypes of
AQP null mice generated and characterized to date. Deficiencies in the AQP family of proteins confirm a role for AQPs in absorptive and/or secretory processes in the kidney, eye, sweat glands, and lung (Table 2). In addition, the KO mouse models provide evidence for additional roles for AQP family members in hearing, cell migration, response to brain injury and fertility (Table 2). Moreover, mice deficient in select GLP family members have begun to provide valuable information about the function of GLPs in vivo. AQP3 null mice show increased urinary concentrating defects, and severe polyuria (Ma et al., 2000B). In addition, the $P_f$ of the basolateral membrane of the cortical collecting duct cells is significantly reduced in AQP3 KO mice compared with wild-type control mice highlighting the physiological role of AQP3 in water reabsorption in the kidney (Ma et al., 2000B). Reduced stratum corneum hydration, defective hydration response to high humidity, delayed wound healing and decreased skin elasticity are phenotypes of AQP3 KO mice (Hara et al., 2002). The $P_f$ of epidermal skin cells and glycerol permeability of keratinocytes, both were significantly reduced in AQP3-KO mice (Ma et al., 2002). The skin abnormalities were corrected, when mice were administered glycerol either topically or systemically, implicating an important role of glycerol in skin hydration (Hara and Verkman, 2003). Impaired glycerol transport in AQP7 KO mice leads to accumulation of glycerol within adipocytes, resulting in adipocyte hypertrophy (Hara-Chikuma et al., 2005). AQP7 KO mice also become obese as adults (Hibuse et al., 2005). Therefore, it has been suggested that the lack of AQP7 in adipocytes might result in a reduction of glycerol release which contributes to an increased production of triglycerides in adipocytes causing obesity (reviewed in Rojek et al., 2008). Plasma glycerol levels are significantly increased in AQP9 KO mice, indicating a reduction in the uptake of glycerol
through the hepatocyte plasma membrane (Rojek et al., 2007). Thus AQP9 provides the primary route for hepatocyte uptake of glycerol required for gluconeogenesis (Jelen et al., 2011). Moreover, mouse erythrocytes lacking AQP9 show a significant reduction in rapid transmembrane glycerol transport in response, indicating a role for AQP9 in the membrane permeability of erythrycotyes to glycerol (AQP9; Liu et al., 2007). Thus several lines of evidence demonstrate a vital role for GLPs in many different physiological functions including production of concentrated urine, fat and glycerol metabolism and skin hydration. Thus, based on these known functions of AQP/GLPs it is possible that AQPs/GLP might play an important role in freeze tolerance through affecting the process of glycerol accumulation during cold-acclimation in FT anurans.

**AQPs in anurans**

Anurans have successfully adapted to living in different environments, ranging from aquatic/semi-aquatic to terrestrial habitats. Expression of AQPs in several tissues including three major osmoregulatory organs (skin, kidney and urinary bladder) is crucial for maintaining fluid homeostasis in anurans (reviewed in Suzuki and Tanaka, 2010). To date, anuran orthologs for AQP0,1,2,3,4,5,7,8,9,10, and 11 have been identified by targeted cloning, shotgun EST sequencing, and/or whole genome sequence (i.e., *Silurana tropicalis*) homology searches. Except for AQP6, and AQP12, orthologs to all other mammalian AQPs have been identified in anurans. In addition, two anuran-specific categories of AQPs, specified as AQPa1 and AQPa2 are not expressed in mammals (reviewed in Suzuki and Tanaka, 2010; Table 3). Type AQPa1 includes *X. laevis* AQP and AQPxlo from *X. laevis* oocytes (Virkki et al., 2002). The class of type AQPa2 is
composed of AQP-h2 (Hasegawa et al., 2003), AQP-h3 (Tanii et al., 2002) from *H. japonica* and AQP-t2 and AQP-t3 from *Bufo marinus* (Table 3).

The gene regulatory mechanisms that control the expression of AQPs in anurans are similar to mammals. In *H. japonica* AQP-h2K which is expressed in the principal cells of the kidney collecting duct has two phosphorylation sites, a PKC phosphorylation site at Ser-236 and a PKA phosphorylation site at Ser-262 (Ogushi et al., 2007). In addition, AQP-h2 expressed in principal cells of central pelvic skin has one PKA phosphorylation site at Ser-256 (reviewed in Suzuki and Tanaka, 2009). In response to dehydration, antidiuretic hormone arginine vasotocin (AVT), AQP-h2K through cAMP-PKA signaling pathway (similar to AQP2 activation in mammalian kidney collecting duct cells) is translocated from intracellular vesicles to the apical membrane where it functions to mediate water influx from tubular fluid and may exit through AQP-h3BL localized on basolateral membrane (Akabane et al., 2007; Ogushi et al., 2007). In *H. chrysoscelis*, HC-2 expressed on apical surface and HC-3 on the basolateral surface of renal collecting duct cells similar to that observed in *H. japonica* might participate in AVT-dependent reabsorption of water (Pandey et al., 2010).

The nucleotide and amino acid sequence of HC-1, -2 and -3 from *H. chrysoscelis* showed strong identity with human AQP1,-2 and -3 (Zimmerman et al., 2007). The amino acid positions at P1-P5 differ significantly between HC-1, -2 and HC-3 and hence HC-1 and HC-2 are categorized as classical AQPs and HC-3 as GLP. Functional analysis using *X. laevis* oocytes confirmed water permeability for HC-1 and HC-2, whereas HC-3 showed high glycerol permeability and low water permeability (Zimmerman et al., 2007). Both water and glycerol (HC-3 alone) permeabilities were inhibited in the presence of 0.3
mM HgCl$_2$. Real time PCR and immunofluorescence analysis confirmed the expression of *H. chrysoscelis* HC-1, -2 and -3 in several tissues. HC-1 mRNA is expressed in multiple tissues including brain, lung, liver, skin, fat body, kidney, eye, skeletal muscle, gut and bladder in both warm and cold-acclimated frogs. However, HC-2 mRNA and protein expression are restricted to the osmoregulatory organs including skin, kidney and urinary bladder (Zimmerman et al., 2007; Pandey et al., 2010). HC-3 mRNA is expressed in brain, lung, liver, skin, fat body, kidney, eye, skeletal muscle, gut and bladder (Zimmerman et al., 2007). Increased levels of glycerol accumulated during cold-acclimation period parallels with high expression of HC-3 mRNA in liver, muscle and bladder, indicating that HC-3 may participate in glycerol transport during cold acclimation (Zimmerman et al., 2007). Immunofluorescence further confirmed HC-3 protein expression in skin, liver, kidney, skeletal muscle, gut, brain, bladder and erythrocytes of *H. chrysoscelis* (Pandey et al., 2010). HC-3 protein is more abundant in erythrocytes from cold-acclimated treefrogs compared with warm-acclimated treefrogs (Goldstein et al., 2010). In addition, erythrocytes expressing HC-3 show high glycerol permeability, which is abolished by HgCl$_2$, a known AQP/GLP inhibitor, suggesting that glycerol permeability is facilitated through GLPs (Goldstein et al., 2010). These findings further suggest a role for HC-3 in mediating transmembrane glycerol flux during the process of cold-acclimation in preparation for freezing.

**Corroborating evidence supporting a role for AQP/GLP in FT**

AQP/GLP expression in other systems has already been implicated in FT. For example studies in FT insects demonstrated the role of these channels in mediating transmembrane
transport water/glycerol indicating that AQP/GLPs may participate in the physiology of FT (Philip et al., 2011; Yi et al., 2011). Enhanced AQP/GLP expression correlates with improved FT in baker’s yeast (Tanghe et al., 2002) and artificial AQP/GLP expression improves viability following cryopreservation of fish embryos (Hagedorn et al., 2002) and mouse oocytes (Edashige et al., 2003), suggesting facilitated glycerol transport through AQP/GLP. Taken together, these data strongly implicate a role for AQP/GLP, specifically HC-3 in FT of *H. chrysoscelis*.

**Hypothesis**

Based on previous results, it was hypothesized that HC-3, an ortholog of mammalian AQP 3, enhances membrane permeability to glycerol which acts as a natural cryoprotectant to regulate osmotic gradients formed by extracellular ice crystal formation during freezing. The objectives of this dissertation were:

1) to establish an *in vitro* cell culture model using erythrocytes from *H. chrysoscelis* to examine dynamic changes in HC-3 protein expression and subcellular localization observed in warm- vs cold-acclimated frogs (Chapter II).

2) to develop a method to knockdown HC-3 expression in cultured erythroctres (Chapter III).

3) to determine AQP/GLP-dependant responses to hypotonic challenge in cultured erythrocytes as it correlates with AQP/GLP expression (Chapter IV).
Significance

Although previous studies showed that glycerol transport is mediated by GLPs, the regulation of expression of these proteins, the cellular processing that leads to expression in the membrane, and the definitive role of these proteins in osmoregulation during FT remains poorly understood. The use of erythrocytes from *H. chrysoscelis* provides a simplified system to study the regulation of GLP, HC-3 expression and also analyze the molecular mechanisms that regulate these expression levels. In studying these processes, the mechanisms of amphibian FT can be elucidated in particular, but more generally will contribute to the understanding of cryoprotection.
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Mutations in aquaporin-1 in phenotypically normal humans without functional

Aquaglyceroporins serve as metabolic gateways in adiposity and insulin resistance

Severe urinary concentrating defect in renal collecting duct-selective AQP2


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<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Phylogenetic AQP/GLP</th>
<th>Functional Permeability</th>
<th>Tissue/Cellular Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP0</td>
<td>AQP</td>
<td>Water</td>
<td>Lens of the eye</td>
</tr>
<tr>
<td>AQP1</td>
<td>AQP</td>
<td>Water</td>
<td>Kidney (proximal tubule and thin descending limb of the loop of Henle), erythrocytes, capillary endothelium, choroid plexus, corneal epithelium, ear, lung, GI tract, skeletal muscle, heart, muscle</td>
</tr>
<tr>
<td>AQP2</td>
<td>AQP</td>
<td>Water</td>
<td>Kidney (Principal cells of the collecting duct and connecting tubules; apical surface and subapical vesicles)</td>
</tr>
<tr>
<td>AQP3</td>
<td>GLP</td>
<td>Urea and Glycerol; Water</td>
<td>Kidney (Principal cells of the collecting duct and connecting tubules; basolateral surface), airways, lung, GI tract, brain, ear, urinary bladder, cornea, skin, erythrocytes (human, rat)</td>
</tr>
<tr>
<td>AQP4</td>
<td>AQP</td>
<td>Water</td>
<td>Kidney (collecting duct principal cells; basolateral), retina, ear (rat), airways, lung, GI tract, fast-twitch skeletal muscle, glial cells at blood brain barrier, astrocytes, ovaries</td>
</tr>
<tr>
<td>AQP5</td>
<td>AQP</td>
<td>Water</td>
<td>Salivary gland, lacrimal gland, trachea, epithelia of nasopharynx and airways, alveolar type 1 cells, ear (rat), eye, placenta, pancreas</td>
</tr>
<tr>
<td>AQP6</td>
<td>AQP</td>
<td>Anions (NO$_3$ and Cl$^-$); Water</td>
<td>Kidney (intracellular vesicles in type A intercalated cells of the collecting duct), parotid acinar cells</td>
</tr>
<tr>
<td>AQP7</td>
<td>GLP</td>
<td>Urea and Glycerol; Water, Arsenite</td>
<td>Testis, sperm, kidney (proximal tubule), adipose tissue, skeletal muscle, eye, cardiac tissue</td>
</tr>
<tr>
<td>AQP8</td>
<td>AQP</td>
<td>Urea and NH$_3$ Water</td>
<td>Testis, sperm, ovaries, GI tract, placenta, kidney (proximal tubule and collecting duct), airways, liver, salivary glands (rat), glial and neuronal cells, pancreas</td>
</tr>
<tr>
<td>AQP9</td>
<td>GLP</td>
<td>Urea and Glycerol; Water, Arsenite</td>
<td>Liver, testis, sperm, spleen, brain, leukocytes, kidney, lung, brain (astrocytes and ependymal cells), eye, skin, erythrocytes (murine)</td>
</tr>
<tr>
<td>AQP10</td>
<td>GLP</td>
<td>Urea and glycerol; Water</td>
<td>Duodenum, jejunum</td>
</tr>
<tr>
<td>AQP11</td>
<td>SuperAQP</td>
<td>Water</td>
<td>Kidney, liver, testis (rat), brain, eye, salivary gland</td>
</tr>
<tr>
<td>AQP12</td>
<td>SuperAQP</td>
<td>Unknown</td>
<td>Pancreas (acinar cells)</td>
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### Table 2: Phenotypes of MIP-deficient mouse strains

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Phenotype of MIP Deficient Mouse Strains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP0</td>
<td>Cataracts</td>
<td>Shiels and Bassnett, 1996</td>
</tr>
<tr>
<td>AQP1</td>
<td>Polyuria, defective proximal tubule fluid absorption</td>
<td>Ma et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Decreased osmotic water permeability across endothelium</td>
<td>Bai et al., 1999</td>
</tr>
<tr>
<td>AQP2</td>
<td>Severe polyuria; failure to thrive</td>
<td>Rojek et al., 2006</td>
</tr>
<tr>
<td>AQP3</td>
<td>Urinary concentrating defect—NDI</td>
<td>Ma et al., 2000 B</td>
</tr>
<tr>
<td></td>
<td>Reduced skin hydration &amp; elasticity</td>
<td>Ma et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Delayed wound healing</td>
<td>Hara et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Impaired tumorigenesis in skin</td>
<td>Hara-Chikuma and Verkman, 2008</td>
</tr>
<tr>
<td>AQP4</td>
<td>Mild urinary concentrating defect</td>
<td>Ma et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Reduced injury-induced brain edema</td>
<td>Manley et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Hearing defects</td>
<td>Li and Verkman, 2001</td>
</tr>
<tr>
<td></td>
<td>Defective folliculogenesis- Reduced fertility</td>
<td>Sun et al., 2009</td>
</tr>
<tr>
<td>AQP5</td>
<td>Impaired salivary secretion</td>
<td>Ma et al., 1999; Krane et al., 2001 B</td>
</tr>
<tr>
<td></td>
<td>Airway hyperresponsiveness to cholinergic stimulation</td>
<td>Krane et al., 2001 A</td>
</tr>
<tr>
<td></td>
<td>Impaired stimulated sweat secretion</td>
<td>Nejsum et al., 2002</td>
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<tr>
<td></td>
<td>Decreased osmotic water permeability across alveolar epithelium</td>
<td>Ma et al., 2000 A</td>
</tr>
<tr>
<td></td>
<td>Impaired secretion in airway submucosal glands</td>
<td>Song and Verkman, 2001</td>
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<tr>
<td>AQP6</td>
<td>Unknown</td>
<td></td>
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<tr>
<td>AQP7</td>
<td>Increased body fat with adipocyte hypertrophy</td>
<td>Hara-Chikuma et al., 2005</td>
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<tr>
<td></td>
<td>Increased body weight and age-dependent insulin resistance</td>
<td>Hibuse et al., 2005</td>
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<tr>
<td></td>
<td>Cardiac hypertrophy and heart failure</td>
<td>Hibuse et al., 2009</td>
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<tr>
<td>AQP8</td>
<td>Mild hypertriglyceridemia</td>
<td>Yang et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Reduced apoptosis of granulosa cells- Increased fertility</td>
<td>Su et al., 2010</td>
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<tr>
<td>AQP9</td>
<td>Defective glycerol metabolism in liver</td>
<td>Rojek et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Increased resistance to malarial infection</td>
<td>Liu et al., 2007</td>
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<tr>
<td>AQP10</td>
<td>Unknown</td>
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<tr>
<td>AQP11</td>
<td>Polycystic kidney disease (proximal tubule)</td>
<td>Morishita et al., 2005</td>
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<td>AQP12</td>
<td>Acute Pancreatitis</td>
<td>Ohta et al., 2009</td>
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<td>Mammalian AQP</td>
<td>Anuran AQP</td>
<td>Anuran Species</td>
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<td>----------------</td>
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<tr>
<td>AQP0</td>
<td>AQP0</td>
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<td>AQP1</td>
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<td>AQP-x5</td>
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<td>AQP9</td>
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<td>AQP10</td>
<td>Silurana tropicalis</td>
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<td>AQP11</td>
<td>Silurana tropicalis</td>
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<tr>
<td>Anuran specific</td>
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<td>AQP</td>
<td></td>
<td>Xenopus laevis</td>
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<td>AQPxlo</td>
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<td>Bufo marinus</td>
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<td>Hyla japonica</td>
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<tr>
<td>AQP-t3</td>
<td></td>
<td>Bufo marinus</td>
</tr>
</tbody>
</table>
Under cold temperatures formation of ice begins in the extracellular fluid (ECF). As water freezes, solutes are excluded and the osmolarity of the remaining fluid increases drastically resulting in cell shrinkage as water is withdrawn from the cell. In an unprotected state, rapid growth of ice crystals cause damage to membrane and cell death. Freeze tolerant organisms use cryoprotectant glycerol which acts by minimizing the cell volume reduction and limiting the size of the ice crystals. Figure adapted from Storey, (2004).
Figure 2: *Aquaporin structure.*

Protein conformation analysis of the AQP family suggests a six transmembrane spanning topology connected by five loops (A-E) with cytosolic amino carboxy termini. Highly conserved NPA (Asparagine, proline, alanine) motifs are present in loops B and E. The cysteine residue located in loop E confers mercury sensitivity in the majority of AQP proteins. Amino acid positions P1-P5 differ between members of AQP and GLP subfamilies. Figure taken from Krane and Goldstein (2007).
CHAPTER II

DYNAMIC REGULATION OF AQUAGYCEROPORIN EXPRESSION IN ERYTHROCYTE CULTURES FROM COLD- AND WARM- ACCLIMATED COPE’S GRAY TREEFROG, *HYLA CHRYSOCELIS*.

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Abstract

Cope’s gray treefrog, *Hyla chrysoscelis*, is a freeze tolerant anuran which accumulates and distributes glycerol as a cryoprotectant prior to 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°C 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.
**Introduction**

*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 (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; Tani 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 upon 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 two 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 four weeks prior to 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 non-recovery 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*, collected in heparinized capillary tubes. In order to attain sufficient number of erythrocytes for each experiment, and to control for the inter-individual variation that naturally exists between non-inbred individual gray treefrogs collected from the wild, blood was pooled from 3-4 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, Carlsbad, CA), 5% fetal bovine serum (Fisher Scientific, Hanover Park, IL)}. Blood samples were centrifuged at 1000 x g for 10 min. Approximately 5x10^6 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 cm² 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°C or 4°C in flasks positioned upright at a 45 degree angle on a plate shaker with constant rotation (190 rpm). Media was 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 manufacturer’s instruction (Invitrogen, Carlsbad, CA).

**Western blot analysis**

HC-3 protein expression was analyzed by Western blotting using established methods (Krane et al., '99). Approximately 1x10^6 erythrocytes were collected from cultures at selected time points and centrifuged at 1000 x 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 1:1000 dilution of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 2 mg/ml phenylmethylsulfonyl fluoride (PMSF), and lysed by three consecutive freeze-thaw cycles (1 minute on dry ice: 1 minute 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 (PVDF) membranes (SequiBlot; Bio-Rad, Hercules, CA). Western hybridization was carried out overnight at 4°C using a peptide-derived, monospecific rabbit polyclonal antibody raised against sixteen C-terminal amino acids of HC-3 (0. 44 µg/ml; Goldstein et al., 2010), or mouse-anti-β-Actin antibody, (diluted 1:5000; Sigma-Aldrich, St. Louis, MO, USA) followed by incubation with horseradish peroxidase (POD)-conjugated goat anti-rabbit secondary antibody (1:1000; Santa Cruz Biotech, CA, USA) or goat anti-mouse secondary antibody (1:1000; 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 pre-absorption control was performed by pre-incubating the HC-3 primary antibody with a 200-fold molar excess of the immunizing peptide for 1 hr at room temperature prior to 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, St. Louis, MO). Twenty micrograms of protein was pre-incubated 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 (PNGaseF; New England BioLabs, Ipswich, MA) for 1 hr at 37°C as per manufacturer’s instruction. Control and enzyme-treated proteins were size fractionated by 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 1x1 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 (PLP; 4%
paraformaldehyde, 75 mM lysine, 37.5 mM sodium periodate, and 10 mM Na$_2$HPO$_4$, 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 1:1000
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 1:1000 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 manufacturer’s instruction (Sigma-Aldrich, St.
Louis, MO). Immunofluorescence was analyzed using the Olympus Fluoview 1000 Laser
Scanning Confocal Microscope. A pre-absorption control was performed by pre-
incubating primary antibody with a 200-fold molar excess of the immunizing peptide for
1 hr at room temperature prior to application to the slide.

**Statistics**

The abundance of HC-3 protein expression (normalized to β-actin) in freshly isolated
erthrocytes 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 the current 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 to warm-acclimated frogs (Fig. 3A, B). Western blots from
erthrocyte proteins obtained from cold-acclimated frogs also showed high molecular
weight immunoreactive species which appear as a smear ranging from >65 kDa to 120 kDa on the Western blot (Fig. 3A). 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. 4A). 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. 4B, C). Pre-incubating the primary antibody with immunizing peptide abolished all fluorescent signal, indicating that the fluorescence observed is HC-3 specific (Fig. 4D). Taken together, these data indicate that HC-3 protein expression is markedly up-regulated in erythrocytes from cold-acclimated frogs vs. warm-acclimated frogs, and is predominantly localized to the plasma membrane in erythrocytes from cold-acclimated treefrogs.

**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 erythrocytes in culture by exploring cell viability in response to the frequency of media replacement, shaking vs. non-shaking 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 to those without media replacement at the 24 or 48 hr time points. However, by 72 hours, cell viability in cultures receiving daily media replacement was significantly different from the viability of cells in culture without media replacement (Fig. 5A; Table 4).

Next we compared cell viability in shaken vs. static cultures, when media was 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 hrs (Fig. 5B; Table 4). 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. 5C).
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°C and 20°C for 12, 24, 48 and 72 hr. A repeated measures ANOVA indicated that there was no significant interaction between time and temperature when comparing native HC-3 protein expression in erythrocytes cultured at 4° vs. 20°C (Fig. 6A, C). Thus, native HC-3 expression remained constant throughout the 72 hr time course, regardless of culture temperature.

Likewise, the abundance of glycosylated HC-3 expression (>65 kDa-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 to the abundance of glycosylated HC-3 protein in cells cultured at 4°C for the same amount of time (Fig. 6A, E). There was a >10X fold increase in glycosylated HC-3 expression erythrocytes cultured for 24 and 48 hr at 20°C as compared 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 6A 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 C-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°C or 20°C at any time point (Fig. 6B, D). But the expression levels of the high molecular weight bands (between >65 kDa-120 kDa) was significantly higher in erythrocytes cultured at 20°C at 24, 48 and 72 hr compared to the cells cultured at 4°C. Glycosylated HC-3 abundance was increased by 3X-5X fold at 24, 48 and 72 hr in erythrocytes cultured at 20°C as compared to expression in freshly isolated erythrocytes (Fig. 6B, 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 (>65kDa-100 kDa) relative to the 4°C cultures (Fig. 6A, B, E, 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. 7). 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 are consistent with those previously published for PNGaseF treatment of protein lysates from treefrog kidney in which the deglycosylated HC-3 protein was observed at ~ 25 kDa (Pandey et al., 2009). Results from similar experiments conducted on AQP3, the mammalian orthologue 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. Non-specific bands were observed at 65 kDa in the presence and absence of the enzyme, whereas an additional non-specific band of 55 kDa appeared in the presence of the enzyme (Fig. 7, 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. 8A, B, C, 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. 8A, C) or warm-acclimated frogs (Fig. 8B, 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 kDa-120 kDa) was significantly different at 24 and 48 hr in erythrocytes cultured in glycerol containing media as compared to 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 to expression in control media (Fig. 8A, 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 to expression in those cultured for 48 hr in control media (Fig. 8B, F). The level of glycosylated HC-3 protein increased by 2.2-fold at 48 hr in glycerol-containing media as compared to control media (Fig. 8B, 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. 6B, 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. 8B, 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 inter-individual 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. 3A, B), erythrocytes from cold-acclimated treefrogs cultured at 4°C displayed prominent membrane expression. Immunofluorescence revealed a pronounced increase in fluorescence intensity in the membrane when cells were cultured in media containing glycerol (Fig. 9). The shift in HC-3 localization to the membrane is concomitant with a reduction in expression of green fluorescence in the internal membranous structures of the cytoplasm in both cold- and warm-acclimated cells (Fig. 9). 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. 9B, D, F, H). This effect was especially prominent in erythrocytes harvested from warm-acclimated treefrogs, cultured
for 24 hr in glycerol-containing media (Fig. 9F) and was absent or much reduced in other conditions (e.g., without glycerol; Fig. 9A, C, E, 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. 9H). 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., 1993) and at least one aquaglyceroporin. AQP3 is expressed in human and rat (Roudier et al., 1998) 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; Irwin and Lee, 2003; Layne and Jones, 2001; Zimmerman et al., 2007; Layne and Stapleton, 2009). Gray treefrogs supercool by a few degrees prior to 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; Tanghe et al., 2005; Tanghe et al., 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; Izumi et al., 2007, Edashige et al., 2007; Philip et al., 2008). If freeze-induced damage does occur, aquaporins may be important in cellular recovery and tissue healing (Costanzo et al., '93, Gallagher et al., '97; Hara-Chikuma and Verkman, 2008).
Furthermore, over-expression 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 improves viability following cryopreservation (Hagedorn et al., 2002; Edishige 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 the current 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. 3, 4). 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 ≥96 hours with continuous shaking in culture media, and in media containing glycerol, when the media was replaced every 24 hrs (Fig. 5). Cultured erythrocytes maintained robust native HC-3 expression which did not
significantly differ from expression in freshly isolated erythrocytes (Fig. 6, 8). 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 to 4°C indicating that HC-3 expression is subjected to time and temperature-dependant regulation \textit{in vitro}. Likewise, 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 PNGaseF resulted in the shift of immunoreactive bands toward the native conformer, indicating that a substantial fraction of HC-3 is subject to \textit{N-}linked glycosylation (Fig. 7). \textit{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 non-glycosylated 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 or assembly. However, more recent reports have detailed that, variations in the proportion of glycosylated: non-glycosylated AQP1 is correlated to clinical diagnoses of disease and abnormal cell function (Ticozzi-Valerio et al., 2007). 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 tree-frogs (Fig. 9). 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.
ACKNOWLEDGEMENTS

The authors thank Dr. Wiebke Diestelkamp for assistance with the statistical analyses and Drs. B.K. Kishore and Dan Krane for reviewing the manuscript. This work was supported in part by research grant NSF IOB-0517301 to DLG and CMK, University of Dayton Graduate School Summer Fellowship to VM, and a 2010 American Physiological Society Undergraduate Summer Research Fellowship to MVP.
REFERENCES


Table 4: Percentage of cell viability of cultured erythrocytes from *H. chrysoscelis*.

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Media Replacement</th>
<th>Shaking</th>
<th>Time in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>+ / +</td>
<td>100</td>
<td>98 ± 1.1</td>
<td>96 ± 4.6</td>
</tr>
<tr>
<td>- / +</td>
<td>100</td>
<td>92 ± 1.5</td>
<td>81 ± 7.6</td>
</tr>
<tr>
<td>+ / -</td>
<td>100</td>
<td><strong>92 ± 1.5</strong></td>
<td><strong>81 ± 2.0</strong></td>
</tr>
</tbody>
</table>

Values are represented as percent cell viability ± S.D. as compared to time zero (100%) (n=3 for each time point for each condition.

*Pair-wise comparison of media replacement vs. no media replacement with constant shaking, p<0.05.

**Pair-wise comparison of shaken cultures vs. static cultures with media replacement, p<0.05.
Figure 3: 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 kDa 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 to 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%).
A

Glycosylated

Cold  Warm

100 kDa
75 kDa
65 kDa
50 kDa

HC-3 → 31.5 kDa

β-Act → 42 kDa

B

Average % of normalized HC-3 Expression

<table>
<thead>
<tr>
<th></th>
<th>Cold</th>
<th>Warm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression</td>
<td>250</td>
<td>100</td>
</tr>
</tbody>
</table>

* Indicates a significant difference.
Figure 4: 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 (60X). (B) Immunocytochemistry of HC-3 expression in freshly isolated erythrocytes from warm-acclimated treefrogs appeared to be predominantly in the cytoplasm of erythrocytes (60X). 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.
Figure 5: 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 hr, 48 hr, 72 hr, 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 ± S.D. as compared to time zero (n=3). A p-value < 0.05 was considered statistically significant (*).
Figure 6: **HC-3 protein expression in erythrocytes cultured at 4°C and 20°C.**

Erythrocytes from cold- and warm-acclimated frogs were isolated and cultured at 4°C or 20°C. The relative abundance of native (31.5 kDa) and glycosylated (between >65kDa - 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°C 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°C or 20°C (n=3 observations for each condition). Densitometric analysis of HC-3 expression (>65 kDa – 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°C 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 7: 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 non-specific bands at 55 kDa and 65 kDa.
Figure 8: **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°C 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 hr, 48 hr, and 72 hr. HC-3 abundance (native HC-3 at 31.5 kDa and glycosylated HC-3 between >65 kDa-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.
Figure 9: **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 fluorescein labeled secondary antibody was used to detect HC-3 immunoreactivity (green). Nuclei (red) are counterstained with propidium iodide. Scale bars represent 50 µm. Arrows indicate HC-3 membrane expression. Arrowheads indicate areas of apparent polarized expression.
CHAPTER III

ENDO-PORTER MEDIATED DELIVERY OF PHOSPHORODIAMIDATE MORPHOLINO OLIGOS (PMOS) IN ERYTHROCYTE SUSPENSION CULTURES FROM COPE’S GRAY TREEFROG HYLA CHRYSOCELIS.

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Abstract

Cope’s gray treefrog, Hyla chrysoscelis, is a freeze tolerant anuran that accumulates cryoprotective glycerol during cold acclimation. H. chrysoscelis erythrocytes express the aquaglyceroporin HC-3, which facilitates transmembrane glycerol and water movement. Aquaglyceroporins have no pharmacological inhibitors, and no genetic knockout tools currently exist for H. chrysoscelis. Therefore a phosphorodiamidate morpholino oligo (PMO)-mediated expression knockdown approach was pursued to provide a model for testing the role of HC-3. We describe a novel procedure optimized for specific, efficient knockdown of HC-3 expression in amphibian erythrocyte suspensions cultured at non-mammalian physiological temperatures using
Endo-Porter. Our protocol includes three critical components; pre-incubation at 37°C; two rounds of Endo-Porter and HC-3 PMO administration at ~23°C; and continuous shaking at 190 rpm. This combination of steps resulted in 94% reduction in HC-3 protein expression (Western blot), substantial decrease in HC-3 expression in >65% of erythrocytes, and no detectable expression in an additional 30% of cells (immunocytochemistry).

Cope’s gray treefrog, *Hyla chrysoscelis*, is a freeze tolerant amphibian that accumulates cryoprotective glycerol during cold acclimation (1, 2). Control of intracellular and extracellular water content, and glycerol distribution, promotes extracellular freezing while protecting intracellular structures from ice damage. Regulation of those water and glycerol fluxes is enhanced by aquaporins (AQPs) and aquaglyceroporins (GLPs), integral membrane proteins that facilitate water and, in some cases, glycerol flux across cell membranes (3). Of special interest to the physiology of freeze tolerance is the GLP HC-3, cloned and functionally characterized from *H. chrysoscelis* (2). HC-3 is expressed in erythrocytes and other tissues (2, 4, 5), and HC-3 protein abundance in erythrocytes increases during cold-acclimation (5). Although previous studies have shown a correlation between enhanced aquaglyceroporin expression, cryoprotection and freeze tolerance (2, 5-8), the functional role of these proteins in conferring freeze tolerance remains poorly understood. To address this question, an *in vitro* cell culture model system of HC-3 expressing cells was developed. Suspension cultures of erythrocytes from *H. chrysoscelis* were chosen because they are nucleated, metabolically active and therefore capable of regulating gene expression as
well as peptide-mediated endocytosis, and can be repetitively harvested as a highly homogenous cell population.

Since there are no specific inhibitors for AQPs/GLPs, and tools for gene knockout technology do not exist for *H. chrysoscelis*, an antisense phosphorodiamidate morpholino (PMO) knockdown approach was pursued. Antisense PMOs act on RNA targets by sterically blocking the translation initiation complex (9). Techniques available for delivery of PMOs to cells in culture include syringe or scrape loading, lipsomes, cationic polymers, free uptake from the media, and electroporation (9). Electroporation has also been used in adult axolotl for studying tissue regeneration (10) whereas microinjection and electroporation are widely used for delivery of PMOs into non-mammalian developmental models including sea urchin, *Xenopus*, zebrafish, and *Drosophila* embryos (11). Endo-Porter (GeneTools®, Philmoth, OR, USA) is a novel peptide that mediates PMO delivery through non-specific endocytosis, is effective in a wide variety of cell types, is easy to use, and is non-toxic to cells (12).

Endo-Porter has been used *in vitro* to deliver PMOs to mammalian adherent and non-adherent cells cultured at 37°C (12,13) and *ex vivo* in tissue explants (14) resulting in an average range of 50-80% targeted expression knockdown. One recent study used Endo-Porter mediated PMO delivery to target endogenous gene knockdown in cultured carp macrophages (15). Endo-Porter has also been used successfully to deliver PMOs *in vivo* in newts (16). To date, however, Endo-Porter has not been widely used in non-mammalian cell culture systems. The objective of this study was to determine under what conditions and with what efficiency, Endo-Porter mediated PMO delivery could be
used to knockdown targeted gene expression in non-mammalian cell suspension cultures, specifically in amphibian erythrocytes.

The HC-3 PMO was selected using the criteria defined for custom PMO design available through GeneTools (GeneTools®). The HC-3 antisense PMO sequence (5’-CCCATGTGTGCTGAGCCTCTAGGTC-3’) was designed based on complementarity to the following region in HC-3, indicated in brackets on the sense strand, with start codon in parentheses:

ACTACTCAGCCGCGCAGCATCACAGCTCTCCCC[GACCTAGGGGCTAGCAAC(ATG)GG]GCAGGAGGTTCTCA. The commercially available standard control oligo (5’-CCTCTTACCTCAGTTACAATTTATA-3’; Gene Tools®) with no known target in *H. chrysoscelis* was used as a PMO control.

The optimized procedure is as follows: Whole blood from *H. chrysoscelis* was collected in heparinized capillary tubes, transferred to 15 ml conical tubes containing 5 ml complete cell culture media (CCCM; 250 mOsM, RPMI 1640 medium supplemented with L-glutamine, 100 units/ml of penicillin and streptomycin, 0.25 µg/ml of amphotericin B (Invitrogen, Carlsbad, CA, USA), 5% fetal bovine serum (Fisher Scientific, Hanover Park, IL, USA), centrifuged at 1000 x g for 10 minutes, and resuspended in CCCM. Approximately 2x10⁶ cells were incubated in 200 µl of media in round-bottom polypropylene tubes (Fisher Scientific) at 37°C for 2 hrs while shaking at 190 rpm on a titer plate shaker (Lab-Line Instruments Inc., Melrose Park, IL, USA) . The suspension volume was increased to 1 ml followed by addition of one of the following treatments: A.) no treatment (control); B.) 10 µM Endo-Porter alone (EP); C.) 3 µM HC-
3 PMO alone (HC-3 M); D.) 10 µM Endo-Porter and 3µM HC-3 PMO (EP + HC-3 M) and E.) 10 µM Endo-Porter and 3 µM standard control PMO (EP + control M). Endo-Porter and standard or HC-3 PMO were sequentially added to the erythrocyte cultures. Cultures were maintained at 22-25°C within the range of basal body temperature of live treefrogs for 24 hrs while shaking. After 24 hrs, media was replaced with 200 µl fresh CCCM and erythrocytes were again incubated at 37°C for 2 hrs while shaking. At the end of 2 hrs, 800 µl of CCCM was added to each culture. The relevant cultures were spiked with additional 3µM control or HC-3 PMO and/or 10µM Endo-Porter and maintained for an additional 24 hrs at room temperature after which the media was replaced; cells were cultured for a total of 76 hrs. Initial optimization experiments showed that cell viability at 76 hrs (~98%) remained unchanged between untreated erythrocytes and erythrocytes cultured in Endo-Porter alone (6-10 µM), HC-3 PMO alone (3-10 µM), or Endo-Porter and HC-3 PMO (each at 10 µM) (data not shown). At 76 hrs, total cellular proteins were isolated, size fractionated by SDS-PAGE, and subjected to Western hybridization using a peptide-derived, monospecific rabbit polyclonal antibody raised against sixteen C-terminal amino acids of HC-3 (0. 44 µg/ml), or mouse-anti-β-Actin antibody, (diluted 1:5000; Sigma-Aldrich, St. Louis, MO, USA) followed by incubation with horseradish peroxidase (POD)-conjugated goat anti-rabbit secondary antibody (1:1000; Santa Cruz Biotech, CA, USA) or goat anti-mouse secondary antibody (1:1000; Santa Cruz Biotech) as previously described (5). Relative band intensities were determined by densitometry using Vision works software on BioSpectrum® Imaging System (UVP, Upland, CA). Averaged HC-3 protein abundance (normalized to β-actin) was expressed as a percentage of the no treatment control group (A).
HC-3 protein abundance was reduced by 94% in treated cultures (treatment D) and showed no change in control cultures (treatments A–C and E) after 76 h, compared with no-treatment controls (Figure 10, A and B), indicating that HC-3 knockdown was specific to the Endo-Porter plus HC-3 PMO treatment. Immunocytochemistry was performed (as previously described; 5) on erythrocytes harvested at 76 hrs to examine the efficiency and degree of HC-3 knockdown at the cellular level (Figure 11). HC-3 protein abundance was reduced by 94% in treated cultures (treatment D) and showed no change in control cultures (treatments A-C and E) after 76 hrs, compared with no treatment controls (Figures 10A, B), indicating that HC-3 knockdown was specific to the Endo-Porter plus HC-3 PMO treatment. Pre-incubation of the primary antibody with the immunizing peptide resulted in no immunoreactive signal (Figure 10A). Treatment D (Figure 11D) showed an overall 75% reduction in HC-3 expression, assessed semi-quantitatively from combined cellular fluorescence (corrected for cell number) of experimental relative to control groups (Figure 11A-C and E). Moreover, ~30% of erythrocytes showed complete HC-3 knockdown as assessed by the absence of fluorescence (Figure 11D), while >65% of erythrocytes had substantial reduction in HC-3 expression compared with treatment controls (Figure 11A-C and E). Pre-incubation of the primary antibody with the immunizing peptide resulted in no immunoreactive signal (Figure 10A and Figure 11F) indicating that the signal observed in panels A-E is immunospecific for the HC-3 antibody. Thus, a high efficiency (>95%) of complete or substantial HC-3 knockdown was achieved using this method.

The optimized procedure described in this report is novel in three important ways. First, cell viability and expression knockdown was dependent on shaking the cultures at
190 rpm throughout the procedure. Whereas the manufacturer recommends immediate swirling after Endo-Porter administration, it is likely that continual shaking throughout the incubation period contributed to the high efficiency of Endo-Porter mediated PMO delivery by increasing cell exposure to these reagents. Thus, continual shaking of adherent as well as non-adherent cell cultures from any source (mammalian and non-mammalian) may also result in improved PMO mediated expression knockdown through increased delivery efficiency. Second, while frog erythrocytes do possess endocytic capacity (17,18), initial attempts at administering Endo-Porter and HC-3 PMO to erythrocytes cultured at ~23°C (which is within the range of normal physiological temperature for treefrogs) failed to achieve successful HC-3 expression knockdown. Therefore, we implemented a pre-incubation step at 37°C prior to the administration of Endo-Porter and PMO in an attempt to recapitulate the kinetic and thermal conditions for which the delivery vehicle was created. In addition, this 37°C pre-incubation step likely functions to increase the membrane fluidity of the cells, thereby improving the efficiency of peptide-mediated endocytosis (19). Third, preliminary experimental trials indicated the need for a second round of Endo-Porter and PMO administration for efficient knockdown. This is consistent with prior studies (20) and with manufacturer’s recommendations (personal communication). In the procedure used here, a second 37°C incubation step was added prior to a second PMO administration, thereby coupling the effects of second round PMO exposure with improved membrane fluidity. The combination of all three optimization steps was required to achieve targeted gene knockdown in our system. By using this improved and optimized approach, we achieved >95% targeted expression knockdown.
In conclusion, a novel procedure for efficient Endo-Porter mediated HC-3 PMO delivery and expression knockdown in cultured amphibian erythrocytes has been described. This model will be used in future experiments to determine HC-3 function in freeze tolerance. However, the utility of this optimized delivery method is not restricted to amphibian erythrocytes. This novel procedure is likely to be of special interest and utility to investigators using non-mammalian systems (e.g. *Xenopus*, zebrafish, sea urchin, fish, newt, axolotl) who may not have previously attempted to use Endo-Porter due to concerns about its efficacy at non-optimal temperatures. Moreover, the combination of optimized steps can be broadly applied to mammalian as well as non-mammalian systems in order to improve overall knockdown efficiency. Finally, this report demonstrates that Endo-Porter mediated PMO expression knockdown can be optimized for use in models for which genetic knockout tools do not currently exist.

ACKNOWLEDGMENTS

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COMPETING INTERESTS

The authors declare no competing interests.
REFERENCES


Figure 10: Western blot analysis of HC-3 protein expression in cultured erythrocytes.

(A) Western blotting was performed as previously described (5), on total cellular proteins isolated after 76 hrs from erythrocytes subjected to the following culture treatment combinations: Lane 1: no treatment (control); Lane 2: 10 µM Endo-Porter alone (EP); Lane 3: 3 µM HC-3 PMO alone (HC-3 M); Lane 4: 10 µM Endo-Porter and 3µM HC-3 PMO (EP + HC-3 M); Lane 5: 10 µM Endo-Porter and 3 µM standard control PMO (EP + control M). An affinity purified peptide-derived anti-rabbit polyclonal antibody directed against the final 16 amino acids of the C-terminus of the predicted *H. chrysoscelis* HC-3 protein (previously described; 5) was used to detect native and glycosylated HC-3 protein at 33 kDa (arrow) and >65 kDa respectively. Lane 6: Pre-absorption control: Pre-incubation of the HC-3 polyclonal antibody with 200 fold excess of the immunizing peptide blocked HC-3 immunospecific reactivity at 33 kDa and >65 kDa. A non-specific band at 65 kDa not blocked by antibody pre-absorption with the immunizing peptide appears in all lanes. Expression of the housekeeping gene β-actin (42 kDa) as determined by Western blotting with a mouse anti-β-actin antibody is shown as a gel loading control. (B) HC-3 and β-actin expression was quantified by densitometry. For each treatment combination HC-3 protein expression (normalized to β-actin) is expressed as a relative percentage vs. normalized HC-3 expression in erythrocytes with no treatment. HC-3 protein expression in treated cultures (EP+HC-3 M) is reduced by an average of 94% with a range from 92-98% (n=4).
Figure 11: Immunocytochemistry of HC-3 expression in cultured erythrocytes.

Immunocytochemistry was performed on cultured erythrocytes using a primary peptide-derived polyclonal antibody directed against the C-terminus of *H. chrysoscelis* HC-3. (A) No treatment (control). (B) 10 µM Endo-Porter alone (EP). (C) 3 µM HC-3 PMO alone (HC-3 M). (D) 10 µM Endo-Porter and 3µM HC-3 PMO (EP + HC-3 M). (E) 10 µM Endo-Porter and 3 µM standard control PMO (EP + control M). (F) Pre-absorption Control. HC-3 expression is shown as a green signal due to the use of a fluorescein labeled secondary antibody. Nuclei appear red after RNase treatment and propidium iodide staining. Approximately 30% of erythrocytes treated with EP+HC-3 M (Panel D) showed complete HC-3 knockdown as assessed by the absence of fluorescence while >65% of erythrocytes had substantial reduction in HC-3 expression compared with treatment controls (Panels A-C and E) (n=3 independent trials for each condition). No HC-3 signal (green) was seen in the pre-absorption controls (Panel F), where the HC-3 primary antibody was pre-incubated with 200-fold excess of the antigenic peptide, indicating that the HC-3 immunoreactivity seen in panels A-E is specific for the HC-3 antibody. Images were taken using an Olympus Fluoview 1000 Laser Scanning Confocal Microscope with 60X objective. The scale bar indicates 50 µm.
CHAPTER IV

AQUAGLYVEROPORIN EXPRESSION AND ERYTHROCYTE OSMOREGULATION IN CULTURES FROM FREEZE TOLERANT ANURAN, COPE’S GRAY TREEFROG, *HYLA CHRYSOSCELIS.*

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(Unpublished data)

Abstract

Cope’s gray treefrog, *Hyla chrysoscelis* is a freeze tolerant anuran that accumulates glycerol during cold acclimation. We hypothesize that the osmoregulatory capabilities of erythrocytes to tolerate changes in cell volume and to effect rapid water and solute fluxes are critically important during the events of freezing (when water leaves cells and solutes become concentrated) and thawing (when melting ice exposes cells to a hypotonic environment), and are dependent upon the expression and function of HC-3, an ortholog of the human aquaglyceroporin AQP3. Using erythrocytes as an *in vitro* cell culture model system, we have previously shown that HC-3 protein expression and membrane localization are dynamically regulated. The present study explores the consequences of
those changes in expression for osmoregulatory capabilities of erythrocytes. Erythrocytes cultured for 48 hrs in media made hyperosmotic (400 mOsM) through the addition of 150 mM glycerol or urea showed enhanced membrane localization of HC-3 compared with those cultured in control medium (CM) or medium made hypertonic by addition of 150 mM sorbitol or 75 mM NaCl. In order to examine whether culture condition affects erythrocyte response to hypotonic challenge, erythrocytes were placed in solutions that produced either moderate or severe osmotic stress. Erythrocytes subjected to moderate water-induced hypotonic stress (70 mOsM final osmolarity) in the absence of 0.3 mM HgCl$_2$, underwent a series of shape changes as they swelled, initially elongating, then becoming swollen and round. For cells cultured in CM+ 150 mM glycerol, the initial rate of cell elongation ratio (ratio of erythrocyte length to width) was statistically significantly higher than that of cells cultured in CM, CM+150 mM urea, CM+150 mM sorbitol or CM+75 mM NaCl (p<0.05). In addition cells cultured in CM, CM+150 mM glycerol or urea reached maximum elongation faster than cells cultured CM+150 mM sorbitol or CM+75 mM NaCl (p<0.05). After five minutes post-challenge, the two-dimensional shape of cells cultured in CM, or CM+150 mM glycerol or urea was significantly different than cells cultured in CM+150 mM sorbitol or CM+75 mM NaCl (p<0.05). Moreover, cells cultured in CM or CM+150 mM glycerol became round after 5 min, whereas cells cultured in CM+150 mM urea returned to their original shape. In contrast cells cultured in sorbitol or NaCl remained elongated. When erythrocytes were pre-incubated with HgCl$_2$, cells retained normal dimensions, suggesting the importance of aquaporins in conferring water and solute permeability in these cells. When subjected to severe hypotonic challenge (10 mOsM final osmolarity achieved by the addition of
water), 100% of erythrocytes cultured in CM lysed within 15 min compared with 60% of erythrocytes cultured in media containing 150 mM glycerol or urea. Hypotonicity-induced cell lysis was completely blocked by pre-incubation with HgCl₂. We conclude that HC-3 protein expression is enhanced in erythrocytes cultured in the presence of glycerol or urea, which appears to confer a degree of protection against severe osmotically-induced cellular stress responses that likely arise during the freezing and thawing processes. Combined, these data further suggest a functional role for aquaglyceroporins in freeze tolerance in *H. chrysoscelis*. 
Introduction

Mammalian erythrocytes are subjected to severe osmotic perturbations with concomitant cell volume changes in response to fluctuations in extracellular osmolarity (reviewed in Cossins and Gibson, 1997). For example, erythrocytes in kidney are exposed to wide variations in osmotic gradients ranging from 300 mOsm to 1200 mOsm in the vasa recta during which they experience hypertonic stress. Under such conditions, erythrocytes employ adaptive responses either by regulatory volume increase (when cells shrink) or regulatory volume decrease (when cells swell) and activate transport pathways (reviewed in Cossins and Gibson, 1997; reviewed in Okada, 2004). Erythrocytes respond to osmotic stress by activating regulatory mechanisms in order to maintain their shape, volume and surface area (Garcia-Romeu et al., 1991; Groulx et al., 2006). Membrane stretching during swelling triggers mechanosensitive channels which may act as volume sensors leading to regulatory volume decrease or increase (reviewed in Hoffman et al., 2009). Hypotonic swelling in erythrocytes activates many membrane transport systems that are essential to maintain the regulatory volume changes during osmotic challenges. For example symporters (K⁺Cl⁻, Na⁺-K⁺Cl⁻), ion channels (K⁺, Cl⁻), antiporter (Na⁺/H⁺), Na⁺/K⁺ pump, glucose (GLUT1) and urea (UT-A,UT-B) transporters including water channels (AQP/GLPs) all have been shown to participate to regulate cell volume of erythrocytes (Garcia-Romeu et al, 1991; Buemi et al., 2002, Yang and Verkman, 2002). Exposure of human red blood cells (RBCs) to moderate anisosmotic solutions cause transitions from the normal ‘discocyte’ to hyposmotically swollen ‘spherocytes’ with subsequent hemolysis, or hyperosmotically shrunken ‘echinocytes’ (Hoffman, 1987). The lipid bilayer membrane of RBCs along with the rigid cytoskeletal network beneath it,
composed of cytoskeletal proteins including spectrin, ankyrin and band 3 determine the shape changes of RBCs (Zhang and Brown, 2008).

Hypotonicity-induced changes in erythrocyte volume and shape are the result of activation of several membrane proteins that trigger osmotically driven rapid transport of water through the membrane bilayer via AQPs (Cho et al., 1999; Pribush et al., 2002). Various techniques have been used to study these shape changes, including stop flow cytometry which measures the spherical index of deformed RBC (Piagnerelli et al., 2007), laser diffractometry which uses the diffraction patterns of elliptical shaped RBC (Hardeman et al., 2001), and an optical interferometric technique where the mechanical changes in RBCs are measured as they change their shape (Park et al., 2010). The above mentioned methods provide details of cell shape in all three dimensions.

Human erythrocytes express AQP1 and AQP3, while murine erythrocytes express AQP1 and AQP9 (Agre et al., 1993; Roudier et al., 2002; Liu et al., 2007). Functionally, human AQP3 and murine AQP9 have been shown to contribute to erythrocyte glycerol permeability (Liu et al., 2007). GLP-mediated erythrocyte glycerol permeability ($P_{\text{gly}}$) can be measured using a variety of different methods. The timecourse of radioactive glycerol efflux from pre-loaded cells can be used to determine membrane glycerol permeability (Roudier et al., 1998). In stopped flow light scattering experiments, an erythrocyte suspension is mixed with glycerol to create an inward glycerol gradient and the $P_{\text{gly}}$ is measured by passing the cells through the apparatus, where the light scattering signal due to influx of glycerol is calculated (Liu et al., 2007). In the osmotic lysis method, an erythrocyte suspension mixed with a 0.2M glycerol in PBS induces osmotic lysis of cells. The uptake of water and glycerol followed by cell lysis is determined
spectrophotometrically by a decrease in light absorption at 625 nm. From the absorption data a single exponential fit curve is derived to calculate the percentage of glycerol induced osmotic lysis of erythrocytes and this equation can then be used to calculate the percentage of erythrocyte lysis (Goldstein et al., 2010).

In general, the osmotic fragility of erythrocytes is affected by their size, membrane composition and presence or absence of a nucleus (Aldrich and Saunders, 2001). Amphibian erythrocytes are larger in size and have a more rigid membrane-cytoskeletal framework compared to mammalian erythrocytes (Aldrich et al., 2006; Sauviat et al., 2006). The nucleus in amphibian erythrocytes is linked to the membrane-cytoskeleton through several intermediate filaments which provide additional support to the RBC membrane. Thus amphibian erythrocytes have a relatively enhanced resistance to hypotonicity-induced lysis compared to mammalian erythrocytes (Aldrich and Saunders, 2001). The erythrocyte cytoskeleton also affects dynamic cell shape changes that occur due to osmotic stress (reviewed in Svetina, 2012). In addition the cytoskeleton is linked to various transmembrane proteins in the erythrocyte membrane restricting the mobility of transmembrane proteins (reviewed in Svetina, 2012). During hypotonicity-induced cell swelling, a rapid influx of water causes frog erythrocytes to undergo complex shape transformations which have been described as ‘ovate spheroids’, ‘biconcave’ or ‘disc shaped’ and relative volume changes can calculated by measuring cellular dimensions (i.e., cell length, width and thickness, Maroney, 1961). In anurans, since the nucleus is connected to the erythrocyte membrane through intermediate filaments, erythrocyte shape changes also affects the shape of the nucleus.
Erythrocytes from the freeze-tolerant anuran, Cope’s gray treefrog, *Hyla chrysoscelis* express mRNA of both HC-1 (an ortholog of mammalian aquaporin AQP1) and HC-3 (an ortholog of mammalian aquaglyceroporin AQP3) (Goldstein et al., 2010), but only HC-3 protein is expressed (Goldstein et al., 2010; Mutyam et al., 2011). HC-3 confers membrane permeability to both water and glycerol, and thus may be important for both transmembrane water and glycerol flux (Zimmerman et al., 2007). Functionally cold-acclimated *H. chrysoscelis* erythrocytes exhibit an increase in glycerol permeability at 20°C that is inhibited by pre-incubation with 0.3 mM HgCl₂, a potent aquaporin inhibitor (Goldstein et al., 2010). In addition, erythrocytes from cold-acclimated frogs show increased HC-3 protein expression and enhanced membrane localization as compared to erythrocytes from warm-acclimated treefrogs (Goldstein et al., 2010; Mutyam et al., 2011A). These data suggest a role for HC-3 in transmembrane glycerol flux that occurs during the process of freeze tolerance, during which glycerol serves as a cryoprotectant.

Based on these data, we hypothesize that HC-3, an ortholog of mammalian aquaporin 3, enhances membrane permeability to glycerol which acts as a natural cryoprotectant to regulate osmotic gradients formed by extracellular ice crystal formation during freezing. Previously, we have shown that, in erythrocytes from cold-acclimated treefrogs and in erythrocytes cultured in glycerol-containing hyperosmotic media, HC-3 protein undergoes enhanced glycosylation and membrane localization (Mutyam et al., 2011). However the cellular response to changes in membrane permeability properties during freeze/thaw cycles in *H. chrysoscelis* is poorly understood. In this study, cultured erythrocytes were subjected to moderate hypotonic stress and dynamic cell shape changes were examined over a timecourse to determine if culture condition and/or HC-3
expression had an effect on 1.) maximum cell elongation, 2.) rate of cell elongation, 3.)
time needed to reach maximum elongation, and/or 4.) erythrocyte shape at 5 min post-
challenge. To compare the potential effects of culture condition on erythrocyte osmotic
fragility, cells were also subjected to severe hypotonic stress, and the percentage of intact
cells determined over a timecourse of exposure. Our results indicate that glycerol and
urea have a stabilizing effect on erythrocytes, as evidence by a time-dependent resistance
to hypotonicity-induced cell lysis. In addition both glycerol and urea enhance HC-3
localization to the membrane, and thus may influence membrane permeability to water
and glycerol transport important for freeze tolerance.

Materials and methods

Animals

Male gray treefrogs, *Hyla chrysoscelis*, were identified based on trill frequency and
collected at breeding grounds in Greene County, OH, USA. Treefrogs were maintained in
the laboratory at a controlled temperature of 21 °C and exposed to a 12 h light: 12 h dark
cycle in standard cages as approved by the Institutional Animal Care and Use Committee
(IACUC) at Wright State University. Animals had continuous access to fresh water and
were fed crickets twice per week (Zimmerman et al., 2007; Goldstein et al. 2010).

Erythrocyte cultures

Whole blood (~200µl) was drawn from the brachial artery of *H. chrysoscelis*, collected in
heparinized capillary tubes and transferred to 15 ml conical tubes containing 5 ml
complete cell culture media (CCCM; 250 mOsM, RPMI 1640 medium supplemented
with L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml
of amphotericin B, 5% fetal bovine serum) (Invitrogen, Carlsbad, CA). Blood pooled from 2-3 frogs was centrifuged at 1000 x g for 10 min. Cells were resuspended in 10 ml of CCCM and a total cell count performed using a hemocytometer. Trypan blue exclusion dye staining was used to determine cellular viability (n=2 trials). Approximately 5x10⁶ cells were resuspended in CCCM or control media (CM; 250 mOsm). The CCCM was made hyperosmotic (400 mOsm) by addition of 150 mM glycerol or 150 mM urea (CM+150 mM glycerol or urea) and hypertonic (400 mOsm) by addition of 150 mM sorbitol or 75 mM NaCl (CM+150 mM sorbitol or 75 mM NaCl) in a total volume of 5 ml and placed in 25 cm² Corning tissue culture flasks. These suspension cultures were maintained at 20°C for 48 hrs in flasks positioned at a 45° angle on a plate shaker with constant rotation (190 rpm). Cells were given CCCM every 24 hr to provide optimal cell viability.

**Immunocytochemistry**

Erythrocytes cultured for 48 hrs in CM or in CM made hyperosmotic or hypertonic were centrifuged at 1000 g for 10 min. On gelatin-coated slides 10 μl of cell suspension was smeared with a 1x1 inch coverslip and incubated at room temperature (RT) until completely dry. For immunofluorescence assay, cells were fixed in a PLP-fixative (periodate-lysine-paraformaldehyde solution: 4% paraformaldehyde, 75 mM lysine, 37.5 mM sodium periodate, and 10 mM Na₂HPO₄, pH 7.2) at R.T for 20 minutes. Serial washes were carried out in a phosphate-buffered saline (PBS, pH 7.2) solution 5 minutes (x2) to remove the PLP fixative. Cells were then permealized with 0.2% Triton X-100 for 15 min. After 15 minutes of incubation in a 1% glycine PBS solution followed by 15 min incubation in 0.1% sodium borohydride, slides were washed in PBS for 5 min (x3). Cells
were then blocked in a 10% blocking serum (10% normal goat serum, 4% bovine serum albumin and 0.05% Tween-20 in PBS) for 1 hour at R.T and incubated overnight at 4 °C with HC-3 rabbit ployclonal primary antibody (0.44 µg/ml) diluted 1:100 in 1% blocking serum (1:100 dilutions) (Goldstein et al., 2010). After three PBS washes for 5 min (x3), slides were incubated for 1 hr at RT with a fluorescein-conjugated, goat anti-rabbit secondary antibody diluted 1:1000 dilution in 1% blocking serum in PBS. Finally, cells were washed thrice in PBS for 5 min each and counterstained with of propidium iodide nucleic acid stain for 5 minutes in the presence of RNase (4 mg/ml; Promega, Madison, WI) and mounted with Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA) to prevent bleaching. Subcellular localization of HC-3 was analyzed via confocal immunofluorescence microscopy using an Olympus Fluoview 1000 Laser Scanning Confocal Microscope and a 60x objective lens. Images were shown with fluorescein and propidium iodide channels merged. A pre-absorption control was performed by pre-incubating primary antibody with a 200-fold molar excess of the immunizing peptide for 1 hr at room temperature prior to application to the slide.

**Hypotonic-induced changes in cell shape**

After 48 hr, cultured cells were imaged (T₀) under phase contrast using a Nikon microscope (40X) and MetaMorph version software (Molecular Devices). In order to examine cell morphology changes to hypotonicity, erythrocyte cultures were centrifuged at 1000 g for 5 mins at RT to pellet the cells. Erythrocytes cultured in CM were resuspended in 500 µl isosmotic PBS (250 mOsm) and cells cultured in glycerol, urea, sorbitol and NaCl media were equilibrated in 500 µl of PBS made hyperosmotic compared to CM, by addition of 150 mM glycerol or 150 mM urea (PBS+150 mM NaCl).
glycerol or urea) and hypertonic by addition of 150 mM sorbitol or 75 mM NaCl (PBS+150 mM sorbitol or 75 mM NaCl). The cell suspension (25 µl) was placed as a single drop on a 100 mm tissue culture treated plate at RT (T<sub>300</sub>) and imaged. The PBS solution (75 µl) with or without 0.3 mM HgCl<sub>2</sub> was mixed with the erythrocyte suspension (25 µl). Images were captured every 10 sec for 5 min. To induce a moderate hypotonic challenge, 300 µl water placed as four equal sized droplets (75 µl) around the erythrocytes suspension (+/-HgCl<sub>2</sub>) was then mixed with the cell droplet and images were captured every 10 sec for an additional 5 min (T<sub>610</sub>-T<sub>900</sub>). The image captured immediately prior to hypotonic challenge (T<sub>600</sub>) was used to determine if incubation with HgCl<sub>2</sub> alone had an effect on cell shape.

**Erythrocyte lysis assay**

Cells cultured in CM were resuspended in 500 µl isosmotic PBS (250 mOsm) and cells cultured in 150 mM glycerol or urea media were resuspended in 500 µl of PBS made hyperosmotic (400 mOsm) compared to CM, by addition of 150 mM glycerol or 150 mM urea (PBS+150 mM glycerol or urea). The cell suspension (12.5 µl) was placed as a single drop on a 100 mm tissue culture treated plate and imaged. The erythrocyte suspension was mixed with PBS alone (37.5 µl) or PBS containing 0.3 mM HgCl<sub>2</sub> and incubated for 5 min with image capture every 15 sec. To induce hypotonicity induced cell lysis, 1500 µl of water was placed as four equal sized droplets (375 µl) around the erythrocyte suspension (37.5 µl PBS +/- HgCl<sub>2</sub> +12.5 µl erythrocyte suspension) and mixed. For cells cultured in media containing 150 mM glycerol or 150 mM urea, cell lysis was induced by mixing the erythrocyte suspension with 150 mM glycerol in water (1500 µl) or 150 mM urea in water (1500 µl), respectively. Post-challenge images were
captured every 15 sec for 25 min. Cell lysis was indicated by membrane rupture and formation of erythrocyte ghosts (based on visual inspection of disappearance of intact cell membrane). The percentage of intact erythrocytes (n=40-50 cells at T0) was determined as a function of time and represented graphically.

**Data analysis**

In order to determine the sub-cellular localization of HC-3 protein, fluorescence plot profiles were used. Fluorescence amplitudes along the line scans were displayed graphically below each image (a.u. is arbitrary units). The line expression profiles were generated and analyzed using the Image J software program (http://rsb.info.nih.gov/ij/). The line profile covered the cross section of the cell which traversed the plasma membrane and the cytosol of erythrocyte with a constant distance from nucleus maintained.

For hypotonicity induced cell shape analysis, the cellular long axis (LA) and short axis (SA) ratio was measured using ImageJ software (National Institutes of Health, Bethesda, MD). The ratio of LA: SA was calculated for each cell at each timepoint throughout the time course. The average LA: SA ratio (n=6-8 cells) for each culture and challenge condition at each time point was used to graphically represent two-dimensional dynamic cell shape changes throughout the time course. For statistical analysis, four defined variables were chosen: (a) the initial rate of elongation ratio was represented as the slope (cell elongation ratio in first 10 sec after hypotonic challenge [(LA: SA 610-600)/10], (b) the magnitude of the maximum elongation ratio (LA: SA max-LA: SA600) was computed based on the difference between maximum elongation ratio and the ratio at T600 secs prior to challenge, (c) Tmax was defined as the time taken for cells to reach
maximum LA: SA ratio, and (d) the magnitude of LA: SA ratio at 900 secs (LA: SA 900- LA: SA 600) which compared the elongation ratio at time 900 sec vs. the elongation ratio prior to challenge. The differences in the above mentioned variables for different culture conditions were statistically analyzed using repeated measures analyses of variance. A $P$-value below 0.05 was considered statistically significant. The statistical analyses were performed by Dr. Wiebke Diestelkamp, Associate Professor, Department of Mathematics, University of Dayton.

**Results**

**Enhanced membrane localization of HC-3 in erythrocytes cultured in hyperosmotic glycerol and urea**

Previously, we have shown that glycerol enhances the membrane localization of HC-3 in cultured *H. chrysoscelis* (Mutyam et al., 2011). To determine if HC-3 membrane localization is regulated by glycerol specifically, or by hyperosmolarity/hypertonicity in general, erythrocytes were cultured for 48 hours in CM, or CM containing 150 mM glycerol, 150 mM urea, 150 mM sorbitol, or 75 mM NaCl. Immunofluorescence results show that HC-3 membrane localization was enhanced in erythrocytes cultured in media made hyperosmotic in the presence of 150 mM glycerol or 150 mM urea (Fig. 12F, H) as compared to erythrocytes in CM (Fig. 12D). The increased fluorescence intensity in the membrane is displayed as two visible peaks (Fig. 12F, H). In addition, cells cultured in glycerol or urea containing media after 48 hr formed cellular aggregates (Fig. 12F, H) compared with cells cultured in CM, sorbitol or NaCl (Fig. 12D, J, L). Interestingly, polarized expression of HC-3 along the membranes of cells contacting each other within
these aggregates was observed only in cells in media with glycerol (Fig. 12F), but not in urea containing media (Fig. 12H). The absence of such aggregation in any other culture condition, suggests that this effect is not a technical artifact due to overlapping cells, but rather a result of hyperosmotic stress specifically in the presence of glycerol. The overall fluorescence intensity is reduced in cells cultured in sorbitol after 24 and 48 hr (Fig. 12I, J) compared to cells cultured in CM, glycerol, urea or NaCl (Fig. 12 C-F and K, L). This indicates that subcellular localization of HC-3 is diminished in the erythrocytes cultured in media made hypertonic by adding sorbitol. However for erythrocytes cultured in media made hypertonic in the presence NaCl, there was no change in the overall fluorescence intensity of HC-3 similar to that of erythrocytes cultured in CM.

**Mercuric chloride (HgCl₂) inhibits hypotonic-induced erythrocyte cell shape changes**

Erythrocytes cultured in CM or media made hyperosmotic with 150 mM glycerol or urea, or hypertonic with 150 mM sorbitol or 75 mM NaCl were each subjected to hypotonic challenge with water. Hypotonicity-induced morphological changes of erythrocytes were quantified by measuring the LA: SA ratio (Fig 13A). The first image captured was at time zero indicated on the graph by LA: SA 0, to observe the LA: SA ratio of cells cultured in vitro. There were no statistically significant differences in erythrocyte shape in erythrocytes from different cultures (p=1.00). The initial ratio was consistently ~1.68 (Fig. 13B, 14, 15). Pre-incubation of erythrocytes in the presence of 0.3 mM HgCl₂ prior to hypotonic challenge did not affect erythrocyte morphology which remained at LA:SA 600 ~1.68 (Fig. 13B, 14). However, when subjected to moderate hypotonic challenge in the absence of 0.3 mM HgCl₂, cultured erythrocytes underwent a series of time-
dependent cell shape changes (Fig. 13B). The hypotonic-induced shape changes indicated in Figure 13B represents LA: SA ratio at 0, 300 sec (Fig. 13B, I, II), LA: SA ratio at 600 sec and the slope of the curve which represents the rate of changes in cell elongation in the first 10 sec \[(LA: SA_{610-600})/10\] and is indicative of rapid water influx (III). The LA: SA max represents the maximum elongation ratio. Tmax is the time taken to reach the maximum elongation ratio, LA: SA max. The ΔLA: SA max is the magnitude of the difference between the maximum elongation ratio and LA: SA at 600 (Figure 13B, IV). The ratio of cells 900 secs is indicated by LA: SA 900 (Fig 13B, V). These hypotonic-induced cell shape changes were inhibited in the presence of 0.3 mM HgCl₂, a known inhibitor of water transport through aquaporins (Goldstein et al., 2010) (Fig. 13B).

**Hypotonic-induced dynamic cell shape changes are affected by culture treatment**

The immunocytochemical data show increased HC-3 membrane localization in erythrocytes cultured in urea and glycerol, as compared to cells cultured in control media or media containing NaCl or sorbitol. To determine whether enhanced HC-3 membrane localization affects hypotonically-induced cellular responses, cultured erythrocytes were subjected to hypotonic challenge with water, and cell shape was monitored throughout a five min timecourse. Regardless of culture condition, addition of 0.3 mM HgCl₂, a water channel inhibitor, blocked all hypotonicity-induced cell shape changes (Fig 14). The rate of cell elongation in the first 10 sec for cells cultured in CM+150 mM glycerol is ~2-5 times that of cells cultured in CM, CM+150 mM urea, CM+150 mM sorbitol or CM+75 mM NaCl (p<0.05). Thus, cells cultured in the presence of glycerol have a statistically higher initial rate of hypotonicity-induced cell elongation as compared to cells from other cultures, likely due to greater membrane water permeability (Fig. 14, Table 5). The
maximum elongation ratio of cells cultured in CM is significantly higher compared with cells cultured in CM+150 mM urea, CM+ 150 mM sorbitol and CM+ 75 mM NaCl (p<0.05), but not different from cells cultured in glycerol-containing media. The time taken to reach the maximum elongation ratio (Tmax) is significantly different in cells cultured in CM or CM+ 150 mM glycerol vs. cells cultured in CM+150 mM sorbitol or CM+ 75 mM NaCl (p<0.05) (Table 5). In addition, the Tmax for cells cultured in CM+150 mM glycerol is significantly different from cells in CM+150 mM urea. These results indicate that the cells cultured in hypertonic media do not elongate to the degree achieved by cells cultured in control or hyperosmotic media, and require significantly more time to reach the maximum elongation ratio. Cells cultured in CM and CM+150 mM glycerol reach a spherical shape by the end of 900 secs and cells in urea media returned to their original shape. In contrast, cells cultured in hypertonic media in the presence of 150 mM sorbitol or 75 mM NaCl continued to stay in elongated shape. Thus, the difference between cell shape at 900 sec and 600 sec (LA:SA 900-LA:SA 600) is significant between cells cultured in CM, CM+150 mM glycerol or CM+150 mM urea and cells cultured in CM+150 mM sorbitol or CM+ 75 mM NaCl. Overall, these results suggest that erythrocytes cultured in hypertonic media show reduced hypotonically-induced morphological changes, suggestive of low water permeability.

**Cell shape changes due to water and/or glycerol or urea fluxes**

To examine, whether the observed hypotonic induced cell shape changes are due to water influx or glycerol/urea efflux or both, cells cultured in CM and CM+150 mM glycerol or CM+150 mM urea were hypotonically challenged with water or 150 mM glycerol in water or 150 mM urea in water results in water influx. The above conditions caused
hypotonic-induced changes in cell shape due to influx of water (Fig. 15A, B, C).
However, cells cultured in media containing 150 mM glycerol or urea and placed in
isosmotic 250 mM PBS resulting in likely glycerol efflux (Fig. 15C) or urea efflux (Fig.
15D) did not show any cell shape changes. Similarly, cells cultured in CM and placed in
150 mM glycerol in PBS or 150 mM urea in PBS which creates a glycerol or urea inward
gradient resulting in probable glycerol (Fig. 15E) or urea influx (Fig. 15F) also had no
effect on cell shape. These results show that glycerol or urea fluxes alone do not initiate
any changes in cell shape.

**Osmotic tolerance of erythrocytes**

To examine the osmotic behavior of cells under an increased degree of hypotonic
challenge, a cell lysis assay was performed. In the cell lysis assay, the degree of
hypotonicity is 7-fold greater (10 mOsM final osmolarity of the solution that contains
1500 µl water, 75 µl PBS or PBS +150 mOsM solute, and 25 µl cell suspension) as
compared to the final osmolarity of 70 mOsM obtained in the hypotonicity-induced cell
shape assay. The number of intact erythrocytes in each frame of the images captured
throughout the 25 min time course (1 frame/15 sec) was recorded, and converted to a
percentage based on the number of cells present in the frame at time zero (Figure 16A, B,
C; Table 6). For erythrocytes cultured in CM and challenged with water, no intact cells
were visible at 15 min (Fig. 16A). In contrast ~40% of erythrocytes cultured in CM+150
mM glycerol or CM+ 150 mM urea were still intact at 15 min when challenged with 150
mM glycerol in water (Fig. 16B) or 150 mM urea in water (Fig. 17C), respectively. Thus
cells cultured in CM elongate faster and lyse at a faster rate compared to cells cultured in
media containing glycerol or urea. However, for cells cultured in CM+150 mM urea,
none of the erythrocytes had intact membrane by 30 mins, whereas 20% of erythrocytes cultured in CM+ 150 mM glycerol remained intact. Thus, cells cultured in glycerol and urea were more resistant to hypotonicity-induced osmotic stress.

Discussion

The cryopreservation and freeze-thaw responses of human erythrocytes have been studied for decades (Lovelock, 1954). Early on, it was shown that human erythrocyte freeze/thaw damage is substantially reduced by incubating cells in suspension medium containing 1500 mM glycerol (Lovelock, 1954). When incubated in 1500 mM glucose or 1500 mM glycerol, erythrocytes from the freeze tolerant wood frog R. sylvatica further enhanced their cryoprotectant ability (Costanzo and Lee, 1991). The cryoprotectant, glycerol that minimizes the freeze injury to human erythrocytes is naturally produced and accumulates in the freeze tolerant anuran H. chrysoscelis at a concentration of ~100 mM during cold acclimation (Zimmerman et al., 2007). Glycerol is likely synthesized in the liver of H. chrysoscelis and circulates to other parts of the body, where it acts as a cryoprotectant by colligatively reducing the amount of extracellular ice formed while stabilizing membrane structures during freezing. Cellular uptake of glycerol results in the creation of an osmotic equilibrium between the intracellular fluid (ICF) and ECF that moderates the degree of cellular water loss during extracellular freezing. We propose that the cellular acquisition of glycerol occurs through facilitated diffusion via HC-3, an aquaglyceroporin. The results of this study help to support this proposal. The immunocytochemistry results show enhanced HC-3 membrane expression. Thus, membrane localization may have a direct effect on water and solute permeability as well.
as hypotonicity-increased cell volume and resultant cell shape changes that occur during hypotonic challenge.

*In vivo* erythrocytes from *H. chrysoscelis* are subjected to severe hypotonic stress during thawing as ice melts. As a result, erythrocytes likely require aquaporins for mediating rapid transmembrane water flux. Erythrocytes cultured in glycerol media had significantly higher elongation rate measured in the first 10 sec of hypotonic challenge compared to other cultures. This increased elongation rate may be attributed to increase in membrane permeability and enhanced HC-3 localization to the membrane. The role of hypotonicity induced AQP1 localization regulating rapid water flow has been documented in mammalian cells (Conner et al., 2012). Rat astrocytes, under hypotonic stress directly trigger AQP1 translocation within seconds to regulate cell volume (Conner et al., 2012). Similar trafficking mechanisms might exist for HC-3 in cells of *H. chrysoscelis* which needs to be explored. Studies have shown that AQPs may act as membrane sensors to changes in cell volume by interacting with other volume-sensitive ion channels in the membrane to regulate water and solute transport (Chen and Duan, 2011). Under hypo-osmotic shock, yeast cells activate the GLP, Fps1p, which facilitates both uptake and release of glycerol, acting as primary osmoregulator, reducing osmotic stress on the cell (Tamas, 1999). Thus, it is also possible that HC-3, in coordination with other membrane ion channels may function as an osmosensing channel during cell swelling under hypotonic challenge.

The method we employed in this study does not provide information about the hypotonicity-induced cell volume changes since we only measured length and width of erythrocytes. Once the erythrocytes have attained an LA: SA ratio of 1:1, they are likely
spherical in shape and cell volumes can be calculated from the area measurements (Maroney, 1961). Erythrocyte lysis assay showed that once the cells reach spherical shape, their intact membrane is lost when subjected to higher degree of hypotonic shock indicating cell lysis (Fig. 16A, B, C). Cells cultured in media contacting glycerol and urea were able to tolerate such increased hypotonicity where 40% of erythrocytes were intact after 15 mins compared to complete cell lysis in cells cultured in control media (Fig. 16A, C). This can be correlated to alterations in cell shape, where cells remained elongated for a longer period before they became spherical in media containing glycerol (Fig. 14), indicating that glycerol has a stabilizing effect on cell shape. Thus, cell morphology and lysis phenomena are complex processes which are influenced by the nature of cryoprotectant, temperature and pH in addition to membrane proteins (Aldrich et al., 2006).

Apart from the role of HC-3 in facilitating water and glycerol flux, glycerol itself and its interactions with membrane and intracellular proteins may be an important component of freeze tolerance. Glycerol, by hydrophobically interacting with the membrane lipid bilayer, stabilizes the membrane and alters cell shape in human erythrocytes (Bakaltcheva et al., 1996). In animal cells, glycerol has been shown to effectively correct the misfolded proteins as well act as a stabilizing agent in rectifying temperature-sensitive protein defects (Brown et al., 1997).

Overall, our data suggest that glycerol may act both to regulate the localization and therefore the function of HC-3 in the membrane, and may contribute to moderating the dynamic cell shape/volume changes that occur during freezing and thawing in vivo, thus mechanistically contributing to freeze tolerance.
ACKNOWLEDGMENTS

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REFERENCES


Table 5: Erythrocyte elongation ratio changes under moderate hypotonic stress (n=6-8 cells) in the absence of 0.3 mM HgCl₂

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<td>CM (-Hg)</td>
<td>0.0095±0.002</td>
<td>0.485±0.11*</td>
<td>720±34*</td>
<td>*-0.495±0.13</td>
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<tr>
<td>Glycerol (-Hg)</td>
<td>*0.0239±0.001</td>
<td>0.288±0.03</td>
<td>668±37*</td>
<td>*-0.492±0.08</td>
</tr>
<tr>
<td>Urea (-Hg)</td>
<td>0.0038±0.003</td>
<td>0.251±0.16</td>
<td>760±62</td>
<td>*-0.076±0.06</td>
</tr>
<tr>
<td>Sorbitol (-Hg)</td>
<td>0.0048±0.004</td>
<td>0.150±0.04</td>
<td>818±61</td>
<td>0.148±0.03</td>
</tr>
<tr>
<td>NaCl (-Hg)</td>
<td>0.0003±0.0007</td>
<td>0.215±0.10</td>
<td>784±32</td>
<td>0.142±0.07</td>
</tr>
</tbody>
</table>

*Pair-wise comparison of different culture conditions, p<0.05.
Table 6: Percentage of intact erythrocytes under severe hypotonic stress in the absence of 0.3 mM HgCl₂

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Culture condition</th>
<th>CM</th>
<th>Glycerol</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Water</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>40</td>
<td>55</td>
<td>66</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0</td>
<td>41</td>
<td>39</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>0</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>0</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 12: **Immunofluorescence of HC-3 in erythrocytes.**

Immunocytochemistry was used to examine the subcellular localization of HC-3 in erythrocytes isolated from warm-acclimated frogs (A), or in cells cultured for 24 or 48 hrs at 20°C in control media (C,D), or in media containing (E,F) glycerol, (G,H) sorbitol, (I,J) urea, or (K,L) NaCl. The fluorescence intensity (green) was quantified using Image J (Plot Profile) along the line scans (in white on the image) and the results are displayed graphically beside each image of HC-3 immunoreactivity. Nuclei are counterstained with propidium iodide (red). (B) HC-3 immunoreactivity was blocked by preabsorption of the HC-3 antibody with the immunizing peptide. Scales bar represents 50 µm. Arrows indicate HC-3 membrane expression. Arroheads indicate polarized expression of HC-3 protein.
Figure 13: **Hypotonic-induced changes in erythrocyte cell shape.**

Membrane water permeability was determined by examining acute changes in cell shape after subjecting cells to hypotonic challenge. Changes in cell shape were examined by measuring the long-axis (LA) and the short-axis (SA) of erythrocytes (n=6-8 cells) (A), graph indicating the LA: SA ratio over the time course of 15 minutes (B). Cells were initially preincubated in the presence or absence of 0.3 mM HgCl₂ for 5 minutes and subjected to hypotonic challenge with water. Images were captured every 10 seconds for 10 minutes. In the absence of 0.3 mM HgCl₂, cells underwent a series of shape changes as they swelled, initially elongating, then becoming swollen and round. When aquaporins were blocked by HgCl₂, cells retained normal dimensions.
B

I LA:SA 0 .......... LA:SA at time 0
II LA:SA 300 .......... LA:SA at 300 sec
III LA:SA 600 .......... LA:SA at 600 sec
Slope .......... LA:SA 610- LA:SA 600
IV LA:SA max .......... Maximum LA:SA
T max .......... Time to reach maximum LA:SA
ΔLA:SA max .......... LA:SA max- LA: SA 600
V LA:SA 900 .......... LA:SA at 900 sec
Figure 14: **Hypotonic induction of dynamic cell shape changes in cultured erythrocytes.**

Erythrocytes cultured for 48 hrs in CM or media made hyperosmotic by the addition of 150 mM glycerol or urea, or in media made hypertonic by the addition of 150 mM sorbitol or 75 mM NaCl, were subjected to hypotonic challenge with water. The LA: SA ratio (n=6-8 cells) of hypotonically-induced cell shape changes were investigated at each time point and graphed as a function of time and statistically analyzed using repeated measures analyses of variance. A $P$-value below 0.05 was considered statistically significant.
Figure 15: **Effect of water and/or glycerol or urea fluxes on cell shape.**

Cells cultured in CM or CM+150 mM glycerol or CM+150 mM urea were placed in (A) water, (B) 150 mM glycerol in water, (C) 150 mM urea in water, (D) 250 mM PBS or (E) 250 mM PBS+150 mM glycerol, (F) 250 mM PBS+150 mM urea and cell shape was monitored. Regardless of culture condition, hypotonicity induced dynamic cell shape changes that were completely blocked by preincubation with HgCl₂ (A, B, C). Cells placed in solutions which imposed a transmembrane glycerol or urea gradient did not change shape (D, E, F).

![Hypotonic challenge with water](chart1)

![Hypotonic challenge with 150 mM glycerol in water](chart2)
C  Hypotonic challenge with 150 mM urea in water

D  Cells placed in isotonic 250 mM PBS
Cells placed in 150 mM glycerol in PBS

Cells placed in 150 mM urea in PBS
Figure 16: Erythrocyte lysis assay.

Erythrocytes cultured in CM or media made hyperosmotic by the addition of 150 mM glycerol or urea were subjected to hypotonic challenge with (A) water or (B) 150 mM glycerol in water or (C) 150 mM urea in water and % of intact erythrocytes were graphed as a function of time.
Cell lysis: Hypotonic challenge with 150 mM urea in water
CHAPTER V
SUMMARY AND FUTURE STUDY

According to August Krogh “For many problems there is an animal on which it
can be most conveniently studied” (Krogh, 1929). The phenomenon of freeze tolerance is
a complex trait which is influenced by both genetic and environmental factors. At the
whole organismal level, *H. chrysoscelis* is ideally suited to use as a model to explore the
mechanism of freeze tolerance. The physiological, evolutionary and molecular
adaptations this organism has employed to survive under extreme temperatures is of
special interest to comparative physiologists as well as environmental and evolutionary
physiologists. The goal of this research was to evaluate regulation of HC-3 expression
and explore the effects of expression changes by *in vitro* manipulation of cells isolated
from *H. chrysoscelis*. Understanding the nature of HC-3 expression changes and
regulation at the cellular level will give better insights into how this protein contributes to
whole animal freeze tolerance. To our knowledge, this is the first study to use
erthrocytes as a cell culture based model to study the role of GLPs in freeze tolerance.

Previous studies showed significantly high HC-3 mRNA expression levels in select
tissues of cold-acclimated *H. chrysoscelis* which correlates with increased accumulation
of glycerol (Zimmerman et al., 2007). Erythrocytes *in vivo* during cold-acclimation show
upregulation of native and glycosylated HC-3 protein, in addition to enhanced
localization of HC-3 to the membrane (Chapter 2; Mutyam et al., 2011A). However we
do not know the potential association between glycerol accumulation and HC-3
expression changes. In vivo, several factors might be responsible for glycerol
accumulation within the ECF spaces of tissue such as changes in temperature or
photoperiod and metabolic changes associated with availability of food. In the current
study, by manipulating the cultured erythrocytes in vitro which is independent of
photoperiod, metabolic and thermal cues, we were able to understand the possible role of
glycerol in regulating HC-3 expression during cold-acclimation. One of the factors that
influenced HC-3 expression in vitro is presence or absence of glycerol in the media. It is
well documented that aquaporins can be up-regulated in response to osmotic stress (Jenq
et al., 1998; Sugiyama et al., 2001; Umenishi and Schrier, 2002; Arima et al., 2003). In
cultured erythrocytes, addition of glycerol to the media resulted in higher abundance of
glycosylated HC-3 and enhanced membranous expression. However, the native HC-3
protein expression remained constant (Chapters 2 and 4). This indicates that cues that
trigger the expression of HC-3 protein during cold-acclimation might be through glycerol
independent mechanisms. Accumulation of glycerol may be regulating the expression of
HC-3 through post-translational and membrane trafficking mechanisms. Moreover,
membrane localization of HC-3 is also effected by urea, where erythrocytes in media
containing urea showed enhanced HC-3 membrane localization compared to erythrocytes
cultured in CM or media containing hypertonic sorbitol or NaCl (Chapter 4). This implies
that HC-3 localization is enhanced by hyperosmolarity in the presence of glycerol and
urea.
We also observed variation in the patterns of HC-3 glycosylation that can be attributable to interindividual variations that exist in the physiology and genetics of wild caught frogs (Chapter 2; Mutyam et al., 2011A). We have also noticed such differences in glycosylated HC-3 expression in wild caught treefrogs collected in different seasons (data not shown) indicating that seasonal variations might influence HC-3 expression. Freeze tolerant insects (*Eurosta solidaginis*) showed such seasonal variations in AQP expression which were dependent upon glycerol accumulation (Philip and Lee, 2010).

Rapid redistribution of water and solute is not only important during freezing, but is also essential during thawing. As ice melts, extracellular fluids become diluted, and to prevent excessive water uptake and cell lysis, solutes (e.g., glycerol) must rapidly exit the cell (reviewed in Mazur, 1984). Therefore, GLPs may also play an important role in the rapid redistribution of glycerol during thawing. During these events, cells either swell or shrink and change their shapes which may in turn depend upon membrane permeability. The studies of these cellular events during FT are poorly understood. The results from hypotonicity-induced shape changes showed that erythrocytes cultured in media containing glycerol responded rapidly with significantly higher elongation rate and at the same time tolerated hypotonic-induced shape changes compared to controls (Chapter 4). Glycerol has been shown to act as a good stabilizing agent providing structural support to erythrocyte membrane especially at low temperature (Cunha et al., 2007). Thus in addition to glycerol, temperature also plays an important role in determining the shape of cells. Osmotic fragility studies of erythrocytes indicate the extent to which they can tolerate hypotonic stress before their membrane is disrupted and have also shown to influence the shape and size of erythrocytes (Troiano et al., 1998). The examination of
intact erythrocyte membrane under extreme osmotic stress is an attempt to correlate to their shape changes. Cells cultured in glycerol and urea containing media were able to tolerate increased osmotic stress to a greater degree compared to cells cultured in CM (Chapter 4). These osmotic tolerance capabilities are likely dependent on the functionality of aquaporins, which are critical for rapid transmembrane fluxes that occur during freezing and thawing. Using an in vitro cell culture model, our study demonstrated that enhanced HC-3 glycosylation and localization is regulated by glycerol and urea-mediated hyperosmotic stress in addition to their role in moderating dynamic cell shape and lysis and thus may contribute to increased membrane permeability. Combined, these data suggest that enhanced GLP expression may contribute to rapid water and glycerol redistribution during cold acclimation and may promote freeze tolerance.

**Future study**

Erythrocytes in humans express AQP1 and are highly permeable to water (Agre et al., 1993). In contrast erythrocytes in *H. chrysoscelis* did not show any HC-1 protein expression even though weak mRNA signal was detected (Goldstein et al., 2010). The absence of HC-1 might be compensated by HC-3 expression, which permeates both water and glycerol. We do not know if erythrocytes from *H. chrysoscelis* also express other AQP/GLPs GLP, HC-9, which might participate in glycerol transport. Therefore, future experiments need to address the possibility of the presence of additional AQP/GLPs in erythrocytes.

The molecular, biochemical and physiological mechanisms that are involved in the FT process have been well defined in the freeze tolerant wood frog *R. sylvatica* (reviewed in Storey, 2004). The environmental cues that initiate FT in *R. sylvatica* are
thermal and day-light cycle cues in addition to metabolic changes and accumulation of cryoprotectant within their extracellular fluid (reviewed in Storey and Storey, 2005). The cues that initiate glycerol production in *H. chrysoscelis* are currently not known and require more investigation. The environmental signals that govern the physiological responses, including regulation of aquaporin expression are poorly understood. To address the link between the environmental cues and the physiological responses, experiments to address a seasonal timeframe of cold-acclimation and corresponding metabolic and endocrine responses need to be conducted. It is possible, that a combination of changes in food availability, water intake, diurnal cycles, and night time temperature work together to induce the physiological changes that occur during cold-acclimation. It is also possible, that an individual factor may be solely responsible for initiating a cascade of responses. Endocrine mediators such as arginine vasotocin, epinephrine, and/or cortisol may be released *in vivo* in response to environmental cues. Therefore, using the erythrycyte culture model established as part of this dissertation work, it may be possible to establish a direct correlation between these mediators and HC-3 protein abundance, cellular/subcellular localization and its glycosylation state.

It is also unknown if posttranslational modifications such as glycosylation including membrane trafficking of HC-3 protein result in increased membrane permeability to water and glycerol. Furthermore it is not understood whether glycerol is a direct or indirect mediator of HC-3 regulation. Future studies would be needed to determine the regulatory mechanisms associated with changes in HC-3 expression. Inhibition of glycosylation and membrane trafficking pathways by adding specific
inhibitors that block these pathways, would help in better understanding their role in HC-3 expression.

The specific contribution of aquaporins to the process of freeze-tolerance would be most convincingly demonstrated by comparing properties of cells with and without functional aquaporins. A novel method for morpholino-mediated HC-3 knockdown has been developed for this purpose (Mutyam et al., 2011B; Chapter 3). The role of HC-3 protein in freeze tolerance can be evaluated by comparing the post-freeze viability of erythrocytes that express HC-3 vs. those that do not. Also hypotonic-induced erythrocyte shape changes can be tested in HC-3 morpholino-treated vs. control erythrocyte cultures. We predict that the knockdown of HC-3 will compromise functional properties of treefrog erythrocytes including freeze tolerance, water and glycerol permeability, and cell volume control. If HC-3 knockdown does not have any effect on erythrocyte membrane transport to water and glycerol, it is possible that expression of other AQP/SLPs might compensate for the absence of HC-3 in permeating water/glycerol transport. Thus by using the in vitro erythrocyte cell culture system, the key players that regulate HC-3 expression can be identified and also the functional role of HC-3 protein for its ability to tolerate freezing can be determined.

**Broader impacts**

Cope’s gray treefrog, serve as a natural model for elucidating the biology of GLPs and for exploring mechanisms of freeze tolerance that may have broad applicability to the general field of cryobiology. Since the natural lifecycle of this organism involves climate changes and below freezing temperatures, understanding the physiology of these animals may prove to be important to conservation efforts which are focused on the effects of
climate change on declining amphibian species. Increased understanding of their physiological processes will assist in developing cryopreservation techniques that may be adapted to other organisms. Also, the cryopreservation of cells and tissues for clinical use in transplants is currently most successful with small or single-celled tissues. Therefore, insights into how a multicellular organism survives freezing could yield important clues to the cryopreservation of larger tissues and organs, even those from mammals. Thus, by understanding the physiology of this species, we are advancing our knowledge in the fields of comparative physiology, biomedicine, and potential conservation ecology as novel conservational methods to preserve germ cells or embryos from a variety of endangered species may eventually be developed.
REFERENCES


