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Strategies for exploration of freeze responsive gene expression: advances in vertebrate freeze tolerance☆

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Abstract

Winter survival for many cold-blooded species involves freeze tolerance, the capacity to endure the freezing of a high percentage of total body water as extracellular ice. The wood frog (*Rana sylvatica*) is the primary model animal used for studies of vertebrate freeze tolerance and current studies in my lab are focused on the freeze-induced changes in gene expression that support freezing survival. Using cDNA library screening, we have documented the freeze-induced up-regulation of a number of genes in wood frogs including both identifiable genes (fibrinogen, ATP/ADP translocase, and mitochondrial inorganic phosphate carrier) and novel proteins (FR10, FR47, and Li16). All three novel proteins share in common the presence of hydrophobic regions that may indicate that they have an association with membranes, but apart from that each shows unique tissue distribution patterns, stimulation by different signal transduction pathways and responses to two of the component stresses of freezing, anoxia, and dehydration. The new application of cDNA array screening technology is opening up a whole new world of possibilities in the search for molecular mechanisms that underlie freezing survival. Array screening of hearts from control versus frozen frogs hints at the up-regulation of adenosine receptor signaling for the possible mediation of metabolic rate suppression, hypoxia inducible factor mediated adjustments of anaerobic metabolism, natriuretic peptide regulation of fluid dynamics, enhanced glucose transporter capacity for cryoprotectant accumulation, defenses against the accumulation of advanced glycation end products, and improved antioxidant defenses as novel parts of natural freeze tolerance that remain to be explored.
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Natural freeze tolerance is key to winter survival for many species. Multiple adaptations allow organisms to endure the accumulation of ice in extracellular fluid spaces and the many consequences of freezing including long term ischemia caused by the interruption of blood flow and major changes in cell volume, ionic strength, and osmolality caused when 65% or more of total body water is locked in ice. For over twenty years, research in my laboratory has focused on the metabolic adaptations that underlie natural freezing

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survival in animals using insect, molluscan, and lower vertebrate model animals. Recent reviews summarize these studies [24,27,28]. In particular, we have a primary interest in vertebrate freezing survival and have focused most of our efforts on the biochemical adaptations that support freezing survival by the wood frog, *Rana sylvatica* [29]. One of the prominent features of wood frog freeze tolerance is the production of huge amounts of glucose as the cryoprotectant. Triggered within minutes when freezing begins, high rates of liver glycogenolysis pump out glucose for delivery to all organs, raising glucose in blood and core organs to 200–300 mM, compared with ~5 mM in 5 °C control animals. This extreme hyperglycemia can be compared with the case of human diabetics that suffer multiple metabolic injuries from uncontrolled glucose levels ranging from 10 to 50 mM.

In recent years we have become very interested in the role of gene expression in freezing survival and have used a wide range of techniques in molecular biology to explore the freeze-responsive up-regulation of genes and synthesis of proteins. Note that our focus is on freeze-responsive genes/proteins as opposed to cold-induced or seasonally expressed genes/proteins which also play important roles in the winter freeze tolerance of wood frogs (e.g., enhanced levels of glycogenolytic enzymes to support cryoprotectant synthesis are put in place well before freezing begins). Interestingly, when we have looked for genes that are up-regulated by freezing in the freeze tolerant insect, *Eurosta solidaginis*, we have actually found very few. We postulate that this is because changes in gene/protein expression that support freeze tolerance are primarily put in place as part of the annual life cycle of this univoltine species, facilitated by photoperiod and temperature cues during an autumn cold-hardening period.

Wood frogs, however, show quite an impressive array of freeze-responsive changes in gene and protein expression during the several hours that it takes for the animals to freeze. This different strategy may recognize the fact that individual frogs may not always freeze in any given winter. Because wood frogs hibernate on the forest floor under an insulating layer of leaf litter and snow, freezing may not always be an inevitable event of winter. Hence, some of the protein adaptations that support freezing survival would not be needed if freezing did not occur and, therefore, may only be triggered when freezing starts. For example, ectothermic vertebrates and hibernating mammals suppress the levels of clotting factors in their blood over the winter months to reduce the probability of spontaneous clot formation at the low blood perfusion rates that occur when body temperature is near 0 °C. One of the freeze-responsive genes that we have identified is fibrinogen [1]. Synthesis of this protein (and perhaps other clotting factors as well) recognizes the probable need for enhanced clotting capacity to deal with any bleeding injuries to frog tissues that are detected after thawing but constitutively elevated clotting capacity may have negative consequences for frogs during the long winter hibernation if freezing does not occur. Hence, it makes sense for the fibrinogen genes to be freeze up-regulated.

Over the years we have used multiple technologies to provide insights into freeze-responsive gene and protein expression by wood frogs. Initial studies searched for freeze or thaw responsive proteins by using ^{35}S-methionine to label proteins either in vivo after intraperitoneal injection or during in vitro translation of mRNA isolated from control versus frozen frogs [30,31]. Both studies illustrated freeze–thaw and organ-specific differences in protein biosynthesis in wood frogs with prominent synthesis of some 15–20 kDa proteins, but both were limited the inability to identify individual proteins. Interestingly, major recent advances in proteomics technology including 2-dimensional electrophoresis coupled with LC-MS (liquid chromatography-mass spectrometry) of peptide fragments might now make a return to such studies fruitful.

Approaches to freeze-responsive gene expression

To gain a handle on the protein adaptations that support natural freeze tolerance, we turned instead to molecular techniques for gene screening. Over the last eight years we have explored multiple approaches including the construction and screening of cDNA libraries, cDNA array

screening, differential display polymerase chain reaction (dd-PCR), and reverse transcription polymerase chain reaction (RT-PCR). In general, dd-PCR proved to be an unsatisfactory method for gene discovery in our systems but we now use RT-PCR widely as a method of quantifying relative changes in mRNA levels between control and experimental samples, largely replacing our previous use of Northern blots for this purpose. We also use RT-PCR to allow us to examine the expression of selected specific genes in response to freezing and other stresses (e.g., genes for proteins in the same family or the same pathway as a protein that we know to be freeze up-regulated). Using the rich databases of gene sequence information now available for many different organisms, we can design primers for highly conserved regions of the mRNA sequences of such proteins and, in most cases, we can find and amplify the corresponding wood frog mRNA with relative ease.

The first genes that we identified as freeze up-regulated in wood frogs were identified from screening of a cDNA library prepared from liver of frozen frogs. Studies by Dr. Q. Cai reported increased levels of mRNA transcripts encoding fibrinogen α and γ subunits, the ADP-ATP translocase (a mitochondrial membrane transporter), and a novel gene that we called *fr10* [1–3]. Further screening of the liver library revealed a number of other freeze-responsive genes including the mitochondrial inorganic phosphate carrier (PiC) [5], NADH-ubiquinone oxidoreductase subunit 4 (ND4) and elongation factor 1 gamma subunit (EF-1γ) (S. Wu and K. Storey, unpublished data) and two more novel genes that we named *li16* and *fr47* [19,20]. The ability to detect and retrieve mRNA transcripts that encode novel genes that may have a specific adaptive role in the stress under study is a key advantage of cDNA library screening. Other examples of novel proteins that are specific to cold- or freeze-tolerant animals and plants are well-known including the antifreeze proteins of coldwater fish and cold-hardy insects [9] and the COR proteins of cold-acclimated plants [10]. The functions of the former are well-known, but the latter are not, although they are the subject of intense study. Researchers studying biochemical adaptation were formerly restricted to identifying a phenotype characteristic (e.g., a thermal hysteresis between plasma freezing and melting temperatures) that was unique to the organism/stress under study and then following that down to the protein level to find the molecular basis of the adaptation. With cDNA library screening technology, however, a whole new approach is possible—working from the gene level upwards—progressing through gene–protein expression, protein structural studies, protein regulatory and subcellular localization analysis, and finally to a protein function. Interestingly, our use of cDNA screening technologies, to date, has only rarely identified genes/proteins that we can directly link with the major phenotype characteristic that we have been most concerned with for many years—the extreme hyperglycemia that occurs in frozen frogs. Most of the freeze-responsive genes that we have identified seem to be involved with a range of other cell functions, never before been linked with freezing survival. This is particularly so of our new studies using cDNA arrays, as discussed further below.

Over the last couple of years our lab has moved to the use of cDNA array screening technology to identify genes that are up-regulated in response to stress in a number of animal systems. We have tested different types of arrays but have gathered our largest and most comprehensive data sets using the 19K human cDNA chips (containing over 19,000 cDNAs for known genes or expressed sequence tags) produced by the Microarray Center of the Ontario Cancer Institute (http://www.uhnres.utoronto.ca/services/microarray/index.html) to screen for freeze responsive genes in wood frogs, hibernation responsive genes in ground squirrels and bats [6], and anoxia responsive genes in intertidal marine gastropods [17]. Advantages of array screening include: (1) the majority of the genes are already identified, (2) transcripts that are present in low copy number can be detected (versus library screening which favours abundant transcripts), and (3) responses by groups of genes can be assessed (e.g., a family of heat shock proteins, or consecutive members of a signal transduction cascade); this latter advantage is extremely useful for developing a "big picture" of stress-induced metabolic responses. However, heterol-

ogous probing (array from one species, RNA from another) has the disadvantage that due to sequence variation between species, cross-hybridization is not 100%. The potential for false negatives is quite high; i.e., genes that may be stress-responsive but do not have sufficient sequence identity to hybridize with their human counterparts. This can be partially overcome with some initial optimization of hybridization and washing conditions and, ultimately, we have achieved high levels of cross-hybridization, especially within vertebrate groups. Cross-hybridization was 85–90% with human cDNA arrays and cDNA from hibernating mammals (ground squirrel or bat) [6], somewhat lower at 60–80% for frog tissues, and just 18.35% for hepatopancreas of the marine snail *Littorina littorea* (a freeze- and anoxia-tolerant intertidal species) [17]. Consider, however, that with littorines, even though only 10.6% of the genes that hybridized were designated as putatively up-regulated, this still provided us with over 300 anoxia-responsive genes that can be followed up.

To date we have used cDNA arrays to analyze freeze-responsive gene expression in nine different organs of wood frogs. Results for heart, for example, produced over 200 genes that were putatively up-regulated during freezing by at least 1.5-fold with substantial numbers that showed 4–7-fold higher expression in samples from frozen versus control heart. These are discussed more fully below. In addition, a wide range of protein kinases and protein phosphatases and various related regulatory subunits of these enzymes also showed putative up-regulation during freezing which stresses the importance of signal transduction mechanisms in producing coordinated adaptive changes during freezing.

Novel proteins and frog freeze tolerance

Fig. 1 outlines the process of discovering and characterizing freeze responsive novel genes using *li16* as the example [19]. We began with screening of a liver cDNA library and the isolation of a clone that showed differential hybridization with mRNA probes prepared from liver of frozen versus control frogs. After insert isolation from the clone, northern blotting was used to confirm freeze responsive up-regulation and to show the pattern of mRNA transcript accumulation during a freezing event. Sequencing of the *li16* insert revealed an incomplete 5′ end of the gene so the technique of 5′RACE was applied and the full open reading frame was retrieved. Northern blots showed elevated transcript levels which are generally the result of increased gene transcription but can also arise from other factors such as changes in transcript stability under different cellular conditions. The nuclear run-off assay provides the way to confirm that the rate of gene transcription actually changes under an experimental condition. Fresh nuclei are isolated from control and experimental tissues (in this case from liver 5 °C-acclimated and 24 h frozen frogs) and the mRNA transcripts of genes that are being actively transcribed are allowed to elongate in vitro in the presence of ^{32}P-UTP. Total RNA is then extracted and relative levels of ^{32}P-labeled transcripts are determined in dot blots using the *li16* probe and probe for one or more constitutively active genes. The results demonstrated that levels of actively transcribing *li16* mRNA transcripts were 2.4-fold higher in nuclei from frozen frogs versus controls, which confirms active transcription of *li16* during freezing. Finally, Western blotting was undertaken to determine whether the Li16 protein was accumulated during freezing; i.e., active transcription of a gene does not always mean increased translation. In this case, however, the increase in Li16 protein levels paralleled the increase in *li16* mRNA over the time course of freezing demonstrating that the protein is actively synthesized in liver during freezing and indicating that transcriptional control of the *li16* gene is likely the primary factor that regulates Li16 protein levels.

The three novel freeze-responsive genes isolated from wood frog liver show no sequence similarity to any known gene (or reported expressed sequence tag) when subjected to BLAST searches nor do they appear to be related to each other. However, they show some interesting features and we are currently trying to elucidate the function of each protein. Details of the sequence, functional groups, and time/stress dependent expression of both mRNA transcripts and proteins are available in individual publications for FR10, Li16, and FR47 [2,19,20],

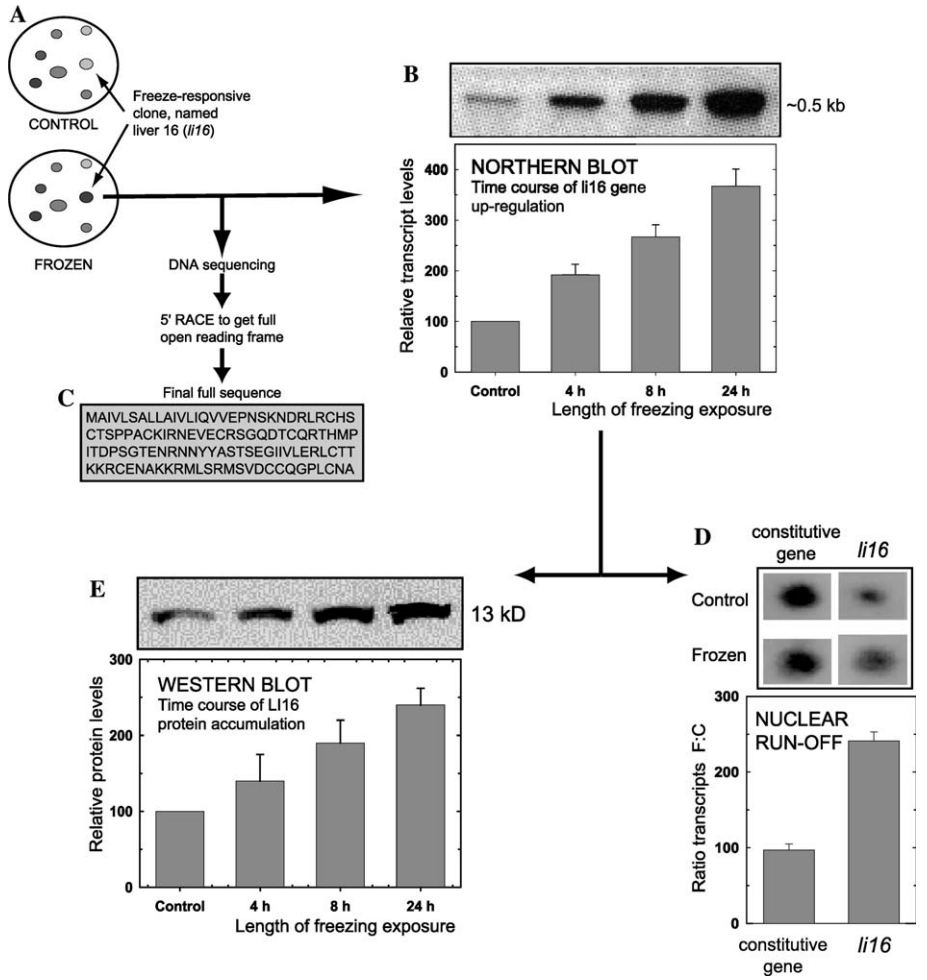

Fig. 1. Schematic showing the isolation, sequencing, and characterization of a freeze responsive gene from liver of wood frogs. (A) A cDNA library was constructed from liver of 24 h frozen frogs and screened with cDNA probes made from liver mRNA of control versus frozen frogs. Comparison revealed a freeze-responsive clone that was named *liver 16* (*li16*). (B) Total mRNA was isolated from control versus frozen liver and hybridized with *li16* probe. These Northern blots confirmed an increase in *li16* transcript levels over a time course of freezing. (C) The *li16* insert was isolated, sequenced, and found to be missing its 5′ end. The technique of 5′ RACE was used to acquire the full open reading frame and then a final sequence was produced, subjected to a BLAST search and shown to have no sequence similarity to any known gene. (D) Fresh nuclei were isolated from liver of frozen frogs and incubated in vitro with α-^{32}P-UTP to label elongating nascent mRNA molecules. These nuclear run-off assays confirmed that the increased *li16* mRNA transcript levels in liver of frozen frogs was the result of increased rates of gene transcription (not other mechanisms such as increased transcript stabilization in frozen animals). (E) Antibodies were raised against a synthetic peptide representing the C-terminal decapeptide of the Li16 protein. Western blotting confirmed increased translation of this 13 kDa protein over the time course of freezing exposure. Data are compiled from McNally et al. [19].

but here I want to emphasize comparisons. Table 1 summarizes some of the properties of the three proteins. Apart from being up-regulated during freezing in liver, the only other shared characteristic that we know of to date is the presence in all three of a hydrophobic region of 21 amino acids in length. In FR10 and Li16 this is an N-terminal region whereas in FR 47 the hydrophobic region is near the C-terminus. In both FR10 and Li16 the region contains four leucine and four valine residues (although they

Table 1
A comparison of the properties of three novel proteins that are freeze-responsive in wood frog liver

	FR10	Li16	FR47
Molecular weight (kDa)	10	12.8	45.7
Number of amino acids	90	115	390
Sequence characteristics	N-terminal hydrophobic region, aa 1–21, possible nuclear exporting signal	N-terminal hydrophobic region, aa 1–21	hydrophobic region near C terminal, aa 350–370
Transcript response to stress (relative to unfrozen control = 100)[a]			
24 h freeze (2 h thaw)	320 (110)	370	510
20% dehydration (full rehydration)	↑↑↑ (↑↑)	802	220
24 h anoxia (1 h recovery)	↓↓↓↓ (n.a.)	760	620
Protein response to stress (relative to unfrozen control = 100)[a]			
24 h freeze (2 h thaw)	n.a.	240 (840)	160 (350)
20% dehydration (full rehydration)	n.a.	175 (110)	65 (125)
24 h anoxia (1 h recovery)	n.a.	440 (130)	60 (90)
Transcript response to in vitro tissue incubation with second messengers[b]			
dibutyryl-cAMP	n.a.	n.s.	n.s.
dibutyryl-cGMP	n.a.	↑↑	n.s.
PMA	n.a.	n.s.	↑↑
Organ distribution	all organs tested	liver, gut, heart, and liver	
Genbank Accession Nos.	U44831	AF175980	AY100690

[a] Values for liver mRNA transcript and protein levels are relative to levels in controls (set to 100); data show effects of the stress with numbers in brackets for the relative level in recovery (2 h thaw, full rehydration sampled after 24 h, or 1 h back under aerobic conditions); the numbers are mean values from quantification of band densities on 3–5 blots whereas for FR10 responses to freezing and anoxia, the arrows show results of $n = 1$ trials.

[b] For in vitro incubations, liver slices from control frogs were incubated with varying concentrations of second messengers (20–2000 μM db-cAMP, db-cGMP, or 2–200 μM PMA), or for varying times (1–10 h) with a set concentration (200 μM) of second messenger. PMA is phorbol 12-myristate 13-acetate. n.a., not available; n.s., not significant change. Data are compiled from [2,19,20].

are not alike in sequence). Such hydrophobic regions are not uncommon in proteins and quite likely represent a transmembrane segment which suggests that all three proteins may associate with membranes. Our original analysis of FR10 suggested that this was a putative nuclear exporting signal [2].

Transcript levels of all three proteins were elevated by 3–5-fold in liver after 24 h of freezing at −2.5 °C and Western blotting with antibodies raised against the C-terminal peptide of Li16 and FR47 confirmed that levels of both proteins were also elevated in liver of frozen frogs (by 2.4- and 1.6-fold, respectively after 24 h). Interestingly, both Li16 and FR47 levels continued to increase over the first 2 h of thawing at 5 °C to maximum levels of 8.4- and 3.5-fold higher than control values (note: after 2 h at 5 °C frogs are only about half-thawed, heart beat has not resumed, and liver is still visibly shrunken) before returning to near control levels by 8 h thawed. This suggests the production of these proteins is continued while the liver is still under stress (i.e., still ischemic, still under cell volume stress), but their disappearance by 8 h thawed (when heart beat and breathing have resumed and liver appears to be visibly restored to normal size) indicates that they are no longer needed once recovery is well advanced. Similarly, although protein levels of FR10 were not measured, *fr10* transcripts in liver quickly returned to control values after thawing. This argues for definite roles for the three novel proteins in dealing with the stresses associated with freezing.

To characterize the function of an unknown protein, pieces of information can be gathered from

a variety of sources. For instance, the organ distribution of the transcripts suggests quite different roles for the three protein products. Transcripts of *fr47* were found only in liver, *li16* was prominent and strongly up-regulated only in liver, heart and gut whereas *fr10* appeared in all eight organs that we tested; hence, functions of the three proteins can be interpreted as liver-specific, restricted to selected organs (which may share a common characteristic), or universal. Furthermore, FR47 protein was detected in liver of two other freeze tolerant frogs, *Pseudacris crucifer* and *Hyla versicolor*, but not in freeze intolerant *Rana pipiens*, or *Scaphiopus couchii*. This species-specific distribution strongly implicates FR47 as being freeze-specific since tolerances for other stresses (anoxia, dehydration) that are components of freezing are just as high in leopard frogs and spadefoot toads as they are in freeze tolerant species.

As mentioned above, two of the main components of freezing stress are ischemia due to the interruption of heart and lung function when ice encases the organs and plasma freezes and cellular dehydration caused by the outflow of a high percentage of organ water to join extracellular ice masses. We first noted a differential responsiveness of freeze tolerance adaptations to anoxia (mimicking the oxygen deprivation part of ischemia) versus dehydration when studying the control of cryoprotectant biosynthesis. Glucose output from wood frog liver was stimulated just as strongly when frogs were dehydrated as when they were frozen, but anoxia exposure had no effect on glucose levels [26]. It is not surprising that the role of glucose as a colligative cryoprotectant is triggered and regulated from cell volume signals, but we have also found that virtually every gene expression response to freezing can also be categorized as responding to anoxia versus dehydration signals. In this regard, the three novel proteins follow suit. Transcripts of *fr10* were strongly up-regulated by dehydration whereas both *li16* and *fr47* transcripts responded strongly to anoxia exposure. Li16 protein was also very high in anoxia-exposed frogs (24 h under N_2 gas at 5 °C). However, although *fr47* transcripts responded to anoxia, the protein did not which suggests that additional controls are involved in its expression; an anoxia/ischemia signal may have been "co-opted" to trigger gene expression during freezing but the protein is actually produced only when the frog is freezing.

Knowledge of the signal transduction pathways involved in regulating the expression of a gene can also provide hints about the metabolic role of the protein product and about the initiating signal, such as a hormone, that may have triggered the signal transduction cascade involved. For instance, the cryoprotectant response of extreme hyperglycemia was rather easily traced to the activation of β-adrenergic receptors on wood frog hepatocyte membranes followed by increased production of adenosine 3′5′ cyclic monophosphate (cAMP) and the activation of cAMP-dependent protein kinase (PKA) to stimulate liver glycogenolysis [26,29]. In vitro incubation of liver slices with different second messenger signaling molecules was used to derive similar information about the regulation of *li16* and *fr47* genes. Neither gene responded to tissue incubations with the cAMP analogue, dibutyryl cAMP, but *li16* transcripts were up-regulated during incubation with cGMP (guanosine 3′5′ cyclic monophosphate). This response to cGMP is very interesting in light of results from cDNA array screening that are presented in the next section; these indicate that the adenosine receptor signaling pathway is up-regulated during freezing, a pathway that is mediated intracellularly by cGMP. By contrast, *fr47* responded to phorbol 12-myristate 13-acetate, a stimulator of the Ca^{2+}, and phospholipid dependent protein kinase C. We have previously shown that inositol 1,4,5-trisphosphate (IP_3) levels, an intracellular second messenger of PKC, rose progressively over time during freezing in wood frog liver reaching about 3- and 11-fold higher than control values after 4 or 24 h of freezing [12]. By contrast, IP_3 rose by 8-fold within 30 min in liver under anoxia but was much less responsive to dehydration (about 3-fold higher in 20% dehydrated animals) [13]. Since IP_3 responds strongly to anoxia and since freezing involves the progressive development of anoxia/ischemia, it would appear that the rise in IP_3 levels during freezing is in response to developing anoxia/ischemia, and that one of the consequences of this may be up-regulation of the *fr47* gene.

Overall, then, we have assembled a considerable amount of information about each of these novel genes and, although we do not yet know their function, we are continuing to move forward on several fronts studying, for example, the subcellular localization of the proteins, and their capacity to improve the freezing survival of cells transfected with each of these genes.

cDNA array screeningnew horizons in freeze tolerance

Table 2 lists selected genes that were identified by cDNA array screening as putatively up-regulated in wood frog heart during freezing. The initial results from heterogeneously probed chips must always be treated with caution and all "hits" must be verified by other techniques (e.g., RT-PCR or Northern blotting) before any further studies are undertaken. A small possibility of false positive matches exists (i.e., frog cDNA that binds to a human cDNA that is not its homologue). To date, however, we have never encountered this in our downstream analysis of the genes/proteins identified from array screening. We have specifically confirmed up-regulation of six of the proteins shown in the table, including three that we know from past studies are freeze up-regulated (ATP/ADP translocase, glucose transporters, and glucose-6-phosphatase) [3,15,25]. Overall, then, we are confident that these genes represent good leads for future studies of vertebrate freeze tolerance.

The data in Table 2 suggest a variety of new research ideas for expanding our knowledge of the adaptive mechanisms that support freeze tolerance. Interpreted broadly, these gene responses indicate that frogs are activating multiple forms of defense against potential damage due to ischemia and are responding metabolically to the challenges of extreme hyperglycemia. Suites of genes controlled by at least two responses elements are up-regulated: the antioxidant response element and the hypoxia response element. These array screening data also illustrate one of the key advantages of this technique—the ability to identify groups of proteins/enzymes that can have interrelated functions. For example, several of the genes listed in Table 2 are related to signaling and response to ischemia; in isolation, each might be difficult to interpret, but together several interesting patterns appear. Several of the new leads that we are following are outlined below.

Adenosine receptors and 5′ nucleotidase

Adenosine A1 and A2A receptors were putatively up-regulated during freezing in frog heart as was 5′ nucleotidase, the enzyme that synthesizes adenosine from AMP. Adenosine accumulates quickly in the brain of anoxia tolerant turtles when animals are exposed to anoxia and acts via adenosine A1 receptors to suppress the activities of ATP-dependent ion channels as part of the overall metabolic rate depression that ensures anoxia survival [21]. Overexpression of adenosine A1 receptors is also known to increase myocardial tolerance of ischemia in transgenic mice [16]. Hence, these data from array screening give us a clear indication that as wood frogs freeze they activate a set of ischemia-protective signals and receptors. Furthermore, the study of Lankford et al. [16] used cDNA array screening to identify genes with altered expression as the result of adenosine A1 receptor overexpression. Significantly, these included the GLUT 4 glucose transporter and Na^+K^+ATPase, both included in Table 2.

Atrial natriuretic peptide (ANP) receptor

ANP is a hormone produced by heart that has a major function in regulating intravascular volume and inhibiting the hypertrophic response by cardiomyocytes. Volume expansion and pressure overload increase ANP expression and secretion; its primary targets are the kidney where ANP binding increases glomerular filtration rate and inhibits tubular sodium resorption [8] and the vasculature where ANP causes a potent vasodilation. Clearly, freezing causes a huge upset of normal fluid dynamics in the frog and ANP may have a role in orchestrating responses by all organs (even heart) to these changing fluid conditions. Up-regulation of the receptor for ANP in heart argues for a role for ANP in freeze tolerance. Notably, ANP works through a cGMP-mediated

Table 2
Genes identified as putatively freeze up-regulated in wood frog (*R. sylvatica*) heart as determined from cDNA array screening using human 19K cDNA gene chips (Ontario Cancer Institute) and cDNA prepared from heart of control (5 °C acclimated) and frozen (24 h at −3 °C) frogs

Genbank Accession #	Name	Details
Signaling related		
R84726	Adenosine A1 receptor	
W15613	Adenosine A2A receptor	
R47859	Natriuetic peptide receptor	A trial isoform
H82585	5′ Nucleotidase	
Glucose-related		
R11726	Facilitated glucose transporter	Type 4, insulin responsive, solute carrier family 2
R02365	Glucose-6-phosphatase	Liver type
AA129587	Aldo-keto reductase	Family 1, member A1:NADPH-aldehyde reductase
W74536	Receptor for advanced glycosylation end products (RAGE)	
Transmembrane carriers and ion motive ATPases		
N93936	Monocarboxylic acid transporter	Solute carrier family 16, member 3, MCT2
R33450	Adenine nucleotide translocator	Mitochondrial carrier, solute carrier family 25
H14143	Na^+-K^+ ATPase	α3 subunit
Hypoxia related		
W47003	Hypoxia-inducible factor 1	α subunit
N43981	ATP synthase, F_o–F_1 complex	F_o subunit c, isoform 3
Antioxidant defense related		
R81846	Ferritin	Light chain
R37412	Glutathione-*S*-transferase	θ1 isozyme
AA136700	Thioredoxin	
W78010	Metallothionein 1G	
AA213449	Glucose-6-phosphatase dehydrogenase	

Total RNA was isolated from frog heart using Trizol reagent and then fluorescently-labeled cDNA was prepared using Cy3 or Cy5 labeled dCTP to label cDNA from control versus freeze-exposed samples, respectively. Labeled probes were hybridized with arrays for 18 h in a microscope slide holder using conditions as defined by the manufacturer, followed by washing with decreasing concentrations of SSC (5×, 2×, 1×, and 0.5×) containing 0.1% SDS, and spin drying. Fluorescence intensity and wavelength were quantified in a fluorescence gene chip readers and quantified using Arraypro software.

pathway (known to stimulate *li16* expression) and has also been shown to reduce ischemia/reperfusion damage in several organs. Clearly, ANP is a target for further study.

Hypoxia-inducible factor 1 (HIF-1)

HIF-1 is the transcription factor that is primarily responsible for activating gene expression in vertebrate organs in response to hypoxia; typically, HIF-1 mediates two gross responses to hypoxia—it increases the capacity for glycolytic ATP output and it stimulates increased oxygen delivery to tissues by increasing production and release of red blood cells, and stimulating capillary growth. The up-regulation of HIF-1 during freezing is likely triggered by the progressive hypoxia/ischemia that develops during freezing. Many of its gene targets are now well known which will facilitate the exploration of HIF-1 mediated gene expression during freezing. For example, HIF-1 is well known to stimulate expression of the GLUT1

transporter, the ubiquitous glucose transporter of all vertebrate organs, so this action might be key for cryoprotectant loading into frog organs. Another typical action of HIF-1 is to stimulate increased expression of glycolytic enzymes, but activities of these enzymes are not elevated in frog organs during freezing [4] so the normal link between increasing glucose uptake capacity by GLUT1 and increasing glycolytic capacity must be dissociated during freezing in order for organs to accumulate the cryoprotectant pool.

Glucose related proteins

GLUT4 is the insulin-responsive glucose transporter found mainly in muscles and adipose tissue. Multiple studies have correlated improved ischemia protection of mammalian heart with the enhanced expression and increased presence in the sarcolemmal membrane of GLUT4 which allows increased uptake of the anaerobic fuel, glucose [18,22]. Wood frogs may have co-opted this mechanism, not so much for ischemia protection since frog organs seem to rely on endogenous glycogen for anaerobic glycolysis in the frozen state, but as a probable means for enhancing their uptake of cryoprotectant from the bloodstream into muscle tissues. Glucose-6-phosphatase (G6Pase) is also putatively up-regulated in heart and its activity is known to increase during freezing in liver and skeletal muscle of wood frogs [25]. In liver, the enzyme is clearly essential to the synthesis the cryoprotectant, glucose, from the glycolytic intermediate, glucose-6-phosphate (G6P), but other organs don't seem to make their own cryoprotectant. However, to retain a static pool of cryoprotectant throughout the freeze it may be necessary to (a) strongly suppress the activity of hexokinase (that reconverts glucose to G6P), and (b) elevate G6Pase activity to recycle any G6P produced by hexokinase back to glucose. Indeed, skeletal muscle shows just this response—elevated G6Pase and suppressed hexokinase [4,25].

Receptor for advanced glycation end products (RAGE)

Persistent high glucose, such as in diabetes, causes damage to tissues for two main reasons: (1) the nonenzymatic glycation of proteins impairs their function and leads to the accumulation of damage products called advanced glycation end products (AGEs), and (2) the pro-oxidant actions of glucose, glycated proteins, and AGEs stimulate the production of reactive oxygen species that cause further damage to macromolecules. The accumulation of high levels of glucose as a cryoprotectant in wood frogs sets up the animals for potential damage due to AGE accumulation during freezing and over the 1–2 weeks that it takes for thawed frogs to clear the huge glucose load. Indeed, we suspect that the damage that could accrue from sustained high glucose is a major reason why cryoprotectant biosynthesis by frogs is triggered only when the animals actually begin to freeze. Up-regulation of RAGE in heart of frozen frogs suggests that AGEs do form during freezing; indeed, the RAGE receptor is known to be up-regulated by its own ligands, including AGEs (it is a multiligand receptor). Typically, the up-regulation of RAGE exacerbates the cellular damage done by AGEs and studies with kidney show that when RAGE is blocked or in RAGE null mice, accumulated diabetic damage to the glomerulus is reduced despite continued hyperglycemia [23]. Furthermore, an secretory version of RAGE has just been found that is able to capture AGE ligands and neutralize their actions on endothelial cells of blood vessels (a major target of glycation damage in diabetic vasculopathy) [23]. So, with respect to frogs, we would hypothesize that two possibilities exist, both with exciting possibilities for future research: (a) the normal vertebrate response of RAGE up-regulation occurs in response to AGEs generated as a result of cryoprotectant accumulation but the damage that can be triggered by RAGE is ameliorated in frogs; or (b) frog organs may up-regulate the splice variant secretory form of RAGE [23] during freezing as a scavenger of AGEs.

Antioxidant defenses

Wood frogs have well-developed antioxidant defenses [14]. Activities of six main antioxidant enzymes are much higher in the tissues of wood frogs than in freeze intolerant frogs (*R. pipiens*)

and so are the levels of the glutathione. Wood frog antioxidant defenses are high for an ectothermic vertebrate and similar to values for anoxia-tolerant turtles [11]. Furthermore, selected peroxidase activities are significantly elevated during freezing but return to near control values after 24 h thawing [14]. We have suggested that high antioxidant defenses are needed for two reasons: (1) to defend against glucose-mediated oxidative damage; and (2) as in other anoxia-tolerant species, to defend against the burst of reactive oxygen species (ROS) that are formed when oxygen is reintroduced into the anoxic system. Indeed, a major source of metabolic damage to mammalian organs from ischemia is really due to ROS generation in the reperfusion phase. Array screening revealed several proteins involved in antioxidant defense as putatively up-regulated during freezing and this again emphasizes the need for strong antioxidant defenses as an important part of freezing survival. In particular, the heart must be well defended against ischemic- or glucose-mediated damage since the reactivation of heart function is absolutely vital to recovery after thawing. The proteins cited from array screening include: (1) ferritin, an iron-binding protein that sequesters iron to lessen iron-mediated free radical generation; (2) thioredoxin, a low molecular weight peptide reducing agent; (3) metallothionein, a metal binding protein that may also act directly as an antioxidant and is up-regulated by freezing exposure in other systems [7]; (4) glutathione-*S*-transferase, one of the core enzymes of antioxidant defense; and (5) glucose-6-phosphate dehydrogenase, which has a critical role in the generation of NADPH to maintain reduced pools of glutathione and thioredoxin.

In summary, then, integrated approaches, utilizing the multiple techniques of gene discovery and analysis, coupled with modern technology in proteomics, and new advances in understanding how enzymes are regulated will continue to define the molecular adaptations that underlie natural freeze tolerance in the wood frog and other animals. Organ specific changes in genome and proteome functions are the next frontier in our drive to identify the unifying principles that Nature has used to allow animals to defy ice's deathly grip on living tissues.

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