Beyond gene chips: transcription factor profiling in freeze tolerance

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Abstract

The Wood Frog, *Rana sylvatica*, is one of several terrestrially hibernating anurans that display natural freeze tolerance. The multifaceted biochemical responses to the cellular stresses imposed when ~65% of total body water is converted to extracellular ice have been extensively explored by my laboratory. Much of our recent work focuses on freeze-induced gene expression and the signal transduction mechanisms that mediate it. This chapter describes a new approach to gene discovery by identifying the transcription factors that are activated during freezing which, in turn, implicate the suites of genes controlled by them as potentially important to freeze tolerance. New findings for liver are reported with a focus on the transcription factor, nuclear factor kappa B (NF-κB), and its potential role in cell survival, antioxidant defence and anti-apoptosis during freezing.

Introduction

One of the most amazing phenomena in comparative biochemistry is freeze tolerance, the ability of organisms to endure ice formation in extracellular fluid compartments and accommodate the range of cellular stresses that this imposes. These include ischaemia/anoxia that arises due to freezing of plasma, the associated ATP limitation in all organs, major cell volume reduction due to the outflow of intracellular water into extracellular ice masses, potential physical damage by ice to delicate tissues, and the long-term cessation of vital functions, including heart beat, breathing, and muscle movement. Research in my lab has uncovered many molecular mechanisms of natural freeze tolerance in both vertebrate and invertebrate systems (Storey & Storey 1996). Our main model for vertebrate freeze tolerance is the Wood Frog, *Rana sylvatica*, a woodland frog that hibernates on the forest floor; the species ranges from the north-eastern USA, across the boreal forests of Canada and up to the treeline in the Yukon and Alaska (see www.carleton.ca/~kbstorey for more information). These frogs endure the conversion of 65–70% of total body water into ice and their most prominent biochemical response to freezing is a rapid breakdown of liver glycogen leading

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to the production and export of large amounts of glucose that is taken up by all organs and used as a cryoprotectant (Storey & Storey 2004).

Our recent studies of vertebrate freeze tolerance have focused mainly on two topics: freeze-induced gene expression and the signal transduction mechanisms that trigger and coordinate adaptive responses (Storey 2004a; Storey & Storey 2007). Gene expression studies have used both cDNA library screening and DNA microarray screening (and related immunoblotting) to identify a range of genes, and their protein products, that are upregulated during freezing (Storey 2004a,b), despite a strong overall suppression of the rates of transcription and translation in frozen frogs. Included are selected novel proteins whose functions we do not yet know and, critically, many proteins representing cellular functions that have never before been associated with freezing survival. Hence, gene-screening studies have substantially expanded our ideas of what it takes to adjust and coordinate cell functions to regulate freeze/thaw, deal with the consequences of freezing, and sustain long-term viability in the frozen state. For example, we now know that freezing survival includes several cell preservation mechanisms that are commonly found in hypometabolic states across phylogeny (Storey & Storey 2007) such as elevated antioxidant defences, iron-binding proteins, heat-shock proteins, glucose-regulated proteins, and serine protease inhibitors (serpins) (Storey 2004a). Gene screening also suggested adaptive strategies that seem to be particularly important for freezing survival, including elevated fibrinogen levels to enhance clotting capacity and deal with physical damage by ice to microcapillaries, natriuretic peptide for regulation of fluid dynamics, enhanced glucose transporter capacity to efficiently distribute cryoprotectant, and defences against the accumulation of advanced glycation end products caused by non-enzymatic damage to proteins by high glucose (Storey 2004a,b). Dozens of other leads remain to be considered in the huge data files that are produced from DNA array screening of multiple organs, not just among the upregulated genes that we have focused on to date, but also among the downregulated genes that have not yet received any follow-up.

Transcription factor cassettes and the functional organisation of metabolism

A transcription (or transactivating) factor (Tf) is a DNA-binding protein that regulates gene expression, typically controlling a specific subset of genes in response to a particular signal (e.g. hormones, nutrients, environmental factors like temperature, oxygen, osmolality). A single transcription factor, by modulating the transcriptional activity of a suite of genes, can provide a "seamless" coordinated cellular response to an imposed stress. Tfs act like a "capo" in the Mafia or a sergeant in the army to marshal and coordinate a specific group of men (genes) to accomplish the many different jobs (protein products) that are necessary for a defined result. In many cases, the downstream genes under the control of a given Tf are now well-defined from both experimental studies (e.g. effects of overexpression or knockout of the Tf) and bioinformatic searching for the unique sequence of the binding response element of a Tf in the upstream untranslated region of genes. Hence, the regulation and coordination of gene expression given by Tfs provides an important level of functional organisation to

metabolism. Many transcription factor cassettes have now been assembled and we can predict with considerable accuracy which suites of genes will show enhanced transcription if, for example, the Heat Shock Factor (HSF) or the Hypoxia-Inducible Factor (HIF) or the Nuclear Factor kappa B (NF-κB) are activated. Thus, we now have a new paradigm with which to explore biochemical adaptation to environmental stress. If we can identify Tfs that are activated in response to a given stress (e.g. freezing), then we can predict that the protein products of the genes regulated by those Tfs are involved in the adaptive response. Furthermore, a Tf analysis may be just what's needed to sort out gene expression data from DNA microarray studies. Categorising upregulated genes with respect to what Tfs they respond to will provide valuable insights into how and why particular genes are upregulated in response to a given stress. How then do we identify Tf activation in response to stress?

Tf control of genes can be briefly summarised as follows (Willmore 2004). All Tfs show two distinct domains, one that binds to a conserved DNA sequence (element or enhancer) upstream of the transcriptional start site of a gene and a second that affects transcriptional activity (activation or repression) by interaction with the basal transcription machinery. Most Tfs also contain a dimerisation domain to allow formation of homo- or heterodimers that are a key regulatory feature for their function. Other Tfs have a ligand-binding domain for interaction with specific activators (e.g. steroids). Many transcription factors are activated by phosphorylation of specific residues by Ser/Thr or Tyr kinases, by cleavage reactions, or by actions that cause bound inhibitory proteins to dissociate from a Tf-inhibitor complex. Most Tfs are localised in the cytoplasm while inactive and, after activation, migrate to the nucleus where they then bind to DNA. Hence, changes in the total amount of a Tf protein (assessed via immunoblotting), or in the amount of phosphorylated Tf (assessed using phosphopeptide antibodies) or cleaved Tf (migrates differently on an SDS gel), and changes in the cytoplasmic vs nuclear distribution of a Tf are all useful indicators of Tf activity status. However, the ultimate predictor is the amount of a Tf bound to DNA.

Methodologies for assessing transcription factor binding to DNA

An original technology for assessing transcription factor binding to DNA is the Electrophoretic Mobility Shift Assay (EMSA). The principle is that a protein–DNA complex will show retarded mobility during electrophoresis as compared with free DNA alone. A specific DNA probe is designed that mimics the response element for the Tf of interest and is end-labelled with ^{32}P or by a non-radioactive method. The probe is mixed with a cell/nuclear extract, incubated and then separated on a non-denaturing polyacrylamide gel. Free DNA and protein-bound DNA are detected as separate bands using autoradiography or a phosphoimager. Various controls are required to ensure specificity and the identity of bound protein can be further confirmed by a "supershift" assay in which a specific antibody for the Tf is included in selected samples; antibody binding further reduces mobility on the gel, further shifting the ^{32}P labelled band. We have used this method to evaluate ATF4 binding to a DNA probe designed from the promoter of rat glucose-regulated protein, *grp78*, in order to determine if ATF4 could regulate the hibernation-responsive elevation of GRP78 chaperone in ground-squirrel tissues (Mamady & Storey 2006).

EMSA assays are quite time-consuming, messy and low throughput, but several commercial methods now provide substantial improvements for assessing Tf binding in nuclear extracts of cells/tissues. We have used three of these for Tf profiling of Wood Frog liver:

The functional equivalent of EMSA supershift assays can be carried out in 96-well microplates using an ELISA-based method from Active Motif Inc. (Carlsbad, CA; www.activemotif.com). The assay uses a consensus-binding site oligonucleotide for a given Tf that is immobilised in each well. Activated nuclear extract is then added and, after incubation to allow Tf binding, a primary antibody recognising an epitope on the bound and active form of the Tf is added, followed by secondary antibody and then plate development for either colorimetric or chemiluminescent readout.

A microplate-based method from Marligen Biosciences Inc. (Ijamsville, MD; www.marligen.com) can assess 50 of the main metabolic stress-responsive Tfs at once. The assay uses biotinylated DNA probes that bind Tfs in nuclear extracts. Unbound probe is then digested (bound probe is protected) and remaining Tf-bound probe is bound to Luminex beads and quantified on a luminometer.

Tf arrays developed by Panomics Inc. (Fremont, CA; www.panomics.com) analyse 345 Tfs using a different assay principle. Biotin-labelled DNA binding probes are mixed with a nuclear extract to form DNA-Tf complexes. Passage through a spin column removes unbound probe and then eluted DNA-Tf complexes are denatured and the liberated probes are hybridised to an array of Tf consensus binding sequences. The intensity of hybridisation detected for each consensus sequence is proportional to the amount of its Tf that was present in the nuclear extract.

All three of these methods can be used to generate important leads on the Tfs that are putatively activated in response to a stress and point the way to potential expanded studies of selected genes under these Tfs as contributors to environmental stress adaptation.

Nuclear factor-kappa B (NF-κB) in Wood Frog liver

Nuclear factor-kappa B (NF-κB) is ubiquitous in eukaryotic cells and has a central role in regulating genes that control cell proliferation and cell survival (Kumar *et al*. 2004). It is also well-known as an inhibitor of apoptosis, regulating both inhibitors of caspases and anti-apoptotic Bcl-2 family members (Luedde & Trautwein 2006). NF-κB helps to mediate cellular responses to stimuli including stress, cytokines, oxygen free radicals, UV irradiation, and bacterial or viral antigens. It is one of a group of "rapid-acting" Tfs that are constitutively expressed but held in an inhibited form so that it is available for immediate activation in response to an activating signal. Inactive NF-κB in the cytoplasm is a heterodimer of p50 and p65 subunits bound to an inhibitor protein (IκB) (all three proteins have multiple isoforms). Phosphorylation of IκB triggers its dissociation (and subsequent ubiquitin-mediated proteolysis) and the p50 and p65 subunits then translocate to the nucleus to activate target genes.

We assessed the participation of NFκB in organ response to freezing by multiple methods. Two measures of NF-κB function were evaluated in Wood Frog liver over a time course of freeze/thaw (DeCroos 2003): (a) the amount of phosphorylated IκB, and (b) DNA binding by the p50 subunit of NF-κB. The latter was assessed with the NF-κB assay kit from ActiveMotif

(see above). Figure 1 shows that the amount of phosphorylated IκB (pIκB) increased rapidly by 2.7-fold within 2 hr after body fluids began to freeze. This suggests a corresponding elevation of the amount of free active NF-κB subunits. The binding assay then showed that p50 subunit binding to DNA also increased significantly by 2-fold after 2 hr freezing. Overall, then, both of these findings provide strong evidence that NF-κB-mediated gene expression is upregulated

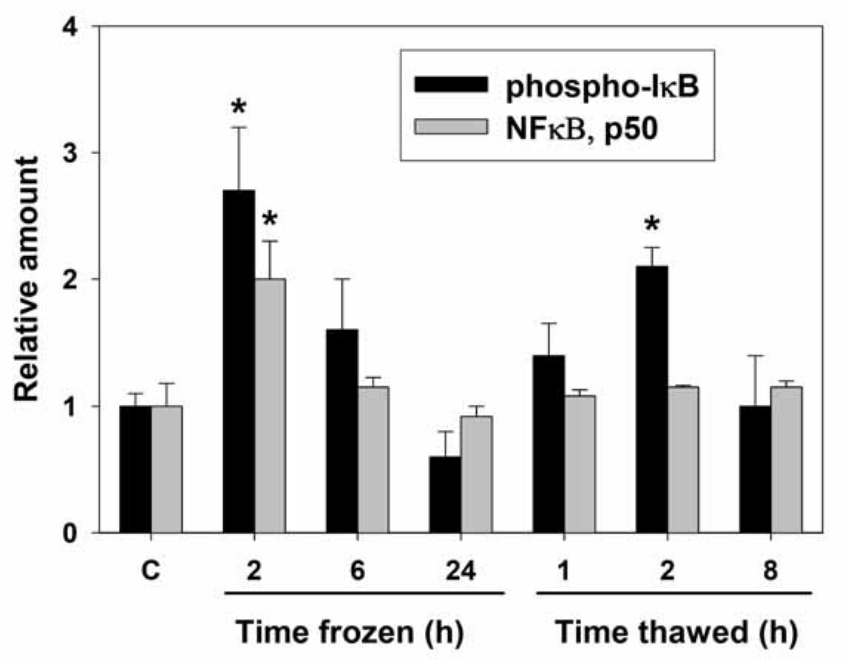

Figure 1. Effect of freezing and thawing on NF-κB transcription factor binding to DNA and the levels of the phosphorylated form of its inhibitor, IκB, in wood frog liver over a time course of 24 hr freezing at -3°C, followed by 8 hr thawing at 5°C. Data are expressed relative to control values for frogs held constantly at 5°C. Black bars show relative levels of pIκB in liver extracts over time. Protein was quantified by immunoblotting using a polyclonal antibody raised against a rabbit source (Cell Signaling) that crossreacted with a single band at ~41 kDa. Bands were visualized by chemiluminescence and quantified using the ChemiGenius BioImaging System (Syngene, Frederick, MD) and the associated GeneTools software. Equal loading of lanes was confirmed by Coomassie blue staining of the blots. Intensities of a strong blue band that did not change across all samples were also quantified and then pIκB band intensities were then normalized against the intensity of the corresponding Coomassie-stained band intensity in the same well. Gray bars show the relative DNA binding activity by the p50 subunit of NF-κB in liver extracts. Samples were assayed using the DNA binding kit for NF-κB from Active Motif (Carlsbad, CA) with the absorbance of bound NF-κB measured at 455 nm. Data are means ± SEM, n = 3 independent samples prepared from different animals. * Significantly different from the corresponding control, $p < 0.05$. (Modified from DeCroos 2003).

as an early response to freezing and suggest that genes under NF-κB control may contribute to cell survival of freezing. Figure 1 also shows that when frogs were thawed there was a second peak of pIκB expression but without a corresponding elevation of NF-κB binding. This may suggest that, within the timeframe of early thawing, NFκB may also have a role in readjusting cells for normal function.

Enhanced transcriptional activity by NF-κB was also confirmed when we analysed the results from multi-Tf analysis methods. In these cases, we compared liver samples from control frogs acclimated at 5°C with frogs given 4 hr of freezing exposure at -2.5°C. This short freezing time was chosen based on the assumption that freeze-responsive gene expression would still be highly active at this time prior to the restriction of blood flow and of tissue oxygenation that occurs as more and more ice accumulates in the body. Results from the analysis of 50 Tfs using the microplate assay from Marligen showed a strong 1.8-fold increase in NF-κB binding to DNA in 4-hr-frozen frogs. The array method from Panomics produced similar results; NF-κB binding to DNA was 2.6–4.2-fold higher in extracts from liver of frozen vs control frogs. Hence, all three methods were consistent in showing enhanced NF-κB binding to DNA in liver during the early hours of freezing exposure. This implies that the expression of various genes under NF-κB control are important for freezing survival by liver (and likely other organs as well). NF-κB modulates the expression of a very large number of genes (Kumar *et al.* 2004) and certainly not all would be needed for freezing survival. However, among the known gene targets of NF-κB are several that we have identified previously from gene screening as putatively freeze upregulated (Storey 2004a). These include ferritin (an iron-binding protein), antioxidant enzymes (glutathione *S*-transferase, Mn-superoxide dismutase), the A1 adenosine receptor, and the receptor for advanced glycosylation end products (RAGE). All have potentially important roles in freezing survival. Up-regulation of iron binding proteins and antioxidant enzymes is proving to be a conserved response across phylogeny among organisms entering hypometabolic states, including freeze-tolerant animals (Storey & Storey 2007). Extracellular adenosine is a well-known trigger of metabolic arrest in anoxia-tolerant vertebrates, its effects being transmitted to cells via the A1adenosine receptor (Buck 2004). Up-regulation of A1 receptors emphasizes the importance of adenosine signaling for regulating stress-induced metabolic arrest, a key feature of long term life extension. RAGE up-regulation in freeze-tolerant Wood Frogs remains to be explored by us but we believe that it will have a unique role in this species in the detection and scavenging of AGEs which are products of high glucose damage to proteins (Sakurai *et al.* 2003). The extreme glucose levels that provide cryoprotection for frog tissues also require that these animals have novel solutions to deal with the multiple damaging effects of long-term hyperglycaemia, which are well-known in diabetes.

Freeze-responsive transcription factors

Screening of Wood Frog liver extracts using the two multi-Tf analysis methods also showed strong increases in the levels of various other Tfs in extracts from 4-hr freezing-exposed frogs, compared with 5°C controls. Using the Marligen 50-plex microplate method, we identified eight Tfs (in addition to NF-κB) that showed freeze-responsive increases of 1.5–2.0-fold. These were MEF2, NF1, STAT, ETS, SREBP, ISRE, PPAR, and PBX. The TranSignal Protein/DNA

array analysis (Panomics) assessed 345 Tfs and showed a surprisingly high number of activated Tfs; 79 Tfs (22.9%) were elevated by 1.5-fold or higher in liver from frozen frogs vs controls and 58 Tfs (16.8%) increased by 2-fold or more. These findings argue that the cellular preparations and defences needed for freezing survival are extensive and require the regulated expression of many different genes involved in many different cell functions with the mediation of a large number of Tfs. Apart from NF-κB, other Tfs that showed enhanced DNA binding included ATF2, AP-1, AP-2, p53, Ets, STAT and multiple isoforms of PAX (paired box gene), HNF (hepatic nuclear factor), and C/EBP (CCAAT/enhancer binding protein). Several of these are well-known to have broad roles in cell survival. The majority of the Tfs listed above are involved in central cellular survival responses, anti-apoptosis, and other actions that could contribute to cell preservation over long term freezing. For example, C/EBPs, PAXs and STATs (signal transducer and activator of transcription) have major roles in directing cellular proliferation, differentiation, and resistance to apoptosis (Nerlov 2007; Lang *et al*. 2007; Calò *et al.* 2003). Putative enhancement of gene expression under these Tfs may be important for the extensive reorganisation of metabolism that has to be accomplished for long term viability in the frozen state including orchestrating overall metabolic rate suppression, inhibition of cell cycle, growth and differentiation responses, ischaemia resistance, and dealing with greatly increased intracellular osmolality. Furthermore, SREBPs, PPARs and HNFs have key roles in regulating carbohydrate and lipid metabolism in liver, several aspects of which need to be reorganised for life in the frozen state (Ferré & Foufelle 2007; Jump *et al*. 2005). For example, SREBPs mediate the insulin signal in liver to induce a variety of enzymes of fatty acid synthesis when high glucose loads are experienced. Frozen frogs, however, must somehow override this response since their extremely high glucose levels need to be retained for cryoprotection. Since the initial evidence from Tf profiling is that SREBP binding to DNA is enhanced during freezing, then we would predict that there must be other interacting factors that prevent SREBP-induced expression of fatty acid synthesis in the unique Wood Frog system. Exploration of the actions and interactions of these Tfs in the control of natural freeze tolerance will provide us with many more opportunities to unravel the secrets of freezing survival.

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