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Expression of freeze-responsive proteins, Fr10 and Li16, from freeze-tolerant frogs enhances freezing survival of BmN insect cells

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ABSTRACT **To date, two novel freeze-responsive proteins, Fr10 and Li16, have been discovered in the wood frog, *Rana sylvatica*, and likely support freezing survival. Although previous studies have established tissue distribution of each protein, there have been no studies that explore their functional consequences in intolerant cells. To assess the ability of Fr10 and Li16 to confer freeze tolerance, we transfected each protein into a freeze-intolerant silkworm cell line (BmN). Selected controls were the transfection of an unrelated protein (CAT) and a no-transfection sample. Li16 and Fr10 showed 1.8 ± 0.1- and 1.7 ± 0.2-fold, respectively, greater survival after freezing at −6°C for 1 h than did transfection controls. To investigate how these novel proteins protect cells from freezing damage, protein structures were predicted from primary amino acid sequences. Analysis of the structures indicated that Fr10 is a secreted protein and may be a new type IV antifreeze protein, whereas Li16 may have intracellular membrane associated functions. This study shows that freezing protection can be provided to intolerant cells by the overexpression of transfected Li16 and Fr10 frog proteins. Results from this study will provide new insights into adapting intolerant cells for medical organ cryoprotection using a natural vertebrate model of tolerance.—Biggar, K. K., Kotani, E., Furusawa, T., Storey, K. B. Expression of freeze-responsive proteins, Fr10 and Li16, from freeze-tolerant frogs enhances freezing survival of BmN insect cells. *FASEB J.* 27, 3376–3383 (2013). www.fasebj.org**

Key Words: cryobiology · silkworm

MANY ORGANISMS LIVING AT high latitudes or altitudes on Earth must have effective strategies for enduring prolonged exposures to temperatures below the freezing point of their body fluids. For some of these animals, this means enduring the conversion of a high percentage of their total body water into extracellular ice masses. Freeze-tolerant animals include hundreds of insect species, various other terrestrial and intertidal invertebrates, and several species of terrestrially hibernating amphibians and reptiles (1, 2). The wood frog, *Rana sylvatica*, is the most extensively studied of the freeze-tolerant vertebrates and is capable of surviving the conversion of ~65% of total body water into extracellular ice (3). The characterized physiological and biochemical responses and adaptations of this animal to freezing include cryoprotectant biosynthesis, control over ice nucleation and ice propagation through tissues, ischemia resistance, regulation of cell volume reduction due to water loss to extracellular ice masses, and regulated suppression of energy-expensive metabolic functions in the frozen state (2–4).

During studies of gene expression responses to freezing in the liver of wood frogs, differential screening of cDNA libraries identified several freeze-responsive genes, including those encoding fibrinogen α and γ subunits, mitochondrial ADP/ATP translocase, and the inorganic phosphate carrier (5–7). Novel upregulated genes were also found, including those encoding a 10-kDa protein designated freeze-responsive protein clone 10 (Fr10; ref. 8) and a 13-kDa protein designated liver protein clone 16 (Li16; refs. 9, 10). Tissue-, time-, and stress-specific (freezing, dehydration, anoxia) responses by these proteins have been traced, but to date, their functions remain elusive. Fr10 was expressed in liver, heart, and gut of *R. sylvatica* in response to freezing or dehydration stresses, whereas Li16 was expressed in multiple tissues under freezing, anoxia, and dehydration conditions (9, 10). Expression was reduced again after thawing.

Abbreviations: AFP, antifreeze protein; CAT, chloramphenicol acetyl transferase; Fr10, freeze-responsive protein clone 10; Li16, liver protein clone 16; LS-12, longhorn sculpin antifreeze protein 12; MOE, molecular operating environment; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NT, nontransfected; ORF, open reading frame; SV5, simian virus 5 protein

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One way to explore the functions of Li16 and Fr10 is to utilize a transgenic approach of expressing the proteins in a freeze-intolerant cell system and then assessing their ability to impart freezing survival to the cells. For example, a transgenic approach has been taken in multiple studies with antifreeze proteins, generally with the goal of imparting freeze resistance to species of importance in agriculture or aquaculture (11–13). The present study explores the possibility that the novel frog proteins, Li16 and Fr10, can confer freeze tolerance in intolerant eukaryotic cells. In addition, *ab initio* protein structure predictions were carried out to further characterize these novel freeze-responsive proteins. These functional and structural analyses of Fr10 and Li16 not only help to identify new biochemical adaptations that support natural freeze tolerance, but will also provide new insights into mechanisms that can be used to provide cryoprotection to intolerant cells.

MATERIALS AND METHODS

Prediction of *ab initio* protein structure and membrane interaction

Structure prediction for Fr10 (GenBank: AAC60284.1) and Li16 (GenBank: AAG33070.2) were conducted using the Quark method, as outlined by Xu and Zhang (14). Quark is an *ab initio* structure prediction program that uses atomic-level knowledge-based force-field and replica-exchange Monte Carlo simulation to generate protein structures. Following structure prediction, all protein models were protonated and optimized by energy minimization using the MMFF94s force-field model in Molecular Operating Environment (MOE) 2011.10 software (Chemical Computing Group, Montreal, QC, Canada). Solvent accessibility was determined using the PredictProtein server (15). Cellular localization of Fr10 and Li16 was predicted using PSORT II, a program that detects sorting signals in proteins and predicts their subcellular localization (16). Membrane boundaries and interactions were obtained from the PPM server and visualized in MOE (17). Amino acid characteristics for Fr10 and LS-12 were visualized using helical wheel plots generated by the EMBOSS pepwheel tool (EMBOSS; http://emboss.bioinformatics.nl/cgi-bin/emboss/pepwheel).

Cell culture

The BmN cell line derived from the silkworm, *Bombyx mori*, was cultured in ESF921 medium (Expression Systems, Davis, CA, USA) supplemented by 10% fetal calf serum (Invitrogen, Burlington, ON, Canada) at 25°C (18).

Construction of expression vectors and transfection

Open reading frame (ORF) regions of Li16 and Fr10 were cloned into the pIZ/V5-His plasmid vector (Invitrogen). For this study, we designed expression vectors encoding Li16 or Fr10 fused with the paramyxovirus simian virus 5 protein (SV5) epitope and His6 tag at the C-terminal ends (GKPIPNPLLGLDSTHHHHHH) for easy detection of protein products by a specific monoclonal antibody raised against the additional sequence. The cDNA encoding the Li16 ORF (9) with *Eco*RI and *Xho*I sites artificially added at the N-terminal and C-terminal regions, respectively, was amplified from single-stranded cDNAs from wood frogs frozen for 24 h by fidelity PCR using primers for the N terminus (5′-GAATTCGAGATGGCGATCGTACTGAG-3′) and C terminus (5′-CTCGAGTCGCAGGCATTGCAGAGCG-3′), and PLX DNA polymerase (Gibco BRL, Carlsbad, CA, USA). The cDNA encoding the Fr10 ORF (8), with an additional *Eco*RI site at the N-terminal region and a *Xho*I site at the C-terminal region, was amplified using primers for the N-terminal region (5′-GAATTCACATGGAAGTCTTGGCCCTC-3′) and C-terminal region (5′-CTCGAGCTCAGTTTTTCTTTGCT-3′). Since authentic Fr10 mRNA does not have a putative Kozak consensus sequence, the authentic sequence, CATCATGA around the start codon ATG of Fr10, was modified to CACATGG during primer design (19). DNA fragments for both proteins were ligated into the *Eco*RI-*Xho*I site of the pIZ/V5-His vector. Nucleotide sequences of the ORF regions of both vectors were stringently reaffirmed to be the same as designed.

Cotransfection of plasmid DNA and *B. mori* BmTRN-1 antisense oligonucleotide (299–322 nt position of BmTRN-1), which has been shown to vigorously enhance foreign protein production in BmN cells (20), was performed by a chemical method as follows. Each mixture of 1 μg of purified plasmid DNA, 0.5 μg of BmTRN-1 antisense oligonucleotide, 6 μl of Fugene 6 reagent (Roche Applied Science, Indianapolis, IN, USA) and 100 μl of ESF921 medium was incubated at 25°C for 15 min, and then added to an $\sim 0.7 \times 10^6$ BmN cell monolayer on each plastic dish (35 mm in diameter). After incubation at 25°C for 5 h, the medium of each culture (1.5 ml) was renewed and then incubated for 3 d at 25°C.

Protein gel electrophoresis and immunoblot analysis

The product of Fr10 and Li16 fusion proteins with SV5-His6 (Fr10/SV5-His6 and Li16/SV5-His6) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 12% gels (21). Proteins were electroblotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by semi-dry transfer in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% (v/v) methanol. Western blotting using the mouse anti-SV5 antibody (Invitrogen) was carried out as recommended by the antibody supplier (1:5000 dilution), and results were visualized by an ECL detection system (Amersham, Baie d'Urfe, QC, Canada).

Freezing protocol

For freezing exposure, cells expressing Fr10/SV5-His6, Li16/SV5-His6, or chloramphenicol acetyl transferase (CAT)-SV5/His6, as well as nontransfected (NT) cells, were placed in an incubator with an air temperature of −6, −8 or −10°C. After cell dishes were cooled for 60 min, the upper cover of each dish was touched with a flat metallic fragment previously cooled in liquid nitrogen, and this triggered nucleation of the culture medium (medium volume of 1.5 ml for each sample). The culture dishes were held at each temperature for set times of freezing exposure, and were then immediately replaced at 4°C. The medium thawed within 1 h at 4°C and then was allowed to recover at 25°C, the original culture temperature.

Assessment of freeze tolerance for BmN cells

We compared the total number of cells attached onto the dish in 6 randomly selected marked circles (each 1 mm diameter) on each dish by observation under microscopy (×40). Mean ± SD normalized cell counts were calculated for all experimental samples, and significance was tested using a 1-way analysis of variance followed by Tukey's test ($P<0.001$).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (22) was used to compare the surviving cells of control and Fr10/SV5-His6- and Li16/SV5-His6-expressing cells. At 1 d after freezing exposure, floating and agglutinated cells in the dishes were removed. The attached cells were twice rinsed with 1 ml of ESF921 medium, and then the dishes were covered with 1.5 ml of ESF921. MTT (200 μl, 5 mg/ml) in sterile PBS was added to each culture dish, and after 2 h of incubation at 37°C, 1 ml of extraction buffer (20% w/v SDS and 50% *N,N*-dimethyl formamide, pH 4.7) was added. After an overnight incubation at 37°C, the optical densities at 570 nm were measured using a spectrophotometer. Mean ± SD normalized optical densities were calculated for all experimental samples, and significance was tested using a 1-way analysis of variance followed by Tukey's test ($P < 0.05$).

RESULTS

Prediction of protein structure, localization, and membrane interaction

Molecular models of protein structure were predicted from the previously published primary amino acid sequences of Fr10 and Li16 from *R. sylvatica* (**Fig. 1**). Models were created *ad initio* by the Quark server. Both Fr10 (LGscore=3.45) and Li16 (LGscore=1.58) models were validated using the ProQ protein quality predictor, where LGscore > 1.5 indicates a good prediction. Finally, all protein models were protonated and optimized by energy minimization using the MMFF94s force-field model in MOE. Fr10 was predicted to have a high probability of an extracellular localization, as estimated by PSORT II, whereas Li16 was predicted to have a high probability of an intracellular location.

Interactions between Fr10 and Li16 and the cell membrane were modeled in an attempt to explore potential mechanisms of freeze protection. Predicted membrane interactions are presented in **Fig. 2**. The models predicted the extracellular localization of Fr10 with a minimal interaction with the extracellular surface of the cell membrane. However, Li16 was predicted to significantly interact with the intracellular surface of the cell membrane.

Freeze tolerance in BmN cells

Fusion proteins of Li16 or Fr10 with SV5 antigen plus a His6 tag (Li16-SV5/His6 and Fr10-SV5/His6) were transfected into the *B. mori* BmN insect cell line, and subsequent studies tested whether the transfected cells acquired freezing survival. As shown in **Fig. 3**, Western blotting using an anti-SV5 antibody revealed that a ~16.2-kDa protein consistent with Li16-SV5/His6 and a ~13.4-kDa protein consistent with Fr10-SV5/His6 were produced by the cells and detected in cell lysates (note that the SV5-His6 tag adds 3.4 kDa to the molecular mass). Fr10-SV5/His6 was also abundant in the culture medium of Fr10-transfected cells.

Cells transfected with and expressing Li16-SV5/His6, Fr10-SV5/His6, or CAT-SV5/His6, as a transfection control, were cultured for 72 h and then given a 1-h freezing treatment at −6, −8 or −10°C. BmN NT cells (transfected with no plasmid) were also used as untreated controls. **Figure 4** shows the number of living cells after various freezing treatments, counted after thawing for 24 h. In preliminary studies, we found that cells killed by freezing exposure were not attached to the bottom of the plates, but were floating within the culture medium, agglutinated with each other and remarkably shrunken. Living cells that survived freezing at −6°C showed shapes similar to normal BmN monolayer cells after 24 h thawing at 25°C and were attached to the bottom of the plate. Therefore, the number of attached cells on the bottom of the plate was used as a simple measure of freeze survival. There were no significant differences in the number of surviving cells in the different transfection groups when freezing treatments were at −8 or −10°C; in all groups, cell

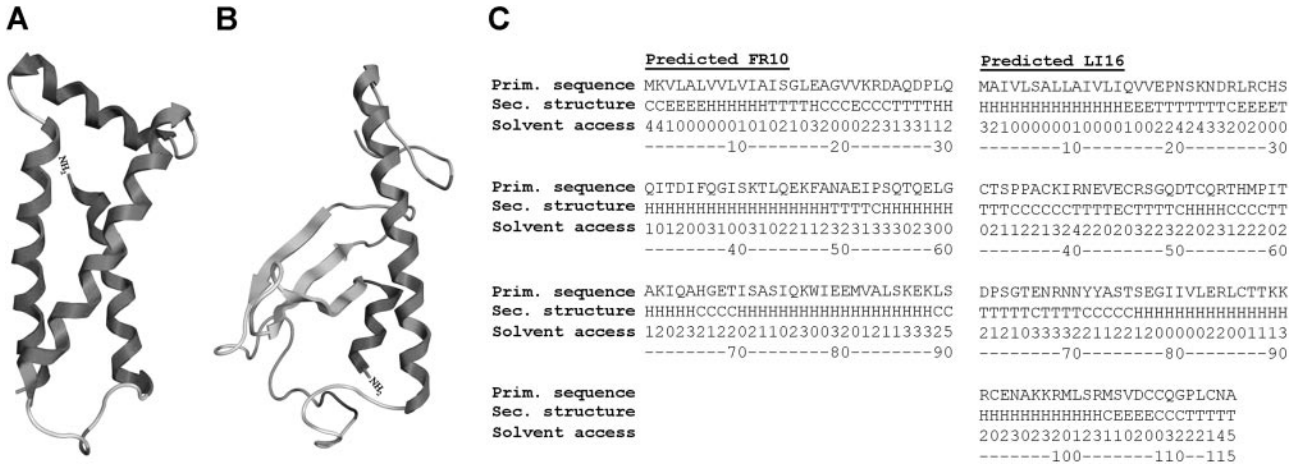

Figure 1. Predicted *ad initio* structure of novel freeze responsive proteins Li16 and Fr10. *A*, *B*) Structures of Fr10 (*A*) and Li16 (*B*) were predicted using Quark and optimized with MOE. *C*) Primary amino acid sequence, predicted secondary structure, and predicted solvent accessibility of Li16 and Fr10. Secondary structure is indicated as follows: C, coil; H, helix; E, sheet; T, β turn. Solvent accessibility is ranked with scores varying from 0 to 9, where 0 indicates buried and 9 indicates exposed amino acids.

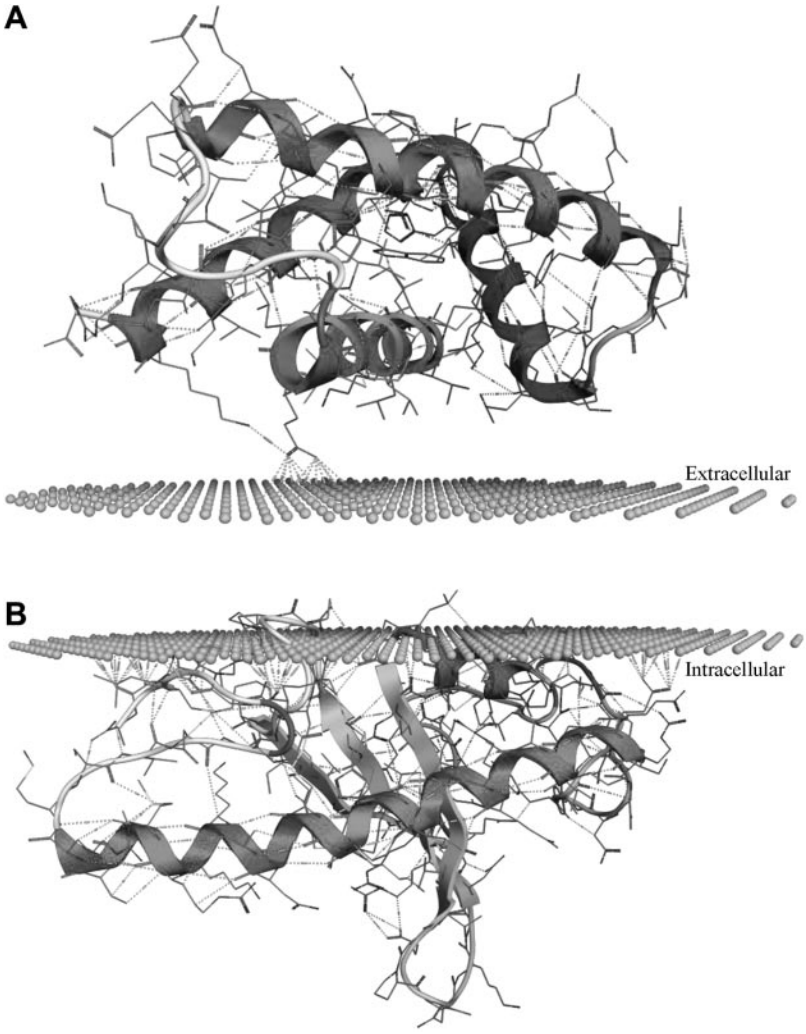

Figure 2. Predicted membrane interactions of novel freeze responsive proteins Li16 and Fr10. Structures of Fr10 (*A*) and Li16 (*B*) are shown with potential interactions with either the extracellular or intracellular membrane boundary (dots). Structures were predicted using the PPM web server and visualized with MOE.

survival measured 24 h after freezing exposure was very low. However, survival of −6°C freezing exposure was very much higher for Li16-SV5/His6-expressing cells as compared with CAT-SV5/His6-expressing cells or with NT cells (1.8±0.1- and 2.3±0.1-fold higher, respectively; $P < 0.001$). Similar responses were seen in cells expressing Fr10-SV5/His6 as compared with CAT-SV5/His6-expressing cells and NT cells (1.7±0.2- and 2.2±0.3-fold higher, respectively; $P<0.001$).

To further investigate the effect of Li16-SV5/His6 and Fr10-SV5/His6 expression on the viability of the cells after freezing, cells were treated to increasingly longer periods of freezing (1, 2 or 3 h) at −6°C and, after freezing, were allowed to recover at 25°C for 24, 48 or 72 h (**Fig. 5*A*, *B*, C,** respectively). Relative cell viability after each freeze-thaw treatment was assessed using an MTT viability assay. Unfrozen control groups from all conditions showed high cell survival that remained virtually constant over 24-, 48-, or 72-h periods and between the different transfection groups. Oppositely, all groups showed low viability after 3 h of freezing at −6°C, with no significant differences among the treatment groups but with marginally better viability after 24 h thawing recovery than 48 or 72 h postfreeze. By contrast, cells expressing Li16-SV5/His6 or Fr10-SV5/His6 that were frozen for 1 or 2 h and allowed to recover for 24 h showed significantly higher survival than the corresponding cells expressing CAT-SV5/His6 or cells that were not transfected (Fig. 5*A*). After 1 h freezing at −6°C and 24 h recovery, viability of CAT-SV5/His6-expressing cells was only 57 ± 11 and 60 ± 12% of the comparable values for Li16-SV5/His6 or Fr10-SV5/His6 transfected cells, respectively ($P<0.05$). Similarly, NT control cells showed viabilities of 43 ± 10 and 46 ± 11% as compared with the comparable Li16-SV5/His6 or Fr10-SV5/His6 survival values, respectively ($P<0.05$). After 2 h freezing (and 24 h recovery), cells expressing Li16 or Fr10 still displayed a survival advantage. Viability of CAT-SV5/His6-expressing cells was 43 ± 6 and 55 ± 8% of the comparable values for Li16-SV5/His6 or Fr10-SV5/His6 transfected cells, respectively ($P<0.05$; Fig. 5*A*). Similarly, NT control cells showed viability values of just 39 ± 5 and 50 ± 7% of the comparable Li16-SV5/His6 or Fr10-SV5/His6 survival values, respectively ($P<0.05$). These experiments show that the expression of Li16 or Fr10 proteins in BmN cells significantly increased survival in 1- and 2-h frozen cells, assessed after 24 h of thawing. However, cells transfected with Li16 or Fr10 did not

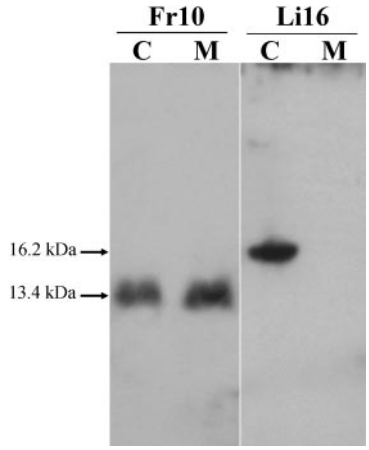

Figure 3. Immunodetection of Li16-SV5/His6 and Fr10-SV5/His6 expressed in the BmN cell lysate and culture medium. Bands of ~16.2 kDa for Li16-SV5/His6 and 13.4 kDa for the intracellular form of Fr10-SV5/His6 are indicated by arrows in the cell lysate (lane C). In the culture medium (lane M), an immunoreactive band for the extracellular form of Fr10-SV5/His6 is indicated. Both protein bands were detected using anti-SV5 antibody as described in Materials and Methods.

show a survival advantage after 48 or 72 h of thawing (Fig. 5*B*, *C*).

DISCUSSION

Identification of the biochemical adaptations that support freezing survival by selected amphibians and reptiles not only provides a greater understanding of this unusual natural phenomenon but can suggest possible molecular strategies that could be applicable in medical cryopreservation. Both Li16 and Fr10 are novel proteins that were discovered by differential screening of wood frog liver cDNA libraries and have been previously proposed to support freezing survival. These novel proteins differ substantially in their organ distribution, the pattern of response over freeze-thaw cycles, and response to other stresses (anoxia and dehydration) (8–10). However, there have been no previous studies that explored the functional consequences of expressing either Fr10 or Li16 protein in freeze-intolerant eukaryotic cells.

To assess the function of these novel freeze-responsive frog proteins in living eukaryotic cells exposed to freezing, we created an experimental system utilizing the BmN insect cell line derived from the silkworm, *B. mori*. Both Fr10 and Li16 are novel proteins produced in the wood frog that, to date, have no known homologues in any insect species, as assessed by our extensive searches in both nucleotide and protein databases. Because of this, it is highly likely that the improved freeze survival responses seen after transfection with either protein is a mimic of the cryoprotective action that they have naturally in frogs, and not of an enhancement of the action of natural *B. mori* homologues, such as inhibition of endogenous cell death-promoting pathways in the BmN cell line. Previous studies have attempted to impart freeze tolerance to intolerant insect larvae. For example, a recent study was able to successfully increase freeze tolerance in *Drosophila melanogaster* larva by low-temperature conditioning and incorporation of free amino acid proline, a low-molecular-weight cryoprotectant (23).

Using BmN cells, fusion proteins of Li16 or Fr10 with SV5 antigen plus His6 tag (Li16-SV5/His6 and Fr10-SV5/His6) were transfected, and subsequent studies determined whether transfected cells showed improved freezing survival as compared with NT cells or with cells transfected with CAT-SV5. BmN cells expressing Li16-SV5/His6 or Fr10-SV5/His6 that were frozen for 1 or 2 h at −6°C showed significantly higher survival than the corresponding cells expressing CAT-SV5/His6 or NT cells, following a 24 h thawing recovery period (Fig. 5*A*). These findings suggest that both Li16 and Fr10 provide some protection against freezing in intolerant cells. Interestingly, when cells were frozen for longer periods of time at −6°C (3 h) or at lower temperatures (−8 or −10°C), ~90% of all cells (transfected or not) did not survive. This may suggest the occurrence of a cell death mechanism that was initiated by some severe and irreparable cellular damage attributable to extended freezing exposure (24). However, the expression of both Li16 and Fr10 proteins clearly enhanced 1- and 2-h freezing survival, suggesting that each protein addressed a cryoprotective need of the cells. The relatively high abundance of Fr10 protein released into the extracellular medium suggests that Fr10 has an extracellular action in cryprotection, whereas Li16 may be limited solely to intracellular action, as it was not found to be secreted. Previous studies have suggested that Fr10 may function as a nuclear export protein, increasing the movement of specific freeze-induced mRNA

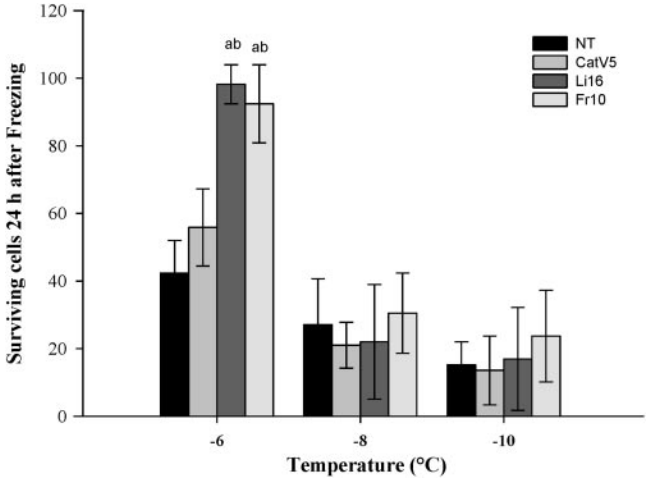

Figure 4. Cell survival after 24 h recovery from 1 h freezing at −6, −8 and −10°C comparing NT control cells, CAT-SV5/His6-expressing cells, and cells transfected with Li16-SV5/His6 or Fr10-SV5/His6. Histogram shows the numbers of surviving cells; data are means ± SD, $n = 10–12$ independent samples. $^{a}P < 0.001$ *vs.* NT cells; $^{b}P < 0.001$ *vs.* CAT-SV5/His6-expressing cells.

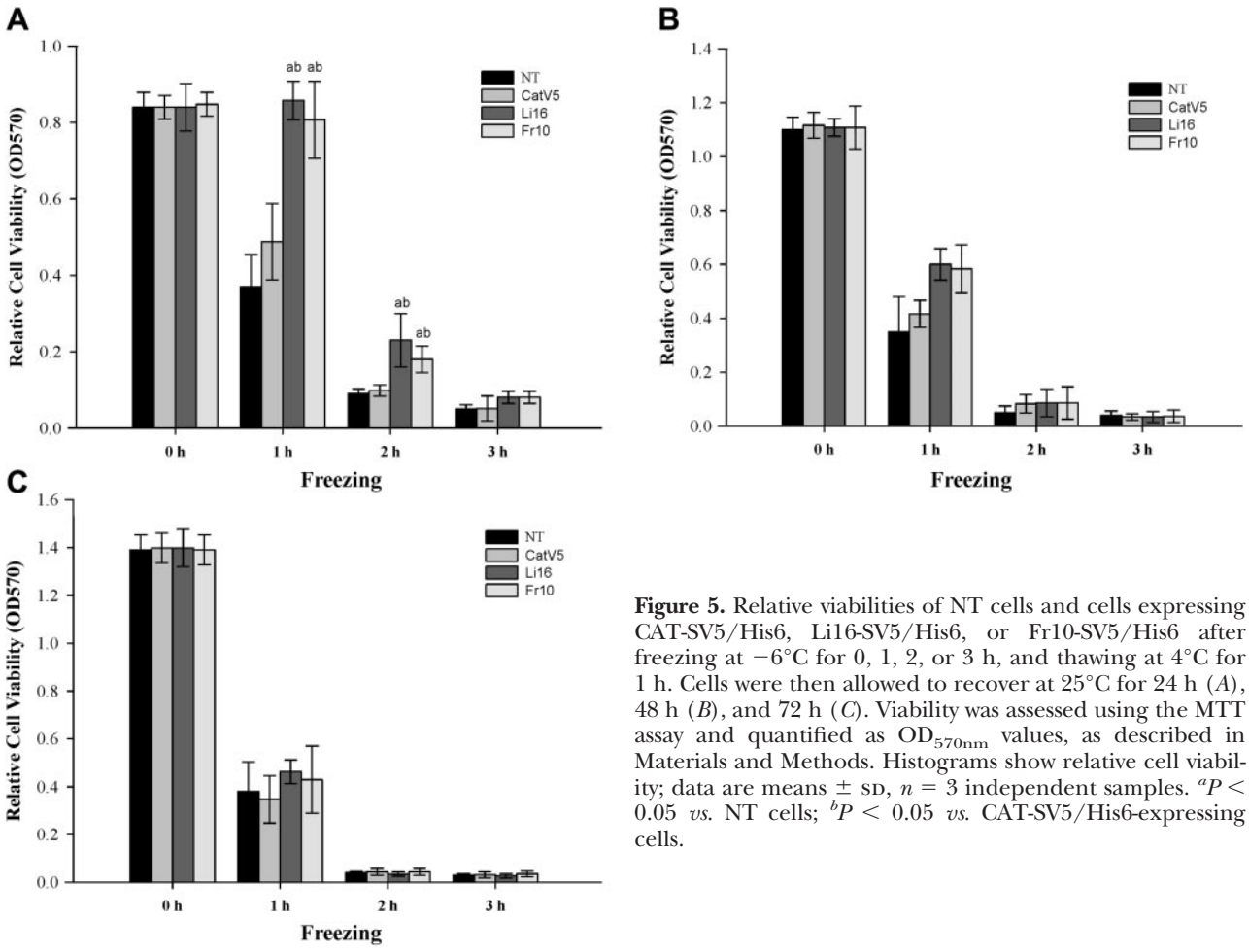

Figure 5. Relative viabilities of NT cells and cells expressing CAT-SV5/His6, Li16-SV5/His6, or Fr10-SV5/His6 after freezing at −6°C for 0, 1, 2, or 3 h, and thawing at 4°C for 1 h. Cells were then allowed to recover at 25°C for 24 h (*A*), 48 h (*B*), and 72 h (*C*). Viability was assessed using the MTT assay and quantified as OD_{570nm} values, as described in Materials and Methods. Histograms show relative cell viability; data are means ± SD, $n = 3$ independent samples. $^{a}P < 0.05$ *vs.* NT cells; $^{b}P < 0.05$ *vs.* CAT-SV5/His6-expressing cells.

from the nucleus to cytoplasm and that Li16 may be involved in a signaling pathway necessary for cryoprotection responses, but these proposals have not been followed up experimentally and, at least for Fr10, are not consistent with the action and location of the protein as revealed in the present study (8, 12).

To help elucidate possible roles of Li16 and Fr10 in frozen frogs, we used a bioinformatics computer-modeling approach in the present study to examine protein structure (*ab initio*), cellular localization, and potential membrane interactions of these two proteins. The predicted structure of Fr10 indicated a protein with a highly hydrophobic core, perhaps allowing Fr10 to translocate to the extracellular environment for freeze-related functions important for survival (Figs. 1 and 2). This study also discovered the presence of Fr10 protein in the extracellular medium (Fig. 3), and bioinformatic analysis also predicted an extracellular localization by PSORT II (25). This finding suggests a possible extracellular function for Fr10 in freeze tolerance. Intriguingly, our structural analysis of Fr10 suggests that the protein is highly similar to type IV AFPs. The predicted structure of Fr10 displays similar structural characteristics to the longhorn sculpin antifreeze protein 12 (LS-12) type IV antifreeze protein (AFP), found in the longhorn sculpin (*Myoxocephalus octodecimspinosis*; **Fig. 6*A, B*** and ref. 26). This AFP is uniquely characterized by the presence of a 4-α-helix bundle and an approximate molecular mass of ~12 kDa. To identify the helical characteristics of both proteins, helix wheel diagrams were used to visualize the relative positions of hydrophobic and hydrophilic amino acids. Interestingly, both Fr10 and LS-12 consist of a hydrophobic core and a primarily hydrophilic surface, with the exception of a hydrophobic region present on the outer surface of the first α helix (Fig. 6*C, D*). Protein Basic Local Alignment Search Tool (BLAST; U.S. National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov) inquiries for both Fr10 and the LS-12 type IV AFP suggest that both proteins, although displaying virtually no amino acid similarity to each other, show sequence similarity to apolipoprotein type A-II (*E* values of 1e-04 and 2e-04, respectively). It could be hypothesized that both Fr10 and LS-12 have evolved independently from preexisting parental apolipoprotein genes. Interestingly, similar helical structures exist for human apolipoproteins. Apolipoproteins are able to form complexes with phospholipids through the alignment of the nonpolar surface of the α helixes to hydrophobic phospholipid chains and the polar surfaces of the α helixes to water (27). Given the relatively low degree of interaction between Fr10 and the outer side of the cell membrane (Fig. 2), it seems unlikely that Fr10 functions by interacting with the lipid membrane. Instead,

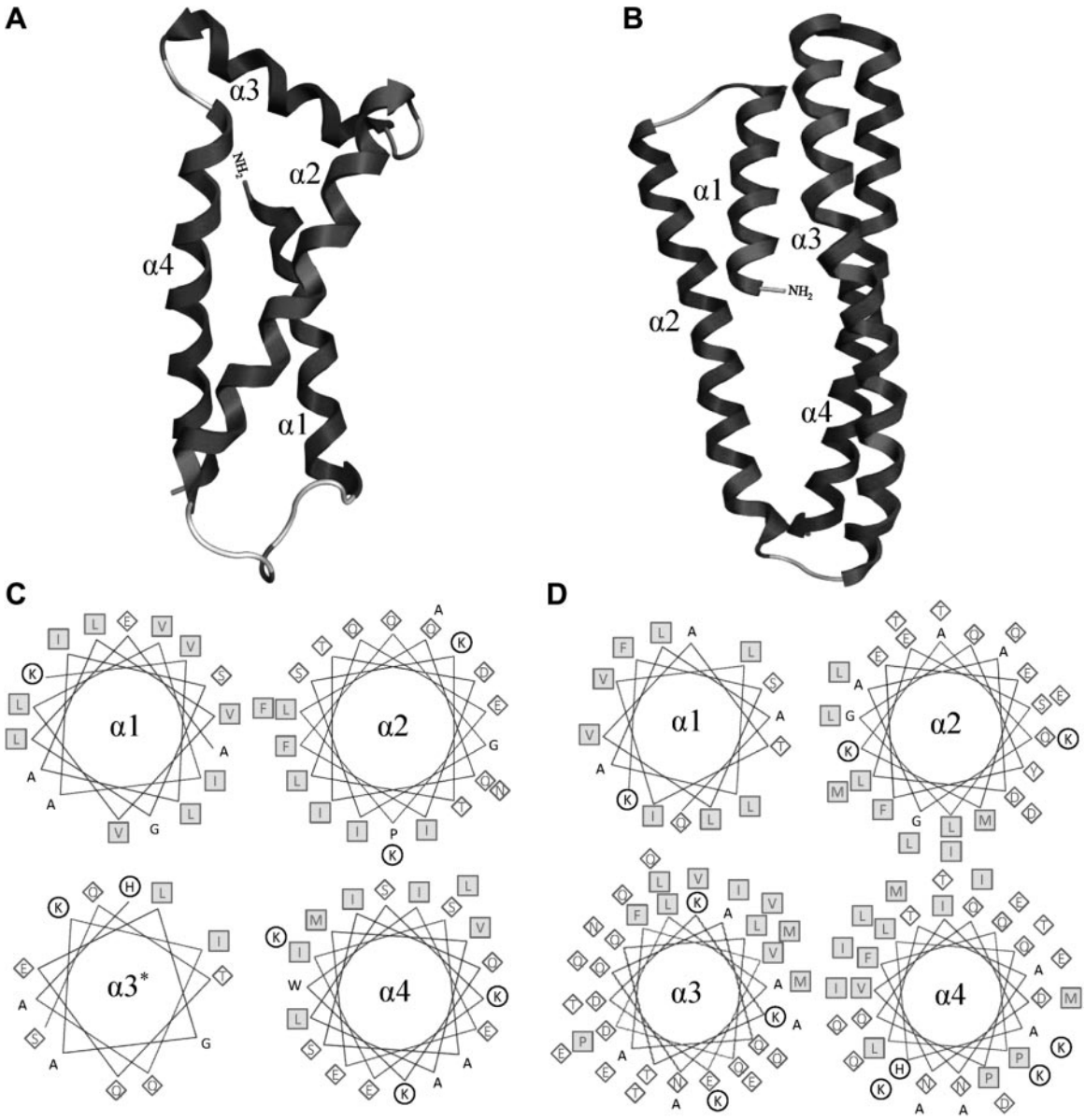

Figure 6. Structural and electrostatic comparison between two freeze-related proteins. *A*, *B*) Comparisons are between LS-12 (*A*), a known AFP, and Fr10 (*B*), a protein with a predicted similar function. Helixes are numbered sequentially from N to C terminus. *C*, *D*) Amino acid characteristics for Fr10 (*C*) and LS-12 (*D*) were visualized using helical wheel plots. Shaded squares identify hydrophobic amino acids, diamonds identify hydrophilic amino acids, circles identify basic amino acids.

the helixes of Fr10 may interact with each other, forming helical bundle structures and perhaps creating a specialized ice crystal binding surface that could impede ice growth. Although the presence of an AFP-like protein in a freeze-tolerant frog might seem contradictory, numerous recent studies have documented the presence of AFPs in freeze-tolerant insects and plants. All AFPs have dual characteristics: thermal hysteresis (the ability to depress the freezing point of a solution below the melting point) and inhibition of the restructuring of ice into larger crystals (28). It is the second action, ice recrystallization inhibition, that is important to freeze-tolerant animals, and this could prove to be the natural action of Fr10 in wood frogs.

Structure prediction and solvent-accessibility analysis of Li16 indicated a strong hydrophobicity near the N terminus, suggestive of a possible transmembrane region (Figs. 1 and 2). In addition, further prediction of Li16 subcellular distribution indicated a high probability of intracellular localization (estimated by PSORT II). Given that Li16 is able to impart freeze tolerance to unrelated insect BmN cells, it is highly unlikely that this protein functions as previously hypothesized, an integral member of a preestablished signaling network present in *R. sylvatica* (10). However, it is possible that Li16 functions as a peripheral membrane protein during periods of freezing (Fig. 2). Although the function of Li16 is unknown, the up-regulation of peripheral membrane proteins during periods of cold stress has been previously documented (29). These proteins typically include repair proteins, osmotic stress-related proteins, proteolysis-associated proteins, and many pro-

teins with unknown functions. Therefore, it is highly likely that Li16 has a membrane-associated role with a function that is unclear at this time.

This study shows that significant freezing protection can be provided to cultured silkworm BmN cells by the overexpression of transfected frog proteins known to be induced naturally during freezing exposure in the freeze-tolerant wood frog. Further analysis of the gene products (including frog Li16 and Fr10) induced during natural freeze tolerance not only will help to identify the full range of cold adaptations that support freeze tolerance but also will provide new insights into adapting intolerant cells for the development of medical organ cryoprotection technology. FJ

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