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Hsp70 and Hsp90 mediated proteasomal degradation underlies TPI^{sugarkill} pathogenesis in *Drosophila*

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Abstract

Triosephosphate isomerase (TPI) deficiency is a severe glycolytic enzymopathy that causes progressive locomotor impairment and neurodegeneration, susceptibility to infection, and premature death. The recessive missense TPI^{sugarkill} mutation in *Drosophila melanogaster* exhibits phenotypes analogous to human TPI deficiency such as progressive locomotor impairment, neurodegeneration, and reduced lifespan. We have shown that the TPI^{sugarkill} protein is an active stable dimer, however, the mutant protein is turned over by the proteasome reducing cellular levels of this glycolytic enzyme. As proteasome function is often coupled with molecular chaperone activity, we hypothesized that TPI^{sugarkill} is recognized by molecular chaperones that mediate the proteasomal degradation of the mutant protein. Co-immunoprecipitation data and analyses of TPI^{sugarkill} turnover in animals with reduced or enhanced molecular chaperone activity indicate that both Hsp90 and Hsp70 are important for targeting TPI^{sugarkill} for degradation. Furthermore, molecular chaperone and proteasome activity modified by pharmacological or genetic manipulations resulted in improved TPI^{sugarkill} protein levels and rescue some but not all of the disease phenotypes suggesting that TPI deficiency pathology is complex. Overall, these data demonstrate a surprising role for Hsp70 and Hsp90 in the progression of neural dysfunction associated with TPI deficiency.

Keywords

Molecular Chaperones; TPI deficiency; Triosephosphate Isomerase; Glycolytic enzymopathy; *Drosophila*

Introduction

Glycolytic enzymopathies are severe heritable human diseases that result from specific mutations affecting enzymes involved in anaerobic metabolism. Familial triosephosphate isomerase (TPI) deficiency is an autosomal recessive disorder that manifests in patients as anemia, progressive locomotor impairment and neurodegeneration, and premature death

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(Schneider et al., 1965; Valentine, 1966). However the pathogenesis of this disease is not well understood. Previous research identified a recessive missense mutation in the *TPI* gene in *Drosophila* named *sugarkill*(*sgk*) that exhibits progressive locomotor impairment, neurodegeneration and reduced longevity. Interestingly, these phenotypes are exacerbated by exposure to elevated physiological temperatures and *TPI^{sugarkill}* animals exhibit rapid paralysis when acutely shifted to 37-39°, the basis of which is unknown (Celotto et al., 2006; Palladino et al., 2002; Seigle et al., 2008b).

In humans, inheritance of TPI deficiency is recessive and caused by several specific missense mutations. The most common homozygous human mutation, Glu104Asp, also exhibits a temperature-sensitive phenotype *in vitro* (Arya et al., 1997; Daar et al., 1986), suggesting that the underlying pathogenesis in *TPI^{sugarkill}* may be similar to human disease-causing mutations. Previous research has shown that *TPI^{sugarkill}* is degraded and that animals have significantly reduced TPI levels after 48h at 29° (SEIGLE *et al.* 2008). Furthermore, the proteasome, a large protein complex that destroys misfolded proteins, has been shown to contribute to *TPI^{sugarkill}* degradation (Seigle et al., 2008b). Protein turnover mediated by the proteasome is often regulated by the ubiquitin-proteasome pathway (UPP) and the actions of molecular chaperones, a class of proteins that recognize and bind misfolded proteins (Bukau and Horwich, 1998; Frydman, 2001; Goldberg, 2003; Hartl and Hayer-Hartl, 2002; McClellan et al., 2005). We hypothesized that *TPI^{sugarkill}* interacts with molecular chaperones and these chaperones regulate its turnover in a proteasomal-dependent manner.

Molecular chaperones are involved in a variety of processes during protein biogenesis, including protein translocation, folding, maintenance of native conformation, preventing protein aggregation, catalyzing post-translational modifications, and targeting misfolded substrates to the UPP for degradation (Becker et al., 1996; Fewell et al., 2001; Hohfeld et al., 2001; McClellan and Frydman, 2001). The three main classes of chaperones are: heat shock proteins (*e.g.*, Hsp70, Hsp40, Hsp110, and Hsp90); ER lectins (*e.g.*, calnexin); and thiol oxidoreductases (*e.g.*, PDI). Importantly, chaperone activity has been linked to several neurodegenerative diseases, such as Huntington's and Alzheimer's diseases, and changes in chaperone activity have been shown to modulate the progression of these disorders (Adachi et al., 2009; Auluck et al., 2002; Auluck et al., 2005; Gong and Golic, 2006; Liao et al., 2008). This work focuses on the role of two key cytosolic chaperones, Hsp90 and Hsp70, and their role in regulating *TPI^{sugarkill}* protein using the *Drosophila* TPI deficiency model.

Hsp90 is one of the most abundant proteins in the cell, comprising 1-2% of the total protein present in unstressed cells (Lai et al., 1984; Welch and Feramisco, 1982). This chaperone helps fold a diverse set of client proteins, such as transcription factors, steroid hormone receptors, and protein tyrosine kinases. For example, Hsp90 has been found to be essential for the proper folding of the glucocorticoid receptor and vSrc in both mammalian and yeast systems (Goeckeler et al., 2002; Nathan and Lindquist, 1995; Smith et al., 1990a; Smith et al., 1990b; Smith et al., 1992; Smith and Toft, 1993). Hsp90 does not act alone in regulating the folding of these client proteins, but rather functions in a series of complexes with other chaperones and co-chaperones to achieve proper folding of the polypeptide (Dittmar et al., 1997; Kosano et al., 1998; Smith and Toft, 1993; Smith et al., 1995; Wegele et al., 2004). One other chaperone that often associates with Hsp90 in the mature complex is Hsp70 (Scheufler et al., 2000). Hsp70 participates in a diverse set of cellular processes such as protein translocation into organelles, protein folding, rearranging multi-protein complexes, disaggregation and targeting misfolded substrates to the UPP (Flaherty et al., 1990; Park et al., 2007; Zhu et al., 1996). The folding of newly formed TPI occurs in the cytoplasm (Olah et al., 2002). We hypothesized that interactions of *TPI^{sugarkill}* with the cytoplasmic molecular chaperone machinery help to stabilize mutant protein, possibly decreasing proteasome-dependent degradation, and increasing TPI function. In support of this hypothesis, we have detected an interaction between

the Hsp70 and Hsp90 molecular chaperones with the TPI^{sugarkill} protein. Surprisingly, data collected using complementary pharmacological and genetic experiments indicate that both Hsp70 and Hsp90 are important for targeting TPI^{sugarkill} for degradation. We have conducted stress sensitivity assays of locomotion and analyzed TPI^{sugarkill} protein levels in order to ascertain whether pharmacologic inhibition of the proteasome or the molecular chaperone Hsp90 results in a decreased or exacerbated phenotype. In addition to the pharmacological experiments, we have generated double mutant flies bearing TPI^{sugarkill} and previously established *Hsp90* and *Hsp70* mutants and transgenes (Auluck et al., 2002; Auluck et al., 2005; Elefant and Palter, 1999; Yue et al., 1999). These *Hsp90* and *Hsp70* genetic reagents allowed us to examine the effect reducing Hsp90 or Hsp70 or increasing Hsp70 activity has on protein turnover. We were able to assess the affect these manipulations had on the animal using characterized TPI^{sugarkill} phenotypes. We have observed a decrease in TPI^{sugarkill} turnover in animals with compromised proteasome, Hsp90 or Hsp70 function. In addition, animals with high levels of Hsp70 activity exhibited an increase in TPI^{sugarkill} turnover. Furthermore, the mechanical stress sensitivity in TPI^{sugarkill} animals with impaired proteasome, Hsp90 or Hsp70 activity was surprisingly improved suggesting that mutant TPI^{sugarkill} protein retains function and that chaperone mediated proteasomal degradation underlies pathogenesis. These data identify Hsp70 and Hsp90 as factors that assist with targeting TPI^{sugarkill} for degradation and are involved in the neurodegenerative disease pathogenesis of TPI^{sugarkill} animals.

Materials and Methods

Drosophila stocks and culture

Standard cornmeal molasses fly media was used. Flies were maintained at room temperature, unless otherwise noted. The TPI^{sugarkill} mutation is maintained as a homozygous viable *ve e sgk* strain. Wildtype controls are *ve e* homozygotes, unless otherwise noted. The mutant and control strains used in all experiments contained the more common TpiS variant (Oakeshott et al., 1984).

Pharmacology

Geldanamycin (Sigma) and MG132 (Sigma) were dissolved in DMSO and 3-methyladenine was dissolved in DMF. Mifepristone (Sigma) was used at 500uM to activate the geneswitch GAL4 (Shen et al., 2009). All drugs were diluted in water and administered to a semicircle of filter paper placed on top of the standard media. Animals were administered the drug for the indicated period of time and analyzed. No pre-incubation of animals with the drug was performed. For long-term analyses, life span and stress-sensitive paralysis, fresh media and drug were provided every other day. The molecular chaperone lines used for these studies are listed in Supplemental Table 1.

Western blot analyses of TPI protein

Ten fly heads were ground by pestle in 50μL 2X SDS PAGE sample buffer (4%SDS, 4%β-mercaptoethanol, 130mM Tris HCl pH 6.8, 20% glycerol). Proteins were resolved by SDS PAGE and transferred onto nitrocellulose. Following treatment in 1% milk PBST the blots were treated with anti-TPI (1:5000; rabbit polyclonal FL-249; Santa Cruz Biotechnology) or anti-Beta-tubulin (1:1000; rabbit polyclonal D-140; Santa Cruz Biotechnology). The loading control is beta-tubulin. ATPalpha antibody was obtained from the Developmental Studies Hybridoma Bank (1:5000; mouse monoclonal alpha5). The blots were washed in PBST, incubated in the appropriate HRP- conjugated secondary antibody, and developed with an ECL kit (Pierce), as previously described (Seigle et al., 2008a). Quantification of the scanned films was performed digitally using ImageJ software available from the National Institutes of Health.

Chaperone analyses and immunoprecipitation

Molecular chaperone levels were quantified using both western blot analysis and quantitative real time RT-PCR. Western blot analyses were conducted as described above but using anti-Hsp90 (N-17, 1:1000) and anti-Hsp70 (dA-17, 1:100) serum from Santa Cruz Biotechnology. The protocol for quantitative real time RT-PCR was previously described in (Celotto et al., 2006) using previously described Hsp70 and Hsp90 primers (Fujikake et al., 2008). Immunoprecipitation experiments were performed on fly head extracts similar to a previously described protocol (Hrizo et al., 2007) using the TPI antibody covalently conjugated to resin (Pierce Co-Immunoprecipitation Kit). Precipitates were analyzed by western analysis as described above.

Cellular fractionation

Sub-cellular fractionation was conducted using a modification of a previously published protocol (Kabani et al., 2002). Briefly, 1mL of flies were homogenized with an IKA Ultra-Turrax T8 for 30 seconds (sec) in 1.5mL PLB (20mM Hepes pH 7.4, 100mM NaCl, 20mM MgCl₂) supplemented with fresh 1mM PMSF. Large debris was removed from the suspension by two successive 5000g 5 minute (min) spins at 4°. The supernatant was centrifuged at 16000g for 15 min at 4°. The pellet from this spin (P1) was resuspended in 200ul PLB, 200ul of supernatant (S1) was set aside for analysis. The remaining S1 was centrifuged at 150 000xg for 15 min at 4°. The supernatant (S2) from this spin was reserved for analysis and the pellet (P2) was resuspended in 200ul PLB. Western analyses for TPI and ATPalpha were conducted as described above. Protein levels were equalized by Bradford assay prior to centrifugation. ATPalpha was utilized as a membrane protein control for a pellet enriched fractionation pattern. For the western blots, the wells and stacker of the gel were included in the transfer and analysis to ensure that large protein aggregates were not being discounted from the fractionation pattern.

Locomotor function

Stress sensitivity (a.k.a., bang sensitivity) was assayed by vortexing flies in a standard media vial for 20 sec and measuring the length of paralysis, similar to a previously described protocol (Ganetzky and Wu, 1982). Temperature sensitivity was assayed by acutely shifting animals to 39° and observing time to paralysis, similarly to methods previously described (Palladino et al., 2003; Palladino et al., 2002). Temperature sensitivity assays utilized animals heterozygous for *TPI^[JS10]*, except those involving *UAS-Hsp70* transgenes, which were homozygous. MG132 and GA were administered to the flies as described above.

Statistics

A Student's t-test was used to evaluate all data except those in Figures 4, 5 and Supplemental Figure 1. For these data a one-way ANOVA with a Dunnetts post-hoc test was used. For pharmacological studies statistical comparisons were made between the various treatment groups and vehicle (DMF or DMSO) controls. For all figures error given is SEM, * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.001$.

Results

TPI^{sugarkill} degradation is enhanced by exposure to high temperature and is proteasome dependent

TPI^{sugarkill} animals exhibit significantly reduced TPI protein levels compared to wildtype animals (~50% reduction in TPI levels at 25°, Supplemental Figure 1A, B). To understand better the connection between temperature and protein stability and observe the dynamics of this process we examined TPI^{sugarkill} in a time course following a temperature shift. While the reduced TPI^{sugarkill} protein levels are relatively constant at 25°, steady state protein levels

significantly diminish following acute exposure to 29° (Figure 1). However, wildtype TPI exhibits no change in endogenous protein levels in animals exposed to the higher temperature (Supplemental Figure 1C). Previous data suggested the possibility that TPI^{sugarkill} protein might be regulated by the proteasome (Seigle et al., 2008b). To directly test the hypothesis that TPI^{sugarkill} is degraded by the proteasome animals were acutely administered the proteasome inhibitor MG132 (Meulener et al., 2005) and TPI^{sugarkill} protein turnover was examined. TPI^{sugarkill} was stabilized at 29° when animals were treated with the proteasome inhibitor, but not the vehicle control (Figure 1B).

As the UPP is not the only means of cytosolic protein degradation in eukaryotic cells, we also examined autophagy (lysosomal degradation) for a role in TPI^{sugarkill} turnover. The cellular targets of autophagy are usually but not always aggregation prone cytosolic proteins (Ding and Yin, 2008) and TPI^{sugarkill} does not appear to be aggregation prone (see below). We observed no change in TPI^{sugarkill} degradation when the flies were administered autophagy modulating drugs rapamycin and 3-methyladenine (Bjedov et al., 2010; Riedel et al., 2010) suggesting that the pathway is not involved in or at least is not the major mechanism of mutant TPI^{sugarkill} protein turnover (Supplemental Figure 2). These data coupled with the previously published results demonstrate that the proteasome contributes significantly to the temperature-dependent stability of TPI^{sugarkill}.

Altered protein solubility of TPI^{sugarkill} does not underlie pathogenesis

While no high molecular weight bands were detected on our western blot analyses of TPI^{sugarkill} extracts (data not shown), we wanted to examine the protein for a change in solubility. We examined the fractionation pattern of TPI and TPI^{sugarkill} at both 25° (Figure 2) and 29° (Supplemental Figure 3) using a previously established protocol to identify aggregated proteins (Kabani et al., 2002) and no change in solubility was detected. As a control the fractionation pattern of a membrane protein (Na, K ATPase alpha subunit, a.k.a. ATPalpha) was included to ensure robust pellet enrichment of a membrane protein could be detected. Furthermore, we did not observe any high molecular weight complexes of TPI or ATPalpha present in the wells or stacker of the gel when analyzed by western blot analysis (data not shown). In addition, because the phenotypes of TPI^{sugarkill} animals worsen as they age (Celotto et al., 2006), we examined the solubility of TPI and TPI^{sugarkill} in aged animals (Day 20) and again observed no change in the fractionation pattern (Supplemental Figure 3). These data support the conclusion that the loss of the TPI^{sugarkill} protein from the cell extracts is due to proteasomal degradation and not due to aggregation and that the increase in TPI turnover rather than aggregation underlies pathogenesis.

Hsp70 and Hsp90 are up-regulated in TPI^{sugarkill} animals and associated with TPI and TPI^{sugarkill}

Proteins are often targeted for proteasomal degradation by a class of proteins called molecular chaperones. These chaperones recognize misfolded proteins, try to refold them and when they cannot, direct the misfolded protein to the proteasome (Bukau and Horwich, 1998; Frydman, 2001; Goldberg, 2003; Hartl and Hayer-Hartl, 2002; McClellan et al., 2005). The expression of molecular chaperones is often up regulated in cells expressing a mutant protein that is degraded by the proteasome (Fujikake et al., 2008; Shi et al., 1998). We examined the levels of two molecular chaperones, Hsp70 and Hsp90, in TPI^{sugarkill} animals at both 25° and 29°. At 25° higher levels of each chaperone protein were detected in TPI^{sugarkill} animals when compared to the wildtype (Figure 3A, B). At 29° higher levels of Hsp90 were detected in TPI^{sugarkill} animals when compared to the wildtype (Figure 3A, B). Importantly, both chaperones showed a strong increase in expression in wildtype animals at 29° over 25°, consistent with temperature-induced chaperone activation. Thus, either temperature or expression of mutant TPI^{sugarkill} protein was capable of chaperone activation but the combined

conditions did not generally cause a significant further activation of chaperone expression. These findings are in accord with chaperone mRNA levels observed under the same conditions (Supplemental Figure 4). Together these data demonstrate induced expression of both Hsp70 and Hsp90 in cells expressing the mutant TPI protein. Additionally, when TPI immunoprecipitates were probed for the presence of Hsp70 and Hsp90, both molecular chaperones were found in the precipitate with wildtype and mutant TPI proteins suggesting that the molecular chaperones regulate the folding of both wildtype and mutant TPI proteins (Figure 3C).

Hsp70 and Hsp90 target TPI^{sugarkill} for degradation

To determine whether Hsp90 or Hsp70 contribute significantly to TPI^{sugarkill} stability, we examined TPI and TPI^{sugarkill} in animals treated with an Hsp90 inhibitor, and in animals bearing previously published *Hsp70* or *Hsp90* mutations or transgenes (Auluck et al., 2002; Auluck et al., 2005; Elefant and Palter, 1999; van der Straten et al., 1997; Yue et al., 1999). Hsp90 activity was altered in three ways: administration of the inhibitor geldanamycin (GA) (Auluck et al., 2005), a reduction in Hsp90 protein levels by p-element insertion (*PPZ* mutant; (Yue et al., 1999), or transgenic expression of a dominant-negative Hsp90 (*E6A* mutant; (van der Straten et al., 1997). In all cases, TPI^{sugarkill} was stabilized when Hsp90 activity was diminished (Figure 4A,B). In addition, TPI^{sugarkill} was stabilized in animals expressing a dominant-negative Hsp70 mutant (K17S) via the UAS-GAL4 system ((Auluck et al., 2002); Figure 4C). The geneswitch GAL4 was utilized to tightly regulate temporal expression of the transcription factor and provide robust widespread expression (Shen et al., 2009). Furthermore when functional Hsp70 levels were increased TPI^{sugarkill} degradation was enhanced (Figure 4C). These data suggest that the interaction of TPI^{sugarkill} with Hsp70 and Hsp90 increases protein turnover.

While changes in TPI^{sugarkill} stability were observed in response to altered Hsp90 and Hsp70 activity, no changes in wildtype TPI stability were observed (Supplemental Figure 1C,E). In addition, modulation of chaperone activity did not result in a restoration of TPI^{sugarkill} levels back to that of wildtype (Supplemental Figure 1A,B,D). The overexpression of the wildtype and mutant Hsp70 constructs were driven by a mifepristone inducible GAL4 geneswitch system. We determined that mifepristone administration had no effect on the stability of TPI or TPI^{sugarkill} (Supplemental Figure 1 D,E). Furthermore, chaperone overexpression (~2 fold increase, *UAS-Hsp70* (WT and K71S)) and reduction in chaperone levels (~50% reduction in *Hsp90-PPZ*) was confirmed by western blot analyses (Supplemental Figure 5).

TPI^{sugarkill} exhibits normal solubility, however, altering protein turnover may provide a highly sensitized system in which to detect otherwise subtle changes in TPI^{sugarkill} solubility. To examine this possibility cellular fractionation experiments were conducted on the most severe chaperone mutant *Hsp90-PPZ* (Kabani et al., 2003; Yue et al., 1999). No change was observed in the fractionation pattern of TPI or TPI^{sugarkill} suggesting that altering chaperone function and TPI^{sugarkill} levels does not affect the solubility of the protein in the cell (Supplemental Figure 6).

Stabilizing mutant protein improves TPI^{sugarkill}

To determine whether the increased turnover of TPI^{sugarkill} observed is a protective mechanism or underlies pathogenesis we assessed the affect of altering TPI turnover on the mechanical stress sensitivity and temperature-sensitive paralysis phenotypes of TPI^{sugarkill} animals (Celotto et al., 2006; Seigle et al., 2008b). We examined the severity of these phenotypes in animals where improved TPI^{sugarkill} levels were observed. The mechanical stress sensitivity of TPI^{sugarkill} animals was significantly improved in animals treated with MG132 and GA, compounds that result in the stabilization of TPI^{sugarkill} (Figure 5A,B) and in animals with

reduced Hsp90 (*PPZ* and *E6A*) or reduced Hsp70 activity (*K71S*) (Figure 5 C,D). Additionally, in animals with increased Hsp70 activity where a reduction in *TPI^{sugarkill}* protein was observed, a corresponding worsening of the mechanical stress sensitivity was observed (Figure 5D). While altering *TPI^{sugarkill}* degradation through modulating chaperone and proteasome activity resulted in changes in mechanical stress sensitivity, no significant change was observed in temperature-sensitive paralysis (Supplemental Figure 7). In addition, we examined lifespan and conducted histological analysis of neurodegeneration and did not see significant improvement in either phenotype (data not shown). The mechanical stress sensitivity experiments suggest that *TPI^{sugarkill}* retains function and that the degradation of functional *TPI^{sugarkill}* underlies pathogenesis in these animals. These data are consistent with previous findings using transgenic overexpression of mutant protein (Celotto et al., 2006; Seigle et al., 2008b). However the lack of rescue of the temperature-sensitive paralysis and lifespan suggests that the stress- and temperature-sensitive paralysis result from a loss of discrete TPI functions or that improved temperature sensitivity requires more than the modest increase in protein levels observed with these genetic and pharmacologic manipulations.

Discussion

Studies of TPI deficiency disease pathogenesis have previously focused on dimer stability and catalytic activity of the mutant enzyme (Olah et al., 2002; Orosz et al., 2001; Ralser et al., 2006; Rodriguez-Almazan et al., 2008; Rodriguez-Almazan et al., 2007; Schliebs et al., 1997; Seigle et al., 2008b). Several labs have shown that some, but not all of the human disease causing TPI alleles, have reduced dimerization and isomerase function using *in vitro* assays (Olah et al., 2002; Orosz et al., 2001; Ralser et al., 2006; Rodriguez-Almazan et al., 2007; Schliebs et al., 1997). However, previously published data suggests that *TPI^{sugarkill}* dimer stability is not compromised (Seigle et al., 2008b). As the *TPI^{sugarkill}* protein does not appear to aggregate (Figure 2) and increased levels of the *TPI^{sugarkill}* protein rescues the mutant phenotypes (Figure 5; (Celotto et al., 2006; Seigle et al., 2008b), these data cumulatively suggest that the mutant protein can conform to a functional shape and likely retains significant activity.

Hsp70 and Hsp90 are molecular chaperones that have been implicated in the progression and amelioration of other neurodegenerative diseases (Adachi et al., 2009; Auluck et al., 2002; Auluck et al., 2005; Fujikake et al., 2008). However, *TPI^{sugarkill}* is a unique cytosolic model protein for the study of these chaperones and their role in mutant protein turnover and disease pathogenesis as it is a soluble protein that is not aggregation prone. Other neurodegenerative models can be rescued with increased activity of the molecular chaperones as they help target the misfolded protein for degradation before toxic cellular protein aggregates can form (Adachi et al., 2009; Auluck et al., 2002; Auluck et al., 2005; Fujikake et al., 2008). The role of Hsp90 and Hsp70 in *TPI^{sugarkill}* pathogenesis is distinct from the previously mentioned examples, as decreased chaperone activity reduces pathogenesis for some mutant phenotypes and *TPI^{sugarkill}* protein does not appear to cause the toxic cellular aggregates observed in other neurodegenerative diseases. In the case of *TPI^{sugarkill}* the molecular chaperones are targeting a protein for degradation before it can function within the cell, thus reducing the function of the enzyme and contributing to the disease states. TPI is folded in the cytoplasm and previous labs have studied TPI protein folding rates *in vitro* and have found them to be rather rapid (Olah et al., 2002). The mechanism of chaperone identification and targeting of *TPI^{sugarkill}* for degradation is not known. For example, the cytosolic *TPI^{sugarkill}* protein may be recognized by molecular chaperones and targeted for degradation due to a slower folding rate than the wildtype protein, as is the case with the ER secretory CFTR Δ F508 mutant protein (Younger et al., 2006; Younger et al., 2004). Overall, while other studies have shown that up-regulation of molecular chaperones may prove beneficial for reducing neurodegeneration caused by formation of toxic aggregates (Adachi et al., 2009; Auluck et al., 2002; Auluck et al., 2005;

Fujikake et al., 2008), our study corresponds with previously published work that also suggests that excessive up-regulation or unregulated chaperone and proteasome activity may lead to undesirable side-effects and that a balance of chaperone and proteasome activity may be required for neuronal function and health (Mosser, 2004; Watts et al., 2003).

We observed that modulation of molecular chaperone activity alone was not sufficient to restore TPI^{sugarkill} protein to wildtype levels. The results are in line with what is expected for modest hypomorphic or increased expression conditions examined. Transgenic overexpression of mutant TPI^{sugarkill} protein also rescued TPI^{sugarkill} phenotypes (Celotto et al., 2006; Seigle et al., 2008b), consistent with the interpretation that the mutant protein retains function. Thus far any means examined of altering steady state TPI^{sugarkill} protein, either by increasing rate of synthesis or decreasing proteasomal-dependent degradation, results in the predicted affect on TPI^{sugarkill} phenotypes. These data demonstrate that TPI^{sugarkill} degradation is not a protective mechanism, as has been seen with aggregation prone protein models (Adachi et al., 2009; Auluck et al., 2002; Auluck et al., 2005; Fujikake et al., 2008), but rather leads to a loss of functional protein that underlies pathogenesis.

While the proteasome and molecular chaperones have been shown to be involved in TPI^{sugarkill} turnover, it is not clear how TPI^{sugarkill} is recognized and targeted for degradation. The majority but not all proteasomal-targeted proteins are polyubiquitinated prior to recognition and subsequent degradation by the proteasome (Basbous et al., 2008; Kalejta and Shenk, 2003; McClellan et al., 2005; Naidoo et al., 1999; Schork et al., 1995; Seigle et al., 2008b). Further studies will be needed to clarify the recognition and targeting mechanisms.

Overall, these experiments suggest that TPI^{sugarkill} retains activity and that by modulating TPI turnover disease pathogenesis can be altered. In addition, a new mutant substrate that interacts with molecular chaperones and is degraded by the proteasome has been identified for study. Previous studies of cytosolic protein targeting for protein degradation have focused on ER proteins (Park et al., 2007) and exogenous mutant proteins such as VHL tumor suppressor (McClellan et al., 2005). TPI^{sugarkill} is a unique protein quality control substrate, the further study of which will yield important insight into the mechanisms of identification and targeting of cytosolic proteasomal substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. Temperature enhanced turnover of *TPI^{sugarkill}* is stabilized with proteasome inhibition

A: TPI protein was assessed from *TPI^{sugarkill}* animals acutely shifted to 25° (yellow line) or 29° (red line). TPI levels were visualized by western blot. N = 5. Wildtype TPI levels do not change in response to exposure to 29° (data not shown). B: TPI protein levels were assessed from *TPI^{sugarkill}* animals treated with 30uM MG132 (red line) or the vehicle control (0.03% DMSO, blue line) and acutely shifted to 29°. N = 3.

FIGURE 2. TPI and TPI^{sugarkill} have similar fractionation patterns

A: Extracts from *TPI* and *TPI^{sugarkill}* animals at 25° were fractionated. The pellet (P) and supernatant (S) fractions were assessed for TPI by western blot analysis. B: ATPalpha control demonstrates a pellet enriched fractionation pattern. C: Representative western blots of TPI, TPI^{sugarkill}, and ATPalpha. N = 3.

FIGURE 3. Hsp70 and Hsp90 levels are increased in *TPI^{sugarkill}* animals

A: Hsp90 protein was assessed from *TPI^{sugarkill}* animals acutely shifted to 25°C and 29°C for 24 hours. Hsp90 levels were visualized by western blot with anti-Hsp90a/b. N = 7. B: Hsp70 protein levels were assessed as in A but using anti-Hsp70. N = 7. C: TPI and *TPI^{sugarkill}* were immunoprecipitated using anti-TPI conjugated resin. Precipitated proteins were visualized by western blot. N = 3.

FIGURE 4. *TPI^{sugarkill}* is stabilized in animals with reduced Hsp90 or Hsp70

A: TPI protein levels were assessed in *TPI^{sugarkill}* animals treated with 5uM geldanamycin (GA, red line) or the vehicle control (0.03% DMSO, blue line) and acutely exposed to 29°. N = 3. B: TPI protein levels were assessed from *TPI^{sugarkill}* animals wildtype (+) or mutant (E6A or PPZ) for Hsp90 and that were exposed to 25° or 29° for 24hr. TPI levels were compared to *TPI^{sugarkill}* animals with wildtype (+) Hsp90. N = 5. C: TPI protein levels were assessed from *TPI^{sugarkill}* animals with no UAS transgene (+), *UAS-Hsp70* construct, or *UAS-Hsp70 [K71S]*. The animals were exposed to 25° or 29° for 24hr. TPI levels were compared to *TPI^{sugarkill}* animals with no transgene. N = 6.

FIGURE 5. Stabilizing TPI^{sgk} reduces the progressive mechanical stress sensitivity of TPI^{sgk} animals

The time required for recovery of mobility following mechanical stress is indicated. Animals were age matched (day 20). A & B: Vehicle only control (DMSO) and untreated (–) controls were also examined. A: MG132 administered at 10uM or 30uM improved locomotion in *TPI* [*sgk*] and did not alter wildtype animals. N = 12. B: Geldanamycin (GA) administered at 2uM or 5uM improved locomotion in *TPI* [*sgk*] and did not alter wildtype animals. N = 12. C: The *Hsp90*[*E6A*] and *Hsp90*[*PPZ*] mutations improved locomotion in *TPI* [*sgk*] and did not alter wildtype animals. N = 12. D: Wildtype Hsp70 overexpression (UAS-Hsp70) worsened locomotor function, whereas, expression of dominant-negative Hsp70[K71S] (UAS-Hsp70 [K71S]) improved locomotion. Expression of these transgenes did not affect wildtype animals. N = 5.