Regulation of ubiquitin-proteasome system-mediated degradation by cytosolic stress

by

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A Thesis

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CERTIFICATE OF APPROVAL

This is certify that the Master's Thesis of

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List of Abbreviations

aa	amino acid
α	alpha
β	beta
BFA	brefeldin A
C-	carboxyl-
CHX	cycloheximide
CFTR	Cystic fibrosis transmembrane conductance regulator
Cx32	Connexin 32
Cx43	Connexin 43
Cys	cysteine
DSC	Differential Scanning Calorimetry
DTT	dithiothreitol
ε	epsilon
ER	Endoplasmic Reticulum
ERAD	ER-associated degradation
F (protein context)	phenylalanine
fs	frame-shift

GFP	green fluorescent protein
h	hour
H_2O_2	hydrogen peroxide
H/O stress	hyperthermic/oxidative stress
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
IgG	gamma immunoglobulin
κ	kappa
λ	Lambda
KD	kilo Dalton
K (protein context)	lysine
μg	microgram
μM	micromolar
mM	millimolar
N-	amino-
NaCl	sodium chloride
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
R (protein context)	arginine

ROS	reactive oxygen species
S	Svedberg units
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
-SH	sulfhydryl
TGN	trans Golgi network
TM	trans-membrane
UPS	ubiquitin-proteasome system
WT	wild-type
Δ	delta
°C	degree Celsius

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ABSTRACT

ER-associated, ubiquitin-proteasome system (UPS)-mediated degradation of the wild-type gap junction protein connexin32 (WT Cx32) is inhibited by mild forms of cytosolic stress at a step prior to its dislocation into the cytosol. We show that the same conditions (a 30 min, 42°C heat shock or oxidative stress induced by arsenite) also reduce the ER-associated turnover of disease-causing mutants of Cx32 and CFTR, as well as that of WT CFTR and unassembled Ig light chain. Stress-stabilized WT Cx32 and CFTR, but not the mutant/unassembled proteins examined, could traverse the secretory pathway. Heat shock also slowed the otherwise rapid UPS-mediated turnover of the cytosolic proteins MyoD and GFPu, but not the degradation of an ubiquitination-independent construct (GFP-ODC) closely related to the latter. Analysis of mutant Cx32 from cells exposed to proteasome inhibitors and/or cytosolic stress indicated that stress reduces degradation at the level of substrate polyubiquitination. These findings reveal a new link between the cytosolic stress-induced heat shock response, ER-associated degradation, and polyubiquitination. Stress-denatured proteins may titer a limiting component of the ubiquitination machinery away

from pre-existing UPS substrates, thereby sparing the latter from degradation.

Chapter 1

Introduction

Ubiquitin-Proteasome System

In all eukaryotic tissues, the majority of intracellular proteins are degraded by the ubiquitin-proteasome system (UPS) (Rock *et al.*, 1994). The UPS targets normal short-lived proteins and misfolded or damaged proteins for degradation. UPS-mediated protein degradation allows for post-translational modulation of steady-state levels of proteins regulating key cellular processes such as resistance to stress, cell cycle progression, signal transduction, chromosomal silencing, and cellular differentiation (Pickart, 2004).

A key step in UPS-mediated degradation is the linkage of the polypeptide ubiquitin onto an amino group of target proteins to mark them for specific downstream events. This is typically the ε-amino group of an internal lysine residue, although for at least two substrates, the ubiquitin chain can added to the α-amino group at the substrate's N-terminus (Breitschopf *et al.*, 1998; Bloom *et al.*, 2003). Ubiquitin is a highly conserved 76 aa protein differing at only 3 of 76 residues between yeast and humans that can be linked to substrates as a monomer, or as isopeptide-linked polymers (polyubiquitin chains) whose structure can influence the substrate's fate (as reviewed in (Pickart and Eddins, 2004)). The length of the polyubiquitin chain is ultimately determined by both the

activity of the ubiquitin conjugation machinery as well as that of the deubiquitinating enzymes that remove ubiquitin molecules from the chain (Lederkremer and Glickman, 2005). Polyubiquitin chains, consisting of at least four ubiquitin molecules linked through the K48 of ubiquitin, target the substrate for degradation by the 26S proteasome (Thrower *et al.*, 2000), the large multicatalytic protease complex that degrades ubiquitinated proteins to small peptides. The physiological occurance of ubiquitin linkages other than K48, such as K63-linked chains, have been well established and have been implicated in a number of diverse functions such as endocytosis of cell surface receptors, DNA repair, and activation of NFκB signaling (Glickman and Ciechanover, 2002).

With the identification of hundreds of substrate protein targets of the UPS and the multitude of processes that these targets are involved with, the importance of the UPS under normal and pathological conditions has become increasingly clear. A further understanding of its function and regulation is imperative.

The biochemistry of ubiquitin conjugation

The conjugation of ubiquitin to substrates involves three steps: an initial activation step catalyzed by the E1 enzyme, an intermediate step in

which ubiquitin is covalently linked to a conjugating enzyme (E2), and a final step in which the ubiquitin is attached to an amino group on the substrate. This last step is usually mediated by a ubiquitin ligase enzyme (E3). In all genomes studied to date, a single functional E1 enzyme, between 15-20 E2 enzymes, and hundreds of E3 enzymes have been identified (Glickman and Ciechanover, 2002).

The E1 enzyme is an abundant 110 kD enzyme that uses ATP to generate a ubiquitin thiolester, a highly reactive form of ubiquitin. This activation reaction consists of the initial formation of a ubiquitin-adenylate intermediate, followed by reaction of this intermediate with a cysteine residue to form the E1–ubiquitin thiolester. The ubiquitin is then transferred to an active-site cysteine residue of an E2 enzyme. Individual E2s dictate the specific biological function of ubiquitin because the specificity of the E2/E3 interaction limits the final destination (substrate) of the ubiquitin carried by a given E2. All E2s share an active site ubiquitin-binding Cys residue and are also distinguished by UBC domains that mediate interaction with E3 enzymes. The enormous diversity of substrates for ubiquitin conjugation necessitates the existence of a large number of E3 enzymes that confer specificity and allow for regulation of substrate modification. The known E3s belong to three families:

Homologous to E6AP Carboxyl Terminus (HECT), Really Interesting New Gene (RING), and UFD-2 homology (U-box). The HECT, RING, and U-box domains share the common biochemical property of binding both an E2 and a UPS substrate. For the HECT domain E3s, the ubiquitin is transferred from the E2 to an active site Cys residue on the E3 before its final transfer to the substrate. The other two classes of E3s guide the direct transfer of the ubiquitin from the E2 to the substrate (Pickart and Eddins, 2004).

Regulation of substrate ubiquitination and hence degradation has been documented to commonly be at the level of E3 or E2/E3 complex recognition of the substrate. This can occur by activation of the E3 by post-translational modification so that it may now recognize the substrate (Kornitzer and Ciechanover, 2000). Alternatively, it can be the substrate that is modified either biochemically (i.e. by phosphorylation) or by an ancillary protein (such as a molecular chaperone), which recruits the E2/E3 complex (Connell *et al.*, 2001; Murata *et al.*, 2003).

Proteasomes

Proteasomes are found within the cytoplasm and nucleus of all eukaryotic cells and function to degrade intracellular proteins that have

been modified with polyubiquitin chains. The 26S proteasome is comprised of the 20S core particle, and either one or two 19S regulatory particles that cap the 20S core particle at its ends. The core particle can also be modified with 11S activators instead of the 19S cap, which in part confers proteolytic activity towards antigen processing (Wolf and Hilt, 2004). Within eukaryotes, the 20S core is a 28-mer of 14 different subunits arranged in a stack of four heptameric rings ($\alpha\beta\beta\alpha$). The α- and β- rings are each composed of seven distinct subunits (α1-7 and β1-7). This arrangement forms a cylindrical barrel-shaped complex that houses three proteolytic activities within a chamber: trypsin-like provided by the β2 subunit, chymotrypsin-like provided by the β5 subunit, and β1-supplied peptidyl-glutamyl-like activities, cleaving after acidic amino acids (Wolf and Hilt, 2004). Proteolysis of substrates by the proteasome is processive, generating peptides of between four and fourteen amino acids (Nussbaum *et al.*, 1998). These different proteolytic activities are inhibited to different extents by a number of compounds, including the peptide aldehydes ALLN and MG132, which reversibly bind to the active sites and inhibit cleavage of hydrophobic or acidic substrates (Rock *et al.*, 1994; Oberdorf *et al.*, 2001; Groll and Huber, 2004). Another example is the bacterial metabolite lactacystin, which covalently modifies threonine

residues within the active site of the β5 subunit of the proteasome, irreversibly inhibiting this activity (Fenteany *et al.*, 1995). The 19S cap consists of at least 18 subunits that make up two substructures, the base and lid (Kohler *et al.*, 2001a). The base consists of a hexameric ring comprised from the subunits Rpt1-6, all of which harbor AAA- ATPase activity. Polyubiquitinated substrate recognition by the proteasome is mediated by the polyubiquitin-binding proteins Rpn10 and Rpt5, both found within the 19S cap (Deveraux *et al.*, 1994; Wilkinson *et al.*, 2000; Elsasser *et al.*, 2002; Lam *et al.*, 2002; Elsasser *et al.*, 2004). Several other proteins (Rad23, Dsk2) that have ubiquitin-binding capabilities (UBA or ubiquitin-association domains) and/or UBL (ubiquitin-like) domains interact with the proteasome, and serve to deliver polyubiquitinated substrates to the proteasome (Ortolan *et al.*, 2000; Rao and Sastry, 2002). After binding to substrates, the 19S cap is responsible for several processes, including the unfolding of the substrate via a chaperone like activity (Braun *et al.*, 1999; Liu *et al.*, 2002), cleaving polyubiquitin chains from the substrate (mediated by deubiquitination activity by ubiquitin-specific isopeptidases associated with the Rpn10 subunit) (Verma *et al.*, 2002; Yao and Cohen, 2002), gating the axial pores of the 20S particle which allows substrate entry into the

proteolytic chamber (Groll *et al.*, 2000; Kohler *et al.*, 2001b), and threading of the unfolded substrate into the 20S core particle.

ERAD

ER-associated degradation (ERAD) is a degradative pathway within the UPS that is involved in the degradation of both soluble and membrane-integrated nascent polypeptides within the secretory pathway. In most cases, this is for quality control purposes, for instance when a protein is unable to efficiently attain its native tertiary or quaternary structure. The capacity to sort proteins by conformational criteria prevents the potential deleterious transport of defective gene products. ERAD requires the retro-translocation (also termed dislocation) of these polypeptides from the ER lumen or membrane into the cytosol via a proteinacious conduit whose identity is still debated (see figure I-1). There is evidence that the Sec61 channel responsible for import of nascent polypeptides is also involved in the dislocation of ERAD substrates (Plemper *et al.*, 1998; Huyer *et al.*, 2004). Another protein called Derlin-1 has also been implicated in the dislocation of several ERAD substrates (Lilley and Ploegh, 2004; Ye *et al.*, 2004). When at least part of the ERAD substrate is exposed to the cytosol, E2 ubiquitin conjugating

enzymes along with E3 ligases attach polyubiquitin. E2/E3 pairs involved in the ubiquitination of a number of ERAD substrates have been identified, and are either constituents of the ER membrane or can be recruited to the ER membrane from the cytosol, depending on the substrate. Concurrent with, or subsequent to, dislocation, the ubiquitinated polypeptides become degraded by cytosolic proteasomes. It has been demonstrated for a number of ERAD substrates that their dislocation from the ER membrane or lumen is in fact dependent upon their ubiquitination (de Virgilio *et al.*, 1998; Shamu *et al.*, 2001; Jarosch *et al.*, 2002). Although there are reports of ubiquitin-independent degradation of a small number of ERAD substrates based on lysine mutagenesis approaches, the possibility that these substrates can be alternatively modified at their N-termini was not addressed by most of those studies (Yu *et al.*, 1997). Ubiquitin chains on ERAD substrates serve as a recognition signal that mediates binding of the cytosolic AAA (ATPases Associated with various cellular Activities) ATPase p97 after recruitment of p97 to the ER membrane via interaction with ubx2 and derlin-1 (Schuberth and Buchberger, 2005; Ye *et al.*, 2005). Here, p97 along with its cofactors Ufd1 and Npl4 serves as the driving force, at least in part (Carlson *et al.*, 2006), to extract the polypeptide from the ER membrane

or lumen (Ye *et al.*, 2001; Jarosch *et al.*, 2002). Inactivation or depletion of the p97complex has been shown to reduce the degradation rate of a number of ERAD substrates (Bays *et al.*, 2001; Gnann *et al.*, 2004; Huyer *et al.*, 2004; Carlson *et al.*, 2006). The 19S cap of the proteasome, which has the capacity to bind to polyubiquitin chains and contains AAA ATPase activity conferred by subunits Rpt1-6, has also been implicated in the dislocation of at least one ERAD substrate, the lumenal substrate pro-alpha factor, which was sufficiently dislocated from ER-derived microsomes by purified 19S cap (Lee *et al.*, 2004; Brodsky, 2005). Proteasomal-mediated extraction of substrates would imply coupled dislocation and degradation of substrates, and require that proteasomes be closely associated with the ER membrane, which has been observed (Mayer *et al.*, 1998; Xiong *et al.*, 1999). Recent data for the direct association of proteasomes with Sec61p has been reported (Kalies *et al.*, 2005; Ng *et al.*, 2007).

Heat Shock Response

The heat shock response (HSR) is a highly conserved, ordered genetic response triggered by a wide variety of toxic conditions that lead to the accumulation of non-native proteins. These stressors include

elevated temperatures (hyperthermia), oxidative stress induced by a variety of heavy metals and small molecules, amino acid analogs, and small molecule chemical toxicants. The HSR can also become activated by some viral infections (Westerheide and Morimoto, 2005). Activation of the HSR leads to the rapid induction of genes with cytoprotective functions. This includes proteins that aid in the refolding of non-native conformations (folding chaperones and co-chaperones) and proteins involved in degradative pathways (ubiquitin, ubiquitin-conjugating enzymes). Some chaperones (Hsp70) play both refolding and degradative roles, depending on their association with co-factors (Murata *et al.*, 2003). These heat shock proteins (HSPs) provide protection against, and aid in the recovery from, cellular damage associated with the accumulation of misfolded proteins, commonly by holding non-native proteins as soluble intermediates in a refolding-competent state, which prevents the formation of off-pathway intermediates and aggregates. The heat shock gene superfamily includes the Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small heat shock protein (sHSP) families, each named for their molecular size. Another effect of the HSR is to temporarily attenuate the translation of the majority of proteins in order to relieve the chaperone

machinery from the task of folding nascent proteins (as reviewed (Clemens, 2001)).

The heat shock response is regulated at the transcriptional level by trimerization of heat shock factors (HSF) and their subsequent translocation from the cytosol to the nucleus. Here, they bind with high affinity to consensus sequence heat shock elements (HSE). While three HSF's (HSF-1, -2, and –4) have been identified from the human genome, HSF-1 is essential to the heat shock response and has been the most extensively characterized. HSF-1 normally exists as an inactive monomer within the cytosol, bound to Hsp70 and other chaperones (Westerheide and Morimoto, 2005). It is generally accepted that upon cellular stress, the acute increase in non-native polypeptides sequesters Hsp70 from HSF-1, resulting in trimerization of the transcription factor, its translocation to the nucleus, binding to HSE's upstream of Hsp genes, and transcription of Hsp genes. Trancsription of HSP genes may also involve non-HSF transcription factors that bind to DNA elements other than HSEs (Pi *et al.*, 2003).

Heat shock and its effects on cellular proteins

One of the major effects of heat shock on cells is the thermal denaturation of a subset of cellular proteins. Heat shock increases the amount of detergent insoluble protein compared to cells held at 37°C (Beckmann *et al.*, 1992), and it has been reported that simply injecting denatured protein into cells is sufficient to induce Hsp synthesis (Ananthan *et al.*, 1986; Mifflin and Cohen, 1994). The heat shock response is not an all-or-nothing phenomenon, but rather a graded response that depends on the severity of the conditions. The effects of heat stress are dependent upon both the temperature and exposure time; as temperature increases by 1°C, the time required for the same extent of heat shock response is decreased two-fold (Dewey, 1989, 1994; Park *et al.*, 2005). Heat shock sensitivity also depends on biological factors such as cell type and developmental stages (Ferlito and De Maio, 2005). Several criteria have been proposed to distinguish between mild and severe heat stress, including the extent of parameters such as protein denaturation, HSF-1 activation/HSP synthesis, acquisition of thermal tolerance, and apoptosis. Ultimately, the major distinction in cellular response to mild and severe heat stress is either the adaptation to growth conditions (mild) versus cell death or morbidity (severe) (Park *et*

al., 2005). It is important to note that many studies focus on cellular responses to severe heat stress (43-45°C, 15-20 minutes), which leads to transient arrest of the cell cycle (Kuhl and Rensing, 2000) and in many cell types can lead to apoptosis (Punyiczki and Fesus, 1998). Mild heat stress within febrile range (39-42°C 10-30 min), on the other hand, can be beneficial to the organism (Hasday and Singh, 2000).

The effects of oxidative stress on proteins

Oxidative stress is caused by imbalances in biochemical processes, resulting in production of reactive oxygen species (ROS). One of these, the hydroxyl radical, is highly reactive with all biomacromolecules at diffusion-controlled rates. This ROS is most commonly generated physiologically by the Fenton reaction between reduced transition metals (Iron (II) or copper (I)) and hydrogen peroxide (H_2O_2) (Sayre *et al.*, 2001). Damage to proteins under oxidative stress conditions can be via hydroxyl radical-mediated cleavage of protein side chains, resulting in the formation of carbonyl groups (Stadtman, 1992; Berlett and Stadtman, 1997). Proteins can also be modified with adducts of products of glycoxidation and/or lipoxidation (Sayre *et al.*, 2001). Oxidative stress also increases the GSSG/GSH ratio within the cytosol of cells. This leads

to an increase in glutothiolynation of many sulfhydryl-containing proteins, leading to their inactivation and/or unfolding (Giles and Jacob, 2002). Not surprisingly given the reducing environment of the cytosol, it was found that regardless of the oxidant used, cytosolic proteins are the most sensitive to oxidative modification (Jung *et al.*, 2006).

Using differential scanning calorimetry (DSC) to measure protein stability under oxidative stress conditions induced by diamide, it was found that thiol oxidation events lower the thermal stability of certain otherwise thermally stable proteins so that they denature at 37°C rather than at higher temperatures (Freeman *et al.*, 1999). Conversely, conditions that stabilize protein structure such as glycerol treatment block induction of the heat shock response under conditions of oxidative stress (Edington *et al.*, 1989). Taken together, these findings support the concept that oxidative stress leads to protein misfolding, which induces the heat shock response.

Sodium arsenite as in inducer of oxidative stress

Arsenic (As) is a metalloid widely distributed in the environment that normally exists in two oxidative states: trivalent arsenite (As^{+3}) and pentavalent arsenate (AS^{+5}) (Del Razo *et al.*, 2001). The trivalent As

species is the most toxic (Delnomdedieu *et al.*, 1993; Styblo *et al.*, 1999), and acts by directly attacking –SH groups or through generation of a diverse group of reactive oxygen species (ROS) including hydrogen peroxide (Wang *et al.*, 1996; Chen *et al.*, 1998; Shi *et al.*, 2004), hydroxyl radical species (Wang *et al.*, 1996; Garcia-Chavez *et al.*, 2003), nitric oxide (Gurr *et al.*, 1998), and superoxide anion (Barchowsky *et al.*, 1999; Shi *et al.*, 2004). The mechanisms for generation of these reactive intermediates are poorly understood but are currently under investigation (Samikkannu *et al.*, 2003). A number of studies have indicated that sodium arsenite induces the stress response by increasing the levels of hydrogen peroxide (Jing *et al.*, 1999; Pi *et al.*, 2003) and that when scavengers of hydrogen peroxide were applied, arsenite-induced toxicity was suppressed (Jing *et al.*, 1999). These findings lend support to a model of toxicity in which treatment of cell with arsenite leads to increases in H_2O_2 production, followed by hydroxyl radical formation by the Fenton reaction (Wang *et al.*, 1996).

Effects of hyperthermic/oxidative stress on the UPS

Given that both thermal and oxidative stress conditions lead to an increase in misfolded proteins, it is important to understand the effects

of these conditions on pathways which serve to resolve or remove damaged and misfolded proteins from the cell. Numerous studies have shown an increase in total ubiquitin-conjugated proteins upon exposure to non-lethal hyperthermic or oxidative stress conditions in many cell types (Carlson *et al.*, 1987; Parag *et al.*, 1987; Fujimuro *et al.*, 1997; Adamo *et al.*, 1999; Ramanathan *et al.*, 1999; Shang *et al.*, 2001; Taylor *et al.*, 2002; Kirkpatrick *et al.*, 2003; Bredfeldt *et al.*, 2004; Fernandes *et al.*, 2006), a phenomenon thought to be due to the increased production of misfolded substrates for polyubiquitination. In support of this contention, it has been shown that carbonyl-containing proteins isolated from cells treated with physiological levels of hydrogen peroxide that induce mild oxidative stress are ubiquitinated. Conversely, the ubiquitinated pool of proteins within cells, exposed to these same levels of mild oxidative stress are enriched in carbonylated proteins (Shang *et al.*, 2001). Several studies have identified specific proteins that become ubquitinated and degraded at an increased rate under various oxidative stress conditions. These include the arsenite-induced degradation of endothelial nitric oxide synthase (eNOS) (Tsou *et al.*, 2005), and of Cdc25 (Chen *et al.*, 2002; Savitsky and Finkel, 2002) and cyclin D1 ubiquitination and degradation after oxidative stress induced by several methods (Fasanaro *et al.*,

2006). Although there have been several reports of direct oxidative modification or negative regulation of specific proteins involved in UPS-mediated degradation, it should be noted that most of these studies were under conditions of strong hyperthermic (≥45°C) or oxidative stress (Parag *et al.*, 1987; Ishii *et al.*, 2005; Noguchi *et al.*, 2005).

Proteasomal substrates used in these studies and their degradation

CFTR

Cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ABC transporter family, is a 1,480-residue membrane glycoprotein that serves as a chloride ion channel. It consists of two transmembrane domains (each containing six TM helices), two cytosolic nucleotide binding domains (NBD's), and a regulatory domain (R-domain) (Riordan *et al.*, 1989). The naturally occurring CFTR ΔF508 mutant lacks F508 in NBD1, a defect that leads to misfolding of 99% of the protein, retention in the ER, and results in the cystic fibrosis phenotype. Although the biogenesis of wild-type CFTR is inefficient in most cell types with 60-

75% of the nascent protein being degraded (Ward *et al.*, 1995; Meacham *et al.*, 2001), recent studies using native epithelia indicate that endogenous CFTR biosynthesis may be more efficient (Varga *et al.*, 2004). The degradation of both forms of the protein requires the cytosolic chaperone Hsp70 working in conjunction with the DnaJ co-chaperone Hdj-2 (Meacham *et al.*, 1999; Zhang *et al.*, 2001) and the E2/E3 pair UbcH5/CHIP (Meacham *et al.*, 2001; Younger *et al.*, 2004). Other E2/E3 pairs have also been identified as functioning in CFTR ubiquitination (Gnann *et al.*, 2004; Younger *et al.*, 2006), and cytosolic chaperones besides Hsp70 have been implicated in the degradation of CFTR ΔF508 in a yeast system, including the small Hsp's Hsp26 and Hsp42 (Ahner *et al.*, 2007).

IgG light chain

The IgG light chain of the T15 anti-phosphocholine antibody (T15L) does not become secreted when expressed in SP2/0 myeloma cells in the absence of its heavy-chain binding partner. Instead, it is retained in the ER prior to becoming degraded in a proteasome-dependent manner (O'Hare *et al.*, 1999). Although never demonstrated for this specific κ light chain, other nonsecreted κ and λ light chains have been shown to associate with

the ER-localized Hsp70 homologue BiP via binding to the variable region of the light chain, an association that positively correlates with their half-lives (Knittler and Haas, 1992; Knittler *et al.*, 1995; Skowronek *et al.*, 1998; Chillaron and Haas, 2000). Ubiquitinated forms of unassembled IgG light chains have not been demonstrated.

MyoD

MyoD is a tissue-specific basic helix-loop-helix transcriptional activator involved in skeletal muscle differentiation (Weintraub *et al.*, 1991). It is localized to the nucleus where it has an extremely short half-life of approximately 30 minutes (Thayer *et al.*, 1989). MyoD degradation is dependent on the ubiquitin proteasome system. The protein can become ubiquitinated at its N-terminus (Breitschopf *et al.*, 1998; Ciechanover *et al.*, 1999) or at an internal lysine (K133) (Batonnet *et al.*, 2004). An SCF-like E3 complex was identified to mediate MyoD ubiquitination in conjunction with the E2 ubiquitin-conjugating enzyme Ubc3 (Song *et al.*, 1998; Tintignac *et al.*, 2005).

GFPu

GFPu is a destabilized form of GFP fused at its C-terminus to the16 amino acid CL1 degron. The CL1 degron was identified in a screen for sequences that destabilize β-galactosidase in yeast, in which its degradation was dependent upon the closely related E2's Ubc6 and Ubc7 (Gilon *et al.*, 1998). GFPu is also degraded by the UPS in mammalian cells (Bence *et al.*, 2001). In these cells, overexpression of a dominant-negative form of Ubc6 had no effect on the turnover of GFPu, suggesting that other E2s can participate in the ubiquitination and degradation of this protein (Lenk *et al.*, 2002). The destabilized protein has been immunoprecipitated in a ubiquitinated form (Bence *et al.*, 2001) and its degradation is impaired by over-expression of dominant-negative K48R ubiquitin (Bennett *et al.*, 2005) as well as under conditions of oxidative stress (Dong *et al.*, 2004).

GFP-ODC

While degradation of most ER and cytosolic proteins by the proteasome requires a polyubiquitin tag, ubiquitin-independent degradation of at least one substrate, ornithine decarboxylase (ODC), has been documented (Hoyt *et al.*, 2003). ODC has a 37 aa degron at its C-

terminus (cODC) that is necessary and sufficient to deliver the protein to the proteasome. It has been demonstrated that simply appending this degron to other proteins causes their degradation by the proteasome in the same ubiquitin-independent manner (Loetscher *et al.*, 1991; Li *et al.*, 1998; Hoyt *et al.*, 2003). Turnover of intact ODC is regulated by the protein antizyme (Hoyt and Coffino, 2004), which binds to and disrupts ODC dimers, promoting the exposure of the C-terminal degron of ODC and thereby enhancing the affinity of ODC for the proteasome by about 10-fold (Zhang *et al.*, 2003). Recognition of ODC/AZ by the proteasome can be competitively inhibited by either ubiquitinated proteins or by free chains of polyubiquitin, indicating that the ODC C-terminal degron and polyubiquitin conjugates engage the same binding site on the proteasome (Zhang *et al.*, 2003).

Connexin 32

Gap junctions are intercellular plasma membrane channels that link the cytosols of adjacent cells. These structures are composed solely of members of a family of highly homologous four transmembrane-spanning integral membrane proteins called connexins. Noncovalent oligomers of six connexin molecules form the hemi-channels or connexons found at the

plasma membranes of cells. The extracellular domains of hemi-channels at the plasma membrane from two apposing cells bind each other with high affinity, leading to the formation of gap junctional channels, which serve as conduits for the intercellular passage of small molecules and ions.

Compared to most other integral membrane proteins, connexins are degraded relatively quickly with half-lives of between 1.5-5 hrs (Musil *et al.*, 1990). It has been demonstrated that for two members of the connexin family, connexin43 and connexin32, both the lysosomal and proteasomal pathways are involved in their turnover. The proteasomal degradation of these connexins takes place at the ER by ERAD (VanSlyke *et al.*, 2000), presumably because biogenesis of connexins, like that of many other trans-membrane proteins, is an inefficient process. The studies that make up this thesis are the first to report on ubiquitinated forms of Cx32, although the E2/E3 enzymes involved remain to be identified.

Thesis rationale and overview

Degradation of connexins by ERAD is decreased by non-lethal hyperthermic and oxidative stress conditions as well as proteasome inhibitors (VanSlyke and Musil, 2002). These H/O stress conditions were

found to inhibit the ERAD of connexins prior to their dislocation into the cytosol. Inhibiting the degradation of newly synthesized wild-type Cx43 and Cx32 at the ER membrane by H/O stress was shown to lead to a substantial increase in gap junctional assembly at the plasma membrane. This increase in gap junctional plaques correlated with an increase in gap junctional-mediated intercellular dye transfer, demonstrating that negative regulation of connexin ERAD by H/O stress might serve a physiological role in allowing for increased gap-junctional signaling under these stress conditions (VanSlyke and Musil, 2002).

The aim of the work described in this thesis was to examine how the degradation of connexins at the ER by ERAD is negatively regulated by H/O stress conditions. In doing so, these studies examined the stress sensitivity of the ERAD of a soluble (lumenal) substrate, unassembled IgG light chain, as well as the effects of these stress conditions on the degradation of cytosolic (GFPu) and nuclear (MyoD) UPS substrates. The possibility that these stress conditions would affect ER quality control retention mechanisms and allow for the transport of misfolded proteins to post-ER compartments was also addressed. Finally, these studies attempted to determine what step in UPS-mediated degradation was affected by H/O stress.

Author's contribution to this work

The author contributed all of the work found in chapter 2, with the exception of figure 3, which was contributed by Dr. Judy Van Slyke.

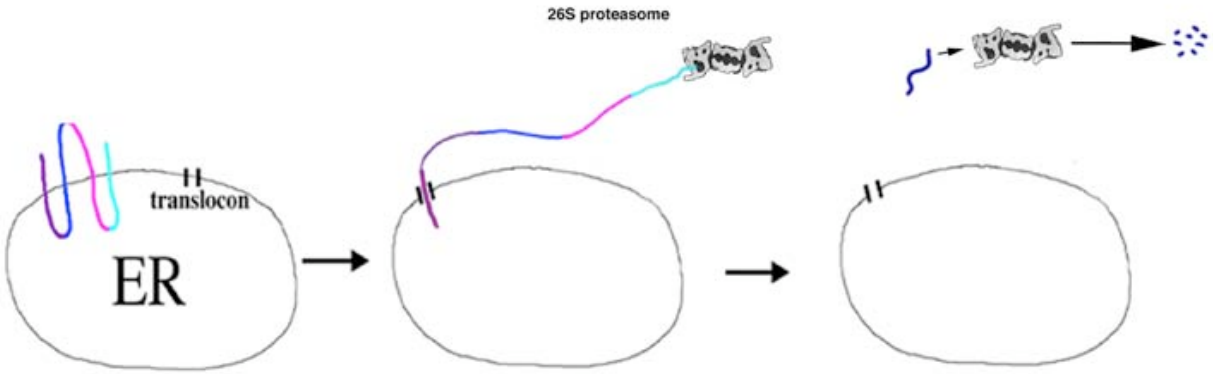

Figure I-1. Dislocation of ERAD substrates from the ER. General schematic of an ERAD substrate, in this case a polytopic membrane protein, becoming dislocated from the ER and degraded by cytosolic proteasomes. The dislocation channel here is labeled translocon, although another channel has been implicated in the dislocation of several ERAD substrates.

Chapter 2

Regulation of ubiquitin-proteasome system-mediated degradation by cytosolic stress

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INTRODUCTION

Although once thought to be restricted to cytosolic and nuclear proteins, degradation by the ubiquitin/proteasome system (UPS) also plays a major role in regulating the level of proteins synthesized within the ER (Meusser *et al.*, 2005). ER-associated degradation by the proteasome (ERAD) can be conceptualized as consisting of four tightly linked steps:

(1) Initial recognition, mediated by proteins (e.g., protein chaperones) that bind to the ERAD substrate because of a feature of the latter that destines it to be destroyed, usually incorrect or incomplete folding or the unmasking of a degron.

(2) Polyubiquitination, in which the first ubiquitin moiety becomes linked through an isopeptide bond to (most commonly) the epsilon amino group of a lysine residue within the UPS substrate and is then extended to form a K48-linked polyubiquitinated chain.

(3) Delivery to the proteasome, which requires the extraction (dislocation) of the ERAD substrate from the ER into the cytosol (Jarosch *et al.*, 2003). With very few exceptions (e.g., yeast mutant pre-pro α factor; (Werner *et al.*, 1996), polyubiquitination is a prerequisite for

complete extraction from the ER and association with the 26S proteasome.

(4) Proteolysis, in which the ERAD substrate is degraded to small peptides within the 20S proteasome core. One or more of the 20S peptidase activities are blocked by compounds such as MG132, epoxomicin, lactacystin, ALLN, and the chemotherapeutic bortezimide (Groll and Huber, 2004). In the presence of such proteasome inhibitors, (Ferlito and De Maio, 2005)polyubiquitinated and unmodified forms due to repeated cycles of polyubiquitination (performed by E1/E2/E3 enzymes) and deubiquitination (carried out by one of the more than 50 enzymes known or predicted to have protein deubiquitinating activity (Shamu *et al.*, 1999; Nijman *et al.*, 2005). Proteasome inhibitors are also known to slow, but do not completely block, dislocation of ERAD substrates (Yu *et al.*, 1997; Story *et al.*, 1999; Mancini *et al.*, 2000; Oberdorf *et al.*, 2006). Although the mechanism of this effect is not known, one possibility is that the ubiquitin-binding sites of proteins that mediate dislocation become occupied by undegradable polyubiquitinated proteins.

With the exception of dislocation, these processes are also required for degradation of UPS substrates located within the cytoplasm or nucleus.

We have studied the ERAD of connexins, the family of nonglycosylated, four-transmembrane domain proteins that form gap junction intercellular channels in virtually all multicellular tissues in animals ranging from tunicates to man (Goodenough *et al.*, 1996; Laird, 2006). Approximately one-half of newly synthesized wild-type connexin43 (Cx43) and connexin32 (Cx32) molecules are rapidly degraded even if their exit from the ER is blocked (VanSlyke *et al.*, 2000). It is likely, but has not yet been definitively established, that degradation of newly synthesized connexins at the ER is a consequence of slow and/or inefficient folding. We have reported that fully ER membrane-integrated Cx32 and Cx43 can be chased into a soluble cytoplasmic pool in full-length form, if (and only if) the degradation of the dislocated molecules is blocked with proteasome inhibitors. Dislocation of connexins, and therefore their proteasome-mediated degradation, is inhibited by agents (80 μM sodium arsenite or a 10-30 min incubation at 42°C) that induce oxidative or hyperthermic stress, respectively (VanSlyke and Musil,

2002). By causing the thermal or oxidative denaturation of proteins in the cytosol, such treatments (hereafter collectively referred to as H/O stress) trigger the heat shock response. Stress stabilization of connexins does not require expression of heat shock proteins, but is instead closely correlated with the stress-induced accumulation of unfolded protein in the cytosol. Rapid degradation of Cx43 is restored 12-18 h after a 30 min, 42°C heat shock, a time at which the level of unfolded protein in the cytosol would be expected to have returned to normal. Several lines of evidence indicate that H/O stress inhibition of connexin ERAD is a specific and potentially physiologically significant phenomenon (VanSlyke and Musil, 2002). First, the treatments that upregulate connexin stability do not kill the cells or block their proliferation. Second, agents that increase the level of unfolded protein in the ER instead of in the cytosol (e.g., tunicamycin; DTT) do not reduce connexin ERAD. Third, H/O stress inhibition of ERAD was observed in all cell types examined. Importantly, wild-type connexin spared from ERAD by H/O stress remains in a full-length, membrane-integrated form capable of folding, multisubunit oligomerization, and transport to the cell surface. This increase in mature connexin was associated with a striking increase in the number of functional gap junctions in cells that would otherwise be gap junction-

deficient (VanSlyke and Musil, 2002), most likely because gap junction assembly is an autofacilitated, self-assembly process (Valiunas *et al.*, 1997; Castro *et al.*, 1999). Given that gap junctions mediate the regulatable cell-to-cell transfer of low molecular weight substances including ions, antioxidants, and second messengers, their upregulation by H/O stress could have important consequences for cell signaling and survival under pathophysiological conditions such as ischemia-reperfusion injury or fever.

These studies were the first to demonstrate that ERAD could be inhibited by physiologically relevant forms of stress to increase the functional pools of a protein on the cell surface. An important question raised by our findings is whether the effect of H/O stress is confined to wild-type connexins. Well over 400 mutations in at least 9 different connexin family members have been shown to cause human diseases including the peripheral neuropathy CMTX (Cx32), ODDD (Cx43), deafness, cataract, and various skin disorders. Several of these mutations have been shown to disrupt transport of the abnormal protein to the cell surface as reviewed by (Laird, 2006). Does H/O stress also inhibit the degradation of such proteins, and if so, does this change their

localization within the cell? We have reported that H/O stress reduces the dislocation of unassembled MHC I heavy chain (VanSlyke and Musil, 2002). Does stress also slow the degradation of other wild-type and mutant UPS substrates? Which step in degradation is blocked by H/O stress? We have addressed these issues using a combination of biochemical and morphological techniques. Our results suggest that H/O stress inhibits the proteasome-mediated degradation of both ERAD and cytosolic UPS substrates via a novel mechanism, most likely by interfering with their ubiquitination.

MATERIALS AND METHODS

Cell culture and transient transfection: CHO-K1 cells were maintained in F-12 medium containing 10% FCS, penicillin G, and streptomycin. T15L L chain-expressing SP2/0 myeloma cells grown in Iscove's Modified Dulbecco Medium (IMDM Gibco # 12440-053), containing 20%FCS, 0.1 mM MEM non-essential amino acids (Gibco # 11140-050) 1 mM sodium pyruvate (Gibco # 11360-070), and 50 μg/mL Gentamicin (Gibco # 15710-064). Transient transfections were conducted one day after plating using FUGENE 6 (Roche Applied Science). For CHO cells, 4 x 10^5 cells were plated on day 0 in a 35 mm dish. Transfections were conducted as follows 24 hrs after seeding: expression plasmids and carrier plasmids were mixed at a 1:5 ratio for a total of 1.6 μg DNA. Serum free media (SFM) containing 4.8 μL Fugene 6 transfection reagent (Roche Applied Sciences) per 1.6 μg DNA was added to the DNA and incubated 15 min at room temperature. 100 μL transfection complexes were added per dish drop wise to cells that were rinsed 1x with SFM and now in complete media without antiobiotics. Cells were then returned to

37°C. Transfection experiments were scaled accordingly to dish size. Cells were analyzed 24 h after transfection. Plasmids used and their sources include: wild-type and 142fs Cx32 (in pcDNA3) from Dr S. Scherer, 175fs and E208K Cx32 from Dr R. Bruzzone, subcloned into pcDNA3, HA-ubiquitin (plasmid pMT123; from Dr. D. Bohmann) (Treier, M., Staszewski, L.M., and Bohmann, D. (1994) Cell 78, 787-98), myoD (in pcDNA3) from Dr. M. Thayer, GFPu (in pEGFP-C1, purchased from ATCC), and the GFP-ODC-encoding plasmids pEGFP-C1-ODC from Drs. R. Kopito and E. Bennet), and pd1EGFP-N1 (BD Biosciences).

Metabolic labeling and treatment with stressors or inhibitors: Adherent cells were rinsed twice with DMEM lacking methionine and cysteine (labeling medium) followed by metabolic labeling by incubation at 37°C with labeling medium supplemented with 5% dialyzed FCS, 2mM glutamine, and 0.1mCi/35mm dish of cells [^{35}S]methionine (EXPRE ^{35}S ^{35}S, Perkin Elmer), scaled proportionately for dishes of other sizes. The radioactive medium was removed after a 20 minute pulse and where indicated, chased samples were rinsed 3x with serum free F-12 (CHO-K1) or Iscove's Modified Dulbecco's Medium (SP2/0) containing 0.5 mM unlabeled methionine and then replaced with complete medium

supplemented with 0.5mM unlabeled methionine, followed by incubation at 37°C in either the absence (control) or presence of the following compounds: 80 μM sodium arsenite (Sigma-Aldrich), 6 μg/ml brefeldin A (BFA, Epicentre), 70 μM cycloheximide (CHX), 2 mM dithiothreitol (DTT), 30 μM MG132 (Calbiochem). Cells were either heat shocked or mock shocked in Leibowitz L15 medium supplemented with 5 mM Hepes buffer solution (Gibco) and 10% FCS. Hypothermic treatments were conducted in water baths in which the cell growing surface of the tissue culture dish was maintained at 42°C (37°C for mock heat treatment) as assessed by continuous monitoring using a thermocouple thermometer to verify that the growing surface of the tissue culture dish was maintained at the desired temperature.

Cell Lysis of Radiolabeled and Non-labeled samples: Cells were rinsed twice with PBS at 4 C and resuspended in lysis buffer (5 mM Tris-HCL, 5 mM EDTA, 5 mM EGTA (pH 8.0)) supplemented with 0.6% SDS, 250 mg/ml soybean trypsin inhibitor, 200 μM leupeptin, 10 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride. Lysates were collected with a 22-gauge needle attached to a 1 mL syringe and transferred to eppendorf tubes and heated at 100°C for 5 minutes (non-

connexin samples). Two modifications to this procedure were made for lysis of Cx32 samples: samples were lysed in SDS at room temperature for 30 minutes rather than 100°C for 5 minutes to minimize aggregation of Cx32, followed by passage of lysates through a 26 gauge needle 3x.

Preparation of Cytosolic and Membrane Fractions: 35mm cultures of adherent cells were rinsed twice with 4°C PBS and then scraped from the dish in 750 μL of homogenization buffer (10mM Tris, 1mM EDTA, 10 mM iodoacetamide, 0.2 mM PMSF, 20 μM ALLN, pH 7.5). After a 20 minute incubation on ice the cells were passed eight times through a 25-gauge needle. The resulting lysate was subjected to a 5 minute centrifugation at 5,000 rpm at 4°C, after which the supernatants were collected and the resulting pellet was resuspended in 250 μL of homogenization buffer and re-extracted by a second round of trituration and centrifugation. The supernatants of the first and second spins were combined, adjusted to 0.25 M NaCl, and separated into cytosolic (supernatant) and membrane (pellet) fractions by centrifugation at 100,000 x g (43, 000 rpm in TLA55 Beckman ultracentrifuge) for 1 hour at 4°C. The pellet was then resuspended in 1 mL homogenization buffer with 0.25 M NaCl. Both supernatant and membrane fractions were brought to 0.6% SDS.

Immunoprecipitation and SDS-PAGE and Western Blotting: For analysis of all proteins other than CFTR, SDS-denatured samples were diluted with 2.5 volumes of Immunoprecipitation Dilution Buffer (100 mM NaCl, 20 mM Na Borate, 15 mM EDTA, 15 mM EGTA, 0.02% NaN3 pH 8.5 supplemented with 0.7 %BSA, 1.2% TX100, 10 mM iodoacetamide, and 2mM phenylmethylsulfonyl flouride). Samples were spun 10 minutes in a microfuge at room temp to remove any precipitate and incubated with immunoprecipitating antibodies overnight at 4°C. Protein/antibody complexes were then bound to sepharose protein A or G beads for 2 hrs at 4°C. Beads were washed 4x with wash buffer 1(1 mL each): Immunoprecipitation Buffer (100 mM NaCl, 20 mM sodium borate, 15 mM EDTA, 15 mM EGTA, 0.02%NaN3 pH 8.5) supplemented with 0.5% BSA, 0.5% Tx100, 0.1% SDS, 10 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride). After removal of the fourth wash, beads were resuspended in 1 mL wash buffer 2 (Immunoprecipitation Buffer supplemented with 0.05% Tx100, 0.1% SDS, 10 mM iodoacetamide, and 2 mM PMSF and transferred to a new microfuge tube. After complete removal of wash 2 with a 27gauge needle attached to an aspirator, samples were solubilized in sample loading buffer containing 2% 2-

mercaptoethanol and incubated at room temperature for 30 minutes (Cx32) or 100°C for 5 minutes (all other proteins). Samples were resolved by SDS-PAGE on 12.5% gels. Radioactive samples were fixed with destain (10% glacial acetic acid, 20% methanol) for 30 minutes, dried and exposed to a phosphoimaging screen. Western blotted samples were transferred to PVDF membranes (Imobilon) for 40–55 minutes. Membranes were blocked 1 hour at room temperature with Odyssey Blocking Buffer and then incubated at 4°C overnight with primary antibodies in Odyssey blocking buffer diluted 1:1 with PBS and 0.1% (final concentration) Tween-20. Membranes were then washed 4x with PBS/ 0.2% Tween -20 at room temperature, followed by incubation with secondary antibodies for 1 hour at room temperature in Odyssey buffer diluted 1:1 with PBS/0.1% Tween-20 and 0.01% SDS. Membranes were then washed 4x for 5 minutes with PBS/0.1% Tween-20 and 1x with PBS to remove residual Tween-20. Membranes were then scanned using the Odyssey Infrared Imaging System. CFTR-expressing cells were lysed for 30 min at 4°C in RIPA buffer consisting of 0.1 M NaCl, 0.1 M Tris, 1 mM EDTA, 6 mM $MgCl_2$, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 8.0. The solubilized cells were scraped from the dish, subjected to a 10 min 15,000 x g centrifugation, and the supernatant incubated

overnight at 4°C with anti-CFTR antiserum directed against amino acids 45-65, a gift from Dr. W. Skach (Xiong et al., 1999). Immune complexes were bound to protein A Sepharose beads and washed four times in RIPA buffer prior to incubation at 37°C for 5 minutes with electrophoresis buffer. Other antibodies used for immunoprecipitation were all from rabbit and included: anti-Cx32 (Sigma #C3470), anti-Cx43 (AP7298; Musil et al., 1990), anti-light chain (Cappel # 50236-36252), and anti-myo D (Santa Cruz # sc-760). Radiolabeled gels were quantitated on a Biorad Personal FX Imager utilizing Quantity One software. Blots were probed with the following primary antibodies: anti-GFP (#632381 from Clontech), anti-Cx32 (M12.13; kind gift from Drs A. Harris and D. Goodenough), anti-ubiquitin (# sc-8017 from Santa Cruz), or anti-HA (Covance #MMS-101R). Immunoreactive protein bands were detected using IRDye800-conjugated secondary antibodies (Rockland Immunochemicals) or Alexa Fluor^{680} (Molecular Probes) and directly quantified using the LI-COR Biosciences Odyssey infrared imaging system (Lincoln, NE) and associated software. Unless indicated otherwise, all experiments were repeated a minimum of three times and representative experiments are shown.

Microscopy: CHO cells grown on glass coverslips were fixed in 2% paraformaldehyde in PBS f or 30 min at room temperature, rinsed for 30 min with PBS, and then incubated for 30 min in PBS supplemented 0.5% normal goat serum, 0.1% bovine serum albumin, 0.2% Triton X-100, and 0.02% sodium azide. Cells were sequentially incubated with anti-Cx32 or anti-light chain antibodies and with Alexa-594 goat anti-mouse (Cx32) or ant-rabbit (T15L) secondary antibodies. Anti-sec61β antibodies (a kind gift from Dr. V. Lingappa), and anti-calnexin (BD Transduction Laboratories #45520). Immunofluorescence images were captured using a Leica DM LD photomicrography system and Scion Image 1.60 software.

RESULTS

Effect of H/O stress on ERAD and intracellular transport of Cx32 mutants

To investigate the effect of H/O stress on disease-associated mutant forms of connexins, we transiently expressed various species of Cx32 in otherwise Cx32-null CHO cells. Cells were metabolically labeled for 20 min with [^{35}S] methionine and then chased under various conditions prior to cell lysis and immunoprecipitation of Cx32 under denaturing conditions. Initial experiments were conducted with wild-type

Cx32 (WT Cx32) in the continuous presence of the intracellular transport inhibitor brefeldin A (BFA) to block exit of the newly synthesized connexin from the ER. Most of the pulse-labeled [^{35}S] met-WT Cx32 was lost within 6 h in a process slowed by the addition of proteasome inhibitors such as MG132, ALLN, or epoxomicin to the chase medium (Fig 1A), as expected for an ERAD substrate (VanSlyke *et al.*, 2000). Turnover of WT Cx32 was reduced to a comparable extent when the cells were subjected to H/O stress (either a 30 min incubation at 42°C immediately after the pulse prior to return to 37°C, or a 6 h chase in the continuous presence of 80 μM sodium arsenite) (Fig 1A). These results are similar to those obtained for the wild-type Cx43 endogenously expressed in CHO cells (VanSlyke and Musil, 2002). E208K Cx32 is a point mutant of Cx32 that causes the peripheral neuropathy X-linked Charcot-Marie-Tooth disease (CMTX) (Fairweather *et al.*, 1994). Under control conditions, E208K Cx32 is completely confined to the ER and rapidly degraded by ERAD even in the absence of an intracellular transport blocker (VanSlyke *et al.*, 2000). Both oxidative and hyperthermic stress, but not the ER stressor DTT, increased the amount of pulse-labeled [^{35}S] met-E208K Cx32 recovered after a 4 h chase to an

extent comparable to that obtained with the proteasome inhibitor MG132 (Fig 1A, B).

Scherer and colleagues have described a subset of Cx32 mutants (hereafter referred to as class 3 mutants) that when stably expressed in tissue culture cells or transgenic mice produce high levels of mutant-encoding mRNA, but no protein product as assessed by Western blot (Deschenes *et al.*, 1997; Abel *et al.*, 1999). We hypothesized that class 3 mutants are translated but are then very rapidly degraded due to their severely abnormal structure, rendering them undetectable when expressed at physiologically relevant levels. This was confirmed after transient overexpression of two such mutants from a CMV promoter-based plasmid in CHO cells. Proteasome inhibitors, as well as H/O stress (but not DTT or the lysosomal inhibitor chloroquine; not shown) significantly slowed the degradation of both the 175fs and 142fs Cx32 mutants (Fig 1A, B). Although H/O stress did not completely block the degradation of any Cx32 species, it stabilized both frameshift mutants to an extent sufficient to markedly increased the number of transiently transfected cells that accumulated 142fs and 175fs Cx32 to levels above the threshold for detection by anti-Cx32 immunofluorescence microscopy (Fig 1C). Control experiments verified that H/O stress did not enhance

the synthesis of any of the constructs under the conditions employed; instead, hyperthermia caused a transient decrease in total protein translation, as expected (Brostrom and Brostrom, 1998).

Under control conditions, transiently expressed E208K Cx32 largely colocalized with a specific marker for the ER (Sec61β) (Fig 2A), in keeping with its distribution in stable transfectants (VanSlyke *et al.*, 2000). A similar result was obtained for the small fraction of cells that synthesized detectable levels of the class 3 mutants 142fs and 175fs Cx32 under basal conditions (Fig 2A). Incubation at 20°C did not alter the staining pattern of the mutants, indicating that their localization in the ER was due to retention instead of retrieval from post-ER compartments (not shown) (VanSlyke *et al.*, 2000). Control experiments demonstrated the expected redistribution of a fraction of the intracellular pool of WT Cx32 upon incubation of cells with chloroquine or brefeldin A, indicating the presence of wild-type connexin in the endolysosomal system or Golgi/TGN, respectively (Musil and Goodenough, 1993; VanSlyke *et al.*, 2000). Neither compound affected the subcellular localization of any of the Cx32 mutants (data not shown). A 30 min, 42°C heat shock followed by a 5.5 h chase in the presence of cycloheximide resulted in the accumulation of presynthesized WT Cx32 in

gap junctional plaques and other post-ER compartments (Fig 2B), as was previously observed for endogenous Cx43 (VanSlyke and Musil, 2002). In contrast, E208K Cx32 spared from ERAD by the same treatment (Fig 2B), or with either arsenite or MG132 (not shown), never formed gap junctions and instead remained colocalized with Sec61β. H/O stress also did not detectably change the localization of the class 3 frameshift mutants (Fig 2B). Note that H/O stress did not cause anti-Cx32 immunoreactivity to accumulate in perinuclear aggresomes, consistent with the concept that these treatments inhibit ERAD of Cx32 prior to its dislocation from the ER.

Effect of H/O stress on non-connexin ERAD substrates

We extended our studies to a wild-type and ER-retained mutant form of another polytopic plasma membrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR). In CHOs and most other cell types, ~75% of wild-type CFTR is degraded by ERAD, as is nearly 99% of the Δ508 CFTR mutant (Younger *et al.*, 2004). The immaturely glycosylated, ER-localized forms of both wild-type and mutant CFTR migrate at ~140 kD on SDS-PAGE and are referred to as the "B form." Subjecting [^{35}S] met-labeled cells to heat stress immediately after the

pulse did not appreciably change the amount of wild-type or mutant [^{35}S] met-CFTR recovered after a 6 h chase (not shown). Proteasome inhibitors are also ineffective in slowing CFTR turnover when present only during the chase period (Gelman *et al.*, 2002), whereas significant stabilization of CFTR is obtained if cells are preincubated with the inhibitor. This behavior is in keeping with reports that CFTR is ubiquitinated and targeted for ERAD cotranslationally (Sato *et al.*, 1998). We therefore incubated cells for 30 min at 42°C, followed by 4 h at 37°C, before subjecting them to pulse-labeling and chase under unstressed conditions. Control experiments confirmed that this "preshock" stabilized endogenous Cx43 to the same extent as when hyperthermia was administered immediately after the pulse (VanSlyke and Musil, 2002) (Fig 3C). This treatment also reduced the turnover of [^{35}S] met-WT CFTR by 2.4-fold +/- 0.22 (n=4), which was recovered in the maturely glycosylated, plasma membrane Mr ~170 kD "C" form (Fig 3A). Δ508 CFTR was also spared from degradation by preshock when chased at either 37°C (not shown) or 27°C (by 3.24-fold +/- 0.35; n= 3; Fig 3B). The latter temperature has been reported to promote the proper folding of Δ508 CFTR, albeit in a process too slow and inefficient to result in detectable recovery of pulse-labeled [^{35}S] met-Δ508CFTR in the C form

(see (Younger *et al.*, 2004). Similar results were obtained if the experiment was repeated in the presence of actinomycin, ruling out a role for stress-induced heat shock proteins in CFTR stabilization (not shown). We conclude that H/O stress reduces the degradation of both wild-type and ER-retained mutant forms of Cx32 and CFTR, but that only wild-type proteins are competent to traverse the secretory pathway.

Secretory proteins lacking transmembrane domains are also substrates for ERAD. When stably expressed in SP2/0 myeloma cells, the T15L immunoglobulin light chain (L chain) cannot be transported to the Golgi due to the lack of its obligatory assembly partner and is instead rapidly degraded by the proteasome (O'Hare *et al.*, 1999). Pulse-chase analysis of these transfectants demonstrated that H/O stress was as potent as a proteasome inhibitor in reducing [^{35}S] met-T15L turnover (Fig 4 A, B). Immunoprecipitable [^{35}S] met-L chain did not accumulate in the medium under any of the conditions tested, indicating it remained transport-incompetent (not shown). Although some forms of unassembled light chain have been reported to be able to accumulate in cytoplasmic aggresomes (Dul *et al.*, 2001), anti-L chain immunocytochemistry revealed no evidence for their formation in either stressed or unstressed SP2/O cells. Instead, the staining pattern of T15L

spared from degradation by cytosolic stress remained colocalized with calnexin in a distribution indistinguishable from that of the ER, and very different from that of the Golgi, as identified by (O'Hare *et al.*, 1999) (Fig 4C).

Effect of H/O stress on degradation of cytoplasmically localized, non-ERAD substrates of the proteasome

The transcription factor myoD is a naturally occurring resident of the cytosol and nucleus that is rapidly degraded by the UPS. As in myocytes and transfected COS cells (Thayer *et al.*, 1989; Abu Hatoum *et al.*, 1998; Breitschopf *et al.*, 1998), the half-life of pulse-labeled [^{35}S]met-myoD in transiently transfected CHO cells was less than 1 h. The addition of MG132 to the chase medium greatly increased the recovery of [^{35}S] met-myoD after a 2-4 h chase, as did subjecting the cells to either oxidative or hyperthermic stress (Fig 5).

The results obtained with MyoD demonstrated that stabilization by H/O stress is not confined to ER-localized proteins. All of the aforementioned stress-sensitive substrates, do, however, have in common that they undergo polyubiquitination and are degraded by the 26S proteasome. To distinguish which of these two processes is the most likely target of H/O

stress, we compared the effect of heat shock on two GFP-based constructs, GFPu and GFP-ODC (Fig 6). GFPu is EGFP destabilized by the addition of the 16-amino acid CL1 degron to its carboxyl terminus, which targets it for polyubiquitination and destruction by the 26S proteasome. It has been widely used as a unregulated reporter of the UPS (Bence *et al.*, 2005). In contrast, degradation of mouse ornithine decarboxylase (ODC) by the 26S proteasome does not require its ubiquitination in mammalian, yeast, or in vitro systems. Fusion of the COOH-terminal 37 amino acid degron of mouse ODC confers rapid, ubiquitin-independent turnover to EGFP, presumably because this domain allows the construct to bind to the 26S proteasome without an obligatory requirement for antizyme or any other targeting protein (Li *et al.*, 1998; Hoyt *et al.*, 2003). GFPu and GFP-ODC were transiently expressed in CHO cells and their degradation monitored by Western blotting after a 4-6 h incubation with cycloheximide (CHX) to block further protein synthesis (in our hands, immunoprecipitation by commercially available anti-GFP antibodies was inefficient). As expected, ~70% GFPu and GFP-ODC was degraded over the chase period in a process partially inhibited by proteasome inhibitors such as MG132 (Fig 6A), but not by blockers of lysosomal degradation or autophagy (not shown). Turnover of GFPu was reduced by a 30 min,

42°C heat shock to an extent comparable to that obtained with MG132. In contrast, this treatment had no effect on the degradation of GFP-ODC constructs in 5/5 trials (Fig 6A). To rule out the possibility that this lack of effect was the result of more rapid targeting of GFP-ODC for degradation than inhibition of this process by hyperthermia, cells expressing either GFPu or GFP-ODC were mock (37°C)- or heat (42°C)-shocked for 30 min prior to being returned to 37°C for 3.5 h. Cells were then lysed either immediately or after a 4 h chase with CHX. As expected from results obtained with metabolically labeled ERAD substrates (Fig 3), "preshock" at 42°C slowed the degradation of GFPu to an extent comparable to, or slightly greater than, that seen under standard assay conditions used in the experiment shown in Figure 6A. In contrast, the turnover of GFP-ODC in preshocked cells was very similar to that in mock preshocked cells (Fig 6B). Given that GFPu (H/O stress-sensitive) and GFP-ODC (stress-insensitive) are virtually identical except for the small destabilizing sequences appended to the carboxyl terminus of GFP, it is highly unlikely that differences other than their dependence on ubiquitination are responsible for these results. This finding further rules out the possibility that H/O stress inhibits UPS-mediated degradation by interfering with the proteasome itself (by, for example, "choking" the

proteasome with degradation-resistant, stress-induced unfolded proteins), because in that case the (proteasome-mediated) turnover of GFP-ODC and GFPu would be expected to be equally affected.

Effect of H/O stress and proteasome inhibitors on the levels of polyubiquitinated E208K Cx32

The finding that H/O stress inhibits the turnover of proteins whose recognition for proteasomal degradation must be mediated by factors located in different subcellular compartments (the cytosol/nucleus for MyoD and GFPu; the ER lumen for T15L light chain) suggests that stress cannot be acting by blocking a common recognition chaperone. For virtually all ER- and cytosolically- localized substrates including connexins (McGee *et al.*, 1996), the next step in proteasomal degradation is polyubiquitination. An exception is GFP-ODC, which we have shown is unique among the proteins examined in being insensitive to hyperthermia (Fig 6). We therefore examined whether H/O stress inhibits ubiquitination using E208K Cx32 as a model protein. This mutant was chosen because it is confined to the ER and thus not subject to lysosomal degradation, contains the carboxyl terminal epitope (missing from 175fs and 142fs Cx32) recognized by the antibody most efficient for immunoprecipitation,

does not appear to be cotranslationally ubiquitinated like wild-type or Δ508 CFTR, and (in contrast to T15L light chain, GFPu, or MyoD) can be reproducibly detected in a polyubiquitinated form under basal conditions in CHO cells. Cells transiently transfected with E208K Cx32 and HA-epitope tagged ubiquitin plasmids were subjected to various treatments prior to preparation of total membrane and cytosolic fractions. The SDS-solubilized fractions were immunoprecipitated with rabbit anti-Cx32 antibodies, and then analyzed by Western blotting for unmodified E208K Cx32 and for polyubiquitinated forms of the protein by probing with either a monoclonal anti-Cx32 antibody or anti-ubiquitin antibodies, respectively (blot-back with anti-ubiquitin instead of anti-Cx32 antibodies prevented any high molecular weight but nonubiquitinated Cx32 aggregates from being misidentified as Cx32-ubiquitin conjugates). Under basal conditions, both non-ubiquitinated and polyubiquitinated E208K Cx32 species were recovered from the membrane fraction (Fig 7A, lane 1M bottom and top, respectively). Plasmid-minus and no antibody controls verified that all signals were specific; anti-HA antibodies yielded results comparable to those obtained with the anti-ubiquitin antibody. Thus Cx32, like other membrane ERAD substrates, becomes ubiquitinated prior to its dislocation into the cytosol. Inhibitors of

proteasome function such as MG132 block protein degradation, but not the activity of protein ubiquitinating and deubiquitinating enzymes. They also slow, but do not block, dislocation of connexins and many other proteins from the ER by an unknown mechanism (Yu *et al.*, 1997; Story *et al.*, 1999; Mancini *et al.*, 2000). As would be predicted from these properties, a 4 h exposure to MG132 increased the level of both ubiquitinated (by 1.96-fold +/- 0.35) and non-ubiquitinated (by 1.3-fold +/- 0.17) E208K Cx32 in the membrane fraction relative to untreated controls (lane 2M). If cells were exposed to H/O stress (in Fig 7A, 80 μM sodium arsenite for 4 h) instead of MG132, the amount of unmodified E208K Cx32 in the membrane fraction was increased (1.5-fold +/- 0.1) to a level comparable to that obtained with the proteasome inhibitor, in keeping with our pulse-chase results (Fig 1). Remarkably, however, the level of ubiquitin-modified E208K Cx32 recovered in the membrane fraction after H/O stress was less than that observed in MG132-treated cells in 5/5 experiments (compare lane 3M to 2M). Given that proteasome inhibitors do not accelerate ubiquitination, the most likely explanation for these results is that H/O stress reduces polyubiquitination of connexins, acting at a step upstream of the site of action of MG132. If so, then it would be expected that treating cells with arsenite plus MG132

would reduce the amount of polyubiquitinated E208K Cx32 recovered in the membrane fraction relative to that obtained after exposure to MG132 alone. This is what was observed (lane 4M). Similar results were obtained when hyperthermia was used as the H/O stress (30 min at 42°C, followed by a 1.5 h chase in the presence of cycloheximide to prevent artifacts due to differences in protein synthesis between the heat-shocked and mock-shocked cells). An H/O stress-induced reduction in the intensity of the anti-ubiquitin signal of immunoprecipitated E208K Cx32 on Western blots could be the result of a decrease in either the number of E208K Cx32 molecules bearing polyubiquitination chains, or in the number and/or length of polyubiquitination chains per molecule. Because ubiquitination is a readily reversible modification and is required for the association of UPS substrates with the 26S proteasome, either type of alteration would be expected to reduce the probability that a substrate becomes productively engaged with the 26S proteasome (Elsasser and Finley, 2005), thereby slowing its rate of degradation. Figures 7 B and C summarize the effects of arsenite, heat shock, and/or proteasome inhibitors on recovery of polyubiquitinated E208K Cx32 in membrane fractions; all data were normalized to unmodified E208K Cx32 recovered from the same sample. It is appreciated that recognition of

polyubiquitinated proteins by Western blot may be inefficient. This caveat would apply to samples prepared from cells exposed to either MG132 or stress and would, if anything, cause us to underestimate the extent to which E208K Cx32 is polyubiquitinated in the presence of MG132 alone. Note that the ratio of ubiquitinated to unmodified E208K Cx32 in the membranes of stressed cells is similar to that from untreated control cells (Fig 7 B, C). This would be expected if E208K Cx32 molecules that attain a higher level of ubiquitination are dislocated from the membrane and degraded. Fewer connexin molecules reach this threshold level of ubiquitination in stressed cells than in controls, accounting for the increased levels of (unmodified) connexin in the membrane under stress conditions.

Analysis of the cytosolic fractions from these experiments supports the contention that H/O stress and proteasome inhibitors have distinct effects on ERAD (Fig 7A). Under control (no stress or MG132) conditions, neither polyubiquitinated nor unmodified forms of E208K Cx32 were detectable in the cytosol, as expected given the rapid rate at which most dislocated proteins are degraded (Fig 7A lane 1C). In MG132-treated cells, E208K Cx32 was recovered in the cytosol, in keeping with our previous data demonstrating gradual accumulation of dislocated

connexin in the cytosol in the presence of proteasome inhibitors (VanSlyke and Musil, 2002) (lane 2C). Unlike E208K Cx32 in membranes (lane 2M), this material is visible by Western blot only as a polyubiquitinated species, although both modified and unmodified forms are detected by the more sensitive radiolabeling/immunoprecipitation technique (see Fig 8). In cells subjected to arsenite plus MG132, ~57% (+/- 5.2; n=4) less (polyubiquitinated) connexin was recovered in the cytosol than from cells treated with MG132 alone. The finding that virtually no connexin was detectable in the cytosol of cells treated with arsenite alone (Fig 7A lane 3C) is consistent with the concept that H/O stress, unlike proteasome inhibitors, does not block degradation within the proteasome core. Comparable results were obtained when hyperthermia was used as the H/O stressor (not shown). Taken together, these findings support a model in which H/O stress inhibits dislocation of E208K Cx32 at least in part by reducing its polyubiquitination. This is distinct from proteasome inhibitors, which slow dislocation without decreasing ubiquitination (Fig 7D).

We next examined the effect of H/O stress on E208K Cx32 no longer associated with membranes (Fig 8). CHO cells doubly transfected with E208K Cx32 and HA-ubiquitin plasmids were metabolically labeled for

4 h in the presence of MG132, conditions under which ER-dislocated Cx32 accumulates in the cytosol (VanSlyke and Musil, 2002). A cytosolic fraction was prepared and analyzed by anti-Cx32 immunoprecipitation followed by SDS/PAGE and phosphorImaging. Both high molecular weight [^{35}S] met-E208K Cx32 (verified as being polyubiquitinated by anti-ubiquitin or anti-HA Western blot) and unmodified E208K Cx32 were recovered from the cytosol (Fig 8A; lane 2). Because polyubiquitination is an obligatory prerequisite for dislocation, the presence of both unmodified and modified E208K Cx32 is likely a consequence of repeated cycles of de- and re- ubiquitination under conditions (i.e., plus MG132) in which the protein cannot be degraded (Shamu *et al.*, 1999; Flierman *et al.*, 2003). Cells pulsed in the presence of MG132 were then chased for 4 h in medium containing cycloheximide under various conditions prior to analysis of E208K Cx32 in the cytosolic fraction. If the chase was conducted in the absence of MG132, both ubiquitinated and nonubiquitinated forms of E208K Cx32 were rapidly lost from the cytosol, as would be predicted from the reversible nature of the inhibitor and because the dislocated connexin is likely to be severely misfolded and therefore a high-affinity target for re-ubiquitination and degradation (lane 3). In cells chased in the continuous presence of MG132 (lane 4), the

ratio of ubiquitinated to unmodified [^{35}S] met-E208K Cx32 recovered in the cytosol was similar to that immunoprecipitated from cells not subjected to a chase (lane 2), suggesting that the activity of ubiquitinating and deubiquitinating enzymes had reached a steady-state during the first 4 hours. If cells were chased with MG132 and also subjected to H/O stress (30 min at 42°C), 56% (+/- 5.3; n = 3) less polyubiquitinated [^{35}S] met-E208K Cx32 was recovered than from cells chased with MG132 but no stress (compare lane 5 to lane 4). Quantitation of the corresponding anti-ubiquitin Western blots revealed a 65% +/- 19% (n=3) reduction in the amount of polyubiquitinated connexin from heat-shocked cells; in 2/2 experiments, an even greater reduction was obtained when arsenite was used as the H/O stress (76% and 81%; not shown). Because the amount of unmodified, ~30 kD [^{35}S] met-E208K Cx32 immunoprecipitated from the cytosol of cells subjected to H/O stress plus MG132 is approximately the same as that from cells treated with MG132 alone (compare lanes 4 and 5), this indicates that H/O stress reduced the fraction of cytosolic connexin in a polyubiquitinated form. Polyubiquitinated [^{35}S] met-E208K Cx32 is metabolically labeled on its ubiquitin as well as its connexin moieties. Deubiquitination of E208K Cx32 results in the loss of the ubiquitin-

derived [^{35}S] methionine signal, likely explaining why the amount of radioactivity in the total anti-Cx32 immunoprecipitant in lane 5 is less than that in lane 4. The simplest explanation is that H/O stress inhibits the reubiquitination of dislocated, MG132-stabilized E208K Cx32, analogous to how treating cells with H/O stress plus MG132 reduces the accumulation of ubiquitinated E208K Cx32 in the membrane fraction relative to cell treated with MG132 alone (Fig 7).

Effect of H/O stress on the level of total cellular ubiquitinated proteins

Finally, we addressed whether the reduction in recovery of E208K Cx32 in a polyubiquitinated form in H/O stressed cells compared to proteasome inhibitor-treated cells reflected a general shut-down of the cellular ubiquitination machinery under stress conditions (Fig 9). Anti-ubiquitin Western blots of total cellular protein lysates revealed a ~2-fold increase in polyubiquitinated conjugates in cells incubated for 4 h with MG132, as expected (Fig 9). An even greater, or slightly smaller, stimulation in total anti-ubiquitin immunoreactivity was observed in cells exposed to either oxidative or hyperthermic stress, respectively. Increased anti-ubiquitin immunoreactivity in lysates from heat-shocked or arsenite-treated cells relative to unstressed cells has also been observed

by others (e.g., (Bond *et al.*, 1988), and is likely due to un/misfolding of a subset of especially thermally or oxidatively sensitive cytosolic and nuclear proteins (Lepock et al., 1993; Senisterra et al., 1997). The increased levels of such high-affinity substrates for polyubiquitination machinery could explain, at least in part, why H/0 stress inhibits the turnover of E208K Cx32 and of the other stress-sensitive proteins examined (see Discussion).

DISCUSSION

We have discovered a previously unrecognized aspect of the heat shock response, namely a reduction in the rate at which multiple proteins are degraded by the UPS. The stresses imposed did not cause cell death or compromise ER quality control, indicating they did not induce general cell dysfunction. They also do not appear to stabilize mutant or unassembled proteins by promoting their folding into a mature, less proteolytically labile state, because in that case T15L light chain and the Cx32 and CFTR mutants would be expected to become competent to traverse the secretory pathway. This was not observed.

Because UPS substrates located in two topologically separate compartments (the ER lumen and cytosol) are both stabilized by H/O stress, H/O stress cannot inhibit degradation by affecting the binding of a common recognition protein. We can also exclude proteolysis in the 20S core as the critical target for H/O stress given that exposure of cells to the levels of H/O stress used in our studies does not appreciably reduce the hydrolysis of proteasome substrate peptides (Pratt *et al.*, 1989; Stanhill *et al.*, 2006). Insomuch as it stabilizes residents of the cytosol,

H/O stress cannot be acting solely by inhibiting dislocation. For almost all substrates, the step in ERAD after recognition and before complete dislocation is polyubiquitination. We found that although degradation of membrane-associated E208K Cx32 was inhibited by exposure of cells to either H/O stress or MG132, the amount of polyubiquitinated E208K Cx32 recovered in both the membrane or cytosolic fractions was markedly greater after treatment with MG132 alone than after H/O stress either by itself or in combination with MG132. Given that there is no evidence that proteasome inhibitors such as MG132 increase the activity of the ubiquitination machinery, the simplest interpretation is that H/O stress perturbs the function of one or more components of this system. Among all of the proteasome substrates examined, the only one that was not stabilized by hyperthermia was the sole protein whose degradation is independent of ubiquitination (GFP-ODC). This finding further supports our contention that H/O stress affects polyubiquitination and not post-ubiquitination processes.

H/O stress rapidly raises the amount of thermally or oxidatively denatured proteins in the cytosol. Such misfolded proteins are highly susceptible to ubiquitination and are responsible for the increased levels

of total polyubiquitinated proteins detected under these conditions (Fig 9) (Parag *et al.*, 1987; Bond *et al.*, 1988). Stress-denatured proteins could titer a limiting component of the ubiquitination machinery away from pre-existing UPS substrates to an extent that depends on the substrate's abundance, how prone it is to deubiquitination, and (especially) its inherent affinity for the ubiquitination machinery. The ability of H/O stress to reduce connexin ERAD is transient, most likely reflecting the time required to return the level of unfolded protein in the cytosol to basal levels (VanSlyke and Musil, 2002). The turnover of UPS substrates with half-lives greater than ~ 8 h would therefore not be expected to be markedly affected by H/O stress, which could explain why such conditions do not substantially reduce the rate of degradation of total cellular proteins (Bond *et al.*, 1988; Westwood and Steinhardt, 1989; Luo *et al.*, 2000).

Ubiquitination of cytosolic as well as ERAD substrates of the UPS requires the activity of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating; Ubc), and, for most proteins, E3 (ubiquitin ligase) enzymes (Glickman and Ciechanover, 2002). Exogenous overexpression of ubiquitin does not appreciably affect stabilization by H/O stress,

indicating that H/O stress does not act by reducing the level of ubiquitin available for polyubiquitination (not shown). Given the high level of substrate specificity of most of the > 500 known E3 enzymes, it is very unlikely that degradation of all of the proteins we have found to be stress-sensitive involves the same E3 ligase. Selective inhibition of the ubiquitination and/or turnover of some, but not the majority, of cellular UPS substrates has previously been shown to result from partial inhibition of E1 or E2 activity (Gonen *et al.*, 1999; Salvat *et al.*, 2000). For the latter, this is likely due to the ability of multiple, but not all, E2s to modify a particular UPS substrate; only if the downregulated E2 cannot be fully compensated for by another E2 is the substrate's ubiquitination (and thus its turnover) reduced (Saville *et al.*, 2004; Younger *et al.*, 2004). Similar to what we have observed after H/O stress (Fig 3), inhibiting the activity of the E2 UbcH5a decreases the degradation of both pulse-labeled Δ508 and wild-type forms of CFTR, but detectably increased the maturation of only the latter during a short (< 8 h) chase (Younger *et al.*, 2004). (Salvat *et al.*, 2000) have reported that levels of E1 that are ~10- 15% of wild-type support the degradation of p53 and the ubiquitination of most other proteins, but not that of another well-characterized UPS substrate, c-jun. Evidence that E1 activity may not be in excess under

basal conditions (Pickart, 2004) and becomes limiting for a subset of ubiquitination substrates after only 10 min at 42°C (Kulka *et al.*, 1988) has been discussed. Moreover, certain E2s, and to a lesser extent E1, are transcriptionally upregulated in yeast by both heat shock and oxidative stressors, suggesting that they become limiting under these conditions (Gasch *et al.*, 2000). Further experiments will be required to determine whether E1 and/or E2s become saturated and/or partially inactivated during H/O stress, and whether the function of deubiquitinating enzymes is altered. Kopito and coworkers have established that expression of aggresome-forming proteins inhibits the degradation of multiple ER-associated and cytosolic UPS substrates. Under these conditions, however, substrates accumulate in a polyubiquitinated form, in some cases (e.g., CFTR) after their dislocation (Johnston *et al.*, 1998). The effect of H/O stress is also distinct from that of knockdown of p97, which inhibits UPS-mediated degradation at a post-ubiquitination step (Wojcik *et al.*, 2006), and from that of ataxin-3 overexpression, which affects the degradation of ERAD, but not fast-turnover cytosolic, UPS substrates (Zhong and Pittman, 2006).

Physiologically, the heat shock response is induced by a variety of conditions including hypoxia/reoxygenation, high fever, circulatory and hemorrhagic shock, energy depletion, acidosis, hypothermia, and viral infection. Moreover, hyperthermia and inorganic arsenite compounds (e.g., arsenic trioxide) at levels comparable to those used in this study have been administered in the treatment of several types of cancer (Dewhirst *et al.*, 2006; (Douer and Tallman, 2005). A well-known aspect of the heat shock response is a temporary inhibition of general protein synthesis (Brostrom and Brostrom, 1998). Stress stabilization might serve as a means to help preserve the function of fast-turnover UPS substrates under conditions in which they cannot be replaced. H/O stress also increases the level of ER-retained, misfolded proteins, which might act as dominant-negative inhibitors of wild-type proteins by binding to them or by otherwise interfering with their function. Either scenario would be a novel means by which genetic and environmental factors interact to influence disease progression.

ACKNOWLEDGMENTS

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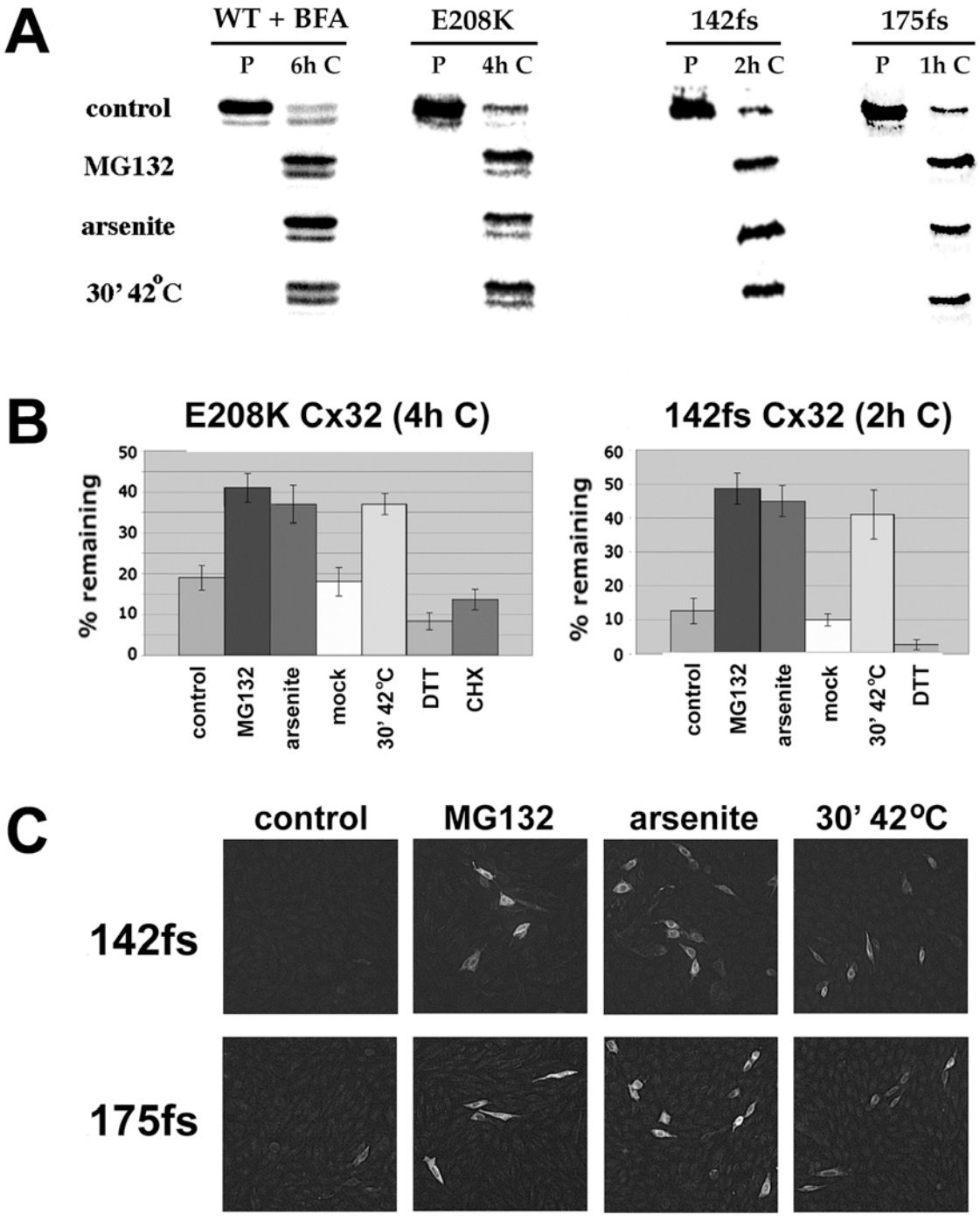
Figure 1. Induction of H/O stress decreases ERAD of wild-type and mutant forms of Cx32. (A) CHO cells transiently transfected with plasmids encoding wild-type (WT), E208K, 142fs, or 175fs Cx32 were metabolically labeled with [^{35}S] methionine at 37°C for 20 min and lysed either immediately (P) or after being chased for the indicated period with no additions (control), 30 μM MG132, 80 μM sodium arsenite, or for 30 min at 42°C immediately after the pulse and returned to 37°C for the remainder of the chase period. For WT Cx32 only, brefeldin A was included in the pulse and chase media. Total cellular Cx32 was immunoprecipitated and analyzed by SDS/PAGE and PhosphorImaging. (B) The amount of [^{35}S] methionine-Cx32 recovered by immunoprecipitation after a 4 h (E208K Cx32) or 2 h (142fs Cx32) chase under the indicated conditions was expressed as a percentage of [^{35}S] methionine-Cx32 immunoprecipitable immediately after the pulse. Mock-shocked cells were treated identically to heat-shocked samples, except that the temperature was maintained at 37°C throughout the experiment. CHX, cycloheximide; DTT, dithiothreitol. For each experimental condition, n=3. (C) CHO cells transiently transfected to express 142fs or 175fs Cx32 were incubated for 4 h at 37°C with no additions (control), MG132, or arsenite as indicated. Heat-shocked cells were incubated at 42°C for 30 min and then returned to 37°C for an additional 3.5 h. All cells were then fixed and immunostained with anti-Cx32 antibodies.

Figure 1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WT + BFA
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142fs
175fs
P
6h C
P
4h C
P
2h C
P
1h C
control
MG132
arsenite
30' 42°C
B
E208K Cx32 (4h C)
142fs Cx32 (2h C)
% remaining
control
MG132
arsenite
mock
30' 42°C
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30' 42°C
142fs
175fs

Figure 2

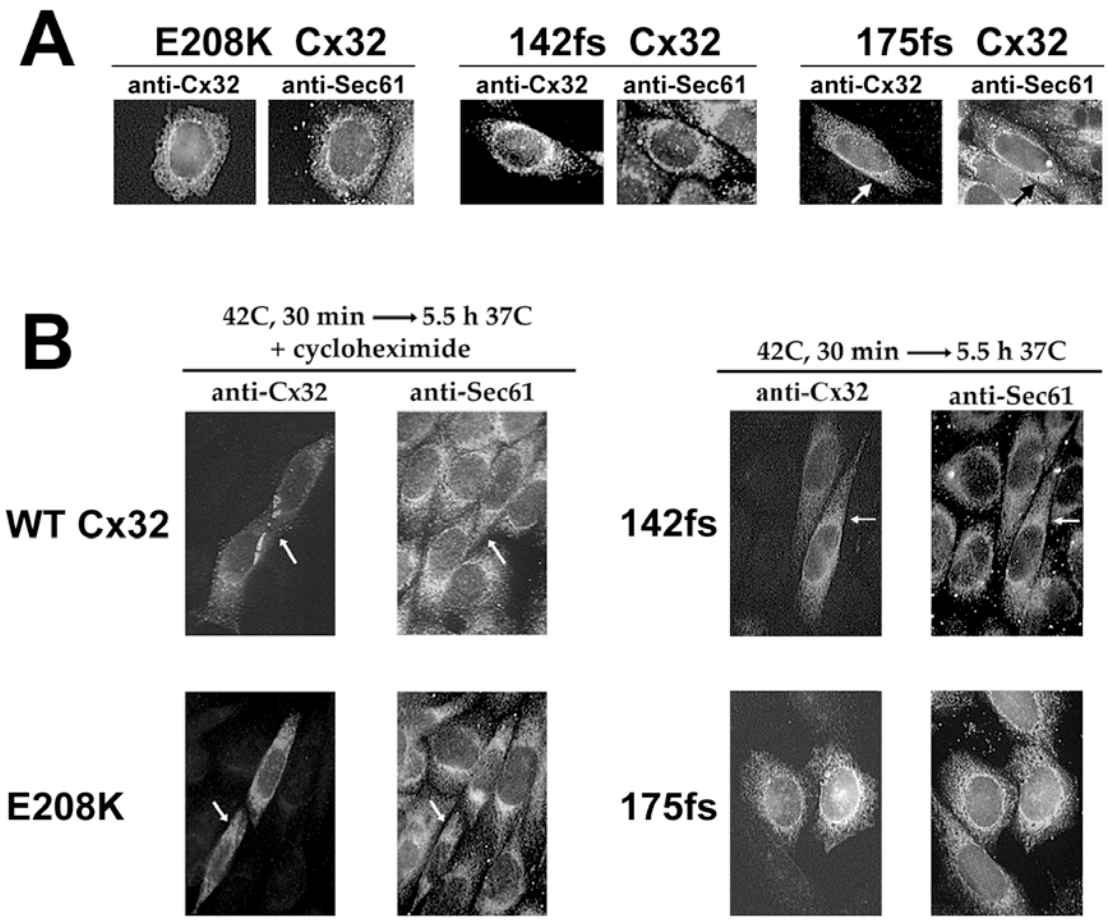


Figure 2. H/O stress does not restore trafficking of mutant Cx32. CHO cells transiently transfected with plasmids encoding the indicated Cx32 species were incubated for 6 h at 37°C with no additions (2A), or at 42°C for 30 min and then returned to 37°C for an additional 5.5 h incubation in either the presence (WT and E208K Cx32) or absence (175fs and 142fs Cx32) of cycloheximide (2B). All cells were then fixed and doubly immunostained for Cx32 and the ER marker Sec61β.

Figure 3

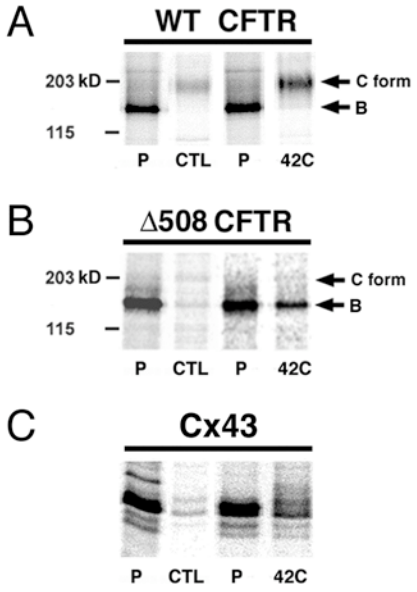
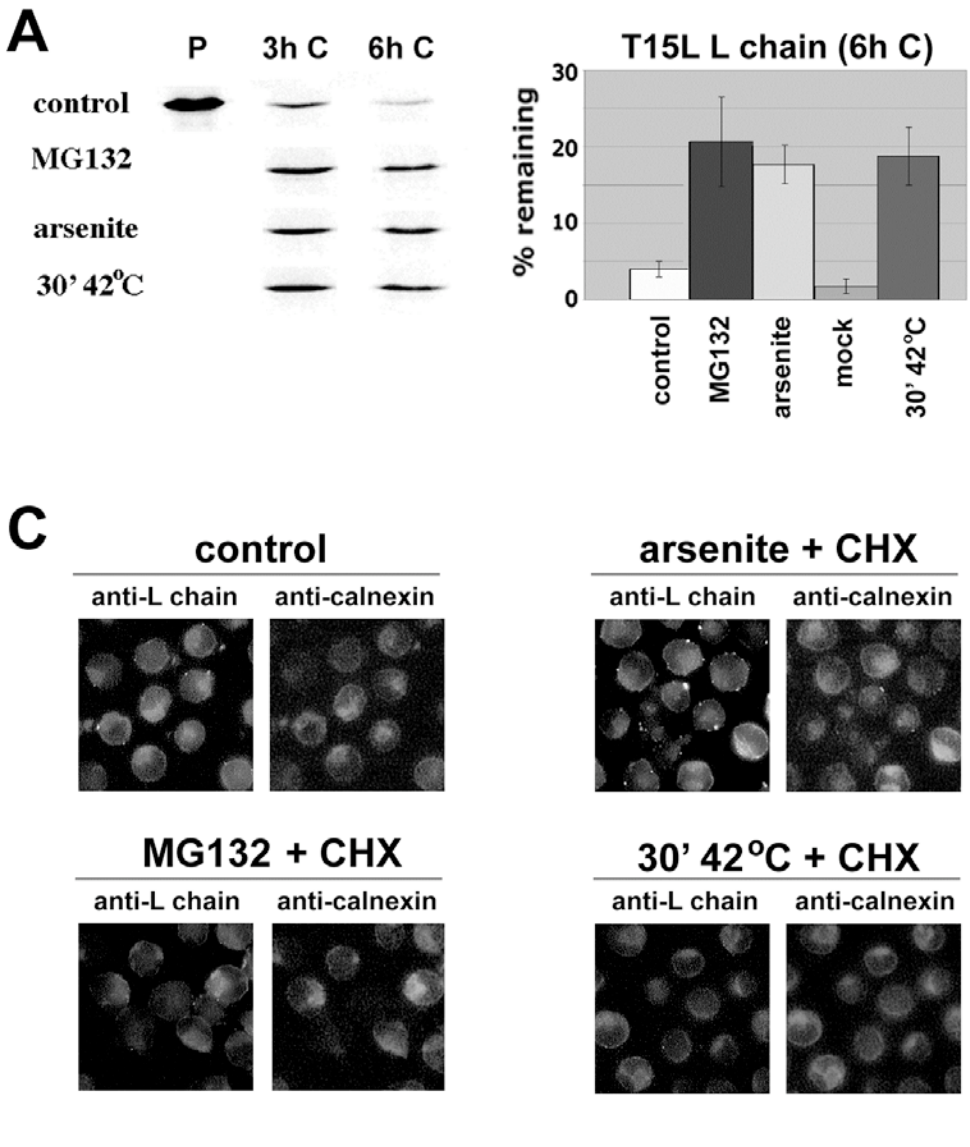


Figure 3. H/O stress decreases the degradation of both wild-type and Δ508 CFTR, but only promotes the maturation of the former. CHO cells stably expressing either wild-type (3A) or Δ508 (3B and C) CFTR were either "preshocked" by a 30 min incubation at 42°C (42C), or mock shocked at 37°C (CTL), and returned to 37°C for 4 h. The cells were then pulsed for 30 min with [^{35}S] methionine and either lysed immediately (P) or chased for 6 h at 37°C (WT) or 27°C (Δ508) prior to immunoprecipitation of CFTR from the RIPA soluble cell lysate (3A and B), or of endogenous Cx43 from the pooled RIPA soluble and insoluble fractions boiled in SDS (3C). The contrast of the image of each pulse-chase pair was adjusted such that the intensity of all of the pulse (P) lanes was approximately equal.

Figure 4. H/O stress promotes the stability, but not the intracellular transport, of a soluble ERAD substrate. (A) SP2/0 myeloma cells stably expressing the unassembled, secretion-incompetent T15L immunoglobulin light chain were metabolically labeled for 20 min and lysed either immediately (P) or after being chased for the indicated period with no additions (control), MG132, sodium arsenite, or for 30 min at 42°C immediately after the pulse and returned to 37°C for the remainder of the chase period. The cells were treated with SDS, and immunoprecipitated with anti-L chain antibodies. (B) The amount of [^{35}S] methionine-L chain recovered by immunoprecipitation from the cells after a 6 h chase under the indicated conditions was expressed as a percentage of [^{35}S] methionine-L chain immunoprecipitable immediately after the pulse. For each experimental condition, *n*=3 or 4. (C) The T15L-expressing cells were incubated for 4 h at 37°C with no additions (control), or for a total of 4 h in the presence of cycloheximide and: MG132, arsenite, or for 30 min at 42°C followed by 3.5 h at 37°C. All cells were then fixed and immunostained with anti-L chain antibodies and antibodies specific for the ER marker calnexin.

Figure 4


A
P
3h C
6h C
control
MG132
arsenite
30' 42°C
T15L L chain (6h C)
% remaining
30
20
10
0
control
MG132
arsenite
mock
30' 42°C
C
control
anti-L chain
anti-calnexin
arsenite + CHX
anti-L chain
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anti-L chain
anti-calnexin

Figure 5

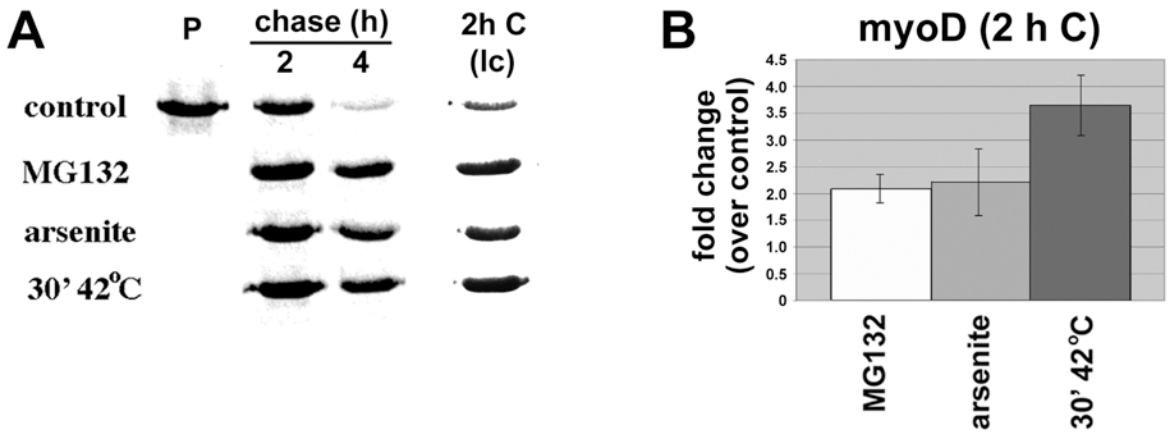
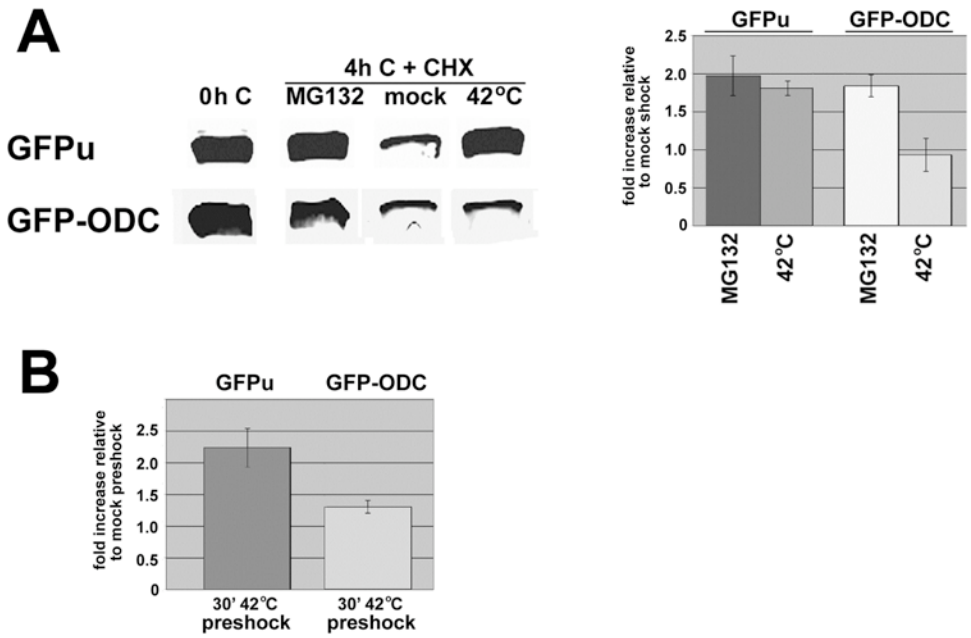


Figure 5. Inhibition of degradation of the cytosolic/nuclear UPS substrate MyoD by H/O stress. CHO cells were transiently transfected with plasmids encoding myoD. (A) Cells were lysed and immunoprecipitated with anti-MyoD antibodies either immediately after a 20 min pulse with [^{35}S]-methionine (P), or after being chased for the indicated period with no additions (control), MG132, sodium arsenite, or for 30 min at 42°C immediately after the pulse and returned to 37°C for the remainder of the chase period. A lower contrast (lc) image of the 2 h chase data is included to indicate the extent to which stress reduced the turnover of MyoD at this timepoint. (B) The amount of [^{35}S] methionine-MyoD recovered by immunoprecipitation from the cells after a 2 h chase under the indicated conditions was expressed as the fold increase relative to that obtained from untreated cells. For each experimental condition, *n*=3.

Figure 6. Heat stress inhibits the degradation of EGFP bearing a ubiquitin-dependent degron, but not that of EGFP with a ubiquitin-independent degron. CHO cells were transiently transfected with plasmids encoding either GFPu or GFP-ODC. (A) Cells were lysed without any treatment (0h C), or incubated in the presence of cycloheximide for: 4 h at 37°C with MG132 (MG132), 30 min at 42°C followed by 3.5 h at 37°C (42°C), or 30 min at 37°C followed by 3.5 h at 37°C (mock). Total cell lysates were analyzed by Western blotting with anti-GFP antibodies. The amount of GFPu or GFP-ODC remaining after a 4 h chase in the presence of cycloheximide under the indicated conditions was graphed as the fold increase relative to that obtained from cycloheximide-treated mock-shocked cells. For each experimental condition, n=5. (B) CHO cells transiently transfected with plasmids encoding either GFPu or GFP-ODC were "preshocked" by a 30 min incubation at 42°C or mock-shocked at 37°C and returned to 37°C for 3.5 h. The cells were then lysed without any treatment (Oh C), or incubated for 4 h with cycloheximide at 37°C and analyzed by Western blotting with anti-GFP antibodies. The amount of GFPu or GFP-ODC remaining after the cycloheximide chase was calculated relative to the corresponding 0h C sample and was graphed relative to the value obtained from cycloheximide-treated mock-shocked cells. For each experimental condition, n=3.

Figure 6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MG132
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GFP-ODC
GFPu
GFP-ODC
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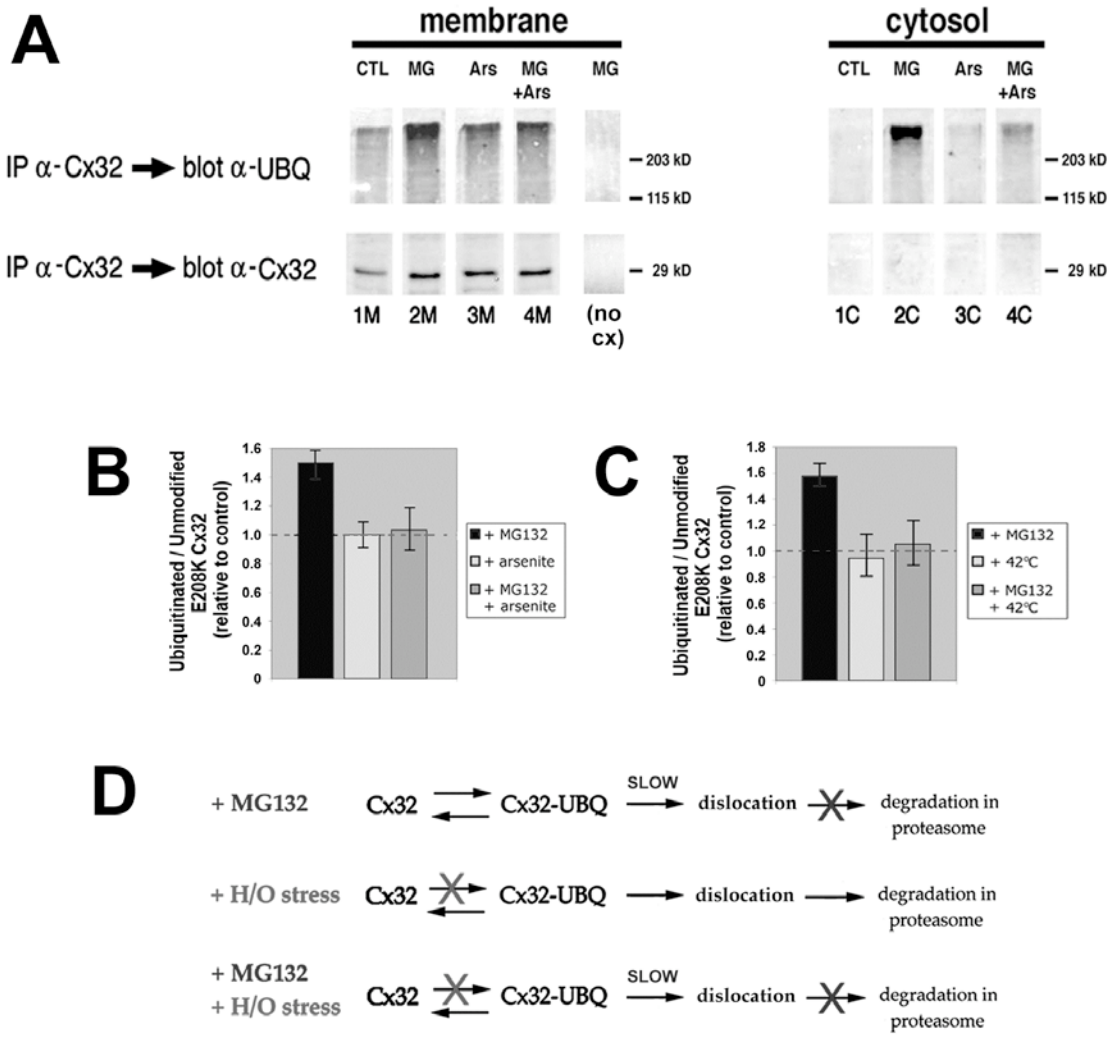
2.0
1.5
1.0
0.5
0
MG132
42°C
MG132
42°C
B
GFPu
GFP-ODC
fold increase relative to mock preshock
2.5
2.0
1.5
1.0
0.5
0
30' 42°C preshock
30' 42°C preshock

Figure 7. Membrane-associated E208K Cx32 spared from ERAD by H/O stress is less highly ubiquitinated than E208K Cx32 saved by proteasome inhibitors. (A) CHO cells were transiently cotransfected with plasmids encoding E208K Cx32 and HA-tagged ubiquitin at a ratio of 1:5. The cells were incubated for 4 h without additions (control; CTL), or with MG132 (MG), arsenite (Ars), or both. All cells were then lysed without detergent and fractionated into cytosolic (C) and membrane (M) fractions prior to solubilization in SDS and immunoprecipitation of Cx32. Immunoprecipitates were subjected to SDS-PAGE on 12% gels and transferred to PVDF membranes. The blots were cut in half, and the lower portion probed for unmodified E208K Cx32 with anti-Cx32 antibodies and the upper portion probed for polyubiquitinated E208K Cx32 with anti-ubiquitin antibodies. No Cx, sample was prepared from cells transfected with ubiquitin-HA only and treated as in lane 2M. (B) For each membrane sample from cells treated with MG132 and/or arsenite, the signal obtained for polyubiquitinated E208K Cx32 was calculated as a percentage of that obtained for polyubiquitinated E208K Cx32 from control cells in the same experiment. A similar calculation was performed for unmodified E208K Cx32 in the membrane. The ratio of the percentage obtained for polyubiquitinated E208K Cx32 to the percentage obtained for unmodified E208K Cx32 was calculated, and graphed relative to the ratio obtained from controls (defined as 100%/100%; 1). Data from three independent experiments. (C) Cells cotransfected with plasmids encoding E208K Cx32 and HA-tagged ubiquitin as in (A) were incubated in the presence of cycloheximide for: 2 h at 37°C with no other additions (control), 2 hr at 37°C with MG132, 30 min at 42°C and returned to 37°C for 1.5 h, or 30 min at 42°C and returned to 37°C for 1.5 h in the continuous presence of MG132. Cells were lysed and analyzed as in (A) and the results graphed as in (B). Data from three independent experiments. (D) Schematic of the effect of MG132 and/or H/O stress on ERAD of E208K Cx32. (Top) Proteasome inhibitors slow, but do not completely abolish, dislocation, and have a stronger inhibitory effect on proteolysis within the 20S proteasome core. Under these conditions, E208K Cx32 accumulates in the membrane in both ubiquitinated and non-ubiquitinated forms (Fig 7A, lane 2M), and is also slowly dislocated into the cytosol after ubiquitination but is not degraded (lane 2C). (Middle) Cytosolic stress reduces ubiquitination of E208K Cx32, thereby inhibiting its dislocation and causing E208K Cx32 to accumulate in the membrane in a less ubiquitinated form than in the presence of proteasome inhibitors (lane 3M). Any E208K Cx32 that does

become ubiquitinated and dislocated is degraded by the proteasome (lane 3C). (Bottom) Ubiquitination of membrane-associated E208K Cx32 is also reduced when cells are subjected to H/O stress in the presence of MG132 (lane 4M). The latter blocks degradation of the small amount of E208K Cx32 that becomes dislocated, allowing it to be recovered from the medium (lane 4C).

Figure 7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cytosol
CTL
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Ars
MG +Ars
MG
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IP α-Cx32 → blot α-Cx32
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115 kD
29 kD
1M
2M
3M
4M
(no cx)
1C
2C
3C
4C
B
Ubiquitinated / Unmodified E208K Cx32 (relative to control)
+ MG132
+ arsenite
+ MG132 + arsenite
C
Ubiquitinated / Unmodified E208K Cx32 (relative to control)
+ MG132
+ 42°C
+ MG132 + 42°C
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SLOW
dislocation
degradation in proteasome
+ H/O stress
+ MG132 + H/O stress

Figure 8

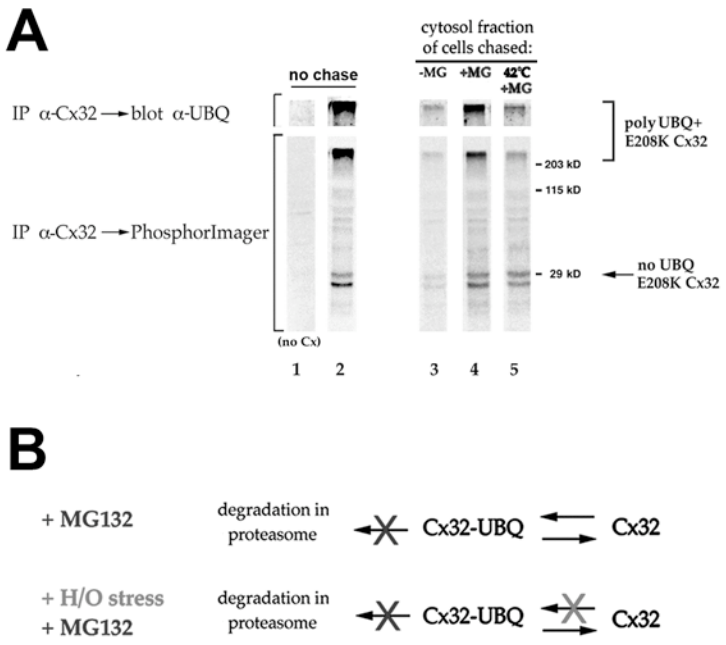

Figure 8. H/O stress promotes the recovery of dislocated, MG132-stabilized E208K Cx32 in a non-ubiquitinated form. (A) CHO cells transiently cotransfected with plasmids encoding E208K Cx32 and HA-tagged ubiquitin were metabolically labeled for 4 h in the presence of MG132. The cells were lysed without detergent and a cytosolic fraction prepared, either immediately after labeling (lane 2) or after chasing in the presence of cycloheximide as follows: for 4 h with no other additions (lane 3), for 4 h with MG132 (lane 4), or for 30 min at 42°C followed by 3.5 h at 37°C in the continuous presence of MG132 (lane 5). Cx32 was immunoprecipitated from the cytosolic fraction, subjected to SDS-PAGE on 12% gels, and transferred to PVDF membranes. The membranes were analyzed for radiolabeled E208K Cx32 by phosphorImaging, as well as probed with anti-ubiquitin antibodies. Only the upper portion of the anti-ubiquitin blot (Mr > 200 kD) showed specific anti-ubiquitin immunoreactivity and is included. Lane 1, sample prepared from cells transfected with ubiquitin-HA only and treated as in lane 2. (B) Schematic of the effect of H/O stress on post-dislocation pools of E208K Cx32. When degradation of dislocated, ubiquitinated E208K Cx32 (Cx32-UBQ) is blocked by MG132, E208K Cx32 accumulates in the cytosol in a less highly ubiquitinated form if the cells are also subjected to H/O stress due to inhibition of connexin reubiquitination by stress.

Figure 9

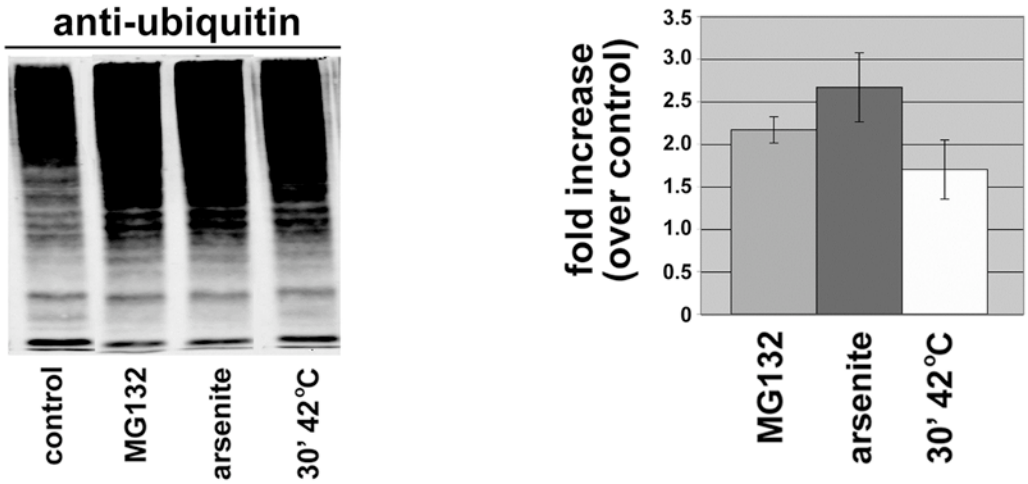

Figure 9. Total cellular ubiquitin conjugates are increased by H/O stress. CHO cells transiently cotransfected with plasmids encoding E208K Cx32 and HA-tagged ubiquitin were incubated for 4 h with no additions (control), MG132, sodium arsenite, or for 30 min at 42°C followed by 3.5 h at 37°C. Equal amounts of total, SDS-solubilized cell lysates were analyzed by Western blotting using anti-ubiquitin antibodies and the results quantitated as the fold increase in anti-ubiquitin immunoreactivity under the indicated conditions relative to untreated controls. For each experimental condition, *n*=3.

Chapter 3

Summary, Conclusions, and Future Studies

The aim of these studies described within this thesis was to gain a better understanding of the inhibitory effects of non-lethal heat and oxidative stress on the degradation of connexins from the ER. In doing so, these studies examined the effects of H/O stress on the degradation of several mutant forms of Cx32 which have drastic differences in their half-lives, and which are retained within the ER under basal (non-stressed) conditions. As was found for wild-type connexins, ERAD of the relatively stable point mutant Cx32 E208K as well as two highly unstable frameshift mutants, Cx32 142fs and 175fs, is inhibited by these stress conditions. This work also compared the stress sensitivities of another polytopic membrane protein, CFTR, to an ER-retained mutant form of the protein, CFTR ΔF508, establishing that the ERAD of both forms of the protein is inhibited by H/O stress. Furthermore, these studies also established that, unlike the case for wild-type connexins and wild-type CFTR, inhibiting the ERAD of mutant forms of these proteins by H/O stress does not allow for increased transport to post-ER compartments, but do cause the mutant proteins to accumulate within the ER. Thus H/O stress does not

overcome quality control retention mechanisms within the ER, at least at the substrate expression levels used in these studies.

In an effort to determine how H/O stress conditions might be negatively regulating the degradation of these proteins, we also examined whether the degradation of other categories of UPS substrates, including the lumenal ERAD substrate, unassembled IgG light chain, were affected by these treatments. Degradation of this soluble ERAD substrate, as well as that of the cytosolic substrate, GFPu, and the nuclear substrate MyoD, was also slowed under conditions of H/O stress. These findings indicated that it is unlikely that H/O stresses are affecting some ancillary protein (e.g. a protein chaperone) commonly involved in linking the recognition of all of these UPS substrates to ubiquitin modification and degradation. The finding that the degradation of UPS substrates that don't require dislocation from or through the ER membrane (GFPu and MyoD) is inhibited by H/O stress indicated that, although these stress conditions result in the inhibition of dislocation of ERAD substrates, it is unlikely that the dislocation machinery is exclusively affected. Because the dislocation of most ERAD substrates examined is contingent upon polyubiquitination of the substrate, these studies led to an examination of whether H/O stress conditions affected the ubiquitination of Cx32 E208K. It was

demonstrated that both hyperthermic and oxidative stress conditions reduce the relative proportion of ubiquitin-modified Cx32 E208K to unmodified Cx32 E208K, as compared to those found in the presence of proteasome inhibitors. This was observed whether Cx32 E208K was membrane associated or after artificial accumulation within the cytosol. In support of the contention that H/O stress negatively regulates ubiquitination, the only proteasomal substrate that was insensitive to hyperthermic stress was the ubiquitin-independent substrate GFP-ODC. Future studies will be required to determine whether ubiquitinated forms of other UPS substrates are decreased by H/O stress. Finally, these studies demonstrated that the mild H/O stress conditions used did not have a global deleterious affect on the ubiquitination machinery, as total cellular ubiquitin conjugates were increased.

One of the most significant effects of these stress conditions on cells is the rapid generation of unfolded/misfolded proteins that are cleared from the cell by the UPS. It is possible that under H/O stress conditions, specific ubiquitination machinery becomes limiting for a subset of proteins as damaged proteins are increased and targeted for ubiquitination and degradation. Evidence that E2 ubiquitin conjugating enzymes can be limiting include the observations that endogenous levels

of the specific E2 Ubc4, but not another E2 were shown to actually be limiting under non-stressed basal condition in rat tissue extracts (Rajapurohitam *et al.*, 2002) as well as in lens cells (Pereira *et al.*, 2003). Overexpression of certain E2 enzymes alone, including those in the Ubc4/5 family, or in combination with E3 enzymes has been shown to enhance stress tolerance in budding yeast (Hiraishi *et al.*, 2006). Finally, both hyperthermic and oxidative stress lead to the transcriptional upregulation of E2 ubiquitin conjugating enzymes and E3 ligases in both budding yeast and mammalian cell lines (Sonna *et al.*, 2002; Li *et al.*, 2003; Dikshit and Jana, 2007) suggesting that these enzymes can become limiting under stress conditions. By a number of accounts, the Ubc4/Ubc5 family seems to be responsible for the ubiquitination of misfolded/damaged proteins (Seufert and Jentsch, 1990; Chuang and Madura, 2005). Similar experiments in which over-expression of specific ubiquitin conjugating enzymes are tested in their ability to negate the stress effect observed on the UPS substrates used in our studies could be utilized to test a titration model. Conversely, mimicking the effect of H/O stress on the degradation of these substrates by over-expression of dominant negative forms of ubiquitin-conjugating enzymes would also

support a model in which stress affects the ubiquitination of these substrates by limiting the availability of these enzymes.

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