HERP REDUCES ER CALCIUM CONTENT BY PROTEASOMAL DEGRADATION OF SERCA

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DEDICATION

I would like to thank the members of my Graduate Committee.

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by

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Herp, an endoplasmic reticulum (ER) stress inducible protein, reduces ER Ca^{2+} content in neurons and prevents their apoptosis. An understanding of the mechanism by which Herp decreases ER Ca^{2+} content requires studies of Herp interacting proteins, which could be SERCA and the proteasome. Herp may recruit the proteasome from the cytosol to the ER membrane, thereby facilitating the ER associated degradation (ERAD) of SERCA. The proteasome recruitment and the subsequent degradation of SERCA reduce ER lumenal Ca^{2+} concentration and the Ca^{2+} release during ER stress which counteracts the activation of apoptosis. This proposal describes how to determine the mechanism through which Herp reduces ER Ca^{2+} content, how to test the proteasomal degradation of

SERCA, how to illustrate the proteasome recruitment to the ER membrane, and how to demonstrate the interaction between Herp and SERCA. The work will provide a new regulatory link between ER stress and Ca^{2+} homeostasis. In addition, studies of the proteasomal degradation of SERCA will broaden our present understanding of the regulation of SERCA. Since dysregulation of Ca^{2+} homeostasis has been implicated in the pathophysiology of several neurodegenerative diseases like Alzheimer's and Huntington's, research focused on Herp may lead to insights regarding therapies for those.

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PRIOR PUBLICATIONS

Y.S. Mao, H.L. Yin. Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases. *Pflügers Arch.* 2007, in press.

Qiu X, Zhu X, Zhang L, **Mao Y**, Zhang J, Hao P, Li G, Lv P, Li Z, Sun X, Wu L, Zheng J, Deng Y, Hou C, Tang P, Zhang S, Zhang Y. Human epithelial cancers secrete immunoglobulin g with unidentified specificity to promote growth and survival of tumor cells. *Cancer Res.* 2003, 63(19):6488-95.

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SPECIFIC AIMS

Herp, an endoplasmic reticulum (ER) stress inducible protein, reduces ER Ca2+ content in neurons and prevents their apoptosis. An understanding of the mechanism by which Herp decreases ER Ca2+ content requires studies of Herp interacting proteins, which could be SERCA and the proteasome. Herp may recruit the proteasome from the cytosol to the ER membrane, thereby facilitating the ER associated degradation (ERAD) of SERCA. The proteasome recruitment and the subsequent degradation of SERCA reduce ER lumenal Ca2+ concentration and the Ca2+ release during ER stress which counteracts the activation of apoptosis. This proposal describes how to determine the mechanism through which Herp reduces ER Ca2+ content, how to test the proteasomal degradation of SERCA, how to illustrate the proteasome recruitment to the ER membrane, and how to demonstrate the interaction between Herp and SERCA. The work will provide a new regulatory link between ER stress and Ca2+ homeostasis. In addition, studies of the proteasomal degradation of SERCA will broaden our present understanding of the regulation of SERCA. Since dysregulation of Ca2+ homeostasis has been implicated in the pathophysiology of several neurodegenerative diseases like Alzheimer's and Huntington's, research focused on Herp may lead to insights regarding therapies for those.

1. Determine the mechanism by which Herp decreases ER Ca^{2+} storage.

Previous studies demonstrated that Herp stabilizes cellular Ca^{2+} homeostasis in neurons during ER stress. The goal of research in this section is to determine the means by which Herp reduces ER Ca^{2+} content. Both indirect and direct measurements of ER Ca^{2+} concentration will be performed in different cell lines to confirm the same role of Herp in non-neuronal cells. In addition, performing Ca^{2+} imaging and uptake experiments to test each of the most probable ways that have been known to decrease ER Ca^{2+} content will lead to the determination of the mechanism Herp uses.

2. Determine if Herp activates proteasomal degradation of SERCA.

Research in this section focuses on understanding of how SERCA is down-regulated by Herp. Western blotting of SERCA in the presence/absence of Herp will elucidate whether the down-regulation of SERCA is on the protein level or on the activity level. Comparison of the amount of SERCA plus/minus the proteasome inhibitors will allow us to identify whether the proteasome degradation is involved in this down-regulation. These studies will be followed by the experiments to confirm the increased ERAD of SERCA by pulse-chase experiments.

3. Determine how Herp activates ERAD of SERCA.

The work in this section will provide us an explanation of how Herp activates ERAD of SERCA. Initial experiments will show that Herp recruits proteasome to the ER membrane using co-immunoprecipitation (IP), immunofluorescence

microscopy, and membrane binding assay. Furthermore, the interaction of Herp with SERCA will be tested and IP in different [Ca^{2+}] conditions will help us to investigate whether this interaction is regulated by Ca^{2+} level.

BACKGROUND AND SIGNIFICANCE

Biology of Herp

Human homocysteine-induced ER protein (Herp) is a 54 kDa protein which encodes 391 amino acid residues. Human and mouse Herp share nearly 90% identity[1-4]. However, there is no Herp homologue found in yeast, worm or drosophila to date. ER stress induced by regularly used unfolded protein response (UPR) inducers or hyperhomocysteinemia, or Aβ–a major component of amyloid deposits in Alzheimer's disease strongly induces Herp expression by over 50 folds[1,4,5]. There are two *cis*-acting elements in the promoter of Herp which account for this considerable increase. One is ER stress–response element (ERSE), which allows the NF-Y–dependent binding of ATF6; the other is novelly discovered and named ERSE-II[2], which allows not only ATF6 binding but also NF-Y–independent XBP1 binding[6].

Immunofluorescence staining and cell fractionation studies showed that Herp is an ER-resident integral membrane protein[1-4]. Both its N-terminus and C-terminus are facing the cytoplasmic side of the ER and this topology makes Herp

unlikely to act as a chaperon in ER lumen (Figure 1A)[1]. Locate in the N-terminus of Herp is a ubiquitin-like domain (UbLD)[1,4]. Recent sequence analysis revealed that the Herp UbLD contains a proteasome-interacting motif (PIM) (Figure 1B)[7]. These data have led to the supposition that Herp may recruit proteasome from the cytosol to the ER membrane during UPR and this translocation of proteasome can facilitate the ERAD[8,9].

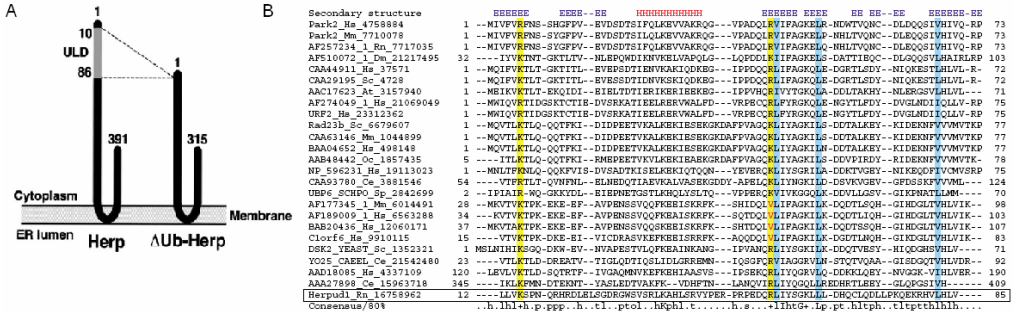

Figure 1. (A.) Schematic representation of Herp and Herp lacking the UbLD (ΔUb-Herp encodes Herp that lacks the residues Val[10]-Cys[86]). Both the N-terminus and C-terminus of Herp are facing the cytosolic side of the ER membrane[10]. (B.) Alignment of sequences containing PIM. Highlighted (yellow, positively charged; blue, hydrophobic) residues represent the potential PIM residues that are conserved in UbLD. Rattus norvegicus Herp protein was boxed[7].

Previous studies showed that a Herp null cell line is more vulnerable to ER stress compared to wild type and the rescue of the phenotype requires the N-terminal UbLD[11]. Furthermore, Chan *et. al.* found that the overexpression of Herp protects neuronal cells against ER stress induced apoptosis[5]. This Herp mediated protective effect is not due to up- or down-regulation of proteins known to regulate neuronal survival upon ER stress induction such as BiP, Grp94, Hsp70, calreticulin, and Bcl-2, but is mediated by the reduction of Ca^{2+} storage in the ER

lumen. This reduced Ca^{2+} load in the ER would make less releasable Ca^{2+} pool upon ER stress induction. Herp-decreased Ca^{2+} release attenuates c-Jun N-terminal kinase (JNK) and pro-caspase 12 activations and might ultimately suppress the Ca^{2+} activation of mitochondrial processes, in particular the release of cytochrome *c*. The mitigation of the apoptotic signaling cascade therefore protects neuronal cells against ER stress-induced cell death. However, the underlying mechanism of stabilizing cellular Ca^{2+} homeostasis by Herp is unclear[5].

Regulation of SERCA

Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is an integral membrane protein of the ER or SR responsible for the ATP-dependent transport of Ca^{2+} from the cytosol to the lumen of ER or SR[12]. In mammals, three genes encode for three highly homologous pumps and different splicing variants give rise to at least seven isoforms. Particular isoforms are associated with certain cell types. SERCA1a/b are exclusively expressed in fast-twitch skeletal-muscle cells. SERCA2a is the main isoform in cardiac-muscle and slow-twitch skeletal-muscle cells while SERCA2b, which has been considered as the house keeping isoform, is ubiquitously expressed in smooth- and non-muscle cells and will be the primary focus in this grant. SERCA3a/b/c are rarely found alone and are usually combined with SERCA2b in a range of specialized cell types (e.g., lymphocytes, mast cells, Purkinje neurons)[13].

Recent studies have shown the importance of the regulation of SERCA as related to Ca^{2+} homeostasis, ER stress, cell proliferation, and apoptosis. ER transmembrane protein phospholamban is considered to be the main regulator, and its binding with SERCA lowers the apparent affinity for Ca^{2+} of SERCA, thereby inhibiting SERCA transport activity[13]. Another regulator of SERCA is the oncoprotein Bcl-2, but some of the published data are contradictory and much debated. Part of the controversy may be explained by cell-specific differences[12]. Bcl-2 overexpression in HeLa cells[14] or LNCaP prostate cancer cells[15] reduces ER Ca^{2+} concentration partially because of the reduction of SERCA2b expression. This reduced releasable Ca^{2+} pool protects cells from apoptosis. However, another report showed that Bcl-2 overexpression in breast epithelial cells stimulates SERCA2 expression, which increases the Ca^{2+} load in the ER, thus promoting cell proliferation counteracting apoptosis[16].

It has been reported that both SERCA1[17] and SERCA2a/b[18,19] could be down-regulated by proteasomal degradation. Ala^{3}-Thr^{9} of SERCA1a was shown to be sensitive to ER quality control, and the deletion of those residues strongly enhances the ERAD of SERCA1a, which suggests that this region is either important for the correct folding or stabilizing the folded state of SERCA1a[17]. Mutations causing Darier's Disease within SERCA2b markedly enhance its proteasome-mediated degradation, therefore affecting its activity[16]. Yoshito ***et. al.*** demonstrated that SERCA2a forms a complex with the overexpressed calreticulin

under the oxidative stress. The formation of the complex initiates the proteasome-dependent degradation of SERCA2a, thus inhibiting the Ca^{2+} uptake into the ER lumen[19].

Summary of hypotheses

We hypothesized that Herp recruits proteasome from cytosol to the ER membrane by its UbLD and this recruitment facilitates the proteasome-mediated degradation of its interacting protein SERCA2b, thus reducing the Ca^{2+} load in the ER, which counteracts apoptosis induced by ER stress (Figure 2). Preliminary experiments of ER Ca^{2+} concentration measurements will be conducted in three different cell lines to confirm that Herp could also reduce ER Ca^{2+} storage in non-neuronal cells. Then we will address if the observed decrease of ER Ca^{2+} content is the effect of down-regulation of SERCA. Other possibilities will be ruled out by performing Ca^{2+} imaging and uptake experiments. We also wish to show the instability and enhanced ERAD of SERCA2b by Herp overexpression. This will be done by comparison of the protein level and half-life of SERCA2b in the presence/absence of Herp. Finally, proteasome recruitment and Herp/SERCA interaction will be demonstrated. Co-IP, immunofluorescence microscopy, and *in vitro* membrane binding assays will be performed to address those questions. Overall, these experiments will provide a better understanding as to how Herp stabilizes cellular Ca^{2+} homeostasis and most importantly a better understanding into the pathophysiology behind diseases of Ca^{2+} homeostasis dysfunction.

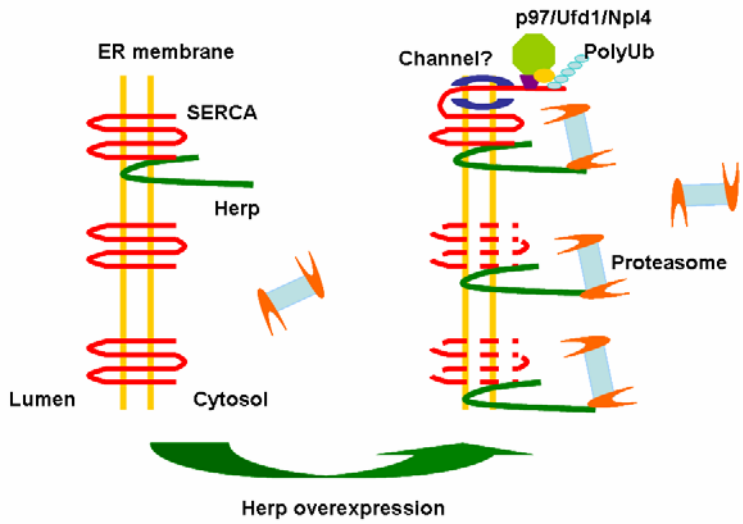

Figure 2. Model for how Herp enhances the ERAD of SERCA. After the overexpression of Herp, the proteasome can be recruited to the ER membrane by binding to the UbLD of Herp. SERCA will probably be retro-translocated through the channel which is unknown by p97/Ufd1/Npl4 complex, poly-ubiquitinated, and finally degraded by the proteasome.

RESEARCH PLANS

1. Determine the means by which Herp decreases ER Ca^{2+} storage.

Increased Ca^{2+} release from ER constitutes the primary trigger for apoptosis during ER stress[20]. Herp prevents ER stress-induced cell death at least partially because of its ability to decrease the Ca^{2+} release. The studies of Chan *et. al.* have demonstrated that Herp overexpression reduces ER Ca^{2+} content and ER stress-induced release in neuronal cells[5]. In order to begin to understand how Herp stabilizes cellular Ca^{2+} homeostasis, we will determine the means by which Herp decreases ER free lumenal Ca^{2+} concentration.

1a. Decrease of ER Ca2+ content in non-neuronal cells

We will create mammalian expression plasmids for Herp wt (WT), Herp without UbLD (ΔUb-Herp), and Herp K15M/I57A (K/I) (K15 is in the PIM; I57 is equivalent to I44 in ubiquitin whose mutation leads to abrogation of proteasome binding[21]) by using pCMV5-myc vector and plasmid for Herp UbLD (UbLD) by using pCMV5-HA vector. Subcellular distribution of these truncated/mutated proteins will be investigated by immunofluorescence staining after the transient transfection of human embryonic kidney (HEK) 293 cells and monkey kidney fibroblast COS cells. According to Sai *et. al.*[10], there should be no significant difference in intracellular localization between WT and ΔUb-Herp or K/I and all of those should mainly localize in the ER membrane. UbLD may show the same staining pattern as that of ubiquitin. In addition, UPR should not be induced by the overexpression of those proteins, which will be confirmed by measuring BiP protein level and PKR-like ER kinase (PERK) activation/phosphorylation. Generation of stable expression cell lines will also be performed in mouse B-lymphoma (A20) cells, and those cell lines will be referred to as VT (vector transfected cells), Herp (Herp stably overexpressing cells), ΔUb-Herp, and K/I.

Chan *et. al.* demonstrated that Herp overexpression reduces the free Ca^{2+} concentration within the ER by addition of Thapsigargin (TG), an inhibitor of SERCA which causes a passive leak of Ca^{2+} from ER lumen into cytoplasm, to PC12 cells which is an indirect measurement of the ER Ca^{2+} content (Figure 3)[5].

The same experiment will be repeated in WT, ΔUb-Herp, and K/I transiently transfected 293 and COS cells as well as stably overexpressing A20 cells using the high affinity Ca^{2+} dye Fura-2 AM (Molecular Probes) to rule out the species, transfection method, and cell type dependencies.

Another approach which directly measures the free lumenal Ca^{2+} concentration will also be performed by using the low affinity Ca^{2+} sensitive dye Fura-2FF AM (Molecular Probes) in those cells[22]. Experimental details of calcium imaging and ER Ca^{2+} measurement was described previously by Chen *et. al.*[23].

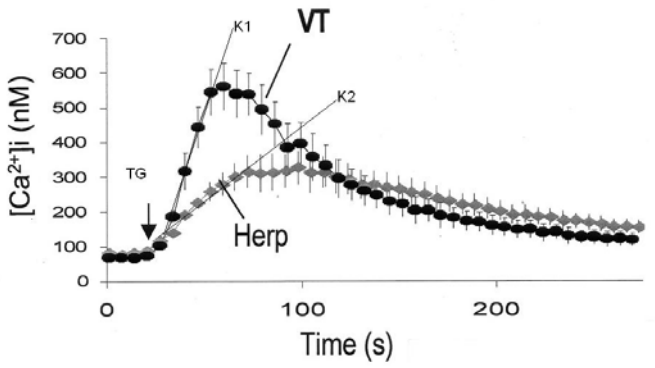

Figure 3. Herp stabilizes ER Ca^{2+} homeostasis during ER stress. Representative recording showing levels of $[Ca^{2+}]_i$ prior to and after exposure of VT and Herp cells to 1 μM TG (arrow indicates time of addition). Values are the mean and S.D. of determinations made in four separate cultures. K_1 and K_2 are the slopes of initial Ca^{2+} release induced by TG in VT and Herp cells respectively[5].

Both measurements should sense the reduction of ER lumenal Ca^{2+} concentration in WT transfected cells and Herp cells but not in mock transfected and VT cells if the function of Herp is consistent ubiquitously. If the UbLD of Herp plays a critical role in recruiting the proteasome and the proteasome is

involved in this process (see Specific Aim 3), no reduction will be found in ΔUb-Herp and K/I cells.

1b. Determination of the means by which Herp decreases ER Ca2+ content

The mechanism by which Herp decreases free ER lumenal Ca^{2+} concentration is unknown. However, there are several mechanisms which might account for that[24,25]: *(i)* Herp enhances the Ca^{2+} permeability of the ER membrane; *(ii)* Herp down-regulates the pumps involved in ER Ca^{2+} reuptake such as SERCA; *(iii)* Herp decreases the Ca^{2+} capacity of ER by down-regulation of sequestration protein such as calreticulin; *(iv)* Herp reduces capacitative Ca^{2+} entry (CCE); and *(v)* Herp increases mitochondrial Ca^{2+} uptake (Figure 4).

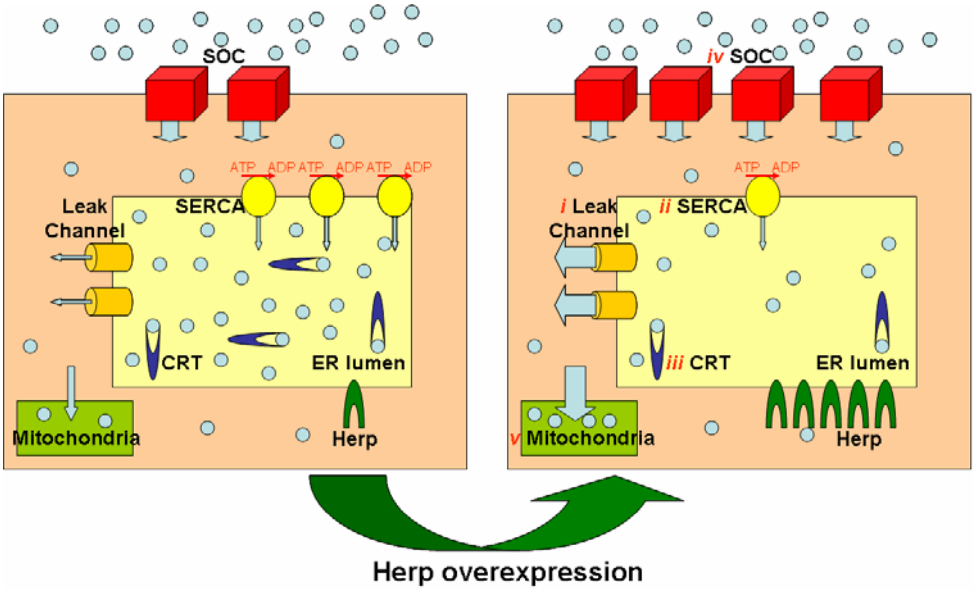

Figure 4. Schematic diagram showing in simplified form the possible major pathways that Herp overexpression could stabilizes ER Ca^{2+} homeostasis. The left panel presents the control conditions characterized by the low levels of Herp expression and the basal expression of the ER leak channel, SERCA pump, lumenal calreticulin (CRT), mitochondrial uniporter, and plasma membrane store operated channel (SOC).

The overexpression of Herp (right) results in lowered ER lumenal Ca^{2+} concentration could due to (*i*) the enhanced Ca^{2+} leak via ER leak channel, the down-regulation of (*ii*) SERCA pump and (*iii*) CRT, (*iv*) the enhanced mitochondria Ca^{2+} uptake, and (*v*) the decreased Ca^{2+} SOC entry.

Chan *et. al.* have already ruled out (*iii*) and (*iv*) by showing that the expression level of calreticulin did not change significantly during ER stress in the presence of Herp and CCE was not inhibited in Herp stably expressing PC12 cells[5].

The initial slope (K) of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase induced by TG in the presence of EGTA roughly represents ER Ca^{2+} permeability. As shown in Figure 3, K in Herp cells is 40% smaller than that of VT cells which makes (*i*) not possible here. To further confirm this, the kinetics of TG-induced Ca^{2+} store depletion will be studied by adding 1μM ionomycin (IM) (Calbiochem) at defined time interval after TG addition in the presence of EGTA. The amount of Ca^{2+} release induced by IM will then be calculated for various time-points as a percentage of the total pool which is the amount of Ca^{2+} release in response to the concomitant addition of TG and IM. If the Ca^{2+} permeability of the ER membrane is not enhanced by Herp overexpression, the percentage calculated in Herp cells should be comparable to that in VT cells. While that in Bcl-2 overexpressing A20 cells, which will be used as a positive control, should be much smaller[26].

Due to the subcellular localization of Herp, it is unlikely that the decreased ER Ca^{2+} content is caused by an increased mitochondrial Ca^{2+} uptake. If our

assumption is right, treatment of Herp cells with a mitochondrial Ca^{2+} uniporter inhibitor–RU360 (Sigma)[27], will not significantly increase the ER lumenal Ca^{2+} concentration.

A $^{45}Ca^{2+}$ uptake assay will be conducted by using the purified microsomes to confirm that (*ii*) is the bona fide mechanism. For the preparation of microsomes, cell homogenate will be centrifuged and ultracentrifuged. The final pellet which contains microsomes will be resuspended for the following experiments. The Ca^{2+} uptake will be measured radiometrically using the Millipore filtration technique as described previously[19]. The Ca^{2+} uptake rate in the microsomes from Herp cells should be significantly impaired compared to those from ΔUb-Herp or K/I cells.

It is possible that Herp stabilizes Ca^{2+} homeostasis through different mechanism(s), for example, enhancement of plasma membrane Ca^{2+} ATPase (PMCA). However, given the fact that Herp localizes only in the ER membrane, and the obvious decrease of ER Ca^{2+} does not seem like secondary effect caused by the perturbation of plasma membrane, this is unlikely. It is also possible that more than one mechanism discussed above combine. If we find that this is the case, we will primarily focus on the down-regulation of SERCA.

2. Determine if Herp activates proteasomal degradation of SERCA.

Following the studies described in part 1, we will proceed to the determination of how Herp down-regulates SERCA during ER stress. Based on the fact that the down-regulation of SERCA pump can be achieved by ERAD[17-19],

we hypothesize that the down-regulation of SERCA by Herp is using the same means. The decrease of the SERCA protein expression will be proved in this section. Furthermore, enhanced ERAD of SERCA by Herp overexpression will be confirmed by pulse-chase experiments.

2a. Destabilization of SERCA by Herp overexpression

Two possibilities, i.e. (*i*) decreased protein expression and (*ii*) decreased transport activity, could explain the down-regulation of SERCA and those can not be distinguished by SERCA functional assay. However, they will be easily tested by biochemical experiments. Whole cell lysates from VT, Herp, ΔUb-Herp, and K/I cells will be subjected to SDS-PAGE and then western blotted and probed with a monoclonal anti-SERCA2 antibody (clone 2A7-A1, Sigma) to detect whether there is a significant decrease of SERCA2 in Herp cells compared to VT, ΔUb-Herp, and K/I cells. After the confirmation of (*i*), further experiments will be done to decide which mechanism accounts for the decreased SERCA protein. Still, there are two possibilities, one is the decreased synthesis including inhibited transcription and retarded translation and the other is the increased degradation. Northern blotting of SERCA transcripts may help to estimate if the transcription of SERCA is interfered. Moreover, if the observed decrease of SERCA protein can be blocked by lactacystin (50μM, 4hrs) and epoxomycin (300nM, 4hrs), both of which are specific proteasome inhibitors, we can conclude that enhanced degradation of SERCA caused by Herp overexpression is mediated by the

proteasome. Leupeptin (100μM, 4hrs), an inhibitor of lysosomal cysteine and trypsin-like proteases, will be used as a negative control.

2b. Facilitated ERAD of SERCA

Proteasomal degradation of SERCA might be enhanced in the presence of Herp, which will be further investigated in this section by assessment of the turnover of SERCA. The stability of SERCA will be checked in VT, Herp, ΔUb-Herp, and K/I cells by pulse-chase experiments. HEK293 cells will be transfected with mock, WT, ΔUb-Herp, and K/I respectively, then metabolically labeled with [^{35}S] methionine/cysteine for 1hr, followed by different time intervals of chase in the presence of non-radiolabeled medium. The cells will then be harvested, lysed and subjected to anti-SERCA2 IP. Under these conditions, we expect to observe that SERCA is unstable and degrades more rapidly and exhibits a much shorter half-life after transfection of WT compared to mock, ΔUb-Herp, or K/I. Moreover, this increased turnover rate might be achieved by proteasome recruitment to ER membrane by Herp UbLD which will be confirmed in part 3b. Thus we predict that this increase should be blocked by adding proteasome inhibitors (lactacystin and epoxomycin) and by the co-overexpression of WT with UbLD which acts as a dominant negative form of Herp.

3. Determine how Herp activates ERAD of SERCA.

Herp could promote ERAD of SERCA by several ways and they are most likely (*i*) increase of SERCA polyubiquitination level, (*ii*) enhancement of the

retro-translocation of SERCA, and (*iii*) increase of the proteolysis of SERCA (Figure 5). There is no E2 (ubiquitin-conjugating enzyme) or E3 (ubiquitin ligase) binding domain predicted in Herp. Therefore, (*ii*) and (*iii*) are more preferable than (*i*). It has been shown that the 19S complex of the proteasome might facilitate the retro-translocation of some ERAD substrates[28]. In mammalian cells, the 26S proteasome was found evenly in cytosol, microsome and nucleoplasm and the dislocation of the substrate protein from the ER into the cytosol to gain access to the proteasome is an indispensable event of ERAD[29-31]. Furthermore, the UbLD in the N-terminal region of Rad23 and Dsk2 are involved in the delivery of polyubiquitinated substrate to the proteasome[32-35]. Herp, which also contains the UbLD, thus may recruit the proteasome from the cytosol to the ER membrane and probably enhance the retro-translocation and the proteolysis of SERCA, which interacts with Herp.

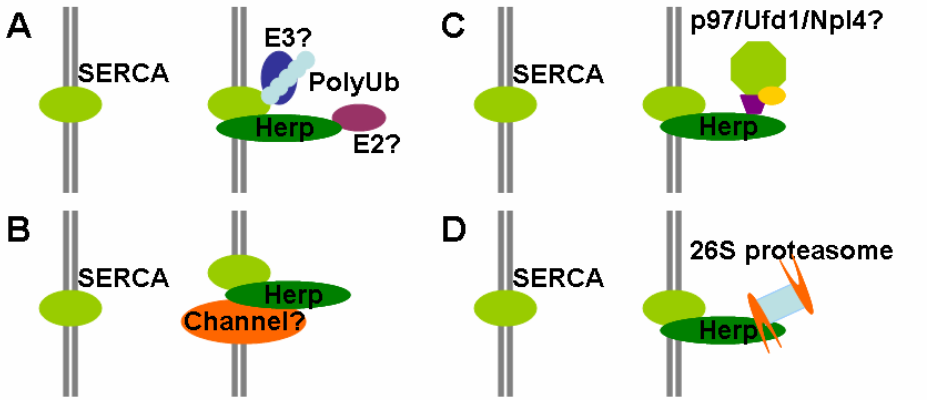

Figure 5. Possible models for how Herp could enhance ERAD of SERCA. (A) Herp could increase polyubiquitination level of SERCA by either recruitment of E2 (Ubc7 or Ubc1) or E3 (Hrd1, Hrd3, Doa10, or Herp itself may serve as an E3). But the fact that there is no predictable E2 or E3 interacting domain in Herp makes it less possible.

(B) Herp may directly link SERCA to some certain channel thus facilitate the retro-translocation of SERCA. Several lines of evidence suggest that Sec61[36] and Derlin-1[37] could be the channels responsible for the retro-translocation of MHCI heavy chain which is a bitopic membrane protein. However, whether the ERAD of politopic membrane protein requires a channel and what is the molecular identity of the channel are still mysterious. (C) Herp may bring the p97/Ufd1/Npl4 complex to the proximity of SERCA and therefore enhance the retro-translocation and the proteasome presentation. However, p97 preferentially binds tetra-ubiquitin chains and show little affinity for mono-, di-, and tri-ubiquitin molecules[38]. Therefore, p97 may probably show little affinity for the UbLD which makes this model less possible. (D) SERCA could be constitutively degraded in a very low rate and the recruitment of the proteasome to the ER membrane by Herp may shift the equilibrium of this degradation and increase the rate.

3a. Polyubiquitination of SERCA

Though SERCA has been already known to be degraded by proteasome, its polyubiquitination was never shown before. We would like to show it and demonstrate that the polyubiquitination level of SERCA is not enhanced by Herp overexpression. Polyubiquitination of endogenous SERCA will be first tested in the presence/absence of proteasome inhibitors. HEK293 cells will be treated with/without lactacystin, lysed and subjected to anti-SERCA2 (Sigma) IPs. IPs will then be immunoblotted with rabbit anti-ubiquitin antibodies (Upstate) to analyze the polyubiquitination level. It will not be surprising to see that the polyubiquitination of SERCA can be increased by lactacystin. In addition, HEK293 cells will be transfected with/without Herp and then the polyubiquitination levels of SERCA2 will be compared to rule out the possibility that Herp overexpression enhances SERCA polyubiquitination.

3b. Translocation of proteasome to the ER membrane

Rad23 and Dsk2 both bind to S5a, a subunit in 19S regulatory complex of 26S proteasome, through their UbLD[8,39,40]. We hypothesize that Herp interacts with the same subunit of proteasome: S5a, which contains a ubiquitin-interacting motif (UIM)[41]. HEK293 cells will be transfected with WT, lysed and immunoprecipitated with an anti-myc antibody (9E10, Santa Cruz), and the IP will be western blotted to detect the endogenous S5a to demonstrate their binding. The polyclonal anti-S5a antibodies can be obtained by immunonization of rabbits with recombinant S5a proteins[39]. IP using ΔUb-Herp and Herp K/I will allow me to identify the interactive region and critical amino acids for binding. If our hypothesis is right, ΔUb-Herp should totally lose S5a binding ability, while Herp K/I may lose it partially or totally.

S2, a subunit of the 8-subunit base of the 19S regulatory cap, was another subunit which has been shown to bind to Rad23 and Ddi1[42,43]. Herp therefore my also bind to this subunit and similar analysis could be performed.

The recruitment of proteasome to the ER membrane from the cytosol will be further confirmed by immunofluorescence microscopy. An anti-19S regulatory complex S7 monoclonal antibody and anti-20S proteasome C7 rabbit polyclonal antibodies (Calbiochem) will be used to monitor the distribution of proteasome. The proteasomes in Herp cells will preferentially colocalize with Herp and a mouse anti-KDEL antibody (StressGen Biotechnologies) which is an ER maker, while those proteasomes in VT, ΔUb-Herp, and K/I cells will not show that

pattern. Transfection of UbLD to Herp cells will disrupt the ER localization pattern of proteasome.

Considering the abundant amount of proteasome in mammalian cells (approximally 0.6% of total cell proteins in HeLa cells[44]), the images from the above experiments will be hard to quantify, so an alternative *in vitro* membrane binding assay can be performed. Purified microsomes from VT, Herp, ΔUb-Herp, and K/I cell lines will be incubated with purified 26S proteasome as well as 20S proteasome (which can be obtained from DeMartino Lab downstairs) and then sedimented by centrifugation. Supernatant fractions and pellets will be analyzed by SDS-PAGE. More 26S proteasome than 20S proteasome should co-sediment with Herp microsomes because both S5a and S2 subunit are in the 19S regulatory complex. The greater binding of the 26S proteasome with Herp microsomes than with ΔUb-Herp, and K/I microsomes should also be detected by this method. To decrease the binding background mediated by other peripheral membrane proteins, purified microsomes will be washed with high salt before applied to this assay.

3c. Interaction of Herp with SERCA

The interaction between Herp and SERCA will be determined and it will be interesting to detect whether elevated Ca^{2+} level regulates this interaction.

To interprete why Herp overexpression enhances the ERAD of SERCA, we wish to see the interaction of Herp with SERCA. To determine whether Herp

associates with SERCA, co-IP will be performed in mock, WT, ΔUb-Herp, and K/I transiently transfected HEK293 cells in a detergent condition (0.5% Tween-20). IPs with anti-myc (Santa Cruz) will be western blotted and probed with an anti-SERCA2 antibody to detect associated endogenous SERCA2. Given the hypothesis (tested earlier in part 3b) that PIM in the UbLD of Herp is critical for recruiting proteasome, it is unlikely that Herp binds SERCA using the same region. Expected results are that all those proteins (WT, ΔUb-Herp, and Herp K/I) can bind SERCA and probably more SERCA will be detected in ΔUb-Herp and Herp K/I IPs than in WT IP because of the increased input of SERCA in those IPs.

Alternatively, blue native-PAGE (BN-PAGE) and following SDS-PAGE/western blotting can be performed by using the ER membrane isolated from WT and ΔUb-Herp transfected HEK293 cells to determine this interaction *in vivo*[45]. Purified ER membrane will be applied to BN-PAGE, and the protein complexes stained by Coomasie blue will be excised, separated by SDS-PAGE, and analyzed by western blotting with anti-myc and anti-SERCA2. More Herp binding candidates could be tested by using this method.

As association of SERCA with phospholamban can be regulated by Ca^{2+} level, it will be of interest to see whether it is the case in SERCA and Herp association. Elevation of $[Ca^{2+}]_i$ will triggers SERCA up-regulation and may destroy the physical interaction of Herp and SERCA. To quantify Herp/SERCA

interactions following the elevation of $[Ca^{2+}]_i$, microsomes from Herp, ΔUb-Herp, and K/I cells will be mixed with reaction mixture containing various concentrations of $CaCl_2$ (10^{-8} to 10^{-5}M). After 5min at room temperature, lysis buffer will be added to the reaction mixture, and the IP will be carried out as described earlier in this grant. Elevation of $[Ca^{2+}]_i$ from *p*Ca 8 to *p*Ca 5 should result in a concentration-dependent decrease of the amount of SERCA2 that is co-immunoprecipitated with an anti-myc antibody (Santa Cruz) against tagged Herp WT and mutants.

CONCLUSION

Research in this proposal integrates the tools of cell biology and biochemistry to better understand Herp function as it relates to the regulation of SERCA and stabilization of Ca^{2+} homeostasis. By studying the most probable ways which could decrease ER Ca^{2+} content one by one, we will gain insight into the means used by Herp. Detailed analysis of the ERAD of SERCA may reveal novel regulatory mechanisms and will further our understanding of Ca^{2+} homeostasis. Research on the interactions of Herp with proteasome and SERCA will explain how Herp fulfills its function. Together, the combined information derived from these studies will provide a better understanding of Herp, SERCA, and Ca^{2+} homeostasis. Such advancements could have significant applications in

development of therapeutic agents that act on this SERCA-Herp-proteasome pathway in several neurodegenerative diseases.

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