AN ENGINEERED RECEPTOR COUPLED WITH A SMALL MOLECULE SYSTEM ACTIVATES A STRESS SIGNALING PATHWAY CRITICAL TO THE ERAD RESPONSE

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ABSTRACT

Endoplasmic reticulum (ER) stress is caused by the accumulation of misfolded proteins in the ER. IRE1, an ER localized transmembrane protein kinase, is a sensor of unfolded proteins and plays an important role in regulating the cellular rescue response to ER stress. We have engineered FV2E-IRE1β, a drug controlled receptor that exploits the IRE1 cell rescue pathway. Addition of the drug AP20187 induces stress-uncoupled activation of FV2E IRE1 and causes IRE1 signaling. Our results indicate that the use of this system has utility in regulating the cytoprotective IRE1-ERAD pathway independently of other ER stress-induced signals, such as proapoptotic JNK signaling.
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BACKGROUND

ER Stress and the Unfolded Protein Response

The Endoplasmic Reticulum (ER) is the cellular organelle where newly synthesized proteins obtain their uniquely folded conformations. As a result of managing the multitude of enzymes and other proteins produced in the body, the ER’s inter-luminal protein concentration may reach up to 100 mg/ml (Horwich et al, 2002). Subsequently, these proteins must be effectively packaged and folded in order for normal cellular function to occur or else the cell could be eliminated through apoptosis via a self-induced caspase cascade. In order to manage these significant protein concentrations, the ER employs a system of chaperone molecules which aids in protein folding and handling (Patil and Walter, 2001). At the transcriptional level, the ER employs a system known as the unfolded protein response (UPR) which increases the natural folding capacity of the ER by upregulation of target genes, as well as decreasing the translational burden in the ER by translational attenuation (Urano et al, 2000a).

The ERAD System

It is also possible for misfolded proteins to move across the ER lumen and be destroyed by the ubiquitin proteosome pathway. This system is known as endoplasmic reticulum associated protein degradation (ERAD) and often enables the cell to be “rescued” from deadly ER stress. The ubiquitin proteasome pathway is a multi-step process that begins with the initial activation of the protein “ubiquitin” by a Ubiquitin activating enzyme known as E1. The activation occurs when ATP hydrolysis is coupled with the genesis of an E1-ubiquitin conjugate formed by a thiolester bond to the C-terminal glycine residue on ubiquitin (Pickart, 2000). Immediately following this linkage, the activated ubiquitin molecule is transferred to a ubiquitin conjugating enzyme (E2). The ubiquitin-E2 conjugate is only an intermediate step that aides in attaching the ubiquitin to the substrate via a ubiquitin protein ligase, or an (E3). The role of the E3 is to specifically recruit the substrate, for example a misfolded protein, and attach the ubiquitin via its C-terminal glycine to the target’s free lysl e-amino group. These three steps will occur repeatedly on the same protein until the target acquires a “ubiquitin tail.” (Vierstra, 2003).
Currently, five different E3 types have been recognized and classified based on their subunit composition and chemical mechanisms. These groups are classified as follows: HECT, Ring/U-box, SCF, VBC-Cul2, and APC. All five E3 ligase classes demonstrate similarity in that they all form an arched scaffold that participates in recruitment of the E2-ubiquitin product and the subsequent transfer of ubiquitin to the substrate. In order to assure specificity in ubiquitination of targets, there are usually a great number of E3 components in every cell (Vierstra, 2003).

Once target proteins acquire a ubiquitin tail, they become targets for destruction in an ATP dependent manner via the 26 S proteasome system. The 26 S proteasome consists of a 20S core protease and a 19S regulatory particle. The proteasome’s specificity is controlled by the 19S subunit which recognizes and processes poly-ubiquitinated substrates. Once recognized, the 19S particle also unfolds, deubiquitinates, and ushers the target into the 20S core protease for imminent destruction (Vierstra, 2003).

**HRD1: A Novel Component of the ERAD System**

With regard to endoplasmic reticulum stress, this ERAD system is very important as it decreases the cellular stress load by degrading unwanted and misfolded proteins. Of particular importance is a novel ER resident E3 ubiquitin ligase called HMG Co-A reductase degradation 1 (HRD1). This E3 ligase is a member of the RING family of E3 ligases since it possesses a RING/H2 domain. This RING/H2 domain is located in the cytoplasm while the rest of the protein resides in the ER. The RING/H2 domain of HRD1 conveys in vitro Ubiquitination activity in the presence of the E2 Ubiquitin conjugating enzyme UBC7 (Kikkert et al, 2004).

**HRD1 Protects Cells from ER Stress**

Unpublished recent studies in our lab using the Akita diabetes model mouse have demonstrated the importance of the HRD1 system in promoting cellular survival. The Akita mouse is a C57/B6 mouse possessing a heterozygous mutation in the Insulin 2 gene (Ins2^WT/C96Y). The mutation occurs when tyrosine is substituted for Cysteine as the 96th amino acid in the insulin 2 protein. This mutation is detrimental to the function of the Insulin 2 protein since Cysteine is important for forming the disulfide bonds common to many folded proteins. Thus, the result of the Ins2^WT/C96Y mutation is a protein that is misfolded. Consequently, it has
been demonstrated that the mouse’s resultant diabetes is a direct effect of ER Stress caused by misfolded proteins in the pancreatic β-cells of Akita mice. The subsequent stress produced by this misfolded insulin eventually kills pancreatic β-cells in Akita mice leading to diabetes.

In considering the important role of Hrd1 in rescuing cells from ER stress through ERAD activation we have explored the role of HRD1 in Akita mice. Preliminary data from real time PCR (Figure 1A) and westerns (Figure 1B) conducted on these mice demonstrates that HRD1 mRNA and protein production are upregulated in Akita mice in comparison to control mice (Figure 1B). Additionally, HRD1 upregulation has also been shown to protect the β-cells of Akita mice from apoptosis through ERAD activation.

**IRE1, PERK, and ATF6: Central Components of the UPR**

These results suggest that enhancement of ERAD in cells undergoing stress may increase cellular survival. In order to effectively enhance the ERAD response, discovery and control of a central upstream regulator of this process is the most important factor. The discovery of the transmembrane protein inositol-requiring 1 (IRE1) as a sensor of unfolded proteins, as well as an activator of the UPR was a critical development leading to the creation of a cell rescue system based on ERAD (Yoshida et al, 2003). IRE1 comes in both an α and a β form, where IRE1α is localized in the pancreas and IRE1β is localized in the gut (Urano et al, 2000a). Apart from their different locations, their structures and functions are nearly identical. In wild type cells, IRE1 activation enhances HRD1 activity but in IRE1 α-/- cells HRD1 activity is negligible (Figure 1B). This indicates that the IRE1 is a critical component in the ERAD system.
Figure 1. Relative Expression Levels of HRD1 mRNA.
A: Upon treatment of wild type murine cells with tunicamycin followed by real time PCR analysis, we found that the E3 ubiquitin ligase HRD1 is highly upregulated. The upregulation of HRD in wild type cells demonstrates its role in managing the increased stress load in the cell. When IRE1α -/- cells were treated with tunicamycin, however, a negligible stress-related HRD1 response was observed, indicating that HRD1 expression is linked to IRE1 activation.
B: Measuring HRD1 levels in the islets of Akita diabetes mice demonstrates that HRD1 levels are highly upregulated in order to deal with large amounts of misfolded insulin. SEL1L and BiP, two other components in ER stress management are also upregulated (data, courtesy of the Urano lab)

Because of its role as a sensor of unfolded proteins, Ire1 is implicated to be a major part of the cell stress cycle. Though the stress cycle is a very complex system, it is regulated by three different sensors. Ire1 is a transmembrane protein localized within the outer wall of the ER and is only one part of the cell’s tripartite system for stress management. The two other components of the ER stress pathway include PKR-like ER kinase (PERK) and activating transcription factor
6 (ATF6) (Harding et al, 1999; Yoshida et al, 1998; Yoshida et al, 2000). In utilizing one, or a combination of these three components, the cell is able to protect itself from a good deal of both normal and abnormal physiological stresses (Figure 2A).

**Figure 2 Three Systems in the Endoplasmic Reticulum That are Essential in the Unfolded Protein Response.**

A: The endoplasmic reticulum has three ways to deal with unfolded proteins. First, activation of the ER resident transmembrane kinase PERK initiates general translational attenuation and upregulation of target UPR genes. Second, ATF6 activation initiates protein degradation as well as upregulation of UPR target genes. Third, the activation of IRE1 causes both cell rescue and cell death through XBP-1 mRNA splicing resulting in XBP-1 transcription factor production. B: Because of IRE1’s important role in the ER stress response, harnessing its cell rescue capability through XBP-1 while bypassing its cell death function through Caspase-12 and ASK-1 is an effective way to decrease cell death.
ATF6 comes in two forms, ATF6\(\alpha\) and ATF6\(\beta\), both of which are ER resident transmembrane proteins containing a bZIP domain. Upon activation of the UPR due to ER stress, both forms of ATF6 are cleaved by site-1 and site-2 proteases. Following cleavage, their bZIP domains are transported to the nucleus where they bind to ER stress response elements (ERSE) (Wang et al, 2000; Yoshida et al, 2000). The ERSEs are cis-acting elements containing the consensus sequence (5’)CCAAT-N9-CCACG(3’). With the binding of a transcription factor to ERSEs comes upregulation of chaperone production and activation of other UPR genes (Haze et al, 1999; Yoshida et al, 1998).

Another method that the cell employs to deal with stress is to stop general protein translation. Activation of PERK causes phosphorylation of the \(\alpha\) subunit of eukaryotic translation factor 2 (eIF2\(\alpha\)). Phosphorylation of eIF2\(\alpha\) transforms eIF2 into an inhibitor of its guanine nucleotide exchange factor eIF2B thus effectively stopping translation (Dever, 2002; Kaufman 2002). While the apparent benefit of this phosphorylation is a reduced protein load on the cell, UPR genes are also upregulated since one-third of them require eIF2\(\alpha\) phosphorylation for activation (Scheuner et al, 2001).

IRE1 Regulates the Only Pathway Conserved Among All Eukaryotes

Though PERK and ATF6 are important to the cell’s ability to handle ER stress, our research focuses mainly on IRE1; because of its more diverse role in the UPR by initiation of ERAD as well as cell death in more severe cases. Additionally, IRE1 regulates the only pathway conserved among all eukaryotes. IRE1 is composed of an N-luminal domain (NLD), transmembrane domain, Kinase domain, and an RNase domain. It has been demonstrated that the NLD plays a critical role in the effective activation of IRE1 (Liu et al, 2003). The chaperone protein BiP also plays an important role in the UPR. As a chaperone protein, BiP binds to misfolded and aggregated proteins and aides in their management. Because of its function, BiP is bound to IRE1’s N-luminal domain in normal non-stress conditions, thus stabilizing the receptor (Marcu et al, 2002). In the presence of unfolded proteins, however, BiP is released from the NLD and attaches to unfolded or misfolded protein to aide in effectively managing the threat to the cell.

In normal cells, release of BiP from the NLD of IRE1 results in its receptor dimerization and trans-autophosphorylation signaling transition into the active state. Upon activation, the
RNase domain of IRE1 splices a 26 bp segment of X-box binding protein 1 (XBP-1) mRNA. By splicing this 26 bp segment out of XBP-1, a frameshift is created. The result of this frameshift is the highly active transcription factor XBP-1, which enters the nucleus and leads to up-regulation of the UPR genes (Calfon et al, 2002; Urano et al, 2002). According to recent research, the three receptors, PERK, ATF6 and IRE1 work in a time dependent manner. Since ATF6 activation occurs before XBP-1 production, ATF6 causes only refolding of misfolded proteins. Yet upon XBP-1 production, both refolding and the ERAD response occur as a response to the more serious cases of protein misfolding.

The action of IRE1 follows a much different path when the most severe cases of ER stress are experienced within the cell. Unlike the previously mentioned cases, where a manageable protein load is encountered resulting in IRE1 augmented cell rescue, severe stress actually causes IRE1 mediated apoptosis. This apoptotic mechanism occurs when the normal rescue pathway is overridden by burgeoning ER stress. In this case, IRE1 recruits TNF-Receptor Associated Factor 2 (TRAF-2). This in turn causes activation of apoptosis signaling kinase 1 (ASK-1). Activation of ASK-1 leads to apoptosis through induction of c-Jun N-terminal protein kinase (JNK) and caspase dependent apoptosis (Leppa and Bohmann, 1999; Nishitoh, 2002; Urano et al 2000).

It is hypothesized that cell death caused by ER stress is mediated by caspases, a family of cysteine proteases that are effectors of apoptosis. In cases of ER stress induced apoptosis, Caspase 12 has been implicated as a major component. Caspase 12 is an ER localized caspase activated solely under conditions of ER stress such as challenge with tunicamycin, an inhibitor of N-linked glycosylation. In support of this finding, Nakagawa et al. report that upon tunicamycin treatment of renal epithelial cells from wild type and caspase-12 -/- mice, severe damage occurs only in wild type cells (Nakagawa et al., 2000). Since a negligible amount of damage occurs in caspase 12 -/- mice, the authors postulate that caspase 12 must mediate cell death via apoptosis. Activation of the caspase-related death pathway occurs when TRAF2 is initially recruited by Ire1. In non-stress conditions TRAF2 is bound to procaspase-12 but upon TRAF2 recruitment procaspase-12 gathers at the ER triggering its cleavage and subsequent activation (Yoneda et al, 2001).
PROJECT PURPOSE

FV2E-IRE1β: A Drug Inducible System that Activates XBP-1 Splicing

Based on the aforementioned information, we postulate that the key to mitigating ER induced apoptosis is to harness and control the IRE1-ERAD/XBP1 pathway independent of ER-stress. Our aim is to override the natural tendency towards apoptosis by allowing the cell to deal with very high amounts of stress without effecting TRAF2 induction (Figure 2B). To mitigate this ER stress related apoptotic response, we postulate that small molecule regulated dimerization of an engineered IRE1 homolog would prevent the cell’s stress regulatory pathway from being overridden. This could be accomplished by the creation of an engineered construct of IRE1 that could be used to rescue the cells from imminent death under stress conditions by enhancing the UPR. The strategy that we have devised to selectively induce dimerization of IRE1 involves the use of the ARGENT regulated homodimerization kit from Ariad Pharmaceuticals (www.ariad.com/regulationkits). The homodimerization kit, supplied by the company includes a plasmid (PC4M-FV2E) containing two homodimerization domains called FKBP (FV) domains. The FKBP domains can be cut out of the plasmid and inserted into any transmembrane protein using recombinant DNA methods. Once constructed, a receptor bearing an FKBP domain can dimerize to an adjacent FKBP domain when treated with the homodimerization drug AP20187, an extremely efficient third generation ligand suitable for in vivo use (Figure 3). Using a small molecule dimerization system ensures safe, accurate, and precise receptor regulation without interfering with endogenous receptors.

In order to make our construct, the two homodimerization domains were cut out of the plasmid using simple restriction digestion. The resulting digested product was then subjected to a series of modifications resulting in a final modified construct of the beta form of Ire1: FV2E-IRE1β. In order to assure that no background XBP-1 activity was present the construct was introduced into Ire1 knockout fibroblast cell lines, using a retroviral vector. Since there is naturally no XBP-1 splicing in these knockout cell lines, they can be treated with AP20187 and the efficacy of dimerization tested by monitoring the XBP-1 splicing levels.
Figure 3. Utilizing the IRE1 Signaling Pathway to Rescue Cells From Apoptosis.
A: The ER resident receptor IRE1 is a sensor of unfolded protein and consists of an N-luminal domain (NLD), transmembrane domain (TM), kinase domain and an RNase domain. Under non-stress conditions, the chaperone protein BiP is attached to IRE1’s NLD. Under stress, however, BiP is released from IRE1 effecting dimerization and either splicing of XBP-1 mRNA or activation of JNK. B: In order to rescue cells from death, a new form of IRE1 was created in this project by ligating two “FV” homodimerization domains at the 3’ end of the NLD. Upon treatment with the homodimerization drug AP20187, the XBP-1 signaling pathway can be activated without activating the JNK death pathway.
Mutations in the Kinase Domain of IRE1 Often Cause Unnatural Effects

Recently, Papa et al reported that in various mutant forms of IRE1, kinase activity could be bypassed using an ATP competitive drug 1-tert-butyl-3-naphthalen-1-ylmethyl-1H-pyrazole [3, 4-d] pyrimidi-4-ylemine (1NM-PP1). Point mutations were created in the kinase domain that successfully weakened, in varying degrees, the kinase activity of IRE1 and consequently its function as an activator of the UPR. These mutations also created an active site pocket sensitizing the mutant IRE1 to 1NM-PP1. Based upon data from similar experiments in other kinases, treatment with the “new” preferred kinase substrate 1NM-PP1 should have effectively nullified any remaining kinase activity (Papa et al, 2003).

Upon 1NM-PP1 treatment of IRE1 kinase dead mutants, however, the expected results were not observed. Contrary to their hypothesis, 1NM-PP1 treatment of kinase dead mutants actually restored even the most crippled kinases to 80% activity (RNA splicing ability) in comparison to wild type IRE1. Thus, creation of these 1NM-PP1 sensitized mutants bypasses the normal kinase activity and effectively uncouples the kinase and RNase functions of IRE1; activating the UPR apart from a stress signal. The reason for this “stress-free” activation is not completely clear but explorations into the 1NM-PP1 mechanism developed the theory that either ATP or ADP may be IRE1’s natural substrate. If this is so, then IRE1 may sense the intricate ADP/ATP balance within the cell switching on the UPR when cellular nutrients are low based upon the amount of specific adenosine nucleotides within the cell (Schuit et al, 1999). Based upon this research and because we were using a modified form of IRE1 as well, we tested the sensitivity of FV2E-IRE1 to varying levels of ATP as well as combinations of ATP and AP20187.

The function of the IRE1 receptor is very important in the clinical realm since it plays an important role in enhancement of the ER-associated protein degradation system or ERAD. Adapting the FV2E-IRE1β system to perhaps rescue beta cells could potentially facilitate a more effective way to control certain forms of diabetes. With enhanced survivability of beta cells in stressed conditions, the patient’s remaining beta cells would be allowed to regain their normal cycle of neogenesis and replication. This in turn would create a level of beta cells sufficient to maintain normal insulin production as well as facilitation of euglycemia.
METHODS

Isolation of FV2E and Ligation into pCRIITOPO Vector.

In order to create a construct “FV2E-Ire1β,” it was necessary to isolate the FV2E fragment from the vector, pC4M-Fv2E (see figure 4A), provided by Ariad pharmaceuticals (Cambridge, Ma). The plasmid contains two FV’ domains, hence the designation of the dimerization domain as FV2E, an amino terminal myristoylation signal, and a carboxyl-terminal epitope tag flank the 600 bp FV2E fragment. The fragment is also designed with an amino acid wobble in one of the FV segments in order to decrease recombination when using retroviral vectors. The plasmid pC4M-Fv2E was then amplified using PCR primers FV1S (5’-AAAGCTTCTAGAGGCGTCCAAGTC-3’) and FV2AS (5’-CTGCTAGCGTTCCAGTTTTAGAAG-3’). The annealing temperature was 57°C for 30 seconds, with a 68°C extension cycle for 40 seconds. The program was set to repeat for twenty cycles. The PCR primers added a HindIII (5’) overhang and a NheI (3’) overhang to the FV2E fragment. After the PCR, the resulting plasmid was purified using phenol chloroform extraction followed by ethanol precipitation, and reconstituted with 10 µl of TE. This fragment was then ligated into pCRIITOPO using 2 µl of PCR sample, 1 µl of MgCl₂ NaCl, and 1 µl of TOPO vector (Figure 4A). After a 30-minute room temperature incubation, the ligation product was transformed into TOP10 bacteria for overnight incubation. Since pCRIITOPO carries the Lac-Z marker, we were able to select the colonies positive for the insert using blue-white colony selection. After overnight culture, the resulting plasmid was purified using a Qiagen mini-prep kit. The isolate was reconstituted with 50 µl of TE and then restricted for two hours with HindIII and NheI. This fragment was subjected to agarose gel electrophoresis on a 1.2% gel and the 600 bp band isolated using a Qiagen gel purification kit.

Creation of an FV2E-IRE1β Construct.

A tube of mIreβ in pBS-SK (26→37) was obtained, courtesy of David Ron at NYU, and restricted with 1 µl of NheI and HindIII (Figure 4B). This restriction removed a fragment of 46 bp from the 3’ end of the N-luminal domain adjacent to the transmembrane domain. This small
fragment removal should be insignificant enough that no dimerization interference results from NLD distortion. Initially, ligation using T4 DNA ligase was conducted with 11.5 µl of FV2E insert and 1 µl of mIre1β-pBS-SK at room temperature for thirty minutes. Prior to ligation, a comparison of the intensity of the Ire1β versus the Fv2E fragments was conducted by running 5 µl of each on a 1% agarose gel. Transformation of the ligation product into DH5α was carried out using 7.5 µl of ligation product. The ligation was unsuccessful. The purity of FV2E-PCRIITOPO was assayed using sequencing and restriction. The fragment and vector were found to be satisfactory. Subsequent ligations were conducted using different ratios of Fv2E to Ire1β in the following order: 11.5:1, 1:20, 1:9, 0.3:10, 0.5:12, and 0.8:11.7. Due to the unsuccessful ligations, Ire1β was grown on an ampicillin resistant plate and new colonies were grown overnight in order to provide a fresh source of plasmid. The resulting plasmid, purified using the Qiagen miniprep kit, was cut with HindIII and NheI, and purified using phenol chloroform extraction and ethanol precipitation. New ligation concentrations were used; 15:1, 15:0.5, 10:0.5, these ligations were carried out at 16°C in the thermal cycler overnight. The ligation product was then transformed into DH5α. The results of colony PCR showed that the only ratio that produced colonies was 15:1. Results of sequencing showed that the construct was complete and satisfactory except for a mis-sense mutation in a codon for proline. Fortunately, this mutation resulted in no effects since it did not change the amino acid produced.

**Insertion of FV2E-IRE1β into pcDNA3.**

In order to express the engineered construct in mammalian cells, it was necessary to ligate it into the mammalian expression vector pcDNA3 (Figure 4C). The vector pcDNA3 includes a CMV promoter as well as ampicillin and neomycin resistance markers. Restriction enzymes chosen to cut the construct out of pBS-SK were EcoRI and XhoI. It was originally thought that these enzymes were unique to both the insert and the vector. Yet, upon independent restriction tests with each enzyme and target, it was discovered that there is an XhoI site located in the Fv’ domain of the construct. These sites were unique in the vector plasmid, pcDNA3 yielding an isolable 5.3 kb fragment. Thus, before ligation, Fv2E-Ire1β underwent complete digestion with EcoRI, phenol chloroform precipitation, and then partial digestion with XhoI. Following these steps, the 3.5 kb fragment of Fv2E-Ire1β was isolated. As in all previous
ligations, relative ratios of insert and vector were checked prior to the ligation. In the previous ligation of FV2E into Ire1βpBS-SK the molecular mass of Ire1β was considerably greater than that of FV2E. In the FV2EIRE1β-pcDNA3 ligation, the molecular masses of insert and vector are similar, making this ligation particularly difficult. With this in mind, many different ratios for ligation were experimented with. All ligation products were transformed into DH5α high transformation efficiency bacteria. The following ligation ratios of insert to vector were attempted; 14:2, 15:1 (both multiple times with newly isolated insert and vector), 10:0.5. Upon ligation with ratio of 10:0.5, 20 colonies were observed and a colony PCR was conducted. No insert was detected, probably a result of the vector self-ligating. This most likely resulted because the fragment was not isolated from the agarose gel but purified using chloroform phenol extraction. New ligations were also tried with ratio of 18:0.5 resulting in one positive clone. After large-scale culture, a midi-prep was conducted using the Qiagen midi prep kit. Sequencing conducted with T7-2 primers showed that the sequence had ligated into the plasmid backwards. More ligations, with new material proved to give the same backwards ligation effect in all clones, a result of recombination during cloning.

**Insertion of Fv2EIRE1β into pcDNA3.1/hygro**

Since the previous ligations produced unsatisfactory results, a new mammalian expression vector, pcDNA3.1/hygro was chosen (Figure 4C). This vector is similar to pcDNA3, but instead of neomycin resistance, it carries a hygromycin resistance marker. Two unique sites in both insert and vector, NotI and KpnI, were chosen for restriction. Ligation using a ratio of 15:1 was conducted and transformed into DH5α yielding multiple colonies. Upon sequencing, however, the construct was still ligated in the plasmid backwards. Because of this frequent tendency to ligate itself in the wrong direction we decided to use a retroviral vector to express the construct in fibroblasts (Figure 4D).
Figure 4. Construction of FV2EIRE1β.
A: Two “FV” dimerization domains were obtained from the plasmid pC4M-Fv2E by using PCR primers that added a 5’ HindIII site and a 3’ NheI site. The resulting amplified FV2E domain was then ligated into PCRIITOPO and was purified after amplification in bacteria. The plasmid FV2E-PCRIITOPO was then restricted with NheI and HindIII to release the FV2E domain.
B: The FV2E domain was then ligated into IRE1β in pBluescript SK (pBS-SK) and amplified.
C: Ligation and amplification yielded the construct FV2E-IRE1β which was then removed from pBS-SK using EcoRI (5’) and XhoI (3’) sites. Ligations into both pcDNA3 and pcDNA3.1/hygro, using NotI (5’) and KpnI (3’), were unsuccessful due to the complicated nature of the construction.
D: Because these ligations were not successful, a VSV-G retrovirus was created and was used to successfully infect fibroblast cells.
**Transfection Methods and Protein Detection**

In order to transfect the construct into cells, it was necessary to test different methods of transfection with Ire1βpBS-SK. Two different transfection methods were used to introduce the plasmid into mammalian cells. The first involved using the Fugene transfection reagent provided by Roche. In this method, a six well plate of 293-T cells was created. Three wells were controls and three were transfected. To initiate transfection, 9.5 µl of Fugene transfection reagent was added to 300 µl of serum free media in the center of a round bottom tube. Next, 2.5 µl of Ire1β was added to the mixture and then incubated for thirty minutes at room temperature. After this time, the solution was added drop-wise over the cells. After thirty-six hours, the product was collected and protein was isolated. The purified protein was then quantified using a set of standard samples to generate a standard curve. Next, 20 µg of protein was run on a 7% Polyacrylamide-agarose gel for approximately 50 minutes. After electrophoresis, the electrophoresed product was transferred to a nylon membrane using 100 volts for 60 minutes on ice. Incubation with α-IRE1β antibody and then α-rabbit HRP antibody and standard western blot tests were negative for protein expression.

In light of the failed transfection using Fugene, we decided to use the Calcium Phosphate method to transfect another six well plate of 293T cells with Ire1β. A solution of plasmid DNA, water and 2M CaCl2 was created and added to 2X HEPES. This solution was allowed to incubate for five minutes at room temperature and then added drop-wise over the cells. The resulting protein was isolated using Immunoprecipitation, but otherwise the same protocol as above. This time, however, the results were positive for transfection.

**Creation of a Retroviral Vector for Transfer of FV2E-IRE1β into Fibroblasts and MIN6 Cells**

In order to create an adequate amount of virus particles for infection we first plated 2x10^6 293T cells into two plates for future use as a “virus factory.” During the time the cells were multiplying, the plasmids pBabe Puro and FV2E-IRE1βpBS-SK were cut with EcoR1 and Sal1 (Xho1). The two fragments (FV2E Ire1 and pBabe Puro) were then ligated overnight to produce the construct FV2E-IRE1β-pBabe Puro which was then transformed into STBL2 bacteria and isolated using a Qiagen midiprep kit. Using the CaCl2 protocol, the 293T cells were transfected with the following components for retrovirus assembly: 0.4 µg pCMVtatHIV, 1.5 µg, 3 µg pJK3
pL-VSV-G, and 4.5 µg FV2E-IRE1β-pBabe Puro. Before application to the cells, water was added up to 876 µl and then mixed with 124 µl of 2 M CaCl2. After it was thoroughly mixed, 1 mL of 2X Hepes was added to the mixture and the final solution was added to the cells drop wise. The cells were kept away from light at all times so as not to destroy the virus.

**Collection of the FV2E-Ire1β-pBabe Puro Retrovirus and Infection into a Fibroblast Cell Line.** The cells produced above were allowed to grow overnight and the media was changed the following morning. **Note:** In order to assure proper lab safety technique, all cells were **manipulated under a properly ventilated hood and all media and equipment used was bleached to prevent contamination/infection.** New media was added and the following day, 5 mL of media was collected and placed in a foil wrapped tube. New media was added to the plates and the procedure was repeated the following day. The 293T cells were then bleached and discarded. The viral media collection from the two days was pooled and then filtered through a 0.4 µm filter. The filtered media containing the virus was then added dropwise over two plates containing fibroblast cells.

**Creation of a Stable Cell Line Expressing FV2E-Ire1β.**

Infected fibroblast cells were allowed to grow overnight and on the following day, the media was changed and treated with 2.5 µl of puromycin to select the fibroblasts bearing the construct and expression vector. Twelve colonies were picked one week later and allowed to grow in 24 well plates and then transferred to several individual plates. Puromycin selection was carried out each time the media was changed. In order to develop a stable MIN6 cell line expressing FV2E-Ire1β, we used an identical cell culture technique as listed above for fibroblast cells but instead added the virus to MIN6 cells and used 2.0 µl of puromycin for selection.

**Collection of Nuclear and Cytoplasmic Protein Extracts**

Because isolation of both nuclear and cytoplasmic extracts is critical to development of this receptor, we used a combined isolation method for cells used in protein detection. Plates were first washed twice with cold PBS and then plates were placed on ice and 1 mL of cold PBS was added to the plates. These plates were scraped then the cells/PBS was transferred to 1.7 mL tubes and the tubes were spun (at 4°C) at 3,000 rpm for 5 minutes to pellet the cells. The
supernatant was poured off and then the pellet resuspended with 500 µl of harvest buffer (10mM HEPES, 50 mM NaCl, 0.5 M sucrose, 0.1mM EDTA, 0.5% 100X triton plus proteinase inhibitors). After adding harvest buffer, the cells were incubated for 5 minutes on ice and then spun at 1,000 rpm in a swinging bucket rotor in order to pellet the nuclei. The supernatant was transferred to new tubes and spun for 14,000 rpm for 15 minutes to isolate and further purify the cytoplasmic extract. The cytoplasmic extract was then placed in new tubes and stored at -80°C. The pellet from the 1,000 rpm centrifugation was then washed in 500 µl buffer “A” (10mM HEPES pH 7.9, 10 mM KCL, 0.1mM EDTA, 0.1mM EGTA plus proteinase inhibitors) and spun for 5 minutes. After removing and discarding the supernatant, the pellet was resuspended in 50 µl of buffer C (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% IGEPAL (NP40). The resuspended pellet was vortexed for 15 minutes at medium to high speed to break up the pellet. For final isolation of the nuclear extract, the tubes were spun for 10 minutes at 14,000 rpm and the supernatant transferred to new tubes and frozen at -80°C.

Antibodies and Protein Detection

Detection of FV2E-Ire1β. In order to be sure that FV2E-IRE1β was in fact expressed in transfected fibroblast cells, we collected cytoplasmic protein extract from transfected cells and analyzed the result using western blot analysis. To assay for the presence of the insert we used α-IRE1β antibody in a 1:3,000 concentration. Since this antibody was raised against the transmembrane domain of IRE1β it is also effective for FV2E-IRE1β detection since the transmembrane domains are identical. For a secondary antibody we used α–rabbit polyclonal HRP tagged antibody in a 1:3,000 concentration.

Detection of XBP-1 Protein and mRNA. XBP-1 mRNA produces a protein transcriptional activator that is easily detected using western blot analysis. To detect XBP-1 protein, nuclear extract was collected and α-XBP1 antibody was used in a 1:3,000 dilution as a primary antibody. As the secondary antibody we used α-rabbit HRP, in the same dilution. In order to assay for the presence and activity of XBP-1 mRNA, RNA was collected from cells and cDNA was created from these RNA’s. From these cDNA’s we used real time PCR methods as described below to explore the characteristics of XBP-1 transcription.
Detection of GRP-78. Since the chaperone molecule GRP-78/BiP is attached to inactivated IRE-1 detecting high levels of GRP-78 in the cytoplasmic extract indicates activation. As a primary antibody, α-GRP78 antibody was used in a 1:5,000 concentration. Rabbit polyclonal antibody with an HRP tag was used as a secondary antibody.

Detection of HRD1. As previously mentioned, the E3 ubiquitin ligase HRD1 is a major factor in the ERAD response and thus it’s detection in the cytoplasmic isolate indicates the ERAD response is active. In order to detect the protein, α-HRD1 antibody was used in a 1:1,000 concentration. The secondary antibody, α-rabbit HRP, was used in a 1:3,000 concentration.

Detection of JNK. In order to be sure that the construct does not cause cell death, we tested selected nuclear isolates for phosphorylated JNK apoptosis kinase using α–JNK antibody in a 1:3,000 concentration. The secondary antibody was α–rabbit polyclonal HRP tagged antibody.

Detection of Caspase-12. As a secondary indicator of cell death we also observed the activation of Caspase-12 using a 1:3,000 concentration of α-Caspase 12 antibody and α–rabbit polyclonal HRP tagged antibody as a secondary antibody.

Detection of Actin. In order to be sure that western blot results were not flawed due to unequal protein amounts, we incubated all membranes with α-Actin protein in a 1:3,000 concentration after primary and secondary incubation with another specific antibody. In this case we used α–mouse monoclonal HRP tagged antibody in a 1:3,000 concentration.

Detection of Phosphorylated IRE. In order to determine stress levels in MIN6 and Hela cells, we used α-Phospho IRE1 antibody in a 1:1,000 concentration. The secondary antibody was α–rabbit polyclonal HRP tagged antibody.
**Detection of Spliced XBP-1 mRNA**

Spliced XBP-1 mRNA was detected either by real time PCR or with normal PCR methods. Using a 2.5% agarose gel, and 5S (5’-TGGCCGGGTCTGCTGAGTCCG-3’) and 10AS (5’-TCCATGGGAAGATGTTCTGG-3’) primers, splicing was detected by the presence of slightly smaller bands, whereas unspliced mRNA appears as a larger band. This differential detection method is excellent to verify the presence of both spliced and unspliced mRNA. For quantification of splicing, it was necessary to use real time PCR, with two different sets of primers. For detection of spliced sequences, primers 11S(5’-TGAGTCCGAATCAGGTGCAG-3’)-10AS provides a definitive detection method. Use of primers 7S(5’CAGCACTCAGACTATGTGCA-3’)-10AS is sufficient to detect unspliced XBP-1 mRNA.

**Drug Dosage Experiments**

In order to determine the optimum amount of AP20187 to use in our experiments we conducted a series of experiments using different dosages of the drug. Final concentrations, when diluted in 10 mLs of media were as follows: 100 nM, 100 pM, 10 pM, and 1 pM. All of these dilutions were made from a stock solution of 1 mM AP0187. For our ATP dosage experiments, we used a dosage of 100 µM. For stress induction in cells, we found that using 5 µl of tunicamycin was sufficient to elicit a satisfactory stress response.
RESULTS

Developing an Fv2E Construct

Construction of FV2E-pCRIITOPO. As previously stated, the fragment FV2E was developed with an overhang of HINDIII and NheI (Figure 4A) and then ligated into pCRIITOPO. The final construct of Fv2E-pCRIITOPO was a final size of 4.6 kb and contained the Fv2E fragment between the HINDIII and NheI sites (Figure 4B).

Final Construction of Fv2E-Ire1β. As mentioned before the expected results of ligations into pcDNA3 and pcDNA3.1/hygro were backwards. In pcDNA3, the 5’ end of Fv2E-Ire1βPBS-SK should have ligated into the EcoRI site of pcDNA3 and the 3’ end into the XhoI site. About 1.3 kb downstream of this sequence sits the 0.6 kb FV2E fragment flanked by a 5’ HindIII site and a 3’ NheI site. The inserted fragment is located at the 3’ end of the of the N-Luminal domain. Following this insert, the structure of Ire1β is unadulterated and includes from 5’-3, the transmembrane domain, Kinase domain and the RNase domain (Figure 5). As for ligation into pcDNA3.1/Hygro (-), the structure of the insert remains the same, except that the ligation of Fv2E Ire1β was accomplished using NotI (5’) and KpnI (3’). Since this ligation was unsuccessful, however, we instead used a retrovirus expression system yielding successful incorporation of the FV2EIRE1β construct into the ER membrane of IRE1 knockout fibroblast cells.
Figure 5. The Structure of the Final Functional Construct of FV2EIRE1β.

A: Endogenous IRE1β contains an N-luminal domain (NLD) that participates in the stress sensing and dimerization capabilities of IRE. Adjacent to the NLD is a transmembrane domain as well as a kinase domain which participates in trans-autophosphorylation. IRE1’s RNase domain splices XBP1 mRNA in response to stress. B: The construct “Fv2E-Ire1β,” is a modified form of IRE1β in which a small 46 bp fragment is removed from the N-Luminal domain (NLD) and replaced with the dimerization domain “Fv2E.” This alteration of the NLD should not be enough to impede the NLD’s critical role in dimerization.

Detection of XBP-1 in Stressed Cells

In order to verify that our IRE1 knockout cell lines were in fact absent of all XBP-1 splicing we first tested these cell lines for XBP-1 splicing under stress. In experiments using wild type and double knockout Ire1α and Ire1β fibroblast cell lines, we were able to detect XBP-1 splicing in stressed wild type cells. XBP-1 splicing was detected in wild type but not knockout cells when cells were treated with cell stress activators thapsigargin (Tg) and tunicamycin (TM), two strong activators of ER stress (Figure 6).

The Fv2EIRE1β System is a Stress Response Switch

In order to verify that the receptor FV2E-IRE1β is a system that acts independently of cellular stress, our first experiments tested for expression of the transcription factor XBP-1 in FV2E fibroblast cells under various conditions. Using western blot analysis we found that FV2EIRE1β can activate the cellular stress pathway that normally causes expression of XBP-1 protein. Also, since Tunicamycin causes XBP-1 expression in wild type cells we treated FV2E
cells with the drug and analyzed the lysates using western blotting analysis found that there is no XBP-1 expression, indicating that the receptor is controlled solely with AP20187 (Figure 7).

Figure 6. Detection of XBP-1 Splicing.
IRE1α and IRE1β wild type and knockout fibroblast cells were either untreated (UT), or treated to induce stress with 5 ml of thapsigargin (Tg), or 5 ml tunicamycin (TM). Lysates were collected after 4 and 8 hours and RNA was isolated from these lysates. After using reverse transcriptase PCR methods with primers specific for XBP-1 activity the samples were run on a 2.5% agarose gel. In wild type cells treated with TM and Tg, splicing was observed as the presence of two bands on the gel. Only one band was observed in double knockout cell lines indicating that even under stress no XBP-1 splicing had occurred.

Figure 7. Production of the Active Form of XBP-1 Transcription Factor by FV2E-IRE1β.
Immunoblot of XBP-1 in lysates of IRE1 knockout cells expressing FV2E-IRE1β. Cells were treated with either AP20187 or tunicamycin (TM) and lysates analyzed using western blotting with a-XBP-1 antibody. Analysis of the results reveals that the FV2E-IRE1β system effectively uncouples XBP-1 production from stress and that the receptor can induce XBP-1 transcription factor production upon treatment with AP20187.

Discovering Other Important Characteristics of the FV2E-IRE1/AP20187 System
Although we have observed that tunicamycin treatment independent of AP20187 treatment can not induce XBP-1 splicing, additional data demonstrates that FV2E-Ire1β activity is strongly enhanced when AP20187 treatment is combined with tunicamycin dosage. The data gathered on the E3 ubiquitin ligase HRD1 displays conditions identical to those previously mentioned. This data also shows that dosage with ATP does not enhance FV2E activation but may actually hinder it. This could be a result of binding site competition from ATP. In order to be sure that our system is not detrimental to cells, because Ire1 activation can induce ASK1 leading to JNK induced apoptosis, we checked the levels of JNK expression in FV2E treated
cells. Fortunately, AP20187 treatment does not cause JNK kinase induction in FV2E expressing cell lines (Figure 8).

**Table FV2E-IRE1β Fibroblasts**

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*Figure 8. Comprehensive Testing of the FV2E-IRE1β System.* Immunoblot of lysates from IRE1 double knockout fibroblast cells transfected with FV2E-IRE1β. Cells were treated were either left untreated, or treated for 5 hours with either ATP, AP20187, AP20187 and tunicamycin (TM), AP20187 and ATP, or AP20187, ATP, and TM. Lysates were then checked for the presence of XBP-1 protein, HRD-1, and JNK, while actin was used as a loading control. Although treatment with TM alone does not enhance levels of XBP-1 transcription factor production, the combination of AP20187 and TM greatly increases the efficiency of FV2E activation. Production of XBP-1 protein is not, however, enhanced with ATP dosage alone or in combination with AP20187. These characteristics are again observed in upregulation of HRD1. Finally, treatment with AP20187 does not significantly induce activation of JNK kinase.

**The FV2E-Ire1β System is Time and Dosage Dependent**

In order to assure accuracy of results and optimal AP0187 performance, we tested various dosages of AP20187 on FV2E cells. Our research shows that the system demonstrates the best results when the final concentration of AP20187 is 10 picomols (Figure 9B). We postulate that the receptor reaches saturation at this concentration and we have also found that the best activation occurs with 2 hours of treatment (Figure 9A). It should be noted, however, that receptor activation with AP20187 is not as strong as the endogenous stress response in wild type cells.
**Figure 9. FV2EIRE1β Exhibits Activation in a Time and Dosage Dependent Manner.**

A: Immunoblot of lysates from IRE1 double knockout fibroblast cells transfected with FV2E-IRE1β (FV2E fibroblasts) in comparison to wild type and knockout fibroblast cells. FV2E fibroblast cells were either left untreated, or treated with AP20187 for 1, 2 and 5 hours. Wild type and knockout cell lines were left untreated or treated with tunicamycin (TM) for 5 hours. All lysates were then collected and analyzed using western blotting with α-XBP-1. In FV2E fibroblasts, the best XBP-1 protein production was observed at 2 hours of AP20187 treatment but was not as strong as the wild type cell’s XBP-1 response to TM. No XBP-1 transcription factor was produced in IRE1 α-/- cells. B: To test for time dependency, FV2E fibroblasts were treated with AP20187 in concentrations of 100 picomols (pM), 10 pM, and 1 pM. Lysates were then collected and tested using western blotting with α-XBP1 to determine at what concentration peak production of XBP-1 occurs. Optimal XBP-1 transcription factor production occurs when cells are treated with 10 pM of AP20187.

<table>
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<th>FV2E fibroblasts</th>
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![Western Blot](image)

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DISCUSSION

Although much significant data has already been discovered in this research, there are still many important discoveries to be made using this receptor. The most important factor in continuing the project was the discovery that using FV2E-IRE1β instead of endogenous Ire1 uncouples IRE1 activity from cell stress. Thus, XBP-1 splicing can be controlled solely using the inducer AP20187, and is independent of even the strongest stress. This is beneficial as it gives the scientist complete control in eliciting an unconditional stress survival response. Additionally, because XBP-1 splicing is induced upon treatment with AP20187, HRD1 is also activated. Activation of HRD1 is of paramount importance in rescuing cells from stress as it targets misfolded proteins for destruction through the ERAD response via the ubiquitin proteasome pathway. Since our preliminary data shows that FV2EIRE1β activation does not significantly increase activation of the JNK “death kinase” we postulate that the system helps to mitigate cell death most likely through ERAD. We postulate that normal activation of IRE1 may be transient because of the fluctuating amount of stress within the cell. If the transient activation of IRE1 occurs in high frequency, JNK may be activated to deal with the stress. When using AP20187, however, the activation of IRE1 is constant and controlled and thus does not send the cell into the JNK death pathway. In order to test these conclusions, we plan to conduct apoptosis and cell viability assays using AP20187 under a variety of different stresses and time conditions.

Although we have successfully observed AP20187 stress-independent activation, XBP-1 splicing, XBP-1 expression and HRD1 activation our data indicates that these responses are much stronger with the combination of tunicamycin and AP20187. Based on this phenomenon, we hypothesize that there may be other signals important to the full activation of IRE1. Our conclusion is that treatment with only AP20187 does in fact induce dimerization of FV2EIRE1β, but since no stress has caused the release of the chaperone BiP from its binding site on FV2E’s N-luminal domain, BiP may impede drug induced dimerization. Treatment with tunicamycin, however, induces protein misfolding and thus releases BiP from the N-luminal domain to aide in
protein folding. Consequently, we observe that induction of XBP-1 splicing and HRD1 activity drastically increases. Concomitant with this activity, we also see minimal JNK kinase activity which indicates that small molecule induced dimerization of FV2EIRE1β is much more effective than stress induced dimerization of endogenous Ire1β and provides the cell with a method to bypass this death signal. In addition to becoming familiar with these pathways in FV2EIRE1β implanted cells we would also like to develop a gene expression profile of all downstream targets of the receptor using DNA microarray analysis.

In an effort to further test our receptor, we would like to observe its activation in a cell line already under stress. We have recently observed that mouse insulinoma cells (MIN6), a cancerous β-cell line, are already naturally under stress. Because MIN6 cells meet both of the aforementioned requirements, we have recently created a stable MIN6 cell line expressing FV2EIRE1β. This is a very important step since misfolded insulin in pancreatic β-cells may be responsible for misfolded insulin associated apoptosis in some forms of diabetes. Furthermore, we hypothesize that the MIN6-FV2EIRE1β system will yield a good amount of high quality data since the receptor demonstrates peak efficiency under stress conditions.

Following successful studies in MIN6 cells, we will create an animal model using the receptor in order to bring the research into a more clinical realm. In animal studies we plan to create a vector system with a rat insulin promoter, which will target localization of the receptor in β-cells. We will create a strain of mice that can be studied by mating a C57/B6 background mouse positive for the construct infection with an Akita diabetes model mouse. This should produce, in one quarter of the offspring, an Fv2E:Akita/+ strain. In this strain of mice we will leave half as untreated controls, and half will be treated with AP20187. In theory, these diabetic Fv2E mice should display a vast improvement in diabetes and rescue from cell stress when treated with AP20187.

With the development of this receptor, we hypothesize that we can successfully rescue pancreatic β-cells from stress caused by misfolded proteins. Recently, Iwawaki et. al has developed a fluorescence reporter system that helps monitor ER stress by fluorescing in the presence of spliced XBP-1. The system is a fusion of the green fluorescent protein venus and the gene for XBP-1. Upon XBP-1 splicing and resultant XBP-1 protein translation, fluorescence is emitted as an indicator of stress. This system is of particular importance in our research as it can be transfected into a cell line that we are studying. Once transfected the reporter and will yield
fast and accurate data about the level of XBP-1 splicing by fluorescence emission. In the future, this system would be very effective in stress analysis and developing drug half lives. (Iwawaki et al, 2004).

The relative importance of this system has been demonstrated with the development of another FV2E dimerization system. The other FV2E based system, FV2E-PERK, was recently created by Lu et al and successfully rescued cells from stress. Much like our receptor, the system uses AP20187 to effect stress independent dimerization of a recombinant form of PERK containing two FV’ domains. Dimerization of FV2E-PERK induced phosphorylation of eIF2α and caused suppression of general translation. In suppressing general translation, cells were allowed the time to deal with and be successfully rescued from ER stress as well as oxidants and peroxynitrites (Lu et al, 2004). With further study and creation of animal models we should be able to show similar cytoprotective effects using FV2EIRE1β.
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