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Evaluation of the Unfolded Protein Response: PI 3-Kinase Regulatory Subunits as Regulators of the IRE1 α Pathway

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Abstract

The endoplasmic reticulum (ER) consists of an interconnected, membranous network that is the major site for the synthesis and folding of integral membrane and secretory proteins. Within the ER lumen, protein folding is facilitated by molecular chaperones and a variety of enzymes that ensure that polypeptides obtain their appropriate, tertiary conformation (1,2). Physiological conditions that increase protein synthesis or stimuli that disturb the processes by which proteins obtain their native conformation, create an imbalance between the protein-folding demand and capacity of the ER. This results in the accumulation of unfolded or improperly folded proteins in the ER lumen and a state of ER stress. The cellular response, referred to as the unfolded protein response (UPR), results in activation of three linked signal transduction pathways: PKR-like kinase (PERK), inositol requiring 1 α (IRE1 α) and activating transcription factor 6 α (ATF6 α) (3,4). Collectively, the combined actions of these signaling cascades serve to reduce ER stress through attenuation of translation to reduce protein synthesis and through activation of transcriptional programs that ultimately serve to increase ER protein folding capacity. Recently, we and Park et. al have characterized a novel function for the p85 α and p85 β subunits as modulators of the UPR by virtue of their ability to facilitate the nuclear entry of XBP-1s following induction of ER stress (5,6). This chapter describes the recently elucidated role for the regulatory subunits of PI 3-kinase as modulators of the UPR and provides methods to measure UPR pathway activation.

1. The Unfolded Protein Response

The endoplasmic reticulum (ER) is the site for the biosynthesis of lipids, sterols as well as integral and secreted proteins (7,8). The vast majority of nascent polypeptides require folding and/or enzymatic modifications to obtain their appropriate conformation. A variety of pathophysiological states including obesity, hyperlipidemia, nutrient deprivation, hypoxia, infection and inflammation have been shown to disrupt ER homeostasis leading to the development of ER stress(9). In response, signaling pathways of the UPR are activated in an attempt to resolve this condition through attenuation of translation to reduce protein load and through activation of transcriptional programs that ultimately increase protein folding. This coordinated response is mediated by the combined action of three ER stress

sensors: PKR-like kinase (PERK), inositol requiring 1 α (IRE1 α) and activating transcription factor 6 α (ATF6 α) (Figure 1).

PERK is a type I transmembrane protein that is composed of a luminal region that senses the presence of unfolded proteins and a cytoplasmic domain that possesses kinase activity. In the absence of ER stress, the binding of the ER chaperone BiP to the luminal domain of PERK prevents homo-dimerization and activation. Upon induction of ER stress, BiP is sequestered from PERK, allowing for dimerization, trans-phosphorylation and stimulation of kinase activity directed towards cytosolic substrates (10,11). One such substrate is eukaryotic initiation factor 2 (eIF2 α), which when phosphorylated by PERK, reduces the rate of polypeptide chain elongation, leading to a decline in mRNA translation and a subsequent reduction in protein folding demand within the ER (12,13).

The second unfolded protein sensor is the ER transmembrane transcription factor, ATF6 α . Accumulation of unfolded proteins in the ER lumen leads to the trafficking of full-length ATF6 α (p90ATF6) to the Golgi where it undergoes sequential cleavage by site 1 (S1P) and site 2 (S2P) proteases to produce a mature, transcriptionally competent transcription factor (p50ATF6)(14,15). This transcriptionally active form of ATF6 α translocates to the nucleus where it binds directly to the ERSE and cooperates with XBP-1 (x-box binding protein 1) to induce UPR target-genes (14,16).

The final sensor is IRE1 α , which is composed of a luminal domain that serves as an unfolded protein sensor and a cytosolic portion composed of a kinase and RNase domain (17,18). In a fashion similar to the activation of PERK, BiP sequestration following ER stress leads to oligomerization of IRE1 α , trans-phosphorylation and subsequent stimulation of RNase activity(19). This activity is directed towards the XBP1 pre-mRNA to generate two distinct mRNA transcripts (Figure 2a). The first ORF (XBP1u) generates a transcript that encodes for a protein that is devoid of transcriptional activity. The second ORF (XBP1s) is created when IRE1 α removes a 26-nucleotide intron to generate a larger protein containing both a DNA binding and transactivation domain (20,21). XBP1(s) translocates to the nucleus where it binds to the ER stress-response element (ERSE) in the promoters of genes involved in lipid synthesis, ER biogenesis and the UPR including BiP, ERdj4, PDI and p58^{IPK}(22–24).

2. PI 3-kinase Regulatory Subunits as Modulators of the UPR

Class Ia PI3Ks are a family of lipid kinases that regulate multiple cellular processes including metabolism, growth, survival, vesicular trafficking and cytoskeletal rearrangements by virtue of their ability to generate the second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP₃). The enzyme exists as a heterodimer composed of a catalytic and regulatory subunit, both of which occur in several isoforms (25–28). The p85 α and closely related p85 β regulatory subunits are highly homologous and share identical organization of functional domains (Fig. 2). The two src-homology 2 (SH2) domains bind tyrosine phosphorylated YxxM motifs with high specificity, allowing for interaction with substrates downstream of receptors possessing tyrosine kinase activity (29). The region between these SH2 domains serves as the binding site for catalytic subunits(30).

The roles of the SH3, proline-rich and BH domains of p85 α and p85 β are less well defined. In an attempt to identify novel, protein-protein interactions mediated by the NH₂-terminal region of p85 α encompassing these domains, a bacterial two-hybrid screen was performed. One unanticipated finding of this screen revealed an interaction between the NH₂-terminal portion of p85 α and XBP-1.

Additional experiments confirmed this physical association and tentatively mapped the interaction interface on p85 α to the BH domain (5,6). Interestingly, over-expression of p85 α or p85 β led to a significant increase in the nuclear accumulation of XBP-1s, suggesting an endogenous role for p85 regulatory subunits as regulators of XBP-1s nuclear shuttling.

Not surprisingly, subsequent analysis of p85 α -deficient cell lines and cells with reduced p85 α expression mediated by shRNA-mediated gene silencing showed a significant alterations in the UPR, including reduced ER stress-dependent accumulation of nuclear XBP-1s and an associated decrease in UPR target gene induction(6,30). Furthermore, mice with deletion of p85 α in liver (L-Pik3r1^{-/-}) showed a similar attenuation of the UPR after tunicamycin administration, an effect most likely due to a reduced accumulation of XBP-1s in the nucleus (5). Collectively, these data demonstrate an important role for p85 α/β as important regulators of XBP-1s nuclear trafficking.

3. Assessing UPR Pathway Activation

In this section, we will describe specific reagents and outline protocols that will facilitate the evaluation of the PERK, ATF6 α and IRE1 α activation. In addition, secondary endpoints that can be used to assess the extent of pathway activation will be described including eIF2 α phosphorylation, XBP-1 splicing and translocation as well as target-gene expression.

3.1 PERK

Based upon our experience in liver, hepatic cell lines, mouse embryonic fibroblasts (MEFs) and immortalized brown pre-adipocytes, the ability to detect the activation of the PERK pathway by immunoblot analysis using phospho-specific antibodies is fraught with difficulties(5). We speculate that this may be due to differences in protein expression, degree of activation relative to other UPR pathways or the transient nature of PERK activation in any given experimental system. In the event that PERK phosphorylation is not detected in straight lysates, immunoblots performed with anti-PERK antibodies on suitably resolved protein lysates can reveal a molecular weight shift indicative of PERK phosphorylation. Alternatively, immunoprecipitation of PERK followed by immunoblotting with phospho-PERK antibodies has been described as a reasonable approach to detect PERK phosphorylation. Lastly, the phosphorylation-state of eIF2 α is often used as a surrogate to measure the extent of PERK activation. Importantly, Interpretation of these data must acknowledge that eIF2 α is a substrate for a number of enzymes including PKR, GCN2 as well as PERK.

3.1.1 Preparation of Cell Extracts

1. Treat 80–100% confluent cells with vehicle or experimental agents.

2. Wash cells two times with ice cold PBS and snap freeze in liquid nitrogen.
3. Thaw cells on ice until first signs of thawing are identified.
4. Add lysis buffer to obtain lysates with sufficient protein content to perform downstream analysis:
 - Tris-HCl: 50 mM, pH 7.4
 - NP-40: 1%
 - Na-deoxycholate: 0.25%
 - .01% SDS
 - NaCl: 150 mM
 - EDTA: 1 mM
 - Phosphatase inhibitors
 - Protease inhibitors
5. Collect lysates and transfer to 1.5 ml eppendorf tube. Rotate lysates at 4° for 10 minutes.
6. Spin lysates at 14,000 rpm for 15 minutes. Transfer cleared lysates to new tubes.
7. Perform protein assay to determine protein concentration.
8. Resolve protein lysates by SDS-PAGE and perform immunoblot analysis.

Required Materials:

- Cells or tissue of interest: Liver, Huh7, MEFs or brown pre-adipocytes.
- Cell Culture Medium: The above cell lines are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 2% glutamine, penicillin and streptomycin.
- Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO)
- Phosphatase Inhibitor Cocktail 2 & 3 (Sigma Aldrich)
- Phospho-PERK (Thr 980) (16F8) rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA)
- PERK (C33E10) rabbit monoclonal antibody (Cell Signaling Technology)
- Phospho-eIF2 α (Ser 51) (119A11) rabbit monoclonal antibody (Cell Signaling Technology)
- eIF2 α antibody (Cell Signaling Technology)
- BM Chemiluminescence Western Blotting Kit (Roche Applied Science, Indianapolis, IN)

3.2 ATF6 α

Upon induction of ER stress, ATF6 α is released from the ER and is transported to the Golgi where it undergoes cleavage by the S1P and S2P proteases. These cleavage events produce a potent transcription factor that transits to the nucleus to participate in a complex transcriptional program that ultimately serves to relieve ER stress and restore ER protein folding capacity(14). Therefore, an assessment of ATF6 α cleavage and nuclear accumulation serves as an accurate gauge of ATF6 α pathway activation. In our experience, the preparation of nuclear extracts (see section 3.3.2) is often required to detect the cleaved, mature ATF6 α (p50ATF6 α).

Required Materials

- ATF6 α antibody (H-280) (Santa Cruz Biotechnology, Santa Cruz, CA)

3.3 IRE1 α

The degree of IRE1 α pathway activation can be assessed by performing immunoblot analysis with phospho-specific antibodies (5). Unfortunately, the ability to detect the activation of IRE1 α using this approach is dependent on the cell line and experimental context. For example, although the phosphorylation of IRE1 α is easier to assess in pancreatic β -cell lines (i.e. Min6), we have experienced limited success in a variety of additional tissues and cell lines. Alternative approaches to detect IRE1 α activation include evaluation of IRE1 α mobility by SDS-PAGE or immunoprecipitation of IRE1 α prior to immunoblot analysis with phospho-specific antibodies. Due to the difficulty of reliably detecting IRE1 α phosphorylation, the IRE1 α -dependent splicing of the XBP-1 mRNA is often performed as an acceptable surrogate to assess IRE1 α pathway activation. In addition, the accumulation of nuclear XBP-1s is an excellent way to evaluate activation of the IRE1 α pathway or to assess the impact of p85 α / β on XBP-1 nuclear transport.

3.3.1 XBP-1 Splicing Assay—The protocol outlined in this section has been adapted from the original report that first described the IRE-1-dependent splicing of XBP-1 in response to ER stress (22). Splicing of the XBP-1 transcript is detected by amplifying the region of the XBP-1 cDNA encompassing the 26 nt intron that is excised following induction of ER stress (Figure 3 a & b). To distinguish the spliced and un-spliced amplicons, the PCR products can be carefully separated by agarose gel electrophoresis or digested with the Pst I restriction endonuclease that digests only un-spliced cDNA to produce two DNA fragments (Figure 3 b & c).

1. Perform reverse transcription on 1–2 μ g of total RNA isolated from control and experimental conditions using random hexamers to prime the reaction.
2. Dilute the cDNA synthesis reaction 1:10–1:20 with H₂O.
3. Prepare a PCR reaction using 5.0 μ l cDNA and 10 mol of mXBP-1 forward and reverse primers.
4. Perform PCR using the following conditions:

94° 4 minutes

94° 10 sec. (40 cycles)
 66° 30 sec.
 72° 30 sec.
 72° 10 minutes

5. Perform Pst I restriction digest on 10 µl of PCR product.
6. Resolve restriction digest on 2–3% agarose gel.

In most instances, the amount of cDNA or the number of cycles of PCR amplification will need to be adjusted to work optimally in any given experimental system.

Required Materials

- Phospho-IRE1α (Ser 724) rabbit polyclonal antibody (Novus Biologicals, Littleton, CO)
- IRE1α (14C10) rabbit monoclonal antibody (Cell Signaling Technology)
- High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA)
- mXBP-1 for 5' GGCCTTGTGGTTGAGAACCAGGAG 3', mXBP-1 rev 5' GAATGCCCAAAGGATATCAGACTC 3'
- Pst I restriction enzyme (New England Biolabs, Ipswich, MA)
- Reagents for PCR
- Agarose Gel Electrophoresis equipment

3.3.2 XBP-1s Translocation—The protocol outlined in this section allows for an assessment of XBP-1s translocation to the nucleus by performing XBP-1 immunoblot analysis on cytoplasmic and nuclear protein fractions from mammalian cell lines following induction of ER stress.

1. Perform any treatment of cells at ~90% confluency.
2. Wash two times with ice cold PBS.
3. Scrape cells into PBS containing Phosphatase and protease inhibitors.
4. Centrifuge 5 minutes at 4,000 rpm (4°C)
5. Re-suspend cell pellet with 400 µl of buffer A containing protease inhibitors:
 - 10 mM HEPES (pH 7.9)
 - 10 mM KCl
 - 0.1 mM EDTA
 - 0.1 mM EGTA
6. Add 25 µl of 10% NP-40.
7. Vortex tube for 15 seconds.

8. Centrifuge for 5 minutes at 13,000 rpm (4°C)
9. Collect the supernatant as the soluble, cytoplasmic protein fraction.
10. Add 50 µl of buffer B containing protease inhibitors and re-suspend:
 - 20 mM HEPES (pH 7.9)
 - 400 mM NaCl
 - 0.1 mM EDTA
 - 0.1 mM EGTA
11. Rock the tube for 20 minutes at 4°C.
12. Centrifuge for 15 minutes at 13,000 rpm (4°C)
13. Collect the supernatant as the nuclear protein fraction.
14. Perform anti-XBP-1 immunoblots on cytoplasmic and nuclear fractions.

Required Materials

- Cells or tissue of interest: Liver, Huh7, MEFs or brown pre-adipocytes.
- Cell Culture Medium: The above cell lines are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 2% glutamine, penicillin and streptomycin.
- Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO)
- Phosphatase Inhibitor Cocktail 2 & 3 (Sigma Aldrich)
- Anti-XBP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA)
- Reagents for SDS-PAGE

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Figure 1. The UPR Pathway

The pathway consists of three proximal sensors: PERK, IRE1 α and ATF6 α . Their coordinated response serves to restore ER folding capacity by inhibiting protein translation (PERK) and orchestrates a transcriptional response that ultimately increases protein-folding capacity (IRE1 α & ATF6 α). BiP negatively regulates these pathways by binding to the luminal domains to prevent activation. The induction of ER stress and accumulation of misfolded proteins in the ER lumen causes the release of BiP from PERK, IRE1 α and ATF6 α , leading to their subsequent activation. PERK dimerization leads to trans-phosphorylation and activation, resulting in the phosphorylation of eIF2 α and a global decrease in the rate of protein translation. However, some mRNAs, such as ATF4 mRNA, are translated under these conditions, possibly by the presence of open reading frames within the 5' untranslated region. BiP release from IRE1 α permits dimerization and activation of its kinase and RNase activities to permit XBP-1 mRNA splicing, creating a translational frame-shift to produce a transcriptionally competent transcription factor (XBP-1s) that traffics to the nucleus in an p85 α / β -dependent fashion. Lastly, the release of BiP from ATF6 α leads to the translocation of ATF6 α to the Golgi where it undergoes sequential cleavage by S1P and S2P proteases. The resultant transcription factor transits to the nucleus where it activates UPR target genes.

Figure 2. Domain structure of p85α and p85β

The NH₂-terminal half of p85α and p85β consists of an Src-homology 3 (SH3) domain, and two proline rich domains (Pro) surrounding the Bcl2-homology (BH) domain. The interaction of XBP-1s and p85α has been mapped to the region encompassing the BH domain. The C-terminal portion of p85α/β consists of two Src-homology 2 (SH2) domains that bind tyrosine phosphorylated YxxM motifs and an inter-SH2 (iSH2) domain that mediates binding with the catalytic subunit.

Figure 3. XBP-1 Splicing

a) In the absence of UPR pathway activation, the XBP-1 pre-mRNA is not spliced by IRE1 α leading to translation of an 267 a.a. protein lacking a transactivation domain (XBP-1u). In contrast, IRE1 α activation leads to the deletion of a 26 nt intron from the pre-mRNA to produce a translational frame-shift and production of a longer, transcriptionally competent protein product (XBP-1s). b) Schematic representation of the region of the XBP-1u and XBP-1s cDNA's amplified with the oligonucleotides used to detect XBP-1 mRNA splicing. c) Agarose gel electrophoresis of Pst I digested PCR fragments from control, tunicamycin or thapsigargin treated mouse embryonic fibroblasts.