The Unfolded Protein Response Reveals eIF2α Phosphorylation as a Critical Factor for Direct MHC Class I Antigen Presentation

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ABSTRACT

The ability to modulate direct MHC class I (MHC I) Ag presentation is a desirable goal for the treatment of a variety of conditions, including autoimmune diseases, chronic viral infections, and cancers. It is therefore necessary to understand how changes in the cellular environment alter the cell’s ability to present peptides to T cells. The unfolded protein response (UPR) is a signaling pathway activated by the presence of excess unfolded proteins in the endoplasmic reticulum. Previous studies have indicated that chemical induction of the UPR decreases direct MHC I Ag presentation, but the precise mechanisms are unknown. In this study, we used a variety of small molecule modulators of different UPR signaling pathways to query which UPR signaling pathways can alter Ag presentation in both murine and human cells. When signaling through the PERK pathway, and subsequent eIF2α phosphorylation, was blocked by treatment with GSK2656157, MHC I Ag presentation remain unchanged, whereas treatment with salubrinal, which has the opposite effect of GSK2656157, decreases both Ag presentation and overall cell-surface MHC I levels. Treatment with 4μ8C, an inhibitor of the IRE1α UPR activation pathway that blocks splicing of Xbp1 mRNA, also diminished MHC I Ag presentation. However, 4μ8C treatment unexpectedly led to an increase in eIF2α phosphorylation in addition to blocking IRE1α signaling. Given that salubrinal and 4μ8C lead to eIF2α phosphorylation and similar decreases in Ag presentation, we conclude that UPR signaling through PERK, leading to eIF2α phosphorylation, results in a modest decrease in direct MHC I Ag presentation.

INTRODUCTION

The MHC class I (MHC I) Ag presentation pathway allows the body’s cells to transmit information about their protein makeup to the extracellular milieu through the degradation and transport of cellular proteins (1). Endogenous proteins are processed into short peptide fragments, selectively loaded onto MHC I molecules, then transported to the cell surface for presentation to circulating lymphocytes (2). The MHC I peptidome is as an indicator of ongoing protein synthesis and degradation (3–5) within the cell, processes essential for disease recognition during adaptive immunity (6). Healthy cells presenting peptides from degraded “self” proteins are ignored by tolerized CD8+ T cells (7). Intracellular pathogens and oncogenic transformation alter the MHC I peptidome as “nonself” proteins are synthesized and processed for presentation (8, 9). Diseased cells presenting foreign (i.e., virus) or aberrant (i.e., cancer) peptides are recognized and eliminated by CD8+ T cells to prevent dissemination (10, 11).

A fundamental characteristic of cells afflicted with pathogenic infection or neoplastic transformation is stress, specifically endoplasmic reticulum (ER) stress (12). As a site of protein synthesis, the ER is well equipped to synthesize, fold, and posttranslationally modify secretory and membrane proteins during normal physiological states (13). Disease-driven production demands overwhelm the protein folding capacity of the ER, leading to the toxic accumulation
of misfolded and unfolded proteins (14). To prevent cytotoxicity, eukaryotes have an elaborate mechanism of quality control known as the unfolded protein response (UPR) (15).

The UPR acts to increase chaperone folding activity, upregulate ER-associated degradation, suppress protein translation, and prevent protein entry into the ER (16). The UPR can be activated by three different ER stress-sensing transmembrane proteins: inositol-requiring enzyme 1 (IRE1), RNA-dependent protein kinase (PKR)-like ER kinase, and activating transcription factor 6 (ATF6) (17). In nonstressed cells, the luminal domains of IRE1, PKR-like ER kinase (PERK), and ATF6 are bound in an inactive state to the ER chaperone Ig-binding protein (BiP), also known as GRP78 (BiP/GRP78) (18). Accumulated unfolded or misfolded proteins sequester BiP/GRP78 to induce the UPR (19). Upon BiP/GRP78 detachment, monomeric protein kinases IRE1 and PERK undergo dimerization-dependent autophosphorylation to activate their respective signaling cascades (20). Activated IRE1 functions as an endoribonuclease to nonconventionally splice X-box binding protein 1 (Xbp1) mRNA, promoting transcription of ER chaperone genes and inducing ER-associated degradation (21). Activated PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) to attenuate protein translation while concurrently promoting the translation of short open reading frame activating transcription factor 4 (ATF4), which regulates genes involved in amino acid metabolism, oxidative stress, and apoptosis (22, 23). ATF6 is a basic region and leucine zipper protein that translocates to the Golgi apparatus where it is cleaved by site-1 and site-2 proteases to release cytosolic ATF6 (33, 24). Cytosolic ATF6 migrates to the nucleus to promote transcription of genes involved in protein folding, processing, and degradation (25, 26).

The UPR manages ER-localized misfolded and unfolded proteins by directly influencing translation and degradation, key processes involved in MHC I Ag presentation (27, 28). Several studies to date have noted that inducing ER stress diminishes levels of MHC I molecules at the cell surface. Impaired classical and nonclassical MHC I expression has been observed in a variety of cell lines following UPR activation (29–31). Additionally, a study using palmitate and glucose starvation to induce ER stress in mouse cells reduced both total MHC I levels and prevented the presentation of a specific model peptide (32). However, it can be difficult to discern what effects are a direct result of the stressor and what is a result of UPR activation. For instance, tunicamycin is a potent activator of ER stress, but its mechanism of activation is to inhibit protein glycosylation. As MHC I proteins are glycosylated, the loss of MHC I expression following tunicamycin treatment may be directly related to the loss of glycosylation, rather than through subsequent UPR signaling. Additionally, it is unknown to what extent each UPR pathway contributes to the inhibition of MHC I Ag presentation.

In this study, we took advantage of small chemicals that can selectively inhibit the IRE1α or PERK signaling pathways to determine the relative contributions of each pathway to impaired MHC I Ag presentation in both murine and human cell lines, without an extrinsic ER stress inducer. We have found that conditions that induce eIF2α phosphorylation result in decreased MHC I levels and prevent the presentation of a specific peptide from a model antigenic protein. These results suggest that diminished protein synthesis, following PERK-mediated eIF2α phosphorylation, decreased direct MHC I Ag presentation. Interestingly, cell-surface levels of MHC I proteins expressed from the MHC I H-2D gene appear to be unaffected by any of the UPR inhibitors we studied, suggesting certain MHC I genes or alleles may vary in their sensitivity to UPR activation.

**MATERIALS AND METHODS**

**Cell lines and culture**

EL4/SCRAP-mCherry, JY/SCRAP-SVG, and SaI/Ak cell lines have been previously described (33–35). EL4/SCRAP-mCherry and JY/SCRAP-SVG cells were cultured in RPMI 1640 (Life Technologies) supplemented with 7.5% FCS (Atlanta Biologicals), GlutaMAX (Life Technologies), and 10 mM HEPES (Life Technologies) in a humidified incubator at 37°C with 6% CO₂. SaI/Ak cells were cultured in IMDM (Life Technologies) supplemented with 7.5% FCS (Atlanta Biologicals) and 10 mM HEPES (Life Technologies).

**Abs and reagents**

The following mAbs were from eBiosciences: allophycocyanin-W6/32 (HLA-A,B,C), allophycocyanin-2SD1-16 (Kβ-SIINFEKL), allophycocyanin-AF6-88.5.5.3 (H-2Kβ), FITC-KH95 (H-2Dβ), Biotin-AF3-12.1.3 (H-2Kβ), and PE-34-1-25 (H-2Dβ). Allophycocyanin-coupled streptavidin was from eBioscences. Alexa 647–coupled RL15A mAb (HLA-A2-SVGGVFTSV) has been previously described (35). The Fc blocking reagents Human TruStain FcX and mAb 93 (CD16/32) were from eBioscences. The rabbit polyclonal Ab targeting phospho-eIF2α (Ser51) was from Cell Signaling Technology. The mouse mAb p97 (clone 58.13.3) was from Fitzgerald Industries International. Secondary Abs IRDye 800LT goat anti-mouse and IRDye 800CW anti-rabbit pAbs (Rockland) was used at a concentration of 10 μg/ml. The protein transport inhibitor brefeldin A (BFA; MP Pharmaceuticals) was used at a concentration of 10 μM. Shield-1 (Clontech) was used at a concentration of 1 μM.

**Ag presentation assays**

Cells were bathed in citric acid buffer (pH 3) and then resuspended in fresh tissue culture media as previously described (34, 36). After acid washing, cells were cultured with various inhibitors and, in particular experiments, with Shield-1 or an equivalent volume of ethanol for the duration of the experiment. Some cells were also cultured in the presence of BFA to determine background staining. Five hours postincubation, 10⁵ cells were harvested and washed in cold HBSS (Life Technologies) supplemented with 0.1% BSA.
(Amresco) prior to Ab staining and flow cytometry analysis. All cells were Fc blocked with anti-mouse CD16/32 or Human TruStain FeX for 20 min at 4°C prior to Ab staining. Cells were then stained with directly coupled Abs for 30 min at 4°C and washed with excess HBSS/BSA. For staining Ki67, cells were first labeled with biotin-coupled AF3-12.1.3 mAb for 30 min at 4°C followed by streptavidin-allophycocyanin for 30 min at 4°C. Cells were then washed in excess HBSS/BSA. Fluorescence from Ab binding and Shield-1 treatment were evaluated using an Accuri C6 flow cytometer (BD Biosciences). A minimum of 10,000 cells were analyzed in each sample, and the mean fluorescence intensity (MFI) of the entire population of cells was recorded. MFI was determined using the BD Accuri C6 software. Levels of fluorescence at the initial time point were treated as background and subtracted from the final time point MFI. Final time point samples were analyzed in triplicate. All Ag presentation experiments were independently repeated a minimum of three times.

Calculations for Ag presentation
Presentation of peptides from both nondefective ribosomal products (DRiP) and DRiP-derived substrates was done as previously described (36). Briefly, the background staining of cells with their respective Ab (allophycocyanin-coupled 25D-1.16 for EL4/SCRAP-mCherry cells or Alexa 647-coupled RL15A for JY/SCRAP-SV4 cells) was determined by measuring the MFI of the sample immediately after acid washing of the cells. This value was subtracted from each MFI value determined at the end of the Ag presentation experiment. To calculate DRiP-specific Ag presentation, the MFI of cells treated with BFA was subtracted from each MFI value determined at the end of the Ag presentation experiment. To calculate DRiP-specific Ag presentation, the MFI of cells treated with Shield-1. To calculate non-DRiP Ag presentation, the MFI of cells treated with Shield-1 was subtracted from the MFI of cells treated with ethanol alone.

Quantitative real-time PCR
EL4/SCRAP-mCherry cells were pelleted at 100 x g for 4 min then resuspended in fresh tissue culture media at 10^6 cells per milliliter prior to culture with indicated concentrations of UPR inhibitors. To induce ER stress, 0.1 µg/ml tunicamycin was added to cells 1 h after UPR inhibitor treatment. Cells were further cultured in the presence of tunicamycin for 5 h to induce ER stress. For assays without tunicamycin, cells were cultured with UPR inhibitors for 5 h. After incubation, 3–5 x 10^6 cells were harvested and washed in cold PBS, and then total RNA was extracted using NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. Five micrograms of total RNA from each treatment was reverse transcribed to cDNA using the RNA to cDNA EcoDry Premix (Takara). Twenty nanograms of cDNA from each treatment was used to perform quantitative real-time PCR (qPCR) using FAST SYBR Green Master Mix (Applied Biosystems). Amplification and detection were performed on the StepOnePlus Real-Time PCR System (Applied Biosystems). All measurements were obtained in triplicate and normalized to β-actin. Results were obtained by the 2^(-∆∆Ct) method and expressed as fold change in respect to DMSO controls.

TABLE I. Primers used for qPCR in this study

<table>
<thead>
<tr>
<th>Primers for qPCR in this study</th>
<th>Direction</th>
<th>Sequence</th>
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<tr>
<td>XBP1</td>
<td>Forward</td>
<td>5’-AAGAAGACCCGGGATGAGC-3'</td>
</tr>
<tr>
<td>XBP1s</td>
<td>Reverse</td>
<td>5’-ACGCGTGTTCTTGAGCTGAC-3’</td>
</tr>
<tr>
<td>β2m</td>
<td>Forward</td>
<td>5’-GGGTGGAACTGTGTTACGTAG-3’</td>
</tr>
<tr>
<td>MHC I (H-2K)</td>
<td>Reverse</td>
<td>5’-CAGACACAGAAGCGAAGG-3’</td>
</tr>
</tbody>
</table>

Western blot analysis
Cells were processed as described for quantitative real-time PCR analysis. For assays without tunicamycin, cells were cultured with UPR inhibitors for 3 h. For tunicamycin combination treatments, 0.1 µg/ml tunicamycin was added to cells 1 h after UPR inhibitor treatment, then further cultured for 3 h in the presence of tunicamycin to induce ER stress. After incubation, 10^6 cells were harvested, washed in cold PBS, and then lysed in cold PBS supplemented with 0.5% Triton X-100 containing protease and phosphatase inhibitors (Pierce) on ice for 30 min. Lysates were centrifuged at 15,000 rpm for 10 min at 4°C to pellet the cellular debris. Bolt LDS sample buffer (Invitrogen) was added to the clarified lysate at one-fourth the volume of the lysate along with 10 mM DTT. Lysates were heated to 96°C for 10 min, resolved by SDS-PAGE using 4–12% Bolt Bis-Tris gel (Invitrogen), and blotted onto an nitrocellulose membrane (Invitrogen). Membranes were blocked with 5% nonfat dehydrated milk in TBS with 0.1% Tween 20 (TBST) for 30 min. Primary Abs (diluted 1:400–1:10,000) in TBST supplemented with 5% BSA were incubated with membranes overnight. Membranes were washed in TBST for 5 min, then incubated with secondary Abs (diluted 1:10,000) in TBST containing 0.5% milk for 1 h. After two 5-min washes in TBST and dH2O, membranes were imaged with an Odyssey infrared imager (LI-COR). Signals were quantified by densitometry using the instrumental software (Image Studio Lite; LI-COR Biosciences). The results represent a minimum of three independent replicates.

Statistics
All statistical analyses were performed using Prism software (GraphPad). For all flow cytometry experiments, unpaired Student t test was used to compare drug-treated samples from control treated samples (triplicate experimental repeats). For Western blot analysis, paired Student t tests were used to compare drug-treated samples to control samples in multiple experiments. For comparisons of Xbp1 mRNA splicing, the average 2^(-∆∆Ct) values for the
FIGURE 1. Outcome of UPR modulation in EL4/SCRAP-mCherry cells.

(A) A conceptual diagram for IRE1α and PERK signaling pathways. Dimerization of IRE1α leads to splicing of Xbp1 mRNA, which is blocked by the action of 4μ8C. Dimerization of PERK leads to phosphorylation of eIF2α, which is blocked by treatment with GSK2656157. Salubrinal inhibits the dephosphorylation of eIF2α, a phenotype similar to PERK activation. (B) EL4/SCRAP-mCherry cells were treated with either 10 μM 4μ8C, 1 μM GSK2656157, or 10 μM salubrinal and cultured for 5 h prior to RNA extractions. Levels of both spliced and unspliced Xbp1 mRNA were quantified, and the ΔΔ cycle threshold of each was calculated from DMSO control cells. The ratio of spliced to unspliced Xbp1 was then calculated. Only 4μ8C treatment resulted in a statistically significant change from DMSO-treated controls (**p < 0.01). (C) Same as in (B), except cells were cotreated with tunicamycin to induce the UPR. Tunicamycin-treated cells had a statistically significant increase in the ratio of spliced to unspliced Xbp1 mRNA (*p < 0.05) compared with DMSO-treated cells. Splicing was blocked by 4μ8C treatment (*p < 0.05). (D) EL4/SCRAP-mCherry cells (Continued)

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ratio of spliced $Xbp1$ from three individual experiments was compared with a theoretical mean of one (by definition, DMSO-treated) for each drug treatment.

**RESULTS**

**Alterations to UPR signaling following treatment with UPR inhibitors**

To explore IRE1α and PERK pathway signaling, we used small molecules known to interfere with specific downstream targets of each pathway. Upon accumulation of unfolded proteins within the ER, IRE1α dimerizes, which leads to the splicing of $Xbp1$ mRNA and transcription of UPR target genes (38). Inhibiting the IRE1α pathway can be achieved with the small molecule 4μ8C, which modifies the IRE1α RNase active site, abolishing its ability to splice $Xbp1$ mRNA (39). Similar to the IRE1α pathway, PERK signaling occurs when PERK dimerizes, leading to the phosphorylation of translation initiation factor eIF2α and diminished protein translation (40). The ATP-competitive inhibitor GSK2656157 prevents the phosphorylation of eIF2α (41), negating PERK signaling. An opposite result can be obtained by treating cells with salubrinal. Salubrinal inhibits the phosphatase PP1 responsible for dephosphorylation of eIF2α (42), and treatment of cells with salubrinal mimics prolonged PERK signaling. Fig. 1A summarizes each signaling pathway and the mechanism of small molecule inhibition.

To confirm the activity of each chemical on their respective signaling pathway, we treated EL4/SCRAP-mCherry cells with each small molecule inhibitor and examined the effect on both splicing of $Xbp1$ mRNA and eIF2α phosphorylation. The ratio of spliced $Xbp1$ to nonspliced $Xbp1$ was significantly lower in 4μ8C-treated cells compared with DMSO control treated cells (Fig. 1B, $p < 0.01$), whereas treatment with GSK2656157 and salubrinal did not significantly change the ratio of spliced to nonspliced $Xbp1$ mRNA. Cells were then treated with a low dose of tunicamycin to induce the UPR signaling pathways while simultaneously being treated with 4μ8C. Tunicamycin treatment of cells resulted in an increase in the ratio of spliced to nonspliced $Xbp1$ (Fig. 1C, $p < 0.05$) compared with DMSO controls. However, the splicing of $Xbp1$ was again blocked by treatment with 4μ8C, consistent with the known role of 4μ8C in blocking $Xbp1$ splicing (Fig. 1C, $p < 0.05$). Concurrent treatment with either GSK2656157 or salubrinal during tunicamycin treatment did not alter the ratio of $Xbp1$ splicing (Fig. 1C).

Next, we examined the effect of drug treatment on eIF2α phosphorylation, the hallmark of the PERK signaling pathway. Treatment with salubrinal resulted in increased phosphorylated eIF2α levels (Fig. 1D, $p < 0.05$) at concentrations as low as 1 μM. GSK2656157 treatment resulted in a lower, but not statistically significant, decrease in phosphorylated eIF2α in cells (Fig. 1E), although the basal level of phosphorylated eIF2α is quite low in cells and a subtle loss in phosphorylated eIF2α is difficult to detect. However, when cells were treated with tunicamycin to induce the UPR with simultaneous GSK2656157 treatment, a measurable decrease in phosphorylated eIF2α was apparent (Fig. 1E, $p < 0.05$). Interestingly, treatment of cells with 4μ8C led to an increase in phosphorylated eIF2α levels (Fig. 1F, $p < 0.005$ at both concentrations). This finding would suggest that as the IRE1α pathway is inhibited, there is enhanced signaling through PERK, leading to an increase in eIF2α phosphorylation.

These data demonstrate that the small molecules used in this study behave as previously predicted, with GSK2656157 and salubrinal having opposite effects on eIF2α phosphorylation, whereas 4μ8C blocks IRE1α signaling, leading to a loss of $Xbp1$ mRNA splicing. However, 4μ8C treatment does have a potential side effect of enhancing eIF2α phosphorylation in a manner similar to salubrinal. Fig. 1G summarizes the observed effects of the drug treatments on EL4/SCRAP-mCherry cells.

**4μ8C and salubrinal treatment suppress synthesis of a model protein**

We next examined the effect of the UPR-modifying small molecules on protein synthesis by taking advantage of the model protein expressed by EL4/SCRAP-mCherry cells. Phosphorylation of eIF2α upon UPR stimulation is known to diminish global protein synthesis (43–46), including in EL4 cells (32). Furthermore, salubrinal treatment by itself can globally suppress protein synthesis (42, 47). We therefore suspect that treatment with 4μ8C and salubrinal, which increase eIF2α phosphorylation, will suppress the synthesis of a model protein serving as a source of antigenic peptides. SCRAP-mCherry consists of a protein destabilization domain, the antigenic peptide SIINFEKL (which can be presented by the murine MHC I molecule Kk), and finally the fluorescent protein mCherry (34). The destabilization domain renders the protein unstable and rapidly degraded within EL4 cells; however, the process can be reversed by the addition of a small molecule termed Shield-1, which facilitates folding of the destabilization domain (48). Cells subsequently gain fluorescence as the newly synthesized protein accumulates in the presence of Shield-1. We therefore treated EL4/SCRAP-mCherry cells with 1 μM Shield-1 and either 4μ8C, GSK2656157, or salubrinal for 6 h and assessed mCherry fluorescence by flow cytometry. As shown in Fig. 2A, treatment with 4μ8C and salubrinal resulted in a statistically significant decrease in mCherry fluorescence, whereas treatment with GSK2656157 resulted in a very small, but nonsignificant, increase in fluorescence compared with cells

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were treated with the indicated concentrations of salubrinal and analyzed by Western blot for phosphorylated eIF2α. Immunoblotting for p97 was used as a loading control. (E) Cells were treated with 1 μM GSK2656157 in combination with tunicamycin (0.1 μg/ml) and analyzed for phosphorylated eIF2α as in (D). (F) Cells were treated with indicated doses of 4μ8C and analyzed for phosphorylation of eIF2α as in (D). (G) A summary of the outcomes from each drug treatment.
treated with DMSO alone. We confirmed these observations in a second cell line expressing a similar model protein. The human lymphoblastoid cell line JY expressing a different form of SCRAP, termed SCRAP-SVG, has been previously described (35). SCRAP-SVG contains the same destabilization domain as SCRAP-mCherry but has a different antigenic peptide, SVGGVFTSV, derived from the West Nile virus E protein and can be presented by the human MHC I molecule HLA-A2. Additionally, SCRAP-SVG contains Venus fluorescent protein (VFP) rather than mCherry. Similar to EL4/SCRAP-mCherry cells, treatment of JY/SCRAP-SVG cells with Shield-1 in combination with either 4μ8C or salubrinal resulted in lower levels of fluorescent protein accumulation, whereas treatment with GSK2656157 did not alter the fluorescent protein level (Fig. 2B). Taken together, these data indicate that treatment with 4μ8C and salubrinal, which increase levels of phosphorylated eIF2α but have differential effects on Xbp1 mRNA splicing, partially inhibit protein synthesis. Conversely, GSK2656157 treatment, which prevents eIF2α phosphorylation, had a minimal impact on protein synthesis.

**4μ8C and salubrinal treatment decrease direct MHC I peptide presentation from DRiP- and non-DRiP–specific peptide sources**

After determining that IRE1α and PERK signaling perturbations alter protein synthesis, we investigated the effect on direct MHC I Ag presentation. The EL4/SCRAP-mCherry and JY/SCRAP-SVG model cell lines allow us to measure the presentation of peptides derived from two potential forms of the same “self” protein: DRiPs and non-DRiP substrates (49, 50). DRiPs represent newly synthesized proteins that are rapidly degraded as a result of being inherently defunct in function or conformation (51). Non-DRiP substrates are defined as functional proteins ultimately degraded based on their unique metabolic half-life (52, 53) or, in this case, from lack of Shield-1 stabilization. Previous studies from our

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**FIGURE 2. 4μ8C and salubrinal treatment decrease protein synthesis.**

EL4/SCRAP-mCherry (A) or JY/SCRAP-SVG (B) cells treated with 30 μM 4μ8C, 10 μM salubrinal, or 1 μM GSK2656157 in the presence of 1 μM Shield-1. Fluorescent protein expression was measured in triplicate by flow cytometry, and the average MFI for each fluorescent protein was reported. Unpaired Student t test was used to determine statistical differences between drug-treated and control cells. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
FIGURE 3. 4µM 8C and salubrinal treatment decrease direct MHC I peptide presentation.

EL4/SCRAP-mCherry or JY/SCRAP-SVG cells were acid stripped to remove existing peptide–MHC complexes at the cell surface and then cultured in the presence or absence of 1 µM Shield-1 while being simultaneously treated with 30 µM 4µ8C (A and B), 10 µM salubrinal (C and D), or 1 µM GSK2656157 (E and F). Postincubation, EL4/SCRAP-mCherry cells were stained with allophycocyanin-coupled 25D-1.16 mAb, and JY/SCRAP-SVG cells were stained with Alexa 647-coupled RL15A mAb. Cells were stained in triplicate, and the average MFI was calculated. DRiP presentation was calculated by subtracting the MFI of BFA-treated cells from the MFI of Shield-1–treated cells. Non-DRiP presentation was calculated by (Continued)
laboratory and others have found that a subset of newly synthesized SCRAP protein is not saved from destruction despite the presence of Shield-1, resulting in the efficient direct MHC I presentation of the antigenic peptide embedded in SCRAP (54, 55). We have defined peptide presentation from this subset of SCRAP proteins as DRiP-specific peptide presentation, and it can be simply measured by comparing treated cells with Shield-1 to cells treated with a global inhibitor of MHC I Ag presentation, such as BFA (56, 57). Presentation of peptides from natural protein turnover can be calculated by measuring the difference in levels of peptides presented in the presence of Shield-1 to cells treated without Shield-1. This experimental setup has been used in numerous studies to identify potential differences in the presentation of peptides from DRiP and non-DRiP precursor proteins (34, 48). To determine if presentation from either precursor protein is influenced by the IRE1α and PERK pathways of the UPR, we treated cells with UPR-modifying chemicals in the presence or absence of Shield-1 to simultaneously measure direct MHC I Ag presentation from both DRiP and non-DRiP substrates.

Both EL4/SCRAP-mCherry cells and JY/SCRAP-SVG cells were bathed in a mild citric acid buffer to remove existing peptide–MHC I complexes. Cells were then incubated with small molecules in the presence or absence of Shield-1 for 6 h prior to peptide–MHC I cell-surface quantification by flow cytometry using Abs that specifically recognize peptide–MHC complexes (58, 59). Both the presentation of DRiP and non-DRiP substrates was decreased in EL4/SCRAP-mCherry cells when treated with either 4μ8C (Fig. 3A) or salubrinal (Fig. 3C). In JY/SCRAP-SVG cells, the presentation of peptides from both classes of substrates was decreased following 4μ8C (Fig. 3B) or salubrinal (Fig. 3D) treatment, but only diminished DRiP presentation reached the level of statistical significance. Treatment with GSK2656157 did not alter the presentation of peptides from either DRiP or non-DRiP substrates in either cell line (Fig. 3C, 3P). Representative histograms depicting the differences in DRiP-, non-DRiP-, and BFA-treated cells following acid wash are shown in Fig. 3G and 3H. These data indicate that conditions that lead to the phosphorylation of eIF2α results in decreased presentation of peptides derived from both DRiP and non-DRiP substrates.

4μ8C and salubrinal treatment reduce total cell-surface MHC I levels

Having examined the effect of UPR-modifying compounds on the presentation of specific peptide Ags, we next determined what effect drug treatment had on total MHC I levels. EL4/SCRAP-mCherry cells were washed in a mild citric acid buffer to lower cell-surface MHC I levels and then treated with drugs for 6 h prior to measuring MHC I recovery. A representative histogram of cells immediately after acid washing compared with acid-washed cells cultured for 6 h with DMSO or BFA is depicted in Fig. 4A and 4B. Similar to our observations with specific peptide presentation, treatment with either 4μ8C or salubrinal reduced the cell-surface levels of the MHC I molecule Kβ in EL4/SCRAP-mCherry cells, whereas GSK2656157 treatment had no effect on total Kβ (Fig. 4C). However, levels of a second MHC I molecule, Dβ, were unaffected by treatment with any of the UPR drugs tested (Fig. 4D). To determine if this observation was specific to EL4 cells or to the Dβ molecule itself, we repeated the experiment with another murine tumor cell line, the sarcoma SaI engineered to express the MHC class II molecule I-Ak (SaI/Ak) (33). SaI/Ak expresses the MHC I molecules Kβ and Dβ. Acid washing of SaI/Ak cells reduced levels of both MHC I molecules, which recovered after 6 h in culture (Fig. 4E, 4F). SaI/Ak cells treated with 4μ8C and salubrinal had lower levels of Kβ on the cell surface, whereas treatment with GSK2656157 had no noticeable effect (Fig. 4G). Cell-surface levels of Dβ were not altered by any drug treatment (Fig. 4H), similar to what we observed with Dβ levels in EL4 cells.

To confirm that loss of MHC I expression was not due to transcript level variation, MHC I (H-2K allele) and β 2-microglobulin transcript levels were compared in EL4/SCRAP-mCherry cells treated with drugs for 5 h. H-2K transcript levels remained unaltered in cells treated with 4μ8C (Fig. 5A), salubrinal (Fig. 5B), and GSK2656157 (Fig. 5C). The β 2-microglobulin transcript levels remained relatively consistent, with the one exception being a slight increase in cells treated with GSK2656157 (Fig. 5C).

Finally, we also measured total MHC I levels in JY/SCRAP-SVG cells using a pan-HLA-A,B,C Ab. JY/SCRAP-SVG cells were acid washed to remove existing peptide–MHC I complexes and recultured for 6 h with DMSO or BFA to allow MHC I levels to recover (Fig. 6A). Consistent with our previous results, levels of total MHC I were diminished upon 4μ8C (Fig. 6B) or salubrinal (Fig. 6C) treatment yet were not altered by GSK2656157 treatment (Fig. 6D). Taken together, these data suggest that treatment with salubrinal and 4μ8C, which lead to phosphorylation of eIF2α, can reduce total MHC I levels at the posttranslational level, but the effect may be limited to particular MHC I genes or alleles.

**DISCUSSION**

UPR signaling is an important physiological process within the cell, and perturbed UPR signaling has been implicated in numerous diseases, such as cancer, diabetes, and pathogenic infection (60, 61). UPR signaling undoubtedly will impact Ag
processing and presentation, both because MHC I molecules are folded within the ER and because peptide Ags are often derived from newly synthesized proteins whose synthesis will likely be disrupted by UPR signaling. In this study, we sought to manipulate specific signaling pathways of the UPR using small molecule inhibitors to determine if one particular pathway had a direct impact on Ag presentation. Importantly, this strategy allows us to probe basal level UPR activity without the need to induce a robust UPR signal, such as stimulation with tunicamycin, which could complicate data interpretation. Our data largely corroborate the works of others showing that UPR signaling can diminish MHC I levels at the cell surface and direct presentation of peptides (29–32).

Our data indicate that treatment with 4μ8C, an inhibitor of the IRE1α pathway, prevented specific Ag presentation and generally...
lowered MHC I levels in both mouse and human cell lines. Although this would suggest that signaling through IRE1α might prevent Ag presentation, we also noted that upon 4μ8C treatment, not only was Xbp1 mRNA splicing inhibited, but a buildup of phosphorylated eIF2α was also detected. Why this occurred is unknown, but it may be possible that blocking IRE1α signaling led to enhanced splicing through the PERK pathway, resulting in increased phosphorylated eIF2α. An increase in phosphorylated eIF2α was also observed when cells were treated with salubrinal, a drug that prevents dephosphorylation of eIF2α but, as we show in Fig. 1, did not alter Xbp1 mRNA splicing. Both 4μ8C and salubrinal treatment dampened de novo protein synthesis and had nearly identical Ag presentation phenotypes. Conversely, GSK2656157 treatment, which can block eIF2α phosphorylation (Fig. 1), did not impact protein synthesis nor did it alter Ag presentation. Taken together, these data suggest that signaling through PERK, and the subsequent phosphorylation of eIF2α, can diminish direct MHC I Ag presentation, whereas signaling through IRE1α, which leads to Xbp1 mRNA splicing, has little to no impact of direct Ag presentation.

The IRE1α signaling pathway is believed necessary for MHC I cross-presentation by dendritic cells (DCs) (62). Impairment of Xbp1 activity in DC resulted in diminished cross-presentation of cell-associated Ags both in vitro and in vivo. Follow-up studies revealed that the endonuclease activity of IRE1α was critical for cross-presentation of cell-associated Ags (63). Interestingly, Medel et al. (63) noted a slight but significant loss of K^b^ molecules at the cell surface of 4μ8C-treated cells as we reported in this article, but treatment had no impact on direct MHC I Ag presentation. This discrepancy may be due to differences in cell type (i.e., DCs compared with tissue culture cell lines) or the method of measuring Ag presentation as Medel et al. (63) used transgenic T cell activation to measure peptide presentation, whereas our work used mAbs to detect peptide–MHC complexes. It is well known that the T cells are more sensitive than mAbs for the detection of low levels of specific peptide–MHC complexes; however, as the number of such complexes increases on the cell surface, detecting small differences between treatments is more easily accomplished with fluorescently tagged mAbs. Thus, it would appear that cross-presentation requires signaling through the IRE1α pathway, whereas direct Ag presentation is inhibited by signaling through PERK.

Although our data demonstrate a general trend by which Ag presentation can be impacted by eIF2α phosphorylation, there are some important contradictory points that must be discussed. In EL4/SCRAP-mCherry cells, both DRiP and non-DRiP sources of the SIINFEKL peptide appear to be impacted by eIF2α phosphorylation (Fig. 3), and the loss of K^b^-SIINFEKL presentation seems to be roughly equivalent. However, in JY/SCRAP-SVG cells, the presentation of the SVG peptide from both DRiP and non-DRiP substrates trended lower but was only statistically significant when presentation was restricted to DRiPs, suggesting that DRiP presentation may be more sensitive to eIF2α phosphorylation than presentation of non-DRiP substrates. This is not surprising as DRiP presentation is highly dependent on ongoing protein synthesis; however, we should caution that in both cell types, the relative reduction in Ag presentation from both sources of peptides is relatively modest, and parsing out any differences in the presentation of peptides is difficult.
It would be satisfying to extend our findings to MHC I in general; however, different MHC I molecules may be differentially affected by UPR signaling. In murine cells it appears as that MHC I proteins encoded by the H-2K gene but not the H-2D gene are decreased following eIF2α phosphorylation. This result stands in contrast to the work of Granados et al. (32), which found that both Kβ and Dβ levels were diminished following treatment of EL4 cells with palmitate or cultured without glucose, both of which induce ER stress. One possible explanation for the disparate results could be the intensity of ER stress, which was at basal levels under the conditions we tested but will be much higher when cells are treated with stress-inducing compounds. Future work should address if other MHC I molecules, encoded by different genetic loci or particular alleles within a locus, are impacted by UPR signaling and to what extent.

DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


