Dynamic Changes in Ribosome-Associated Proteome and Phosphoproteome During Deoxynivalenol-Induced Translation Inhibition and Ribotoxic Stress

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Deoxynivalenol (DON), a trichothecene mycotoxin produced by Fusarium that commonly contaminates cereal-based food, interacts with the ribosome to cause translation inhibition and activate stress kinases in mononuclear phagocytes via the ribotoxic stress response (RSR). The goal of this study was to test the hypothesis that the ribosome functions as a platform for spatiotemporal regulation of translation inhibition and RSR. Specifically, we employed stable isotope labeling of amino acids in cell culture (SILAC)-based proteomics to quantify the early (≤30 min) DON-induced changes in ribosome-associated proteins in RAW 264.7 murine macrophage. Changes in the proteome and phosphoproteome were determined using off-gel isoelectric focusing and titanium dioxide chromatography, respectively, in conjunction with LC-MS/MS. Following exposure of RAW 264.7 to a toxicologically relevant concentration of DON (250 ng/ml), we observed an overall decrease in translation-related proteins interacting with the ribosome, concurrently with a compensatory increase in proteins that mediate protein folding, biosynthesis, and cellular organization. Alterations in the ribosome-associated phosphoproteome reflected proteins that modulate translational and transcriptional regulation, and others that converged with signaling pathways known to overlap with phosphorylation changes characterized previously in intact RAW 264.7 cells. These results suggest that the ribosome plays a central role as a hub for association and phosphorylation of proteins involved in the coordination of early translation inhibition as well as recruitment and maintenance of stress-related proteins—both of which enable cells to adapt and respond to ribotoxin exposure. This study provides a template for elucidating the molecular mechanisms of DON and other ribosome-targeting agents.

Key Words: ribotoxic stress response; ribosome; deoxynivalenol; translation; proteomics.

The ribotoxic stress response (RSR) is a process in which mitogen-activated protein kinases (MAPKs) are activated by natural ribosome-targeting translational inhibitors produced by fungi (eg, deoxynivalenol [DON], T-2 toxin), bacteria (eg, anisomycin, Shiga toxin) and plants (eg, ricin) (Iordanov et al., 1997; Laskin et al., 2002). The trichothecene DON, has been widely used as a model to study the molecular mechanisms of RSR (Pestka, 2010b). This mycotoxin is of public health significance because it is a common contaminant in human and animal foods.

DON targets the mononuclear phagocytes of the innate immune system (Pestka and Smolinski, 2005), stimulating pro-inflammatory gene expression at low or modest concentrations that cause partial translation inhibition, but eliciting leukocyte apoptosis at higher concentrations that approach complete translational arrest (Pestka, 2010a). The immunomodulatory effects of DON are believed to be mediated through RSR, primarily via the activation of kinases associated with the ribosome, a primary cellular target of DON (Zhou et al., 2003a). Following binding to the ribosome, DON activates the ribosome-associated MAPK p38 (Bae and Pestka, 2008; Bae et al., 2009). Two kinases that function upstream of DON-mediated MAPK activation are double-stranded RNA-dependent protein kinase (PKR) and hemopoietic cell kinase (Hck) (Zhou et al., 2003b, 2005). These kinases constitutively associate with the ribosome and are rapidly phosphorylated (≤5 min), thus serving as early sentinels for DON exposure (Bae and Pestka, 2008; Bae et al., 2010). In addition, DON initiates recruitment to the ribosome and phosphorylation of other upstream components in the MAPK cascade, ASK1 and M KK6 (Bae et al., 2010).

DON-induced translation inhibition has been well documented in vivo and in vitro (Azcona-Olivera et al., 1995; Zhou et al., 2003b), and can occur in at least 4 ways. First, DON can interfere with peptidyl transferase function on the ribosome with consequent impairment of initiation and elongation
(Ehrlich and Daigle, 1987). Second, DON can cause the degradation of 18S and 28S rRNA via apoptotic-related pathways (He et al., 2012). Third, DON can induce the activation of ribosome-associated kinase PKR, which when activated, phosphorylates eIF2α, thereby inhibiting translation (Zhou et al., 2003b). Finally, DON can upregulate a large number of microRNAs (miRNAs) which potentially target mRNAs for translational inhibition, most notably for ribosomal proteins (He et al., 2010).

Although the ribosome is considered to be an immensely complex molecular machine dedicated to protein translation, it has been proposed that the composition and post-translational modifications of ribosomal proteins might impart specific regulatory capacities in cells of different phenotypes or developmental stages (Xue and Barna, 2012). For example, ribosomal protein S3 (RPS3) is a 65-binding component of NF-κB complex and is essential for the function of the complex as a transcription factor (Wan et al., 2007). In addition, there is growing recognition that the ribosome can orchestrate other fundamental aspects of cell function by interacting with myriad non-ribosomal proteins (Xue and Barna, 2012). Notably, some members of the Akt/mTOR (mTOR) pathway, including 3-phosphoinositide-dependent protein kinase-1 (PKD1), AKT, mammalian target of rapamycin, and p70 S6 kinase (p70S6K), bind to the ribosomes (Lee et al., 2010; Ruggero and Sonenberg, 2005; Zinzalla et al., 2011). Non-ribosomal proteins can potentially interact with 40S and 60S subunits, monosomes or actively translating polysomes. Identification and functional characterization of such proteins that interact on the ribosome landscape could provide significant insight about their cellular roles during responses to environmental stimuli upon exposure to trichothecenes and other ribotoxins.

Many questions remain about DON-induced translational inhibition and RSR with regard to its impact on the ribosome, the identity of other proteins that are recruited to the ribosome and/or activated, the role of the ribosome in organizing the extensive phosphorylation events upon RSR (Pan et al., 2013b), and how this might impact overall cell function. The goal of this study was to test the hypothesis that the ribosome functions as a platform for spatiotemporal coordination of DON-induced translational inhibition and RSR. Specifically, stable isotope labeling with amino acids in cell culture (SILAC)-based quantitative proteomics was utilized to analyze the alterations in ribosome-associated proteins and phosphoproteins during early RSR (≤30min) induced by DON at a concentration known to partially inhibit translation and evoke robust RSR (Azcona-Olivera et al., 1995; Moon et al., 2003; Zhou et al., 2003b). RAW 264.7 cells are a well-established murine macrophage model (Hambleton et al., 1996; Raschke et al., 1978) that has been used to extensively to investigate the molecular mechanisms of the RSR (Pestka, 2010a). The results revealed that DON broadly affected both composition and phosphorylation status of ribosomal and associated non-ribosomal proteins involved in translational and other biological processes. These findings provide a foundation for further studies of the ribosome’s role in coordinating partial translational arrest and the activation of stress-related proteins for repair and recovery.

**MATERIAL AND METHODS**

**Experimental design.** Changes in the ribosome-associated proteome and phosphoproteome during DON-induced RSR were measured in RAW 264.7 cells (American Type Tissue Collection, Rockville, MD) by a multitiered approach exploiting (1) SILAC for quantification, (2) isoelectric focusing (IEF) for peptide fractionation for ribosome-associate proteome analysis, (3) TiO$_2$ chromatography for phosphopeptide enrichment for ribosome-associate phosphoproteome analysis, and (4) high-accuracy mass spectrometric characterization (Olsen et al., 2006) (Fig. 1). SILAC has been successfully used to characterize the signaling and subcellular compartmentalization for global delineation of macrophage behavior (Dhungana et al., 2009; Rogers and Foster, 2007). Schematic view of SILAC-based relative quantification is shown in Supplementary Figure 1. Briefly, RAW 264.7 cells were labeled with 1-arginine and 1-lysine (Arg6, Lys6), 1-arginine-U-$^{13}$C$_2$-$^{15}$N$_2$, and 1-lysine-$^{12}$C$_{18}$H$_{16}$N (Arg6, Lys4), or 1-arginine-U-$^{13}$C$_2$-$^{15}$N$_2$, and 1-lysine-U-$^{13}$C$_{18}$H$_{16}$N$_2$ (Arg6, Lys4) (Cambridge Isotope Laboratories, Andover, MA) (12 plates of 80% confluent cells per labeling condition) in Dulbecco’s Modified Eagle Medium (DMEM, from SILAC Phosphoprotein ID and Quantitation Kit, Invitrogen, Grand Island, NY). Since SILAC requires sufficient proliferation for the full incorporation of labeled amino acids into the cellular proteome, metabolic labeling was performed for 6 cell doubling times (>108h) during cell passage. The defined mass increments introduced by SILAC among the three RAW 264.7 populations resulted in characteristic peptide triplets that enabled measurement of their relative abundances.

Labeled RAW 264.7 cells were treated with 250ng/ml of DON for 0, 5, and 30 min (Set 1). A second, identically labeled set of cells were treated with DON for 1, 15, and 30 min (Set 2). To account for biological and technical variability, each time course set was repeated in 3 independent experiments (n = 3). In addition, a separate set of unlabeled RAW 264.7 cells were treated with 250ng/ml of DON for 0, 1, 5, 15, or 30 min for confirmatory Western blot analysis. The toxin concentration selected was toxicologically relevant because it has been shown to partially (≈50%) inhibits translation in RAW 264.7 cells (Dhungana et al., 2003b). The time window selected encompasses both initiation and peak of DON-induced RSR in RAW 264.7 cells as reflected by MAPK activation (Zhou et al., 2003b).

**Ribosome isolation.** Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl ice-cold polysome extraction buffer (PEB) (50mM KCl, 10mM MgCl$_2$, 15mM Tris–HCl ([pH 7.4]), 1% (v/v) Triton X-100, 0.1 mg/ml cycloheximide, 1 mM DTT, protease inhibitor (Sigma, St. Louis, MO), and phosphatase inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA)) (Bae et al., 2010). Sucrose solutions (10% and 50% w/v) were prepared prior to use by dissolving sucrose into RNase-free water with 50mM KCl, 10mM MgCl$_2$, 15mM Tris–HCl (pH 7.4), 0.1 mg/ml cycloheximide and protease inhibitor. Cell lysates were centrifuged at 16 000 x g, 4°C, for 15 min to remove nuclei, mitochondria, and cell debris.

SILAC-labeled lysates containing ribosomes from three different time points within each replicate were pooled equally based on protein content as determined by BCA Protein Assay (Pierce, Rockford, IL). These resultant mixtures (4ml) were layered on a 28% linear sucrose gradient solution (10%–50%, w/v) prepared using an ISCO 160 Gradient Former and held at 4°C in an 36 ml Sorvall centrifuge tube and centrifuged at 28 000 x g, 4°C for 16h in Sorvall AH-629 rotor. 40S, 60S, monosome (80S, composed of associated 40S and 60S) and polysome fractions were isolated by fractionating gradient at a rate of 1 ml per min into 2 ml tube by upward displacement using an ISCO Density Gradient Fraction Collector, consisting of a needle-piercing device.
with a syringe pump connected to an EM-1 UV monitor for continuous measurement of the absorbance at 254 nm (Teledyne ISCO, Lincoln, NE).

Proteins from each fraction were precipitated by slow addition of trichloroacetic acid to a final concentration of 10% (w/v) followed by overnight incubation at 4°C. Pellets were recovered by centrifugation (10 000 × g for 15 min), washed with cold acetone twice, and air dried. Proteins were suspended in 6M urea, 2M thiourea, 10mM HEPES, pH 8.0 and digested in solution with trypsin (Olsen et al., 2006). Peptides were desalted with 100 mg reverse-phase tC18 SepPak solid-phase extraction cartridges (Waters, Milford, MA), and two equal aliquots were dried in a Speedvac (Thermo Scientific, Asheville, NC) separately for determination of ribosome-associated proteome and phosphoproteome, respectively.

**Isolation of peptides for ribosome-associated proteome and phosphoproteome analysis.** For isolation of the ribosome-associated proteome, one peptide aliquot was resuspended in 200 µl of IEF buffer (7M urea, 2M thiourea, 0.05M dithiothreitol [DTT], 2% 3-(3-cholamidopropyl)-dimethylammonion] propanesulfonate [CHAPS], 2% ASB-14, 0.2% Biolyte pH3-10, 0.1% bromophenol blue), applied to Bio-Rad IPG strips, 8cm, pH 3-10 (Biorad, Hercules, CA) and allowed to rehydrate overnight at 25 °C. The strips were then electroforesed at 250V for 15 min followed by 8 000V for 30 min. Peptides were harvested by slicing strips into 5 equal sections and placed into individual microfuge tubes. Each of these sections was further chopped into approximately 5 mm pieces and sequentially extracted by sonication in 0.1% trifluoroacetic acid (TFA), 0.1% TFA/30% acetonitrile (ACN), 0.1% TFA/70% ACN.

For isolation of the ribosome-associated phosphophosphate, a second aliquot of peptides was enriched for phosphopeptide with Titansphere PHOS-TiO Kit (GL Sciences, Torrance, CA) following the manufacturer’s instructions. For both the ribosome-associated proteome and phosphoproteome, extracted peptides from both preparations were then separately dried in a Speedvac. Dried samples were reconstituted in blank solution (2% ACN/0.1% TFA) to 100 µl and purified by solid phase extraction using OMIX tips (Varian, Palo Alto, CA) according to manufacturer’s recommendations.

**Mass spectrometry.** Purified peptides were dried in a Speedvac and reconstituted in blank solution to 20 µl. Using a Waters nanoAcquity Sample Manager (Waters, Milford, MA), 10 µl peptide aliquot was automatically loaded onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm × 20mm) at 4 µl/min in 2% ACN/0.1% formic acid for 5 min. The bound peptides were then eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% water/0.1% formic acid, Buffer B = 99.9% ACN/0.1% formic acid) onto a Michrom MAGIC C18AQ column (3µ, 200 Å, 100 U × 150 mm, Michrom, Auburn, CA) and eluted over 120 min with a gradient of 5% B to 30% B in 106 min, ramping up to 90% B by 109 min, held there for 1 min, returned to 5% B at 110.1 min and kept there for the remainder of the run. Solvent flow was kept at a constant rate of 1 µl/min.

Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (Thermo, Hudson, NH) using a Michrom ADVANCE nanospray source with an ionization voltage of 2.0 kV. Survey scans were taken in the FT (50 000 resolution determined at m/z 400) and the top five ions in each survey scan were then subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resulting data files were processed into peak lists using MaxQuant (Cox and Mann, 2008), v1.2.2.5, and searched against the IPI rat database v3.78 using the Andromeda (Cox et al., 2011) search algorithm within the MaxQuant environment. All SILAC quantitation was performed using MaxQuant and the data exported to the program Perseus, v1.2.0.16 (www.maxquant.org) for statistical analysis.
**Data analysis.** Peak list generation, protein quantitation based upon SILAC, extracted ion chromatograms (XIC) and estimation of false discovery rate (FDR) were all performed using MaxQuant (Cox and Mann, 2008), v1.2.2.5. MS/MS spectra were searched against the IPI mouse database, v3.72, appended with common environmental contaminants using the Andromeda searching algorithm (Cox et al., 2011). Further statistical analysis of the SILAC labeled protein and peptide ratio significance was performed with Perseus (www.maxquant.org). MaxQuant parameters were protein, peptide and modification site maximum FDR = 1%; minimum number of peptides = 1; minimum peptide length = 6; minimum ratio count = 1; and protein quantitation was done using all modified and unmodified razor and unique peptides. Andromeda parameters were triplex SILAC labeling; light condition (no modification), medium condition (Arg6, Lys4), heavy condition (Arg10, Lys8); fixed modification of carbamidomethylation (C), variable modifications of oxidation (M), phosphorylation (STY) and acetyl (protein N-term); Maximum number of modifications per peptide = 5; enzyme trypsin max missed cleavage = 2; parent ion tolerance = 6 ppm; fragment ion tolerance = 0.6 Da and reverse database search was included. For each identified SILAC triplet, MaxQuant calculated the three extracted ion chromatogram (XIC) values, and XICs for the light and medium member of the triplet were normalized with respect to the heavy common 30 min time point of DON treatment, which was scaled to one. A significance value, Significance B, was calculated for each SILAC ratio and corrected by the method of Benjamini and Hochberg using Perseus (Cox and Mann, 2008). Significance B values of a peptide in experiment 1 and experiment 2 were calculated independently, and the significance cutoff was set as significance B value <0.05 in at least one set of time course (Cox et al., 2009). Median values of multiple peptides originating from the same protein or multiple measurements of the same phosphopeptide were used to represent the abundance of proteins or phosphopeptides relative to the time zero (0 min) abundance, respectively (Ong and Mann, 2006). Given that both sets contain the common time point of 30 min, relative quantification was first performed in relation to the abundance of each peptide at 30 min in separate sets. The relative quantification ratios from the two sets were subsequently combined, with the assumption that the abundance of one particular peptide at 30 min after DON treatment remains stable throughout the replications of the two sets. Such a relative quantification scheme of 5 time points was first proposed in (Olsen et al., 2006), and has employed in many systems including the RAW 264.7 cells (Sharma et al., 2010). Phosphosites were mapped with PhosphoSitePlus (Hornbeck et al., 2012).

**DAVID analysis.** Analyses for gene ontology (GO) annotation terms were performed with the functional annotation tool DAVID (http://david.abcc.ncifcrf.gov/). Uniprot accession identifiers of significantly regulated phosphopeptides were submitted for analysis of GO biological processes and KEGG pathways, with a maximal DAVID EASE score of 0.05 for categories represented by at least two proteins.

**Western blot.** Immunoblotting analysis was performed on selected proteins as described previously (Bae and Pestka, 2008) to verify phosphoproteome quantitation. Antibodies against the following proteins were used: p38, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAP kinase, phosphop42/p44 MAP kinase (Thr202/Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling, Danvers, MA), RPL7, RPS6 (Bethyl Labs, Montgomery, TX). Blots were scanned on the Odyssey IR imager (LICOR, Lincoln, NE) and quantification was performed using LICOR software v3.0.

**RESULTS AND DISCUSSION**

**Proteomic Profile of Ribosome-Associated Proteins**

The typical mammalian ribosomal protein is small (mean molecular weight 18.5 kD) and very basic (mean isoelectric point 11.05) (Wool et al., 1995). To resolve less abundant non-ribosomal proteins from the highly abundant ribosomal proteins, we employed off-gel IEF, which enables fractionation of peptides by pI. This reduced sample complexity and prevented peptides originating from ribosomal proteins from overwhelming the mass spectrometer, thus facilitating identification of ribosome-interacting proteins. Using this approach, SILAC-labeled RAW 264.7 cell extracts were fractionated into 40S, 60S, monosome and polysome fractions and further analyzed for the associating proteins (Fig. 2). The ribosome-associated proteome was found to contain many proteins involved in other biological functions, that extended beyond canonical ribosomal, translation- and ribosome biogenesis-related proteins (Fig. 2A). It should be emphasized that these data reflect

![Image](https://via.placeholder.com/150.png?text=FIG. 2. Impact of DON on the ribosome-associated proteome. A. The ribosome-associated proteome includes extensive non-ribosomal proteins. Counts of ribosomal proteins, translation-, ribosome biogenesis-related proteins, and other proteins found in different fractions of the ribosome. Number of unique proteins (B) and peptides (C) associated with the ribosome (light + dark grey) that were significantly affected by DON exposure (FDR < 5%, light grey).)
the counts of proteins in the ribosomal fractions following IEF, as opposed to the relative abundance in the cell extract per se, because the most abundant proteins in the ribosomal fractions are still would be ribosomal and translation-related factors.

At an accepted FDR of 1%, hundreds of unique proteins (Fig. 2B), corresponding to thousands of unique peptides (Fig. 2C) identified in the LC-MS/MS, were found in each ribosomal fraction. Approximately 70% of the proteins were identified with two or more independent peptide measurements. Among the unique proteins identified, 91% of the known murine ribosomal proteins (http://ribosome.med.miyazaki-u.ac.jp/) were represented, including 29 of the 32 small subunit and 43 of the 47 large subunit proteins. Translation-related proteins included initiation/elongation factors, tRNA synthetases, and ribosome biogenesis-related proteins that drive ribosome nuclear transport and function, such as ribosomal RNA methyltransferase. Also identified were other ribosome-interacting proteins that do not directly affect translation. These might play alternative roles such as serving as staging sites for signaling or metabolic pathways. With Benjamini–Hochberg corrected FDR <5% as the significance cutoff, 17% and 10% of the unique proteins and peptides for all four fractions of the ribosome were significantly altered, respectively, confirming that DON markedly impacted on protein association with the ribosome (Figs. 2B and 2C).

To discern the potential impact of DON on ribosome-associated proteome, proteins exhibiting significant differential association with the ribosome were related to specific biological processes using Gene Ontology annotation in DAVID. Based on changes in protein association with the ribosome, early DON-induced effects mainly involved modulation of translation, biosynthetic pathways, and macromolecular complex assembly (Fig. 3).

DON-Induced Changes in Ribosome-Associated Proteins—Impact on Translation

Gene Ontology analysis revealed that the process most affected was translation, consisting of 20% or more of the ribosome-associated proteins impacted by DON across all four fractions of the ribosome. Indeed, while some proteins were consistently associated with the ribosome (Figs. 4D–F), there was an overall reduction in the levels of significant differentially associated ribosomal and translation-related proteins during the first 30 min of DON exposure (Figs. 4A–C).

Although the total protein amount in each respective ribosomal fraction for different time points remained constant, there was reduction in the levels of selective ribosomal proteins across the fractions (Fig. 4A), suggesting increased ribosome degradation/disassembly and/or decreased ribosome biogenesis/assembly. Consistent with the latter, we have previously observed in intact RAW 264.7 cells that DON exposure alters phosphorylation of proteins involved in ribosome biogenesis, including NOP56, NOP58, NPM1, and PDCD11 (Pan et al., 2013b). Among the ribosomal fractions, there was a transient (1 min) increase of translation-related proteins in the 80S fractions, which might be indicative of polysome disassembly. Taken together, these data indicate that ribosomal protein depletion likely contributes to DON-induced translational inhibition.

Multiple subunits of eukaryotic initiation factor 3 (eIF3A, B, C, E, F, L), eIF4A and eIF4G had reduced association with 40S ribosomal subunit (Fig. 4B). EIF3 plays a key role in recruiting the pre-initiation complex to the mRNA and also forms a protein bridge to the mRNA by interacting with eIF4G. EIF3 and eIF4G are likely retained on the ribosome during elongation of small uORFs to make them available for renewed scanning following termination. EIF3 also stimulates re-initiation of translation of a long uORF (Sonenberg and Hinnebusch, 2009). Cap-dependent initiation requires interaction of eIF4E with the mRNA 5’ cap structure, which forms the eIF4F helicase complex together with RNA eIF4A helicase and eIF4G.

In addition to the ribosome-association alteration shown here, eIF4G has been shown to be differentially phosphorylated in whole RAW 264.7 cells treated with DON (Pan et al., 2013b) and in the ribosomal fractions. Accordingly, displacement of translation-related factors very likely contributes to translation arrest induced by DON.

Discrepancies observed between transcriptome and proteome data underlines the importance of translational control of gene expression in addition to the better understood transcriptional regulation (Sonenberg and Hinnebusch, 2009). DON impairment of translation possibly liberates translational machinery from pre-existing translation-competent transcripts thus enabling newly made mRNAs to more effectively compete for the translational machinery. This shift in translation, known as “translational reprogramming” (Ron and Walter, 2007), may allow for a bias in the synthesis of proteins that enable the macrophage to combat ribotoxic stress and respond to danger by evoking an innate immune response. Indeed, such biased translatome, as compared to transcriptome, has been recently documented for several proinflammatory genes induced by DON in RAW 264.7 cells (He et al., 2013).

DON-Induced Changes in Ribosome-Associated Proteins—Impact on Protein Folding

Disruption of normally well-coordinated translation might generate improperly folded proteins and therefore impact the unfolded protein response. Thus, it was notable that DON exposure altered HSC70, HSP90 and GRP78 among the ribosome interacting proteins (Fig. 5). Ribosome-associated complex (RAC), which consists of heat shock protein HSP40 and HSP70 proteins, supports de novo folding pathways (Wegrzyn and Deuerling, 2005). Heat-shock cognate HSC70 has intrinsic ATPase activity, and could serve as a chaperone with RAC (Jaiswal et al., 2011). There was an increased ribosome association of HSC70 and other chaperones involved in protein folding, which could boost co-translation folding (Craig et al.,
HSP90 is known to interact with RPS3 and RPS6, and such interaction protects the two ribosomal proteins from ubiquitination and proteasomal degradation (Kim et al., 2006). HSP90 exhibited transiently increased association with 40S, 80S, and polysome, thereby possibly protecting of ribosomal proteins RPS3 and RPS6. Ribosome association of HSP90 decreased at 30 min, which could coordinate the global reduction of ribosomal proteins in response to DON. In addition to the induction of chaperones, weakened translation, as suggested by reduced ribosomal availability in early DON-induced RSR, might decrease the pool of chaperone substrates and thereby increasing the capacity of the folding system (Meriin et al., 2012). Finally, the ER chaperone and signaling regulator glucose-regulated protein 78 (GRP78) showed increased association with the polysome within 30 min of DON treatment, which could be mediated via ERj1 (ER-resident J-domain protein 1) (Benedix et al., 2010), and might further enhance with protein folding.

**DON-Induced Changes in Ribosome-Associated Proteins—Impact on Biosynthesis and Cellular Organization**

Other proteins altered in their ribosome interaction following DON exposure were capable of impacting biosynthesis and cellular organization processes (Figs. 6 and 7). Proteins involved in glucose, ATP, and nucleotide biosynthesis showed an overall increased association with the ribosome (Fig. 6). On the cellular level, enhanced energy metabolism in early DON-induced RSR has been previously implicated in RAW 264.7 by the phosphorylation of AMP-activated protein kinase (AMPK) (Pan et al., 2013b). There are approximately 15 000 ribosomes in a single cell, and they make up about 25% of the dry weight of cells (Luisi and Stano, 2011). Accordingly, translation uses...
FIG. 4. Changes in the levels of ribosome-associated proteins involved in translation, including significant differentially associated (A–C) and constantly associated (D–F) ribosomal proteins (A, D), translation factors (B, E) and tRNA synthases (C, F). Heat map depicts the log 2 transformed relative protein abundance, and grey represents missing values.

FIG. 5. Changes in the levels of ribosome-associated proteins involved in protein folding. Heat map depicts the log 2 transformed relative protein abundance, and grey represents missing values.
a significant proportion of the cell’s energy which needs to be replenished to restore translation of stress-related proteins in DON-induced RSR.

Dysregulation of cellular organization, including the cytoskeleton and chromatin assembly processes, has been suggested to occur at the protein phosphorylation level during early DON-induced RSR in RAW 264.7 (Pan et al., 2013b). Multiple proteins involved in cellular organization showed increased association with the ribosome, including lymphocyte cytosolic protein 1 (LCP1) and multiple chains of tubulin (TUBs) across different ribosomal fractions (Fig. 7). It has been proposed that upon translation perturbation, stressed ribosome complex aggregation and dissolution requires anchorage of cytoskeleton to the ribosomes and the activity of motor molecules for active transport (Anderson and Kedersha, 2008; Kwon et al., 2007). Thus, enhanced association of proteins in cell organization might facilitate transportation of the aforementioned proteins to and from the ribosome.

**Proteomic Profile of Ribosome-Associated Phosphoproteins**

It has been previously demonstrated that DON induces p38 mobilization to the ribosome and its subsequent phosphorylation (Bae and Pestka, 2008). A pilot immunoblot experiment was performed to ascertain if (1) the RSR is induced in the SILAC-labeled RAW 264.7, (2) previously reported ribosome association and phosphorylation of p38 is reproducible under the ribosome isolation conditions used here, and (3) other MAPKs characteristic of DON-induced RSR are also

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**FIG. 6.** Changes in the levels of ribosome-associated proteins involved in biosynthesis, including generation of precursor metabolites and energy (A) and nitrogen compound biosynthetic process (B). Heat map depicts the log 2 transformed relative protein abundance, and grey represents missing values.

**FIG. 7.** Changes in the levels of ribosome-associated proteins involved in cellular organization, including macromolecular complex assembly (A) and cytoskeleton organization (B). Heat map depicts the log 2 transformed relative protein abundance, and grey represents missing values.
associated with the ribosome. MAPKs, including p38, ERK, JNK, were found to constitutively bind to the ribosome, with RPS6 and RPL7 as markers of the ribosome (Fig. 8). At 30 min, which represents the peak of DON-induced RSR, MAPKs were moderately recruited to the ribosome fractions, with overall levels of phosphorylation markedly increased. The results presented here provide additional evidence for association of p38, ERK, and JNK MAPKs with the ribosome.

Due to the low abundance of phosphoproteins, ribosome-associated phosphoproteome was enriched using TiO\textsubscript{2} chromatography after sucrose density fractionation of the ribosome and protein digestion. More than 92% of identified peptides were found to harbor at least one phosphorylation residue, indicating that phosphopeptide enrichment by TiO\textsubscript{2} was highly efficacious (Supplementary Dataset 5). The distribution of phosphorylated serine, threonine, and tyrosine sites was 77%, 18%, and 5%, respectively (Fig. 9A). Fifteen of the identified phosphoproteins were ribosomal (eg, RPS6, RPLP2), translation- (eg, eIF3, eIF4G1) and ribosome biogenesis-related (eg, NPM1, BYSL) with known and novel phosphorylation events. However, like the ribosome-associated proteome, the majority of phosphoproteins interacting with the ribosome were involved in non-ribosomal processes (Fig. 9B).

Using the same set of criteria for protein identification and significance that were used for the ribosome-associated proteome (FDR < 5%), approximately 16% of the total phosphopeptides identified were considered significantly altered by DON, with total and significantly altered unique phosphoproteins (Fig. 9C) and phosphopeptides (Fig. 9D) identified in each ribosomal fraction. Based on PhosphoSitePlus annotation, the ribosome-associated phosphoproteome changes occurring in the macrophage after DON exposure encompass both known and yet-to-be functionally characterized phosphosites.

**DON-Induced Changes in Ribosome-Associated Phosphoproteins—Impact on Translation**

GO analysis revealed that ribosome-interacting phosphoproteins with phosphorylated status significantly altered by DON were primarily involved in translation, regulation of transcription, RNA processing, and cell morphogenesis (Fig. 10). Eukaryotic protein translation is mainly controlled at the level of initiation, a process of elongation-competent 80S ribosome assembly from 40S and 60S subunits. Translation initiation involves multiple protein phosphorylation events (Jackson et al., 2010). Following DON exposure, ribosome-associated phosphorylation events observed include those on ribosomal proteins, as well as translation initiation and elongation factors (Figs. 11–14). For example, RPS6 interacts with the 5′ cap complex required for translation initiation. RPS6 undergoes inducible phosphorylation in response to mitogenic and cell growth stimuli (Ruvinsky and Meyuhas, 2006), and such phosphorylation occurs on a cluster of five serine residues at the carboxyl terminus of RPS6, that is, Ser-235, Ser-236, Ser-240, Ser-244, and Ser-247. Differential phosphorylation
of RPS6 at the first 3 of these residues was detected during DON-induced RSR, which might modulate RPS6’s affinity for the cap and mRNA translation initiation (Hutchinson et al., 2011). Besides the effect on translation, RPS6 phosphorylation has been implicated in attenuating DNA damage (Khalaileh et al., 2013).

Another example, platform protein eIF4G is the binding partner for the cap-binding factor during translation initiation. Increased phosphorylation of eIF4G1 at Ser187 and Ser1189, as found transiently in the 60S ribosome at 15 min during DON-induced RSR, enhances its interaction with MNK, an eIF4E kinase implicated in DON-induced RSR in vivo (Pan et al., 2013a). Binding to eIF4G approximates MNK to its substrate eIF4E and facilitates eIF4E phosphorylation and translation initiation (Dobrikov et al., 2011).

DON-Induced Changes in Ribosome-Associated Phosphoproteins—Impact on Transcriptional Regulation

From the perspective of protein phosphorylation, transcriptional regulation has been shown to be the primary target during early DON-induced RSR in RAW 264.7 cells (Kirat et al., 2009; Pan et al., 2013b; Wong et al., 2002). Ribosome-associating phosphoproteins involved in transcriptional regulation were mainly found to interact with the 40S, 60S, and 80S (Figs. 11–13). These overlapped with those previously described in intact RAW 264.7 (eg, epigenetic regulators TRIM28 and DNMT1, and transcription cofactors LYRIC and MYBBP1A) (Pan et al., 2013b), as well as included new ones not previously demonstrated in DON-induced RSR (eg, PRKRA).

Tripartite motif protein 28 (TRIM28) phosphorylation at Ser473, has been shown to compromise its interaction with heterochromatin protein 1 (HP1) and activate cell cycle regulatory genes (Chang et al., 2008). Phosphorylation of TRIM28 at this site increased in 40S and 60S ribosome at 30 min (Figs. 11 and 12), following the same pattern in the whole cell (Pan et al., 2013b). Relatedly, TRIM28 is a universal co-repressor that induces cell differentiation in the monocytic cell line U937 via its interaction and activation of C/EBP beta (Rooney and Calame, 2001), a transcription factor also activated by DON (Shi and Pestka, 2009).

Myb-binding protein 1A (MYBBP1A), found to be rapidly dephosphorylated at 30 min, is known to interact with and activate several transcription factors such as c-JUN, while repressing others such as PPARγ co-activator 1α (PGC-1α), NFκB, and c-MYB (Fan et al., 2004; Owen et al., 2007; Yamauchi et al., 2008). MYBBP1A associated with 60S ribosome dephosphorylated at Ser6 at 30 min after DON treatment (Fig. 12). This protein was observed to be upregulated at the protein level by DON (150 ng/ml) at 6 h in a prior proteomic study in EL4 mouse thymoma cells (Osman et al., 2010).

DON has been suggested to alter transcription via miRNA-based mechanisms (He et al., 2010). PKR-associated protein X (PRKRA) is required for miRNA production by Dcr-1 homolog (DICER1) and for subsequent miRNA-mediated post-transcriptional gene silencing (Patel and Sen, 1998). It can also regulate gene expression on the translation level. PRKRA could activate PKR in the absence of double stranded RNA (dsRNA), leading to eIF2α phosphorylation and inhibition of translation. Such activation requires phosphorylation of PRKRA at Ser18

FIG. 9. Impact of DON on the ribosome-associated phosphoproteome. A, Distribution of phospho-serine, threonine, and tyrosine identified in peptides from all ribosomal fractions. B, The ribosome-associated phosphoproteome includes extensive non-ribosomal proteins. Counts of ribosomal proteins, translation-, ribosome biogenesis-related proteins, and other proteins found in different fractions of the ribosome. Number of unique phosphoproteins (C) and phosphopeptides (D) associated with the ribosome (light + dark blue) that were significantly affected by DON exposure (FDR < 5%, light blue).
**FIG. 10.** Gene ontology analysis of ribosome-interacting phosphoproteins significantly regulated affected by DON exposure by DAVID biological process terms (DAVID EASE score < 0.05). Data are presented as a histogram of the relevant biological processes identified and shown as a percentage of the total identified proteins that fall within each category. Numbers in parentheses represents the number of proteins in the ribosome fraction significantly altered by DON treatment.

**FIG. 11.** Phosphorylation changes of proteins associated with 40S ribosome during DON-induced RSR. Heat map depicts the log 2 transformed relative abundance of the protein phosphorylation, and grey represents missing values.
Pan et al. (2004), which was observed to be increased in 60S at 30 min after DON treatment (Fig. 12). However, in DON-induced RSR, PKR phosphorylation is observed before phosphorylation of PRKRA (between 1 and 5 min) during DON-induced RSR, suggesting that PRKRA might primarily regulate transcription via modulation of miRNA expression.

**DON-Induced Changes in Ribosome-Associated Phosphoproteins—Impact on Stress-Related Signaling**

When coupled with the global phosphoproteome alterations identified in the intact RAW 264.7 (Pan et al., 2013b), ribosome-associated phosphoproteome changes suggest linkages to a stress signaling network consisting of MAPK, AKT, and NFκB pathways. Therefore, the ribosome could function as a hub for stress-related signaling. Besides phosphorylation of the aforementioned MAPKs p38, ERK, and JNK (Fig. 8), a downstream component in the MAPK pathway cytosolic phospholipase A2 (cPLA2), which associated with the 40S (Fig. 11), was dephosphorylated at Ser437 over time. Phosphorylation of cPLA2 has been reported during DON-induced RSR in RAW 264.7 (Pan et al., 2013b) and is required for the release of arachidonic acid, and has been implicated in the initiation of inflammatory responses (Pavicevic et al., 2008). RPS6 represents a point of regulatory convergence for MAPK- and

<table>
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<th>Function</th>
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**FIG. 12.** Phosphorylation changes of proteins associated with 60S ribosome during DON-induced RSR. Heat map depicts the log 2 transformed relative abundance of the protein phosphorylation, and grey represents missing values.
AKT-linked pathways known to mediate DON-induced RSR (Pan et al., 2013b). RPS6 can be phosphorylated by p70 ribosomal protein S6 kinase (S6K) via the AKTmTOR pathway and p90 ribosomal protein S6 kinases (RSK) via the MAPK pathway (Ruvinsky and Meyuhas, 2006). In 40S, 80S, and polysome fractions, RPS6 was differentially phosphorylated at Ser235, Ser236, or Ser240, possibly due to the different activities of RSKs on these fractions. Thus, the phosphorylation status of RPS6 is determined by the two pathways, and could control translation efficiency in response to environmental perturbations such as DON-induced RSR.

MYBBP1A associated with 60S (Fig. 12) and LYRIC associated with 80S (Fig. 13) are two inhibitors of p65 and negative regulators of the NFκB signaling, a pathway also known to be evoked by DON (Pan et al., 2013b). These two phosphoproteins also link the RSR with transcriptional regulation during DON-induced RSR.

While these proteins could function in other compartments of the cell, their transient association with the ribosome renders the ribosome indicates that this organelle might indeed function as a platform for stress-related signaling in addition to that for translational and transcriptional regulation.

Limitations of the Methodology

Basic techniques to isolate ribosomes were developed in the 1960s and 1970s, and are still widely used with relatively minor modifications (Mehta et al., 2012). Most of these techniques require differential or density gradient ultracentrifugation of cell lysates to yield ribosomes or ribosomal subunits. Previously, proteins identified in the ribosomal fractions without a known role in ribosome-related function have been simply dismissed as contaminating proteins from complexes that comigrate with ribosomal fractions in sucrose gradient. However, factors that associate with actively translating ribosomes might interact transiently with a wide range of affinities. Protein–ribosome interactions exhibit a range of salt sensitivities. Higher stringency approaches might cause the dissociation of specific ribosome-surface associated factors. While low stringency salt conditions allow most specific ribosome-binding proteins to remain associated with the ribosome, it could result in higher background signal (Mehta et al., 2012). After comparing different published studies (Blobel, 1971; Thiébeaud et al., 2009; Tsai et al., 2012), we chose moderate salt conditions in this study and evaluated the ribosome association of MAPKs using immunoblot as a pilot study (Fig. 8). The present proteomic study resolved proteins with altered association with the ribosome upon DON treatment that function in processes other than protein synthesis and folding at moderate stringency. We recognize that this does not exclude the possibility of the inclusion of contaminating proteins as discussed above. However, if these proteins are indeed comigrating contaminants with the ribosomal fractions in sucrose gradient centrifugation, they should have remained constant regardless of DON treatment because different treatment groups with different SILAC labels were combined prior to sucrose gradient centrifugation and fractionation. The observation that DON affected the ribosome-associated proteome and phosphoproteome suggest that these have true physiological impact.

While affinity tagging and immunoprecipitation might be an alternative to confirm the interactions identified in the study (Inada et al., 2002; Zanetti et al., 2005), these methods might require verification that (1) the tag does not interfere with the biological function, and (2) over-expression does not generate misleading non-specific interactions. We have attempted to express affinity-tagged ribosomal proteins in RAW 264.7 macrophages. However, expression and ribosomal incorporation of these tagged proteins were inefficient, possibly due to the nature of the macrophages’ inherent capacity to combat exogenous material.

Another caveat with regards to the ribosome purification method used was the overlap among the four ribosomal
fractions—an inevitable result of sucrose density gradient fractionation. In addition, it is also possible that proteins found are nascent peptide chains produced by the actively translating polysome, adding to the complexity of the polysome-associated proteome. Finally, there is very likely some sample loss during the ribosome isolation, fractionation, and digestion procedures, which might have removed some less abundant proteins, and reduced the sensitivity of the method. This could explain why this study failed to detect some of the ribosome-associated proteins and phosphoproteins identified by Western blot analysis (e.g. PKR, p38, ERK) (Fig. 8), an approach known to be more sensitive than mass spectrometry-based proteomics used here (Mann, 2008).

CONCLUSIONS

In summary, the quantitative proteomic analysis presented herein revealed that upon exposure to DON, proteins critical in mediating translation inhibition and the RSR showed altered interaction and/or phosphorylation in the ribosome. Our proteomics approach also identified large numbers of proteins and phosphoproteins in the ribosome fractions that are not directly associated with the functions of the ribosome, such as translation or ribosome biogenesis. Overall these findings set a foundation for the versatile extraribosomal functions of the ribosome that is summarized in (Fig. 15). Key observations were that after DON exposure, there was (1) an overall decrease in translation-related proteins interacting with the ribosome, (2) concurrent compensatory increase in protein folding, biosynthetic pathways, and cellular organization, and (3) alteration in ribosome-associated phosphoproteome that could fine-tune regulation of translation and transcription. As a whole these changes converged on known signaling pathways. Changes in ribosome-associated proteome and phosphoproteome correspond to composition and post-translational modifications of ribosomal and its interacting proteins, which could offer additional levels of regulation. Therefore, in addition to its role in protein synthesis, the ribosome could function as a platform for translation-related and other processes. Such a structural role for the ribosome could facilitate spatiotemporal coordination of cell behaviors to respond and adapt to the ribotoxic stress caused by DON. Taken together, this investigation enhances our understanding of the role of the ribosome under stress conditions posed by agents like DON that directly impact the ribosome. This could ultimately provide new insights into the molecular mechanisms, at the subcellular level, of DON and other ribotoxic agents, many of which are public health threats or chemotherapeutic agents.

Using the same strategy, future investigations should compare the effects non-ribotoxic stressors (e.g., LPS, UV, and oxidation) with in this model with those of DON to understand how generalized stress might impact the ribosome-interacting proteome and phosphoproteome. In addition, DON has been classically categorized as an elongation-type translational...
Dynamic changes in Ribosome-associated Proteome and Phosphoproteome

inhibitor (Cundliffe et al., 1974; Ehrlich and Daigle, 1987). It will be of interest in the future to compare the effects of elongation-type (eg, DON, trichodermin) and initiation-type (eg, nivalenol, T-2 toxin, and verrucarin A) translational inhibitors to discern whether specific components of the ribosome-associated proteome and phosphoproteome are differentially affected by various types of small molecule translational inhibitors.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


FIG. 15. Summary of ribosome-associated proteome (blue) and phosphoproteome (red) changes in the four ribosomal fractions during DON-induced RSR. Upon DON exposure, there was (1) an overall decrease in translation-related proteins interacting with the ribosome, (2) concurrent compensatory increase in protein folding, biosynthetic pathways, and cellular organization, and (3) alteration in ribosome-associated phosphoproteome that could fine-tune regulation of translation and transcription.


