Small open reading frames and cellular stress responses

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Small open reading frames (smORFs) encoding polypeptides of less than 100 amino acids in eukaryotes (50 amino acids in prokaryotes) were historically excluded from genome annotation. However, recent advances in genomics, ribosome footprinting, and proteomics have revealed thousands of translated smORFs in genomes spanning evolutionary space. These smORFs can encode functional polypeptides, or act as cis-translational regulators. Herein we review evidence that some smORF-encoded polypeptides (SEPs) participate in stress responses in both prokaryotes and eukaryotes, and that some upstream ORFs (uORFs) regulate stress-responsive translation of downstream cistrons in eukaryotic cells. These studies provide insight into a regulated subclass of smORFs and suggest that at least some SEPs may participate in maintenance of cellular homeostasis under stress.

smORFs and bacterial stress responses

Early evidence for the regulated expression of SEPs during cellular stress came from the study of prokaryotes, and a number of stress-response bacterial SEPs have been characterized both at the phenotypic and molecular levels.3,21,32 In this section, we discuss SEP expression during various stress responses, then detail the functions and mechanisms of selected stress-response SEPs in both Gram-negative and -positive bacteria.

Regulated smORF expression during cellular stress in bacteria

Bacterial responses to extracellular stress are governed both transcriptionally and post-transcriptionally.25-36 Transcriptional responses are mediated by dedicated transcription factors, such as σ70/RpoS in Gram-negative and σ38/ SigB in Gram-positive bacteria, which are required for the general stress response (reviewed in ref. 35 and 36, respectively). Post-transcriptional regulatory mechanisms include small regulatory RNAs (sRNA),34 RNA conformational changes,37 and RNA binding proteins; unique among bacterial stress responses, the cold shock response is largely mediated by post-transcriptional mechanisms.38 These transcriptional and post-transcriptional responses govern alterations...
Escherichia coli including heat shock, oxidative stress, and low pH, exhibited differential expression during stress responses, cold shock (blue),23,24 heat shock (red),21,25 antibiotic stress-inducible (light blue). (3) Stress-responsive smORFs discussed in this review, by color: (dark blue). (2) Annotated coding sequences within the complement strand represent: (1) annotated coding sequences within the forward strand E. coli proteins revealed an unannotated peptide mapping to a putative sensitive to envelope stress, and the three of these smORFs, yqcG, ybhT, and sgrS expressed during glucose 6-phosphate accumulation.27 These peptides map to two unannotated, intergenic sequences downstream of cold shock genes cspG and cspI, respectively. YmcF and YnfQ are upregulated by cold shock by up to a factor of 10, and exhibit 66% sequence identity, suggesting possible functional overlap. Interestingly, both of these cold-inducible smORFs initiate at AUU start codons, consistent with regulated expression.39 Subsequent work by Hemm and coworkers identified an additional 21 amino acid smORF, ynfR, downstream of ynfQ, that is also cold-inducible.24

SEPs are stress-inducible in diverse bacterial species. For example, three smORFs (sbrABC) recently discovered in Staphylococcus aureus are expressed in a SigB-dependent manner.40 sbrA and sbrB encode SEPs that are 26 and 38 amino acids, respectively, while sbrC may encode a sRNA. In a second case, transcriptomic analyses of the photosynthetic cyanobacterium Synechocystis sp. PCC 6803 revealed three SEPs, NsiR6, HliR1, and NorF1, that were induced by stress conditions, including transfer of the cyanobacteria from light to darkness.41 The nsiR6 and hliR1 transcripts (nitrogen stress-induced RNA 6 and high light inducible RNA 1) were previously annotated as noncoding RNAs.

Antibiotic stress
Antibiotics activate several bacterial stress pathways and can induce the stringent response via (p)ppGpp signaling.42 Certain antibiotics and therapeutics such as ciprofloxacin and mitomycin C induce the SOS response.43 A key antibiotic stress response linked to development of resistance is expression of drug efflux pumps.26 The 49 amino acid membrane-bound AcrZ interacts with the AcrAB–TolC drug efflux pump, which exports some classes of antibiotics to confer resistance (Fig. 2a).26 For example, strains lacking acrZ are sensitive to chloramphenicol and tetracycline, but not to erythromycin or rifampicin. While the mechanism of AcrZ is not fully characterized, AcrZ interacts directly with AcrB, which is hypothesized to lead to a conformational change in AcrB and export of specific antibiotics.26

Nutrient sensing and utilization
Specific pathways have evolved to maintain homeostasis during nutrient stress, which can arise from either nutrient limitation or accumulation.4 An early report linking smORF expression to nutrient status showed that the 227 nt sgrS sRNA in E. coli is expressed during glucose 6-phosphate accumulation.27 sgrS also encodes the 43-amino acid SEP SgrT (Fig. 2b).27 The bifunctional sgrS/sgrT gene inhibits the glucose permease PtsG at both the RNA and protein level. Under conditions of high intracellular glucose 6-phosphate, the sgrS sRNA inhibits translation of the ptsG mRNA, while the SgrT SEP binds to PtsG and inhibits glucose uptake. Overexpression of SgrT renders cells incapable of translocation of sugars.
The stressosome is a 1 MDa cytosolic complex that regulates the general stress response in Gram-positive bacteria. The stressosome senses extracellular stress and, through a prescan sequence, initiates intracellular signaling. Prli42 therefore provides a model of a SEP–protein interaction that regulates stress-response signaling in bacteria.

SmORFs and eukaryotic stress responses

Upstream SmORFs (uORFs) and translational regulation during stress

Translational regulation of the proteome is an important component of eukaryotic stress responses and may occur more rapidly than transcriptional responses; more expression-level changes occur at the protein level (several thousand genes) than at the mRNA level (hundreds of genes) during stresses such as glucose and oxygen deprivation. Generally, global protein translation is downregulated during cellular stress, while translation of a subset of stress-response proteins remains constant or increases. A specific class of eukaryotic SmORFs – upstream ORFs (uORFs) – play a role in stress-dependent translational regulation of downstream cistrons. Recent global profiling studies in yeast, plants and mammals have shown that uORF translation is widespread, especially following cellular stress. Ribosome profiling of oxidatively stressed yeast results in rapid accumulation of ribosomes on transcripts bearing uORFs following five minutes of hydrogen peroxide exposure. This observation is paralleled in human cells affected by oxidative stress, as well as oxygen and glucose deprivation.

The prevailing model of uORF-mediated translational regulation holds that translating a uORF prevents scanning and/or re-initiation at the downstream coding sequence. Re-initiation is dependent on the distance between the uORF and downstream cistron. While uORFs were initially reported to act as cis-translational inhibitors of downstream coding sequences within the same mRNA, it has become clear that uORFs can either down- or up-regulate downstream protein translation depending on the uORF start codon. AUG-initiated uORFs typically compete for translation with their downstream ORFs under normal growth conditions. In contrast, accumulation of Mn can be toxic to cells. The SEP MsfS is repressed by the manganese-dependent transcriptional regulator MntR at high manganese, and overexpression of MntS leads to increased manganese sensitivity. MntS may function to increase intracellular Mn at low Mn concentrations.
inhibition of downstream protein translation,74 and the weak eIF2 competitor eIF2A is de-repressed and delivers initiator tRNA to selected sites75 including non-AUG codon-initiated uORFs,76 driving their translation during stress (Fig. 3). For example, eIF2A drives translation of two uORFs initiating with UUG and CUG start codons and induces expression of the downstream cistron encoding binding immunoglobulin protein (BiP), an ER-resident chaperone vital for the activation of the integrated stress response.76 This mechanism also operates in squamous cell carcinoma tumorigenesis, in which eIF2A-dependent translation drives a 1.8-fold increase in uORF occupancy by ribosomes.77

uORFs are generally thought to compete for scanning ribosomes, which can then only initiate translation of downstream coding sequences via leaky scanning or re-initiation,73 implying that the regulatory function of uORFs should depend only on their translation and therefore be independent of their sequences. In a few cases, however, the specific amino acid sequence of a uORF is required for its regulatory activity.78–80 An early report of this phenomenon described a uORF in the 5′ untranslated region (UTR) of-DDIT3, which encodes the CHOP protein, a transcription factor that promotes a switch from stress response signaling to cell death.81 Translation of the uORF alone is insufficient to recapitulate translational downregulation of CHOP, as introduction of nonsense and missense mutations within the uORF alleviated translational repression of CHOP, whereas silent mutations did not.81 Further mutational analysis defined an IPI motif within the uORF that promotes ribosome stalling to inhibit CHOP translation in cis.82 Fungal uORFs in the 5′ UTR of arginine biosynthetic genes ARG2 and CPA1 also regulate downstream protein production in cis in a sequence-dependent manner via ribosome stalling.83–87

Taken together, these studies show that uORF translational regulation plays a key role in proteomic reprogramming during cellular stress responses. While several uORFs have been reported to sequence-specifically induce ribosome stalling, translated products of uORFs have generally been assumed to lack function at the polypeptide level (though the uORF-encoded MIEF1 microprotein, which binds to and regulates the mitochondrial ribosome, presents a counterexample74). In contrast, conserved smORFs encoded in dedicated transcripts have been proposed to be functional,20 and a number of these smORFs are involved in mediating stress responses.76

**Functional stress-response smORFs in eukaryotes**

Characterization of SEPs that function in eukaryotic cellular and organismal stress responses is dramatically accelerating. Several recent reports have implicated SEPs in response to infection and innate immunity. First, ribosome profiling of influenza virus-infected human lung cancer cells identified 19 novel smORFs in long non-coding RNAs (lncRNAs) and other non-coding RNAs that were either up- or downregulated during infection.88 Among these, a SEP translated from the host gene for mir-22, MIR22HG, was upregulated during infection with both wild-type influenza and N51-mutant influenza that is rapidly cleared from cells due to interferon responses, suggesting that the MIR22HG SEP may respond to cellular stress due to viral particle exposure. More recently, ribosome profiling was applied to identify differential translation of lncRNA-encoded smORFs in lipopolysaccharide (LPS)-treated mouse macrophages.89 An LPS-upregulated smORF within the lncRNA Aw112010 encodes a CUG-initiated SEP that drives interleukin-12 beta expression. Characterization of a knockout mouse demonstrated that the Aw112010 SEP is essential for mucosal immunity during both Salmonella infection and colitis. While the molecular mechanisms of the MIR22HG and Aw112010 SEPs remain uncharacterized, these studies provide a link between SEP expression and infection in cells and in vivo.

The SEP humanin has been reported to protect cells from stress-induced apoptosis. Humanin was first discovered in 2001 as a neuroprotective factor in Alzheimer’s disease, conferring neuronal resistance to apoptosis by a disease variant of the amyloid precursor protein.90 Humanin has subsequently been reported to play additional intracellular roles in suppressing stress-induced apoptosis via Bax binding and inactivation.91 While these functions suggest that humanin is protective against apoptosis downstream of cellular stress, it remains unclear how humanin is produced in cells, as its coding sequence may map to either mitochondrial or genomic DNA.91

Extensive work has identified SEPs that participate in muscle regeneration following injury. DWORF,92 a 34-amino acid SEP that localizes to the sarcoplasmic reticulum membrane, was identified in an lncRNA exhibiting heart- and muscle-specific expression (Fig. 4a). DWORF is downregulated at the protein and mRNA level during ischemic heart failure.92 DWORF normally functions to increase Ca2+ uptake into the sarcoplasmic reticulum via interaction with the Ca2+-ATPase SERCA and displacement of three other polypeptide inhibitors.93–95
Decreased contractility observed during heart failure can be caused by reduced Ca^{2+} levels in the sarcoplasmic reticulum resulting from insufficient activity of the SERCA pump. Activation of SERCA through DWORF overexpression restored calcium levels and heart contractility in a mouse model of heart disease. Another example is SPAR, a SEP encoded by IncRNA LINC00961 which is downregulated upon muscle injury (Fig. 4b). SPAR normally localizes to the endosome/lysosome membrane to promote association between lysosomal v-ATPase, Ragulator, and Rag GTPases, preventing mTORC1 activation. Upon muscle injury, SPAR downregulation promotes mTORC1 activation and muscle regeneration. Conversely, Minion or Myomerger is a SEP which is transcriptionally upregulated in muscle tissue regeneration and development (Fig. 4c). Skeletal muscle development and regeneration following injury proceeds through temporally regulated stem cell activation and differentiation, myoblast fusion and subsequent maturation into myofibers. CRISPR/Cas9 knockdown of Minion results in defects in myoblast fusion, while homozygous mutants are unviable, most likely due to the inability to form multinucleate myotubes. In summation, differential expression of a suite of SEPs is required for response to injury in both cardiac and skeletal muscle.

Conclusion

Mounting evidence supports regulatory (in eukaryotes) and functional (in both prokaryotes and eukaryotes) roles for smORF translation in cellular stress responses. A future direction will be elucidation of the functional, molecular, and phenotypic roles of dozens of yet-uncharacterized SEPs that have been identified as differentially regulated during various stress conditions in a wide variety of organisms. While dozens of SEPs have been implicated as differentially expressed at the RNA or protein level during stress responses, post-translational regulation of SEPs, especially via post-translational modifications (PTMs), has remained largely unaddressed. Given the importance of PTMs in stress signaling, identification of stress-regulated PTMs may be informative in elucidation of SEP functions. Finally, it is tempting to speculate that the small size of smORFs allows rapid translation, consistent with a need for rapid response to external stressors; measurements of the dynamics and abundance of SEP expression relative to the rate of production of known stress response proteins could test this hypothesis. Taken as a whole, the growing literature demonstrating roles for SEPs in cellular stress provides one testable hypothesis for characterization of newly discovered smORFs, and has also improved our understanding of the full complement of regulatory factors in stress response pathways.

Conflicts of interest

There are no conflicts to declare.

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