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Review Article

Methods for the characterization of stress granules in virus infected cells

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ABSTRACT

Stress granules are induced in many different viral infections, and in turn are inhibited by the expression of viral proteins or RNAs. It is therefore evident that these bodies are not compatible with efficient viral replication, but the mechanism by which they act to restrict viral gene expression or genome replication is not yet understood. This article discusses a number of methods that can be employed to gain a more complete understanding of the relationship between cellular SGs and viral RNA and protein synthesis in cells infected with diverse viruses.

1. Introduction

1.1. Stress granules

Stress granules (SG) are dynamic assemblies of ribonucleoprotein particles (mRNPs), which are formed in the cytoplasm of cells under many types of environmental stress [1,2]. The sequestration of mRNAs into these translationally-stalled cytoplasmic foci results from the rapid redirection of translation from housekeeping proteins to heat-shock proteins and other stress response factors. Upon detection of stress conditions, housekeeping mRNAs are triaged into SGs to conserve metabolic energy and to allow newly transcribed mRNAs for stress response factors to be efficiently translated.

Translation initiation under normal conditions requires the concerted activity of many cellular proteins known as eukaryotic initiation factors (eIFs). In brief, translation is enhanced when capped mRNAs are circularized by the cap-binding complex (eIF4F) and PABP, promoting their recruitment to the eIF3/40S ribosomal subunit complex. Subsequent recruitment of the 60S subunit and translation initiation at an AUG codon requires the ternary complex of eIF2, GTP and the methionyl initiator tRNA (eIF2–GTP–$tRNA_i$Met). SGs are formed when translation is blocked at the initiation stage by a number of different conditions [3], for example, inhibition of eIF4F activity or by eIF2α phosphorylation, which reduces the levels of eIF2–GTP–$tRNA_i$Met. When initiation is stalled, RNA binding proteins such as TIA-1 and TIAR can bind to the abortive complexes and relocalize them to form SGs [3]. When initiation factors become available again, for example when stress is relieved, SGs are disassembled as translation resumes. Assembly of the granules is also dependent on the Ras-GAP SH3 domain binding proteins 1 and 2 (collectively referred to as G3BP) [4]. G3BP proteins contain a nuclear transport factor-2 (NTF2)-like domain and the N-terminus and RNA binding domains closer to the C-terminus, both of which are necessary for SG formation [4]. The NTF2-like domain binds a number of partners, such as USP10 and CAPRIN-1, and also mediates dimerization. The ability of G3BP to nucleate SGs is regulated by phosphorylation of serine 149 [4]. CAPRIN-1 is necessary for normal progression through the G1-S cell cycle restriction point [5] and ubiquitin specific protease 10 (USP10) promotes the stability of a number of important proteins, including p53 [6]. Complexes containing G3BP and its many binding partners are thus involved in numerous cellular processes and likely involved in many signaling pathways [7].

In this review, we will discuss some methods for characterization of SGs in virus-infected cells. We discuss microscopy-based methods for revealing the localization of cellular and viral proteins and RNAs, as well as a newly-developed assay for SG protein solubility. We provide detailed protocols and reagent details for selected assays.

2. Importance of stress granules in viral infection

The first hint that SGs are important during viral infection came from the observation that the SG protein TIAR was bound and

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sequestered by Sendai virus RNAs [8]. We subsequently showed that Semliki Forest virus (SFV) infection induces a transient wave of SGs early in infection that are triggered by eIF2α phosphorylation, but that SGs were not detected later in infection despite continued eIF2α phosphorylation [9]. The activation of the responsible eIF2α kinase, double stranded RNA activated protein kinase (PKR), is caused by viral dsRNA replication intermediates present in the cytoplasm in the initial stages of infection. The resulting SGs are subsequently disassembled by a virus-specific mechanism as viral RNA replication progresses [9]. Since those initial reports, studies have shown that all the major families of RNA viruses as well as several DNA viruses inhibit the induction of the SG response very soon after infection (reviewed in [10]). A common theme is their transient induction at early times in infection, followed by virus-directed block in formation of SGs to relieve the restriction on viral gene expression. Poliovirus disrupts SG assembly by mediating cleavage and degradation of G3BP1 [11], while other picornaviruses block SGs by different mechanisms [12]. Our recent work showed that the alphaviruses, including SFV and Chikungunya virus, disassemble SGs via the binding and sequestration of the SG proteins G3BP1 and G3BP2 mediated by the viral non-structural protein 3 (nsP3) [13–15]. Importantly, we showed that a viral mutant (SFV-F3A) unable to bind and sequester G3BP1/2 was attenuated for growth in vitro, but grew to titers equal to those of WT SFV in cells unable to form phospho-eIF2α-dependent SGs. This confirms that the main function of G3BP sequestration is to inhibit SGs, and that without this, SFV replicates poorly in WT cells. Clearly, SGs are an important facet of the early cell-intrinsic resistance against infection, but many details about their activity remain to be revealed. Such knowledge would help to identify targets for therapeutic intervention.

2.1. Special considerations for studying SGs in virus infected cells

The special and diverse environments in virus-infected cells present some particular considerations for the study of SGs. Virus replication alters the cellular environment in ways that promote viral genome replication, transcription, particle assembly and immune evasion. Often, cellular proteins are sequestered by viral factors and reassigned to functions that support rather than inhibit virus replication. SG proteins are no exception to this, with several examples of these proteins being co-opted by viruses to their own ends [13,15–18]. With this in mind, investigators need to concurrently use more than one marker to distinguish SGs from other foci, such as of accumulation of viral proteins with their cellular binding partners. It is advisable to double- or triple-stain with an SG marker such as TIA-1/R OR G3BP-1/2 in addition to an initiation complex protein such as eIF3, eIF4G or PABP. The dissolution of SGs by cycloheximide or emetine treatment can also be used to distinguish bona fide SGs from other foci of protein accumulation [19].

A common strategy for demonstrating viral inhibition of SG assembly has been to challenge virus-infected cells with an exogenous stress inducer, such as sodium arsenite (SA) to determine if virus-encoded factors act to block the induction of SGs by the exogenous inducer. SA activates the heme-regulated inhibitor (HRI) kinase, which phosphorylates eIF2α, in turn inducing SGs [20,21]. However, this approach is complicated by the activation of another eIF2α kinase (PKR), triggered by diverse virus infections. In cells infected with viruses that induce phosphorylation of eIF2α, the addition of arsenite is redundant and does not lead to formation of SGs in infected cells, even if there is no virus-encoded mechanism to block their formation. For example, in SFV-infected cells, PKR becomes activated early in infection by the presence of dsRNA replication intermediates. Despite the consequent high and sustained levels of phosphorylated eIF2α, translation of the viral sub-genomic mRNA is efficient due to the presence of a translational enhancer in the capsid coding region [9]. To avoid this complication, researchers should consider using a phospho-eIF2α-independent SG inducer (see Table 1), such as pateamine A (Pat A, [22]) or hippuristanol [23], which inhibit the RNA helicase eIF4A, a component of the eIF4F complex, leading to SG formation independently of eIF2α Cells infected with WT SFV do not form SGs in response to either SA or Pat A, but a mutant which does not inhibit SG formation does form SGs in response to 50 nM Pat A, but not to SA [15].

3. Immunostaining – classical method for identification of SGs

SGs contents are in rapid flux in and out of the structures [19], confounding attempts to biochemically isolate them. The

Table 1
Commonly used SG inducing treatments.

Treatment/reagent	Stock solution, if applicable	Conditions	Effects	Comments
Sodium arsenite Sigma S7400	65 mg/ml makes 0.5 M stock in PBS	0.5-1.0 mM for 30–60 min	Induces SGs, phospho-eIF2α dependent	Commonly used SG inducer, works in all cells except eIF2α S51A mutants
Thapsigargin Sigma T9033	0.65 mg/ml makes 1.0 mM stock in DMSO	1.0 μM for 30–90 min	Induces SGs, phospho-eIF2α dependent	Does not work on HeLa, DU145, and COS7 cells
Pateamine A Jerry Pelletier (McGill University)	DMDA-PatA 2.0 mM stock in DMSO	50 nM for 30–60 min	Induces SGs, phospho-eIF2α independent	Blocks eIF4A helicase activity
Hippuristanol Jerry Pelletier (McGill University)	Stock 10 mM in DMSO	1 μM for 30–60 min	Induces SGs, phospho-eIF2α independent	Blocks eIF4A helicase activity
2-Deoxy-D-glucoseSigma D8375	164 mg/ml makes 1 M stock in H_2O	250 mM in HBSS or PBS 60–90 min	Induces SGs, phospho-eIF2α independent	Can be combined with FCCP for total energy starvation
Oligomycin Sigma 75351	7.9 mg/ml makes 10 mM stock in DMSO	10 μM for 60 min	Induces SGs, phospho-eIF2α independent	Blocks F1Fo ATPase
FCCP Sigma C2920	20 mg/ml makes 78 mM stock in DMSO	78 μM for 1–2 h in glucose-free media	Induces SGs, phospho-eIF2α independent	Mitochondrial protein gradient abolished
Heat shock	Preheated incubator	42–44°, 30–45 min	Induces SGs, phospho-eIF2α independent	Cells adapt and SGs can disappear within 1 h
Puromycin Sigma P9620	10 mg/ml makes 18.4 mM in H_2O	20 μg/ml for several hours	Induces large SGs	Disassembles ribosome, only ~20% cells respond; effects vary with cell line
Emetine Sigma E2375	100 mg/ml makes180 mM stock in H_2O	180 μM for 1–2 h	Disassembles SGs; blocks elongation and stabilized polysomes	Useful SG diagnostic
Cycloheximide Sigma C7698	50 mg/ml makes 177 mM stock in DMEM	17–68 μM for 1–2 h	Disassembles SGs; blocks elongation and stabilized polysomes	Useful SG diagnostic