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The stress granule component G3BP is a novel interaction partner for the nuclear shuttle proteins of the nanovirus pea necrotic yellow dwarf virus and geminivirus abutilon mosaic virus

Susanna Krapp[a], Eva Greiner[a], Bushra Amin[b], Uwe Sonnewald[a], Björn Krenz[a,*]

[a] Universität Erlangen-Nürnberg, Lehrstuhl für Biochemie, Department Biologie, Staudtstr. 5, 91058 Erlangen, Germany
[b] Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA

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ABSTRACT

Stress granules (SGs) are structures within cells that regulate gene expression during stress response, e.g. viral infection. In mammalian cells assembly of SGs is dependent on the Ras-GAP SH3-domain–binding protein (G3BP). The C-terminal domain of the viral nonstructural protein 3 (nsP3) of Semliki Forest virus (SFV) forms a complex with mammalian G3BP and sequesters it into viral RNA replication complexes in a manner that inhibits the formation of SGs. The binding domain of nsP3 to HsG3BP was mapped to two tandem 'FGDF' repeat motifs close to the C-terminus of the viral proteins. It was speculated that plant viruses employ a similar strategy to inhibit SG function. This study identifies an *Arabidopsis thaliana* NTF2-RRM domain-containing protein as a G3BP-like protein (AtG3BP), which localizes to plant SGs. Moreover, the nuclear shuttle protein (NSP) of the begomovirus abutilon mosaic virus (AbMV), which harbors a 'FVSF'-motif at its C-terminal end, interacts with the AtG3BP-like protein, as does the 'FNGSF'-motif containing NSP of pea necrotic yellow dwarf virus (PNYDV), a member of the *Nanoviridae* family. We therefore propose that SG formation upon stress is conserved between mammalian and plant cells and that plant viruses may follow a similar strategy to inhibit plant SG function as it has been shown for their mammalian counterparts.

1. Introduction

Stress granules (SGs) are cytoplasmic-localized dynamic structures that quickly form when external stresses are applied, which leads to a general decline in translation. On the other hand, SGs disperse and gene expression resumes, when cellular stress conditions abate (reviewed in Buchan and Parker, 2009). The scenario most often described for SG formation follows when oxidative, nutrient or heat stress activates one of the eIF2α kinases, which phosphorylate the alpha subunit of eIF2 and abortive translation initiation complexes form around mRNA molecules (Kedersha et al., 1999). Assembly of the granules is dependent on the RNA-binding ability of the Ras-GAP SH3 domain–binding protein (G3BP; Tourrière et al., 2003). G3BP is a multifunctional RNA-binding protein that is present in three forms in humans, G3BP-1, -2a and -2b. SGs have been shown to be induced by a number of viral infections and have been implicated in cellular defense against infection. In many cases, viral gene products were shown to inhibit SG assembly such that their formation is transient or undetectable in wild-type virus infections (Beckham and Parker, 2008; Lloyd, 2012; White et al., 2007). McInerney et al. (2005) reported that early events in Semliki Forest virus (SFV) infection induce the formation of SGs in the cytoplasm of infected cells, but that the SGs are rapidly disassembled in the vicinity of newly formed viral RNA replication complexes as the infection progresses. The C-terminal domain of the viral nonstructural protein 3 (nsP3) of SFV forms a complex with G3BP and sequesters it into viral RNA replication complexes in a manner that inhibits the formation of SG on viral mRNAs (Panas et al., 2014, 2012). Emerging evidence indicates that plant cells utilize SGs for posttranscriptional gene control similar to mammalian cells. SG-like structures were identified by cellular localization studies of eukaryotic initiation factor 4E (eIF4E), oligouridylate-binding protein 1 (UBP1), poly-A binding protein (PABP) and small ribosome subunit proteins (Pomeranz et al., 2010; Weber et al., 2008). In Arabidopsis, a tandem zinc finger protein, AtTZF1, shuttles into SG-like structures. TZF proteins recruit and activate the mRNA decay machinery in mammalian cells (Lykke-andersen and Wagner, 2005) and may nucleate mRNA processing bodies (PBs)

* Corresponding author.
E-mail address: bjoern.krenz@fau.de (B. Krenz).

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in conjunction with silencing of mRNAs containing AU-rich elements. Pomeranz et al. (2010) found that AtTZF1 co-localizes with plant SG components and also binds both DNA and RNA. Reverse genetic analyses indicated that AtTZF1 acts as a positive regulator of sugar and abscisic acid (ABA) –mediated stress response, and as a negative regulator of giberellic acid (GA) –dependent growth response. Plants over-expressing AtTZF1 are more compact than wild type, flower later, and exhibit superior cold- and drought-tolerance (Pomeranz et al., 2010).

There are only a few studies, which link plant virus to RNA granules (PBs and SGs). For instance, the replication cycle of brome mosaic virus (BMV), which belongs to the alphavirus-like superfamily, has been studied in yeast and PB constituent proteins were shown to affect its replication. PBs are cytoplasmic protein complexes that are also involved in degradation and translational arrest of mRNA and are functionally linked to SGs (Beckham et al., 2007). Recently, it was demonstrated that the helper component-proteinase (HCpro) of potato virus A (PVA), the potyviral suppressor of RNA silencing, induces the formation of RNA granules (Hafrén et al., 2015). Presence of argonaute 1 (AGO1), UBP1, varicose (VCS) and eIF4E in these potyvirus-induced RNA granules was shown. All these proteins are also components of SGs and PBs. To date, this is the only study that describes the co-localization of a plant SG component (UBP1) and a plant virus protein (HC-Pro) (Hafrén et al., 2015).

Recently, (Panas et al., 2015) proposed further candidates of virus proteins with the ability to bind to G3BP and thereby potentially inhibiting SG function. This assumption is based on the similarity to the G3BP-binding motif 'FGDF' in the nsP3 of SFV, and the authors suggested that several plant viruses may have the potential to bind to G3BP, e. g. the M-Rep of subterranean clover stunt virus (SCSV) that contains an 'FGEF'-motif. SCSV is a member of the *Nanoviridae* family, plant virus pathogens with a multipartite genome of circular single-stranded (ss) DNAs (Vetten et al., 2012). The observation by Panas et al. (2015) that the M-Rep of subterranean clover stunt virus contained an 'FGEF'-motif cannot be extended to the M-Rep proteins of other nanoviruses as their sequences vary at this position. Available data on the three-dimensional structure of the faba bean necrotic yellow virus M-Rep DNA binding domain show that amino acids at this position are part of a conserved beta-sheet in the replication initiator proteins of ssDNA viruses (Vega-Rocha et al., 2007), which would make their implication in G3BP-binding difficult to reconcile. Therefore, we looked for the presence of other potential G3BP-binding motifs in plant virus proteins and found that the $NSP^{nanovirus}$ harbor a well conserved 'F(N/T)GSF'-motif in the central part of the protein and $NSP^{begomovirus}$ a '(F/Y)VS(F/Y)'-motif at the C-terminal end. Hence the question arose whether the NSPs have the ability to bind to the plant homologues of G3BP.

First we wanted to see if plant SGs are functionally similar to their mammalian counterparts and then test if the NSP of pea necrotic yellow dwarf virus (PNYDV), a nanovirus identified in commercially grown pea (*Pisum sativum* L.) in Germany (Grigoras et al., 2010) and the NSP of abutilon mosaic virus (AbMV) have the ability to bind to potential G3BP homologues from plants.

2. Materials and methods

2.1. Microorganisms, plants, and general methods

Material for the construction of PYNDV NSP and AbMV NSP expression vectors were kindly provided by Bruno Gronenborn, Tatjana Kleinow and Holger Jeske, respectively. Material for the construction of GFP_nsP3_31 and GFP:HsG3BP expression vectors were kindly provided by Gerald M. McInerney. *N. benthamiana* plants were grown in an insect-free S1 greenhouse with 16 h supplementary illumination. Recombinant DNA techniques were performed according to (Sambrook and Russell, 2001). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers. DNA sequencing confirmed the correctness of the respective constructs.

2.2. Construction of expression plasmids

The coding sequence of entire open reading frames of NSP^{PNYDV} (NC_023159), NSP^{AbMV} (NC_001929), TZF1 from *Arabidopsis thaliana* (At2g25900), G3BP-like protein from *Arabidopsis thaliana* (At5g43960), and UBP24 from *Arabidopsis thaliana* (At4g30890) was amplified by PCR using the primers listed in supplemental Table 1. The resulting PCR products were inserted into the vector pENTR-D/TOPO (Invitrogen). To generate a translational fusion between the nanoviral proteins and GFP or RFP, the fragments were subsequently recombined into the destination vector pK7FWG2 or pH7RWG2, respectively, (Karimi et al., 2002) using L/R-Clonase (Invitrogen). Constructs for bimolecular fluorescence complementation are based on Gateway® compatible versions of pRB-C-$Venus^{N173}$ and pRB-C-$Venus^{C155}$ (Nietzsche et al., 2014). To create the $NSP^{F54A/F56A/PNYDV}$:GFP expression constructs two overlapping PCR fragments generated with the primer pairs PNYDV_NSPfor/PNYDV_NSPmutrev and PNYDV_NSPmutfor/PNYDV_NSPrev were fused and subsequently inserted into the vectors pENTR-D/TOPO (Invitrogen), pK7FWG2 (Karimi et al., 2002) and pRB-C-$Venus^{N173}$ and pRB-C-$Venus^{C155}$ (Nietzsche et al., 2014). The sequence for the last 31 aa of NSP^{AbMV} was synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) with an additional SacI restriction site at the 3′ end and an overlap of 31nt complementary to the 3′ end of GFP at its 5′ end. GFP_NSP31 was constructed by PCR with primer pair GFP_nsP3_31for and AbMV_NSP31rev, subcloned into pGEM-Teasy (Promega, Mannheim, Germany) and transferred into BamHI/SacI-linearized pBIN-GFP (Haseloff et al., 1997). GFP_NSP31mut was generated by PCR with primer pair GFP_nsP3_31for and AbMV_NSP_31mutrev with GFP_NSP31 as template and finally also inserted into BamHI/SacI-linearized pBIN-GFP. Schematic illustration of the generated constructs can be seen in Suppl. Fig. 1 and all used primers are listed in Supplemental Table 1.

2.3. Agroinfiltration assay

Expression plasmids were transformed into *A. tumefaciens* C58C1 by heat shock. Plasmid-containing agrobacteria were grown overnight, pelleted, resuspended in 10 mM $MgCl_2$ and diluted to an $OD_{600} = 0.1$, and incubated for at least 2 h at room temperature. Source leaves of 5 to 6 weeks old *N. benthamiana* plants were infiltrated with a 1 ml syringe with the respective agrobacteria suspension according to (Morilla et al., 2006). Agrobacteria suspension was mixed in a ratio of 1:1 or 1:1:1 for co-expression experiments. Each agroinfiltration assay was repeated at least three times, where 3 plants were infiltrated and two leaves/plant.

2.4. Western blot analysis

Immune-affinity capture of proteins (GFP-trap® or RFP-trap ®, Chromotek, Munich, Germany) was performed as recommended by the manufacturer and eluted in SDS-PAGE loading buffer (4% SDS, 20% glycerol, 0.5 M DTT, 100 mM Tris–HCl, pH 6.8, 0.005% bromophenol blue). The solution was boiled for 5 min, and separated in 12% Bis-Tris gels. Proteins were transferred to nitrocellulose (GE Healthcare Life Sciences, Amersham TM Protran TM 0.45 μm NC) using a semi-dry transfer assembly