SCD6 induces ribonucleoprotein granule formation in trypanosomes in a translation-independent manner, regulated by its Lsm and RGG domains

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ABSTRACT
Ribonucleoprotein (RNP) granules are cytoplasmic, microscopically visible structures composed of RNA and protein with proposed functions in mRNA decay and storage. Trypanosomes have several types of RNP granules, but lack most of the granule core components identified in yeast and humans. The exception is SCD6/Rap55, which is essential for processing body (P-body) formation. In this study, we analyzed the role of trypanosome SCD6 in RNP granule formation. Upon overexpression, the majority of SCD6 aggregates to multiple granules enriched at the nuclear periphery that recruit both P-body and stress granule proteins, as well as mRNAs. Granule protein composition depends on granule distance to the nucleus. In contrast to findings in yeast and humans, granule formation does not correlate with translational repression and can also take place in the nucleus after nuclear targeting of SCD6. While the SCD6 Lsm domain alone is both necessary and sufficient for granule induction, the RGG motif determines granule type and number: the absence of an intact RGG motif results in the formation of fewer granules that resemble P-bodies. The differences in granule number remain after nuclear targeting, indicating translation-independent functions of the RGG domain. We propose that, in trypanosomes, a local increase in SCD6 concentration may be sufficient to induce granules by recruiting mRNA. Proteins that bind selectively to the RGG and/or Lsm domain of SCD6 could be responsible for regulating granule type and number.

INTRODUCTION
Ribonucleoprotein (RNP) granules, membrane-free RNP particles, are central to the posttranscriptional regulation of gene expression. The cytoplasmic RNP granules include processing bodies (P-bodies), which are constitutively present and contain mRNA degradation enzymes, as well as various types of stress granules, which contain components of the translation initiation machinery (Anderson and Kedersha, 2009).

Although these two types of RNP granules are broadly classified as foci of mRNA decay machinery or as storage particles, the precise functions and composition of most RNP granules are still poorly defined, and much effort has focused on identifying specific core components as entry to an understanding of RNP granule function. By RNA interference (RNAi) depletion of individual granule components, several proteins have been identified in mammals that are interdependently necessary for correct P-body formation, namely GW182, Ccr4, Lsm1, Lsm4, RCK/p54, eIF4E-T, RAP55, and Ge-1/Hedls (Jakymiw et al., 2007). A similar redundancy of core P-body components was observed in yeast (Teixeira and Parker, 2007). In addition to the identified proteins, micro-RNAs (miRNAs) and mRNAs were found to be necessary for P-body formation (Jakymiw et al., 2007).
However, as P-bodies are formed by translationally repressed RNPs, RNAi experiments do not distinguish between core granule-building components, which are directly necessary for P-body formation, and proteins that could be involved in maintaining the pool of nontranslated mRNAs, for example, by repressing translation. To date, only two proteins have been identified as “true” P-body core proteins, based on the fact that their depletion resulted not only in loss of granule formation but did so without affecting translational repression that occurs concomitantly with P-body–inducing glucose deprivation. One is the Lsm protein EDC3; the other is the Lsm4 protein, a subunit of the cytoplasmic Lsm1-7 complex involved in mRNA decapping (Decker et al., 2007). The Q/N-rich domain of Lsm4 appears to be involved in granule formation by self-aggregation via a prion-like mechanism. The Q/N-rich domain of a yeast prion protein can compensate for the equivalent domain of Lsm4 in granule formation. At least 20 P-body proteins contain Q/N-rich domains (Reijns et al., 2008). Self-aggregating domains are also involved in stress granule formation (Anderson and Kedersha, 2008, 2009); for example, the prion-related Q-rich domain of TIA-1/TIAR (Kedersha et al., 1999; Gilks et al., 2004). Recently, low-complexity domains of RNA-binding proteins were identified to be necessary and sufficient for RNP granule assembly induced in vitro by biotinylated isoxazole (Kato et al., 2012).

One protein shown to be essential for P-body formation in vertebrates (Tanaka et al., 2006; Yang et al., 2006), plants (Xu and Chua, 2009), and trypanosomes (Kramer et al., 2012), although not in Drosophila (Eulalio et al., 2007b) or Caenorhabditis elegans (Audhya et al., 2005), is the Lsm domain containing protein RAP55/SCD6. It was initially discovered as a component of cytoplasmic RNP particles in the salamander Pleurodeles waltii (Lieb et al., 1998). The protein and its localization to P-bodies is highly conserved in euukaryotes (Lieb et al., 1998; Audhya et al., 2005; Boag et al., 2005; Wilhelm et al., 2005; Barbee et al., 2006; Squirell et al., 2006; Tanaka et al., 2006; Yang et al., 2006; Pepling et al., 2007; Kramer et al., 2008; Xu and Chua, 2009; Mair et al., 2010). In Xenopus and C. elegans, expression of the RAP55/SCD6 orthologues is restricted to germ cells and early embryos (Lieb et al., 1998; Audhya et al., 2005; Boag et al., 2005; Tanaka et al., 2006), while in Drosophila and humans, it is expressed in both germ and somatic cells (Barbee et al., 2006; Yang et al., 2006; Eulalio et al., 2007a,b). RAP55/SCD6 consists of an N-terminal Lsm domain, a C-terminal FDF motif, several RGG motifs (Marnef et al., 2009), and, in some homologues, Q/N-rich sequences (Supplemental Figure S1). RAP55/SCD6 acts as a repressor of translation (Marnef et al., 2009), likely mediated by an interaction between the RGG motif in RAP55/SCD6 and elf4G (Rajaguru et al., 2012). In addition to elf4G, several other proteins have been identified as interacting with RAP55/SCD6, for instance, the RNA helicase DH11, also known as CGH-1 or p54 (Audhya et al., 2005; Boag et al., 2005; Wilhelm et al., 2005; Tanaka et al., 2006; Triticshler et al., 2009; Nisan et al., 2010; Matsumoto et al., 2012); the decapping enzyme DCP1-DCP2 (Tritschler et al., 2008; Nisan et al., 2010; Fromm et al., 2012); the decapping activator Pat1 (Nissan et al., 2010); and the arginine methyltransferases PRMT1 and PRMT5 (Tanaka et al., 2006; Matsumoto et al., 2012).

The high degree of conservation of SCD6 throughout the eukaryotes, together with its conserved localization to P-bodies and interactions with translation initiation factors and proteins involved in decapping, suggests that SCD6 plays a central role in regulating the formation of RNP granules. A model suggesting that SCD6 might contribute to RNP granule formation by forming a heptameric ring, via interactions between the Lsm domain in a similar manner to the Lsm1-7 or Lsm2-8 complexes, is attractive but currently without supporting evidence. Tra1, the Drosophila orthologue of SCD6, does not form multimers in vitro, and coimmunoprecipitation studies have not revealed interactions of Tra1 with itself or proteins of the Lsm1-7 complex (Triticshler et al., 2008).

Trypanosomes have a large number of different RNP granules, a possible reflection of their almost complete reliance on posttranscriptional regulation of gene expression (De Gaudenzi et al., 2011; Kramer, 2012). At least four different types can be distinguished: P-body–like structures (Cassola et al., 2007; Holetz et al., 2007; Kramer et al., 2008), starvation stress granules (Cassola et al., 2007), heat shock stress granules (Kramer et al., 2008), and nuclear periphery granules (NPGs), which resemble perinuclear germ granules and form after inhibition of trans-splicing (Kramer et al., 2012). Orthologues of many proteins proposed to have core functions in P-body or stress granule formation in metazoan and yeast are not readily identifiable in the trypanosome genome; for example, the P-body components elf4E-T, EDC3, DCP1-DCP2, GW182, and Ge-1/Hedls or the stress granule components TTP, G3BP, TIAR/TIA-1, and CPEB. The cytoplasmic Lsm1-7 complex is also absent, because trypanosomes lack LSM1 (Liu et al., 2004). The only cytoplasmic Lsm domain protein that can be readily identified in the trypanosome genome is SCD6. The protein localizes to P-bodies (Kramer et al., 2008) and is essential for P-body formation (Kramer et al., 2012).

In this paper, we provide evidence that SCD6 is the P-body protein responsible for granule assembly in trypanosomes, a role possibly related to the absence of other Lsm domain proteins in the cytostomplasm. When overexpressed, SCD6-eYFP (enhanced yellow fluorescent protein) aggregates into granules that corecruit other P-body proteins, as well as poly(Al)-binding protein, elf4G5, and mRNAs. Granule formation does not correlate with a repression of translation, and our data provide evidence that SCD6 may recruit its mRNA targets mainly from the nonpolyosomal mRNA pool. In fact, granules can also form when SCD6 is targeted to the nucleus. Granule type and number is dependent on the presence of an RGG motif, seemingly in a translation-independent manner. We propose that the formation of P-bodies and P-body–related granules in trypanosomes is largely determined by the local concentration of SCD6 and mRNA.

RESULTS
Overexpressed SCD6 aggregates into cytoplasmic granules enriched at the nuclear periphery
To investigate the role of SCD6 in trypanosome RNP granule formation, we aimed to overexpress an SCD6-eYFP transgene in a procyclic cell line, using the tetracycline-based expression system described by Wirtz et al. (1999) and Sunter et al. (2012). In the absence of tetracycline, no SCD6-eYFP was detectable by Western blotting, while after 24 and 48 h of induction, SCD6-eYFP was present at about twice the wild-type level (Figure 1A), indicating successful overexpression. The majority of SCD6-eYFP was localized to numerous (typically >20) cytoplasmic granules (Figure 1B). The degree of granular localization is a consequence of overexpression, because expression of SCD6-eYFP from its endogenous locus at native expression levels resulted in only a minor fraction of SCD6-eYFP present in a few (<10) P-bodies (Kramer et al., 2008; Figure 1C).

The expression levels of SCD6-eYFP varied between individual cells more than two orders of magnitude, even though the population was clonal, an effect that had been previously reported (Sunter et al., 2012). This enabled the fraction of SCD6-eYFP localized to granules to be analyzed as a function of expression level (Figure 1D). At low expression levels, only a minor fraction of SCD6-eYFP was localized to granules, and the number and size of...
the granules were similar to P-bodies marked by expression of fluorescein-labeled P-body markers at endogenous levels. With increasing expression, the percentage of SCD6-eYFP in granules increased up to ~75%, and granule number increased from <10 in cells with low SCD6-eYFP expression up to 45 in cells with high expression. At higher expression levels, granules were not equally distributed throughout the cytoplasm but appeared enriched in regions adjacent to the nucleus. Three-dimensional modeling revealed that all granules were extranuclear (Figure 1E and Supplemental Movie S1).

The total amount of endogenous SCD6 protein present in a trypanosome procyclic cell was estimated to be around 60,000 molecules, using a titration of recombinant SCD6 protein (Figure S4). This is an amount similar to the number of rRNA molecules (Dhalla et al., 2006).

The variation in SCD6-eYFP expression between cells prevented an accurate determination of the expression level necessary for granule induction. However, considering that two thirds of the cells were expressing SCD6-eYFP at concentrations high enough to induce granules, and the mean overexpression level is around threefold the endogenous expression (Figure 1A), we can estimate that a threefold overexpression is sufficient for the induction of granules. Therefore, in all subsequent microscopy experiments, an arbitrary SCD6-eYFP expression threshold corresponding to a total fluorescence intensity of 200,000 (at identical image acquisition conditions), which safely corresponds to at least a threefold overexpression of SCD6, was used to define cells "highly" overexpressing SCD6.

The fusion protein SCD6-eYFP was shown to be functional, as a cell line with one SCD6 allele deleted and the second allele replaced with SCD6-eYFP is viable (Figure S2), whereas the lack of SCD6 is lethal, as previously shown by RNAi depletion (Kramer et al., 2012). Moreover, comparable expression levels of eYFP alone did not result in granule formation (Figure S3A). For further confirmation that induction of granules was not an artifact of the eYFP tag, wild-type SCD6 protein was overexpressed. We were unable to detect SCD6 granules by immunofluorescence. One possible reason is that the granule structure prevents antibody access. We have previously found that some types of trypanosome RNP granules are very difficult to detect with antibodies, for instance, P-bodies (Kramer et al., 2008), while others can be detected easily (Kramer et al., 2012). However, overexpression of wild-type SCD6 in a cell line also expressing the P-body marker protein DH1H1 as an mChFP transgene caused localization of mChFP-DHH1 into granules (Figure S3B), similar to that seen with overexpression of SCD6-eYFP did (see results below in Figure 3, A–D). We therefore assume that the phenotypes observed after SCD6-eYFP and SCD6 overexpression are identical.

**Overexpression of SCD6 does not cause major reductions in growth, translation, or global mRNA levels within 24 h**

The aggregation of overexpressed SCD6 into granules could be either a direct effect of the increase in protein concentration or a secondary effect caused by translational repression. To distinguish between these two possibilities, we monitored growth, translation, and steady-state mRNA levels over a time course of SCD6-eYFP induction (Figure 2). Cell proliferation was normal for the first 24 h, by which time the SCD6-eYFP granules had formed (Figure 2A), although there was an almost complete growth arrest at later time points. At 24 h after induction, there was only a small reduction in the aggregation of overexpressed SCD6 into granules could be either a direct effect of the increase in protein concentration or a secondary effect caused by translational repression. To distinguish within these two possibilities, we monitored growth, translation, and steady-state mRNA levels over a time course of SCD6-eYFP induction (Figure 2). Cell proliferation was normal for the first 24 h, by which time the SCD6-eYFP granules had formed (Figure 2A), although there was an almost complete growth arrest at later time points. At 24 h after induction, there was only a small reduction in overall translation, monitored by either polysome gradients (Figure 2B) or [3S]methionine incorporation (Figure 2C). In trypanosomes, total mRNA can be quantified by probing a Northern blot for the mini-exon, the part of the spliced leader (SL) RNA that is transspliced to the 5′ end of all trypanosome mRNAs. There was no significant change in total steady-state mRNA levels up to 48 h of SCD6 overexpression (Figure 2D).

The small reduction in translation after 24 h of SCD6 overexpression is not sufficient to account for the formation of the amount of RNP granules observed, even considering that the unequal
Several proteins were expressed as eYFP fusion proteins from their endogenous loci in cell lines containing an inducible SCD6-CerFP transgene; others were expressed as mChFP fusion proteins in cell lines containing an inducible SCD6-eYFP transgene (Figure 3A). Of the proteins tested, only DHH1, eIF4E1, PABP2, and eIF4G5 were detected in SCD6-induced granules (Figures S6 and 3, A–D). It must be noted, however, that nonrecruitment could be due to impaired functionality of the eYFP or mChFP fusion proteins. Only a small fraction of PABP2 (Figure 3B) and eIF4G5 (Figure S6) localized to SCD6-induced granules, while a larger fraction of the two P-body proteins DHH1 and eIF4E1 was present in granules.

A conspicuous difference between the recruitment of the two P-body markers was that DHH1 was enriched in granules distant from the nucleus (Figure 3C and Movie S1), whereas the distribution of eIF4E1 was very similar to that of SCD6-CerFP (Figure 3D). Of the proteins tested, only DHH1, eIF4E1, PABP2, and eIF4G5 were detected in SCD6-induced granules (Figures S6 and 3, A–D). It must be noted, however, that nonrecruitment could be due to impaired functionality of the eYFP or mChFP fusion proteins. Only a small fraction of PABP2 (Figure 3B) and eIF4G5 (Figure S6) localized to SCD6-induced granules, while a larger fraction of the two P-body proteins DHH1 and eIF4E1 was present in granules.

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Recruitment of other proteins to the SCD6-induced granules is dependent on a granule’s distance from the nucleus

To characterize the granules induced by SCD6 overexpression, we examined whether they recruited proteins associated with translation. Several proteins were expressed as eYFP fusion proteins from their endogenous loci in cell lines containing an inducible SCD6-CerFP transgene; others were expressed as mChFP fusion proteins in cell lines containing an inducible SCD6-eYFP transgene (Figure 3A). Of the proteins tested, only DHH1, eIF4E1, PABP2, and eIF4G5 were detected in SCD6-induced granules (Figures S6 and 3, A–D). It must be noted, however, that nonrecruitment could be due to impaired functionality of the eYFP or mChFP fusion proteins. Only a small fraction of PABP2 (Figure 3B) and eIF4G5 (Figure S6) localized to SCD6-induced granules, while a larger fraction of the two P-body proteins DHH1 and eIF4E1 was present in granules.

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SCD6 granules close to the nucleus are sensitive to inhibition of transcription

Interference with either translation or transcription affects different types of RNP granules in a specific way, providing some information about their possible functions. To further characterize the granules that formed after induction of SCD6-eYFP expression, we used a cell line that constitutively expressed mChFP-DHH1 from the modified endogenous locus in addition to the inducible SCD6-eYFP transgene. After 24 h of induction, cultures were incubated with either actinomycin D, to inhibit transcription, or cycloheximide, a drug that causes a reduction of P-bodies and an increase of polysomes. Over a time course of 120 min, actinomycin D caused the SCD6-eYFP granule distribution to change from perinuclear enrichment to a more even dispersal throughout the cytoplasm (Figure 4A). This change was less obvious for mChFP-DHH1, as DHH1 was already preferentially present in the distant granules (Figure 3, C and E). The percentage of SCD6-eYFP and mChFP-DHH1 within the perinuclear region was determined in the presence and absence of actinomycin D. The perinuclear region was defined as a 7.1-μm circle around the nuclear center. In untreated cells, an average of 73.3% ± 7% of total SCD6-eYFP and 56.6% ± 8% of mChFP-DHH1 was localized close to the nucleus (Figure 4A). In the presence of actinomycin D, the percentage of SCD6-eYFP in perinuclear granules decreased to 67% ± 10%, 60% ± 9%, and 48% ± 10% at 30, 60, and 120 min respectively, while there was no significant change in the distribution of mChFP-DHH1. After 60 or 120 min of actinomycin D treatment, there was no significant difference between the percentage of SCD6-eYFP and mChFP-DHH1 contained in perinuclear granules (Figure 4A).

In contrast, treatment with cycloheximide caused no significant change in the percentage of SCD6-eYFP close to the nucleus and only a minor increase from 57% ± 8% to 64% ± 11% of the perinuclear mChFP-DHH1 fraction after 120 min of incubation. The

Regions of interest are enlarged. (E and F) For an average of 15 randomly selected granules per cell, taken from five individual cells, the content of SCD6-eYFP and mChFP-DHH1 (E) or eIF4E1-mChFP (F) was quantified. The quotient was plotted against granule distance to the center of the nucleus. Cells with an SCD6-eYFP expression level below threshold (see legend for Figure 1D) or more than one nucleus were excluded from the analysis.
In trypanosomes, trans-splicing can be inhibited by sinefungin, an S-adenosylmethionine analogue that prevents methylation of the cap of the spliced leader RNA. In the presence of transcription, inhibition of trans-splicing results in the accumulation of polycistronic mRNAs, which partially leak into the cytoplasm (Kramer et al., 2012). This causes the formation of NPGs. The function of NPGs remains unknown, but one possibility is that they act as a novel cytoplasmic compartment to assess mRNA quality and prevent immature mRNAs from entering translation. NPGs have striking similarities to nucleus-associated germ granules found in gonads of adult animals. Similarities include protein composition (e.g., VASA), localization, dependency on transcription, and stability in the presence of cycloheximide (Eddy and Ito, 1971; Mahowald, 1971; Mahowald and Hennen, 1971; Strome and Wood, 1982, 1983; Hay et al., 1988a,b; Sheth et al., 2010). Both granule types may have a similar evolutionary origin. To investigate whether SCD6-induced granules resemble NPGs, we incubated cells that had been induced for 24 h with the trans-splicing inhibitor sinefungin. There was a significant increase from 73.3% ± 7 to 82% ± 3% of SCD6-eYFP in the perinuclear region (Figure 4A).

SCD6-induced granules contain mRNA
The mRNA content of granules formed after SCD6-eYFP induction was examined by fluorescence in situ hybridization (FISH), using cy3-labeled oligos directed against the poly(A) tail (dT) or the mini-exon sequence (ME) that is trans-spliced to the 5′ end of every trypanosome mRNA. Prior to induction, both oligos were evenly distributed throughout the cytoplasm, whereby the ME oligo also detected an RNA in the nucleus, which is most likely the SL RNA (Kramer et al., 2012). After induction, mRNA was detected in all granules containing SCD6-eYFP, both in the absence and presence of actinomycin D (Figure 5).

The data suggest that the granules formed after SCD6 induction contain intact mRNAs, although it cannot be ruled out that they contain a mixture of partially 5′–3′ degraded mRNAs and partially 3′–5′ degraded mRNAs. Moreover, mRNA is present in both the transcription- and translation-sensitive populations of SCD6-induced RNP granules. The presence of mRNAs with poly(A) tails fraction of SCD6-eYFP in perinuclear granules remained significantly higher than the fraction of mChFP-DHH1 up to 120 min of cycloheximide treatment (Figure 4A).

Taken together, these data indicate that granules close to the nucleus are sensitive to inhibition of transcription but not to an inhibition of translation, and may therefore contain newly transcribed mRNAs.

The induction of transcription-sensitive RNP granules concentrated in a perinuclear compartment is reminiscent of NPGs that form in trypanosomes upon inhibition of trans-splicing (Kramer et al., 2012). In trypanosomes, trans-splicing can be inhibited by sinefungin, an S-adenosylmethionine analogue that prevents methylation of the cap of the spliced leader RNA. In the presence of transcription, inhibition of trans-splicing results in the accumulation of polycistronic mRNAs, which partially leak into the cytoplasm (Kramer et al., 2012). This causes the formation of NPGs. The function of NPGs remains unknown, but one possibility is that they act as a novel cytoplasmic compartment to assess mRNA quality and prevent immature mRNAs from entering translation. NPGs have striking similarities to nucleus-associated germ granules found in gonads of adult animals. Similarities include protein composition (e.g., VASA), localization, dependency on transcription, and stability in the presence of cycloheximide (Eddy and Ito, 1971; Mahowald, 1971; Mahowald and Hennen, 1971; Strome and Wood, 1982, 1983; Hay et al., 1988a,b; Sheth et al., 2010). Both granule types may have a similar evolutionary origin. To investigate whether SCD6-induced granules resemble NPGs, we incubated cells that had been induced for 24 h with the trans-splicing inhibitor sinefungin. There was a significant increase from 73.3% ± 7 to 82% ± 3% of SCD6-eYFP in the perinuclear region (Figure 4A). Single-plane images of deconvolved Z-stacks of nuclei from several untreated and sinefungin-treated cells were compared (Figure 4B). Although NPGs are generally more uniform in size and more evenly distributed around and closer to the nucleus than SCD6-induced granules, the perinuclear subset of SCD6-induced granules resemble NPGs in granule pattern and sensitivity to actinomycin D.
The Lsm domain of SCD6 is necessary and sufficient for granule induction, but the RGG motif determines granule number

A structure-informed deletion analysis was performed to investigate which part of SCD6 is sufficient for granule induction. In total, transgenes encoding 13 different mutants were inducibly expressed in a cell line containing a mChFP-DHH1 transgene at the endogenous locus, resulting in constitutive expression. The mutants included several truncations, as well as a deletion of the N-rich domain and a mutation of one of the RGG motifs (Figure 6). As already observed for the wild-type protein, the expression levels of the SCD6 mutants were nonuniform, and only cells with the previously defined total expression level of >200,000 were included in the microscopic analysis. First, the ability of each mutant to induce granules at overexpression was tested. This was defined as both the ability of the SCD6-eYFP mutant to localize to granules (Figure 6) and an increase in the percentage of mChFP-DHH1 in granules (data not shown).

Granules formed after expression of all transgenes containing the Lsm domain, including the Lsm domain alone (Figure 6K). None of the three mutants lacking the Lsm domain localized to or formed granules. Thus the Lsm domain of SCD6 is necessary and sufficient for the formation of granules.

The number of granules formed after transgene induction varied significantly between the different SCD6 mutants. This was quantified by measuring 1) the number of granules, 2) the percentage of total SCD6-eYFP mutant protein in granules, and 3) the percentage of total SCD6-eYFP mutant protein in the largest granule. Induction of wild-type SCD6-eYFP expression for 24 h resulted in an average of 38 granules containing ∼60% of the protein (Figure 6A). Neither the removal of the N-rich domain nor the mutation of the N-terminal RGG box (RGG1) caused a significant change in either granule number or the percentage localized to granules (Figure 6, B and C). Deletion of the FDF-TFG domain and the C-terminal RGG box (RGG2) resulted in a minor decrease in the number of granules to ∼25–30, and the percentage of SCD6 localizing to granules decreased to ∼37% (Figure 6, D and E). Deletion of the FDF-TFG domain and the RGG2 box combined with mutation of the RGG1 box resulted in a decrease in granule number to an average of 12, and only ∼15% of SCD6 was localized to granules. Thus absence of the RGG1 box significantly interferes with granule induction, but only in the absence of the C-terminus: the RGG2 box might compensate for the mutated RGG1 box. Successful deletions of RGG1 resulted in a further decrease in both the number of granules and in the percentage of the mutant SCD6-eYFP localizing to granules (Figure 6, G–K). Mutations of SCD6 mainly affected granule number, while granule size was in a similar range compared with wild type. There were two exceptions that had significantly smaller granules than the wild type (Figure 6, G and K). It is possible that the truncation interfered with protein folding in these cases. These data show that the Lsm domain is both necessary and sufficient for SCD6 to localize to and induce granules, but the additional presence of an intact RGG motif is required for the induction of many granules, as opposed to only a few.

We observed that certain SCD6 truncations formed granules located at the posterior pole of the cell (e.g., Figure 6K, arrow); mChFP-DHH1 was not recruited to granules in this position (data not shown). This localization resembles that of XRNA, the trypanosome orthologue to XRN1, during heat shock (Kramer et al., 2008) and suggests an association with the ends of the microtubules in the subpellicular array that underlies the plasma membrane in trypanosomes (Robinson et al., 1995). It is possible that this association with the microtubule cytoskeleton is normally transient but becomes
parallel increase in the fraction of mChFP-DHH1 in granules, confirming that SCD6ΔC-eYFP not only localized to granules but also recruited other proteins. The P-body protein eIF4E1 was detectable in SCD6ΔC-granules but not the stress granule marker protein PABP2, in contrast to wild-type SCD6-induced granules, which include both (Figure 7B). This suggests that the composition of the SCD6ΔC granules may resemble that of P-bodies.

Granules formed after overexpression of a C-terminally truncated SCD6 resemble P-bodies

The differences between the few large granules formed by overexpression of SCD6 mutants without an RGG domain and the many granules formed by overexpression of wild-type SCD6 were investigated. The “few granules” were induced by the C-terminally truncated SCD6 (Figure 6I), hereafter named SCD6ΔC.

While wild-type SCD6-eYFP localized to granules even at endogenous expression levels (Figure 1C; Kramer et al., 2008) and well below an expression level of 50,000 (fluorescence intensity units, Figure 1D), the SCD6ΔC-eYFP expression level had to exceed a threshold value of 80,000 before it localized to granules. mChFP-DHH1 was clearly visible in P-bodies at lower expression levels of SCD6ΔC-eYFP, indicating that granule formation per se was not disturbed (Figure 7A). As expression levels increased, the percentage of SCD6ΔC-eYFP in granules increased, and there was an almost parallel increase in the fraction of mChFP-DHH1 in granules, confirming that SCD6ΔC-eYFP not only localized to granules but also recruited other proteins. The P-body protein eIF4E1 was detectable in SCD6ΔC-granules but not the stress granule marker protein PABP2, in contrast to wild-type SCD6-induced granules, which include both (Figure 7B). This suggests that the composition of the SCD6ΔC granules may resemble that of P-bodies.

Cells constitutively expressing mChFP-DHH1 were treated with cycloheximide and actinomycin before and after induction of SCD6ΔC-eYFP expression (Figure 7C), and the fraction of both proteins in granules was quantified. Before induction, 2% of mChFP-DHH1 was detectable in SCD6ΔC-granules but not the stress granule marker protein PABP2, in contrast to wild-type SCD6-induced granules, which include both (Figure 7B). This suggests that the composition of the SCD6ΔC granules may resemble that of P-bodies.

Cells constitutively expressing mChFP-DHH1 were treated with cycloheximide and actinomycin before and after induction of SCD6ΔC-eYFP expression (Figure 7C), and the fraction of both proteins in granules was quantified. Before induction, 2% of mChFP-DHH1 was detectable in SCD6ΔC-granules but not the stress granule marker protein PABP2, in contrast to wild-type SCD6-induced granules, which include both (Figure 7B). This suggests that the composition of the SCD6ΔC granules may resemble that of P-bodies.

The percentage of SCD6ΔC-eYFP in granules was reduced by cycloheximide but remained unaffected by actinomycin D treatment, visible under the experimental conditions. This was not further examined in this study, but the possible role of the microtubules’ association of SCD6 in P-body formation is discussed in Discussion.
formed after SCD6ΔC-eYFP induction did not contain any mRNAs detectable by the same FISH procedure used above (Figure 7D). It is likely that the granules contain little or no mRNA or that any mRNA entering the granules is rapidly degraded. It must also be considered that the granule structure might prevent the penetration of the DNA probe.

In conclusion, the granules formed after induction of SCD6ΔC-eYFP resemble P-bodies in number, composition, sensitivity to cycloheximide, and apparent absence of mRNAs.

We further analyzed the localization of SCD6ΔC-eYFP to other RNP granule types. After 1 h of sinefungin treatment, only a very small percentage of SCD6ΔC-eYFP localized to NPGs (Figure S7A, compare Figure 4A for wild-type SCD6). Similar results were obtained for another SCD6ΔC-eYFP truncation (SCD6ΔC*: Figure 6J and S7B). Thus the Lsm domain does not mediate localization to NPGs. In contrast, an SCD6 mutant lacking the Lsm domain and the N-rich region (Figure 6M; SCD6ΔN) localized to NPGs, although it did not localize to any other cytoplasmic granules (Figure S7C). Thus different requirements exist for the recruitment to NPGs or to other cytoplasmic granules, an observation consistent with P-body but not NPG formation, being dependent on SCD6 (Kramer et al., 2012).

Localization to carbon source–starvation stress granules obtained by culturing the cells in phosphate-buffered saline (PBS; Cassola et al., 2007) was similar to wild-type SCD6 for C-terminally truncated SCD6 and only slightly reduced for the N-terminally truncated version (Figure S7). In comparison with localization to other RNP granules, the localization to starvation stress granules appears to be the least restricted, as almost any RNA-binding protein tested has the ability to localize there (unpublished data). It seems probable that the localization to starvation stress granules occurs via many parallel pathways.

SCD6 and SCD6ΔC induce nucleoplasmic granules when targeted to the nucleus

Induction of SCD6ΔC-eYFP expression caused only a few granules to form, whereas induction of wild-type SCD6-eYFP expression resulted in tens of granules per cell. One possible explanation for this difference could be that wild-type SCD6 is able to induce granules de novo, while SCD6ΔC can only increase the size of the few existing P-bodies. If this were true, SCD6ΔC would be unable to induce granules in the absence of wild-type SCD6. This was tested by simultaneously inducing expression of SCD6ΔC-eYFP and RNAi knockdown
of endogenous SCD6. However, even though a major reduction in endogenous SCD6 protein occurred (Figure S8A), SCD6ΔC-eYFP still localized to granules (Figure S8B).

Because it is possible that the small fraction of endogenous SCD6 that remains present despite RNAi is sufficient to nucleate P-bodies, a different strategy was used. SCD6ΔC-eYFP was targeted to the nucleus, where there is little or no wild-type SCD6 (Kramer et al., 2008). The previously defined NLS (nuclear localization signal) of the La protein (Marchetti et al., 2000) was added to the N-terminus of SCD6-eYFP or SCD6ΔC-eYFP, and the fusion proteins were overexpressed in a cell line expressing SCD6-mChFP from the endogenous loci. A similar strategy has been used to study the interactions between mammalian P-body components (Bloch et al., 2011). Both NLS-SCD6-eYFP and NLS-SCD6ΔC-eYFP localized to the nucleus, while SCD6-mChFP remained cytoplasmic, indicating the nucleus was free of endogenously expressed SCD6 (Figure 8, A and B). The experiments were repeated in a cell line expressing mChFP-DHH1 instead of SCD6-mChFP from the endogenous locus. After induction, both NLS-SCD6-eYFP and NLS-SCD6ΔC-eYFP localized to the nucleus, while mChFP-DHH1 remained cytoplasmic, indicating that no complete P-bodies were formed (data not shown).

NLS-SCD6-eYFP and NLS-SCD6ΔC-eYFP both accumulated in distinct granules in the nucleoplasm and in the nucleolus (Figure 8, A and B). This finding differs from the equivalent experiment performed with the mammalian SCD6 orthologue, which did not aggregate to nuclear granules and was excluded from the nucleoli (Bloch et al., 2011). Granule formation in the nucleoplasm resembled the cytoplasmic process: the number of granules increased with the increase of transgene expression, and SCD6ΔC-eYFP required a higher threshold expression level for aggregation into granules than did the wild-type protein (Figure 8C).

When transcription was inhibited by actinomycin D, nucleoplasmic granules disappeared, and nuclear fluorescence was restricted to one or two small spots that possibly correspond to the remains of the nucleolus (Figure 8D). Cycloheximide did not cause any change in granules (Figure 8D). When a CerFP fusion of elf4E1 was targeted to the nucleus in a similar way, it did not aggregate into granules, although it is possible that the expression level was too low for granule formation (Figure 8E). When either NLS-SCD6-eYFP or NLS-SCD6ΔC-eYFP was expressed at the same time as NLS-elf4E1-CerFP, part of NLS-elf4E1-CerFP colocalized with the granules in the nucleoplasm (Figure 8E).

Taken together, the data show that both wild-type SCD6 and SCD6ΔC can induce the formation of RNP granules in an "artificial" environment free of wild-type SCD6 and DHH1 and probably other P-body proteins. The granules are dependent on active transcription and are therefore most likely to recruit RNA, and they can recruit at least one other granule protein.

**DISCUSSION**

To understand RNP granule function, it is essential to understand granule assembly and identify the core granule components. The search for P-body core proteins in yeast and animals has revealed redundant genetic interactions between many genes, and the process of P-body assembly is complex. The presence of multiple P-body core proteins in different species indicates that P-bodies have evolved to perform similar functions in different organisms. The function of the RGG domain

The function of the RGG domain

The Lsm domain of trypanosome SCD6 is fully sufficient for the induction of RNP granules, but one intact RGG domain is required for all the functions of granule formation, translation repression, and mRNA localization.
while a truncation lacking the RGG domain (SCD6\(\Delta\)C) induces only P-bodies (Rajyaguru et al., 2012). While SCD6\(\Delta\)C is not impaired in mRNA binding, its ability to repress translation is reduced, and the truncated protein cannot interact with eIF4G (Rajyaguru et al., 2012).

Binding of SCD6 to eIF4G is likely to mediate SCD6-induced translational repression by preventing the binding of the 43S initiation complex. This results in the formation of P-bodies and stress granules upon overexpression of SCD6. A similar finding has recently been reported in yeast. Saccharomyces cerevisiae SCD6 induces both P-bodies and stress granules upon overexpression, while a truncation lacking the RGG domain (SCD6\(\Delta\)C) induces only P-bodies (Rajyaguru et al., 2012). While SCD6\(\Delta\)C is not impaired in mRNA binding, its ability to repress translation is reduced, and the truncated protein cannot interact with eIF4G (Rajyaguru et al., 2012). Binding of SCD6 to eIF4G is likely to mediate SCD6-induced translational repression by preventing the binding of the 43S initiation complex.
complex (Rajaguru et al., 2012, Nissan et al., 2010). Therefore the RGG domain of SCD6 seems to be essential for translational repression at the initiation stage via eIF4G binding and for mRNA recruitment to storage granules. It may prevent mRNA degradation by hindering access of DCP2 to the cap (Rajaguru et al., 2012). SCD6ΔC must recruit its mRNA targets, if any, from another source, possibly from translating polysomes.

A significant novel finding of this work is that, in trypanosomes, the RGG domain appears to function independent of translation or any other factor restricted to the cytoplasm. The differences between granules induced by either wild-type SCD6 or SCD6ΔC remain when the proteins are targeted to the nucleus. The data are not necessarily contradictory to the findings in yeast, if one assumes that SCD6 binds to eIF4G-mRNA complexes already in the nucleus. In fact, eIF4G has important nuclear functions in pre-mRNA processing in humans (McKendrick et al., 2001) and yeast (Kafasla et al., 2009).

The minimal requirement for an RNP granule

There is no evidence for SCD6 forming multimers in Drosophila (Tritschler et al., 2008), and it is likely not do so in trypanosomes, as we could not detect any interactions between SCD6 molecules by either coimmunoprecipitation or FRET (fluorescence resonance energy transfer) (unpublished data). Furthermore, the removal of the N-rich region, a candidate sequence for prion-like aggregation (Decker et al., 2007; Reijns et al., 2008), did not prevent RNP granule induction. Therefore RNP granule aggregation is likely to require other factors in addition to SCD6. The sensitivity of both the perinuclear and nuclear SCD6 granules to actinomycin D indicates a dependency on RNA, possibly mRNA, but other RNA types cannot be excluded. The formation of RNP granules in either the cytoplasmor the nucleus shows that RNP granule formation requires neither exclusively nuclear nor cytoplasmic components. Therefore, it is possible that SCD6 and RNA are fully sufficient for the formation of RNP granules. However, we cannot completely rule out that cytoplasmic proteins are corecruited to the nucleus by SCD6, even though both proteins tested in this work, DHH1 and endogenous SCD6, remained cytoplasmic. There also remains the possibility that nuclear-cytoplasmic shuttling proteins are required.

A model of granule formation

One SCD6 molecule has at least two RNA-binding sites: the Lsm domain and the RGG domain. In addition, it can bind to several mRNA-binding proteins, for instance, eIF4G (Rajaguru and Parker, 2012), of which trypanosomes have five isoforms. This multivalence enables SCD6 to act as a connector between two mRNA molecules (either directly or via binding to a molecule bound to the mRNA) and can result in the assembly of a network of mRNAs and SCD6. Other proteins may be “passively” corecruited to the granules, because they are bound to the recruited mRNA. At endogenous expression levels, with about one SCD6 molecule per mRNA, the concentration of involved molecules and binding constants result in the formation of only a few small granules. An increase or decrease in the amount of either the available mRNA molecules or the mRNA linker protein SCD6 will shift the equilibrium and result in an increase or decrease of granules. Experimentally, drugs that interfere with translation can produce changes in the concentration of available mRNAs and RNAi, or overexpression can change the concentration of SCD6. Under natural conditions, changes in the concentration of available mRNAs can occur via stress-induced polysomal repression, for example, stress induced by heat shock (Kramer et al., 2008). Changes in SCD6 protein concentration can occur by a change in SCD6 expression or by a subcellular increase of SCD6 mediated by active transport. Evidence for a possible association of RNP granules with the microtubule system comes from the localization of SCD6 truncations (this study) or XRNA (Kramer et al., 2008) to the posterior pole of the cell, which is where the microtubule plus ends are located in trypanosomes (Robinson et al., 1995). The RGG domain of SCD6 determines the type of RNP granule, P-body, or storage granule, possibly by determining the type of mRNA that is recruited. One possible mechanism is the masking of the RGG domain by an unknown factor that normally prevents SCD6 from recruiting intact mRNAs. This would explain why P-bodies or granules induced by SCD6ΔC do not contain any detectable mRNAs. On SCD6 overexpression, this factor may become limiting, which would now allow the recruitment of intact mRNAs, possibly by binding to eIF4G (Rajaguru and Parker, 2012). Under natural conditions, the binding of such factors would be regulated.

Understanding the regulation of RNP granule formation is essential to unraveling gene expression pathways. In yeast and mammals, this is hampered by the complexity and redundancy in RNP granule core proteins. The unique role SCD6 appears to have in the formation of trypanosome RNP granules and the absence of most RNP granule core proteins suggests that trypanosomes may well serve as model organisms for the study of RNP granule formation.

MATERIAL AND METHODS

Trypanosomes

The cell line Lister 427 pSPR2, which expresses a tetracycline repressor, was used for all experiments (Sunter et al., 2012). Transgenic trypanosomes were generated using standard procedures (McCulloch et al., 2004). All experiments were performed with logarithmically growing trypanosomes at a cell density of <1 × 10^7 cells/ml. The wild-type Lister 427 strain (a kind gift from George Cross, Rockefeller University) was used for Figure S2.

Plasmids used in this work

All plasmids used in this work are summarized in Table S1. Endogenous tagging of proteins was essentially performed as described by Kelly et al. (2007), and inducible overexpression was done based on the 3383 plasmid described by Sunter et al. (2012); in many cases, selectable markers and tags were exchanged/added.

Western blots, Northern blots, polysomes, and [35S]methionine labeling

Western blots were done according to standard protocols. Proteins were detected and quantified by the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Northern blots, polysomes, and [35S]methionine labeling were done as previously described (Kramer et al., 2008).

Drugs

Drugs were used in the following concentrations: cycloheximide, 50 μg/ml; actinomycin D, 10 μg/ml; sinefungin, 2 μg/ml.

RNA FISH

RNA FISH was performed as previously described (Kramer et al., 2012), except that 5% dextran sulphate was included in the hybridization solution, and 1 μM of oligos labeled with Cy3 at both ends were used for detection. Oligo sequences were

- dT:45T;
- dA:45A;
- ME antisense: CAATATAGTACAAACTGGTCTAATAATAGCGTT;
- ME sense: AACGCTATTATAGAACAGTTCAGTACTATAATG.
Microscopic imaging and quantification

Cells were washed with SDM79 without serum and hemin and fixed at a density of 1 × 10^7 cells/ml with 2.4% paraformaldehyde overnight, washed once in PBS, and stained with 4',6-diamidino-2-phenylindole (DAPI). Z-stacks (100 images, 100-nm spacing) were recorded with a custom-built TILL Photonics IMiC microscope equipped with a 100×, 1.4-numerical aperture objective (Olympus, Tokyo, Japan) and a sensicam qe CCD camera (PCO, Kelheim, Germany); deconvoluted using Huygens Essential software (SVI, Hilversum, The Netherlands); and, unless otherwise stated, presented as Z-projections (method sum slices) produced by and quantified with ImageJ. The total expression level of an individual cell was calculated by integration of the total background-subtracted fluorescence of the cell in the 32-bit Z-projections; the percentage of SCδ6-εYFP (or mutants) in granules was determined by adding the integrated fluorescence of all granules (manual threshold) and correlating it to the total fluorescence of the cell. For three-dimensional imaging, the recorded stacks were volume- and surface-rendered using Imaris software version 7.6 (Bitplane, Belfast, Northern Ireland).

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