Survival motor neuron protein facilitates assembly of stress granules

Yimin Hua, Jianhua Zhou*

Department of Medicine and Program in Neuroscience, University of Massachusetts Medical School, 364 Plantation Street, LRB 325, Worcester, MA 01605, USA

Received 3 May 2004; revised 2 July 2004; accepted 9 July 2004

Available online 23 July 2004

Edited by Michael R. Bubb

Abstract The survival motor neuron (SMN) protein forms cytoplasmic granules when overexpressed. We report here that SMN co-localizes with TIA-1/R and G3BP, protein assemblers of stress granules (SGs), and that SMN is co-immunoprecipitated with TIA-1/R, suggesting that SMN granules are SGs. Formation of SMN granules precedes accumulation of TIA-1/R, indicating that SMN serves as a facilitator of SG formation. However, the exon 7 skipping product, SMNΔ7, is largely retained in the nucleus and forms nuclear granules, indicating that exon 7 is critical for SG formation. Our findings reveal a novel SMN function and possible SG involvement in the pathogenesis of spinal muscular atrophy.

Keywords: Survival motor neuron; Spinal muscular atrophy; Stress granule; TIA-1/R

1. Introduction

Stress response is a protective cellular process induced by a variety of environmental stresses including chemical exposure, heat shock and UV irradiation. Transcription and translation of specific stress-induced genes are prioritized in response to stress, while many other genes are silenced. About 50% of total poly(A)$^+$ mRNA is actively recruited and dynamically sorted into stress granules (SGs) [1] in response to stress-induced phosphorylation of eukaryotic initiation factor (eIF) 2α [2]. Phosphorylation of eIF2α inhibits protein synthesis by preventing formation of eIF2-GTP-Met-tRNAi complex. SGs are not translationally competent, but rather serve as local storage and protection compartments for mRNAs under translational arrest. Once the stress is released, the SGs are disassembled, and mRNAs are repacked into translationally competent mRNPs and proteins are synthesized [3].

Efforts have been taken to identify SG components, however, the list is far from complete. The proteins found to accumulate in SGs include the RNA-binding proteins TIA-1 (T-cell internal antigen-1), TIAR (TIA-1-related protein) and PABP (poly[A]$^+$ mRNA binding protein) as well as a subset of eIFs, including elF2, elF2B, elF3, elF4E and elF4G [4,5]. Under stressed conditions, TIA-1/R (TIA-1 and TIAR) shuttle from the nucleus into the cytoplasm and form large particles by self-aggregation of prion-like domains at the C-termini of TIA proteins, then modulating the formation of SGs [6]. This process is facilitated by G3BP, a rasGAP-associated endoribonuclease [7]. Other proteins identified in SGs include 40S ribosomal subunits S3 and S19, and two ARE-binding proteins, the RNA-stabilizing protein HuR and the mRNA-destabilizing protein TTP (tristetraprolin). Alternately, 60S ribosomal subunits L5 and L37 and other ARE-binding proteins, such as hnRNPA1 (heteronuclear RNP-A1) and hnRNPD (heteronuclear RNP-D)/AUF-1 (AU-rich RNA binding factor) are excluded, suggesting that components in SGs are selective [5,6,8].

The survival motor neuron (SMN) protein is ubiquitously expressed and distributed both in the cytoplasm and in the nucleus where it concentrates in gems (gemini of coiled bodies) [9]. There are two S*MN* genes, *SMN1* and *SMN2*, which encode identical proteins. Loss-of-function of *SMN1* is responsible for the development of the autosomal recessive spinal muscular atrophy (SMA) [10–12]. The *SMN*2 copy, which is present in most of SMA patients, produces preferentially exon 7-skipping isoform, SMNΔ7, and inadequate full-length protein that fail to protect motor neurons from the loss of the *SMN1* gene. We have recently observed the localization of SMN protein in granule-like structures in the cytoplasm under stressed conditions [13], consistent with previous reports that SMN protein can form granule-like aggregates in the cytoplasm of human fetal muscle cells and rat motor neurons [14,15], and in other cell types when overexpressed, or under conditions of cellular starvation [16–19]. In this report, we further demonstrate that the SMN granules are stress granules.

2. Materials and methods

2.1. Antibodies

Antibodies were from Santa Cruz Biotech (Santa Cruz, California) (SMN polyclonal antibody H195), BD Transduction Laboratories (Franklin Lakes, New Jersey) (monoclonal antibodies, anti-SMN and anti-G3BP), EMD biosciences, Inc. (San Diego, California) (monoclonal antibody, anti-α-tubulin), Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania) (FITC conjugated goat anti-rabbit secondary antibody) or Molecular Probes (Eugene, Oregon) (Secondary antibodies: highly cross-adsorbed Alexa Fluor 488 goat anti-rabbit and highly cross-adsorbed Alexa Fluor 633 goat anti-mouse). Monoclonal mouse anti-TIA-1/R antibody 3E6 was a generous gift from Dr. Nancy L. Kedersha (Brigham and Woman's Hospital, Boston).

2.2. Cell culture, stress treatment and immunofluorescence

HeLa cells, human neuroblastoma SKN-MC cells, human SMA type I fibroblasts (3813 and 9677) and normal fetal fibroblasts

* Corresponding author. Fax: +1-508-856-6797.
E-mail address: jianhua.zhou@umassmed.edu (J. Zhou).

Abbreviations: SMA, spinal muscular atrophy; SG, stress granule; HA, hemagglutinin; dSMN, *Drosophila* SMN

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.07.010

(IMR-91) were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) at 37 °C in 5% CO_2. Cells growing on glass coverslips were insulted by UV irradiation or exposure to 0.5 or 1.0 mM sodium arsenite, or by floating the plate in a 44 °C pan of water in a CO_2 incubator (heat shock) as described previously [1]. For heat shock experiments, the temperature was gradually increased to 44 °C over a period of 10 min. Immunofluorescence staining was then performed as described [20]. The localization of proteins was visualized with a confocal laser scanning microscope (Leica Microsystems, Inc., Bannockburn, IL).

2.3. Plasmid constructions and transfection

Constructs, that express GFP-SMN and GFP-SMN deletion mutants, were generated by inserting wild-type SMN and truncated SMNs, amplified by PCR, into the polylinker region at *Bgl*II and *Sal*I sites following GFP in the pEGFP-C1 vector (Clontech). Hemagglutinin-survival motor neuron (HA-SMN) cDNA or wild-type SMN cDNA was cloned at *Bam*HI and *Eco*RI sites in pcDNA3 vector (Invitrogen). Plasmids were transiently transfected into cells using the calcium phosphate method. Cells with or without stress treatment were analyzed by immunofluorescence or immunoprecipitation.

2.4. Immunoprecipitation and Western blot analysis

HeLa cells with or without exposure to heat shock at 44 °C for 30 min were mechanically detached from dishes and collected by centrifugation (1500 rpm at 4 °C for 5 min). Cells were then lysed in Buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail [Roche Applied Science]) supplemented with 0.6% NP-40 on ice for 15 min. Cytoplasmic fractions (supernatant) were collected by centrifugation (14 000 rpm at 4 °C for 10 min). The pellets were then resuspended in Buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail) and incubated on ice for 1 h. Nuclear extracts were cleared by centrifugation at 14 000 rpm at 4 °C for 15 min. Presence of SMN and α-tubulin proteins in cytoplasmic and nuclear extracts was detected by Western blot analysis. For co-immunoprecipitation, TIA-1/R were pulled down using 3E6 and protein G beads from HeLa lysates (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 2 mg/ml bovine serum albumin, 1% NP-40, 1 mM PMSF and protease inhibitor cocktail). Western blot analysis was carried out for the presence of SMN.

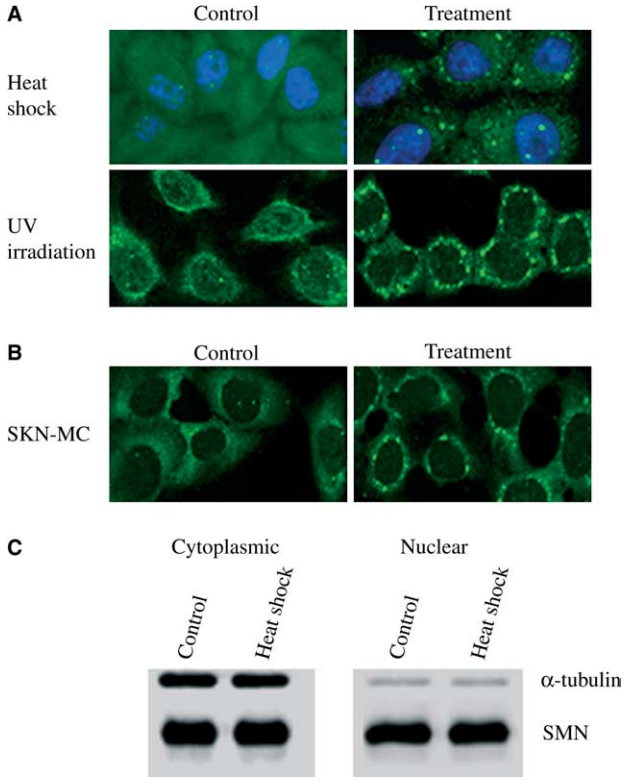

Fig. 1. The endogenous SMN protein forms cytoplasmic granules under stressed conditions. HeLa and SKN-MC cells were insulated by incubation at 44 °C for 30 min or UV irradiation for 20 min. Immunofluorescence analysis was then carried out by using anti-SMN antibody (H195) and a FITC conjugated anti-rabbit secondary antibody (green). Nuclei were counterstained using Hoechest dye (blue). (A) HeLa cells. (B) SKN-MC cells heat shocked for 30 min. (C) Western blot experiments by using a mouse anti-SMN antibody were performed in the cytoplasmic and the nuclear extracts of cells incubated at 44 °C for 30 min.

3. Results

3.1. SMN co-localizes with TIA-1/R in SGs and interacts with TIA-1/R

We have previously shown that SMN forms granules in HeLa cells in response to stress [13]. To investigate the significance of this event, HeLa cells and neuronal SKN-MC cells were insulted with heat shock or UV irradiation. Immunofluorescence experiments were then conducted by using anti-SMN antibody (H195). Our data shows that under harmful conditions, SMN was redistributed and formed cytoplasmic punctuated bodies in both cell types (Fig. 1A and B). We designate the structures as cytoplasmic SMN granules. With H195 antibody, we also detected gems in both normally cultured and stressed cells (Fig. 1). Staining using a mouse monoclonal anti-SMN antibody displayed similar patterns (data not shown), eliminating the possibility of antibody artifacts. Western blot analysis demonstrated that no significant change of relative SMN levels in the cytoplasm and the nucleus (Fig. 1C) was observed, suggesting that the cytoplasmic SMN aggregation is not caused by redistribution of SMN protein from the nucleus into the cytoplasm. To further characterize SMN granules, double immunofluorescence experiments were performed for the endogenous SMN and TIA-1/R proteins, two SG markers that promote the recruitment of untranslated mRNAs to SGs [21]. Confocal microscopy analysis revealed that SMN was mostly co-localized with TIA-1/R when HeLa cells were exposed to either heat shock (Fig. 2A) or UV irradiation (data not shown). The SG distribution of the SMN protein was further confirmed by its co-localization with G3BP (Fig. 2B), a RasGAP interacting protein that was recently described as an assembler for the formation of SGs in cells insulted by heat shock [7]. Furthermore, co-immunoprecipitation experiments from HeLa cells that were transfected with SMN constructs demonstrated that SMN was strongly associated with TIA-1/R (Fig. 2D and E). Arsenite, a widely used stimulant promoting the formation of SGs, also induced co-localization of SMN with TIA-1/R into SGs in both HeLa (Fig. 2C) and SKN-MC cells (data not shown). Moreover, emetine, a protein synthesis inhibitor that has been shown to suppress SG formation [21], dispersed SMN granules (Fig. 2C). Our results indicate that SMN granules are SGs.

3.2. SMN facilitates SG formation

To elucidate the relevance of SMN in formation of SGs, we conducted a time-course experiment. When HeLa cells were incubated at 44 °C for 20 min, a few loose SMN granules began to emerge while the localization of TIA-1/R in the granules was not observed. After incubation at 44 °C for 30 min, almost all of the cells formed SMN granules while TIA-1/

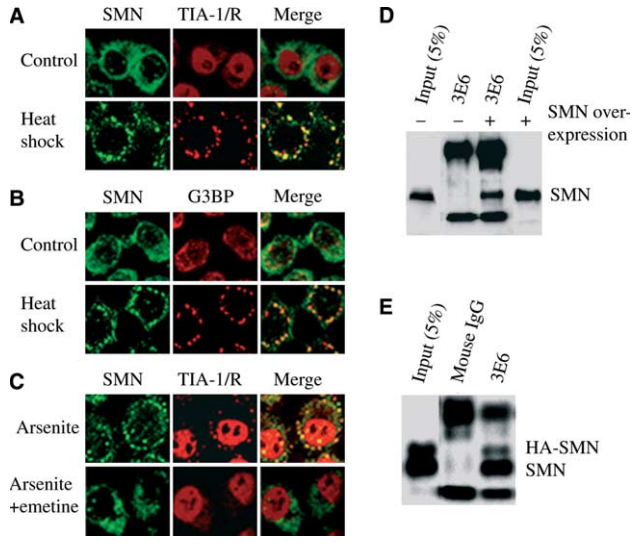

Fig. 2. SMN co-localizes with TIA-1/R and G3BP in SGs and is associated with TIA-1/R. HeLa cells were incubated at 44 °C for 30 min. Double immunofluorescence experiments were performed using anti-SMN antibody H195 and anti-TIA-1/R (3E6) or anti-G3BP, and secondary antibodies against rabbit (Alexa Fluor 488, green) and mouse (Alexa Fluor 633, red). Localization of proteins was monitored by a confocal microscopy. Yellow represents co-localization. (A) Double-staining for SMN and TIA-1/R. (B) Double staining for SMN and G3BP. (C) Cellular distribution of SMN and TIA-1/R in HeLa cells treated with 1 mM of arsenite for 30 min, or with 1 mM of arsenite for 30 min and then addition of 10 μg/ml of emetine for 60 min. Cellular extracts from HeLa cells transfected with SMN (D) or HA-SMN (E) were immunoprecipitated with anti TIA-1/A antibody 3E6 or mouse IgG and then analyzed by western blotting with a monoclonal anti-SMN antibody.

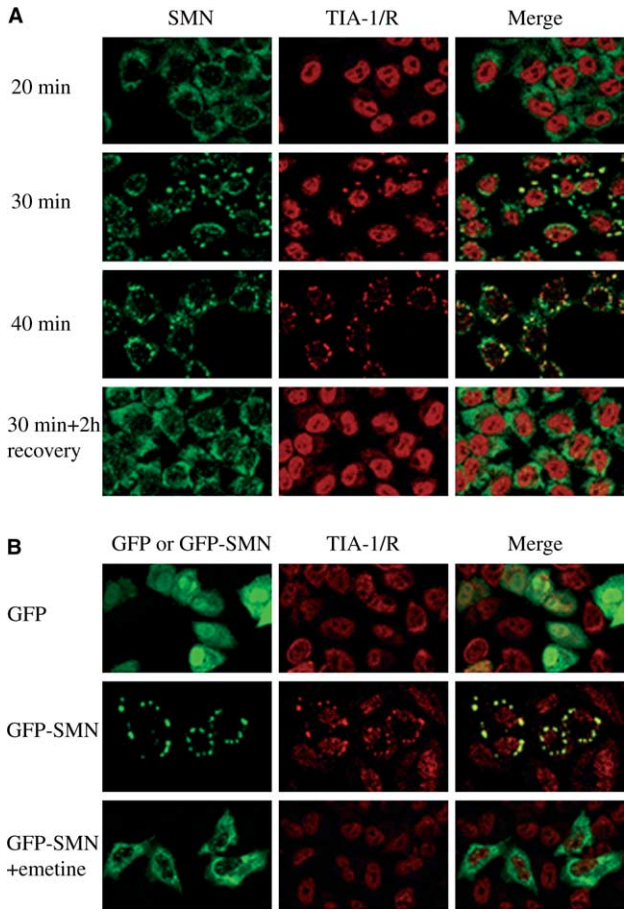

Fig. 3. SMN facilitates the formation of SGs. (A) Formation of SMN granules preceded recruitment of TIA-1/R into SGs. HeLa cells were cultured and incubated at 44 °C heat shock for 20, 30 and 40 min, or re-incubated at 37 °C for 2 h after 30 min heat shock. Immunofluorescence analysis of SMN and TIA-1/R proteins was performed. (B) Overexpression of GFP-SMN fusion protein formed cytoplasmic SMN granules in HeLa cells. TIA-1/R were detected in the granules. GFP protein alone was used as a negative control. Treatment of cells with 10 μg/ml of emetine for 1 h dispersed GFP-SMN granules in HeLa cells.

R were recruited into granules in only about 50% of cells. However, after being exposed to 44 °C for 40 min, all HeLa cells formed cytoplasmic granules with double positive staining of SMN and TIA-1/R (Fig. 3A). These results suggest that the formation of SMN granules precedes recruitment of TIA-1/R with a time-dependent manner, indicating a possible role for SMN as a facilitator for the formation of SGs. This notion is further supported by the observation that punctuated SMN aggregates in the cytoplasm, that were induced by overexpression of SMN contained TIA-I/R in 30–40% of transfected cells (Fig. 3B). We again used emetine and proved that it dispersed SMN granules triggered by overexpression of SMN (Fig. 3B). Our results suggest that the formation of SGs may be initiated and facilitated by SMN aggregated granules.

3.3. Exons 2a–2b are associated with stress induced formation of granules

To determine which motifs affect the localization of SMN to SGs, we generated multiple GFP fusion constructs with the following regions of SMN: exons 1–2a, 1–2b, 2a–2b, 2b, 3–4, 3, 4, 2a–7, 2b–7, 3–7, 4–7, 5–7 or 6–7 (Fig. 4A). The fusion proteins were expressed in HeLa cells and the assembly of cytoplasmic SMN granules was monitored by immunofluorescence. Considering that SGs are structures that accommodate many RNA species and RNA binding proteins, one would expect that exons 2a–2b, an RNA binding domain [22], would play a role in the localization of SMN into SGs. This assumption was supported by the observation that small granules were formed by overexpression of GFP-SMN exons 1–2b, 2a–2b, but not by overexpression of GFP-SMN exons 1–2a or 2b alone (Fig. 4B). However, all fusion proteins that contain either exons 3–4 or exons 4–7 in the absence of exons 2a–2b also automatically triggered the formation of small SMN granules (Fig. 4C and D). Next, we tested whether stress could influence the function of exons 2a–2b, 3–4 or 4–7 in SMN granule assembly by exposing HeLa cells at 44 °C for 30 min. We observed a dramatic increase in the number of cells (from 40% to 90% of transfected cells) to form cytoplasmic granules, when the fusion proteins contained exons 2a–2b, but no increase in the number of cells with granules (30%) when transfected with constructs without exons 2a–2b. These data indicate that while multiple regions of SMN may initiate assembly of small SMN granules, exons 2a–2b operate in response to stress.

3.4. C terminus of SMN modulates the formation of SMN granules

To investigate the roles of SMN C-terminus in SG formation, GFP fusion constructs containing SMN exons 1–5,

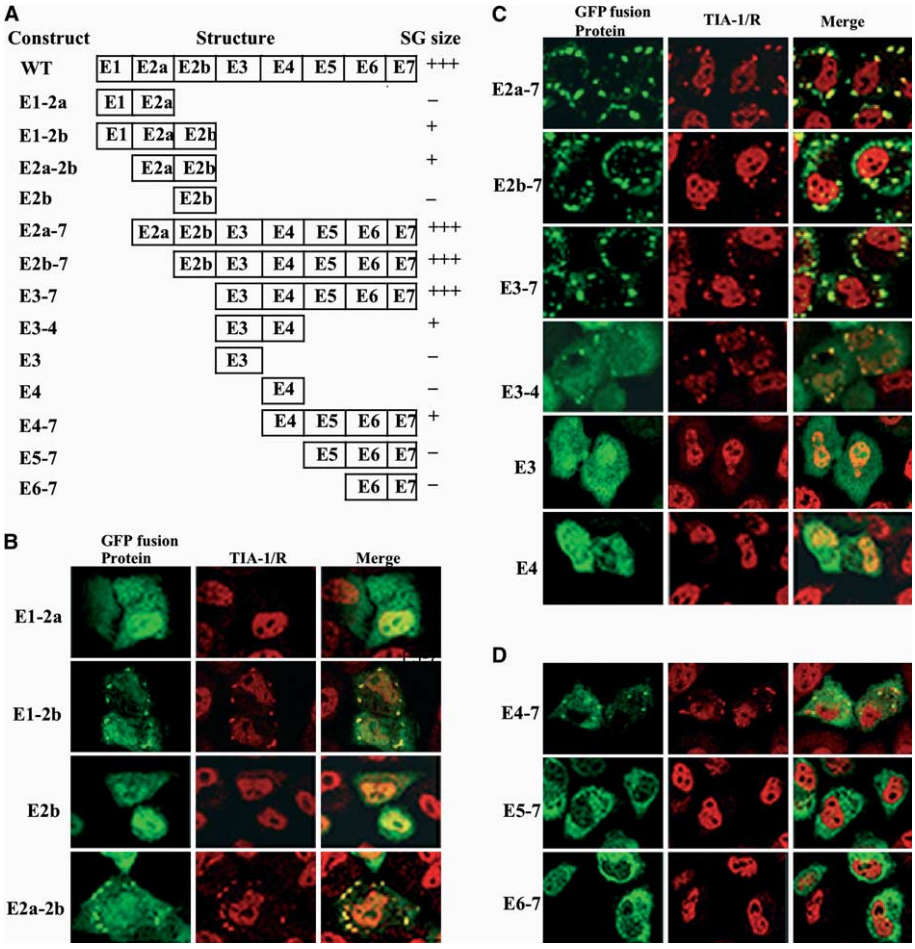

Fig. 4. Multiple regions of SMN are involved in the formation of SMN granules. The GFP fusion constructs with deletion mutant SMNs were generated and transiently transfected into HeLa cells. Distribution of the fusion proteins and the TIA-1/R protein was examined by confocal immunofluorescence, 24 h after transfection under normal growth conditions. (A) Structures of GFP-SMN constructs. In this and the following figures, "Exon(s)" is abbreviated as "E". −: no granules; +: small granules; +++: large granules. (B) GFP fusion proteins with exons 2a–2b but not with exon 2a or 2b alone promoted the formation of granules. (C) GFP fusion proteins with exons 3–4 sufficiently promoted the formation of granules. (D) Exons 4–7 facilitated granule formation.

exons 1–6, or exons 1–6 with Y–G motif deletion (exons 1–6 m) were generated and transfected into HeLa cells (Fig. 5A). To our surprise, GFP-SMN exons 1–6 were primarily localized into highly concentrated nuclear granules while GFP-SMN exons 1–6m, which lack the Y–G domain, exhibited similar distribution patterns as GFP-SMN exons 1–5 (Fig. 5B). These observations suggest that the Y–G motif might promote SMN protein distribution into nuclear granules in the absence of exon 7. In the presence of exon 7, however, the effects of Y–G domain were reversed and the protein of exons 1–7 was redistributed into cytoplasmic granules. Interestingly, when exon 7 was replaced by a peptide of 22 amino acids, which was derived from the multiple cloning site of the vector as GFP-SMN exons 1–6+, distribution of the fusion protein was primarily observed as granules in the cytoplasm (Fig. 5B). We hypothesize that the specific exon 7 sequence is not critical on SMN localization. These results are consistent with the observations that C-termini (exon 7) of human SMN and *Drosophila* SMN are diversified, while Y–G domains within exon 6 are conserved (Fig. 5A). Furthermore, to test the possible relevance of SGs to the pathogenesis of SMA disease, SMA type I fibroblast cells (cell lines 3813 and 9677) and normal control fetal fibroblast cells (IMR-91) were treated with heat shock. Formation of SGs in control cells with both SMN and TIA-1/R staining was evident after prolonged exposure of 44 °C for 1 h, while only drastically smaller and much fainter granules detected by TIA-1/R staining were observed in SMA type I fibroblasts (Fig. 6).

4. Discussion

In mammalian cells, the characteristic feature of SMN protein is the formation of gems in the nucleus. Using immunofluorescence and confocal microscopy, we showed in this report that SMN co-localized with TIA-1/R and G3BP in SGs. This is the first description of endogenous cytoplasmic SMN aggregates related to a known cellular structure, implicating novel functions for SMN. Several lines of evidence indicate that SMN is important for SG assembly. Detailed analysis suggests that multiple regions can initiate SMN self-aggregation. However, to form large and smooth granules, the tudor domain as well as exon 6 and exon 7, are indispensable. These

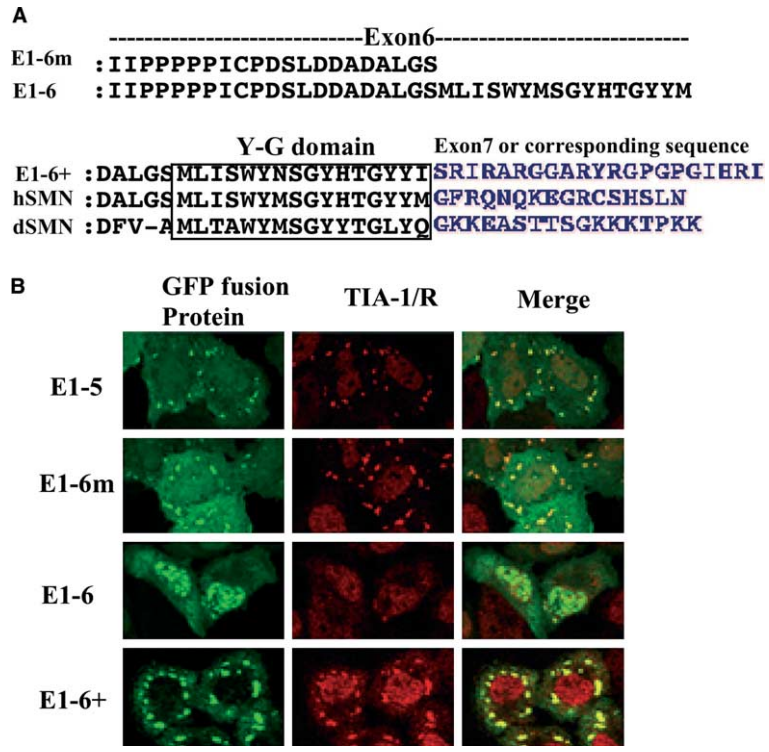

Fig. 5. Formation of SMN granules and distribution of SMN proteins are regulated by the Y–G motif and exon 7. (A) Sequence alignments of C-termini of GFP-SMN constructs, including GFP-SMN exons 1–6m (exons 1–6 with deleted Y–G motif), exons 1–6, and exons 1–6+ (exons 1–6 plus a peptide of 22 amino acids derived from the poly-linker site of the vector). The C-terminus of dSMN was also aligned to show conservation of Y–G motif and diversification of the sequence corresponding human SMN exon 7. (B) Distribution of GFP-SMN exons 1–5, exons 1–6, and exons 1–6+ together with the TIA-1/R proteins was examined in HeLa cells 24 h after transfection under a confocal immunofluorescence microscopy.

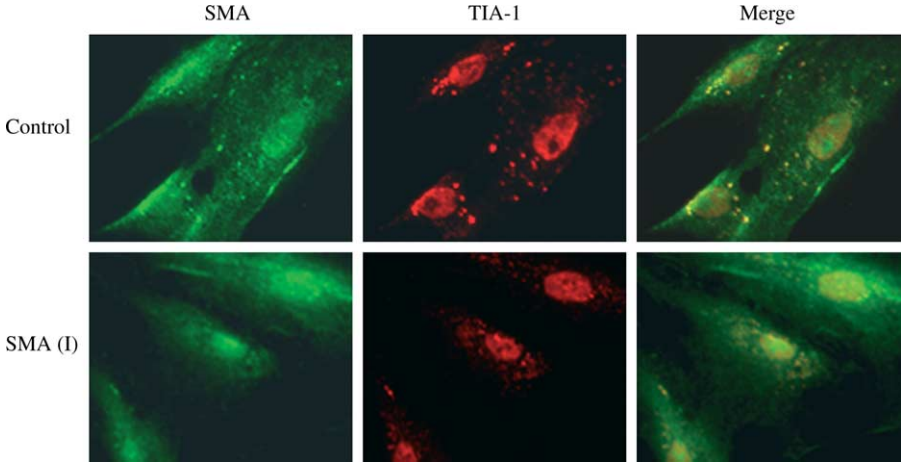

Fig. 6. SMA type I fibroblasts are defective in forming clear SGs. Distribution of endogenous SMN and TIA-1/R proteins was examined by immunostaining using antibodies H195 and 3E6 in SMA type I fibroblasts (cell lines 3813) and normal fetal fibroblasts (cell line IMR-91) after 60-min exposure to heat shock.

observations together with the fact that exons 2a–2b alone are responsible for the initiation of SMN granules under stress suggest synergistic effects among these domains, particularly when cells are under severe environmental stresses. However, since some results are obtained in cell cultures and by using tagged SMN constructs, spontaneous aggregation induced by overexpression of these proteins may not be totally stress related. The tags may induce misfolding that nucleates aggregation of SMN. On the other hand, co-immunoprecipitation of untagged SMN and TIA-1/A indicates that overexpression of SMN indeed induces SGs (Fig. 2D). Furthermore, defects of SG formation in SMA fibroblasts, which express lower levels of SMN protein, support the notion that SMN plays a role in SG formation. However, more direct experiments should be

conducted preferably in motor neurons to examine the possible involvement of SGs in the pathogenesis of SMA.

It is important to point out that the Y–G motif within exon 6 is conserved across species and present in several other RNA binding proteins [23], however, its function has not been well defined. Immunofluorescence analysis clearly showed that exons 1–5 with intact exon 6 were translocated into the nucleus, while deletion of Y–G motif reversed SMN distribution from the nucleus into the cytoplasm, suggesting that Y–G motif is essential for nuclear targeting. Interestingly, addition of extra sequence of exon 7 or an unrelated sequence into exon 6 downstream inhibited the nuclear translocation. We hypothesize that although functions at the C-terminus (exon 7) of SMN are largely unknown, its presence may modulate effects of Y–G motif in exon 6. In contrast to a recent report that a cytoplasmic localization sequence is present in exon 7 [18], we conclude that the specific exon 7 sequence may not be important for the inhibition.

Acknowledgements: We are very grateful to Dr. Nancy L. Kedersha of Brigham & Women's Hospital for anti-TIA-1/R antibody. This work is supported by the Muscular Dystrophy Association (MDA USA) and NIH NS-41665 to JZ.

References

[1] Kedersha, N.L., Gupta, M., Li, W., Miller, I. and Anderson, P. (1999) J. Cell Biol. 147, 1431–1442.
[2] Clemens, M.J. (2001) Prog. Mol. Subcell. Biol. 27, 57–89.
[3] Nover, L., Scharf, K.D. and Neumann, D. (1989) Mol. Cell. Biol. 9, 1298–1308.
[4] Kedersha, N., Chen, S., Gilks, N., Li, W., Miller, I.J., Stahl, J. and Anderson, P. (2002) Mol. Biol. Cell 13, 195–210.
[5] Kimball, S.R., Horetsky, R.L., Ron, D., Jefferson, L.S. and Harding, H.P. (2003) Am. J. Physiol. Cell Physiol. 284, C273–C284.
[6] Kedersha, N. and Anderson, P. (2002) Biochem. Soc. Trans. 30, 963–969.
[7] Tourriere, H., Chebli, K., Zekri, L., Courselaud, B., Blanchard, J.M., Bertrand, E. and Tazi, J. (2003) J. Cell Biol. 160, 823–831.
[8] Brennan, C.M. and Steitz, J.A. (2001) Cell. Mol. Life Sci. 58, 266–277.
[9] Liu, Q. and Dreyfuss, G. (1996) EMBO J. 15, 3555–3565.
[10] Lefebvre, S. et al. (1995) Cell 80, 155–165.
[11] Rodrigues, N.R., Owen, N., Talbot, K., Ignatius, J., Dubowitz, V. and Davies, K.E. (1995) Hum. Mol. Genet. 4, 631–634.
[12] Parsons, D.W., McAndrew, P.E., Iannaccone, S.T., Mendell, J.R., Burghes, A.H. and Prior, T.W. (1998) Am. J. Hum. Genet. 63, 1712–1723.
[13] Hua, Y. and Zhou, J. (2004) Biochem. Biophys. Res. Commun. 314, 268–276.
[14] Burlet, P. et al. (1998) Hum. Mol. Genet. 7, 1927–1933.
[15] Pagliardini, S., Giavazzi, A., Setola, V., Lizier, C., DiLuca, M., DeBiasi, S. and Battaglia, G. (2000) Hum. Mol. Genet. 9, 47–56.
[16] Gangwani, L., Mikrut, M., Theroux, S., Sharma, M. and Davis, R.J. (2001) Nat. Cell Biol. 3, 376–383.
[17] Sleeman, J.E., Trinkle-Mulcahy, L., Prescott, A.R., Ogg, S.C. and Lamond, A.I. (2003) J. Cell Sci. 116, 2039–2050.
[18] Zhang, H.L., Pan, F., Hong, D., Shenoy, S.M., Singer, R.H. and Bassell, G.J. (2003) J. Neurosci. 23, 6627–6637.
[19] Dodds, E., Dunckley, M.G., Roberts, R.G., Muntoni, F. and Shaw, C.E. (2001) FEBS Lett. 495, 31–38.
[20] Ilangovan, R., Marshall, W.L., Hua, Y. and Zhou, J. (2003) J. Biol. Chem. 278, 30993–30999.
[21] Kedersha, N., Cho, M.R., Li, W., Yacono, P.W., Chen, S., Gilks, N., Golan, D.E. and Anderson, P. (2000) J. Cell Biol. 151, 1257–1268.
[22] Lorson, C.L. and Androphy, E.J. (1998) Hum. Mol. Genet. 7, 1269–1275.
[23] Hahnen, E., Schonling, J., Rudnik-Schoneborn, S., Raschke, H., Zerres, K. and Wirth, B. (1997) Hum. Mol. Genet. 6, 821–825.