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QNQKE Targeting Motif for the SMN-Gemin Multiprotein Complex in Neurons

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Spinal muscular atrophy (SMA) is a heritable neurodegenerative disease affecting motor neurons that is caused by the impaired expression of the full-length form of the survival of motor neuron protein (SMN), which may have a specialized function in neurons related to mRNA localization. We have previously shown that a population SMN complexes contain Gemin ribonucleoproteins and traffic in the form of granules to neuronal processes and growth cones of cultured neurons. A QNQKE sequence within exon 7 has been shown to be necessary for both cytoplasmic localization of SMN and axonal function. Here we show that the QNQKE sequence can influence the nucleo-cytoplasmic distribution of the SMN-Gemin complex and its localization into neuronal processes. QNQKE exerted a stronger effect on SMN localization in primary neurons compared with COS-7 cells. By using double-label fluorescence in situ hybridization and immunofluorescence, SMN granules within neuronal processes colocalized with poly-(A) mRNA and PABP. These findings provide further evidence in support of a neuronal function for SMN and motivation to investigate for impaired assembly and/or localization of mRNP complexes as an underlying cause of SMA. © 2007 Wiley-Liss, Inc.

Key words: survival of motor neuron protein (SMN); spinal muscular atrophy (SMA); ribonucleoprotein (RNP); mRNA localization; mRNA transport; motor neuron disease

Spinal muscular atrophy (SMA) is the most common inherited cause of infant death, characterized by a neurodegenerative process affecting primarily α-motor neurons of the lower spinal cord (Frugier et al., 2002). This autosomal recessive disease is caused by deletions or mutations of the survival motor neuron protein gene (*SMN1*) that encodes for the SMN protein (Lefebvre et al., 1995). Numerous studies indicate that SMN forms a ribonucleoprotein (RNP) complex containing seven Gemin ribonucleoproteins, which acts to facilitate the interactions of Sm core RNA binding proteins with uridine-rich snRNAs during the assembly of spliceosomal snRNPs (Terns and Terns, 2001; Paushkin et al., 2002). *SMN1* is an essential gene in divergent organisms, where null mutations are lethal during early development (Frugier et al., 2002). SMN is ubiquitously expressed in all cells and tissues, with high levels in the nervous system and especially spinal cord (Battaglia et al., 1997).

Unlike other species (i.e., mice) that have only one copy of the *SMN* gene, in humans the *SMN* gene is duplicated, having both a telomeric copy *SMN1* and a centromeric copy *SMN2*. As a result of differential splicing, the majority of mRNAs (90%) from *SMN1* encode for the full-length protein, whereas the majority of mRNAs (90%) from *SMN2* encode for a truncated and unstable protein lacking the carboxyl-terminal exon 7 (Wirth, 2000). The most common inherited forms of SMA are caused by large deletions that inactivate the *SMN1* gene, and, although the unique presence *SMN2* in humans can protect against lethality, a neurodegenerative process occurs leading to SMA. Efforts to develop therapeutic strategies for SMA have focused on identifying drugs that increase levels of the full-length SMN protein encoded by the *SMN2* gene, by enhancement of transcription or alteration of splicing (Sumner, 2006).

SMN is localized to both the nucleus and cytoplasm, and considerable attention over the years has focused on concentration of SMN in nuclear dot-like structures termed *gems* and *coiled* (Cajal) bodies (Carvalho et al., 1999; Young et al., 2000; Renvoise et al., 2006). There have been extensive studies, in HeLa cells and other non-neuronal cell lines, on the targeting of SMN to these nuclear substructures and its function in spliceosome/snRNP

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assembly (Meister et al., 2000; Paushkin et al., 2002). However, primary defects in splicing have not yet been revealed in human SMA cells or mouse models (Jablonka et al., 2000; Briese et al., 2005; Monani, 2005). Thus, although SMN involvement in snRNP assembly is clearly an important housekeeping function, it remains uncertain whether there is a causal relationship between impaired snRNP assembly and the neurodegenerative process of SMA (Briese et al., 2005; Monani, 2005). A critical question is whether SMN has distinct functions in neurons that when disrupted could impair neuronal growth, differentiation, and survival.

Previous immunocytochemical studies have localized SMN in dendrites (Bechade et al., 1999; Pagliardini et al., 2000) and axons (Pagliardini et al., 2000) of spinal cord motor neurons in vivo, but the significance of this peripheral cytoplasmic localization has been largely controversial. Insofar as SMN is involved in snRNP assembly, why would a neuron require this activity to occur so far away from the cell soma? We have applied techniques of fluorescence microscopy and digital image analysis to understand better the dynamics and composition of SMN complexes in neuronal processes (Zhang et al., 2003, 2006). We showed that a population of SMN granules (over 40%) contained the ribonucleoproteins Gemin2 and Gemin3 (Zhang et al., 2006).

Because SMNΔ7 is the predominant form expressed in SMA, and the subcellular localization of SMN may play an important part of its function, we have further investigated the role of exon 7 in SMN sorting. Our previous study showed that full-length SMN was distributed in granules along throughout neurites, yet SMNΔ7 was largely confined to the nucleus in form of excess foci (Zhang et al., 2003). We identified a cytoplasmic targeting signal (QNQKE) within exon 7 that could redistribute SMN1–6 from the nucleus to the cytoplasm, where it localized in granules within neurites (Zhang et al., 2003). QNQKE also redirected the nuclear protein D-box binding factor 1 into the cytoplasm (Zhang et al., 2003). Most recently, mutation of the highly conserved glutamine (QNQKE) was shown not to rescue axon guidance defects in a zebrafish model of SMA (Carrel et al., 2006), providing critical evidence that this sequence imparts an axonal function to SMN. In this study, we show that QNQKE can influence the subcellular localization of the SMN-Gemin complex in all cells, but more markedly so in neurons. In addition, we show that SMN granules colocalize with markers for poly-(A) mRNA containing RNA granules in neuronal processes and growth cones. These data suggest that there may be neuron-specific interactions with the QNQKE motif within exon 7 that influence either its subcellular localization and/or its interactions with mRNA important for axonal function.

MATERIALS AND METHODS

Chick Forebrain Culture

Chick forebrain neurons were cultured as described previously (Zhang et al., 2001, 2003). Briefly, chick forebrains (E8) were dissected, trypsinized (0.15% in HBSS) at 37°C for 7 min, and plated on poly-L-lysine (0.4 mg/ml)- and laminin (0.02 mg/ml)-coated coverslips. Cells were inverted onto a monolayer of astrocytes in N_3-conditioned medium with 2% fetal bovine serum (FBS) and cultured for 4 days at 37°C in 5% CO_2. N_3-conditioned medium containing minimum essential medium Eagle (MEM) was supplemented with transferrin (0.2%), ovalbumin (0.1%), insulin (10 μg/ml), putrescine (32 μg/ml), sodium selenite (26 ng/ml), progesterone (12.5 ng/ml), hydrocortisone (9.1 ng/ml), T3 (3,3′, 5′-triiodo-L-thyronine, sodium salt, 20 ng/ml) and bovine serum albumin (BSA; 10 μg/ml). Neurons were fixed in paraformaldehyde (4% in 1× PBS) for 20 min at room temperature and washed in 1× PBS with 5 mM $MgCl_2$ three times.

Fluorescence Protein-Reporter Constructs

Full-length cDNA of the human *SMN1* was subcloned into EGFP-C1 (EGFP-SMN) as previously described (Zhang et al., 2003). In the present study, full-length cDNA of *SMN1* was also subcloned into MRFP-C1 (Campbell et al., 2002) or YFP-C1 vector at HindIII and EcoRI sites (BD Biosciences Clontech, Palo Alto, CA). cDNAs encoding truncated SMN forms, including SMNΔ7 (containing four amino acids from exon 8), SMN1–6, and SMN1–6/TIF, were generated using PCR and fused to EGFP, YFP or CFP, respectively. [TIF denotes targeting motif (QNQKE) identified by Zhang et al. (2003).] Full-length cDNAs of human Gemin2 and PABP were obtained by RT-PCR from total RNA extracts of HEK 293 cells and inserted into CFP-C1 or EGFP-C1 vector using BglII and EcoRI sites. All of the constructs were purified (Qiagen, Valencia, CA) and sequenced to ensure that no frame shift had occurred.

Transfection

Neuronal transfections were performed with DOTAP liposomal reagent (Roche), as described previously (Zhang et al., 2003, 2006). In cotransfection experiments, two DNA constructs were mixed equivalently (2–3 μg in total) and diluted to 100 μl with transfection buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and then incubated with DOTAP (5 μl), as described above.

COS-7 cells were cultured in DMEM containing 10% FBS and followed by briefly washing with prewarmed medium before transfection. EGFP-tagged construct (2–3 μg in total) was diluted to 100 μl in the transfection buffer and then incubated with 5 μl of FuGENE 6 transfection reagent (Roche) for 15 min at room temperature. After incubation with the DNA mixtures for 1 hr, COS-7 cells were cultured in DMEM containing 10% FBS for 24 hr . Protein lysates were prepared using a lysis buffer for Western blot. Transfected COS-7 cells were also imaged using a fluorescence microscope (described below).

Fluorescence Microscopy and Digital Imaging

Neurons were visualized by using a Nikon Eclipse inverted microscope equipped with a ×60 Plan-Neofluar objective, phase optics, 100-W mercury arc lamp, and HiQ bandpass filters (Chroma Tech). Images were captured with a cooled CCD camera (Quantix, Photometrics) with a 35-mm

shutter and processed in IP Lab Spectrum (Scanalytics). Fluorescence images were acquired using narrow bandpass filters, including EGFP, CFP, YFP, or MRFP. Exposure time was kept constant and below gray-scale saturation.

Western Blot

Protein extracts were prepared from the transfected COS-7 cells at 24 hr after transfection and fractionated onto 12% SDS-PAGE gel. Fractionated proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Aslington Heights, IL) at 4°C overnight. EGFP fusion proteins were detected with a monoclonal antibody (1:2,000 diluted in TBS buffer; BD Biosciences) and anti-mouse antibody conjugated with peroxidase (Jackson Immunoresearch, West Grove, PA). The signal was developed using ECL detection reagents (Amersham Biosciences).

RESULTS

Nucleocytoplasmic Sorting of SMN-Gemin Complex Is Modulated by QNQKE

We have recently used immunofluorescence, quantitative digital imaging analysis, and 3D reconstructions to show that over 40% of endogenous SMN granules in neurites and growth cones contain the ribonucleoproteins Gemin2 and Gemin3 (Zhang et al., 2006). Overexpression of SMN and Gemin proteins, tagged with different fluorescent proteins, demonstrates FRET interactions and cotransport (Zhang et al., 2006). The distribution of overexpressed SMN and Gemin proteins, tagged with fluorescent proteins, was also shown by immunofluorescence to colocalize with endogenous SMN and Gemins (Zhang et al., 2006). Moreover, endogenous SMN-Gemin2- + -3-containing granules were observed in processes of a variety of cultured neuronal types that included forebrain neurons, hippocampal neurons, primary motor neurons, and embryonic stem (ES) cell-derived motoneurons (Zhang et al., 2006). These past studies validate the use of overexpression of fluorescently tagged proteins to investigate the trafficking of SMN complexes in neurons.

In this study, we have investigated the role of SMN domains in the formation and sorting of multiprotein complexes in cultured neurons. YFP-SMN and CFP-Gemin2 were cotransfected into cultured chick forebrain neurons, expressed for 3 days, and fixed for analysis by fluorescence microscopy. As shown previously, there was strong colocalization of SMN and Gemin2 within cytoplasmic granules (Fig. 1C). Within the nucleus, there were a few foci, most likely to be gems, which have been previously shown to contain SMN and Gemin proteins. With a scoring method, 99% of the neurons exhibited a predominantly cytoplasmic localization of the SMN-Gemin complex (Fig. 1M). In contrast to the distribution of full-length SMN, when SMN lacking exon 7 (SMN1–6 fused to YFP) was cotransfected with CFP-Gemin2, only 32% of cells were scored as having a predominant cytoplasmic localization (Fig. 1M). SMN1–6 was more frequently enriched in the nucleus, where there were numerous aggregates (Fig. 1E). This nuclear aggregation of SMN1–6

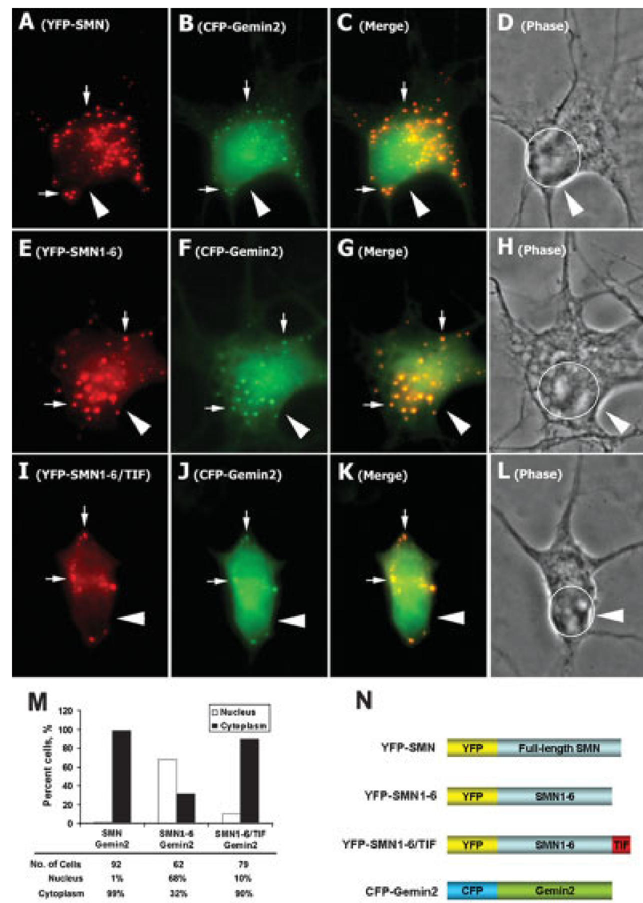

Fig. 1. Nuclear aggregation of Gemin2 by SMN1–6 was rescued by the exon 7 targeting motif. Chick forebrain neurons were cotransfected with CFP-Gemin2 and SMN, SMN1–6, or SMN1–6/TIF (YFP labeled; TIF: targeting motif; QNQKE; see Zhang et al., 2003). Fluorescence images were acquired and overlaid to show colocalization. **A–D:** Full-length SMN (YFP-SMN; A, red) formed granules that colocalized with CFP-Gemin2 (B, green) in cytoplasm and the processes (C, yellow, arrows). A circle indicates the area of the nucleus viewed with phase optics (D, arrowhead). **E–H:** Neuron transfected with YFP-SMN1–6 showed nuclear aggregation (E, red, arrowhead), although some small granules were observed in the cytoplasm (E, arrows). CFP-Gemin2 (F, green, arrowhead) was accumulated in the nucleus at sites of YFP-SMN1–6 (G, yellow, arrowhead). Phase image indicates the nuclear area (H, arrowhead). **I–L:** In contrast, when SMN1–6 fused to the QNQKE motif (YFP-SMN1–6/TIF), the fluorescence granules were excluded from the nucleus and appeared in the cytoplasm (I, red, arrows). There was no major nuclear aggregation in the cells. CFP-Gemin2 was no longer accumulated in the nucleus (J, green, arrowhead), and instead we noted predominant cytoplasmic localization (J, green, arrows), colocalized with YFP-SMN1–6/TIF (K, yellow, arrows). Phase optic shows nuclear area (L, circle, arrowhead). **M:** Cells were visually scored (user blind) for whether the protein distribution was predominantly nuclear or cytoplasmic. Quantitative analysis from over 60 neurons in each group; 68% of the neurons showed nuclear aggregation of CFP-Gemin2 when cotransfected with YFP-SMN1–6. In contrast, CFP-Gemin2 was not accumulated in the nucleus of the neurons cotransfeced of YFP-SMN1–6/TIF. **N:** Schematic of the constructs used for transfection.

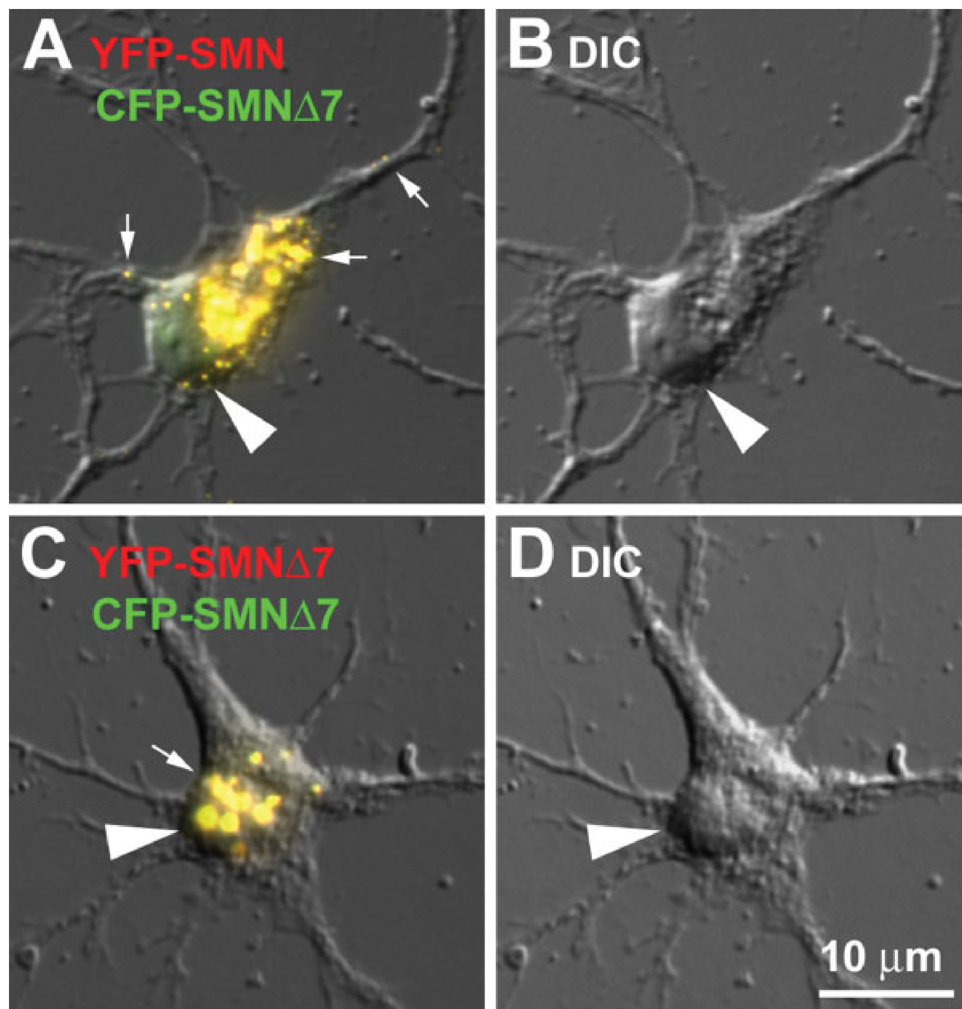

Fig. 2. Heterotypic dimerization of SMN with SMNΔ7 restored the localization of complex to the processes. **A:** Neurons were cotransfected with YFP-SMN (red) and CFP-SMNΔ7 (green). CFP-SMNΔ7 (green) was not accumulated in the nucleus (arrowhead) and instead showed a predominant cytoplasmic distribution, analogous to full-length YFP-SMN. Colocalized granules of YFP-SMN (red) and CFP-SMNΔ7 (green) were detected in the cytoplasm and processes (yellow, arrow). Arrowhead in A denotes position of the nucleus as evident from DIC image in **B**. **C:** Cotransfected YFP-SMNΔ7 and CFP-SMNΔ7 both coaggregated (arrow) in the nucleus (arrowhead), and very little signal was noted in the cytoplasm. These data show that heterotypic dimerization (SMN::SMNΔ7) can restore cytoplasmic localization to exon-7-lacking SMN.

is abnormal in that many SMN foci do not correspond to coiled bodies and are much larger than gems (data not shown). Of interest, CFP-Gemin2 was now also largely restricted to the nucleus, where it colocalized with YFP-SMN1–6 within these aggregates (Fig. 1F,G). The excess nuclear accumulation of the SMN1–6/Gemin2 complex was corrected when the QNQKE sequence was fused in frame to SMN1–6 (Fig. 1I–K). Now, 90% of cells showed predominant cytoplasmic colocalization of SMN-Gemin2 (Fig. 1M).

These data were obtained in neurons that overexpressed tagged proteins for 3 days (to permit optimal imaging of granules), so we repeated these experiments using an overnight expression, conducted here with EGFP- and mRFP-tagged proteins in mouse cortical neurons. This experiment also revealed that full-length SMN and Gemin2 exhibited predominant sorting and colocalization within the cytoplasm (Suppl. Fig. 1A–C, upper row), whereas SMN1–6 coaggregated in the nucleus with Gemin2 (Suppl. Fig. 1F–H, middle row). Fusion of QNQKE to SMN1–6 limited the excess accumulation of SMN1–6 to the nucleus (Suppl. Fig. 1K–M, bottom row). In this experiment, the nucleus was stained with DAPI and clearly shows the accumulation of SMN1–6 in the nucleus (Suppl. Fig. 1F,I), in comparison with full-length SMN and SMN1–6 + QNQKE (Suppl. Fig. 1A,K). Collectively, these two experiments, using different fluorescent tags and different neurons, indicate that the localization of SMN is modulated by a QNQKE sequence within exon 7 and can dictate how Gemin2 is sorted between the nucleus and the cytoplasm.

In addition, we have more recently performed experiments with much shorter expression times in qan effort to validate the differential sorting of SMN forms and truncations. As early as 8 hr posttransfection, full-length SMN was shown to have a predominantly cytoplasmic localization and detection of SMN in a few nuclear foci, likely gems (Suppl. Fig. 2, top row). In contrast, expression of SMN1–6 for 8 hr showed noticeably more SMN foci in the nucleus, and this condition was exacerbated over time. After 24 hr, SMN1–6 was largely nuclear in its distribution, whereas full-length SMN was largely cytoplasmic (Suppl. Fig. 2, middle row). Fusion of the QNQKE motif to SMN1–6 greatly limited the tendency

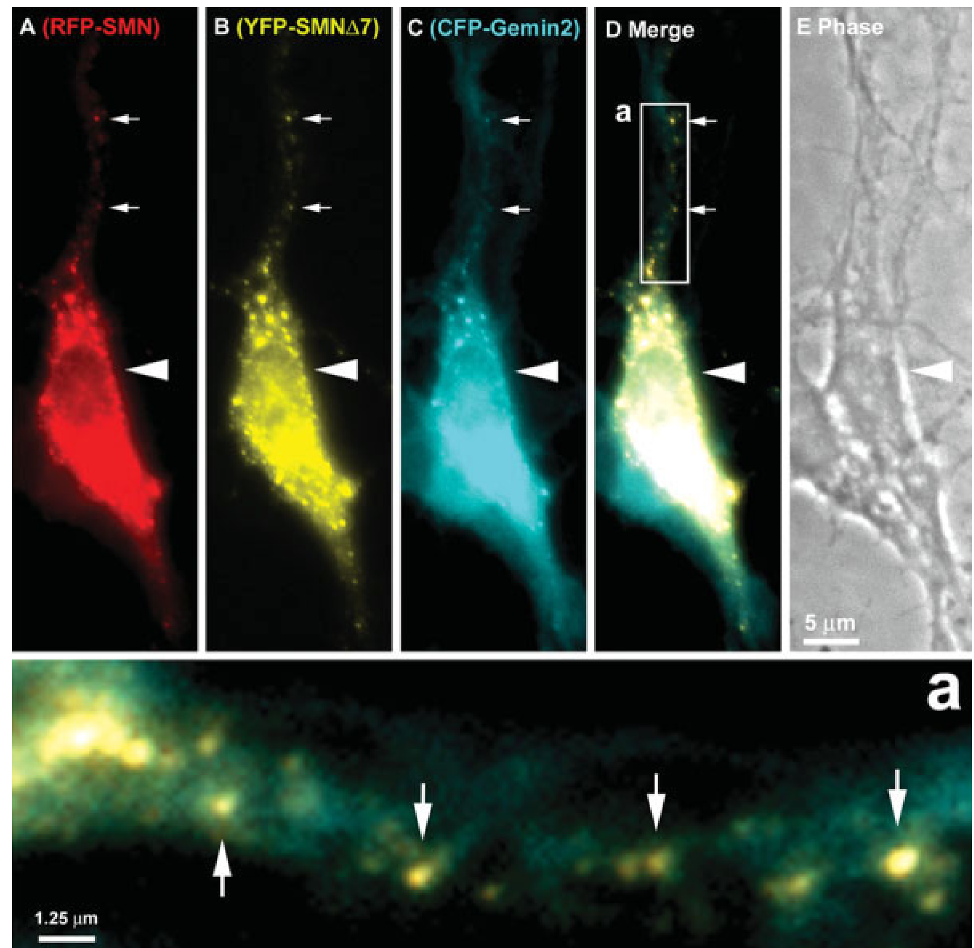

Fig. 3. Gemin2 association and localization with heterotypic SMN complex. Neurons were transfected with triple-labeled proteins, including MRFP-SMN, YFP-SMNΔ7, and CFP-Gemin2. Each fluorescence image was captured with a cooled CCD camera with narrow band-pass filters. **A:** MRFP-SMN (red) formed granules that localized to the cytoplasm and processes (arrows). **B:** YFP-SMNΔ7 (yellow) was also observed in granules in the cytoplasm and processes (arrows). Little nuclear accumulation (arrowhead) was seen when coexpressed with a full-length protein. **C:** CFP-Gemin2 showed granules in the processes (blue, arrows). **D:** Merged images show colocalization of the triple-labeled granules in the processes (white, arrows). At high magnification, colocalized granules were clearly exhibited in the processes (**inset**, white, arrows). **E:** Phase optic.

of SMN1–6 to accumulate in the nucleus over time, although the number of nuclear foci was slightly higher than that observed for full-length SMN (Suppl. Fig. 2, bottom row).

Differential Localization of Heterotypic and Homotypic SMN Complexes With Exon 7 Deletion

Previous evidence indicates that SMN contains a modular oligomerization domain within exon 6 that can promote self-association of SMN into dimeric or oligomeric complexes (Lorson et al., 1998). In a mouse transgenic model of SMA, overexpression of SMNΔ7 was able to form heterotypic complexes with full-length SMN and reduce phenotypic severity (Le et al., 2005). Here we also show that cotransfection of YFP-SMN (full length) and CFP-SMNΔ7 revealed predominantly cytoplasmic localization and a high level of colocalization for this heterotypic complex in granules (Fig. 2A) in comparison with the predominantly nuclear localization and coaggregation observed in cells cotransfected with dually tagged SMNΔ7 proteins (Fig. 2C). Because these overexpressed proteins are present at much higher levels than endogenously, the ability of endogenous SMN proteins to form complexes with the overexpressed SMNΔ7 proteins is likely limited. In triply transfected neurons (MRFP-SMN, YFP-SMNΔ7, CFP-Gemin2), we also show that granules containing the heterotypic complex (SMN/SMNΔ7) were colocalized with Gemin2 in neuronal processes (Fig. 3A–D, inset). These data indicate that heterotypic complexes of SMN can maintain interactions with Gemin proteins and influence their localization in a fashion similar to homotypic complexes of full-length SMN.

Differential Impact of QNQKE on SMN Localization in Neuronal and Nonneuronal Cells

A major objective for the field has been to determine whether SMN has a specific function in neurons that is impaired in SMA. As a step toward this goal, we have investigated whether the exon 7 targeting motif (QNQKE) behaves similarly in neurons compared with nonneuronal cells. In transfected forebrain neurons, over 90% of cells show a predominant cytoplasmic localization with full-length SMN (Fig. 4A,I), which is reduced to 20% in cells that express SMNΔ7 (Fig. 4B,I) and to near zero in cells that express SMN1–6 (Fig. 4C,I). SMNΔ7 differs from SMN1–6, in that SMNΔ7 has an extra four amino acids from exon 8 at the carboxy-terminus. Thus, these four amino acids seem to have a modest affect to

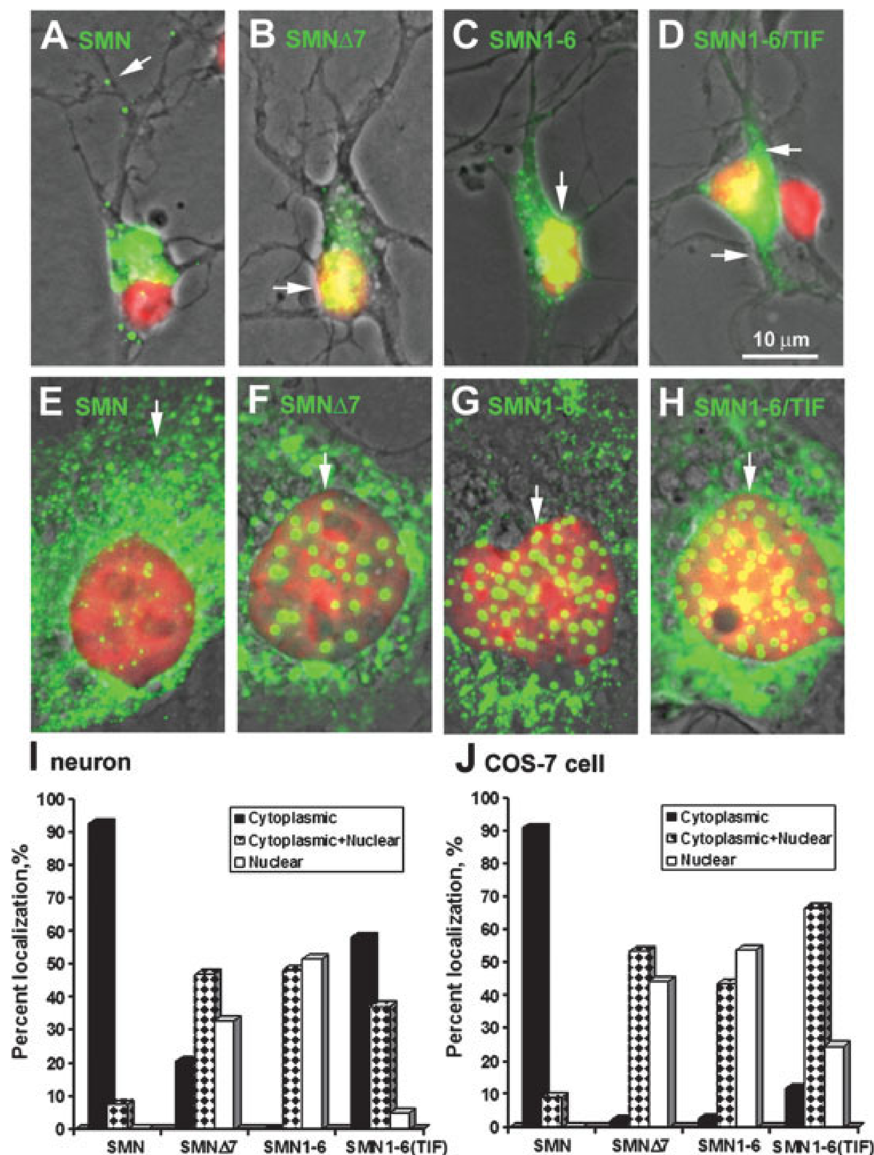

Fig. 4. QNQKE has strong influence on SMN cytoplasmic localization in primary neurons. The distributions of full-length SMN and the truncations (fused to EGFP) were quantitatively analyzed in transfected neurons and compared with these in nonneuronal cells (COS-7 cells). DAPI-stained nucleus is shown in red. **A–D:** Neurons were transfected with EGFP-tagged SMN constructs. Full-length SMN depicted granules in the processes (A, green, arrow). SMNΔ7 (B) and SMN1–6 (C) were observed in aggregations in the nucleus (arrows). In contrast, SMN1–6/TIF showed granules that were excluded from the nucleus and into the cytoplasm (D, arrows). **E–H:** Distributions of the SMN and its truncations in COS-7 cells. EGFP-SMN formed granules that localized in the cytoplasm (E, green, arrow). No nuclear aggregation was found in the cell (E, red), whereas obvious nuclear aggregations of the EGFP-tagged proteins were observed in the cells transfected with SMNΔ7 (F, green, arrow), SMN1–6 (G, green, arrow), or SMN1–6/TIF (H, green, arrow). Cytoplasic localization of SMN1–6/TIF was partially restored by the QNQKE motif. **I:** Quantitative results of the SMN distributions in neurons. Over 30 neurons for each transfection were scored in three categories as indicated. Cytoplasmic localization of SMN1–6/TIF was significantly rescued by fusion to the QNQKE signal. Compared with both SMNΔ7 and SMN1–6, which had major nuclear aggregations, neurons (up to 60%) showed major cytoplasmic localization when SMN1–6 was fused to the QNQKE sequence (SMN1–6/TIF). **J:** Histogram showing distributions of the SMN and its truncations in COS-7 cells. In comparison with SMNΔ7 and SMN1–6, SMN1–6/TIF did not show as dramatic a restoration of the cytoplasmic localization by the QNQKE motif. These results suggest that QNQKE signal has stronger influence on SMN localization in primary neurons compared with COS cells.

limit the severity of nuclear aggregation observed with SMN1–6. Of interest, fusion of QNQKE to SMN1–6 resulted in over 60% of transfected neurons showing a predominant cytoplasmic localization (Fig. 4D,I). When these constructs were expressed for the same length of time in COS-7 cells (triplicate experiments were done in a paral-

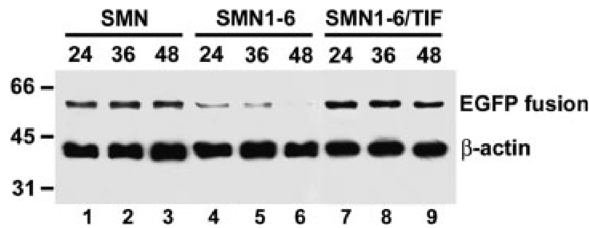

Fig. 5. Restoration of SMN1–6 stability by the QNQKE motif. Western blot was performed on protein extracts from the COS-7 cells transfected with EGFP-labeled SMN constructs. Protein lysates were prepared at indicated times (hours) after the transfection. Membrane was incubated with monoclonal antibodies to GFP and β-actin (as loading control). Compared with the full-length SMN (lanes 1–3), reduction of the SMN1–6 level was seen over the period of posttransfection (lanes 4–6). In contrast, when SMN1–6 fused to QNQKE (SMN1–6/TIF), it became more stable, and levels were now comparable to those of full-length SMN (lanes 7–9).

lel), we observed that the QNQKE targeting motif was markedly less efficient at promoting a predominantly cytoplasmic localization (Fig. 4E–H,J). Only 12% of COS-7 cells showed a predominantly cytoplasmic localization of SMN1–6/TIF, and the majority showed a more uniform distribution of SMN between the cytoplasm and nucleus (Fig. 4H,I).

Stabilization of SMN Levels by QNQKE

Previous studies have indicated that exon 7 can stabilize SMN, which explains observations for very low levels of SMN in SMA (Coovert et al., 1997; Lefebvre et al., 1997; Burlet et al., 1998). We speculate that SMN stability may be at least partially attributed to its subcellular localization. Cells were transfected with full-length SMN, SMN1–6, or SMN1–6 fused with QNQKE (SMN1–6/TIF). All constructs were EGFP fusion proteins, and anti-

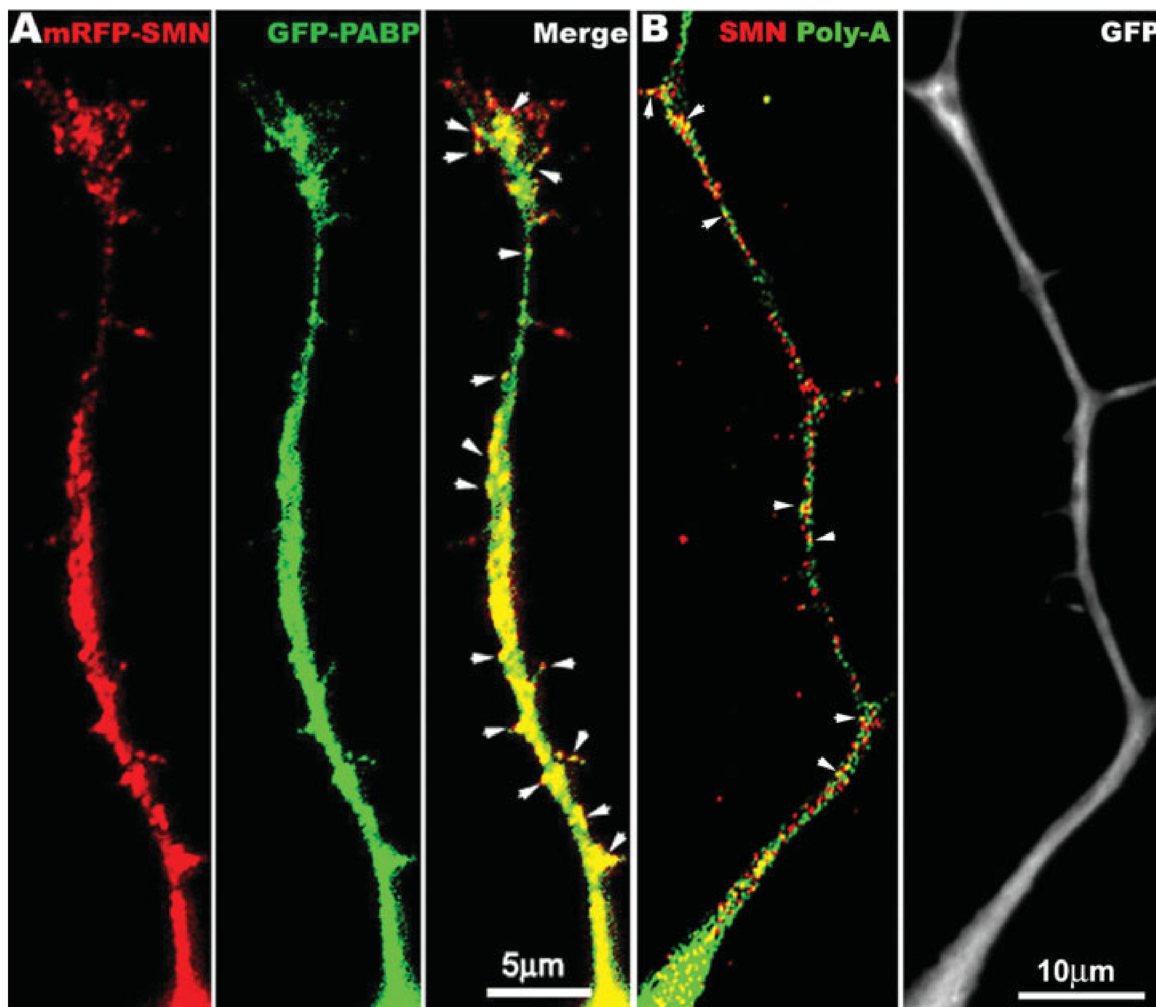

Fig. 6. Colocalization of SMN with poly-(A) binding protein (PABP) and poly-(A) mRNA in granules. **A:** Chick forebrain neurons were cotransfected with mRFP-SMN and EGFP-PABP. Merged signals show frequent colocalization within granules in neurite and protrusions (arrows). **B:** Double label in situ hybridization to poly-(A) mRNA and immunofluorescence detection of SMN revealed frequent colocalization within granules (merged image, arrows). GFP denotes motor neurons expression from HB9 promoter as shown previously (Zhang et al., 2006).

body to EGFP was used for Western blot analysis. Stable expression of full-length SMN was observed over three time points (24, 36, and 48 hr; Fig. 5, lanes 1–3). In contrast, SMN1–6 levels were substantially lower (Fig. 5, lanes 4–6). Addition of the targeting motif (QNQKE) to SMN1–6 resulted in stable SMN levels that were now comparable to those of full-length SMN (Fig. 5, lanes 7–9). These data indicate a correlation between SMN levels and its stabilization within the cytoplasm.

SMN Granules Colocalize With Poly-(A) mRNA and PABP

We have recently shown that axonal SMN-Gemin containing granules are deficient in spliceosomal Sm proteins needed for snRNP assembly (Zhang et al., 2006). These data suggest that SMN granules may serve some other function. One attractive hypothesis is that SMN granules may be involved in the assembly and/or localization of mRNA granules (Briese et al., 2005; Monani, 2005). To obtain further evidence in support of this hypothesis, we investigated whether SMN granules colocalized with markers for mRNA. In chick forebrain neurons cotransfected with MRFP-SMN and poly-(A) binding protein (EGFP-PABP), granules containing both proteins were observed throughout the length of the axon, axonal filopodia, and terminal growth cone (Fig. 6A; see arrows in merged image). We also have performed double-label FISH and IF to show that endogenous poly-(A) mRNA and SMN frequently colocalized in RNA granules along the length of the axon and into the growth cone of ES cell-derived motor neurons (Fig. 6B; arrows in merged image). EGFP is shown as a marker for motor neurons that is expressed from a motor-neuron-specific promoter (Wichterle et al., 2002; Zhang et al., 2006).

DISCUSSION

Function of QNQKE Motif in Exon 7 in SMN Complex Assembly and Sorting

Our previous results and new findings from this study indicate a role of QNQKE to influence the nucleocytoplasmic sorting of SMN, where this motif biases the localization toward the cytoplasm. Deletion of the carboxyl-terminal exon 7 resulted in abnormal nuclear aggregation and loss of cytoplasmic localization (Zhang et al., 2003). Here we show that the differential sorting of SMN, SMN1–6, and SMNΔ7 is observed after only several hours of transfection and can be modulated by QNQKE.

A hallmark observation in SMA is low levels of SMN. Several studies have shown that SMN levels are dramatically reduced in cells from SMA patients (Coovert et al., 1997; Lefebvre et al., 1997; Burlet et al., 1998), which appears to indicate that the SMNΔ7 is a much less stable protein than its full-length counterpart. Also apparent in human SMA cells have been observations of a lack of nuclear gems (Gangwani et al., 2001). Such observations of "no nuclear gems" in a mouse model having a conditional deletion of exon 7 have suggested a possible nuclear targeting defect (Frugier et al., 2000). However, our results argue against a role for exon 7 in nuclear localization and indicate just the opposite; we show that the exon 7 deletion mutant is confined to the nucleus (Zhang et al., 2003). Moreover, we show that fusion of exon 7 or the cytoplasmic targeting motif QNQKE to a nuclear protein, DBF1, resulted in its redistribution from the nucleus to the cytoplasm (Zhang et al., 2003), which provides additional evidence for a role of exon 7 sequences in facilitation of cytoplasmic localization.

In this study, we obtain additional data to demonstrate a correlation between SMN forms, their stability, and their nucleocytoplasmic localization. The QNQKE sequence was shown both to facilitate localization of SMN1–6 to the cytoplasm and to enhance its stability. Thus, to reconcile past observations of low levels of SMN in SMA, we speculate that SMNΔ7 may be degraded in the nucleus during early stages of SMA. Our findings provide a motivation to test this hypothesis.

Although we have identified a role for QNQKE in facilitating the cytoplasmic localization of SMN, we note that other sequences added to the carboxyl-terminus have been shown also to influence cytoplasmic localization and protein stability in nonneuronal cells. In the SMNΔ7 form, the C-terminus is derived from four amino acids encoded by exon 8, which are not encoded by the full-length SMN because of a stop codon at the 5′-end of exon 7 (Lefebvre et al., 1995, 1997). When exon 7 is skipped to generate SMNΔ7, the stop codon is now in exon 8. A recent study has shown that addition of exon 8 to SMN1–6 had a stabilizing effect on protein levels and promotes cytoplasmic localization in HeLa cells (Wolstencroft et al., 2005). Addition of a random stretch of amino acids to SMN1–6 was also able to stabilize the protein and promote cytoplasmic localization in HeLa cells (Hua and Zhou, 2004). These results argue that specific sequences within exon 7, i.e., QNQKE, may be necessary but not sufficient for protein stabilization and cytoplasmic localization. An alternative view of exon 7 is that it inhibits nuclear-directed localization, which has an indirect effect to promote cytoplasmic localization (Hua and Zhou, 2004). Hua and Zhou also showed that a random C-terminal tag of five amino acids could inhibit the occurrence of multiple nuclear foci and promote cytoplasmic localization of SMNΔ7 in nonneuronal cells. Collectively, these studies indicate that a role for exon 7 in regulation of nucleocytoplasmic sorting may be sequence independent; however, this inference is based only on analysis of nonneuronal cells, and these investigators should also analyze the effects of these other sequences on protein distribution and stability in neurons.

We present new findings here to indicate that the QNQKE motif facilitated cytoplasmic localization more strongly in primary neurons in comparison with COS cells. We speculate that neurons may express specific mRNA binding proteins that interact with QNQKE to influence both its sorting to the cytoplasm and its function within axons. We note that the QNQKE sequence within exon 7 is highly conserved across species (Lefebvre et al., 1995; Talbot et al., 1997; Wang and Dreyfuss, 2001). It

appears without substitution in human, dog, and cat, although it is present as QNKKE in mouse. Some amino acids within this QNQKE motif are highly conserved, such as Q (glutamine 282 in human) and E (glutamic acid), which are both present in all of the above species, as well as *Xenopus*, zebrafish, and *Drosophila*.

The need to conduct research in neuronal systems is underscored by most recent findings showing that QNQKE serves an essential function in axons in vivo, insofar as its addition to SMN1–6 rescued axon defects in a zebrafish model of SMA, whereas other random C-terminal sequences (as discussed above) did not (Carrel et al., 2006). These data further indicate that, although there may be a sequence-independent requirement of the C-terminus to influence cytoplasmic localization, the QNQKE motif has a specific axonal function, which we speculate (see below) is related to aspects of axonal mRNA regulation.

Effect of SMN Complex Stoichiometry on Protein Localization and Stability

An important finding is that the stoichiometry of SMN proteins within a macromolecular complex can affect its stability and localization. While SMN1–6 or SMNΔ7 has a predominant nuclear localization, we show here that overexpression of both SMNΔ7 and SMN resulted in its localization to the cytoplasm. This heterotypic complex was now also able to target Gemin2 into the cytoplasm, in contrast to the predominant nuclear localization of Gemin2 in cells that express a homotypic complex lacking exon 7. These results have a number of implications. First, they provide further evidence that an excess of SMNΔ7, which occurs in SMA, has the potential to be stabilized and sorted to the cytoplasm when present at stoichiometric levels with full-length SMN. In support of this idea, Le et al. (2005) crossed transgenic mice expressing high levels of SMNΔ7 onto transgenic mice having a severe SMA-like phenotype (mSMN$^{-/-}$; hSMN2 transgene). In these doubly transgenic mice (SMNΔ7, hSMN2), the addition of SMNΔ7 extended survival of mice from 5 to 13 days (Le et al., 2005). In vitro studies also showed that SMNΔ7 can self-associate with full-length SMN (Le et al., 2005). The speculation is that survival was extended because the presence of high SMNΔ7 levels had shifted the equilibrium such that a population of SMNΔ7 was forced to assemble heterotypic complexes, which resulted in a net increase in functional SMN. Although more research is needed to show a direct relationship between the presence of heterotypic complexes in these mice having extended survival, the data do show that high levels of SMNΔ7 are not detrimental and are consistent with possible recruitment of SMNΔ7 into a heterotypic SMN complex that is stable and localized predominantly to the cytoplasm.

SMN Role in mRNA Localization During Development

SMA is a neurodegenerative process that leads to the loss of spinal cord motoneurons (Frugier et al., 2002). In a zebrafish model for SMA, there is clear evidence for axonal defects in motor axon guidance (McWhorter et al., 2003). Knockdown of zebrafish SMN in vivo using morpholino antisense was shown to result in motor neuron axon guidance defects, such as truncations and abnormal branching beneath the muscle (McWhorter et al., 2003). Time-lapse imaging of defective motor neuron axons in the vicinity of myotome suggests truncation or stalling errors that precede formation of excess axonal branching (McWhorter et al., 2003). These axon-guidance defects were rescued by human SMN but not the nuclear SMNΔ7. Thus, although the loss of SMN may not prevent the axon from finding the target muscle, it is conceivable that delayed or inappropriate innervation patterns may set the stage for longer term consequences, i.e., dying back process during an early postnatal period (McWhorter et al., 2003).

One hypothesis is that axonal defects that occur in SMA models are due to loss of a local function for SMN in the growth cone. Cultured neurons provide an ideal model with which to study mechanisms of local protein function in growth cones. We have shown that SMN is localized to growth cones of developing axons, cultured from embryonic cortex or spinal cord (Zhang et al., 2003). Overexpression of SMNΔ7 in these neurons reduced axon outgrowth, a phenotype that was rescued by addition of the QNQKE cytoplasmic targeting sequence to SMNΔ7, providing an important correlation between the localization of SMN and neurite outgrowth (Zhang et al., 2003).

An extensive analysis of motoneuron differentiation in culture from the SMA transgenic mouse model has been performed (Rossoll et al., 2003). Whereas these SMN-deficient neurons did not show impaired survival in culture when compared with wild-type (WT) and could differentiate axons and dendrites, some axonal-specific phenotypes were noted. Of interest, axons were shorter in length and had smaller growth cones, which contained less β-actin mRNA and protein (Rossoll et al., 2003). The localization of β-actin mRNA to growth cones has been previously shown to influence β-actin protein enrichment necessary for growth cone protrusion and forward motility (Zhang et al., 2001). Because the mRNA binding protein hnRNP-R, which binds SMN directly (Rossoll et al., 2002), coprecipitates radiolabeled β-actin mRNA (Rossoll et al., 2003), the authors suggest that SMN may affect β-actin mRNA localization through its interaction with hnRNP-R. However, previous studies have shown that zip code binding proteins, ZBP1 + -2, are required for binding to the 54-nt zip code in the 3′UTR to promote β-actin mRNA localization (Zhang et al., 2001; Gu et al., 2002), so future research is needed to assess whether SMN-mediated localization of β-actin mRNA may involve hnRNP-R and/or ZBPs. In that ZBP1-dependent localization of β-actin mRNA and local synthesis of β-actin protein was shown recently to be involved in axon guidance (Leung et al., 2006; Yao et al., 2006), future studies to assess possible interactions of SMN with ZBP1 needed for axon outgrowth and guidance are compelling.

ZBP1 is a known shuttling factor, which enters the nucleus and forms a complex with β-actin mRNA (Oleynikov and Singer, 2003), so it will also be interesting to assess whether QNQKE interacts with ZBP1 to affect the sorting of SMN or vice versa.

In this study, we show a high degree of colocalization of SMN granules with markers for total poly-(A), suggesting that SMN may interact with a wide range of mRNPs, in addition to the β-actin mRNP localization complex. Thus, it is critical to assess how the loss SMN may impair localization of many mRNAs, in addition to β-actin mRNA localization. Recent analysis of axonal mRNAs indicates that over 200 proteins can be locally synthesized (Willis et al., 2005). One model to test is that the SMN-Gemin complex, which is known to act as an assembly factor for snRNPs, may play a similar role to facilitate the assembly of mRNP localization complexes, which are termed *granules* (Kiebler and Bassell, 2006).

Nucleocentric and Axonal Viewpoints of SMA

The major emphasis on past SMN research has been to understand its role in snRNP and spliceosome assembly (Paushkin et al., 2002), likely because very early reports hypothesized that SMA is a "splicing" disease following observation of impaired pre-mRNA splicing in extracts from patient fibroblasts (Pellizoni et al., 1998). Consequently, most approaches to elucidate the cellular function of SMN have employed immortalized cells such as HeLa and also lymphoblastoid cell lines or fibroblasts derived from patients. However, our past studies (Zhang et al., 2003, 2006) and data presented here strongly suggest a more diverse role for SMN in the development and maintenance of neurons. Most recently, we showed that, although SMN granules contained Gemin2 and Gemin3, they lacked snRNP components (Zhang et al., 2006). Spliceosomal Sm proteins were not detectable in distal neuronal processes, unlike the SMN-Gemin granules (Zhang et al., 2006). Most recently, in zebrafish, SMN function in motor axons was shown to be independent of functions required for snRNP assembly (Carrel et al., 2006). In particular, mutants defective in snRNP assembly were able to rescue axon-guidance defects in SMN-deficient neurons. In contrast, mutation of the highly conserved glutamine in the QNQKE motif was not able to rescue axon defects (Carrel et al., 2006). This mutation (Q282A) did not impair axon targeting, pointing to an additional function for this motif in axon function. We speculate that the Q282A mutant might have impaired interactions with mRNA binding proteins that are important for mRNA localization and/or translation. Future studies will be needed to determine the molecular details of how SMN interacts with localized mRNPs and whether Gemins can influence these interactions. Our previous findings revealed SMN complexes (granules) with and without Gemins (Zhang et al., 2006), so it will be critical to assess the role of Gemins in RNA regulation and axon function. From this direction, toward understanding SMN function in axonal mRNA regulation, new insights into the disease process in SMA will be gained.

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