Published in final edited form as:

Neurosci Lett. 2008 September 5; 442(1): 54–58. doi:10.1016/j.neulet.2008.06.059.

A SMN Δ 7 read-through product confers functionality to the SMN Δ 7 protein

Virginia Mattis¹, Melissa Bowman², Rashmi Kothary², and Christian L. Lorson^{1*}

¹Department of Veterinary Pathobiology, Life Sciences Center, Room 404, University of Missouri, Columbia, MO, 65211

²University of Ottawa, Tabaret Hall, 550 Cumberland St, Room 246, Ottawa, ON, Canada, K1N 6N5

Abstract

Spinal muscular atrophy (SMA) affects about 1 in every 6000 children born and is the leading genetic cause of infant death. SMA is a recessive disorder caused by the mutation or deletion of *Survival Motor Neuron-1* (*SMN1*). *SMN2*, a nearly identical copy gene, has the potential to encode the same protein as *SMN1* and is retained in all SMA patients. The majority of *SMN2*-derived transcripts are alternatively spliced and therefore encode a truncated isoform lacking exon 7 (SMN Δ 7), which is a defective protein because it is unstable, has a reduced ability to self-associate and is unable to efficiently function in SMN cellular activities. However, we have shown that the SMN C-terminus functions non-specifically, since heterologous sequences can compensate for the exon 7 sequence. Several classes of compounds identified in SMN-inducing high throughput screens have been proposed to function through a read-through mechanism; however, a functional analysis of the SMN Δ 7 read-through product has not been performed. In this report, the SMN Δ 7 read-through product is characterized and compared to the SMN Δ 7 protein. In a series of *in vitro* and cell based assays, SMN Δ 7 read-through product is shown to increase protein stability, promote neurite outgrowths in SMN deficient neurons, and significantly elevate SMN-dependent UsnRNP assembly in extracts from SMA patient fibroblasts. Collectively, these results demonstrate that SMN Δ 7 read-through product is more active than the SMN Δ 7 protein and suggest that SMA therapeutics that specifically induce SMN Δ 7 read-through may provide an alternative platform for drug discovery.

Keywords

Spinal Muscular Atrophy (SMA); Survival Motor Neuron (SMN); Read-through

Introduction

Spinal Muscular Atrophy (SMA) is the leading genetic cause of infantile death and the second most common autosomal recessive disorder [8]. It affects every 1:6000 births and has a carrier frequency of about 1:35. SMA is caused by the loss of alpha-motor neurons, which results in the atrophy of voluntary muscles [6]. *SMN2* is a nearly identical copy gene [10]. By itself, *SMN2* is not able to compensate for the loss of *SMN1* since the majority of *SMN2* transcripts

*Corresponding Author: University of Missouri, 1201 Rollins Rd, 471G Bond Life Sciences Center, Columbia, MO 65211, lorsonc@missouri.edu, (573) 884-2219.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

are alternatively spliced and encode a truncated and unstable protein called SMN Δ 7 [3,13, 17]. However, increasing copies of *SMN2* correlate with milder phenotypes [5,11,18]. Therefore, *SMN2* is a critical disease modifier, making it an ideal target for therapeutic intervention.

Recent reports demonstrate that certain compounds increase SMN protein levels by inducing a read-through event of the SMN Δ 7 protein or through unknown mechanisms [14,15,22]. Unlike the HDAC inhibitors [21] that induce expression of native SMN, the read-through inducing compounds likely induce a novel SMN species: the SMN Δ 7 read-through product. While heterologous sequences, including the proposed read-through product SMN^{RT}, have previously been shown to support normal SMN intracellular localization patterns, the functionality of SMN^{RT} has not been explored. In this report, the SMN^{RT} protein is analyzed for its ability to perform SMN functions in a variety of *in vitro* cellular assays.

Materials and Methods

Cloning of Constructs

All constructs were cloned in frame downstream of the Red2 gene into the pDsRed2-C1 vector (Clontech) fused to the carboxy-terminus of Red2. The inserts were PCR amplified using SMN cDNA with a *Bam* HI/SMN forward primer and insert specific *Xho* I reverse primers with the exception of SMN⁶⁻⁸, and SMN⁷⁻⁸, *Drosophila melanogaster* Smn and *Caenorhabditis elegans* Smn (Supplemental Table 1).

Transfection of Constructs

Constructs were transfected into U2OS and HEK293 cells using 2 μ g DNA and PEI according to manufacturer's directions. Constructs were transfected into 3813, HeLa and pC12 cell lines [1] using 3 μ g DNA and Lipofectamine according to manufacturer's direction. PC12 cells were refed after transfection and again after 24 hours with DMEM plus and 50 ng/ mL media with neuronal growth factor (NGF).

Western Blotting

Cellular extracts and western blotting was performed as previously described [15].

Immunohistochemistry

Cells were plated on glass coverslips and settled for 24 hours before transfection. 24 h after transfection cells were fixed with 1:1 acetone:methanol for 2–3 minutes. Nuclei were stained with DAPI for 5 min, rinsed and mounted using DABCO mounting media (Sigma). Neurite outgrowths of 25 transfected cells for each construct were measured in PC12 cells stably expressing a short hairpin RNA which knocks-down rSmn by 85% [1]. Neurite lengths were quantified using Metamorph software.

Stability

U2OS were transfected and stability assay performed as previously described [12]. Assays were performed at least in triplicate and representative blots are shown. Western blots quantified using Metamorph software.

UsnRNP assembly

Cells were transfected with indicated SMN expression constructs, lysates harvested 24 hours post-transfection and used for *in vitro* snRNP assembly assays as described previously [4]. UsnRNP assembly gels quantified using Metamorph software.

Results

Expression of SMN Constructs

To begin to analyze the activity of the C-terminus of SMN and the SMN read-through product, 12 SMN constructs were cloned in frame into a vector expressing an N-terminal Red2 tag (Fig. 1A). The SMN^{FL} and SMN^{Δ7} clones are identical to cDNAs expressed from the endogenous *SMN1* and *SMN2* genes. While both constructs SMN⁶⁻⁸ and SMN⁷⁻⁸ encode include exon 8, these clones have a translational stop signals located within exon 7. SMN^{ΔQNQKE} is an otherwise full-length cDNA lacking the exon 7 sequences coding for the previously described cytoplasmic localization signal 'QNQKE' [23]. The SMN read-through construct is the SMN^{Δ7} cDNA with the first stop codon in exon 8 mutated to a tyrosine and an intact second stop codon four amino acids downstream. Tyrosine was chosen to replace the stop codon due to the sequence of the wobble position. SMN^{1-6/+4}, SMN^{1-6/+9} and SMN^{1-6/+14} are encoded by SMN¹⁻⁶ cDNA with an additional 4, 9 or 14 amino acids derived from exon 7, respectively. Additionally, the Smn homologs from *Caenorhabditis elegans* or *Drosophila melanogaster* which diverge in their C-termini from the human sequence were included.

To confirm expression levels from the SMN vectors, each construct was transfected into HEK293 (Fig 1B) and U2OS (data not shown) and Western blot analysis was performed using an anti-Red2 antibody and an anti-SMN antibody. All vectors achieved similarly detectable levels of expression and resulted in the production of proteins of migrating at the expected sizes, although minor differences in expression were observed (Fig. 1B).

SMN read-through promotes neurite outgrowth in Smn depleted PC12 cells—To analyze SMN in a more disease-related cellular context, SMN constructs were assayed in a cell-based neurite- outgrowth assay [1]. In this system, rat neuronal PC12 cells were stably transfected with a vector expressing a short-hairpin RNA that specifically knocks-down only the endogenous rSmn by approximately 85%, which have been shown to have reduced outgrowth after differentiation with NGF [1]. As anticipated, SMN^{FL} restored neurite outgrowths to near wildtype levels. Similarly, the SMN^{RT} construct and SMN^{FLΔQNQKE} supported neurite outgrowth lengths comparable to SMN^{FL}. Neurite outgrowths were significantly shorter in SMN^{Δ7} and SMN^{1-6/+9}, and were shorter still in cells transfected with SMN¹⁻⁶, SMN^{1-6/+4}, and empty vector (Fig. 2A). Taken together, these results suggest that SMN^{RT} and SMN^{FL} activity were comparable in their ability to support neurite outgrowth and that SMN exon 7 length impacts SMN functionality.

SMN read-through supports high levels of UsnRNP assembly in SMA-deficient extracts—One of the most well characterized cellular activities of the native SMN protein involves SMN's activity in snRNP maturation [7,16,20]. To determine whether the SMN^{RT} could support snRNP assembly, SMN-deficient cytoplasmic extracts were generated from SMA type I patient cells (3813), transfected with SMN^{FL}, SMN^{RT}, SMN^{Δ7}, or empty vector. Since endogenous levels of SMN are very low in 3813 cells, basal snRNP activity is nearly undetectable while extracts generated from SMN^{FL} transfected cells supported high levels of snRNP assembly (Fig. 2B). Similarly, SMN^{RT} was capable of mediated snRNP assembly levels comparable to SMN^{FL}. SMN^{Δ7} and empty vector did not support detectable levels of snRNP assembly (Fig. 2B).

SMN read-through confers increased protein stability—To examine protein stability of the SMN constructs, cells were transfected with SMN^{RT}, SMN^{FL} and SMN^{Δ7} vectors and treated with cycloheximide to inhibit protein synthesis. At times indicated post cycloheximide treatment, Western blotting was used to evaluate SMN protein stability (Fig. 3). The SMN^{FL} protein (Fig. 3) was significantly more stable than the SMN^{Δ7} protein (Fig. 3). While it was

not as stable as SMN^{FL}, the SMN^{RT} product (Fig. 3) conferred increased stability to the SMN^{Δ7} construct. It is possible that the increased variability seen in SMN^{RT} is due to an intermediate level of stability, however it is clearly more stable than SMN^{Δ7}. Taken together, these results demonstrate that the SMN^{RT} product functions similar to native full-length SMN in a variety of cellular assays, suggesting that compounds designed to induce SMN^{RT} production may result in increased functional SMN protein in SMA contexts that are due to SMN deficiency.

Discussion

There are various proposed SMA therapies currently being studied, working through different modes of action [21]. Some compounds, including the aminoglycoside class, are thought to stabilize the existing SMN^{Δ7} protein by a process known as ‘read-through’. While it has been shown previously that this class of compounds can elevate gem numbers as well as total SMN protein [15,22], the read-through protein product has not been thoroughly examined. This work demonstrates SMN ‘read-through’ isoform’s functionality in a variety of cellular assays.

Since SMN^{Δ7} is ~90% of what is produced from *SMN2*, this, along with its reduced ability to self- or hetero-oligomerize for its ability to form the gemin complex, is thought to be at the basis of SMA. It is shown here that the product of a read-through event produces a more stable isoform than SMN^{Δ7}, pointing to increased functionality of the protein.

We next evaluated the functionality of the ‘read-through’ protein by two cellular assays, the first being the ability of the construct to rescue neurite extension. It has been previously shown in zebrafish that ‘read-through’ was able to enable transport into the axons, but was not able to rescue axonal extension after *Smn* knockdown [2]. Contrary to these findings, we show that this construct was able to significantly increase axonal lengths over the empty vector alone. The differences in the results between these two assays could be explained by either the difference between the two assays and the fact that the “stop” amino acid varies between an alanine in the zebrafish and a tyrosine in this assay [2], or due to the inherent differences between the zebrafish and PC12-based assays. Additionally, we show that SMN^{Δ7} is able to promote significant neurite outgrowth, though not to the levels of SMN^{FL}. This is not surprising when considering addition of the SMN^{Δ7} cDNA transgene to the SMA mouse model *Smn*^{-/-}; *SMN2* ^{+/+} dramatically reduced the severity of the phenotype [9], indicating a positive role for SMN^{Δ7}. Additionally, this may also point to the SMN^{Δ7} transcript is an ideal target for therapeutic intervention.

It is still controversial regarding the specific SMN-dependent pathway that leads to SMA development, SMN’s role in UsnRNP assembly has been well documented [16,19,20]. While ‘read-through’ was less able to bind a neuronal form of the Sm protein (SmN) [2], the ability for the protein to properly assemble UsnRNPs had yet to be examined. We show in an *in vitro* assay that ‘read-through’ is able to increase assembly of the U1snRNP to levels comparable to those observed with full-length SMN. This strongly supports that ‘read-through’ is at least partially functional within the cell.

There are many avenues of therapy currently being proposed for SMA such as *SMN2* activation, exon 7 inclusion, and SMN protein stability, all using different classes of compounds and even gene therapies [21]. Here we show that compounds that stabilize the SMN protein by inducing a read-through event could be an additional potential therapy. To date, a SMN read-through product has not been detected following drug treatment, however, abridged SMN reporter constructs detected a read-through event following treatment with aminoglycosides [15]. The inability to detect the native read-through protein may be a result of the relatively low frequency at which read-through occurs, or the current lack of read-through specific antibodies. In

analysis of the read-through inducing compounds in the SMA mice is critical and will be instrumental in determining their validity in an SMA context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

V.B.M. is supported by the Clinical BioDeceives Program (NIH T90 DK070105). This work was funded by grants from Phi Zeta-Pi Chapter Research Grant (V.B.M.), FightSMA (C.L.L.), the Muscular Dystrophy Association (C.L.L.) and the National Institutes of Health (C.L.L, R01 NS41584; R01 HD054413).

References

1. Bowerman M, Shafey D, Kothary R. Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *J Mol Neurosci* 2007;32:120–131. [PubMed: 17873296]
2. Carrel TL, McWhorter ML, Workman E, Zhang H, Wolstencroft EC, Lorson C, Bassell GJ, Burghes AH, Beattie CE. Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis. *J Neurosci* 2006;26:11014–11022. [PubMed: 17065443]
3. Cartegni L, Krainer AR. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. *Nat Genet* 2002;30:377–384. [PubMed: 11925564]
4. Coady TH, Shababi M, Tullis GE, Lorson CL. Restoration of SMN function: delivery of a trans-splicing RNA re-directs SMN2 pre-mRNA splicing. *Mol Ther* 2007;15:1471–1478. [PubMed: 17551501]
5. Coovert DD, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, Coulson SE, Androphy EJ, Prior TW, Burghes AH. The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet* 1997;6:1205–1214. [PubMed: 9259265]
6. Crawford TO, Pardo CA. The neurobiology of childhood spinal muscular atrophy. *Neurobiol Dis* 1996;3:97–110. [PubMed: 9173917]
7. Fischer U, Liu Q, Dreyfuss G. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* 1997;90:1023–1029. [PubMed: 9323130]
8. Iannaccone ST. Spinal muscular atrophy. *Semin Neurol* 1998;18:19–26. [PubMed: 9562664]
9. Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coovert DD, Gavrilina TO, Xing L, Bassell GJ, Burghes AH. SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet* 2005;14:845–857. [PubMed: 15703193]
10. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 1995;80:155–165. [PubMed: 7813012]
11. Lefebvre S, Burlet P, Liu Q, Bertrand S, Clermont O, Munnich A, Dreyfuss G, Melki J. Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat Genet* 1997;16:265–269. [PubMed: 9207792]
12. Lorson CL, Androphy EJ. An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum Mol Genet* 2000;9:259–265. [PubMed: 10607836]
13. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci U S A* 1999;96:6307–6311. [PubMed: 10339583]
14. Lunn MR, Root DE, Martino AM, Flaherty SP, Kelley BP, Coovert DD, Burghes AH, Man NT, Morris GE, Zhou J, Androphy EJ, Sumner CJ, Stockwell BR. Indoprofen upregulates the survival motor neuron protein through a cyclooxygenase-independent mechanism. *Chem Biol* 2004;11:1489–1493. [PubMed: 15555999]

15. Mattis VB, Rai R, Wang J, Chang CW, Coady T, Lorson CL. Novel aminoglycosides increase SMN levels in spinal muscular atrophy fibroblasts. *Hum Genet.* 2006
16. Meister G, Buhler D, Pillai R, Lottspeich F, Fischer U. A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. *Nat Cell Biol* 2001;3:945–949. [PubMed: 11715014]
17. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH, McPherson JD. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum Mol Genet* 1999;8:1177–1183. [PubMed: 10369862]
18. Monani UR, Sendtner M, Covert DD, Parsons DW, Andreassi C, Le TT, Jablonka S, Schrank B, Rossol W, Prior TW, Morris GE, Burghes AH. The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in *Smn*($-/-$) mice and results in a mouse with spinal muscular atrophy. *Hum Mol Genet* 2000;9:333–339. [PubMed: 10655541]
19. Paushkin S, Gubitz AK, Massenet S, Dreyfuss G. The SMN complex, an assemblyosome of ribonucleoproteins. *Curr Opin Cell Biol* 2002;14:305–312. [PubMed: 12067652]
20. Pellizzoni L, Yong J, Dreyfuss G. Essential role for the SMN complex in the specificity of snRNP assembly. *Science* 2002;298:1775–1779. [PubMed: 12459587]
21. Sumner CJ. Therapeutics development for spinal muscular atrophy. *NeuroRx* 2006;3:235–245. [PubMed: 16554261]
22. Wolstencroft EC, Mattis V, Bajer AA, Young PJ, Lorson CL. A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels. *Hum Mol Genet* 2005;14:1199–1210. [PubMed: 15790598]
23. Zhang HL, Pan F, Hong D, Shenoy SM, Singer RH, Bassell GJ. Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J Neurosci* 2003;23:6627–6637. [PubMed: 12878704]

Fig. 1. Schematic and expression of SMN C-terminal constructs

(A) Schematic of mutant SMN cDNA constructs showing exons 1–6 (black), exon 7 (hatched), exon 8 (grey) and missing regions (white). All constructs are expressed in a pDsRed2-C1 expression vector with an N-terminal Red2 tag. Indicated are the amino acid lengths after exons 1–6. (B) Extracts from transfected HEK293 cells were analyzed by western blot by using an anti-Red2 polyclonal antibody (Clontech) (upper blot). Arrows indicate the sizes of the constructs or endogenous. * indicates nonspecific bands. The blot was stripped and reprobed with anti-SMN 4B7 (lower). Anti-SMN 4B7 has been mapped to exon 1 and therefore constructs lacking human exon 1 (vector, SMN⁶⁻⁸, SMN⁷⁻⁸, *Drosophila* Smn and *C. elegans* Smn) are not recognized.

Fig. 2. SMN^{RT} regains functionality as determined by neurite outgrowth and UsnRNP assembly
 (A) C-terminal constructs in PC12 cell line C59 [1] (85% knock-down rSmn). Cells were plated, transfected and refed with 50 ng/ml NGF for 48 hours. Cells were fixed and DAPI (blue) indicates nucleus. Transfected constructs are indicated by arrows and brightfield is shown. Graphical representation of neurite outgrowth is also shown. The longest extension of an arbitrary transfected cell was measured (averages graphed). Error bars represent SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's T Test). (B) UsnRNP assembly shows functionality of SMN^{RT} (representative film) Wild-type U1snRNA (WT) or U1snRNA lacking the Smith core binding site (Δ Sm), negative control, were transcribed using α p32-labeled UTP. Cytoplasmic extracts of U2OS or 3814 cells (positive controls) or 3813 cells transfected with empty pDsRed2-C1, SMN^{FL}, SMN ^{Δ 7} or SMN^{RT} (average quantification (AU): 96757.760, 19489.650, 2842.730, 8258.345, 1984.380, 21507.490 respectively) were incubated with the U1snRNAs, allowed to assemble, and then, using anti-Y12 antibody, assembled U1snRNPs were pulled down and run out on an 8% Bis-Acrylamide Urea gel.

Fig. 3. SMN^{RT} protein stability is increased over SMN^{Δ7}, but not to the levels of SMN^{FL}. U2OS cells were transfected and 24 hours later treated with 100 mM cycloheximide for 0, 45, 90, 120, or 150 minutes before being harvested, protein quantified to load equal amounts, and run out and examined by western blot using anti-SMN 4B7, then stripped and reprobed for actin as a loading control. Representative western blots are shown for SMN^{FL}, SMN^{Δ7}, and SMN^{RT}. Average of quantification also shown, with error bars representing standard deviation (* significance p < 0.01 as compared to time 0).