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SMN, profilin IIa and plastin 3: A link between the deregulation of actin dynamics and SMA pathogenesis

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ABSTRACT

Spinal muscular atrophy (SMA) is the most common human genetic disease resulting in infant mortality. SMA is caused by mutations or deletions in the ubiquitously expressed survival motor neuron 1 (*SMN1*) gene. Why SMA specifically affects motor neurons remains poorly understood. We have shown that Smn deficient PC12 cells have increased levels of the neuronal profilin IIa protein, leading to an inappropriate activation of the RhoA/ROCK pathway. This suggests that mis-regulation of neuronal actin dynamics is central to SMA pathogenesis. Here, we demonstrate an increase in profilin IIa and a decrease in plastin 3 protein levels in a SMA mouse model. Furthermore, knock-out of profilin II upregulates plastin 3 expression in a Smn-dependent manner. However, the depletion of profilin II and the restoration of plastin 3 are not sufficient to rescue the SMA phenotype. Our study suggests that additional regulators of actin dynamics must also contribute to SMA pathogenesis.

Introduction

Spinal muscular atrophy (SMA) is the number one genetic killer of children under two years of age, affecting approximately 1 in 10,000 live births (Pearn, 1978; Crawford and Pardo, 1996). It is an autosomal recessive disorder defined by severe loss of α-motor neurons in the spinal cord (Crawford and Pardo, 1996). The motor neuron degeneration subsequently causes muscular atrophy of the limbs and trunk, which leads to paralysis and in severe cases, death (Crawford and Pardo, 1996). SMA is caused by homozygous deletions or mutations of the ubiquitously expressed survival motor neuron 1 (*SMN1*) gene (Lefebvre et al., 1995). The *SMN* gene is highly conserved and although all eukaryotic organisms studied to date have only one copy of the gene, humans have two (Lefebvre et al., 1995; DiDonato et al., 1997). The telomeric copy, *SMN1*, produces the full-length protein while the duplicated centromeric copy, *SMN2*, expresses a truncated isoform with a deletion of the C-terminal exon 7, termed Δ7SMN (Lefebvre et al., 1995). The excision of exon 7 is caused by a C to T substitution in the *SMN2* gene at position 6 of exon 7 (Lorson et al., 1999). This leads to the loss of an exon splicing enhancer and/or the gain of a exon splicing silencer, which promotes exon 7 skipping of the *SMN* gene (Cartegni and Krainer, 2002; Kashima and Manley, 2003). Since the *SMN2* gene produces a small amount of functional full-length protein, it can modulate the severity of SMA in a copy number-dependent manner (Lefebvre et al., 1995).

The 38 kDa SMN protein is part of a multiprotein complex, composed of self-oligomerized SMN, Gemin2-8, Unrip and Sm proteins (Paushkin et al., 2002; Carissimi et al., 2006; Gabanella et al., 2007). This complex is responsible for the assembly of small nuclear ribonucleoproteins (snRNPs) and thus, regulates the processing of most pre-mRNAs and ribosomal RNAs (Paushkin et al., 2002; Carissimi et al., 2006; Gabanella et al., 2007). Consequently, many investigations have focused on the housekeeping role of SMN in mRNA splicing and its relevance in SMA pathogenesis. Recently, a correlation between snRNP assembly activity in the spinal cord of SMA mice and severity of the disease has been demonstrated (Gabanella et al., 2007). Also, Smn deficiency causes tissue-specific pre-mRNA splicing defects in the spinal cord, brain and kidney in a SMA mouse model, suggesting that SMA is a general splicing disease (Zhang et al., 2008). However, no causal link has yet been made between the identified aberrantly spliced targets and SMA pathogenesis. Thus, the question of motor neuron specific death in SMA remains unresolved and suggests that additional mechanisms outside of pre-mRNA processing are involved.

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Other studies propose a specific role for Smn in neuronal cells. Work in P19 cells, zebra fish, and neurosphere-derived neural stem cells of severe SMA mice show a role for Smn in neurite outgrowth, neuronal differentiation, axonal pathfinding and neuromuscular maturation (Fan and Simard, 2002; McWhorter et al., 2003; Shafey et al., 2008). Motor neurons isolated from severe SMA mouse models have normal survival but reduced axonal growth. β-actin mRNA and protein staining is reduced in distal axons and growth cones (Rossoll et al., 2003). Interestingly, Smn interacts and co-localizes in the cytoplasm, in neurite-like extensions, and in nuclear gems with the neuronal specific protein profilin II, a small actin-binding protein and key regulator of actin dynamics (Giesemann et al., 1999; Sharma et al., 2005). More recently, Smn has been attributed a role in neuromuscular junction (NMJ) development (Kariya et al., 2008; McGovern et al., 2008; Kong et al., 2009). Indeed, pre-synaptic defects such as poor terminal arborization, intermediate filament aggregates, denervation, and impaired synaptic vesicle release have been identified in SMA mouse models (Kariya et al., 2008; McGovern et al., 2008; Kong et al., 2009). All of these observations therefore suggest a role for Smn in neurodevelopment and/or neuromaintenance. In fact, during the maturation of the human central nervous system, there is a progressive shift from nuclear to cytoplasmic and then to axonal localization of SMN (Giavazzi et al., 2006).

We have shown that Smn knock-down in PC12 neuronal cells leads to defects in neuritogenesis caused by alterations in cytoskeletal integrity (Bowerman et al., 2007). Furthermore, we have provided evidence that these defects are mediated via an increased expression of profilin IIa and an inappropriate activation of the RhoA/ROCK pathway. In the present study, we characterize profilin IIa expression in an intermediate SMA mouse model as well as evaluate the effect of profilin II knock-out on the SMA phenotype. We provide evidence for modulation of actin-binding and actin-regulating proteins in a Smn-dependent manner, highlighting the perturbed actin cytoskeletal pathway as an important player in SMA pathogenesis.

Results

Profilin IIa expression in an intermediate SMA mouse model

Our previous findings indicate that there is increased profilin IIa mRNA and protein in Smn-depleted PC12 cells (Bowerman et al., 2007). This in turn had a negative impact on neuronal outgrowth and neurite formation. To extend this analysis to an *in vivo* context, we wanted to assess profilin IIa protein levels in an intermediate SMA mouse model, termed $Smn^{2B/-}$ (unpublished data; DiDonato et al., 2007). This knock-in transgenic mouse line has a recombined allele harboring mutations within exon 7 of the endogenous *Smn* gene. The 2B allele has 3 nucleotides substituted within the exon splicing enhancer (ESE) of exon 7. This change leads to the alternative splicing of the Smn transcript in a predominant manner, resulting in the production of Δ7Smn mRNA (DiDonato et al., 2001). By comparison, the normal wild type mouse *Smn* gene is never alternatively spliced. The $Smn^{2B/-}$ mice have a phenotype that arises shortly after two weeks of age and at three weeks of age they are significantly smaller than their wild type littermates. The lifespan of $Smn^{2B/-}$ mice is shorter than its wild type littermates with the majority dying by one month of age. RNA analysis shows a significantly smaller ratio of full-length Smn/Δ7Smn transcript while Smn protein expression is significantly reduced in all tissues, with only approximately 15% of the full-length protein still present. The $Smn^{2B/-}$ mice also exhibit a reduction in the number of motor neurons in the brain stem and spinal cord at P21 (close to the end stage of disease), concordant with existing SMA mouse models (Hsieh-Li et al., 2000; Jablonka et al., 2000; Monani et al., 2000). Therefore, these mice represent an intermediate SMA mouse model that can be used to investigate potential pathogenic molecular pathways prior to and at the onset of disease.

We performed immunohistochemistry to get an appreciation of profilin IIa levels within the spinal cord and brain stem of SMA mice. SMI32, an antibody specific for phosphorylated neurofilaments of axonal bodies, was used to specifically identify neuronal cells (Fig. 1A). Upon quantitative analysis, we observed a significant reduction in the number of neurons (SMI32-positive) in the brain stem and spinal cord of $Smn^{2B/-}$ mice (Fig. 1B). However, interestingly, we only observed a decrease in the number of profilin IIa-positive cells in the brain stem but not in the spinal cord of $Smn^{2B/-}$ mice (Fig. 1B). Further quantification indicates that there is no change in the percentage of profilin IIa-positive motor neurons (identified morphologically by size and shape) (Fig. 1C). This suggests that the motor neuron pool is not specifically affected by the observed changes in the number of profilin IIa-positive neurons. Qualitative assessment initially suggested an increase in profilin IIa signal intensity in the SMI32-positive cells in both brain stem and spinal cord of the $Smn^{2B/-}$ mice (Fig. 1A). However, quantitative measurement of profilin IIa immunofluorescence shows that SMI32-positive motor neurons (identified morphologically by size and shape) in the spinal cord but not the brain stem of $Smn^{2B/-}$ mice have a greater signal intensity compared to wild type and $Smn^{2B/+}$ mice (Fig. 1D) (refer to Experimental methods for quantitative assays). Immunoblot analysis shows that there is no difference in profilin IIa protein levels in the brain and spinal cord of $Smn^{2B/-}$ mice when compared to wild type mice (Fig. 1D). This suggests that within the spinal cord of our SMA mouse model, the increase in profilin IIa expression is cell-autonomous and not a general event in the entire tissue. Since SMA mice are characterized by a significant loss of spinal cord motor neurons, a cell-specific increase in profilin IIa expression may thus be muted in whole tissue lysate composed of more than one cell type. As previously mentioned, overexpression of profilin IIa in hippocampal neurons has been shown to negatively regulate neuritogenesis (Da Silva et al., 2003). In addition, it has been proposed that interaction of Smn with profilin IIa could regulate actin dynamics by modulating the inhibitory effect of profilin IIa (Sharma et al., 2005). As such, the increase in profilin IIa in the motor neurons within the spinal cord of $Smn^{2B/-}$ mice might be having an overall negative effect on these neurons.

Plastin 3 levels are decreased in the brain and spinal cord of SMA mice

Plastin 3, also known as T-plastin and T-fimbrin, is expressed in solid tissues, including the spinal cord (Bretscher, 1981; Lin et al., 1988). It is an actin-bundling protein that organizes actin filaments into complex networks but also has an independent role in actin stabilization (Bretscher, 1981; Gimona et al., 2002; Giganti et al., 2005). Furthermore, plastin 3 has recently been identified as a protective gender-specific modifier of SMA, forming a large complex with SMN and actin (Oprea et al., 2008). In the latter study, it was reported that female patients missing the *SMN1* gene were asymptomatic and exhibited significantly higher levels of expression of plastin 3 in lymphoblast cells when compared to their SMA-affected siblings (Oprea et al., 2008). Furthermore, it was demonstrated that plastin 3 is important for axonogenesis since its overexpression rescued the axon length and outgrowth defects associated with Smn depletion in motor neurons derived from SMA mouse embryos and in zebra fish (Oprea et al., 2008). Here, we assessed plastin 3 levels within the brain and spinal cord of the $Smn^{2B/-}$ mice compared to their wild type littermates. Upon immunoblot analysis of mice at the phenotype stage (P21), we found that plastin 3 levels were reduced in the brain and spinal cord of $Smn^{2B/-}$ mice (Fig. 2A). Densitometric analysis shows that the ratio of plastin 3 over actin is significantly greater in both the brain and spinal cord of wild type mice compared to $Smn^{2B/-}$ mice (Fig. 2B). Plastin 3 expression was also assessed qualitatively by immunohistochemistry (Fig. 2C). Quantitative measurement of plastin 3 immunofluorescence in SMI32-positive motor neurons (identified morphologically by size and shape) indicates a significant