The Spinal Muscular Atrophy disease protein SMN is linked to the Rho-kinase pathway via profilin

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ABSTRACT

Spinal muscular atrophy (SMA), a frequent neurodegenerative disease, is caused by reduced levels of functional \textit{SMN} protein. SMN is involved in multiple pathways including RNA metabolism and splicing as well as motoneuron development and function. Here we provide evidence for a major contribution of the Rho-kinase (ROCK) pathway in SMA pathogenesis. Using an \textit{in vivo} protein interaction system based on SUMOylation of proteins, we found that SMN is directly interacting with profilin2a. Profilin2a binds to a stretch of proline residues in SMN, which is heavily impaired by a novel \textit{SMN2} missense mutation (S230L) derived from a SMA patient. In different SMA models, we identified differential phosphorylation of the ROCK-downstream targets cofilin, myosin-light chain phosphatase and profilin2a. We suggest that hyper-phosphorylation of profilin2a is the molecular link between SMN and the ROCK pathway repressing neurite outgrowth in neuronal cells. Finally, we found a neuron-specific increase of F-/G-actin ratio that further support the role of actin dynamics in SMA pathogenesis.
INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a devastating neuromuscular disease leading to a progressive degeneration of the α-motoneurons in the spinal cord (1). With an incidence of 1-6,000 to 1-10,000 and a carrier frequency of 1:35 in the Caucasian population SMA is one of the most common autosomal recessive disorder which leads to an early death in childhood (2-4). SMA is caused by deletion, gene conversion or subtle mutation of the survival of motoneuron 1 (SMN1) gene encoding the SMN protein (5). A second SMN gene, SMN2, modifies the severity of the disease by expressing a small amount of full-length and functional SMN protein; a higher copy number of SMN2 results in milder SMA phenotypes termed SMA type II, III and IV (6, 7). The 40 kDa ubiquitously expressed SMN protein acts as an assembly factor for small nuclear ribonucleoprotein particles (snRNPs) or small nucleolar RNPs (snoRNPs) involved in splicing (8-10). In the nucleus, SMN is found in nuclear foci (11). Several SMN mutations identified in SMA patients disrupt in vitro the binding of SMN to Smith (Sm) antigen proteins, which are components of snRNPs (12). However, no splicing defects are found in presymptomatic stages which are linked to motoneuron death (13).

Recent evidence demonstrates that motoneuron defects in SMA are due to lack of SMN located in axons. SMN colocalizes in ribonucleoprotein granules within neurites and at growth cones (14, 15). Since Sm proteins are lacking in the axonal complexes, SMN might carry out other functions independent from snRNP assembly. SMN interacts with hnRNP-Q/R and promotes the transport of mRNA along the axon (16, 17). It is known from various SMA models that SMN deficiency leads to axonal outgrowth and pathfinding failures as well as neuromuscular maturation defects (14, 16, 18-23). Experiments in PC12 cells as well as in zebrafish showed that neurite outgrowth defects in the SMN depleted cells can be rescued by the C-terminal part of the SMN protein comprising exon 6 and 7 (22, 24), which is not directly involved in snRNP assembly. Additionally, we demonstrated in PC12 cells that
outgrowth defects are accompanied by a hyperstabilization of actin-filaments. Interestingly, others have found concentration of F-actin around the cell body perimeter in SMN knock-down cells, accompanied by accumulation of GAP-43 in membrane ruffles and neurofilaments in swellings of neurites (25). The dynamics of actin cytoskeleton at growth cones in neurons is the driving force of neurite extension. A functional association of SMN with actin-regulatory proteins has been previously reported to upregulate the actin binding protein profilin2a in SMA cells (25, 26). Profilin2a promotes actin polymerization and is a downstream target of Rho-Kinase 2 in the Rho-Kinase (ROCK) pathway, which becomes inhibited during neuronal differentiation (27-29). Additionally, it has been shown that application of the ROCK inhibitor Y27632 rescues SMA symptoms in an intermediate mouse model for SMA. Here, lifespan was prolonged significantly, maturation of the neuromuscular junction improved and muscle fiber size increased (30). Importantly, these data underscore the therapeutic potential of ROCK inhibition in SMA. SMN colocalizes with profilin2a in neurite-like extensions (31). The SMN protein sequence exhibits a poly-proline domain in exon 5 which is assumed to be the binding site of profilin2a (32). In the present study, we characterized the interaction between profilin2a and SMN in a cellular system using a new protein interaction method. With this system, we were able to determine the profilin2a binding domain in the SMN protein. Interestingly, we found that a newly identified SMN patient mutation located next to the binding domain abolished profilin2a binding to SMN. Additionally, we show that ROCK-downstream targets become regulated in SMA by differential phosphorylation. Loss of SMN resulted in hyper-phosphorylation of profilin2a thereby repressing neurite outgrowth in neuronal cells. These results demonstrate that SMN interferes with the ROCK pathway via profilin2a and provides a mechanistic basis to explain axonal defects in SMA pathogenesis.
RESULTS

The actin cytoskeleton in growth cones of SMA motoneurons becomes dysregulated

We have previously shown that SMN regulates neurite outgrowth in PC12 cells and modulates β-actin dynamics, a driving force of neurite outgrowth. In lysates of these cells, a hyperstabilization of actin filaments has been detected by a centrifugation assay (22). To test whether this is also present in SMA motoneurons, we here determined the ratio of filamentous F-actin to globular G-actin in growth cones of explanted motoneurons from SMA mice and controls by fluorescence staining of both actin forms. In a preceding control experiment in PC12 cells, the fluorescence assay showed increase of F-actin after SMN knock-down (Fig. S1) and confirmed our previous biochemical results (22). Motoneurons were first explanted at embryonic stage E14 from SMAΔ7 and control mice and then cultivated on collagen I. After quantification of the fluorescent Phalloidin signal for F-actin and DNaseI signal for G-actin in the growth cones, F-/G-actin ratios were calculated (Fig. 1). The result showed that the F-/G-actin ratio was significantly increased in SMA motoneurons in comparison to controls. Next, we asked whether this dysregulation is motoneuron-specific or a general observation. We analyzed the F-/G-actin ratios by means of a biochemical centrifugation assay in a number of primary fibroblast cell lines obtained from SMA patients and healthy controls (Fig. S2A-D). The results reveal no differences between fibroblasts from patients and controls. This argues that the observed dysregulation of the actin cytoskeleton is restricted to neuronal cells. This is an important finding since only a few neuron-specific consequences of SMN-loss have been described previously.
**SMN binds to profilin2a in vivo**

Actin assembly is promoted by the actin-binding proteins profilin1 and profilin2a. The latter is the neuronal isoform of profilin. A SMN profilin2a interaction has been previously demonstrated, however, a direct interaction in vivo has not been shown yet (30-32). To determine whether SMN and profilin2a are interacting in the motoneuron cell line NSC34, we used a new interaction assay based on SUMOylation of proteins, a posttranslational modification of substrate proteins mediated by the SUMO-conjugating enzyme Ubc9. In this method, called the trans-SUMOylation (TRS) system (33), the putative binding partner is fused to Ubc9 and coexpressed with SUMO1 as well as the protein of interest. An interaction between the proteins tethers the fused Ubc9 in close proximity to the interactor and results in its trans-SUMOylation (Fig. S3A). Hence, trans-SUMOylation reflects the binding of these proteins. An advantage of this system is the detection of in vivo protein interactions (33).

To study the SMN-profilin2a interaction, profilin2a was fused to Ubc9 and coexpressed with EGFP-SUMO and SMN-EGFP. For negative control, cells were transfected with Ubc9 instead of Ubc9-profilin2a. After 24h of coexpression, we detected SUMOylated SMN-EGFP in the extracts analyzed by western blotting (Fig. 2A). SUMOylated SMN migrated slower in SDS-PAGE (Fig. 2A, lane 5; Fig. S3B). As an additional control, SUMOylation of SMN was performed with non-fused SUMO (Fig. S3B, lane 4). As a negative control, expressing Ubc9 alone instead of the fusion protein Ubc9-profilin2a did not result in SUMOylated SMN (Fig. 2A, lane 4). Endogenous SMN was not SUMOylated (Fig. 2A, lane 6) possibly due to lack of modifiable sites. Instead, EGFP in the SMN-EGFP fusion protein was probably the target for the trans-SUMOylation. Moreover, SMN expression levels were comparable between lanes (Fig. 2B). Hence, the in vivo SUMOylation by tethering Ubc9 in close contact to a protein of interest is a specific indicator for the interaction of SMN with profilin2a.
Profilin2a binding to SMN is disrupted by a SMA-patient mutation

Profilins bind to prolin-rich sequences (32). In SMN, three proline-rich stretches are present in the sequence encoded by exon 5. To determine the profilin2a binding site, we generated a mutant for each proline stretch denoted as P1 (P196-198A), P2 (P219-224A) and P3 (P245-247A) by converting 3-5 proline to alanine residues. Expression of the proline mutants was first tested in HEK293 cells to check for normal distribution in the nucleus and in growth cones of NSC34 cells (Fig. 3A). SMN and proline-mutants were located in nuclear bodies and in the perinuclear cytoplasm as well as in the growth cones. The proline mutants showed no change of the SMN distribution in comparison to the wild-type. Then, binding of profilin2a to the SMN mutants was determined in the SUMOylation assay. The analysis showed that the mutation in the first (P1) and in the third (P3) domain did not affect profilin2a binding (Fig. 3B). However, mutations in the second domain (P2) disrupted consistently this interaction reflected by a decrease of SMN trans-SUMOylation. We conclude that the P2 proline stretch is important for binding of profilin2a.

Next, we analyzed whether loss of the profilin2a binding is linked to SMA pathogenesis. Therefore, we generated five point mutants (W92S, E134K, S230L, Y272C, T274I) which harbour SMN missense mutations of SMA patients. S230L is a novel missense mutation in the SMN2 gene (c.689C>T) of a SMA type II patient with two copies of SMN2 (Fig. S4). Expression of the mutants in NSC34 cells revealed no change of SMN distributions in comparison to the wild type (Fig. S5). Then, we performed the SUMOylation assay to determine whether the mutation has an impact on the interaction with profilin2a. The analysis revealed that profilin2a-binding of the mutants was not affected in W92S, E134K, Y272C and T274I mutants but significantly decreased binding in S230L (Fig. 4A). Serine residue 230 is in close proximity to the prolin-sequence 219-224 determined as critical for profilin2a binding.
The interaction of SMN and the SMN S230L mutant with profilin2a, respectively, was further analyzed in vivo by application of Förster resonance energy transfer (FRET) approach. Both, SMN and the S230L mutant, were fused with ECFP (donor), and profilin2a with EYFP (acceptor) to study their interaction in cotransfected NSC34 cells. In samples coexpressing SMN-ECFP and profilin2a-YFP we found the typical intracellular distribution (Fig. 4B). For quantitative analysis of SMN-profilin2a interaction, we applied LuxFRET method (34), which was recently developed in our group. Apparent FRET efficiency \( E_{D} \) was calculated for different datasets at a pixel based manner (Fig. 4B, column four). While wild-type SMN demonstrates specific interaction with profilin2a both in total cells and in neurites, the SMN S230L mutant shows significant less FRET and thus no close proximity to profilin2a (Fig. 4C). This suggests that serine in position 230 represents an important regulatory interaction site of SMN with profilin2a.

**SMN loss in SMA causes phosphorylation changes of ROCK downstream-targets**

Profilin2a directly interacts with ROCK, a key player in actin regulation and neuronal differentiation (28, 35). We hypothesized that loss of the physiological interaction of SMN with profilin2a would elicit functional changes of the ROCK pathway. First, we tested whether depletion of SMN caused any expression differences of ROCK and its downstream targets in PC12 cells. In this system, SMN expression is knocked-down by transient transfection with a shRNA vector. Experiments were carried out after 3 days of neuronal differentiation. At this point, cells are well differentiated and express neuronal markers beta-tubulin and neurofilament 68 (NF68) (22). Extent of knock-down was similar after 3 and 7 days, respectively, of neuronal differentiation (data not shown). After 3 days of differentiation with NGF, we detected no change in profilin2a protein expression in the extracts of shRNA expressing cells in comparison to cell extracts of the empty vector control (Fig. 5A, B).
Similarly, protein expression of the other ROCK downstream targets LIMK1/2, cofilin and MLCP was also not affected.

Next, we analyzed whether SMN depletion had an impact on the phosphorylation of profilin2a. To detect profilin2a phosphorylation in extracts of SMN depleted PC12 cells and controls, a 2D gel electrophoresis (first dimension: isoelectric focusing, second dimension: SDS-PAGE) was performed since a phospho-profilin2a antibody was not available. The western blot revealed at least two phospho-isoforms of profilin2a with a third one occurring in SMN knock-down cells only (Fig. 6A, B). Therefore, SMN down-regulation causes a hyperphosphorylation of profilin2a. To confirm these results in vivo we analyzed spinal cords from end stage (postnatal day 9) of a severe SMA mouse model (36, 37). These mice express a low level of human SMN on a mouse SMN knock-out background. The results demonstrate a significant profilin2a hyperphosphorylation and confirm the results obtained from the cell culture experiments (Fig. 6C, D).

To test whether other ROCK downstream targets are affected after down-regulation of SMN, we analyzed the phosphorylation of LIM domain kinase 1/2 (LIMK1/2) and its downstream target cofilin which binds to actin filaments. We found a significant decrease in cofilin phosphorylation after knock-down using the adequate phospho antibodies. In order to evaluate the effect of either overexpression or down-regulation of SMN in PC12 cells on another downstream target of ROCK, we used an ELISA-based system that determines the activity of ROCK by measuring the phosphorylation of the myosin-binding subunit of myosin phosphatase at threonine 696. Whereas an overexpression of SMN reduced ROCK activity significantly, the down-regulation of SMN mRNA did not influence ROCK activity compared to control conditions (Fig S6B). Additionally, we evaluated the phosphorylation of another interactor of ROCK, the myosin light chain phosphatase (MLCP), at two different threonine residues. Phosphorylation of threonine 696 was not changed in SMN knock-down PC12 cells.
or in spinal cords of postnatal day 5 (p5) and p10 SMA mice (Fig. S6C) as revealed in a ROCK-activity assay employing the recombinant C-terminus of MLCP. In strong contrast, SMN knock-down PC12 cells showed a significant hypophosphorylation at threonine 850 (Fig. 5A, B) known to be phosphorylated by ROCK (38, 39). MLCP expression was not changed under these conditions.

These results demonstrate that depletion of SMN caused significant changes of phosphorylation patterns downstream of ROCK, but not a differential expression. Whereas profilin2a became hyperphosphorylated, MLCP and cofilin displayed hypophosphorylation. These results are consistent with a model where decreased binding of SMN to profilin2a increases targeting of profilin2a to ROCK with the consequence of its subsequent hyperphosphorylation. Remarkably, this is not a transient or immediate effect of SMN depletion, since the observed dysbalance in target modifications was measured after three days. We analyzed the normalized activity of the upstream small GTPases cdc42, Rac and RhoA and could not detect significant changes in differentiated knock-down PC12 cells compared to controls (Fig. S6A). The results strongly argue for ROCK as the critical molecule for functional interaction of SMN with the ROCK-pathway.

**Profilin2a mutants show differential neurite extension**

We aimed to analyze the consequences of profilin2a phosphorylation. Serine 137 has been described as a phosphorylation site in profilin1 (35), which is conserved in the profilin2a sequence. To test whether the profilin2a phosphorylation has an impact on neurite outgrowth we generated the dominant-negative mutant S137A and the constitutively active, phospho-mimic mutant S137D and expressed the constructs in PC12 cells. Wild-type profilin2a, S137A and S137D constructs, respectively, were coexpressed with EGFP using a vector
containing an Internal Ribosomal Entry Site (IRES). Cells were differentiated for 3 days by NGF treatment and neurite lengths of EGFP-positive cells measured subsequently. Quantification showed no statistical significant change between the mutants and the wild type. However, neurite lengths in cells transfected with the phospho-mimic S137D mutant were significantly decreased in comparison to the non-phospho-mimic S137A mutant (Fig. 7A). We conclude that inhibition of neurite extension by profilin2a is dependent on phosphorylation of serine 137. The observed hyperphosphorylation of profilin2a can therefore explain our previous data demonstrating inhibition of neurite extension under SMN knock-down conditions. We tested the effect of wild-type profilin2a expression on neurite outgrowth (Fig. 7B). As expected, profilin2a decreased neurite outgrowth in agreement with previous data showing that profilin2a displays an inhibitory function (28). Co-expression of SMN should bypass this effect by increasing the number of SMN-profilin2a complexes. Indeed, we could show significant longer neurites under this condition (Fig. 7B). Next, we analyzed the consequence of ROCK-inhibition under SMN knock-down conditions. The RhoA inhibitor C3 and the ROCK-inhibitor Hydroxyfasudil – a drug already in clinical use for treatment of vasospasm after subarachnoid hemorrhage (40) – were both employed in outgrowth assays and could rescue the defect caused by loss of SMN (Fig. 7C). This clearly demonstrates the importance of dysregulation of ROCK downstream targets for neurite growth defects.

**SMN mutants display decreased neurite outgrowth**

Next, we evaluated the effect of SMN harbouring the S230L patient mutation on neurite outgrowth. SMN mutant constructs were expressed in PC12 cells and neurite length measured after 3 days of differentiation. We have previously shown that SMN overexpression results in a significant increase of neurite growth in contrast to SMN down-regulation (22). Here we demonstrate that all selected mutations led to significant shorter neurites in comparison to
wild-type SMN but also to the empty vector control (Fig. 7D). This included a profilin2a-binding-deficient mutant of SMN (SMNΔ[221-228]). Hence, the mutations inhibited neurite outgrowth. The strongest negative effect showed mutation S230L. This result supports that neurite defects are implicated in SMA pathogenesis.
DISCUSSION

In the present study, we demonstrated the direct interaction between SMN and the actin binding protein profilin2a using a new interaction method (TRS), which allows detection of protein interactions in a cellular system. A SMN/profilin2a interaction had been already proposed previously showing that SMN binds to profilin2a and with lower affinity to profilin1 in co-immunoprecipitation analysis using a crosslinker (32). Profilin2a was suggested to interact with the poly proline domains spanning AS residue 195-248 of SMN (31, 32). In our study, we showed that exclusively the second proline motif spanning residues 219-224 is critical for profilin2a binding and demonstrate for the first time a clear biological function of this region in the SMN protein. Furthermore, we determined the profilin2a binding ability of different SMA causing mutants including the newly identified mutation S230L. This site localizes adjacent to the proline stretch and specifically disrupted interaction with profilin2a in the TRS assay and in the FRET analysis. S230L affects a putative phosphorylation site which could provide a regulatory function important for profilin2a interaction. Other mutants did not display any changes in profilin2a interaction in our in vivo interaction analysis which is in contrast with a previous in vitro study showing that the ability of mutant Y272C to bind profilin2a is reduced (31). Since this mutation also affects SMN dimerization (41), monomers may indirectly affect profilin2a binding, whereas in our study mutants could form heterodimers with endogenous SMN and could be less sensitive against disruptions of the binding site.

How is SMN linked to the ROCK-pathway? In this study we found striking phosphorylation changes of ROCK downstream targets after knock-down of SMN or in SMA mice. Protein levels were not affected excluding the possibility of expression changes caused by SMN loss. Upstream of the ROCK pathway small GTPases integrate growth factor signals: RhoA-GTPase mediates signals from the p75 NGF-receptor by activating ROCK (42). The other
GTPases Cdc42 and Rac integrate signals by activation of the ROCK-downstream target LIM-kinase (43). An abnormal activation of RhoA and inhibition of the Cdc42 pathway in SMN knock-down cells has been reported previously (25). In addition, RhoA activity is increased in an intermediate SMA mouse model (26). In this study we could not detect any changes of these small GTPases. This discrepancy may be caused by the use of different models, however, all data come to end of an abnormal increase in the ROCK pathway. We could show that the functional integration of SMN’s loss may occur at the level of ROCK: Whereas cofilin and MLCP become hypophosphorylated, profilin2a is hyperphosphorylated if SMN levels are decreased. Profilin2a has been shown to be a direct target of ROCK (28, 35). The observed regulative changes are consistent with a model that employs competition between SMN and ROCK for binding to profilin2a and functionally links SMN to the ROCK pathway (Fig. 8). The other ROCK downstream targets cofilin and MLCP become hypophosphorylated putatively by increased number of ROCK-profilin2a complexes. This is strongly supported by a previous study where an increase of profilin2a/ROCK complexes under SMN knock-down conditions compared to controls was found (25). In the functional context of neurite outgrowth, this model is strongly supported by our observation of increased neurite length after co-expression of both profilin2a and SMN (Fig. 7B). In contrast, profilin2a expression alone has an inhibitory effect on neurite growth. Others have found enhanced expression of profilin2a levels in a SMN-deficient PC12 cell line generated by stable transfection (25) and increased numbers of profilin2a-positive cells in spinal cord (30). Despite the differences of the experimental systems used in the studies compared to our results, all studies show higher levels of profilin2a availability when not bound to SMN and point to the same direction.

In this study we show that hyperphosphorylation of profilin2a in SMA leads to inhibition of neurite outgrowth. However, it is unclear how profilin2a phosphorylation affects actin
polymerization during neurite outgrowth. On the level of expression, increase of profilin2a inhibits actin polymerization (28, 31). ROCK and profilin2a form a complex (25, 28) and the kinase activity of ROCK is important for regulation of profilin2a (28, 35, 44). Phosphorylation of profilin at serine 137 negatively regulates its affinity to G-actin (35). At present, the location of other phospho-sites in profilin2a is unknown. ROCK inhibition, results in decreased profilin2a phosphorylation accompanied by a reduction of F-actin (28). Thus, it can be assumed that phosphorylated profilin2a is the active form, which promotes actin polymerization at the plus end of the active filament. SMN knock-down or loss causes the opposite: hyper-phosphorylation of profilin2a and increase of F-actin. Therefore, ROCK-inhibition could correct the effect of SMN reduction in SMA with regard to an adequate ratio of de-/phosphorylated profilin2a. The ROCK inhibitor Y27632 is in fact able to ameliorate the disease phenotype of SMA mice (26).

We did not observe a general change of ROCK activity and no increase of phosphorylation of other ROCK-targets, e.g. LIMK and cofilin. An increase of the inhibitory profilin2a/ROCK complex with regard to actin polymerization does not necessarily imply an increase in general ROCK activity if a competitive mechanism exists: After SMN loss, more profilin2a becomes recruited and phosphorylated by ROCK, whereas other ROCK targets are hypophosphorylated. Loss of SMN may cause a dysbalance of the system, but not induce a total change. However, addition of an inhibitor changes the situation as described above and drives the system back towards hypo-phosphorylation of profilin2a.

Cofilin and MLCP also contribute to the regulation of neurite outgrowth (45-47). Cofilin counteracts actin polymerization by binding to F-actin and promoting actin severing as well as depolymerization of the filaments. This mechanism ensures dynamics of the actin cytoskeleton at the tip of the growth cone. Cofilin phosphorylation is mediated by LIMK,
which is a direct target of ROCK (Fig. 8). LIMK phosphorylation inactivates cofilin, whereas slingshot dephosphorylates (48). The proper balance of phosphorylation and dephosphorylation is required to regulate cofilin activity during neurite extension (46, 48). An enhanced severing activity of cofilin in SMA increases the number of free barbed actin ends as starting points for new polymerization and could therefore explain the observed shift of the F-/G-ratio towards more F-actin. Phosphorylation of MCLP increases the amount of activated myosin II, which promotes the retrograde flow of F-actin at the tip of the growth cone (49, 50).

In our SMA cell culture model, MLCP is hypophosphorylated which in turn may probably inhibit myosin II and thus actin dynamics. Additionally, we performed a ROCK activity assay in SMN depleted cell lysates and controls. Here, the ROCK activity was measured by phosphorylation of a peptide consisting of the myosin light chain phosphatase subunit (MBS). Surprisingly, phosphorylation of MBS was decreased in lysates of SMN overexpressing cells, whereas no changes were detected in lysates of SMN depleted cells when compared to controls. One explanation for this could be that phosphorylation was measured using an antibody specific for phosphorylation at residue 695 which is referred to regulate catalytic activity of MLCP (51). In contrast, the antibody we used for MLCP phosphorylation detected the phosphorylation at residue 850 which is referred to be important for MLCP-myosin-binding and predominantly phosphorylated by ROCK (39, 51).

The ROCK pathway is a signalling hub that integrates growth factor signals and relays them to a number of downstream targets, which are involved in different actin-and myosin-dependent motility processes. Striking evidence for the important role of actin cytoskeleton regulation in SMA came from a study identifying the actin binding protein plastin 3 as a protective genetic modifier for SMA (52). Interestingly, plastin 3 is functionally linked to the profilin2a/ROCK system in a SMN-dependent manner, since knock-out of profilin2a increases plastin 3 expression (30). As we showed here, interaction of SMN with profilin2a
directly links SMN to the ROCK pathway. We demonstrated in a functional in vitro assay under SMN knock-down conditions that neurite outgrowth can be restored by RhoA and ROCK inhibition.

These results indicate that the ROCK pathway causes actin cytoskeletal perturbation in SMA. Interestingly, actin regulation is important for synaptic vesicle cycling (53) and could be linked to properties observed in SMA like impaired neurotransmission (20, 54). The ROCK pathway is a potential target for pharmacological intervention in SMA. Importantly, a recent in vivo study clearly showed that inhibition of ROCK by Y27632 in an intermediate SMA mouse model dramatically improved lifespan and improved NMJ maturation defects (26). These data demonstrate a therapeutic potential of ROCK inhibition in SMA. These observations are consistent with our in vitro results and point towards the importance of a detailed understanding of SMN-dependent ROCK pathway regulation and actin regulation.
MATERIAL AND METHODS

Mouse strains

Mice of the strain FVB.Cg-Tg(SMN2)2Hung Smn1tm1Hung/J (36) were obtained from the Jackson Laboratory (stock number 005058). Mice developing severe SMA symptoms were obtained following established breeding strategies (37, 55). The mean survival of SMA mice was 10 days. Each litter contained 50% SMA mice (Smn−/−; SMN2tg/+ ) and 50% control carriers (Smn+/−; SMN2tg/+). Genotyping was performed by polymerase chain reaction (PCR) of DNA from tail biopsies. Mice were decapitated, spinal cords dissected and immediately frozen in liquid nitrogen. Spinal cords from SMA mice FVB.Cg-Tg(SMN2)89Ahmb Smn1tm1Msd/J (56) were used for isolation of primary lumbar E14 motoneurons as described previously (57).

Plasmid constructs

Molecular cloning of full-length human pSMN1–294-EGFP was described before (22). pProfilin2a-IRES2-EGFP plasmid was a generous gift from Dr. A. Lambrechts and Dr. C. Ampe, Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Belgium (58). pcDNA3-MCS-Ubc9 and pEGFP-SUMO1 were constructed as described (59, 60). Profilin2a was amplified by PCR by appropriate primers introducing EcoRI/XhoI sites and cloned into pcDNA3-MCS-Ubc9 allowing in-frame fusion with Ubc9. Mutations were generated using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) following the manufacture’s protocol. All constructs were verified by sequencing.
Cell culture and transfection

NSC34, PC12 and HEK293T cells were incubated at 37°C in a 5% CO₂ humidified atmosphere. PC12 cell cultures were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS), 10% (v/v) horse serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 6 mM l-glutamine on poly-l-lysine. PC12 cells for differentiation were plated on a collagen I coated surface. 24 h after transfection, PC12 cells were cultured for 72±2h in PC12-medium without FCS but with 1% (v/v) horse serum and 100 ng/ml NGF to induce neuronal differentiation. NSC34 cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 1 mM glutamine and 100 U/ml penicillin and 0.1 mg/ml streptomycin. For experiments with ROCK inhibition, 10 µM Hydroxyfasudil (Calbiochem) or 100 nM C3 was used. For immunocytochemistry, cells were cultured on a poly-l-lysine coated surface. After transfection cells were cultured in NSC34 medium containing 1% FCS. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin/ 0.1mg/ml streptomycin, 1 mM sodium pyruvate, and 6 mM l-glutamine. Transfections were performed by liposomal transfection with Metafectene Pro (NSC34) or Metafectene (PC12, HEK293T) according to the manufacturer's recommendations (Biontex). Liposomal transfection of NSC34 resulted in at least 60 % transfected cells (n=334 cells). For biochemical analysis, PC12 cells were transfected by nucleofection using the Cell Line Nucleofector Kit (Lonza) followed by geneticin selection/NGF-differentiation for three days. This combination of efficient transfection and selection resulted in 49.4 ± 0.2 % (n=2 experiments, n=660 cells) transfected cells. Primary human fibroblasts were obtained from skin biopsies of SMA patients and unaffected controls and genetically characterized by Dr. B. Wirth, University of Cologne, Germany. Use of cells was approved by the ethical committee of the Hannover Medical School, Germany.
ML35 cells were derived from an unaffected control, ML63 and ML66 cells were from heterozygote carriers. These primary cells were used as controls. ML17 and ML39 derived from SMA type I patients harbouring homozygote deletions of the SMN1 gene. ML17 and ML39 contain two and ML16 three SMN2 gene copies (61). Cells were cultured in HEK293T medium and incubated at 37°C in 8.5% CO2 humidified atmosphere.

**Immunocytochemistry and fluorescence microscopy**

Cells were fixed and processed as described before (62) and mounted in Prolong Gold (Molecular Probes). Neurite length was measured as previously described (22) with an Olympus IX70 microscope after 3 days of neuronal differentiation. Briefly, the length of the longest neurite of a neuron was tracked from the centre of the cell along the neurite to its end. The following protocol was used: (1) minimum length of neurite of 40 µm, (2) only the longest neurite of a cell was measured, (3) this neurite had to be longer than the size of the pericaryon, (4) measurement of at least 50 cells/experiment and at least three replicates of the experiments per condition, (5) measurement by drawing a line from the centre of the pericaryon along the neurite to the end of the neurite (to the end of growth cone). The software analySIS 3.0 (Olympus) was used for these measurements. Images of nuclei and axons were taken with a Leica TCS SP2 confocal microscope equipped with Leica acquisition software at room temperature using oil immersion objectives HCX PL APO CS40 (40x/NA 1.25) and HCX PL APO BL (63x/NA 1.4). Images were assembled with Adobe Photoshop CS2. Images from growth cones were taken with an Olympus BX60 microscope with an UPLAN 100 (100x/NA 1.3) objective equipped with a cooled CCD camera and analyzed with the program analySIS 3.0 (Olympus).
**FRET measurements**

Single cell confocal images were acquired using a custom tailored spinning disk setup (CSU-X1 in combination with a iXon EMCCD DU-897, Andor Technology) placed on an upright Olympus Microscope BX61. Samples where excited alternatingly with 445 nm and 514 nm diode laser lines, the emission is split to the wavelength bands 483/32 nm and 542/27 nm using an OptoSplit II (Cairn Research Ltd, UK). FRET analysis were performed by self-written Matlab scripts following the Lux-FRET algorithms of Wlodarczyk et. al (2008).

**Protein interaction assays and F-/G-actin assays**

For *trans*-SUMOylation assays (33), 5 – 10 x 10⁴ NSC34 cells were seeded in 12 well dishes and transfected after 12-16h with the respective constructs (300ng of the putative interaction partners; 100ng pEGFP-SUMO or unfused pSUMO). 24h after transfection, cells were directly treated with 150 µl of Laemmli buffer (80 mM Tris (pH 6.8), 2% SDS, 5% β-ME, and 0.01% bromophenol blue) and incubated for 5 min at 95 °C. 20µl of the lysates were loaded on a 10% SDS gel and subsequently analyzed by western blot. Centrifugation assays for the determination of F-/G-ratios in primary fibroblasts were performed as described before (22). For quantification of F-/G-actin ratios in growth cones of motoneurons, cells were fixed with 4% PFA and stained with Phalloidin-Alexa546 and DNsaseI-Alexa488 (Invitrogen) and mounted with Prolong Gold (Invitrogen) for image processing.
Antibodies

Antibodies against the following proteins were used: mouse anti-SMN monoclonal (Becton Dickinson GmbH), mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) monoclonal antibody (Chemicon), rabbit anti-Coilin 204/10 polyclonal antibody (generous gift from Angus Lamond, University of Dundee, Dundee, Great Britain), anti-MYPT1 antibody (generous gift from Dr. M. Nakayama and Dr. K. Kaibuchi, Nagoya University, Japan), rabbit polyclonal anti-phospho MYPT1 (T850), chicken anti-profilin2a polyclonal antibody (Abcam) rabbit anti-UBC9 polyclonal antibody (Santa Cruz Biotechnology). For western blot analysis, the following secondary horseradish peroxidase-conjugated antibodies were used: sheep anti-mouse (Amersham), rabbit anti-chicken (Sigma) and goat anti rabbit (Jackson). For immunocytochemistry, a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 and goat anti-rabbit antibody conjugated to Alexa Fluor 555 (Molecular Probes) was applied. For pull-down assays, the following antibodies were used: anti-RhoA (clone 26C4; Santa Cruz), anti-Cdc42 (clone 44, BD Transduction Laboratories) and anti-Rac1 (clone 102, BD Transduction Laboratories).

2D gel electrophoresis

Spinal cords from SMA mice and controls at postnatal day 9 were homogenized in RIPA buffer and dialyzed in a Slide-A-Lyzer MINI Dialysis Unit Kit (7K MWCO, Pierce) for 24 ± 2 h. The protein concentrations were measured by BCA assay (Pierce) and equal amounts of protein (150-250µg) were analyzed by isoelectric focusing using the ZOOM®-IPGRunner system (pH range: 4-7; Invitrogen) according to the manufacture’s protocol. The isoelectric focusing was carried out discontinuously by increasing the voltage from 200 to 2000 V (200 V: 20 min.; 450 V: 15 min.; 750 V: 15 min.; 2000 V: 105 min.) for 2 hours with a constant
power of 1 W and a current of 1 mA. After 15 min incubation in 2x SDS buffer (125 mM Tris-HCl pH 6.8; 140 mM SDS; 20 mM DTT; 0.03 mM bromophenol-blue, 20 % (v/v) glycerol), the IEF strips were placed on the top of the SDS gel and analyzed by SDS/western blotting procedure. For direct western analyses, cells were lysed with RIPA buffer (137 mM NaCl; 50 mM NaF; 20 mM Tris-HCl pH 7.5; 25 mM β-glycerophosphate; 2 mM EDTA; 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride (PMSF); 2 % (v/v) protease-inhibitor Cocktail (EDTA-free, Roche); 1 % (v/v) Triton-X-100, 1 % (w/v) Sodium Deoxycholate; 1 % (v/v) phosphatase inhibitor cocktail 1, Roche), the protein concentrations were measured by a BCA assay (Pierce) and equal amounts of protein (10-30µg) were analyzed by SDS-PAGE/western blot as described before (63).

**ROCK activity assay**

Three days after transfection of PC12 cells with pEGFP, pSMN-EGFP, pSupp.control or pSupp.SMN, respectively, and one day after differentiation with NGF, cells were lysed with RIPA buffer. The protein content of the cell lysate samples was determined using the bicinchoninic acid assay (Pierce). The ROCK activity assay was performed according to manufacturer’s instructions (CycLex, Nagano, Japan). Briefly, equal amounts of protein (50 ng) were applied to the assay plate wells and incubated with ATP-containing kinase reaction buffer at 30°C for 30 min. After washing, incubation with the HRP conjugated detection antibody was performed at room temperature for 60 min. After another washing step, the tetramethylbenzidine containing substrate reagent was added and incubated at room temperature for 10 min until the reaction was stopped with 0.25 M sulphuric acid. Absorbance was measured in a Tecan spectrophotometric plate reader (Tecan, Crailsheim, Germany) at 450 nm single wavelength.
Pull-down assay

Pull down experiments were performed as described before (64). PC12 cells were grown in culture flasks (150 cm²) and SMN knockdown was performed as described above. For pull-down analyses, cells were rinsed twice with ice cold PBS and harvested in 1 ml of ice cold fish buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 1 % [w/v] Nonidet P-40, 0.25 % [w/v] Triton X-100, 5 mM dithiothreitol, 100 µM PMSF). Lysates were gently shaked at 4°C for 5 min followed by centrifugation at 14,000 rpm for 10 min. The supernatant was used for pull-down experiments. Therefore, 20 µl of beads slurry of GST-Rhotekin (C21) or GST-Pak CRIB-domain (constructs were a generous gift from John Collard, Amsterdam, The Netherlands) bearing approximately 20 µg protein each were added to each sample and rotated at 4°C for 30 min. The beads were collected by centrifugation at 10,000 rpm and washed twice with lysis buffer and subjected for SDS-PAGE and immunoblot.
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REFERENCES


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LEGENDS

Figure 1. F-/G-actin ratio in growth cones of motoneurons is increased in SMA mice (A)
Primary embryonic day 14 (E14) motoneurons of SMA mice and wild-type controls were
cultured for 5 days. After fixation with 4% PFA, growth cones were stained with Phalloidin-
Alexa546 (detection of F-actin) and DNaseI-Alexa488 for G-actin. Representative images
showing growth cones of control and SMA motoneurons, respectively. (B) Fluorescence
signals were quantified and the F-/G-actin ratios calculated (Mann-Whitney-Test, n=33 SMA
from 3 mice and n=30 control growth cones from 3 mice, ** p<0.01).

Figure 2. SMN-profilin2a interaction detected by trans-SUMOylation (TRS) system.
SMN-EGFP was coexpressed with or without EGFP-SUMO1 (S1), Ubc9 and Ubc9-
profilin2a, respectively, in NSC34 cells as indicated (lanes 2-6). After 24 hours, cells were
lysed in SDS buffer and analyzed by SDS-PAGE/western blot. (A) SMN-EGFP and
SUMOylated SMN-EGFP (S1-SMN-EGFP) were detected in the lysates by using anti-SMN
antibody. Expression of EGFP-SUMO1 was detected with anti-GFP antibody, Ubc9-
profilin2a with anti-profilin2a and Ubc9 with anti-Ubc9 antibody. S1-SMN-EGFP reflected
the profilin2a-SMN interaction in lane 5 (bandshift indicated by arrow). (B) SMN-EGFP
expression, separated in size from EGFP-SUMO, was verified by using anti-GFP antibody.

Figure 3. Identification of the profilin2a-binding site in SMN. (A) SMN mutants P196-
198A (P1), P219-224A (P2), P245-247A (P3) were generated by site directed mutagenesis
and fused to EGFP. (A) HEK293 and NSC34 cells were transfected with pSMN-EGFP (wild-
type, WT) and the EGFP-fused mutants P1, P2 and P3, respectively. 24h after transfection,
cells were fixed with 4% PFA and nuclei were counterstained with DAPI. Representative images of transfected cells were taken by confocal laser microscopy. EGFP-fused P1, P2, P3 as well as wild-type SMN were detected by EGFP-signals in nuclear foci (HEK293) and in granules at growth cones (NSC34). (B) SMN-EGFP, P1, P2 and P3, respectively, were coexpressed with EGFP-SUMO1 (S1) and Ubc9-profilin2a in NSC34 cells. 24h after transfection, cells were lysed in SDS buffer and analyzed by SDS-PAGE/western blot with anti-SMN antibody. Binding of P1 and P3 to SMN was not changed in comparison to WT. However, P2 displayed a strong decrease of profilin2a binding reflected by a reduction of SMN SUMOylation (indicated by arrow). Shifted bands representing SUMOylated SMN and SMN-EGFP signals were densitometrically analyzed and relative trans-SUMOylation calculated. Only mutant P219-224A showed a significant decrease of profilin2a-binding (t-test; means with standard error of the means; *, p<0.05; n=4 experiments).

Figure 4. SMN missense mutation S230L – a mutation causing SMA – disrupts SMN-profilin2a interaction. SMA causing mutants W92S, E134K, S230L, Y272C and T274I were generated by site directed mutagenesis and fused to EGFP. (A) SMN-EGFP and the EGFP-fused mutants, respectively, were coexpressed with pEGFP-SUMO1 (S1) and pUbc9-profilin2a in NSC34 cells. 24 hours after transfection, cells were lysed in SDS buffer and analyzed by SDS-PAGE/western blot with anti-SMN antibody. W92S, E134K, Y272C and T274I showed no changes in SMN-profilin2a interaction whereas S230L led to a decrease of the interaction reflected by a decrease in SMN SUMOylation (indicated by arrow). (B) NSC34 cells were transfected with plasmids coding for SMN-CFP or the SMN mutant S230L, respectively, and profilin2a-YFP. Förster Resonance Energy Transfer (FRET) was measured in vivo and maximum projections were shown (columns one to three). Apparent FRET efficiency EfD was calculated at a pixel based manner (column four). The lower two lanes
represent details from axons. (C) Quantitative analyses of apparent FRET efficiencies (EFD) in total cells and within regions of interest in neurites of SMN- and S230L-transfected cells. Average EF_D of cells expressing the mutant was set to zero and the difference to wild-type SMN evaluated (Mann-Whitney test; ***, p<0.001; **, p<0.01; n=8 cells for each condition). Cells as well as neurites show significant differences of EF_D between SMN and S230L with regard to interaction with profilin2a.

**Figure 5. Knock-down of SMN causes phosphorylation changes of ROCK-downstream targets cofilin and myosin light chain phosphatase.** (A) Knock-down of SMN in PC12 cells was performed by electroporation with plasmids coding for shRNAs pSupp.control and pSupp.SMN, respectively. Cells were cultured on collagen I coated dishes. 24 h after transfection, cells were differentiated in a medium containing 100 ng/ml nerve growth factor (NGF, differentiation medium), for 72 h. SMN protein level was determined by SDS-PAGE/western blot in cell lysates using anti-SMN antibody and anti-GAPDH antibody as loading control. Expression levels of ROCK downstream targets were analyzed by using the corresponding antibodies. (B) Blots were densitometrically analyzed using GAPDH as loading control. No significant changes of cofilin, MLCP, ROCK, profilin2a and LIMK1/2 expression were detected in SMN knock-down cells compared to controls. However, phosphorylation of cofilin and MLCP was significantly decreased under knock-down conditions (Mann-Whitney-Test, n≥3 independent experiments, * p<0.05; n.s.: non significant).
Figure 6. Decreased SMN protein level leads to hyperphosphorylation of profilin2a in a SMA cell culture and a severe SMA mice model. (A) Phosphorylation of profilin2a was analyzed by 2D gel electrophoresis since a phospho-specific antibody was not available. Isoelectric focusing (IEF) in the range of pH 4.0 – 7.0 was performed followed by a SDS-PAGE/western blot using anti-profilin2a antibody. 1: non phosphorylated, 2-4: phosphorylated forms of profilin2a. (B) Blots were densitometrically analyzed and distributions of the different forms calculated. Profilin2a was significantly hyperphosphorylated under knock-down conditions (Chi-square test; * p<0.05). Data points represent means with standard errors of the mean (SEM). (C) Spinal cords (n≥3) were dissected from SMA mice and heterozygote controls at postnatal day 9. IEF was performed as above. (D) Blots (n=3) were analyzed as above (Chi-square test; n=3 independent experiments, **, p<0.01). Although the pattern of phosphorylation is similar to the in vitro data, phospho-form 2 cannot be differentiated from the main form 1 in vivo.

Figure 7. Neurite outgrowth regulation by the ROCK pathway and SMN. (A) PC12 cells were transfected with pProfilin2a-IRES-EGFP wild-type, the phosphorylation-resistant mutant S137A and the phospho-mimetic mutant S137D, respectively. Measurement of neurite length was carried out after 72 h of differentiation. S137D expressing cells demonstrated decreased neurite length in comparison to S137A (Mann-Whitney test, n≥75 neurites/condition in 3 independent experiments, *** p<0.001). (B) For analysis of the impact of profilin2a and SMN expression on neurite outgrowth, PC12 cells were transfected with pProfilin2a-EGFP and/or with pSMN-FLAG coding for full-length SMN in combination with the respective control plasmids. Expression of profilin2a shows inhibition of neurite outgrowth, whereas SMN increased the length of neurites. However, co-expression of both profilin2a and SMN did not significantly change neurite length indicating that increased
concentration of SMN-bound profilin2a suppresses its inhibitory regulative activity on neurite outgrowth. (C) PC12 cells were transfected with the pSupp.control (shRNA control) and pSupp.SMN (shRNA SMN), respectively, and cultured in differentiation medium with vehicle or inhibitors. The neurite outgrowth defect observed under SMN knock-down conditions was rescued with C3 (RhoA-inhibitor) and hydroxyfasudil (ROCK-inhibitor), respectively. (D) PC12 cells were transfected with different plasmids as stated. Cells were cotransfected with pDsRed2 for visualisation of neurites. After 72 h of differentiation, neurite lengths were measured. As expected, SMN overexpression increased neurite length, SMN knock-down led to a decrease. However, expression of SMN mutants negatively regulated neurite outgrowth. Interestingly, the S230L mutant displayed the strongest effect (Mann-Whitney test, \( n \geq 119 \) neurites/condition in 3 independent experiments, ** \( p<0.01 \), *** \( p<0.001 \) in comparison to pEGFP). Data points represent means with standard error of the mean (SEM).

**Figure 8. Proposed model model for SMN’s functional interaction with the ROCK-pathway.** SMN binds to profilin2a, which becomes phosphorylated by ROCK (dashed lines). Loss of SMN by *Smn1* gene deletion results in a decrease of SMNprofilin2a complexes and stronger interaction of profilin2a with ROCK. As a consequence, other downstream molecules like Myosin Light Chain phosphatase (Myosin-LCPase) and coflin become hypophosphorylated. On a cellular level, the dysregulation of ROCK-downstream targets leads to an increase of the inhibitory activity of profilin2a with regard to neurite outgrowth regulation and putatively other motility processes.
ABBREVIATIONS

SMN, survival of motoneuron protein; ROCK, Rho-kinase; MLCP, myosin light chain phosphatase
Figure 1.
Figure 2.

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anti-SMN

S1-SMN-EGFP

SMN-EGFP

anti-EGFP

EGFP-SUMO1

anti-Ubc9

Ubc9-profilin2a

Ubc9

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anti-EGFP

S1-SMN-EGFP

SMN-EGFP
Figure 3.
Figure 5.
Figure 6.

A

B

C

D

collection of distributions:

+ shRNA control
- shRNA SMN

+ anti-profilin2a

+ anti-profilin2a

control mice

SMA mice

kD

kD

non phosphoryl.
phosphoryl.
phosphoryl.
phosphoryl.

non phosphoryl.
phosphoryl.
phosphoryl.
phosphoryl.

comparison of distributions:

* control mice

** control mice

comparison of distributions:

* shRNA control

** shRNA SMN

comparison of distributions:

* control mice

** SMA mice
Figure 7.