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Post-translational modifications in the survival motor neuron protein

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by a progressive loss of the spinal motoneurons. The SMA-determining gene has been termed survival motor neuron (SMN) and is deleted or mutated in over 98% of patients. The encoded gene product is a protein expressed as different isoforms. In particular, we showed that the rat SMN cDNA produces two isoforms with M_r of 32 and 35 kDa, both localized in nuclear coiled bodies, but the 32 kDa form is also cytoplasmic, whereas the 35 kDa form is also microsomal. To determine the molecular relationship between these two isoforms and potential post-translational modifications, we performed transfection experiments with a double-tagged rat SMN. Immunoblot and immunostaining studies demonstrated that the 32 kDa SMN isoform derives from the full length 35 kDa, through a proteolytic cleavage at the C-terminal. Furthermore, the 35 kDa SMN isoform is physiologically phosphorylated in vivo. This may modulate its interaction with molecular partners, either proteins or nucleic acids.

Keywords: Spinal muscular atrophy; Survival motor neuron; Isoforms; Post-translational modifications

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by the degeneration of spinal cord motoneurons and weakness of limb and trunk muscles.

The primary cause of SMA derives from deletions or deleterious mutations in the survival motor neuron (SMN) gene located in a duplicated region of chromosome 5q13 [1]. Although the SMN gene is present in almost identical centromeric (*SMN2*) and telomeric (*SMN1*) copies, only homozygous (*SMN1*) deletions cause an SMA phenotype.

SMA ranges from a very severe type (Werdnig–Hoffmann disease, onset at birth with a poor prognosis and death usually within the first year) to the intermediate type (children can sit but never walk) and a milder juvenile type (Kugelberg–Welander disease) in which patients have muscular weakness and atrophy but eventually walk [2]. As all three SMA types present the same *SMN1* gene deletion, it has been hypothesized that the different phenotypes may be explained in part by a mechanism of gene conversion from *SMN1* to *SMN2* [3].

SMN1 mRNA is ubiquitously expressed and encodes a protein of still unknown role in motoneuron degeneration [1]. A wealth of studies have been performed in the last few years trying to unveil SMN function(s) in the cell: the protein is involved in the assembly of spliceosomal small nuclear RNPs and in pre-mRNA splicing [4,5]. Furthermore, yeast two-hybrid screening and biochemical interaction studies have shown that SMN interacts with several proteins (e.g., fibrillarin, coilin, gemin2-7, profilins, viral proteins, and FUSE binding protein) and itself [4,6–10].

The expression and distribution of SMN have been studied in cells and tissues. The protein is present in different sub-cellular compartments: in the nucleus, both in novel dot-like structures termed *gemini* of coiled bodies

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(GEMS) [11] and in coiled bodies [8,12], and in the nucleolus [13]. Interestingly, the presence of SMN in the nucleolus has raised the possibility that the protein may be involved in rRNA maturation/ribosome synthesis [13].

SMN is also significantly expressed in the cytoplasm: subcellular fractionation studies which have demonstrated its presence in the cytosol as a soluble protein [8] and bound to microsomal membranes in insoluble complexes [8,14]. Moreover, recent evidence also indicates that SMN may be implicated in the dendritic transport and in RNA processing in motor axons [14,15].

This complexity in SMN cellular distribution is paralleled by the existence of different molecular isoforms, which have each a specific subcellular localization [8,16–19]. In particular, we have recently shown that two isoforms of SMN with M_r 32 and 35 kDa are produced by the same cDNA, and are expressed in primate and rodent cells [8]. Further, the autoradiographic analysis of the in vitro-synthesized rat SMN showed that the 35 kDa isoform corresponds indeed to the full length protein.

The 32 kDa isoform is enriched in the cytosol, whereas the 35 kDa isoform segregates in the microsomal fraction. Both forms co-localize in coiled (Cajal) bodies in cultured motoneurons but not in GEMS. Interestingly, this newly discovered nuclear organelle appears to be abundant in a subtype of HeLa cells and is infrequently seen in other cell types [8,20].

Recently, it has been suggested that a shorter form of SMN, engineered to be truncated at the C-terminal, or the full length protein with a point mutation (SMN Y272C) shows a pro-apoptotic activity, whereas the full length form might instead play an anti-apoptotic role [21,22].

In the present work, we studied the post-translational modifications of SMN in cultured cell lines and embryonic rat spinal motoneurons. Transient transfection of COS-7 cells with a double-tagged SMN led to the demonstration that the 32 kDa SMN isoform is cleaved at the C-terminal. Furthermore, we show that full length SMN is phosphorylated in vivo. These results confirm that SMN undergoes post-translational changes that might relate to specific functions of this protein in motoneurons, the cells selectively affected in SMA.

Materials and methods

Antibodies. A polyclonal rabbit anti-rat SMN (pαSMN) was raised against a synthetic peptide corresponding to the first 15 aminoacids of the N-terminal region of the rat protein coupled to ovalbumin. The antibody was purified and characterized as described elsewhere [18]. A monoclonal mouse anti-human SMN (mαSMN) was purchased from Transduction Laboratories (Lexington, KY, USA). Other antibodies used in this study were monoclonal mouse anti-c-Myc (9E10, Santa Cruz Biotech, Santa Cruz, CA, USA) and anti-FLAG (Stratagene, La Jolla, CA, USA).

Cell cultures. COS-7 cells and 3T3 mouse fibroblasts were seeded on glass coverslips, 100 mm plastic dishes, or 75 cm^2 Falcon flasks and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Embryonic rat spinal motoneurons were purified in a double-step procedure of a density gradient (metrizamide) centrifugation followed by immunopurification by indirect magnetic cell sorting [23]. Purified motoneurons were plated either in 35 mm dishes or glass coverslips. Culture medium was Neurobasal with 2% B27 supplement (Invitrogen SARL, Cergy Pontoise, France), 2% horse serum, L-glutamine (0.5 mM), L-glutamate (25 μM), and 2-mercaptoethanol (25 μM). Recombinant rat GDNF (1 ng/ml) and rat CNTF (10 ng/ml) (Sigma, St. Louis, MO, USA) were added at the time of seeding.

Plasmid construction and transient transfection of COS-7 cells. Rat *SMN* cDNA was subcloned in pCMV-Tag1 vector (Stratagene, La Jolla, CA, USA), an epitope tagging vector designed for gene expression in mammalian cells. The cytomegalovirus (CMV) promoter allows high levels of expression of the cloned DNA in several mammalian cell lines.

SMN cDNA was inserted in the MCS region of the pCMV-Tag1 vector between FLAG and c-MYC sequences to give a SMN fusion protein with FLAG epitope at the N-terminal and c-MYC epitope at the C-terminal (FLAG-SMN-c-MYC, Fig. 1A). The primers used were: *bg1-rsmn* 5′ TAAGATCTGTATGGCGATGGGCAGCGGC and *Xho-rsmn* 3′ TGCTCGAGATTTGTATGTGAGCACTTCTTTC.

The sequence of this construct was checked. We also verified the protein expression by an in vitro assay consisting of ^{35}S-labelled methionine/cysteine and the TNT-coupled transcription/translation rabbit reticulocyte lysate system (Promega, Madison, WI, USA).

Transient transfection was performed by incubating COS-7 cells at 70–80% confluence with 8–10 μg of double-tagged SMN-expressing vector (pCMV-Tag1-SMN construct, termed in Fig. 1B as pT-S) or control plasmid (pCMV-Tag1, termed in Fig. 1B as pT) in DOTAP liposomal transfection reagent (Boehringer–Mannheim, Indianapolis, IN, USA) for 10 h, and then allowed to grow until complete confluence.

Protein extracts. Cultured cells were washed with PBS, collected, and homogenized in TET buffer [8]. Homogenates were then incubated at 4 °C for 1 h and then centrifuged at 800*g* for 10 min at 4 °C. The supernatant (crude extract) was stored at −80 °C until further use.

When motoneurons were used, given their low number per dish, the homogenates were concentrated by precipitation in 2.5 vol methanol at −20 °C and then resolubilized in buffer for further analysis.

Alkaline phosphatase assay. For analysis of the phosphorylation state of the SMN isoforms, motoneuron and 3T3 fibroblast extracts were treated for 30 min at 37 °C with 500 U/ml calf intestine alkaline phosphatase (Invitrogen SARL, Cergy Pontoise, France). Incubation was stopped by adding SDS–PAGE sample buffer. Samples were then electrophoresed, blotted, and probed with appropriate primary and secondary antibodies (see below).

SDS–PAGE and Western blotting. Protein extracts were thawn on ice, mixed with SDS–PAGE sample buffer, and boiled for 5 min. Low-range unstained MW markers were used in all experiments, and 12% SDS–PAGE electrophoresis and Western blotting were performed as described [18].

Primary antibodies were used at the following final dilutions: pαSMN, 1:5–1:10; mαSMN, 1:3000–1:5000; monoclonal anti-FLAG, 1:2000-1:4000; monoclonal anti-c-MYC, 1:1000-1:2000. Secondary anti-rabbit or anti-mouse HRP-conjugated antibodies were used at 1:7000 dilution. Detection of the specific bands was performed using BM chemioluminescence blotting substrate (Boehringer–Mannheim, Indianapolis, IN, USA).

Immunocytochemistry and confocal image analysis. All cells used for immunofluorescence experiments were cultured on glass coverslips [8].

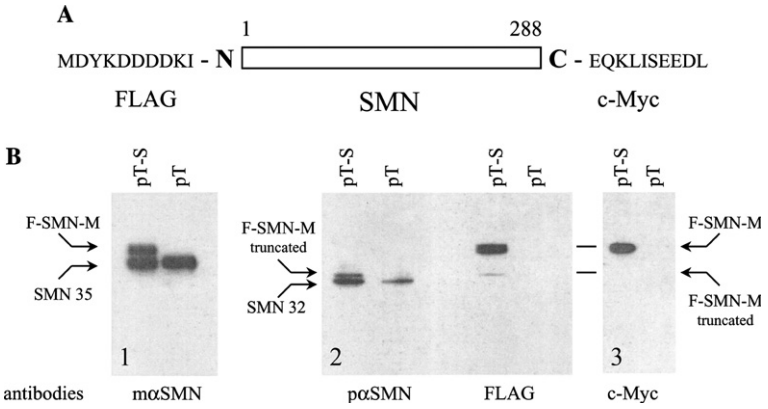

Fig. 1. (A) Schematic representation of the recombinant rat SMN fused to FLAG peptide (at the N-terminal) and c-Myc peptide (at the C-terminal). The fused protein was termed FLAG-SMN-c-Myc. (B) Immunoblots of extracts of COS-7 cells transfected with either a plasmid containing a double-tagged rat *smn* cDNA (pT-S) or the plasmid alone (pT). (Panel 1) Filter probed with mαSMN. The extract from FLAG-SMN-c-Myc (F-SMN-M) over-expressing cells shows two bands (first lane), the upper one corresponding to the double-tagged SMN. (Panel 2) Filter probed with pαSMN (left) and FLAG Ab (right). pαSMN recognizes two closed bands, the upper one corresponding to the double-tagged SMN (first lane). FLAG Ab stains two bands (third lane) with identical electrophoretic mobility as the two upper bands stained by mαSMN and pαSMN, respectively. (Panel 3) Filter probed with c-Myc Ab. This Ab stains only one band which corresponds to the upper bands recognized by mαSMN and FLAG Abs, respectively.

For double immunofluorescence staining, primary antibodies [anti-SMN (pαSMN and mαSMN), anti-FLAG, and anti-c-MYC] were incubated overnight at 4 °C, and secondary antibodies (donkey anti-rabbit IgG coupled to Cy3 and donkey anti-mouse IgG coupled to FITC (Jackson Immunochemicals, West Grove, PA, USA) were incubated for 45 min at RT.

Laser confocal microscopy was performed with a Zeiss LSM 400 confocal microscope, with argon ion lasers set at 548 nm for Cy3 excitation and 488 nm for FITC excitation. Digitized series of optical axial sections were recorded separately from each channel, images for each staining reconstructed, and then files merged.

Results

The 32 kDa SMN isoform is truncated at the C-terminal

We recently demonstrated that the two major SMN isoforms (i.e., 32 and 35 kDa SMN) originate from the same cDNA and that are specifically recognized by different antibodies [8]. As the full length SMN corresponds to the 35 kDa isoform, the shorter 32 kDa protein should derive from a post-translational modification of the full length protein.

To better define the molecular relationship between the two SMN isoforms, we transiently transfected COS7 cells with a plasmid in which the rat *SMN* cDNA has been inserted between two tags (FLAG at the N-terminal and c-Myc at the C-terminal, Fig. 1A). These tags allowed the expression of the transfected SMN protein to be followed using specific anti-FLAG and anti-c-Myc antibodies. We used also pαSMN and mαSMN antibodies to detect both endogenous and the over-expressed double-tagged SMN.

Fig. 1B shows the immunoblot pattern of expression of the endogenous SMN and the double-tagged protein (FLAG-SMN-c-Myc). Interestingly, we found that over-expression of the double-tagged SMN gave in addition to the endogenous isoforms either a band close to the 35 kDa isoform, and recognized by the mαSMN (Fig. 1B, first panel), or a band close to the 32 kDa isoform, and recognized by pαSMN (Fig. 1B, second panel, left). Therefore, the transfected FLAG-SMN-c-Myc construct reproduced the same pattern of expression as the endogenous SMN, indicating that the double-tagged protein also underwent a post-translational modification.

When using the anti-FLAG antibody, the two new bands were revealed in the transfected cell extracts (Fig. 1B, second panel, right). Conversely, anti-c-Myc detected only one band with an electrophoretic mobility identical to that identified by mαSMN, i.e., close to 35 kDa (Fig. 1B, third panel). As c-Myc is attached to the C-terminal of the tagged SMN, these experiments demonstrate that the shorter isoform of the rat SMN is post-translationally cleaved at the C-terminal. The cleaved fragment with the c-Myc tag should be around 4 kDa, but we could never detect it in our Western blot experiments. It is possible that this fragment is degraded in the cell or, alternatively, that the monoclonal anti-c-Myc antibody does not recognize it in a standard Western blot.

The expression of the double-tagged SMN was also analyzed in transfected COS-7 cells by double immunofluorescence (double IF) and confocal image analysis. In these cells, the over-expression of FLAG-SMN-c-Myc formed large aggregates in the cytoplasm, mainly in the perinuclear zone. Double IF with pαSMN and monoclonal c-Myc showed that only the cytoplasmic patches were actually co-stained by both antibodies, whereas the fine granular staining of the cytoplasm and many SMN-containing dots were only detected by pαSMN (Figs. 2A, A′, and A″). To the contrary, a double IF using pαSMN and anti-FLAG antibodies

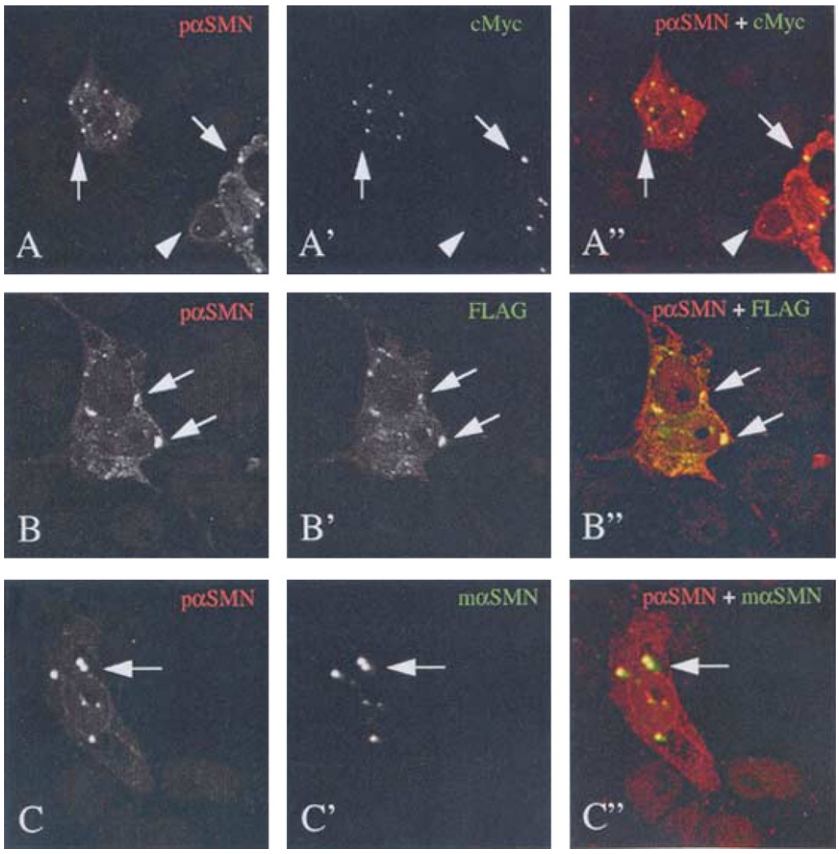

Fig. 2. Expression of endogenous SMN isoforms and the FLAG-SMN-c-Myc construct in COS-7 cells transiently transfected with pCMV-Tag1-SMN. Laser confocal microscopy images of double immunofluorescence experiments performed with pαSMN and c-Myc (A, A′, and A″); pαSMN and FLAG (B, B′, and B″); and pαSMN and mαSMN (C, C′, and C″). Fixed cells were co-incubated with primary antibodies followed by Cy3-donkey anti-rabbit IgG (detects pαSMN, red) and FITC-donkey anti-mouse IgG (detects c-Myc, FLAG, and mαSMN, green). The FLAG-SMN-c-Myc construct form large aggregates in the cytoplasm and in the perinuclear zone that are stained by all antibodies (arrows). Note that several SMN-containing dots stained by pαSMN are not detected by c-Myc Ab (A, A′, and A″, arrows heads).

showed that the same structures, both in the cytoplasm and in the nucleus, were recognized by the two antibodies (Figs. 2B, B′, and B″). Finally, double-staining with pαSMN and mαSMN showed that both antibodies stained the cytoplasmic aggregates and the coiled bodies, whereas only pαSMN stained the cytoplasm with the typical fine granular pattern (Figs. 2C, C′, and C″).

We conclude that the over-expressed double-tagged SMN is mainly present in the newly formed perinuclear aggregates, even though its distribution largely overlaps that of the endogenous SMN (Fig. 2B′). Moreover, we further confirm that the shorter SMN isoform is cleaved at the C-terminal, since c-Myc antibodies could not stain either the cytoplasm or several of the nuclear bodies (Fig. 2A′), structures which are strongly stained by pαSMN, identifying the 32 kDa isoform [8].

The 35 kDa SMN isoform is phosphorylated

A computer-assisted search suggested that rat SMN might undergo specific post-translational modifications, e.g., glycosylation and phosphorylation, which could theoretically contribute to the different localizations and functions of the two main isoforms.

To test whether the 32 and 35 kDa SMN would be phosphorylated, we incubated extracts from rat embryonic spinal motoneurons with alkaline phosphatase.

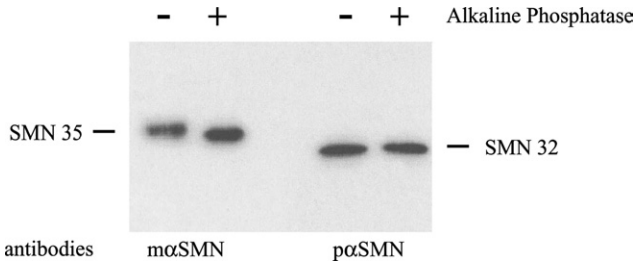

Fig. 3. Phosphorylation analysis of the SMN isoforms. Protein extracts were prepared from cultured rat motoneurons and immunoblots were performed with mαSMN (detects the 35 kDa isoform) and pαSMN (detects the 32 kDa isoform). Treatment with alkaline phosphatase induced a significant shift in the electrophoretic mobility of the 35 kDa SMN, indicating that this isoform is phosphorylated in vivo.

Immunoblot experiments showed that the 35 kDa isoform shifted significantly its electrophoretic mobility after dephosphorylation in vitro (Fig. 3). Conversely, the migration of the 32 kDa band was apparently unchanged. Similar results were obtained when extracts from 3T3 fibroblasts or COS7 cells were used (data not shown).

We conclude that the 35 kDa SMN isoform is normally phosphorylated in most cells; it remains possible that other post-translational changes of the two isoforms, such as glycosylation and lipid modifications, may occur in vivo.

Discussion

After demonstrating that rat SMN is expressed in the cell as two major isoforms with MW of 32 and 35 kDa, respectively [8], we show here that the 35 kDa SMN (i.e., the full length isoform) is post-translationally cleaved at the C-terminal, giving rise to the shorter 32 kDa isoform. Furthermore, the full length SMN is normally phosphorylated, an event that may drive its ability to interact with specific molecular partners, either proteins or nucleic acids.

Rat SMN mRNA does not undergo alternative splicing [18,24], thus in rodents, only one protein is expected. In human cells, SMN isoforms could theoretically be produced from alternatively spliced *SMN2* mRNA transcripts [25]. Interestingly, immunoblots of spinal cord extracts from both normal subjects and SMA-affected patients never revealed any significant SMN2-related band [26,27], indicating that the SMN2-derived proteins might be highly unstable and quickly degraded in the cell. However, as several studies have suggested the existence of multiple isoforms of SMN both in rodent and human tissues [8,16–19], these isoforms must be the result of post-translational modifications.

Our transfection experiments with a double-tagged recombinant rat SMN protein have clarified that indeed, the 32 kDa isoform is a C-terminal-truncated form of the 35 kDa isoform. The two isoforms have different localizations in the cell [8], the 32 kDa isoform being enriched in the cytosol, whereas the 35 kDa SMN enriched in the microsomal fraction, thus suggesting different functions. However, while multiple functions have been suggested for the full length SMN (e.g., spliceosome complex formation, pre-mRNA splicing, anti-apoptotic protein, etc.), no specific role has been identified for the shorter 32 kDa protein, which was also found in cell lines of human origin, such as HeLa cells, and, more recently, in human leukocytes [18,28]. In these cells of human origin, the 32 kDa isoform could have been related to a product of the *SMN2* gene. The alternative splicing of the *SMN2* mRNA theoretically produces three different SMN2 protein isoforms with a MW of 31 kDa (SMN—exon 7), 29 kDa (SMN—exon 5), and 27.5 kDa (SMN—exons 5 and 7). None of these isoforms could be detected in our immunoblot experiments, despite the fact that our SDS–PAGE method can resolve ~1 kDa difference between proteins [18]. Therefore, in human too, the 32 kDa isoform is probably a cleavage product.

Proteolytic cleavage of SMN in human and rodent has been shown to occur in cells undergoing apoptosis or after ischemic injury [21,22]. The expression of a shorter protein, truncated at the C-terminal and with a MW of about 29 kDa, increases when apoptosis is induced. To test whether the 32 kDa SMN could play the same role, we exposed human lymphoblastoid cells to an apoptosis inducer, daunomycin: no change in the 32 kDa isoform level was observed (V. La Bella, unpublished observations). Thus, the proteolytic cleavage at the C-terminal is the only similarity between the two isoforms, and in fact, the 32 kDa protein is physiologically expressed in normal cells, whereas the 29 kDa isoform appears only when apoptosis is induced.

The role of the 32 kDa SMN in the cell, if any, is still unknown. As this isoform is soluble in the cytosol and does not form complexes with other molecules [8], it could represent a by-product of the 35 kDa SMN. This hypothesis is indirectly supported by the evidence that only the full length SMN is critical for the cell survival. In SMA, in fact, the severity of the phenotype decreases with increased expression of full length SMN [26].

The biochemical analysis of some putative post-translational modifications of SMN indicated that the 35 kDa SMN undergoes significant phosphorylation in vivo. This finding is novel, and potentially interesting for the function of this isoform.

A computer-assisted search revealed that at least three different serine/threonine kinases, i.e., cyclic nucleotide-dependent protein kinase, protein kinase C, and casein kinase II, could potentially be involved in the phosphorylation of SMN. Phosphorylation of the 35 kDa isoform is apparently not cell-specific: we tested total extracts from 3T3 fibroblasts or embryonic rat spinal motoneurons and found a significant electrophoretic shift of the protein band when extracts were incubated with alkaline phosphatase.

Phosphorylation of proteins alters their charge leading to a change in their ability to interact with other molecules, e.g., nucleic acids, cyclic nucleotides, and proteins. This might be the case for SMN, known to interact both with proteins and nucleic acids, and phosphorylation could reinforce and stabilize these interactions. Future work will aim at identifying the specific phosphorylation sites of the 35 kDa SMN and at determining the importance of phosphorylation for the cellular function of SMN.

Conclusions

In the present work, we demonstrated that SMN, the protein implicated in SMA, undergoes a post-translational rearrangement. In physiological conditions, the protein is proteolytically truncated at the C-terminal, a putative intermediate step in its degradation pathway. SMN is also phosphorylated, and this may modulate its ability to interact with its molecular partners, either proteins or nucleic acids.

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