Mutation in the $Q^{28}SDD^{31}SD$ site, but not in the two SQ sites of the survival of motor neuron protein, affects its foci formation

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Abstract. The survival of motor neuron (SMN) protein forms a multiprotein complex (SMN complex) with Gemin proteins. The complex is known to play a crucial role in RNA metabolism. Several lines of evidence show that SMN is phosphorylated at serine and/or threonine residues. In this study, we hypothesized that SMN is phosphorylated at two kinds of serine residues, the $Q^{28}SDD^{31}SD$ site and two SQ sites (^{80}SQ and ^{163}SQ). A FLAG-tagged wild-type construct (SMNfull) and three FLAG-tagged mutant constructs were made: an SMNAQ mutant with two AQ sites instead of two SQ sites at residues 80 and 163, an SMNQADDAD mutant with QADDAD instead of $Q^{28}SDD^{31}SD$, and an SMNAQ/QADDAD mutant with the two AQ sites and QADDAD. We expressed these mutants in HeLa cells and analyzed their phosphorylated bands by immunoblotting, the protein stability using cycloheximide, binding to Gemin 2 and foci formation. Mutations in $Q^{28}SDD^{31}SD$, but not in two SQ sites reduced the intensity of phosphorylation bands, indicating that $Q^{28}SDD^{31}SD$ is the major phosphorylation site in SMN. Mutations in the two SQ sites and $Q^{28}SDD^{31}SD$ did not affect protein stability and binding to Gemin 2. Whereas mutations in the two SQ sites did not cause apparent changes in foci formation, mutations in $Q^{28}SDD^{31}SD$ resulted in a reduced number of large foci in the cytosol. We demonstrated that phosphorylation in $Q^{28}SDD^{31}SD$ may be important in cytosolic foci formation.

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Abbreviations: SMN, survival of motor neuron; SMA, spinal muscular atrophy; RNA, ribonucleic acid; RNP, ribonucleoprotein; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CT, acetoacetyl-CoA thiolases; DAPI, 4'-6-Diamidino-2-phenylindole; ANOVA, analysis of variance; PIKK, phosphatidylinositol 3-kinase-like kinase

Key words: survival motor neuron, spinal muscular atrophy, phosphorylation, foci, mutation

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by loss of α-motor neurons in the spinal cord and atrophy of muscles (1). The responsible genes of SMA are survival of motor neuron 1 (SMN1) and SMN2; the former produces full-length transcript (SMNfull), the latter generates transcripts lacking exon 7 and a small amount of SMNfull. A reduced level of SMNfull, which results from loss or mutation of SMN1, causes SMA, since SMNfull derived from SMN2 is not able to compensate for it (2,3).

SMN is a ubiquitous protein of 294 amino acids and constitutes a multiprotein complex (SMN complex) with Gemins 2-8. The SMN complex plays essential roles in RNA metabolism, such as biosynthesis or the dissolution of small nuclear (sn)RNA or RNP (4,5). Recent studies have revealed that the SMN complex is phosphorylated in cytoplasm, but it is hypophosphorylated in the nucleus and, in addition, phosphorylation might control the function and localization of the SMN complex (6,7). Although it has been shown that a unique phosphorylation site, $Q^{28}SDD^{31}SD$, is the major phosphorylation site in SMN (6), the mechanism of its phosphorylation is still unknown.

We made several mutant expression vectors to study the functional importance of possible phosphorylation sites, and analyzed the difference of protein stability, interaction and localization between wild-type and mutant SMN proteins.

Materials and methods

Plasmids. Total RNA was extracted from control human fibroblasts using Isogen kits (Nippon Gene, Tokyo, Japan) and first-strand cDNA synthesis was done with SMN-specific antisense primer SMN3 (5'-^{977}CCAGTTATCTTCTATAA CGC^{958}-3'). The human SMNfull was amplified using Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland) and primers as follows, SMN1 (sense, Hind III tagged) 5'-TCCG GAAGCTT^{1}ATGGCGATGAGCAGCGGCGGCAGTGGT-3' SMN2 (antisense, EcoRI tagged) 5'-CGTTTGAATTC^{922}TG

GTGTCATTTAGTGCTGCTCTATC^{898}-3'. PCR-amplified fragments were subcloned into pUC118 by Mighty Cloning Kit (Blunt End) (Takara Bio, Tokyo, Japan). After subcloning, we selected a clone of wild-type SMN (SMNfull) with no PCR errors by sequencing. We made three mutant cDNAs by *in vitro* mutagenesis. The SMNAQ mutant cDNA encoded two AQ sites instead of two SQ sites at 80 and 163. The SMNQADDAD mutant cDNA encoded QADDAD instead of $Q^{28}SDD^{31}SD$ and the SMNAQ/QADDAD mutant cDNA encoded the two AQ sites and QADDAD. After confirmation of no PCR errors, these cDNAs were subcloned into a pFLAG-CMV-6a expression vector (Sigma-Aldrich, St. Louis, MO) after EcoR I/Hind III digestion. They were referred to as pFLAG-SMNfull, pFLAG-SMNAQ, pFLAG-SMNQADDAD and pFLAG-SMNAQ/QADDAD, respectively. The plasmids were purified using Plasmid Purification (Qiagen, Hilden, Germany) for use in transfections.

Cell cultures. HeLa cells were cultured in Eagle's minimum essential medium containing 10% fetal calf serum.

Transient expression analysis. HeLa cells were transfected with 9.0 μg of pFLAG-SMNfull or pFLAG-SMNAQ or pFLAG-SMNQADDAD or pFLAG-SMNAQ/QADDAD, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 h of incubation, cells were washed with phosphate-buffered saline (pH 7.4) (PBS) and harvested.

Immunoblot analysis. Cells were freeze-thawed and homogenized with an extraction buffer (50 mM sodium phosphate (pH 8.0), 0.1% Triton X-100, 200 mM NaCl, protease inhibitor cocktail (Roche, Mannheim, Germany), then centrifuged at 10,000 x g for 10 min. The protein concentration was determined in the supernatant by the method of Lowry using bovine serum albumin (BSA) as the standard. Samples were boiled for 5 min with a 2X Laemmli sample buffer. Protein samples between 2.5-10 μg were separated on 10% XV Pantera Gel (DRC, Tokyo, Japan), and were then transferred onto nitrocellulose membranes using an iBlot gel transfer system (Invitrogen). Membranes were blocked and incubated with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) (dilution 1:10,000). Proteins were finally detected with a donkey anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution 1:4,000), and visualized with an ECL Western blotting analysis system (GE Healthcare, Little Chalfont, UK).

Alkaline phosphatase assay. For analysis of the phosphorylation state of the SMN protein, the transfected HeLa cells were homogenized with a TET buffer [10 mM Tris-HCL, pH 7.4, containing 1 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail (Roche)]. The extracts were treated at 37˚C with 500 U/ml calf intestine alkaline phosphatase (Takara Bio) for 0, 5, 10, and 30 min. Incubation was stopped by adding a 2X Laemmli sample buffer. Samples were analyzed by immunoblotting using an anti-FLAG antibody.

Evaluation of protein stability using cycloheximide. Protein stability was analyzed by incubation in the presence of cycloheximide. For transient expression of each plasmid, we used four flasks of cells grown at 30˚C for 48 h. Following the addition of cycloheximide (final concentration 25 μM) to the medium to inhibit protein synthesis, proteins were harvested at indicated time points (0, 6, 24, and 48 h after addition) (8). At each time point, protein extracts, corresponding to 5 μg of protein at 0 h, were loaded and analyzed by immunoblotting using an anti-FLAG antibody. An anti-human cytosolic acetoacetyl-CoA thiolase (CT) antibody was used as the internal control for loading (9). The levels of FLAG-tagged SMNfull, SMNAQ, SMNQADDAD, SMNAQ/QADDAD, and CT proteins were evaluated by densitometric analysis.

Immunoprecipitation analysis. HeLa cells in a 100-mm dish were transfected with 3.0 μg of pFLAG-SMNfull, pFLAG-SMNAQ, pFLAG-SMNQADDAD and pFLAG-SMNAQ/QADDAD, using Lipofectamine 2000. After 48 h of incubation, cells were washed with cold PBS and harvested with a cell lysis buffer [20 mM HEPES buffer (pH 7.4) with 100 mM NaCl, 250 mM KCL, 0.5% Triton-X100, protease inhibitor cocktail (Roche)], rotating for 30 min at 4˚C. After centrifugation at 10,000 x g and 4˚C for 10 min, the supernatant was obtained, proteins of which (200 μg) were rotated for 2 h with 5 μg of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), which were pre-bound to Protein A Sepharose Beads (GE Healthcare). After rotation, the protein complex samples were solubilized with a 2X Laemmli sample buffer after washing four times with a cell lysis buffer. These samples were analyzed by immunoblotting using an anti-Gemin 2 polyclonal antibody (Santa Cruz Biotechnology) and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich).

Immunofluorescence analysis. HeLa cells were seeded at a density of $1.0x10^5$/ml per well of a 4-chamber slide (Thermo Fisher Scientific, Waltham, MA). pFLAG-SMNfull, pFLAG-SMNAQ, pFLAG-SMNQADDAD and pFLAG-SMNAQ/QADDAD (0.25 μg) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h incubation, cells were fixed with 3.7% paraformaldehyde in PBS for 15 min, and then washed with 10 mM glycine in PBS. Fixed cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min at room temperature, then transferred to PBS containing 1% BSA and 0.1% Triton X-100 for 1 h at room temeprature for blocking. These cells were treated with an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and anti-Gemin-2 polyclonal antibody (Santa Cruz Biotechnology) as primary antibodies and the binding was detected by incubation with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen), respectively, as secondary antibodies. They were then treated with a Vectashield Mounting Medium using DAPI (4'-6-Diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA) as a shield and a nuclear stain. We used a fluorescence microscope for observation (BZ-9000, Keyence, Osaka, Japan). In all cases, specificity of staining was confirmed by omission of the primary antibody in the controls.

Statistical analysis. Statistical analysis was performed using Prism 5.0 (GraphPad Software, San Diego, CA) statistical software. We employed a non-parametric Kruskal Wallis ANOVA test and Dunn's multiple comparison test for evaluation of size distribution of the foci. A P-value <0.05 was considered statistically significant.

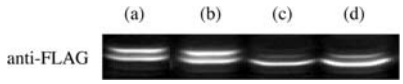

Figure 1. Transient expression analysis. Transient expression of FLAG-tagged SMNfull (a), SMNAQ (b), SMNQADDAD (c) and SMNAQ/QADDAD (d) expression vectors were done in HeLa cells. Immunoblotting was performed using an anti-FLAG antibody as the first antibody. Protein (2.5 μg) was applied.

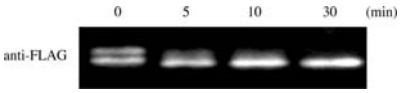

Figure 2. Alkaline phosphatase assay. FLAG-tagged SMNfull were treated with alkaline phosphatase for 0, 5, 10, and 30 min. Immunoblotting was performed using an anti-FLAG antibody as the first antibody. The upper band disappeared by alkaline phosphatase treatment.

Results

SMN phosphorylation. We performed a transient expression analysis of wild-type SMN in the pFLAG-CMV-6a expression vector. Two bands were observed with similar intensity in immunoblot analysis using an anti-FLAG antibody as the first antibody (Fig. 1a). The upper and lower bands were confirmed as phosphorylated and unphosphorylated FLAG-tagged SMNfull, respectively, from the fact that the upper band disappeared by alkaline phosphatase treatment (Fig. 2). We repeated this experiment twice and similar results were obtained. The molecular weight of FLAG-tagged SMNfull was calculated to be 33.2 kDa from the cDNA sequence.

Possible serine/threonine phosphorylation sites in SMN. Transient expression of FLAG-tagged SMNfull, SMNAQ, SMNQADDAD and SMNAQ/QADDAD expression vectors were done in HeLa cells (Fig. 1). In the cases of FLAG-tagged SMNfull (Fig. 1a) and SMNAQ (Fig. 1b) expression, two bands with similar intensity were observed using an anti-FLAG antibody. In the cases of FLAG-tagged SMNQADDAD (Fig. 1c) and SMNAQ/QADDAD (Fig. 1d) expression, two bands were also identified, but their upper band intensities were much lower than those of FLAG-tagged SMNfull and SMNAQ. We also repeated this experiment twice and similar results were obtained. This indicated that the major phosphorylation site in SMN was $Q^{28}SDD^{31}SD$.

Evaluation of protein stability using cycloheximide. Cycloheximide inhibits translation. Hence, the stability of accumulated FLAG-tagged wild-type and mutant SMN proteins can be examined when the FLAG-tagged SMN proteins are chased with cycloheximide after transient expression analysis. These experiments were performed three times and similar results were obtained. In the case of transient expression of FLAG-SMNfull (Fig. 3a), FLAG-tagged SMNfull was stable even after chasing for 48 h. FLAG-tagged SMNAQ (Fig. 3b), SMNQADDAD (Fig. 3c), and SMNAQ/QADDAD (Fig. 3d) were stable, as well as FLAG-tagged SMNfull. This indicates that mutation at these possible phosphorylation sites does not change the stability of the SMN protein.

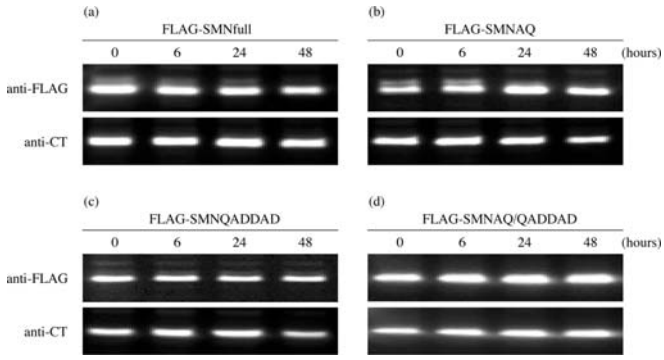

Figure 3. Stability of FLAG-tagged SMNfull (a), SMNAQ (b), SMNQADDAD (c) and SMNAQ/QADDAD (d). Cells transfected with these vectors were expressed at 30˚C and further incubated in cycloheximide (25 μM) at 37˚C for the times indicated prior to SDS-PAGE and immunoblotting with an anti-FLAG antibody and anti-CT antibody for controls.

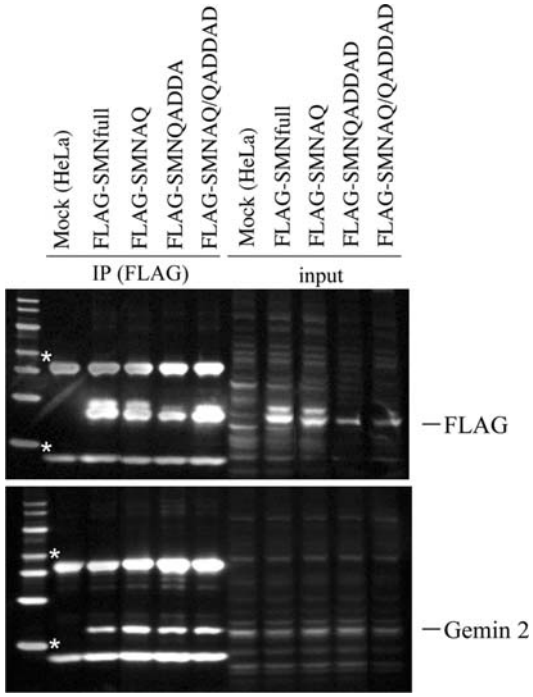

Figure 4. Immunoprecipitation analysis. Immunoprecipitation analysis using an anti-FLAG antibody following transient expression of FLAG-tagged SMNfull, SMNAQ, SMNQADDAD and SMNAQ/QADDAD in HeLa cells was performed. Immunoblotting was done using an anti-FLAG antibody and anti-Gemin 2 antibody as the first antibody. The input lanes contain 5% of the amount used in the binding reactions. Gemin 2 was co-immunoprecipitated in all cases. *IgG heavy and light chains.

Immunoprecipitation analysis. SMN forms an SMN complex with Gemin proteins. Among them, Gemin 2, 3 and 8 interact with SMN directly. An immunoprecipitation assay was done to determine whether overexpressed FLAG-tagged SMNAQ, SMNQADDAD and SMNAQ/QADDAD affect binding to Gemin 2. As shown in Fig. 4, Gemin 2 was co-immunoprecipitated by an anti-FLAG antibody in all cases. This result

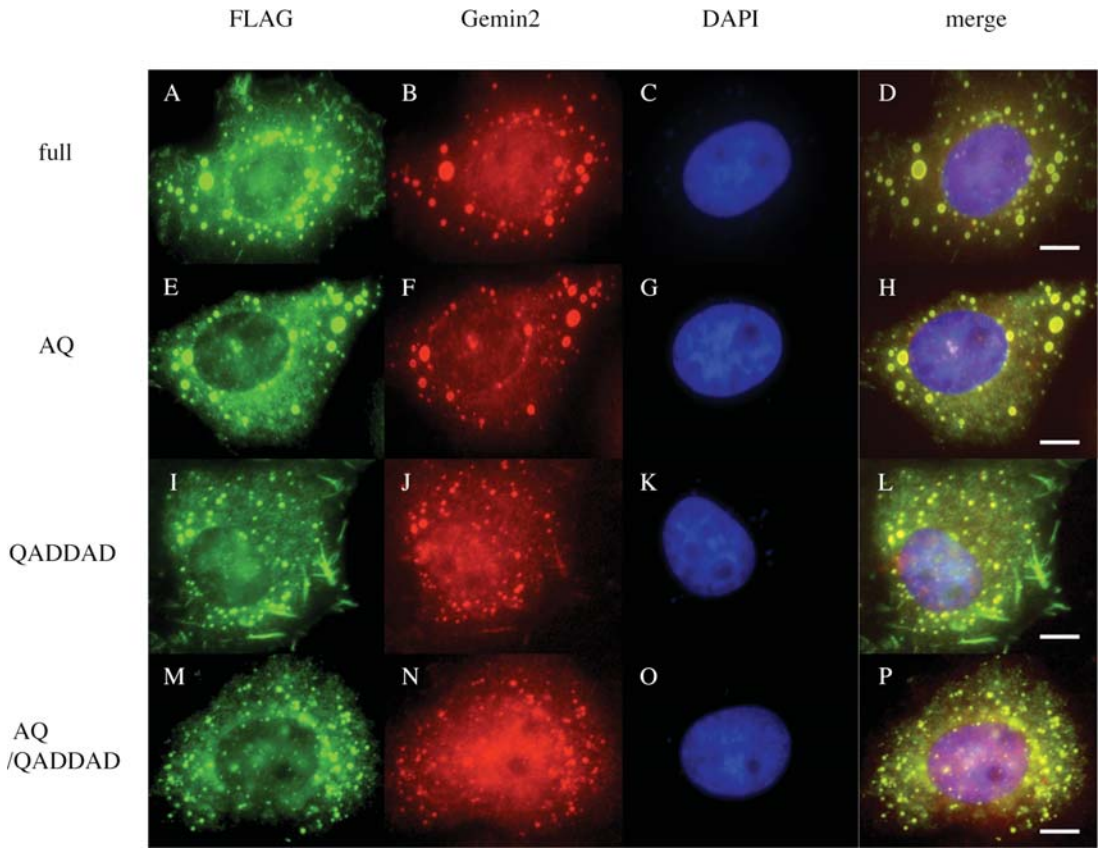

Figure 5. Immunofluorescence analysis in HeLa cells. Immunofluorescence analysis following transient expression of FLAG-tagged SMNfull, SMNAQ, SMNQADDAD and SMNAQ/QADDAD in HeLa cells was performed. Transfected with a vector expressing (A-D) FLAG-tagged SMNfull, (E-H) SMNAQ, (I-L) SMNQADDAD, and (M-P) SMNAQ/QADDAD. FLAG fluorescence is shown in green (A, E, I, M). Gemin 2 is labeled in red (B, F, J, N). Nuclei were stained with DAPI (4'-6-Diamidino-2-phenylindole) (C, G, K, O). Merged images are shown in (D, H, L, P). Scale bars, 10 μm.

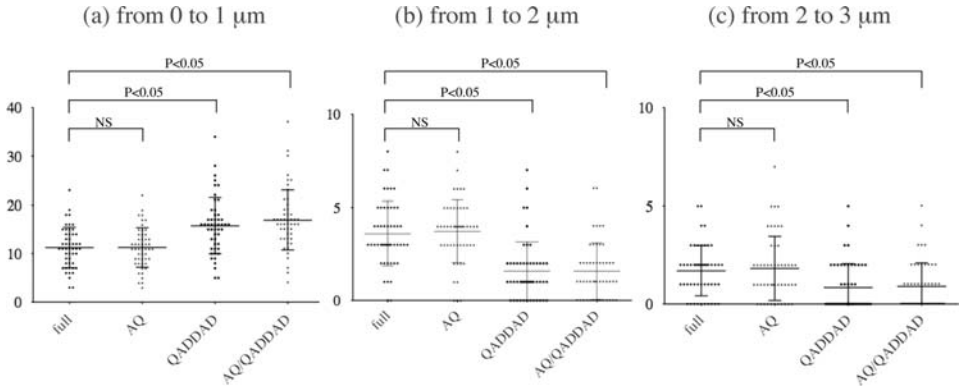

Figure 6. Statistical analysis of size of foci. We measured the number and diameter of foci located in the cytosol of 50 HeLa cells expressing FLAG-tagged SMNfull, SMNAQ, SMNQADDAD and SMNAQ/QADDAD. The diameters of the foci were classified into 3 categories, 0-1 (a), 1-2 (b), and 2-3 μm (c). The numbers of foci per one transfected cell are shown. We employed a non-parametric Kruskal Wallis ANOVA test and Dunn's multiple comparison test for evaluation of size distribution of the foci. A P-value <0.05 was considered statistically significant. NS, not significant.

indicates that mutations at the possible phosphorylation sites do not influence the interaction of Gemin 2.

Immunofluoresence analysis. SMN forms foci in both the nucleus and cytosol in immunofluorescence analysis. Immunofluorescence analysis following transient expression of FLAG-tagged SMNfull, SMNAQ, SMNQADDAD and SMNAQ/QADDAD in HeLa cells was performed (Fig. 5).

In the case of transient expression of FLAG-SMNfull in HeLa cells, many cytosolic foci with a small number of nuclear foci were observed using anti-FLAG antibodies (Fig. 5A). Gemin 2 was co-localized with FLAG-SMNfull (Fig. 5B and 5D). In the case of transient expression of FLAG-SMNAQ, anti-FLAG staining had a pattern similar to the expression of FLAG-SMNfull (Fig. 5E). Gemin 2 was also co-localized with FLAG-SMNAQ (Fig. 5F and 5H).

In the case of transient expression of FLAG-SMNQADDAD, anti-FLAG staining had a pattern quite different from the expression of FLAG-SMNfull and SMNAQ. FLAG-SMNQADDAD expression obviously exhibited fewer large cytosolic foci (Fig. 5I), whereas Gemin 2 was co-localized with FLAG-SMNQADDAD (Fig. 5J and 5L). In the case of transient expression of FLAG-SMNAQ/QADDAD, similar results were obtained with the use of FLAG-SMNQADDAD (Fig. 5M). Gemin 2 was also co-localized with FLAG-SMNQADDAD (Fig. 5N and 5P).

To evaluate the difference in the foci formation pattern in the immunofluorescent analysis, we measured the number and diameter of foci located in the cytosol of 50 HeLa cells expressing FLAG-tagged SMNfull, SMNAQ, SMNQADDAD and SMNAQ/QADDAD. The diameters of the foci were classified into 3 categories, 0-1, 1-2, and 2-3 μm. As shown in Fig. 6, the sizes of the foci in cells expressing Flag-tagged SMNAQ did not differ from those in cells expressing FLAG-tagged SMNfull. However, the cells expressing Flag-tagged SMNQADDAD and SMNAQ/ QADDAD had significantly more 0-1-μm foci and less 1-2-μm and 2-3-μm foci than cells expressing FLAG-tagged SMNfull.

Discussion

The SMN protein forms a multiprotein complex with Gemin proteins, the SMN complex, which is known to play a crucial role in RNA metabolism, and is highly phosphorylated when it is in cytoplasm, but it is hypophosphorylated in the nucleus. The phosphorylated form in cytoplasm is thought to be a much more active state of SMN than that in the nucleus. Previous studies have revealed that phosphorylation might control the localization and function of the SMN complex, and it has been suggested that phosphorylation does not affect the composition of the SMN complex but only its activity (6).

Several lines of evidence show that SMN is phosphorylated at serine and threonine residues (10). In this study, we hypothesized that SMN is phosphorylated at two kinds of serine residues and made expression constructs for their mutant SMN proteins. One site was the $Q^{28}SDD^{31}SD$ site, as reported (6), and the others were two SQ sites (^{80}SQ and ^{163}SQ). These SQ sites are possible substrates for phosphatidylinositol 3-kinase-like kinases (PIKKs). Since PIKKs regulate important steps such as cell cycles, RNA decay, and apoptosis (11,12), these SQ sites might be important for the SMN function. When the serine residues were substituted for alanine in the two SQ sites we found, i) no significant reduction of phosphorylated SMN bands in immunoblot analysis; ii) no significant changes in the stability of the mutant SMN in binding with Gemin 2, and in foci formation. Based on these data, we could not obtain any evidence for the importance of phosphorylation in these two SQ sites.

On the contrary, it has been shown that a unique phosphorylation site, $Q^{28}SDD^{31}SD$, is the major phosphorylation site in SMN (6). Evaluation of protein stability using cycloheximide and immunoprecipitation analysis revealed that mutations at the possible phosphorylation sites do not influence the stability of protein or interaction with Gemin 2. Meanwhile, our immunofluoresence analysis demonstrated that SMNQADDAD and SMNAQ/QADDAD do not form such large foci as in the case of SMNfull, which is statistically significant, as shown in Fig. 6. SMN forms an oligomer to stabilize itself, as most of the binding proteins will not associate with monomeric SMN (13), and it has been reported that SMNfull proteins were observed in cytoplasm both diffusely and in large cytoplasmic aggregates (14). One speculation about this result is that the hypophosphorylation of SMN inhibits self-oligomerization, leading to forming smaller foci.

We demonstrated that the dual mutations of two serines (amino-acid positions 28 and 31) to alanine, named SMNQADDAD, affect the foci formation of SMN. Although the meaning of these results is still unknown, there is a possibility that foci formation of SMN is related to its self-oligomerization. This result might be helpful for further experiments with SMN phosphorylation.

Acknowledgements

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