A Direct Interaction between the Survival Motor Neuron Protein and p53 and Its Relationship to Spinal Muscular Atrophy*

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Mutations in the SMN1 (survival motor neuron 1) gene cause spinal muscular atrophy (SMA). We now show that SMN protein, the SMN1 gene product, interacts directly with the tumor suppressor protein, p53. Pathogenic missense mutations in SMN reduce both self-association and p53 binding by SMN, and the extent of the reductions correlate with disease severity. The inactive, truncated form of SMN produced by the SMN2 gene in SMA patients fails to bind p53 efficiently. SMN and p53 co-localize in nuclear Cajal bodies, but p53 redistributes to the nucleolus in fibroblasts from SMA patients. These results suggest a functional interaction between SMN and p53, and the potential for apoptosis when this interaction is impaired may explain motor neuron death in SMA.

Spinal muscular atrophy is a neurodegenerative disorder characterized by loss of the α-motor neurons of the spinal cord, resulting in progressive atrophy of the voluntary muscles of the limb and trunk (1). There are three forms of the childhood disease, types I, II, and III, with type I being the most severe. All three forms are due to mutations of the telomeric copy of the SMN1 (survival motor neuron 1) gene (2). The centromeric copy of the SMN gene (SMN2) differs from SMN1 by only 11 nucleotides (2, 3). One of these is a CT transition located in exon 7 and results in different pre-mRNA splicing patterns; SMN1 produces mainly full-length product, whereas the majority of SMN2-derived transcripts lack exon 7, SMNΔ7 (2–4).

SMN is a ubiquitously expressed protein localized within the cytoplasm and the nucleus (2). Nuclear SMN localizes within Cajal bodies (5). Cajal bodies are defined by the presence of p80 coilin (6). Although the precise nature of the motor neuron-specific defect in SMA† is not known, the SMN protein has been implicated in several pathways, including nucleo-cytoplasmic transportation, UsnRNP assembly, transcription, and apoptosis (7). SMN can serve as an anti-apoptotic factor in neuronal cells, whereas SMNΔ7 and C-terminal missense mutations promote apoptosis (8). The means by which SMN achieves pro- and anti-apoptotic activities are not known. SMN self-association is a prerequisite for essentially all SMN functions (9–13). The SMN protein self-associates independently through regions encoded by exons 2b and 6, and the ability to homodimerize correlates with disease severity (14, 15). The primary product of SMN2, SMNΔ7, lacks exon 7, and the resultant protein has a reduced ability to self-associate, accounting for the inability of SMN2 to compensate for the loss of SMN1 (15).

The tumor suppressor protein p53 is multifunctional factor involved in cell cycle control (16), DNA repair (17, 18), transcription activation (19, 20), and apoptosis (21). p53 can induce apoptosis through several pathways, including mechanisms that are dependent or independent of p53-mediated transcriptional activity (21, 22). Under normal physiological conditions, p53 protein levels are maintained at relatively low levels through a negative feedback loop mediated by HDM2 (23–25). HDM2 inhibits p53 transcriptional activity by binding to the p53 transactivation domain and also targets p53 for ubiquitin-mediated proteolysis (26, 27). In the developing nervous system, p53 performs a critical role by regulating neuronal apoptotic death (28).

We now show that SMN interacts directly with p53 and that the region encoded by SMN exon 2 mediates the SMN-p53 association. SMN single amino acid substitution mutations that cause SMA inhibited SMN-p53 complex formation. The degree of inhibition correlated with the effects of each mutation on SMN dimerization and SMA disease severity. Following endogenous p53 activation, SMN and p53 accumulated in Cajal bodies. In contrast, in SMA patient-derived fibroblasts with very low SMN protein levels, p53 was localized within the nucleolus and not in Cajal bodies. Increased availability of pro-apoptotic p53 when SMN levels are very low suggests itself as a possible mechanism for loss of motor neurons in SMA.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—Wild-type SMN, SMN isoform lacking exon 7 (SMNΔ7), point mutated SMN, and SMN exon subconstructs were cloned into the glutathione S-transferase (GST) expression vector pGEX3X (Amersham Biosciences, Inc.). p53 cDNA was cloned in to the pRSET-C vector (Invitrogen). Transformed bacteria were induced with 1mM isopropyl-thio-D-thiogalactoside for 4h at 37 °C. Expressed recombinant proteins were purified from inclusion bodies by sequential extraction with increasing urea concentrations (2, 4, 6, and 8 M) and in phosphate-buffered saline as previously described (29).

Cellular Extracts and Immunoprecipitations—Cellular extracts from ∼1 × 10⁶ U2OS cells (human osteosarcoma cells; ATCC HTB96) tran-

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† The abbreviations used are: SMA, spinal muscular atrophy; GST, glutathione S-transferase; HA, hemagglutinin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; LMB, leptomycin B; BIA, biomolecular interaction analysis; HBS, HEPES-buffered saline; NAIP, neuronal apoptosis inhibitory protein; TRITC, tetramethyl rhodamine isothiocyanate; His₆-p53, hexahistidine-tagged p53; PML, promyelocytic leukemia; SMN-WT, full-length SMN.

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Resin was washed three times with binding buffer and incubated with bound fractions were resolved by SDS-PAGE. Protein concentrations when indicated, the cells were treated with 10 nM ALLN for 6 h, 10 mM cycloheximide for 4 h, 10 mM leptomycin B for 6 h, or 10 mCi/mm in 4 h prior to harvesting cellular extracts. LMB binds directly to CRM1 and results in nuclear accumulation of p53 (30), the ALLN peptide inhibits caspase-mediated p53 degradation, whereas cycloheximide and UV treatment stimulates p53 gene expression.

**Biomolecular Interaction Analysis (BIA)**—The BIAcore biosensor detects changes in total mass at the surface of a sensor chip by measuring variations of the critical angle needed to produce total internal refraction. The binding in critical angle is proportional to the amount of bound protein and is expressed as resonance units. In Fig. 1C, the shift in resonance units, or response, is plotted against time and is displayed as a sensorgram. Injected samples contain protein, urea, imidazole, and HEPES-buffered saline (HBS) and therefore are denser than HBS running buffer. This increase in medium density during protein injections produces the large vertical spikes seen on the sensorgram (the initial vertical line indicates the start of an injection, and the second vertical line indicates the end of an injection). The amount of bound protein following injection is indicated by the decrease in the horizontal baseline. A Cm5 sensor chip was covalently linked with rabbit anti-mouse IgG by the amino-coupling protocol according to the manufacturer's instructions (BIAcore AB, Stevenage, UK). A BIAcore-X apparatus was used with an operating flow rate of 5 μl/min. All injected volumes were 5 μl. Concentrations of stock recombinant proteins were determined for each reaction prior to injection. The proteins were diluted in HBS to 10 μg/ml unless otherwise stated.

To construct the monomer SMN chip, SMN was bound directly to a Cm5 chip (14). Stock SMN solutions in 8 mM urea, 10 mM 2-mercapto-ethanol were subjected to gel filtration on a calibrated Superdex G-15 column in coupling buffer to remove all traces of urea and 2-mercapto-ethanol in preparation for amino coupling to the chip. SMN eluted in the excluded volume was coupled immediately to the chip. After coupling, the chip was pulsed with HBS containing 0.1% SDS followed by 10 mM HCl, 8 mM urea to remove all noncovalently bound protein and leave monomeric SMN on the chip. Successful coupling was confirmed by binding of previously described SMN-specific mouse monoclonal antibodies. MANSMA1–3 (29).

For all BIA experiments control proteins, emerin and neuronal apoptosis inhibitory protein (NAIP), that do not interact with SMN were used to determine the background binding values. All of the reactions were performed at least in triplicate, and the plotted values represent the means from the individual experiments. All recombinant proteins used were exposed to a Cm5 chip with no coupled protein to ensure that nonspecific interactions were not occurring on the chip matrix. All stepwise reactions were repeated three times. Each reaction was repeated at least three times, i.e. for the SMN-WTP53 binding study the primary run lacked MANSMA1 to ensure that the Cm5 rabbit-anti-Mouse IgG chip did not capture SMN or p53. The secondary run lacked SMN WT, thus demonstrating MANSMA1 did not capture p53. Once these controls were performed and nonspecific interactions were eliminated, the actual experiment was performed, with stepwise injections of MANSMA1, SMN-WT, and p53.

**Immunohistochemistry**—Subconfluent U2OS and skin fibroblast cell lines were plated and treated with ALLN, LMB, cycloheximide, or UV light as described above. The cells were washed three times with phosphate-buffered saline, fixed for 1 min with 50% acetone, 50% methanol, and then washed an additional three times with phosphate-buffered saline. The cells were then analyzed by double-label immunofluorescence (29, 31). Images were captured using TRITC and fluorescein isothiocyanate filter sets and a 100× oil immersion objective in conjunction with a TCS-NT confocal microscope.

**In Vitro Binding Assays**—GST and His<sub>6</sub> binding resins (Novagen) were washed three times in binding buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.8, and 0.2% Nonidet P-40) and incubated with recombinant protein diluted to 100 μg/ml in binding buffer for 1 h at 4 °C. The resin was washed three times with binding buffer and incubated with the respective binding protein diluted to 100 μg/ml in binding buffer for 1 h at 4 °C. Resin was washed three times with binding buffer, and the bound fractions were resolved by SDS-PAGE. Protein concentrations were confirmed by Coomassie Blue staining.

**RESULTS**

**SMN Complexes with p53 in Vivo and in Vitro**—SMN can function as an anti-apoptotic factor, although its mode of action is unclear. Because the tumor suppressor p53 protein performs a critical role in monitoring neuronal apoptosis of the developing and mature nervous system, we examined whether SMN was involved in a p53 pathway. To determine whether SMN and p53 interact in vivo, the cells were transfected with a HA epitope-tagged SMN and treated with ALLN. ALLN prevents p53 degradation by inhibiting calpain activity and has been used to study endogenous p53 localization patterns (30). Co-immunoprecipitation reactions were performed with anti-p53 monoclonal antibody. In extracts of U2OS cells treated with ALLN, p53 co-precipitated with HA-tagged SMN (Fig. 1A, left panel). In contrast, SMN was not immunoprecipitated from untreated extracts (Fig. 1A, right panel). A direct interaction between SMN and p53 in vitro was shown using recombinant SMN and p53 proteins (Fig. 1B). BIA was used to confirm the specificity of the SMN/p53 interaction. SMN captured on a sensor chip bound high levels of p53 while failing to interact with control proteins, emerin and NAIP (Fig. 1C).

Using BIA, the p53-SMN complex was also detected in cellular extracts from cells pretreated with ALLN peptide (Fig. 1D). Endogenous p53 and its complexes were captured on a sensor chip using an anti-p53 monoclonal antibody, and an anti-SMN polyclonal antibody was used to detect complexed SMN. Extracts from untreated cells contained little p53, whereas ALLN-treatment resulted in an 8–10-fold increase in p53 levels (Fig. 1D), p53 complexes captured from ALLN-treated extracts contained SMN (Fig. 1D), but SMN was not detected in untreated control extracts (data not shown). Similar results were obtained with three other treatments that raise p53 levels: LMB, which binds directly to CRM1 and results in nuclear accumulation of p53 (30); cycloheximide; or UV light (data not shown).

To identify the domain(s) of SMN that mediated SMN-p53 complex formation, in vitro binding experiments were performed using recombinant H<sub>6</sub>-tagged p53 protein and SMN subdomains (peptides encoded by exons 1, 2, 3, 4, 6, or 7) expressed as GST fusion proteins. The domain encoded by SMN exon 2 was necessary and sufficient to bind to p53 (Fig. 2A, left panel). Similarly, hexahistidine-tagged p53 (His<sub>6</sub>-p53) captured on agarose-linked nickel beads precipitated SMN fragments containing exon 2-encoded sequence but failed to associate with a fragment encoded by exons 4–7 (Fig. 2A, right panel). Quantitative BIA studies showed that SMN exon 2 was sufficient to mediate the p53 interaction and that use of full-length SMN and larger SMN peptides (SMN exons 1–2 and 1–4) did not enhance p53 binding (Fig. 2B). Under increasing NaCl concentrations, full-length SMN protein (SMN-WT), SMN exons 1–2, exons 1–4, and SMN exon 2 displayed similar binding capacities for p53 (Fig. 2C).

**SMA Patient-derived SMN Mutations Decrease the SMN/p53 Interaction**—To determine whether disease-causing SMN mutations disrupt the SMN-p53 complex, three patient-derived SMN mutations consisting of individual single amino acid substitutions (Y272G, T274I, and G279V) were assayed for their ability to interact with p53. Additionally, we assessed the ability of SMN7 to interact with p53. SMN7 is the primary product of SMN2 and is present in SMA patients. Compared with SMN-WT, the SMN missense mutations and SMN7 displayed a dramatically reduced ability to bind p53 (Fig. 3A). The decrease in p53 binding correlates with disease severity; mutations 262 and 279 are isolated from severe type I SMA and have the greatest impact on p53 binding, whereas mutation 274 was isolated from a less severe patient and has an inter-
Fig. 1. Detection of the SMN-p53 complex. A, SMN-p53 complexes are co-immunoprecipitated in extracts from U2OS cells pretreated with ALLN peptide (left panel) but not in untreated extracts (right panel). Complexes were immunoprecipitated with an anti-p53 monoclonal antibody, and bound fractions were detected by probing with an anti-HA monoclonal antibody followed by chemiluminescence. Transfected (+) and untransfected (−) lysates, the position of Ig heavy chain, and a cross-reacting endogenous band (∗) are indicated. B, recombinant SMN and p53 form a complex. Immobilized GST-SMN fusion protein specifically interacts with His6-p53 fusion protein. Bound fractions were detected by Western blotting with an anti-p53 monoclonal antibody. C, BIA of SMN-p53 binding. SMN captured on a rabbit anti-mouse sensor chip using an anti-SMN mouse monoclonal antibody specifically interacts with p53 but not two negative controls, emerin and NAIP. Levels of captured anti-SMN monoclonal antibody (spectrum A), recombinant SMN (spectrum B), emerin (spectrum C), NAIP (spectrum D), and recombinant p53 (spectrum E) are all indicated. D, BIA detection of endogenous SMN-p53 complexes. SMN-p53 complexes were immunoprecipitated from total protein extracts from U2OS cells treated and untreated with ALLN peptide. Complexes were captured using an anti-p53 monoclonal antibody. The presence of SMN within the complex was confirmed using a rabbit polyclonal antibody specific for SMN. Levels of captured anti-p53 antibody (spectrum A), untreated protein extract (spectrum B), p53-SMN from ALLN-treated extracts (spectrum C), and anti-SMN rabbit polyclonal (spectrum D) are indicated.
mediate capacity to bind p53 (Fig. 3A). The missense mutations and SMNΔ7 disrupt SMN dimerization to an extent that parallels p53 binding and disease severity (Fig. 3B).

Functional SMN exists in at least a dimeric state, and the ability to self-associate correlates with disease severity (14, 15). Consistent with the requirement for self-association, monomorphic SMN failed to associate with p53 (Fig. 3C). The monomorphic state of the SMN captured on the sensor chip was confirmed by reprobing with wild-type SMN protein as previously described (Fig. 3C) (14). Following dimerization of SMN on the sensor chip, p53 binding was increased 6-fold compared with p53 binding by monomorphic SMN (Fig. 3C).

**SMN and p53 Co-localize in Cajal Bodies following p53 Activation**—Nuclear SMN is enriched in Cajal bodies and within the nucleolus (5, 29, 31, 32). Although total SMN protein levels and SMN-positive Cajal body numbers correlate with disease severity, a connection to motor neuron loss has not been established (33). Recently it has been reported that following treatment with ALLN or LMB, p53 accumulates in nuclear structures consistent with Cajal bodies (24, 34). To determine whether these structures are Cajal bodies, double label immunolocalization was performed on untreated, UV-, cycloheximide-, ALLN-, and LMB-treated U2OS cells (Fig. 4). In control cells, a typical SMN distribution was observed, with diffuse cytoplasmic staining and nuclear SMN accumulating in both Cajal bodies and the nucleolus (Fig. 4). Low levels of diffuse nuclear p53 were detected. However, SMN and p53 did not co-localize (Fig. 4). In contrast, following treatment of U2OS
cells with the p53-inducing stimuli, p53 accumulated in SMN-positive bodies. The four treatments did not visibly alter either the cytoplasmic or nuclear distribution of SMN. The p53-SMN-positive structures also contained p80 coilin, confirming that these structures are Cajal bodies (data not shown).

Following activation or stabilization of p53 by agents such as ALLN or LMB, p53 has been shown to partially localize within PML bodies (30, 35). To determine the relationship between Cajal bodies and PML bodies, immunolocalization was performed on U2OS cells using antibodies against SMN and PML.

As expected, there were significantly more PML bodies than Cajal bodies as indicated by the presence of PML compared with SMN-positive foci (Fig. 5). There was, however, a small subset of PML-positive nuclear bodies that also contained SMN, demonstrating that a fraction of Cajal bodies are coincident with PML bodies in untreated U20S cells.

**SMN and p53 Fail to Co-localize in SMA-derived Fibroblasts**

Double label immunolocalization was performed on human cultured skin fibroblasts from an unaffected carrier (control) and a type I SMA individual (33). In untreated control
fibroblasts, diffuse p53 staining within the nucleus was present, whereas SMN was detectable in discrete nuclear bodies. Following ALLN treatment, p53 co-localized in SMN-positive nuclear bodies (Fig. 6), consistent with the distribution patterns observed in U2OS cells.

In SMA fibroblasts, endogenous SMN levels were dramatically reduced and SMN-positive nuclear bodies were not detected (Fig. 6). In contrast to control fibroblasts, following treatment with ALLN peptide, p53 accumulated primarily in the nucleolus and not in nuclear bodies (Fig. 6) Nucleolar localization was confirmed by viewing the cells under phase contrast and with an anti-nucleolin monoclonal antibody (data not shown).

DISCUSSION

We have shown that SMN directly associates with p53 in vivo and in vitro through a series of immunoprecipitations, in vitro bindings, and BIA experiments. The SMN/p53 interaction is mediated through the region encoded by SMN exon 2. Several lines of evidence suggest that the SMN/p53 interaction is functionally significant: 1) SMN mutations isolated from SMA patients disrupt p53 binding in a manner that reflects the severity of disease, and a functional SMN oligomer is essential for the p53 interaction. 2) SMN exon 2 is highly conserved through numerous divergent organisms (36). 3) SMN prevents neuronal apoptosis in vivo and in vitro (8); however, the mechanism behind this protection is unclear. Interestingly, the C-terminal deletions or the missense mutations that inhibited SMN-mediated anti-apoptotic activity also inhibited the ability of SMN to interact with p53.

Mutations that disrupt self-association also disrupt SMN functional interactions, including self-association (14). In this study we show that SMN C-terminal mutations prevent binding to p53. SMNA7 and the C-terminal point mutations (Y272C, T274I, and G279V) likely destabilize SMN exon 6-mediated dimerization, resulting in reduced SMN self-association even when an intact SMN exon 2 dimerization domain is present in cis. Self-association through the SMN exon 6 domain is significantly stronger than self-association mediated by the SMN exon 2b domain and likely functions as an initial anchor that facilitates additional SMN intra- and inter-molecular interactions (14). Therefore, the inability of the C-terminal mutants to bind p53 suggests that the exon 2b interaction is also destabilized. However, the inability of the mutant proteins to associate with p53 is likely not due to gross protein defects, because these mutant proteins retain nearly all of their biochemical properties, although at reduced levels.

p53 accumulates in PML nuclear bodies following the inhibition of p53 degradation and by blocking CRM1-mediated p53 translocation (24). These bodies also contained Sm core proteins and U1 snRNP auxiliary factor (U1A) (24). An average mammalian cell contains 10–20 PML bodies, and these bodies dissociate in cells from human acute promyelocytic leukemia patients (6, 37). In a broad number of cell lines and in differentiated adult mammalian tissue, SMN and p80 coilin co-localize in another class of nuclear bodies termed Cajal bodies (31, 38). Here we demonstrate that following activation of p53 by ALLN, cycloheximide, or ultraviolet light treatment, p53 co-localized with SMN and p80 coilin in Cajal bodies. Additionally, a small subset of Cajal bodies spatially co-localized with PML bodies. Although Cajal bodies and PML bodies have been reported as exclusively independent structures (6), several lines of evidence show that nuclear bodies and their components are highly dynamic (29, 31, 37, 39). Furthermore, spatial distribution and localization of nuclear bodies are transitory and can be altered under specific physiological conditions (39).

The interaction between p53 and SMN suggests that the two proteins have an associated function. However, to date p53 has no role in RNA processing. SMN has been shown to have a role in the induction of apoptosis; however, the mechanism of apoptotic regulation is unclear (8, 40). The lower levels of SMN in SMA patient-derived fibroblasts paralleled the nucleolar localization of p53 in patient fibroblasts, suggesting that high levels of SMN are required either directly or indirectly for p53 localization within nuclear bodies. This could be a mechanism for regulating p53 function, potentially by physical sequestration of p53 in a cellular compartment that inhibits p53 function. Nucleolar accumulation of p53 has recently been shown (41, 42). SMN can also accumulate within the nucleolus of adult neuronal cells and all fetal cell types (31, 32). ZPR1 facilitates...
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SMN localization within Cajal bodies; however, it is unclear whether ZPR1 is required for the structural maintenance of Cajal bodies (43).

The exact nature of the motor neuron-specific defect associated with SMA is not known. Interestingly, SMN can function as an anti-apoptotic factor in neuronal cells and has been reported to functionally interact with the anti-apoptotic factor Bcl-2 in a tissue culture model (8, 40). In contrast, C-terminal deletions and missense mutations converted SMN to a pro-apoptotic factor (8). An anti-apoptotic function of SMN may be significant, with regards to disease, because surviving motor neurons from SMA patients display signs of apoptosis, such as chromatolysis and swelling (44, 45). Although a role for p53 has not been established in these activities, our results are consistent with a model in which wild-type SMN prevents p53-mediated apoptosis. In the disease state, SMNΔ7 or missense mutations fail to associate with p53, allowing activation of p53-dependent apoptotic pathways. p53 can stimulate apoptosis through multiple mechanisms, including pathways that are dependent and independent of p53-transcriptional activation (21). Current studies are underway to determine what role SMN plays in p53-dependent apoptotic pathways and to determine the relevance to motor neuron death observed in SMA.

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FIG. 6. p53 nuclear localization is altered in SMA fibroblasts. Nuclear distribution of SMN and p53 in cultured skin fibroblasts from an unaffected carrier (3184; Ref. 33) and a SMA type I patient (3185; Ref. 33) was detected using an anti-SMN polyclonal (fluorescein isothiocyanate, green) and an anti-p53 monoclonal antibody (TRITC, red). The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole-2HCl (DAPI). Cajal bodies lacking (white arrows) and containing p53 (yellow arrows) and nuclear p53 (green arrows) are indicated. The insets show nuclear regions of interest magnified an additional 2×. The bar represents ~50 μm.
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