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Differential Subcellular Localization of the Survival Motor Neuron Protein in Spinal Cord and Skeletal Muscle

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To compare the expression pattern of the survival motor neuron (SMN) protein in spinal cord and skeletal muscle, we generated a sheep polyclonal antibody against a bacterially expressed human SMN-fusion protein. On Western blots, the affinity purified anti-SMN antibody recognized a ~38 kDa protein band in extracts prepared from the mouse skeletal muscle, spinal cord, and brain that co-migrated with the bacterially expressed SMN protein. In immunohistochemical studies, the anti-SMN antibody labeled mostly the cytoplasm of the motor neurons in the anterior horn of mouse spinal cord. In contrast, predominant uniform labeling of the nuclei was observed in the mouse skeletal muscle. Thus, our results for the first time demonstrate that the SMN protein is differentially localized in mouse spinal cord and skeletal muscle.

The autosomal recessive neuromuscular disease, spinal muscular atrophy (SMA), affects ~1 in 6000 births and is the second most common genetic cause of childhood death after cystic fibrosis (1,2). This disease is associated with degeneration of the anterior horn cells in spinal cord and is characterized by proximal symmetrical weakness and atrophy of limb muscles (3). The primary determining gene of SMA is the survival motor neuron (SMN) gene, which is mutated in over 98% of all SMA patients (4). The SMN mRNA is ubiquitously expressed in both adult (4) and fetal (5) human tissues including the heart, brain, lung, liver, kidney, spinal cord, and skeletal muscle. The SMN gene encodes a putative 294 amino acid protein that is highly conserved through the mammalian species (6,7). However, SMN has no overall homology to any known protein. Although different studies suggest that SMN is involved in RNA biogenesis (8,9) and synergistically enhances the anti-apoptotic effect of Bcl2 (10), its exact function is unclear.

Although SMA is considered primarily a neuronal disease, constitutive muscular abnormalities have also been shown in severe phenotypes of SMA using an *in vitro* nerve-muscle model (11,12). To date, no studies have analyzed the expression of the SMN protein in the skeletal muscle using immunolocalization techniques. Since skeletal muscle is also affected in SMA, it is important to localize the SMN protein in this tissue. In the present study, we investigated the subcellular localization of the SMN protein in mouse spinal cord and skeletal muscle.

MATERIALS AND METHODS

Materials. All chemicals used were ultrapure or ACS grade. The following reagents were purchased as described. Blocking Grade Non-Fat Dry Milk was from BioRad Laboratories (Richmond, CA). Normal donkey serum, normal goat serum, normal rabbit serum, peroxidase and alkaline phosphatase conjugated AffiniPure donkey anti-sheep IgG (H+L), and peroxidase conjugated AffiniPure goat anti-mouse IgG (H+L) were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). Balb-C mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The anti-SMN monoclonal antibody 2B1 was a generous gift from Dr. G. Dreyfuss (University of Pennsylvania, Philadelphia, PA).

SMN expression and purification. The SMN cDNA was PCR amplified (13) from a human fetal brain cDNA library (Clontech, Inc., Palo Alto, CA) and was completely sequenced (14) to ensure the sequence integrity. The SMN cDNA was then subcloned in-frame into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA) and the pGEX-KT vector (ATCC, Rockville, MD). The recombinant plasmids, pMAL-SMN and pGEX-SMN, were used to transform chemically competent *E. coli* DH1αF′ (15). To overexpress MBP-SMN and GST-SMN fusion proteins, the respective transformants were grown in Luria Broth and subsequently induced with 0.5 mM isopropyl-ß-D-thiogalactopyranoside (15) at room termperature for 7h.

Bacterial lysates containing MBP-SMN and GST-SMN were incubated with pre-equilibrated amylose resin (New England Biolabs,

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Abbreviations used: BSA, bovine serum albumin; GST, glutathione S-transferase; MBP, maltose binding protein; NS, normal serum; PBS, phosphate-buffered saline; SMA, spinal muscular atrophy; SMN, survival motor neuron.

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MA) and glutathione agarose (Sigma Chemical Co., St Louis, MO), respectively. After washing with column buffer (20 mM Tris, pH 7.4, 200 mM NaCl, and 1 mM EDTA), MBP-SMN was eluted with 10 mM maltose in column buffer and GST-SMN was eluted using the method of Frangioni and Neel (16).

To cleave SMN from the fusion protein, MBP-SMN was incubated with Factor Xa protease (New England Biolabs, Beverly, MA) at a ratio of 200:1 (w/w) in column buffer. After rocking for 3h at room temperature, 2 mM EGTA and 1 mM PMSF was added to stop the cleavage reaction.

Anti-SMN antibody production and purification. A sheep polyclonal antibody against the MBP-SMN fusion protein was generated using the standard services offered by Pel-freeze Biologicals (Rogers, AR). The antisera were titered by ELISA using MBP-SMN and GST-SMN. An affinity column composed of GST-SMN coupled to Affigel-10 agarose (BioRad Laboratories, Richmond, CA) was prepared according to the manufacturer's instructions. To purify SMN specific antibodies, the SMN antiserum was applied to the GST-SMN affinity column at 4°C. After extensive washing, the adsorbed SMN antibodies were eluted with 100 mM glycine, pH 2.5 and collected as one ml fractions directly into 100 μl of 1 M Tris, pH 8. Pooled fractions containing specific antibodies were concentrated using a Biomax-50K Ultrafree centrifugal filter unit (Millipore Co., MA), and the buffer was exchanged for 10 mM Tris, pH 7.5 containing 1 mg/ml bovine serum albumin (BSA) and 0.1% sodium azide. The purified antibody was stored at 4°C.

SDS-PAGE and Western blot analysis. Proteins from bacterial lysates, mouse tissue extracts, and HeLa cell homogenates were resolved by SDS-PAGE (12% polyacrylamide) according to the method of Laemmli (17). The separated proteins were then electrotransferred at 4°C to an Immobilon-P membrane (Millipore Co., Bedford, MA) as previously described (18). The transfer membranes were blocked with 5% normal serum (NS, either from donkey or goat) then used for Western blots (18). The primary antibody used was a 1:3000 dilution of anti-SMN polyclonal antibody or 1:1000 dilution of anti-SMN monoclonal antibody 2B1 in 1% NS/PBS. Peroxidase conjugated to either donkey-anti sheep IgG (to detect the anti-SMN antibody) or goat anti-mouse IgG (to detect mAb 2B1) was used as the secondary antibody. Finally, the membranes were incubated with SuperSignal Substrate (Pierce, Rockford, IL) for 5 min and then exposed to X-ray films.

Immunohistochemistry. The upper hind leg muscle and spinal cord from Balb-C mice were removed at the time of sacrifice and transferred immediately to 3.7% formalin/2% ammonium bromide, which is recommended as the primary fixative for brain and neurofibrils (19). After fixing overnight, the tissues were processed for routine paraffin-embedding and sectioning. The paraffin-embedded transverse sections (4 μm) were deparaffinized and hydrated. To unmask the SMN antigen, the sections were treated with 0.4% pepsin in 0.01 N HCl (20) for 8 min at 37°C then washed with PBS. Any endogenous peroxide in the sections was quenched by incubating each section with 0.03% hydrogen peroxide in PBS for 10 min at room temperature. After washing with PBS, the sections were blocked with 3% normal rabbit serum for 2 h at room temperature and then incubated overnight at 4°C with a 1:300 dilution of anti-SMN antibody or preimmune IgG in 1.5% normal rabbit serum/PBS. The sections were washed with PBS and then processed with the Vectastain Elite ABC kit for sheep IgG (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's directions. Bound anti-SMN antibody was detected after incubating with ImmunoPure Metal Enhanced 3,3′-diaminobenzidine tetrahydrochloride substrate (Pierce, Rockford, IL). The stained sections were then examined with a Zeiss light microscope and photomicrographs were taken at 100x magnification with a 35 mm camera using Kodak Tmax 100 film (Kodak, Rochester, NY).

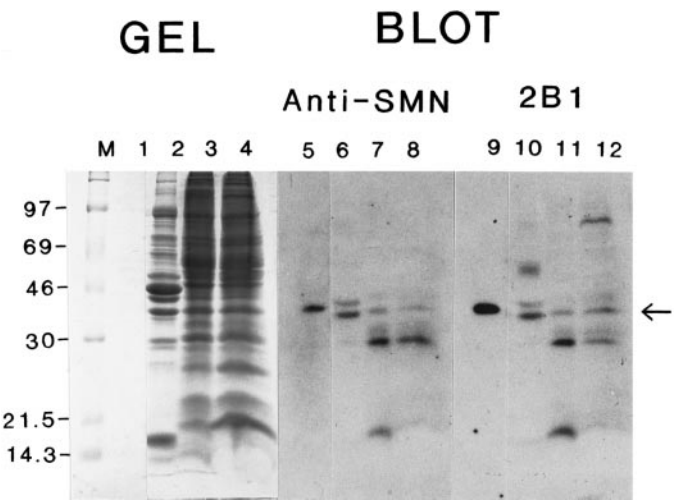

FIG. 1. Detection of SMN in mouse tissue extracts. Bacterially expressed purified SMN and mouse tissue extracts were separated by SDS-PAGE and either stained with Coomassie blue or transferred to Immobilon-P membranes. The transferred proteins were then immunoblotted with the anti-SMN antibody or monoclonal antibody 2B1, as indicated. Lanes 1, 5, and 9, cleaved MBP-SMN; Lanes 2, 6, and 10, mouse skeletal muscle extract (30 μg); Lanes 3, 7, and 11, mouse brain extract (100 μg); and Lanes 4, 8, and 12, mouse spinal cord extract (100 μg). Arrow indicates the 38 kDa SMN protein band.

RESULTS

A sheep polyclonal antibody against the SMN protein was prepared using MBP-SMN as the antigen and purified from the serum using a GST-SMN affinity column. In Western blot analysis, the purified anti-SMN antibody recognized the intact MBP-SMN protein and the SMN protein cleaved from the fusion protein using Factor Xa, but not the MBP protein (data not shown). No bands were detected in the control blot incubated with preimmune serum (data not shown). To determine if skeletal muscle and spinal cord express SMN protein, extracts prepared from mouse tissues were analyzed by Western blot using the anti-SMN antibody. As shown in Fig. 1, the antibody recognized bacterially expressed purified SMN protein (lane 5). A protein band that comigrated with the bacterially expressed SMN protein band was also detected in mouse skeletal muscle, brain, and spinal cord extracts (Fig. 1; lanes 6–8). Lower molecular weight proteins were also detected in the brain and spinal cord extracts (lanes 7 and 8) and are probably proteolytic fragments of SMN. Similar Western blot results were obtained with the 2B1 monoclonal antibody (Fig. 1; lanes 9-12), which was previously generated by Liu and Dreyfuss (21). Thus, we believe that our purified polyclonal anti-SMN antibody specifically recognizes the SMN protein expressed in the mouse tissues.

To identify the subcellular localization sites of the SMN protein, immunohistochemical studies were performed on paraffin-embedded transverse sections of

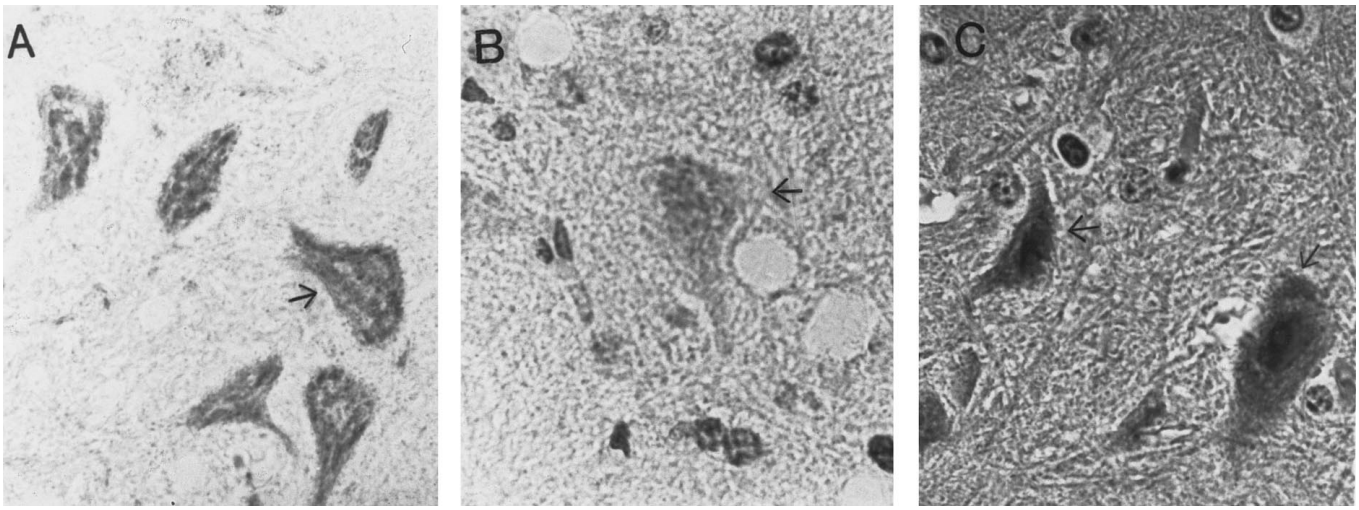

FIG. 2. Immunolocalization of SMN in spinal cord. The mouse spinal cord (A) immunostained with the anti-SMN antibody, (B) immunostained with preimmune sera, and (C) stained with hematoxylin and eosin. Arrows indicate motor neurons. Magnification: 100×.

mouse spinal cord and skeletal muscle. In mouse spinal cord, SMN specific immunostaining was detected in the motor neurons of the anterior horn. As shown in Fig. 2A, the anti-SMN antibody detected SMN mostly in the cytoplasm of the spinal cord motor neurons. In contrast to spinal cord, SMN specific immunostaining was detected predominantly in the nucleus of the skeletal muscle and the nuclear immunostaining was fairly uniform (Fig. 3A). For both spinal cord and for skeletal muscle, reduced immunostaining was detected when the sections were incubated with preimmune serum (Fig. 2B and 3B) or when the anti-SMN antibody was preincubated with excess antigen or was omitted (data not shown). For comparison, the spinal cord (Fig. 2C) and skeletal muscle (Fig. 3C) sections were also independently stained with hematoxylin and eosin to distinguish the nucleus and cytoplasm, respectively, in each tissue.

DISCUSSION

The afflicted tissues in SMA disease are the spinal cord and skeletal muscle. It is believed that the primary cause of SMA is motor neuron degeneration and that muscle weakness and atrophy occur secondarily. In SMA I and II patients, however, there is evidence that an intrinsic defect present in their skeletal muscle contributes to the disease phenotype (11,12). These

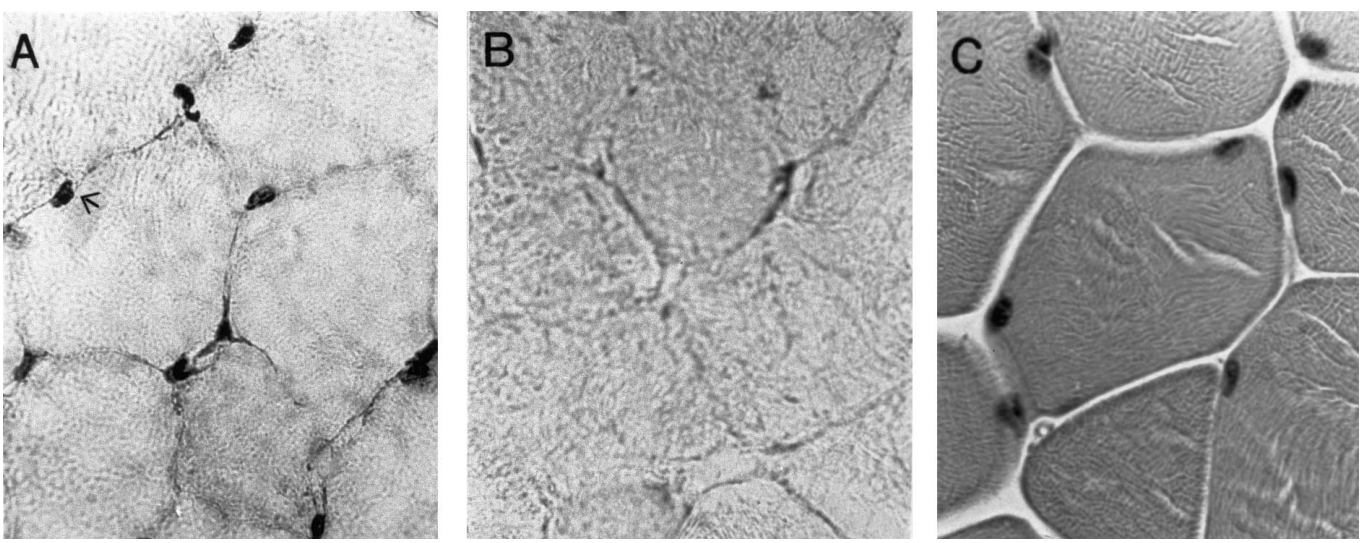

FIG. 3. Immunolocalization of SMN in skeletal muscle. Mouse skeletal muscle (A) immunostained with the anti-SMN antibody, (B) immunostained with preimmune sera, and (C) stained with hematoxylin and eosin. Arrow indicates nucleus. Magnification: 100×.

studies suggest that constitutive muscular abnormalities also contribute to the pathogenesis of SMA.

To study the expression pattern of the SMN protein in both spinal cord and skeletal muscle, we generated a polyclonal antibody against the MBP-SMN fusion protein that contained the entire human SMN protein. The polyclonal antibody was then purified using an affinity column composed of GST-SMN fusion protein. Both the sheep anti-SMN polyclonal antibody generated in our laboratory and the SMN specific monoclonal antibody 2B1 generated by Liu and Dreyfuss (21) detected the same protein bands in Western blots of mouse tissue extracts. This result suggests that our sheep polyclonal antibody recognized the mouse SMN protein, which is 82% identical to its human homologue at the amino acid level (6,7).

Using our purified polyclonal antibody, we observed SMN specific immunostaining mostly in the cytoplasm of the motor neurons of the mouse spinal cord. This result is contrary to the observation by Francis et al. (22), who used a polyclonal antibody generated against a synthetic peptide corresponding to amino acids 60–76 of SMN to show SMN immunostaining exclusively in the nucleus of mouse neurons. However, our results are in good agreement with the results of Battaglia et al. (23), who used a polyclonal antibody generated against the N-terminus of SMN to demonstrate SMN immunostaining mostly in the cytoplasm of lower motor neurons in rat, monkey, and human spinal cords. Similar strong cytoplasmic SMN immunostaining was also reported in COS-7 (23) and in 3T3 mouse fibroblasts (21). Taken together, these studies are consistent with our results.

Our immunostaining results in mouse skeletal muscle are markedly different from that of mouse spinal cord and show predominant, uniform SMN immunostaining of the nucleus and little or no staining of the cytoplasm (Fig. 3). Previously, Liu and Dreyfuss (21) used the 2B1 monoclonal antibody to localize SMN in HeLa cells. They observed diffused cytoplasmic labeling as well as strong labeling of discrete structures in the nucleus named gems. In our case, it was difficult to discern whether gems were stained because of the uniform SMN immunostaining in the nucleus. SMN has also been localized to the nucleoplasm through subcellular fractionation studies in HeLa cells permanently expressing hemagglutinin-tagged SMN protein (10). The uniform nuclear labeling we observe could be due to the ability of our polyclonal antibody to recognize both the nucleoplasm and the gem associated forms of SMN.

Since there is only one SMN isoform expressed in mouse (6,7), the differential labeling patterns in spinal cord and skeletal muscle are not due to the detection of alternatively spliced SMN isoforms. Lower molecular weight proteins (~29-30 kDa) recognized by the SMN antibody on Western blots were detected only in spinal cord and brain extracts and not in the skeletal muscle extract. These smaller proteins are probably due to proteolysis of SMN. If this proteolysis resulted in the loss of the putative nucleic acid binding domain in exon 2 of SMN (24), it could account for the cytoplasmic location in the neurons. Alternatively, the differential localization may be due to tissue specific proteins which interact with SMN. Currently, we do not know which of these two possibilities could better explain our results.

In summary, we have localized SMN at the subcellular level in skeletal muscle and spinal cord. The differential localization of SMN in each of these tissues suggests potentially different tissue specific functions and/or regulation, but the exact significance remains to be elucidated. Detailed analysis of SMN and possible tissue specific factors which associate with SMN protein will be required in the future to fully understand its role in the SMA disease.

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