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The gemin2-binding site on SMN protein: Accessibility to antibody

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ABSTRACT

Reduced levels of SMN (survival-of-motor-neurons) protein are the cause of spinal muscular atrophy, an inherited disorder characterised by loss of motor neurons in early childhood. SMN associates with more than eight other proteins to form an RNA-binding complex involved in assembly of the spliceosome. Two monoclonal antibodies (mAbs), MANSMA1 and MANSMA12, have been widely-used in studies of SMN function and their precise binding sites on SMN have now been identified using a phage-displayed peptide library. The amino-acid residues in SMN required for antibody binding are the same as the five most important contact residues for interaction with gemin2. MANSMA12 immuno-precipitated SMN and gemin2 from HeLa cell extracts as efficiently as mAbs against other SMN epitopes or against gemin2. We explain this by showing that SMN exists as large multimeric complexes. This SMN epitope is highly-conserved and identical in human and mouse. To explain the vigorous immune response when mice are immunised with recombinant SMN alone, we suggest this region is masked by gemin2, or a related protein, throughout development, preventing its recognition as a "self-antigen". The epitope for a third mAb, MANSMA3, has been located to eight amino-acids in the proline-rich domain of SMN.

1. Introduction

SMN (survival-of-motor-neurons) is the protein affected by mutation in Spinal Muscular Atrophy (SMA), a neuromuscular disorder characterised by loss of motor neurons, and the severity of SMA is inversely related to functional SMN levels [1,2]. The SMN core complex, consisting of SMN with seven additional gemins, gemin2–8, and unrip (unr interacting protein), assembles U snRNPs in the cytoplasm and transports them into the nucleus for assembly into the spliceosome [3], although a different function of SMN, transport of mRNA along axons of motor neurons, may be involved in causing SMA [4]. Gemin2 (also known as SIP1) has a major role, since one face of the protein interacts with SMN [5] while the opposite face interacts with a pentamer of the Sm core proteins, D1/D2/F/E/G, which bind snRNA to form the U snRNP [6].

Since they were first produced over 10 years ago [7,8], the monoclonal antibodies (mAbs), MANSMA1 (also known as 11F3) and MANSMA12 (also known as 2E6), against human SMN have been used for immunolocalisation [9], western blotting [10] and immunoprecipitation of SMN for proteomics [11,12] and have been applied to ELISA measurements on SMA patient samples [13] and mouse [2] and zebrafish [14,15] model studies of SMA. We now report the accurate mapping of the binding sites on SMA for these mAbs, using a phage-displayed library of random peptides [16]. The unexpected finding that both mAbs bind to the highly-conserved SMN sequence involved in gemin2 binding has important implications for the structure and stability of SMN complexes.

2. Materials and methods

2.1. Cell extraction

HeLa cells were extracted as described previously [13]. Briefly, 5×10^8 HeLa cells grown in suspension culture were collected by centrifugation, washed with PBS and homogenised in four volumes of modified RIPA buffer (0.5% sodium deoxycholate, 2% NP-40, 2 mM EDTA, 300 mM NaCl, 100 mM Tris–HCl, pH 7.4, 0.1% bovine serum albumin) using a pellet pestle. The extract was then centrifuged for 10 min at 13,000*g* to remove any insoluble cell debris.

2.2. Immunoprecipitation

Anti-mouse Pan Ig-coated magnetic beads (50 μl) (Dynal, Oslo, Norway) were washed three times with 4% BSA/PBS (using a magnetic collector to separate the beads from solution). The beads were then incubated with the following monoclonal antibodies (10 μg in each case) for 30 min at room temperature with gentle rolling: MANSMA12 against SMN, MANSMA4 against SMN, MANSIP1 against gemin2, or MANEM5 against emerin (as a control for non-specific binding). After washing the beads four times with

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MANSMA1:

SDIWDDTALIK**AYD**K**AVA**SF SMN sequence (aa31-50)

LGCD**D**T**AVA**HWPWVY peptide1

SD**AYD**T**AVA**HWPGFI peptide2

IWDDTA**L**IK**AY**DK**AV**ASF **gemin2 binding residues (from ref. 6)**

MANSMA12:

SDIW**DDT**A**LI**KA**Y**DK**A**VASF SMN sequence (aa31-50)

MPPN**LI**AN**Y**ES**A**LSR peptide3

YESPGFLLSGY**DDT**G peptide4

MANSMA3:

LPPF**PSGP**P**I**I**P**PPPP SMN sequence (aa237-244)

WAAH**PSGP**S**I**V**P**RSL peptide5

Fig. 1. Epitope mapping of three mAbs against SMN using phage-displayed peptides. The amino-acid residues that match between the human SMN sequence and the peptide sequences are shown underlined and in bold face. Also shown underlined are the important amino-acids in SMN for gemin2 binding [5]. The peptides selected by MANSMA1 were not selected by MANSMA12, nor vice versa, showing that MANSMA1 and 12 recognise overlapping epitopes in the same region.

PBS (600 μl), the beads were then incubated for 1 h at room temperature with the HeLa cell extract with gentle rolling. The unbound material was collected from the beads and the immunoprecipitated material was eluted from the beads by boiling for 3 min in SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8).

2.3. SDS–polyacrylamide gel electrophoresis and western blotting

Protein extracts were prepared by boiling in SDS sample buffer (2% sodium dodecyl sulphate-SDS, 5% 2-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8). Proteins were subjected to SDS–PAGE using 12% polyacrylamide gels and transferred to nitrocellulose membranes by diffusion blotting. After blocking non-specific sites with 4% powdered milk solution, membranes were incubated with primary antibodies at a dilution of 1/100 in dilution buffer (PBS, 1% foetal bovine serum, 1% horse serum and 0.1% BSA). Antibody reacting bands were visualised by development with peroxidase-labelled goat anti-mouse Ig (1 μg/ml in dilution buffer) and a chemiluminescent detection system (West Femto, Pierce).

2.4. Epitope mapping with phage-displayed peptides

Epitope mapping using phage-displayed random peptide libraries in filamentous phage was performed as previously described [17] using a modification of an earlier method [16]. Monoclonal antibody mixtures were diluted 1:50 with Tris–buffered saline (TBS) and immobilised onto sterile 35 mm Petri dishes coated directly with 1 ml of 1:200 dilution of rabbit-anti-[mouse Ig] in TBS (DAKOpatts, Denmark). Biopanning was performed using a 15-mer peptide library in phage f88–4, maintained in the K91Kan strain of *Escherichia coli* and generously supplied by G.P. Smith (University of Missouri). Any remaining binding sites on the dishes were blocked using 4% BSA in sterile TBS. A sample of the phage

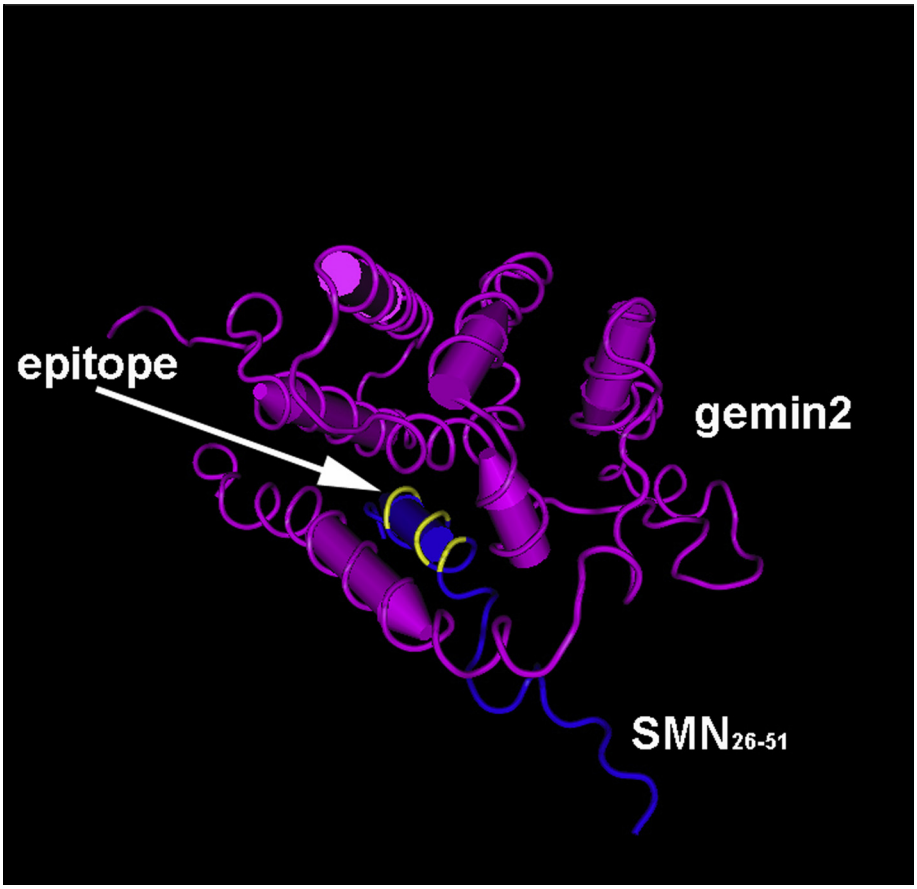

Fig. 2. The interaction site on SMN for gemin2. The published 3D structure of a gemin2-SMN complex [5], NCBI accession number 2LEH, was rotated and highlighted using the NCBI programme, Cn3D. The five most important amino-acids for gemin2 binding are highlighted.

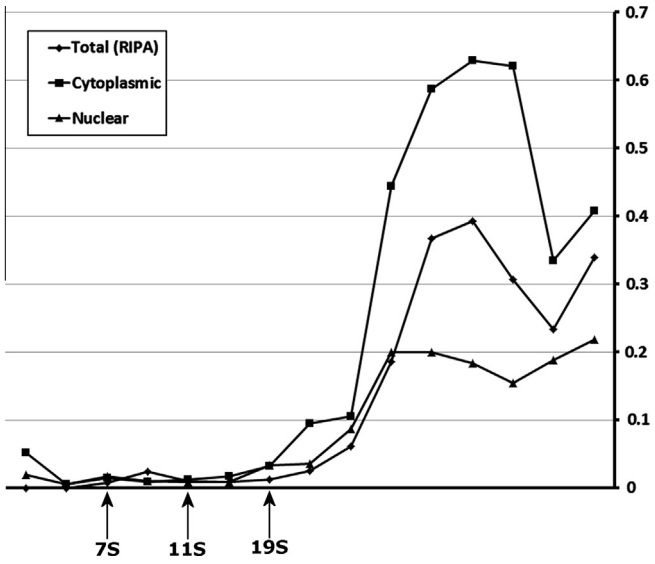

Fig. 3. SMN is present in HeLa extracts as very large, multimeric complexes. RIPA extracts of HeLa cells were analysed on a 35 ml 15%–30% sucrose density gradient in 150 mM NaCl, 50 mM Tris–HCl pH 7.4 and 5 mM $MgCl_2$ by centrifugation in a Beckmann SW28 rotor at 25,000g for 21 h at 4 °C. Thirty fractions were collected and every other fraction was concentrated 10fold using Strataclean resin (Stratagene, Amsterdam, Netherlands) for analysis by ELISA with MANSMA1 anti-SMN mAb. Nuclear and cytoplasmic extracts were prepared as described in [10]. The size markers were immunoglobulin (7S:150 kD), catalase (11S:250 kD) and thyroglobulin (19S:660 kD).

library (10^{13} virions) was pre-incubated in dishes coated with the rabbit anti-mouse antibodies alone to ensure any binding was specific for the target mAbs. Following the first round of biopanning, the bound phage were eluted and amplified by infection of K91Kan *E. coli* cells. Two rounds of biopanning were performed. Individual colonies of the phage-infected cells after the second round were grown on nitrocellulose membrane (BA85) and screened by western blotting to reveal positive clones. Positive clones were subjected to western blotting with individual mAbs from the mixture used for biopanning. After blocking non-specific sites with 5% skimmed milk protein in TBS, membranes were incubated with mAb supernatant (1/100 dilution in TBS). Antibody-reacting clones were visualised following development with biotinylated horse anti-mouse Ig in a Vectastain ABC kit (Vector Labs, Burlingame, CA) and diaminobenzidine substrate (Sigma; 0.4 mg/ml). Phage DNA was purified from positive clones by the phenol/chloroform method and sequenced using primer: 5′-AGTAGCAGAAGCCTGAAGA-3′.

3. Results

We used a mixture of 22 mAbs against SMN [7,8] to select phage particles from a library of random 15-mer peptides expressed in high copy number on the coat protein of fd-88 filamentous phage. We found that only three of these 22 were responsible for the selection process. These mAbs included two of the mostly widely-used for SMA studies, MANSMA1 and MANSMA12, and one mAb against the proline-rich region encoded by exon 5, MANSMA3. Biopanning with the remaining 19 anti-SMN mAbs alone failed to select any further phage clones. Four phage clones were sequenced for each mAb and MANSMA1 and MANSMA12 each produced two unique peptide sequences, but all four clones for MANSMA3 contained the same peptide sequence (Fig. 1). MANSMA1 and MANSMA12 recognise overlapping epitopes near the *N*-terminus (exon 2a) and none of the peptides were recognised by both mAbs (Fig. 1). This is consistent with previous mapping to exon 2 using recombinant SMN fragments [8] and suggests that the important contact residues are different for MANSMA1 and MANSMA12. The MANSMA3 epitope was identified with a single phage peptide sequence but the match is highly significant with six amino-acid identities within an eight amino-acid sequence (Fig. 1). This mAb had previously been mapped to exon 5 by its failure to bind a recombinant SMN isoform lacking the region encoded by exon 5 [8].

A recent study of SMN-gemin2 interaction identified the contact residues in SMN required for gemin2 binding and these were L39, A42, Y43, A46 and V47 (highlighted in Fig. 2). The MANSMA1 epitope includes four of these [A42, Y43, A46, V47] while the MANSMA12 epitope includes three [L39, Y43, A46] (Fig. 1). We were interested in whether MANSMA1 and gemin2 can bind to SMN at the same time, although this seems unlikely when they share a binding site. However, SMN complexes are multimeric and contain many copies of SMN and gemin2, as shown by sucrose density gradient centrifugation of a HeLa cell extract with nearly all the SMN in large complexes near the bottom of the gradient (Fig. 3). This means that it would be possible for MANSMA1 or MANSMA12 to bind to one SMN molecule in a complex and bring down several other SMN molecules with gemin2 still attached. A HeLa cell extract was immunoprecipitated with MANSMA12 and the SMN and gemin2 content of bound and unbound fractions was determined by western blotting. All of the SMN was removed from the extract, but only half of the gemin2 (Fig. 4). This could mean either that gemin2 is being displaced from SMN by MANSMA12 binding or that some gemin2 exists unbound to SMN. However, an anti-gemin2 mAb, MANSIP1A, pulled down an equal amount of SMN and gemin2 (Fig. 4), so there is no evidence for a pool of monomeric gemin2. We hypothesised that a mAb against a different region of SMN might co-immunoprecipitate more gemin2, but MANSMA4, which binds to the exon 4 tudor domain of SMN, gave a similar result to MANSMA12 (Fig. 4).

4. Discussion

The SMN sequence between aa19 and aa62 (all of exon 2a and part of exons 1 and 2b) is identical between human and mouse and

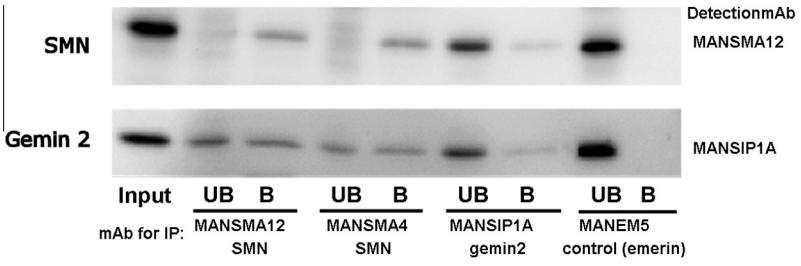

Fig. 4. Co-immunoprecipitation of SMN and gemin2 from HeLa cell extracts. Each of the four mAbs shown was used to immunoprecipitate (IP) from HeLa extracts using Dynabeads. The bound (B) and unbound (UB) proteins were separated by SDS–PAGE and duplicate western blots were developed for either SMN (MANSMA12) or gemin2 (MANSIP1A). The results show that IP with either MANSMA12 (exon 2) or MANSMA4 (exon 4) mAbs against SMN co-precipitates a similar proportion (ca. 50%) of the input gemin2. The control mAb, MANEM5 against emerin [29], does not immunoprecipitate either SMN or gemin2, which are left unchanged in the unbound (UB) fraction.

this contains the binding site for gemin2, MANSMA1 and MANSMA12. Furthermore, 16 additional anti-SMN mAbs also recognise exon 2, though their exact epitopes could not be mimicked by phage displayed peptides, and they also recognise both human and mouse SMN [8]. Such a highly-conserved sequence would not normally be expected to elicit an immune response in mice. However, if the gemin2 binding site is always obscured by gemin2 *in vivo*, the exposed sequence on a recombinant SMN immunogen would be recognised as "foreign" by the immunised mouse and the mechanisms preventing the activation of B-cells against self-antigens would not be engaged [18,19]. The implication is that SMN and gemin2 form a stable heterodimer, shielding the binding site from immune recognition. Gemin2 is known to stabilise SMN and extend its half-life [20,21], possibly by blocking a potential ubiquitinylation site (K41 [22]) that would target it for degradation. Our observation that mAbs against the gemin2-binding site on SMN can immunoprecipitate both SMN and gemin2 (Fig. 4) is a likely consequence of the formation of multimers by SMN (Fig. 3). Thus, one mAb molecule could displace gemin2 from one SMN molecule but still be able to bring down multimeric complexes containing both SMN and gemin2. Another example of a mAb able to do this is MANDAG1 against the dystrophin binding site of beta-dystroglycan; this mAb is able to bind to dystroglycan at the sarcolemma of skeletal muscle sections, provided no cross-linking (e.g., formalin fixation) had occurred to prevent competition with dystrophin [23]. Immunolocalisation of SMN by the mAbs described here is also inhibited by pre-treatment of cells or tissues with cross-linkers [our unpublished data].

Our observation that MANSMA12 brings down all the SMN in a HeLa extract, but only part of the gemin2, could be due to displacement of gemin2 from SMN by the mAb, even though MANSMA4 against a different region (tudor domain) also brought down SMN and a similar proportion of gemin2. Gemin2 binds to 5 members of the seven-membered ring of Sm core proteins [6], but the remaining two members B(B') and D3 have symmetrically-dimethylated RG domains which would enable them to interact with the tudor domain of SMN itself [24]. It is possible, therefore, that mAb binding to the SMN tudor domain could disrupt other interactions within the SMN complex. Further evidence that gemin2 is not irreversibly bound to SMN is the observation that a fibroblast growth factor, FGF-2, can compete with gemin2 for SMN binding [25].

There is an SMA-causing mutation (D44V) within the gemin2 binding site though the evidence for its effect on gemin2 binding is divided: one study found no effect [26] while another found complete loss of gemin2 binding [6]. It is possible that the truth lies between these two extremes, since D44 is not a contact residue for gemin2 [5] and D44V causes only a mild type III form of SMA [26], whereas complete absence of either SMN [27] or gemin2 [28] is embryonic lethal in mice.

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