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# RGG Repeats of PrP-like Shadoo Protein Bind Nucleic Acids

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Supporting Information

**ABSTRACT:** Shadoo (Sho) is a central nervous system glycoprotein with characteristics similar to those of the cellular prion protein $PrP^C$, each containing a highly conserved hydrophobic domain (HD) and an N-terminal repeat region. Whereas $PrP^C$ includes histidine-containing octarepeats, the Sho region N-terminal to the HD includes tandem positively charged "RGG boxes", predicted to bind RNA. Here, we demonstrate that Sho binds DNA and RNA in vitro via this arginine-rich region.

Misfolded proteins that drive prion disease pathogenesis are derived from $PrP^C$, encoded by the *Prnp* gene on mouse chromosome 2. The PrP-like protein Sho was predicted from genomic analyses of fish and a cognate mouse gene *Sprn* identified on chromosome 7.[1] Sho shares characteristics with $PrP^C$, including a central HD, cleavage to metabolically stable "N1" and "C1" fragments, N-linked glycosylation, a GPI anchor, and a partially overlapping pattern of CNS expression.[2−4] Recently, bioinformatics analysis identified an RGG box motif in Sho, predicting an ability to bind RNA.[5] The RGG motif was first identified in the heterogeneous nuclear ribonucleoprotein (hnRNP) U protein[6] but is also found in nucleolin and in Npl3p.[7,8] Here we appraised Sho's RGG box motif and surrounding sequences in the context of nucleic acid binding.

The N-terminus of mouse Sho (amino acids 25−61) was prepared as a glutathione *S*-transferase (GST) fusion protein. Control PrP-based fusion proteins included PrP28−97, which excludes two positively charged regions of residues 23−27 and 100−105, and PrP28−105, which includes one positively charged region (Figure 1). These fusion proteins from *Escherichia coli* lysates were chromatographed on glutathione affinity resin, and released material was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and silver staining (Figure 2A) (see the Supporting Information for the detailed methodology). A gold-colored stain was apparent in material from the Sho fusion protein columns. As silver staining detects nucleic acids in addition to proteins,[9] we inferred that the gold-colored material might represent nucleic acids scavenged from the *E. coli* lysates. To assess this, SDS−PAGE gels were stained with the nucleic acid-intercalating reagent ethidium bromide (EtBr). In Figure 2B, EtBr-stained material was specific to the GST−Sho columns and was absent from the column containing PrP28−97. $PrP^C$ is able to bind nucleic acids in vitro via positively charged regions (amino acids 23−27 and 100−105),[10−12] and when one of these regions was reintroduced in the form of the GST−PrP28−105 fusion protein, a nucleic acid signal was detected (Figure 2C). Because these experiments were performed using a fragment of Sho, it was possible that amino acids in the C-terminus of Sho may interfere with the interaction with nucleic acids. To exclude this possibility, purified full-length recombinant Sho was analyzed by SDS−PAGE and EtBr staining to determine if nucleic acids could be detected; as seen in Figure 2D, a signal was observed. Furthermore, staining of material from the Sho columns disappeared after RNase treatment (Figure 2E), indicating that the bulk nucleic acids bound to Sho from the *E. coli* lysates were RNA.

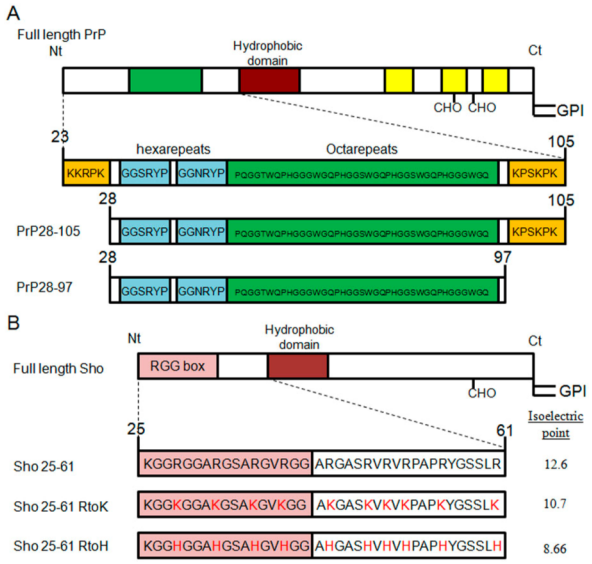


**Figure 1.** PrP (A) and Sho (B) N-terminal fragments fused to GST. The isoelectric points of Sho fusion fragments are shown: Nt, N-terminus; Ct, C-terminus; CHO, glycosylation sites; GPI, glycophosphatidylinositol anchor. All proteins have GST fused to the N-terminus of the fragments.

To determine if Sho could also bind DNA, bacterial lysates containing Sho25−61 protein were treated with cyanase nuclease to degrade all nucleic acids. After the nuclease had been removed, supernatants containing Sho− or PrP−GST fusion proteins were bound to glutathione-linked Sepharose. DNA was added, nonspecific material removed by washing, and material from the column analyzed (Figure 2F). Only the column containing the Sho25−61 fusion protein retained DNA.

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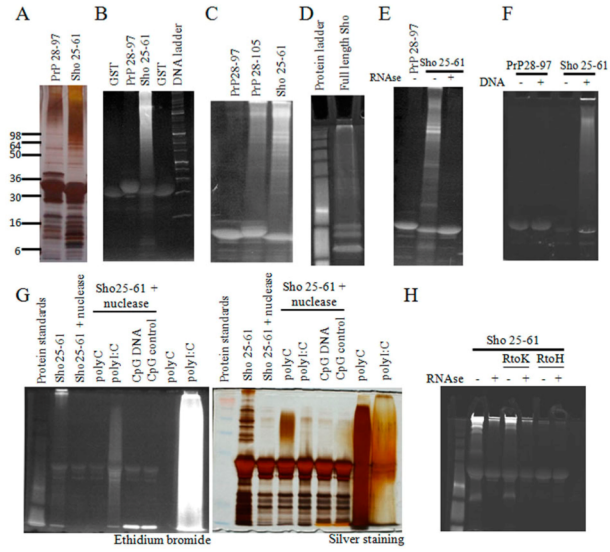


**Figure 2.** Affinity chromatography analysis indicates that Sho binds RNA and DNA. Silver staining (A) or EtBr staining (B) analysis of material released from affinity chromatography matrices loaded with Sho or PrP fusion proteins. EtBr staining analysis of material from a control PrP28−105−GST column (C), purified recombinant full-length Sho (D), and material from columns containing Sho25−61 fusion protein after RNase treatment (E). Material from columns containing fusion proteins pretreated with cyanase nuclease and incubated with DNA (F), polyC, polyI:C, CpG, or CpG control (G) was examined by EtBr staining or silver staining. Analysis of material from columns containing Sho25−61 mutants RtoK and RtoH using EtBr (H).

To further assess the nucleic acid binding ability of Sho, cyanase nuclease-treated bacterial lysates containing Sho25−61 protein were bound to glutathione-linked Sepharose. Synthetic ssRNA polyC or dsRNA polyI:C was then added, and after the column had been washed, material from the column was analyzed. EtBr analysis showed that the Sho column retained dsRNA polyI:C (Figure 2G), while subsequent silver staining indicated that Sho25−61 bound ssRNA polyC as well (Figure 2G). In naturally occurring ssRNA, secondary structure[13] may allow intercalation and detection by EtBr. These structures do not exist in polyC ssRNA, resulting in a lack of signal with EtBr staining. Furthermore, we examined whether Sho was able to differentially bind CpG DNA and its control ("CpG control", containing GpC residues). Bacterial DNA contains CpG motifs at a higher frequency than mammalian DNA, and these motifs are unmethylated, unlike in mammalian DNA.[14] As seen in Figure 2G, the Sho columns retained both unmethylated CpG DNA and a control GpC-containing 22-mer. This is in contrast to Toll-like receptors (TLRs) that can specifically detect unmethylated CpG DNA to initiate an immune response.[15]

The nucleic acid binding ability of Sho could be due to the positively charged arginine (R) residues of the RGG box motif and surrounding amino acids interacting with the negatively charged DNA or RNA. To examine this hypothesis, the arginine residues were mutated to lysine (K) or histidine (H) (Figure 1B). Analysis of material from columns containing these mutants indicated that Sho25−61 RtoK retained an ability to bind RNA, whereas the Sho25−61 RtoH mutant did not (Figure 2H). This was not unexpected because lysine residues are well characterized in peptide−nucleic acid interactions of PrP and other proteins.[10−12,16] While histidine side chains may be positively charged at neutral pH, their role in binding is minimal when nonspecific interactions are occurring (i.e., non-sequence-specific).[16]

Bioinformatics analyses by Corley et al. identified RGG boxes in the N-terminus of Sho,[5] and here, we demonstrate that Sho binds RNA and DNA in vitro. The nucleic acid binding most likely occurs through an electrostatic interaction in which the arginine residues create a positively charged region to bind the phosphodiester backbone of RNA or DNA. Given the involvement of an RGGGGR palindrome in a structure of an "RGG" protein bound to a synthetic RNA,[17] the simple tandem repeat nature of the RGG motifs in Sho, and the properties of known RGG box proteins,[7,8] recognition of specific nucleotide sequences by Sho seems unlikely at this juncture. This is supported by data shown here; Sho was able to bind different forms of DNA and RNA, including polynucleotides. Furthermore, mutation of arginine residues to lysines, which may bind nucleic acids nonspecifically,[16] had no effect; in contrast, mutation to histidines, which are proposed to be involved in specific protein−nucleic acid interactions,[16] interrupted binding.

Interestingly, $PrP^C$ binds both DNA and RNA through its N-terminus,[10,12] most likely via two lysine-rich regions,[18] and in a non-sequence-specific manner.[19] Interactions with nucleic acids may be involved in PrP aggregation, where different aggregates form depending on the size and sequence of the nucleic acid.[19] Others have suggested a strain-specific requirement for RNA in $PrP^{Sc}$ replication.[20] In healthy cells, an innate immunity Toll-like receptor function of $PrP^C$ [21] may involve recognition of viral nucleic acids. Additionally, because nucleic acids can be found in the extracellular matrix and circulating in the blood, they may act as signaling molecules between cells.[22] Although a TLR9-like function for Sho is unlikely, given the binding to CpG control DNA (Figure 2G), the possibility of binding extracellular nucleic acids to initiate downstream functions remains; nevertheless, anatomical−cell biological parameters will place constraints on in vivo events. The bulk of both Sho and $PrP^C$ are GPI-linked, synthesized in the secretory pathway, and displayed at the cell surface.[2,4] In differentiated neurons of the hippocampus, immunohistochemical signals are not notable in cell bodies or nuclei, indicative of axonal and/or dendritic trafficking.[23] However, forms of $PrP^C$ (and perhaps analogous forms of Sho), which enter the cytoplasm through "leaky" signal peptide recognition,[24] have the potential to interact with intracellular nucleic acid pools. Recent data using *Sprn*$^{0/0}$ mice as negative controls have defined staining in the cell bodies of some hypothalamic neurons of wild-type mice, and a portion of wild-type Sho in brain and cultured cells is found in histone-containing fractions in simple subcellular separations.[23] Thus, while effects of the positively charged N-terminus of Sho on protease docking[25] and transit from lipid rafts to clathrin-coated pits[26] cannot be excluded, the seeming exceptions to bulk trafficking are candidates for biologically relevant Sho−nucleic acid interactions. Exploring the neuroprotective properties of Sho[4] in knockout and genetically reconstituted animals may provide a forum for evaluating these ideas.

## ■ ASSOCIATED CONTENT

### Supporting Information

Materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

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## ABBREVIATIONS

CNS, central nervous system; EtBr, ethidium bromide; GPI, glycophosphatidylinositol; GST, glutathione *S*-transferase; $PrP^{C}$, cellular prion protein; $PrP^{Sc}$, infectious prion protein; Sho, Shadoo; TLR, Toll-like receptor; HD, hydrophobic domain.

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