Interaction between Sin Nombre Hantavirus Nucleocapsid protein (N) and Ribosomal protein S19 (RPS19)

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Introduction

Hantaviruses, bunyaviradae family members, cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) with mortalities of up to 15% and 50%, respectively (1). Hantaviruses are enveloped, negative strand RNA viruses with three genomic segments L, M and S encoding viral RNA dependent RNA polymerase (RdRp), glycoproteins (G1 and G2) and nucleocapsid protein (N), respectively. Each of the three segments contain partially complementary nucleotides at the 5' and 3' termini that undergo base pairing and form panhandle-like structures. Nucleocapsid protein (N) plays diverse roles during hantavirus infection. Primarily, N protein is involved in the encapsidation and packaging of viral genome. Hantavirus N protein helps in the preferential translation of viral mRNAs by specifically binding to the 5'UTR of viral mRNAs and recruiting translation initiation machinery (2,3). N protein also binds 5' Cap and facilitates translation initiation by acting as eIF4F surrogate. We have recently found that N protein interacts with 40S ribosomal subunit via ribosomal protein S19 (RPS19) (4). In this study, we mapped N protein for RPS19 binding domain and asked whether N protein deficient in RPS19 binding augments reporter mRNA translation.

Material & Methods

Fluorescence binding: Fluorescence binding studies were carried out as previously reported (5), except the percentage of wild type or variant N bound to panhandle-like RNA structure at each input concentration of the RNA was calculated from equation 1. Percentage bound = $\Delta F/\Delta F_{max}$,100, (Eq.1), Where ΔF is the change in fluorescence signal at 330 nm at each addition of RNA. ΔF_{max} is the same parameter when N is totally bound to the panhandle-like RNA structure. Double reciprocal plot ($1/\Delta F$ versus $1/C_{p,}$) was used to calculate the value of ΔF_{max}, using equation 2. C_p is the input RNA concentration. $1/\Delta F = 1/\Delta F_{max} + K_d/(\Delta F_{max}(C_p)$ (Eq.2). A plot of percent bound N versus RNA concentration was used for the calculation of apparent dissociation constant (K_d), which corresponds to the concentration of RNA required to obtain half saturation, assuming that complex formation obeys a simple bimolecular equilibrium.

Expression and purification of hantavirus N protein Wild type SNVN was expressed in E.coli as C-terminal His tagged protein and purified using NiNTA beads, as previously reported (6). Same procedure was used for the purification of SNVNΔ151-175 mutant (7).

T7 Transcription for RNA synthesis: The panhandle-like structure of viral S-segment RNA, capped and uncapped decameric RNA 5′GAUAUGUGAG3′ were synthesized by *in vitro* T7 transcription reaction (7).

Refer to Ganaie SS *et al.* BJ. 2014 PMC4315616 for other methods.

Flow cytometry: CD measurements
Luciferase assay
Translation assays in rabbit reticulocyte lysates
RNA Filter binding
T7 Transcription for RNA synthesis
Immunoprecipitation analysis

Results

1. Mapping of SNV-N for RPS19 binding domain.

Using deletion mutagenesis approach to generate panel of mutants (Fig. 1 A&D) , we confirmed by co-immunoprecipitation method that RPS19 binding domain is present in the N terminus of N between the amino acids 151 to 175 by (Fig. 1 B,C,E and F).

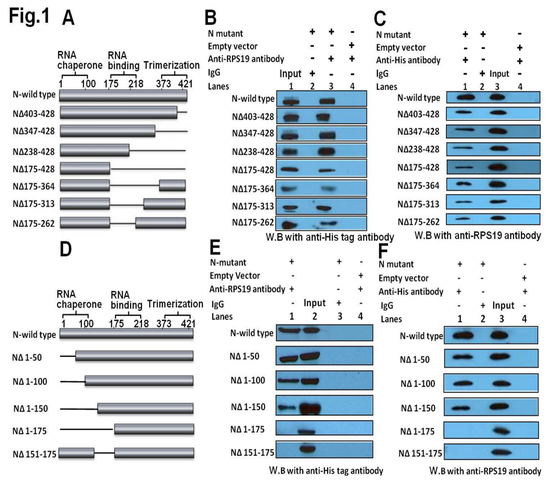

Fig. 1: Diagrammatic representation of wild type N and N mutants used in this study. Thin line represents the deletion pattern (Panel A &D). HeLa cells transfected with either empty vector or plasmid expressing N and N mutants. Cell lysates were used to pull down RPS19 protein using anti-RPS19 antibody or N and N mutants using anti-His tag antibody. On western blot analysis, pulldowns were analyzed for the proteins of interest using either anti-His tag antibody for N and N mutants (panels B and E) or anti-RPS19 antibody for RPS19 protein (panels C and F). (For further information see Ganaie SS *et al.* Biochem. J. 2014 PMC4315616)

2. Structural similarity between wt-N and SNVNΔ151-175 and N151-175 binding to RPS19.

On comparing CD spectra of wt-N and N-mutant, we found that there was no obvious change in the secondary structure of wt-N on deletion of RPS binding domain (Fig 2A&B) and N151-175 was ableto pull down RPS19 when fused at the C terminus of GFP. (Fig 2C)

Fig. 2

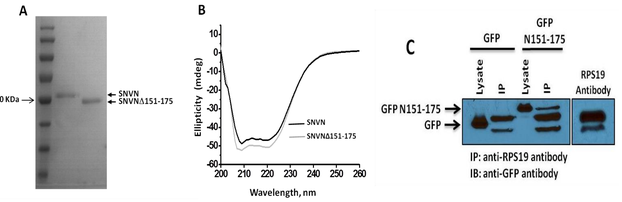

Figure 2: SDS-PAGE showing the purified wild type N and NΔ151-175 variant (Panel A). Far-UV CD spectra of the wild type N (19 μM) and NΔ151-175 variant (13 μM) in phosphate buffer, PH 8.0 at 25 °C (Panel B). HeLa cells were transfected with plasmids expressing either GFP or GFP fused with the N-terminus of RSP19 binding domain of N protein (GFPN151-175). Cells were lysed 48 hours post-transfection and resulting lysates were immunoprecipitated using anti-RPS19 antibody. The immunoprecipitated material (IP) was examined by western blot analysis using anti-GFP antibody. The RPS19 antibody was run as control to show that secondary antibody used in western blot also detected the anti-RPS19 antibody used in immunoprecipitation (7).

4. Characterization of SNVNΔ151-175 for Cap binding.

On studying the interaction between capped or uncapped decameric RNAs with purified wild type N or SNVNΔ151-175 mutant using RNA filter binding assay, we found that N variant lacking RPS19 binding domain binds mRNA 5'cap similar to wild type N (Fig 4)

Fig. 4

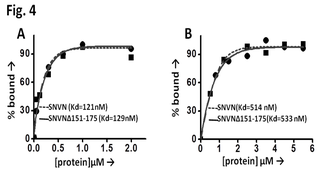

Figure 4: Binding profiles for the interaction of wild type N (filled square) and SNVNΔ151-175 variant (filled circle) with a capped (panel A) and uncapped (panel B) decameric RNA. (For further information see Ganaie SS *et al.* Biochem. J. 2014 PMC4315616)

3. Deletion of RPS19 binding domain and N-mediated translation.

Deletion of RPS19 binding domain abrogated N mediated translation both in-vivo using reporter mRNA (firefly luciferase and GFP) and in-vitro using luciferase mRNA. On inhibiting cap dependent translation (using 4E1 Rcat), deletion of RPS19 domain didn't support translation,

Fig. 3

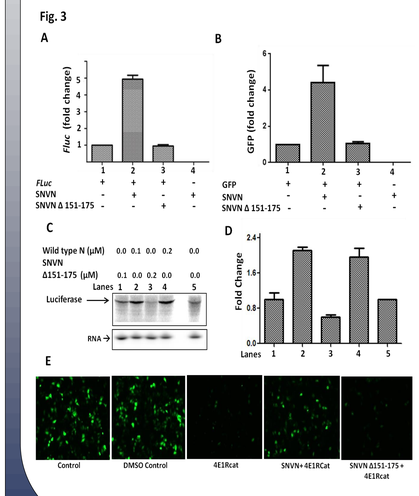

Figure 3:: Panel A and B: HeLa cells expressing firefly luciferase (panel A) or GFP (panel B) and wild type N or SNVNΔ151-175 expressing SNVNΔ151-175 variant of N to show the effect of deleting RPS19 binding domain on N mediated augmentation of reporter mRNA translation. Error bars represent the standard deviation, calculated from three independent experiments. Panel C Translation of luciferase mRNA in rabbit reticulocyte lysates in the absence (lane 5) or the presence of increasing concentrations of either purified wild type N (lanes 2 and 4) or SNVNΔ151-175 variant (lanes 1 and 3). Translation products were radiolabeled with S^{35} Methionine during synthesis and examined by phosphorimage analysis (panel C top). Radiolabeled luciferase mRNA was translated in rabbit reticulocyte lysates without S^{35} Methionine and examined by phosphorimage analysis to determine the integrity of luciferase mRNA in the translation reaction (panel C, bottom). Panel D. The intensity of bands in the Panel C was quantified, normalized to the intensity of the band in the lane 5 and plotted. Data from two independent experiments was used to generate error bars. Panel E: HeLa cells were cotransfected with GFP expression construct along with either empty vector (left three panels) or pSNVN (second panel from right) or pSNVNΔ151-175 (right panel) for the expression of wild type N and SNVNΔ151-175 variant, respectively. Eighteen hours post transfection, cells were incubated with either DMSO control or 4E1RCat for twelve more hours and visualized under fluorescence microscope (7).

5. Characterization of SNVNΔ151-175: Trimerization and viral Panhandle binding

An analysis by semi-native gel revealed that both wild type and SNVNΔ151-175 mutant formed trimers (Fig 5A). Using florescence spectroscopy, it was found that N mutant lacking RPS binding domain bind vRNA panhandle similar to wt-N, the hallmark of functional trimers (Fig. 5C-F).

Fig. 5

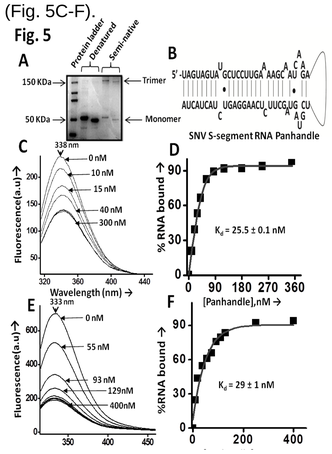

Figure 5 Semi-native PAGE analysis to determine the trimerization of wild type N and SNVNΔ151-175 variant (Panel A). Denatured proteins were loaded in lanes 2-3 and semi-native proteins were loaded in lanes 4-5. Wild type N is shown in lanes 2 and 4 and SNVNΔ151-175 variant is shown in lanes 3 and 5. Panel B: The sequence of SNV S-segment vRNA panhandle-like structure used in fluorescence binding. Purified trimeric wild type N (panel C) and SNVNΔ151-175 variant (panel E) at a concentration of 147 nM each in RNA binding buffer, were excited at 295 nm, and tryptophan fluorescence emission spectra were recorded from 310-500 nm (black line). Binding profile for the interaction of wild type trimeric N (panel D) and SNVNΔ151-175 variant (panel F) with the panhandle-like RNA structure (7)..

Conclusions

- Identification of RPS19 binding domain at the N-terminus of N protein between the amino acids 151 to 175.
- Fusion of N151-175 amino acids with GFP was sufficient to pull down RPS19.
- Wt-N augmented reporter mRNA translation both i*n-vivo* as well as *in-vitro,* while as N-mutant deficient in RPS19 binding didn't.
- Inhibition of cap dependent translation of reporter mRNA (GFP), in particular was rescued only by wt-N, but not the mutant N deficient in RPS19 binding.
- N-mutant deficient in RPS19 binding was able to bind mRNA cap, viral RNA panhandle and formed stable trimers similar to wt-N protein.
- N-mutant deficient in RPS19 binding was structurally similar to wt-N.

Acknowlegements

This work was supported by NIH grants RO1 AI095236-01 and 1R21 AI097355-01. and was published as part of the manuscript in Biochemical journal in 2014 (Ganaie SS et al. Biochem J. 2014 PMC4315616)

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