Structure of the Rift Valley fever virus nucleocapsid protein reveals another architecture for RNA encapsidation

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Rift Valley fever virus (RVFV) is a negative-sense RNA virus (genus \textit{Phlebovirus}, family \textit{Bunyaviiridae}) that infects livestock and humans and is endemic to sub-Saharan Africa. Like all negative-sense viruses, the segmented RNA genome of RVFV is encapsidated by a nucleocapsid protein (N). The 1.93-Å crystal structure of RVFV N and electron micrographs of ribonucleoprotein (RNP) reveal an encapsidated genome of substantially different organization than in other negative-sense RNA virus families. The RNP polymer, viewed in electron micrographs of both virus RNP and RNP reconstituted from purified N with a defined RNA, has an extended structure without helical symmetry. N-RNA species of \(\sim 100\)-kDa apparent molecular weight and heterogeneous composition were obtained by exhaustive ribonuclease treatment of virus RNP, by recombinant expression of N, and by reconstitution from purified N and an RNA oligomer. RNA-free N, obtained by denaturation and refolding, has a novel all-helical fold that is compact and well ordered at both the N and C termini. Unlike N of other negative-sense RNA viruses, RVFV N has no positively charged surface cleft for RNA binding and no protruding termini or loops to stabilize a defined N-RNA oligomer or RNP helix. A potential protein interaction site was identified in a conserved hydrophobic pocket. The nonhelical appearance of phlebovirus RNP, the heterogeneous \(\sim 100\)-kDa N-RNA multimer, and the N fold differ substantially from the RNP and N of other negative-sense RNA virus families and provide valuable insights into the structure of the encapsidated phlebovirus genome.

RNA viruses cause a myriad of human and animal diseases, including measles, poliomyelitis, rabies, the common cold, dengue fever, and Rift Valley fever. Despite tremendous diversity among RNA viruses, all must package a protected RNA genome into virus particles. RNA viruses protect their genome in one of two ways, providing either a protein shell or a protein coat for the genome (1). The process is generally known by the term encapsidation, but functionally and structurally encapsidation takes a variety of forms. Most positive-sense RNA and double-stranded RNA viruses place their genome within a protein shell, known as a capsid. By contrast, the negative-sense RNA viruses encapsidate their genome by coating the length of the RNA with a nucleocapsid protein (N). Although capsid and N all bind RNA, the resulting RNA-protein complexes differ, and it is not possible to make generalizations about the proteins involved in encapsidation across all RNA virus families.

Rift Valley fever is a mosquito- and aerosol-borne disease of livestock and humans in sub-Saharan Africa and is caused by Rift Valley fever virus (RVFV). Rift Valley fever in domestic ruminants results in abortion and high rates of mortality, especially among young animals (2). In humans, Rift Valley fever is typically a self-limited febrile illness, although severe disease, such as hemorrhagic fever and encephalitis, occurs in a small percentage of cases (2). No effective therapeutics exist for treating Rift Valley fever. RVFV has a membrane envelope and a genome composed of three negative-sense RNA segments, termed small (S), medium (M), and large (L) (2). It belongs to the \textit{Phlebovirus} genus in the \textit{Bunyaviridae} family. As with all negative-sense RNA viruses, the genome is bound, or encapsidated, by N. The N of RVFV is a 27-kDa protein encoded by the S segment. Encapsulation of RVFV genomic RNA, as with all negative-sense RNA viruses, plays an essential role in multiple steps within the replicative cycle including transcription and replication by the RNA-directed RNA polymerase (RdRp) and packaging of genome into virions (3). RVFV N is thought to interact with the viral RdRp because N is essential to replication and transcription (4). N also plays a role in virus assembly through interactions with the viral envelope glycoproteins (\(G_N\) and \(G_C\)) (5).

Bunyavirus N binds single-stranded RNA (ssRNA) nonspecifically (6, 7), although some N may have a preference for specific viral RNA sequences (8–10). Studies on some animal viruses within the \textit{Bunyaviridae} family found that encapsidated RNA is resistant to disruption by high salt and Ribonuclease treatment (8, 10, 11). Despite the common property of tight, nonspecific binding to single-stranded RNA, homology of N within the \textit{Bunyaviridae} family is not apparent from sequence data, because N from different genera appear unrelated. However, within a genus, the N are clearly homologous. When RVFV N is compared across the \textit{Phlebovirus} genus, the amino acid identity generally ranges from 50% to 59% and is 36% for Uukuniemi virus, the most divergent clade within the \textit{Phlebovirus} genus. The high degree of sequence identity indicates that the phlebovirus N have similar structures and form similar RNPs. Additionally, the phlebovirus N are distantly related to the N of the \textit{Tenuivirinae}, a genus of negative-sense RNA viral plant pathogens with worldwide distribution (12). Otherwise, the phlebovirus N appear unrelated to N of other negative-sense RNA viruses.

Structures are available for N from several negative-sense RNA viruses, including influenza A virus (FLUA) (13), rabies virus (RABV) (14), human respiratory syncytial virus (HRSV) (15), vesicular stomatitis virus (VSV) (16), and Borna disease virus (BDV) (17). For some of these, ribonucleoprotein (RNP) complexes have been visualized by crystallography or electron microscopy (FLUA) (18), (RABV) (14), (HRSV) (15), (VSV) (16). The crystallized RNP oligomers are high-order ring structures formed by specific contacts of loops or chain termini of neighboring N subunits. In some viruses, the number of subunits in the ring matches the helical repeat of the RNP polymers (15).


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Data deposition: The coordinates and structure factors for the crystal structure have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 3LYF).

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None of the structurally characterized N is from the *Bunyaviridae* family and none has detectable homology with the phlebovirus N. Early electron micrographs of encapsidated bunyavirus genomes reveal a noncondensed, macrocircular form that appears to lack symmetry (19, 20). Nevertheless, negative-sense RNA viruses are assumed to have condensed helical structures based on micrographs of RNP from several virus families (15, 21–24).

Here we report the 1.93-A crystal structure of recombinant RVFV N and views of two forms of RVFV RNP by electron microscopy. N has a novel protein fold that differs substantially from N of other negative-sense RNA viruses. The refolded, recombinant N forms stable multimeric N-RNA complexes of similar appearance to N-RNA multimers released from virus RNP by exhaustive ribonuclease treatment. The N-RNA multimer is heterogeneous with 4–7 N subunits and has an apparent molecular weight of 100 kDa. Authentic virus RNP and RNP reconstituted from refolded N and defined RNA have a similar nonhelical appearance and similar ribonuclease resistance.

Results

**Protein Oligomeric State in Solution.** Purification of recombinant N (recN) under native conditions, including exhaustive ribonuclease treatment, resulted in a discrete complex of the protein and *Escherichia coli* nucleic acid as determined by the ratio of absorbances at 260 nm and 280 nm (Fig. S1 A and B). The protein could not be separated from nucleic acid under native conditions using high salt concentrations and pH extremes. The recombinant N-RNA multimer had an apparent mass of 100 kDa by size-exclusion chromatography. Nucleic acid was extracted from the recombinant RVFV N by denaturation and then treated with either deoxyribonuclease or ribonuclease. The nucleic acid was sensitive only to ribonuclease treatment, demonstrating that N was bound to RNA. The formation of a nonspecific ribonucleoprotein (RNP) complex between recombinant RNA-binding proteins and *E. coli* RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15). Crystal structures of RABV (14), VSV (16), and HRSV (15) RNPs were solved from RNPs bound to RNA is not uncommon (6, 15).

**Cross-Linking of Authentic Virus RNPs and Recombinant N with RNA.** We tested whether the refolded recN could interact with RNA similarly to N in viral RNPs. Purified viral RNPs, refolded recN, and reconstituted N-RNA multimer (recN-RNA) were cross-linked with a homobifunctional amine-reactive cross-linker and then separated by electrophoresis under denaturing conditions (Fig. 1). In the absence of cross-linker (Fig. 1 A, lane 1; Fig. 1 B, lanes 4 and 9), N from all samples migrated as a monomer. When viral RNPs were exposed to increasing amounts of cross-linker, the monomer band decreased in intensity and four higher molecular weight complexes appeared (Fig. 1 A, lanes 2 and 3). Cross-linking of RNA-free, refolded recN resulted in predominant monomer and minor dimer species (Fig. 1 B, lanes 5–8), consistent with the behavior of recN in size-exclusion chromatography (Fig. S1 C). In contrast, when recN-RNA was cross-linked, many species of higher molecular weight were observed (Fig. 1 B, lanes 10–13). The number of N within the dominant cross-linked species created from recN-RNA and virus RNPs (Fig. 1 B, lane 11 and Fig. 1 A, lane 3) was estimated to be 2, 4, 6, and 10 based on a apparent molecular weight of 25 kDa for N. Thus, refolded recN behaves similarly to viral N in its ability to bind RNA and to form multimeric complexes. The cross-linked species formed by both viral RNPs and recN-RNA appear primarily as multiples of two, consistent with a previously reported dimeric association of N (25).

Electron Microscopy of Authentic Virus RNPs and Reconstituted RNPs. We compared RNPs isolated from virus with RNPs reconstituted from refolded N and a large single-stranded RNA. Freshly prepared samples (Fig. S2) were viewed by negative-stain electron microscopy before and after overnight ribonuclease digestion at room temperature. Both authentic virus RNP and reconstituted RNP were ribonuclease-resistant and had a remarkably similar string-like appearance (Fig. 2 A and Fig. S3). No helical symmetry was apparent in either sample. The appearance of phlebovirus RNP is strikingly different from images of similarly prepared RNP from other negative-sense RNA viruses, which have obvious helical symmetry (15, 21–24).

**Heterogenous Multimeric N-RNA Complexes.** Aggressive ribonuclease digestion (3 d at 37 °C) released a multimer from the virus RNP (Fig. 2 B and Fig. S4). In electron micrographs, this particle is similar in appearance to the ribonuclease-resistant, recombinant N-RNA multimer purified from *E. coli* (Fig. 2 C and Fig. S4 B) and to multimers reconstituted from refolded N and defined RNA oligomers (Fig. 2 D and Fig. S4 C and D). Remarkably, the multimers are of similar size distribution (10–12 nm diameter) regardless of the source of RNA. The N-RNA multimers from all sources appear heterogeneous, with 4–7 bright objects per particle (Fig. 2 B–D). The heterogeneity of the recombinant N-RNA multimer explains its inability to crystallize. The recombinant and reconstituted multimers have an apparent molecular weight of 90–100 kDa by size-exclusion chromatography (Fig. S5). Thus, we conclude that the N-RNA multimers contain 4–7 N subunits and an unknown amount of RNA.

The stoichiometry of N and RNA in a reconstituted N-RNA multimer was estimated using known N and RNA extinction coefficients at 260 nm and 280 nm. We used a short RNA decamer in order to enhance the contribution of protein to the total absorbance, which was dominated by RNA. The reconstituted N-RNA multimer had similar behavior upon gel filtration (Fig. S5) and similar appearance in electron micrographs (Fig. S4) to the N-RNA25 multimer. The purified, reconstituted N-RNA multimer had an A260/A280 ratio of 0.94, indicating an average stoichiometry of 5:1 N:RNA, in good agreement with the gel filtration data. This result indicates that each bright spot on multimer images is due to one N subunit.

**Fig. 1.** Similar multimer complexes of authentic virus RNPs and purified recombinant N bound to RNA. (A) Viral RNP. Purified RVFV RNPs were cross-linked with 0.0, 5.0, or 20.0 mM DSP and analyzed by immuno-blot. Asterisks indicate predominant cross-linked species. Molecular weight markers are in the rightmost lane. (B) Recombinant N. N or N bound to U25 single-stranded RNA (N-RNA) was cross-linked using 0.0, 1.0, 5.0, 10.0, or 20.0 mM DSP; separated by SDS/PAGE, and visualized with colloidal Coomassie stain. The dominant cross-linked species are indicated by asterisks.
be a natural dimer because the crystal contains two independent, nearly identical copies of the dimer (RSMD of 0.48 Å for 476 Cα atoms) and because a dimeric species was detected in solution (Fig. 1 and Fig. S1F). The dimer is formed by contacts of residues in helices α1, α7, and α8. The side chain of Trp125 (α7–α8 loop) is buried in the hydrophobic, water-free dimer interface where it contacts Met1, Gln5, Ile9, and Trp125 in the second monomer (Fig. 3D). Ala12, Val120, Val121, Glu124, and Thr131 also form intersubunit van der Waals contacts. The small dimer interface (502 Å² buried surface area per monomer) is consistent with the low proportion of dimeric species in solution (Fig. S1C), nevertheless the dimer is expected to predominate at the high protein concentration in crystals.

Comparison with N of Other Negative-Sense RNA Viruses. Given the rapid rate of virus divergence, we anticipated that the phlebovirus N might resemble the N of other negative-sense RNA viruses even though the sequences are dissimilar. Two different folds for N have been reported, one for FLUA (13) in the family Orthomyxoviridae, and the other for four viruses in the order Mononegavirales [RABV (14), VSV (16), HRVS (15), and BDV (17)]. However, the phlebovirus N fold differs from both these other N folds. Thus at least three different folds exist for N of the negative-sense RNA viruses. Intriguingly, all three folds are predominantly helical and are bilobed. However, the phlebovirus N has a more compact structure. RNA binds in a deep, positively charged cleft between the two lobes of N from both the Mononegavirales and FLUA (14–18). Phlebovirus N lacks a cleft between the N and C lobes (Fig. 3). Another important difference is the lack of protrusions in phlebovirus N. The N and C termini of N of the Mononegavirales protrude from the subunit, as does an extended loop in the N of FLUA. These protrusions contact other N subunits and are important to the structure of the RNP (14–18).

Conservation of Phlebovirus N. Among the highly conserved phlebovirus N (Fig. S7), a total 66 invariant residues map primarily to the core of the structure where they are important for conservation of the overall fold (Fig. S8A). Residues in the dimer interface are not strictly conserved, but a dimer contact appears feasible in all phlebovirus N because compensatory sequence changes accommodate the size and hydrophobicity of the residue corresponding to Trp125. The structure is consistent with published mutagenesis data suggesting that the N terminus of RVFV N is required for dimer formation (25). However, the conserved residues tested in the previous study (Tyr4, Phe11) point away from the dimer interface toward the inside of the monomer where they form stabilizing contacts in the hydrophobic core of the protein. The observed loss of dimer formation of Tyr4Gly and Phe11Gly (25) is likely due to destabilization of helix α1 (residues 3–10) and, indirectly, the dimer interface.

Mutagenesis of the Dimer Interface and C-Terminal Salt Bridge. The structure suggested that Trp125 is critical for dimer formation because of the hydrophobic contacts it makes with Met1, Ile9, and Trp125 of the opposing N monomer. Additionally, a salt bridge between the C-terminal carboxyl group of Ala245 and the Arg178 side chain, which is Arg or Lys in all phlebovirus N, may be important for overall structural integrity. To test the significance of these interactions, three mutant N alleles were generated, Trp125Ala, Arg178Gln, and Arg178Glu, and the functionality of each was analyzed in a cell-based RdRp transcription assay in which RdRp and N were expressed from separate plasmids. When RdRp and N were both present and functional, a luciferase mRNA from a recombinant S segment was transcribed (28). The Trp125Ala allele was severely compromised and activity was only 4% of the wild type allele (Table S2), suggesting that Trp125, and perhaps an N dimer, is essential for transcription. If the salt bridge of Arg178 with the C terminus is critical, then the Arg178Gln allele should retain more function than the
Arg178Glu allele, and this was the observed result (Table S2).

The activity of the Arg178Gln and Arg178Glu alleles was 25% and 7% of wild type, respectively. All alleles expressed protein at a level similar to wild type and all appeared capable of forming higher molecular weight complexes with RNA (Fig. S9).

**Interaction Sites.** We considered whether the RVFV N has an obvious RNA-binding surface. The N lobe has a higher calculated isoelectric point (pI of 9.4 vs. 8.3) and a more positively charged surface (Fig. 4A) than the C lobe. We compared RVFV N with structure-based homology models of N from other clades within the Phlebovirus genus. Based on the high sequence identity, N from all phleboviruses are expected to bind RNA similarly. There is a general trend of greater positive charge on the N lobe than on the C lobe, but the structures lack a common conserved basic surface. We also mapped sequence conservation onto the RVFV N surface (Fig. 4B). The most strongly conserved surface is a hydrophobic pocket at the junction of the N and C lobes formed by a loop (residues 27–35) together with the C-terminal half of α10 and the five succeeding amino acids (residues 198–210) (Fig. 4B and Fig. S8). Residues 27–35 are among the most mobile regions of the N structure (Fig. S8B). The combination of mobility, conservation, and hydrophobicity suggest that this site may be involved in a conserved protein-protein interaction.

**Discussion**

The structure of RVFV N reveals an addition to nature’s repertoire of RNA-binding proteins (Fig. 3). High levels of sequence identity (36–59%) assure that all phlebovirus N possess the RVFV N fold and also suggest that all phlebovirus N bind RNA similarly. The fold may also exist in N throughout the Bunyaviridae family.

This work establishes that RNP organization in the Phleboviruses is different than in other negative-sense RNA viruses. Crystal structures or reconstructions from electron cryomicroscopy have been reported for RNP from four negative-sense RNA viruses (Mononegavirales and Orthomyxoviruses) (14–16, 18). In all cases, RNA binds nonspecifically in an electropositive cleft between the lobes of the N subunit. RNP oligomers from these viruses have a similar architecture in which RNA binds around either the outside or inside of a ring of 9–11 N subunits. In all cases, protrusions from the N subunits make specific contacts with adjacent subunits to maintain the ring structure. In some cases, the number of subunits in the oligomer ring matches the helical repeat of the polymeric RNP, which is apparent in electron micrographs. For HRSV, each N subunit also interacts with other N subunits in the preceding or following turns of the helical nucleocapsid (15).

In contrast to the RNP s of Mononegavirales and Orthomyxoviruses, no helical structure was apparent in any electron micrographs of bunyavirus RNP, which lacked helical symmetry and also suggested that bunyavirus RNP s form large macrocircles (20), probably due to pairing of 10–15 complementary bases at the 3’ and 5’ ends of each genomic segment (29). RVFV RNP macrocircles
were seen in some of our images. Aggressive ribonuclease treat-
ment of RVFV RNP released an N-RNA multimer that appears
heterogeneous in composition and has an average diameter of
11 nm (Fig. 2B and Fig. S4). Multimers of nearly identical
appearance to those from virus RNP were observed both for
ribonuclease-treated recombinant RNP and for RNP reconstit-
tuted with small RNAs (Fig. 2 C and D and Fig. S4). The heter-
genecity of these minimal particles was surprising but is consistent
with the lack of helical symmetry in the RNP. The N crystal
structure is also consistent with the lack of helical symmetry.
The highly compact phlebovirus N has no protruding loops or termini
that could link it to other N molecules in a superstructure like the
rings of 9–11 subunits observed for the Mononegavirales and
Orthomyxoviruses (14–16, 18), although we cannot rule out the
possibility of major conformational changes to N upon RNA
binding. We observed no large superstructure for recombinant
RVFV N in solution, unlike the recombinant rings purified for
N from RABV (14), HRSV (15), VSV (16), and FLUA (18).
All N-RNA multimers that we could test by gel filtration had a
similar apparent molecular weight of ~100 kDa (Fig. S5).

Our working model for the structure of phlebovirus RNP is that
N binds cooperatively to RNA. However, the cooperativity is
limited to 4 N subunits based on the similar size and appearance
of multimers from viral, recombinant and reconstituted RNP and
their similar behavior upon gel filtration (Fig. 2 and Figs. S4
and S5). The RNP lacks a strong helical structure, and N-N contacts
are too weak for ribonuclease treatment to release a specific
N-RNA oligomer from virus RNP. Instead of a tightly associated,
symmetric N-RNA oligomer, limited protein–protein interactions
may lead to oligomers of 4–7 N monomers bound to RNA, result-
ing in the observed mixture of multimer species upon exhaustive
RNase treatment. If there is a weakly associated fundamental
oligomer, it may be a hexamer because our cross-linking data
showed a preponderance of species containing multiples of two
N subunits (Fig. 1). Overall, the organization of phlebovirus
RNP appears less symmetric and with few specific protein–protein
interactions compared to the helical RNP of the Mononegavirales
and Orthomyxoviruses. The evolutionary path to phlebovirus RNP
is not common with the path to helical RNP of some other
negative-sense RNA viruses.

The unusual N-RNA multimer and perhaps the nonhelical
RNP may be a general property of the Bunyaviridae. The observed
RVFV N-RNA multimer species are similar to the reported
109-kDa recombinant RNP from Bunyamwera virus (6). Bunyam-
werawa virus and RVFV belong to different genera within family
Bunyaviridae and their N are not obviously similar at the amino
acid level.

The N lobe of RVFV N was identified as a potential RNA
interaction site because it is more positively charged than the
C lobe in N from all phlebovirus clades. In whatever manner
N binds RNA, it is expected to engage the phosphate backbone
because the multimer is so highly ribonuclease resistant. The
electron micrographs do not permit assignment of RNA to a specific
location in the multimer particles.

The relevance of the N dimer to the RNP is unclear. Based on
the size of multimers in electron micrographs (Fig. 2 and Fig. S4)
and their gel filtration profile (Fig. S5), each bright spot in the
multimer images must be a monomer and not a dimer of N. A
precedent for different oligomer species for RNA-free and
RNA-bound N exists for FLUA where the free protein is a trimer
and the RNP is a nonamer (13, 18). We probed the RVFV N
dimer interface by site-directed mutagenesis at Trp125 but found
cross-linked RNA complexes similar to the wild type (Fig. S9).
However, the Ala substitution revealed an important role for
Trp125 in replication (Table S2). This could be due to any number
of molecular interactions, most obviously with the RdRp.

The most highly conserved surface of phlebovirus N is a hydro-
phobic pocket at the interface of the N- and C-terminal lobes
(Fig. 4 and Fig. S8). The conservation in this region suggests an
important function that is common to phleboviruses, and the
hydrophobicity of the surface suggests that it is not a site for
RNA binding but rather is for interaction with a viral or (uniden-
tified) host protein. Among potential viral protein partners, the
RdRp is an obvious possibility because N is required for transcrip-
tion and replication by the RdRp (4). However, several lines of
evidence suggest that an envelope glycoprotein may be the target
of the conserved hydrophobic pocket on N.

Packaging of RNP into virions occurs at a site of virus assembly
on the Golgi membrane (30). The cytoplasmic tail of the RVFV
envelope glycoprotein Gn was shown recently to recruit the encap-
sidated genome to the Golgi membrane prior to virion assembly
(31). Moreover, three regions within the Gn tail of the Uukuniemi
virus were shown to be important for nucleoprotein binding to the
glycoproteins (5). Genome recruitment is expected to be similar in
all phleboviruses, thus the conserved hydrophobic pocket of N is a
candidate for Gn binding site. This hypothesis is consistent with the
ability of bunyaviruses, both in nature and in vitro, to undergo
reassortment in which progeny have genomic segments that derive
from more than one parental virus (32–34). Reassortment requires
promiscuity in the interaction of N with genomic RNAs from
heterologous viruses and in protein-protein interactions necessary
for assembling virions. All characterized reassortant bunyaviruses
isolated in nature are M segment reassortants (35, 36), demon-
strating that the envelope glycoproteins, which are encoded by
the M segment, are capable of interacting with heterologous
RNP. The hydrophobic character of some regions of the Gn tail,
as well as Pro and Trp residues within it, are conserved among phle-
boviruses (37) and could function in protein–protein interactions
with N. Whether the conserved pocket of N interacts with the Gn

![Image](https://example.com/image.png)

**Fig. 4.** Properties of the RVFV N surface. (A) Electrostatic surface potential. The surface potential from ~−20 kT in red to +20 kT in blue is shown for the front and back of the RVFV N monomer. The ribbon diagrams below show the positions of the N-terminal (blue) and C-terminal (green) lobes and the linker (pink). The image at left is in the same orientation as (Fig. 3A). (B) Conserved hydrophobic pocket. The most conserved surface of N is at the top relative to A. In this view, the N-terminal domain is at left and coloring is by conservation among phlebovirus N, as indicated.
cytoplasmic tail, with the RdRp, or with a host protein, it has potential as a drug target because it is conserved in phleboviruses.

The structure and characterization of phlebovirus N and RNP reveal another paradigm for encapsidation of the genome of negative sense RNA viruses, provide a platform for further studies of virus pathogenicity, and suggest a potential site for development of effective antiviral therapeutics.

Materials and Methods

Detailed methods are given in SI Text.

Production and Purification of Recombinant N. The expression construct for N was designed to contain a 6xHis tag fused to SUMO fused to N. The expression plasmid was transformed into E. coli, and the protein was purified from the soluble fraction of the cell lysate. His-SUMO-N was bound to a metal-affinity column, unfolded in 8 M urea, washed with several column volumes of 8 M urea to remove RNA, refolded in a linear gradient of 8 M to 0 M urea, and eluted with an imidazole gradient. The His-SUMO was cleaved from the refolded protein with SUMO protease (38), a kind gift of C. Lima, Memorial Sloan-Kettering Cancer Center. The cleavage product, N with its full natural sequence, was purified by a second step of metal-affinity chromatography followed by gel filtration (Fig. S1).

Crystallography. The crystal structure was solved by Se-MAD using the SeMet protein. Crystals in space group P1 contain four N polypeptides per unit cell with cell constants a = 67.1 Å, b = 69.6 Å, c = 80.6 Å, α = 78.4°, β = 69.7° γ = 60.9° for the 1.93-Å dataset from a crystal of wild-type N. The final model is of excellent crystallographic and stereochemical quality with R = 0.211, Rfree = 0.254, RMSD = 0.013 Å and 99.6% of residues in allowed regions of the Ramachandran plot.

Cross-Linking. For cross-linking, a recombinant RNP (N-GA) was generated by incubating refolded recombinant N (recN) with a 25-nucleotide polyU RNA oligomer for 30 min at a ratio of 6:1 recN-RNA. The sample was then run on an 8200 size-exclusion column to separate N-RNA from RNA-free recN. The recN and N-RNA were dialyzed against PBS to remove the Tris storage buffer prior to incubation with DSP. Purified recN, N-RNA, or purified viral RNPs (vRNP) were cross-linked by incubating 30 μg of recN at a concentration of 1 mg/μL, or purified vRNPs with 0.0, 1.0, 5.0, 10.0, or 20.0 μM dithiobis(succinimidyl)propionate (Pierce) for 15 min at room temperature. The cross-linking was quenched by addition of Tris pH 6.7 to a final concentration of 100 mM. Protein complexes were analyzed by SDS-PAGE followed by either colloidal Coomassie stain or western blot using a polyclonal rabbit anti-N antibody.

Electron Microscopy. All samples were adsorbed onto a carbon-coated grid and stained with 0.75% uranyl formate using standard protocols. A Morgagni 268D transmission electron microscope equipped with a mounted Orius SC200 CCD camera was used for imaging at room temperature.

RVFV transcription assay. BSR-T7/S cells were plated at 1 × 10⁶ cells/well in 12-well culture plates. After 24 h, cells were transfected using 2 μg/mL TransIT LTI (Mirus Corporation) and plasmids in the ratio 0.25 μg pSTRVFV-SaNaNS::hRLuc:0.50 μg pC75i and 0.75 μg pDRdR. At 48 h posttransfection, the cells were harvested and analyzed by luciferase assay and western blot.

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