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Identification of Two Functional Domains within the Arenavirus Nucleoprotein▽

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Tacaribe virus (TCRV) belongs to the *Arenaviridae* family. Its bisegmented negative-stranded RNA genome encodes the nucleoprotein (N), the precursor of the envelope glycoproteins, the polymerase (L), and a RING finger matrix (Z) protein. The 570-amino-acid N protein binds to viral RNA, forming nucleocapsids, which are the template for transcription and replication by the viral polymerase. We have previously shown that the interaction between N and Z is required for assembly of infectious virus-like particles (VLPs) (J. C. Casabona et al., J. Virol. 83:7029-7039, 2009). Here, we examine the functional organization of TCRV N protein. A series of deletions and point mutations were introduced into the N-coding sequence, and the ability of the mutants to sustain heterotypic (N-Z) or homotypic (N-N) interactions was analyzed. We found that N protein displays two functional domains. By using coimmunoprecipitation studies, VLP incorporation assays, and double immunofluorescence staining, the carboxy-terminal region of N was found to be required for N-Z interaction and also necessary for incorporation of N protein into VLPs. Moreover, further analysis of this region showed that the integrity of a putative zinc-finger motif, as well as its amino-flanking sequence (residues 461 to 489), are critical for Z binding and N incorporation into VLPs. In addition, we provide evidence of an essential role of the amino-terminal region of N protein for N-N interaction. In this regard, using reciprocal coimmunoprecipitation analysis, we identified a 28-residue region predicted to form a coiled-coil domain (residues 92 to 119) as a newly recognized molecular determinant of N homotypic interactions.

The New World group of arenaviruses comprises three phylogenetically distinct clades. One of those clades (clade B) is of particular interest since it includes the known South American pathogens that produce severe hemorrhagic disease in humans: Junín virus (JUNV), which is the etiological agent of Argentine hemorrhagic fever; Machupo virus (MACV); Guanarito virus (GTOV); Sabia virus (SABV); and Chapare virus (10). Clade B also comprises the nonpathogenic Tacaribe virus (TCRV), which is the group's prototype and which displays a close antigenic relationship with and cross-protection against JUNV (10, 39).

Arenaviruses are enveloped viruses with a bipartite negative-sense RNA genome. The large (L) segment encodes two proteins, the viral RNA-dependent RNA polymerase (L protein) and a multifunctional RING finger protein called Z (ca. 11 kDa), which has been shown to act as a matrix protein (8, 48, 56, 62). The small (S) segment encodes two major structural proteins, the glycoprotein precursor (GPC) and the nucleoprotein (N). In both S and L RNAs, the genes are arranged in opposite orientations and are separated by noncoding intergenic sequences with the potential to form stable secondary structures. The coding sequences are expressed from nonpolyadenylated, capped mRNAs transcribed from the 3′ region of the genomes or antigenomes (6). Mapping of the 3′ ends of viral mRNAs within the intergenic region in each segment, along with reverse genetics, has provided direct experimental evidence that intergenic regions contain the *cis*-acting signals necessary for transcription termination (22, 36, 41, 49, 59).

The N protein (ca. 64 kDa) is the most abundant viral polypeptide in both infected cells and virions. N tightly binds to genomic and antigenomic RNAs, forming nucleocapsids, which were earlier thought to act as templates for viral genome transcription and replication, both mediated by the virus polymerase (6). Indeed, more recent evidence demonstrated that N and L proteins are the minimal *trans*-acting factors necessary to drive transcription and full-cycle replication of minireplicons (21, 31, 37). Moreover, the TCRV N has been reported to interact with the L polymerase, and this interaction has been suggested to regulate the polymerase mode of action by facilitating its commitment to replication (25). In addition, evidence of an *in vivo* transcriptional antitermination activity has been reported for JUNV N protein (59).

Besides its involvement in RNA transcription and replication, N seems to play an important role during virion assembly.

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This notion is supported by the fact that N and Z interact with each other both *in vivo* and in viral particles (15, 53), as well as by compelling evidence of the role of Z protein as the main driving force of arenavirus budding (48, 56, 62). Additionally, our previous studies showed that TCRV N can be recruited by JUNV Z into virus-like particles (VLPs) in the absence of other viral components (8), a finding that has been reproduced by coexpressing the homologous Z and N proteins from TCRV, JUNV, Lassa virus (LASV), or Mopeia virus (19, 54, 55). Moreover, we demonstrated that Z-N interactions are required for infectious chimeric VLP assembly, further supporting the idea that Z-N interactions may be critical for nucleocapsid packaging into infectious viral particles (8). However, the protein domains and specific residues within N that are required for these functions are poorly understood.

The aim of the present study was to understand the functional organization of N protein. By performing a mutational analysis of TCRV N, we were able to identify two different functional domains in this viral protein. The C-terminal region was demonstrated to be involved in the interaction with the Z matrix protein, while the N-terminal region was responsible for homotypic N-N interactions. We also found that conserved residues within a putative zinc-finger motif as well as adjacent sequences within the C-terminal domain of N were necessary for binding Z. In addition, a region harboring a newly recognized coiled-coil motif located in the N-terminal domain was demonstrated to be essential for N self-interactions. Altogether, these results provide new molecular details about the N protein, which will help to provide an understanding of its multiple functions in the viral replication cycle.

MATERIALS AND METHODS

Cells and virus. BSR cells (a clone of BHK-21) were grown in Glasgow minimum essential medium (Invitrogen). CV1 cells were cultivated in Dulbecco's modified Eagle medium (DMEM; Invitrogen). Media were supplemented with glutamine (2 mM), 10% fetal calf serum (FCS; Invitrogen), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Invitrogen). Both cell lines were grown in a 5% CO_2 atmosphere at 37°C. Recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase (17), was kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD).

Plasmids. Plasmids pTCRV Z-ha and pJUNV Z-ha express, respectively, TCRV Z and JUNV Z proteins joined at their C termini with a sequence comprising a linker (GS) and an influenza virus hemagglutinin (HA) epitope (YPYDVPDYA). Plasmids pTCRV N-Flag and pTCRV N-myc express the N protein tagged at its N terminus with either the Flag epitope (DYKDDDDK) or a c-Myc epitope (MAEQKLISEEDL), respectively. These pTM1 vector-based plasmids were previously generated and were formerly designated pTacV Z-HA, pJunV Z-HA, pTacV N-Flag, and pTacV N-myc (8). The functionality of the Flag- and c-Myc-tagged versions of TCRV N protein and that of the HA-tagged JUNV Z protein in minigenome replication and infectious VLP formation was previously demonstrated (8).

Plasmid pNΔ334-476, which expresses TCRV N deletion mutant NΔ334-476 (with the numbers corresponding to the deleted amino acid sequence), was generated by digestion of plasmid pTCRV N-Flag with BglII. The DNA fragment containing the truncated N gene was purified and religated. Constructs encoding the rest of the truncated versions of TCRV N were generated by PCR, using plasmid pTCRV N-Flag as the template and specific primers. Internal deletions in mutants NΔ153-332, NΔ461-476, NΔ477-489, and NΔ93-119 were introduced by inverted PCR, and the resulting constructs were designated pNΔ153-332, pNΔ461-476, pNΔ477-489, and pNΔ93-119, respectively. To obtain plasmids pNΔ1-152 and pNΔ1-359, which express the N-terminal-truncated mutants NΔ1-152 and NΔ1-359, respectively, forward primers containing (5′ to 3′) the Flag tag-coding sequence fused to the sequence corresponding to either nucleotides (nt) 457 to 477 or nt 1078 to 1096 of the N open reading frame (ORF), respectively, were designed. To generate C terminal deletion mutants NΔ477-570 and NΔ536-570, reverse primers with (5′ to 3′) stop codon-complementary sequences followed by sequences complementary to positions 1428 to 1411 and 1605 to 1585 of the N ORF, respectively, were used. The resulting plasmids were designated pNΔ477-570 and pNΔ536-570. Point mutations on pTCRV N-Flag were carried out using a QuikChange PCR mutagenesis kit (Stratagene) with primers containing the mutated sequences. All constructs were checked by dideoxynucleotide double-strand DNA sequencing (Macrogen Inc.). All primer sequences and vector maps are available upon request.

Plasmid pCMV-T7pol expresses the bacteriophage T7 RNA polymerase under the cytomegalovirus promoter control (50) and was kindly provided by Martin A. Billeter (University of Zurich, Irchel, Switzerland). All plasmids were purified by use of a tip-100 column (Qiagen Inc.).

DNA transfections. Cell monolayers were transfected with the indicated plasmid combinations using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. BSR cell transfections included, in addition to the indicated plasmids, 1 μg of pCMV-T7pol per well of 12-well plates. In all transfections, the total amount of transfected DNA was kept constant by the addition of vector pTM1 DNA.

VLP generation and purification. BSR cells grown in 12-well plates were transfected with 0.5 to 0.75 μg (per well) of the indicated Z-expressing plasmid and 1.5 μg of either pTCRV N-Flag or each of the N mutant-expressing plasmids. Plasmids were transfected individually, at the same amounts, in control cell monolayers. After 4 h of incubation, the transfection medium was removed, cells were washed twice, fresh medium supplemented with 2% FCS was added, and the incubation was continued. Cells were lysed at 48 h posttransfection in nonreducing SDS-PAGE sample buffer (Invitrogen). Culture supernatants were collected and clarified by low-speed centrifugation, and VLPs were pelleted through 20% (wt/vol) sucrose cushions in TNE buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA), as described before (8). The purified VLPs were resuspended in nonreducing SDS-PAGE sample buffer and analyzed by Western blotting, as indicated below.

Analysis of protein interactions by coimmunoprecipitation. Subconfluent monolayers of CV1 cells grown in 12-well plates were infected with 3 to 5 PFU of vTF7-3 per cell. The inoculum was removed, and the cells were washed and then transfected as follows. For N-Z interaction analysis, 4 μg of either pTCRV N-Flag or each of the N mutant-expressing plasmids and 1 μg of pTCRV Z-ha or pJUNV Z-ha were included in the transfection mix. The amounts of plasmid DNA used for N-N interaction analysis were 1 μg of plasmid pTCRV N-myc and 1 μg of either pTCRV N-Flag or each of the N mutant-expressing plasmids. At 24 h posttransfection, cell monolayers were washed twice with phosphate-buffered saline (PBS) and then lysed by adding TNE-N (0.2% Nonidet P-40 in TNE buffer) containing protease inhibitors (2 μg/ml aprotinin, 20 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml *N*-α-tosyl-L-lysine chloromethyl ketone [Sigma-Aldrich]). Cell lysates were clarified from nuclei and cellular debris by centrifugation at 16,000 × *g* for 20 min at 4°C, and aliquots of cytoplasmic extracts corresponding to about 0.5×10^5 to 2×10^5 cells were immunoprecipitated with either rabbit anti-HA polyclonal antibody (Santa Cruz Biotechnology), mouse anti-Flag M2 monoclonal antibody (MAb; Sigma Aldrich), or rabbit anti-c-Myc polyclonal antibody (Santa Cruz Biotechnology), as indicated, following the procedure previously described (8, 25). Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by Western blotting, as described below.

Western blotting. Immunoblotting was performed according to the protocol previously described (8). Briefly, proteins were resolved by SDS-PAGE, in gels containing 10 to 14% polyacrylamide, and then transferred to a nitrocellulose membrane. After the blots were blocked overnight at 4°C with PBS containing 5% skim milk and 0.1% Tween 20 (Sigma-Aldrich), they were incubated with the primary antibody for 2 h at 37°C. Following incubation with the appropriate horseradish peroxidase-labeled secondary antibody, detection was performed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific), according to the manufacturer's specifications. Finally, blots were exposed to X-ray films, and protein bands were quantified by densitometry, as previously described (8). The following primary antibodies were used: rabbit anti-HA polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-c-Myc polyclonal antibody (Santa Cruz Biotechnology), or mouse anti-Flag M2 MAb (Sigma Aldrich). Horseradish peroxidase-conjugated antimouse or antirabbit secondary antibodies (Jackson ImmunoResearch) were used according to the supplier's specifications.

Indirect immunofluorescence and confocal microscopy. BSR cells grown on glass coverslips in 24-well dishes were transfected with 0.25 to 0.75 μg of each of the indicated plasmids per well. At 24 h posttransfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and then incubated with a mix of primary antibodies consisting of rabbit anti-HA polyclonal antibody (Santa Cruz Biotechnology) and

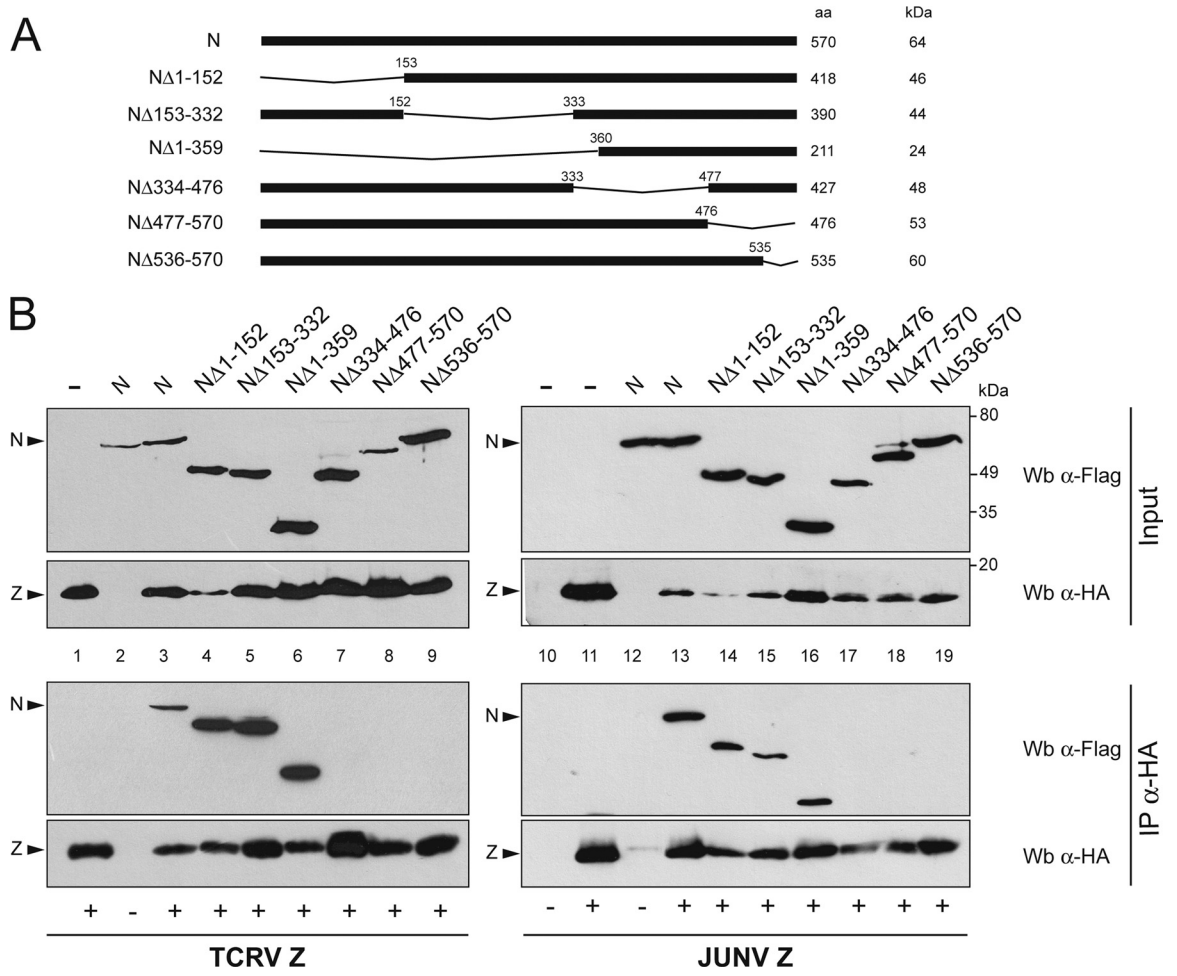

FIG. 1. The C-terminal region of N is essential for N-Z interactions. (A) Schematic representation of wild-type and truncated TCRV N proteins. Coding sequences are shown with bars; lines represent deleted sequences. The numbers above each schema indicate positions on the TCRV N amino acid sequence. The numbers on the right correspond to predicted protein length (in amino acids [aa]) and predicted molecular masses (in kDa). The Flag tag was omitted from the schemes. (B) CV1 cells were transfected to express either TCRV Z-ha or JUNV Z-ha (indicated TCRV Z and JUNV Z, respectively), along with either N-Flag (lanes 3 and 13) or each of the indicated Flag-tagged N mutants (lanes 4 to 9 and 14 to 19). As controls, TCRV Z-ha, JUNV Z-ha, or TCRV N-Flag was expressed individually (lanes 1, 11, 2, and 12). Controls included untransfected cells (lane 10). Cells were lysed at 24 h posttransfection, and aliquots of the cell lysates (Input) were analyzed by Western blotting, using either anti-Flag (Wb α-Flag) or anti-HA (Wb α-HA) antibody. Additional aliquots of the cell lysates were immunoprecipitated using serum against HA (IP α-HA). Precipitated proteins were separated by SDS-PAGE and analyzed by immunoblotting, as indicated on the right. The positions of N-Flag (N) and Z-ha (Z) are indicated by arrowheads. The molecular masses of the markers (in kilodaltons) are indicated on the right. α, anti.

mouse anti-Flag M2 MAb (Sigma Aldrich), as previously described (8). The secondary antibody mixture contained Alexa Fluor 488 chicken anti-mouse immunoglobulin G (Invitrogen) to detect the Flag-tagged N proteins and Alexa Fluor 568 goat anti-rabbit immunoglobulin G (Invitrogen) to detect the HA-tagged Z proteins. After the cells were extensively washed, they were mounted on FluorSave (Calbiochem). Confocal images were collected using a FluoView FV1000 confocal microscope (Olympus Latin America) or an LSM 510 Meta confocal microscope (Zeiss, Jena, Germany), both equipped with spectral detection capacity to select the different emission wavelengths to be detected. A multiline argon laser (458, 488, and 514 nm) and two helium neon lasers (543 and 633 nm) were used in both microscopes. Images were acquired using a ×100 UPlanSApo oil immersion objective (numerical aperture [NA], 1.4; Olympus) or a ×63 Plan-Apochromat oil immersion objective (NA, 1.4; Zeiss). In both microscopes, the pinhole was adjusted to obtain 1 Airy unit (optical slice, 0.8 μm). Emission filters set for fluorescein and rhodamine were previously described (23).

Sequence analysis. Multiple-sequence alignments were done using the ClustalX program (58). Alternatively, the nonredundant protein sequence (nr) database was searched by using the Blastp (protein-protein BLAST) algorithm at the NCBI server (http://blast.ncbi.nlm.nih.gov/). TCRV nucleoprotein secondary structure and coiled-coil predictions were obtained using the PredictProtein (http://www.predictprotein.org/ [51]) and Jpred (http://www.compbio.dundee.ac.uk/~www-jpred/ [11]) servers.

RESULTS

The C-terminal region of TCRV N protein is necessary for its interaction with Z protein and for N incorporation into Z-induced particles. In order to map the region of TCRV N protein required for its interaction with the matrix Z protein, six deletions were introduced throughout the N gene either at the 5′ end, at the 3′ end, or at internal positions. The schematic representation of the deletions is shown in Fig. 1A. Both wild-type and truncated N proteins were expressed as fusion proteins with the Flag tag at their N-terminal end, since we

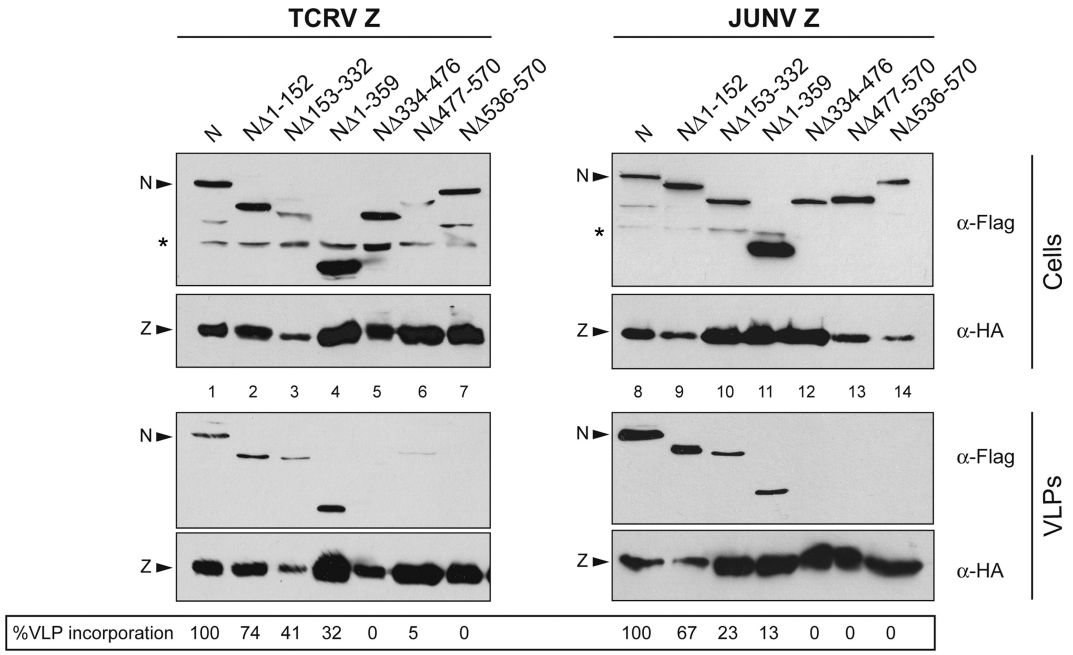

FIG. 2. The C-terminal region of TCRV N is required for its recruitment into Z-containing VLPs. BSR cells were transfected with either pTCRV Z-ha or pJUNV Z-ha along with pTCRV N-Flag or each of the N mutant-expressing plasmids, as indicated. Total cell extracts were prepared, and VLPs were purified from the culture supernatants at 48 h posttransfection, as described in Materials and Methods. Aliquots from both cell extracts and VLPs were analyzed by Western blotting, using either anti-Flag (α-Flag) or anti-HA (α-HA) antibody, and protein bands were quantified by densitometry. Percent VLP incorporation corresponds to the ratio between N protein detected in VLPs and total (VLP and cell lysate) N protein, expressed as a percentage of the values estimated for wild-type N (lanes 1 and 8), set equal to 100%. Mean values from at least two independent experiments are shown. Standard deviations ranged from 4% (NΔ1-359) to 21% (NΔ1-152). Stars denote an unspecific protein band. Arrowheads indicate the positions of Z-ha (Z) and N-Flag (N).

have previously shown that the Flag epitope-tagged version of full-length N is totally functional in a minireplicon-based VLP system (8).

The effect of each deletion on the interaction between N and Z was assessed by coimmunoprecipitation assays. To this end, either wild-type TCRV N-Flag or each of the N mutants was coexpressed along with the HA-tagged version of Z from either TCRV or JUNV in mammalian cells. As a control for protein expression, aliquots of the cell lysates were subjected to SDS-PAGE and subsequent Western blotting. Both full-length N and all the truncated proteins were detected at comparable expression levels, and their apparent molecular sizes were consistent with those predicted from their sequences (Fig. 1B, panels Input). Additional aliquots of the cell extracts were incubated with an anti-HA antibody to pull Z protein down. Precipitated proteins were analyzed by Western blotting using either anti-HA or anti-Flag antibody to detect HA-tagged Z proteins and Flag-tagged N proteins, respectively (Fig. 1B, IP α-HA panels). The results indicated that wild-type N was specifically coprecipitated with either TCRV Z or JUNV Z (Fig. 1B, lanes 3 and 13), since no N protein was detected in lysates from control cells expressing N alone (lanes 2 and 12). Mutants NΔ1-152 and NΔ153-332 were detected in complexes with both Z proteins (lanes 4, 5, 14, and 15). The specificity of the interaction was confirmed by the fact that these mutants were not immunoprecipitated with anti-HA antibody from the lysates of cells expressing them individually (data not shown). In contrast, mutant NΔ334-476 as well as mutant NΔ477-570 failed to coprecipitate with either TCRV Z or JUNV Z (lanes 7, 8, 17, and 18), indicating that deletions of the C-terminal region of N abolished its interaction with Z. Moreover, deletion of the last 35 residues from the C terminus was sufficient to abrogate the capacity of the N protein to bind Z (mutant NΔ536-570, lanes 9 and 19), whereas the truncation of 359 residues from the N terminus did not substantially affect the interaction (mutant NΔ1-359, lanes 6 and 16). These results revealed that the N-terminal moiety of N was dispensable for association with Z, while the C-terminal region of the protein appeared to play a critical role in this interaction.

We have previously shown that, upon coexpression in mammalian cells, JUNV Z protein binds TCRV N and that this interaction is required to drive the incorporation of TCRV N protein into Z-induced particles (8). Therefore, we used a VLP incorporation test to further evaluate the effect of N deletions in the recruitment of N into Z-containing VLPs. Z protein from either TCRV or JUNV was coexpressed with N-Flag or each of the truncated mutants in BSR cells; then both cells and the released VLPs were analyzed for the presence of Z and N proteins by Western blotting. Protein bands were quantified, and the amounts of mutant N incorporated into VLPs were compared to those of wild-type N (Fig. 2). Results revealed that mutant NΔ1-152 was recruited into VLPs directed by either TCRV Z or JUNV Z at levels similar to those for N-Flag (Fig. 2, lower panels; compare lane 2 to lane 1 and lane 9 to lane 8; relative VLP incorporation values are shown at the bottom). In addition, we observed that, as deletion extended to

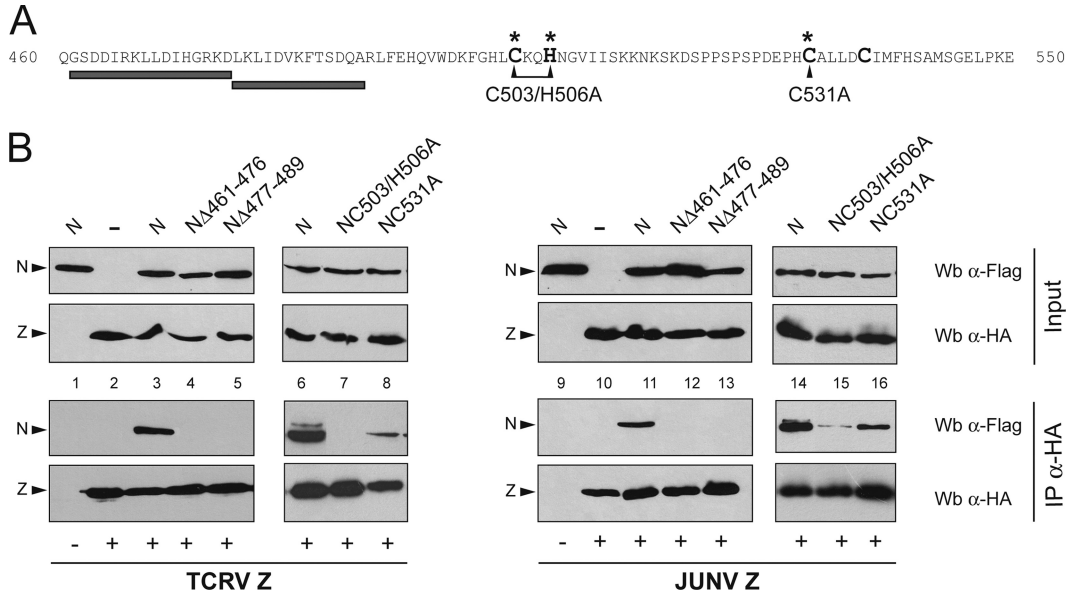

FIG. 3. Role of linear sequences and conserved residues within the C-terminal region of N in N-Z complex formation. (A) Amino acid sequence corresponding to positions 460 to 550 of TCRV N protein. Cysteine (C) and histidine (H) residues predicted to coordinate zinc (47) are shown in boldface. Substitutions of strictly conserved residues (shown by asterisks) are indicated below the sequence. Deleted sequences in mutants NΔ461-476 and NΔ477-489 are indicated with gray bars. (B) CV1 cells were transfected with either pTCRV Z-ha (lane 2), pJUNV Z-ha (lane 10), or pTCRV N-Flag (lanes 1 and 9) or were cotransfected to express the indicated Z protein along with either wild-type TCRV N-Flag or each of the N mutants, as shown. Aliquots of the cell lysates (Input), obtained at 24 h posttransfection, were analyzed by Western blotting using either anti-Flag (Wb α-Flag) or anti-HA (Wb α-HA) antibody. Additional aliquots of the lysates were immunoprecipitated using serum against HA (IP α-HA). Precipitated proteins were separated by SDS-PAGE and analyzed by immunoblotting, as indicated on the right. The positions of N-Flag (N) and Z-ha (Z) are indicated by arrowheads.

the C terminus, reduced but still detectable amounts of either mutant NΔ153-332 or NΔ1-359 were incorporated into VLPs (lanes 3, 4, 10, and 11; with VLP incorporation values being 41 to 23% and 32 to 13%, respectively, those of wild type N-Flag). In contrast, mutants NΔ334-476, NΔ477-570, and NΔ536-570 were almost undetectable in VLPs induced by TCRV Z (lanes 5 to 7) and were absent from VLPs directed by JUNV Z (lanes 12 to 14). In each case, the Z and N proteins were readily detected in the cell lysates (Fig. 2, upper panels). The results were consistent with the data presented above (Fig. 1) and indicated that the region between residues 360 and 570 of N is essential for both N-Z interaction and the recruitment of N into VLPs.

A conserved cysteine-rich motif is involved in N-Z interactions. On the basis of the sequence alignment of a number of arenavirus nucleoproteins, an arrangement of Cys and His residues resembling a classical zinc-finger motif were previously described near the C terminus of the N protein (47). Within this motif, Cys-X_2-His-X_{15-24}-Cys-X_4-Cys (CHCC), the first and the second Cys residues (corresponding to positions 503 and 531 in the TCRV nucleoprotein sequence, respectively), as well as the His residue (position 506 in TCRV N) are strictly conserved in all the arenaviruses examined so far. However, their contribution to the nucleoprotein interactions had not been addressed.

Because we found that the C terminus of N is required for Z binding (Fig. 1 and 2), the relevance of the putative zinc-finger motif in these protein-protein interactions was evaluated. To this end, Ala substitutions of the strictly conserved residues within the zinc-finger motif (C503, H506, and C531) were made in the full-length TCRV N. In addition, to assess the participation of sequences adjacent to the CHCC motif in N-Z interaction, small deletions were introduced within the region immediately N terminal to this motif (Fig. 3A). The ability of the resultant N mutants to bind Z was tested in coimmunoprecipitation experiments (Fig. 3B). Expression of the N mutants and the Z proteins was confirmed by Western blotting (Fig. 3B, panels Input). Analysis of the lysates immunoprecipitated with anti-HA antibody showed that the substitution of amino acid C531 hindered the ability of N to form a complex with either TCRV Z or JUNV Z (Fig. 3B, IP α-HA panels; compare lane 8 to lane 6 and lane 16 to lane 14). Moreover, the double mutation C503/H506A completely disrupted the interaction of N with TCRV Z and critically affected its association with JUNV Z (IP α-HA panels, lanes 7 and 15, respectively), suggesting that the integrity of the putative zinc-finger motif could be required for efficient N-Z complex formation. In addition, deletions of amino acids 461 to 476 or of residues 477 to 489 abolished the interaction of N with Z protein from either TCRV or JUNV (IP α-HA panels, lanes 4, 5, 12, and 13). These results indicated that the sequence spanning residues 461 to 489, adjacent to the putative zinc finger, is also critical for N-Z interaction.

To further support our findings, we used confocal microscopy to examine the subcellular distribution of N mutants either expressed alone or coexpressed with TCRV Z (Fig. 4). As shown before (8), wild-type N-Flag displayed a cytoplasmic distribution with a punctuated pattern when it was expressed alone (Fig. 4B). The same pattern was essentially exhibited by mutants NΔ461-476, NΔ477-489, and NC503/H506A (Fig. 4C

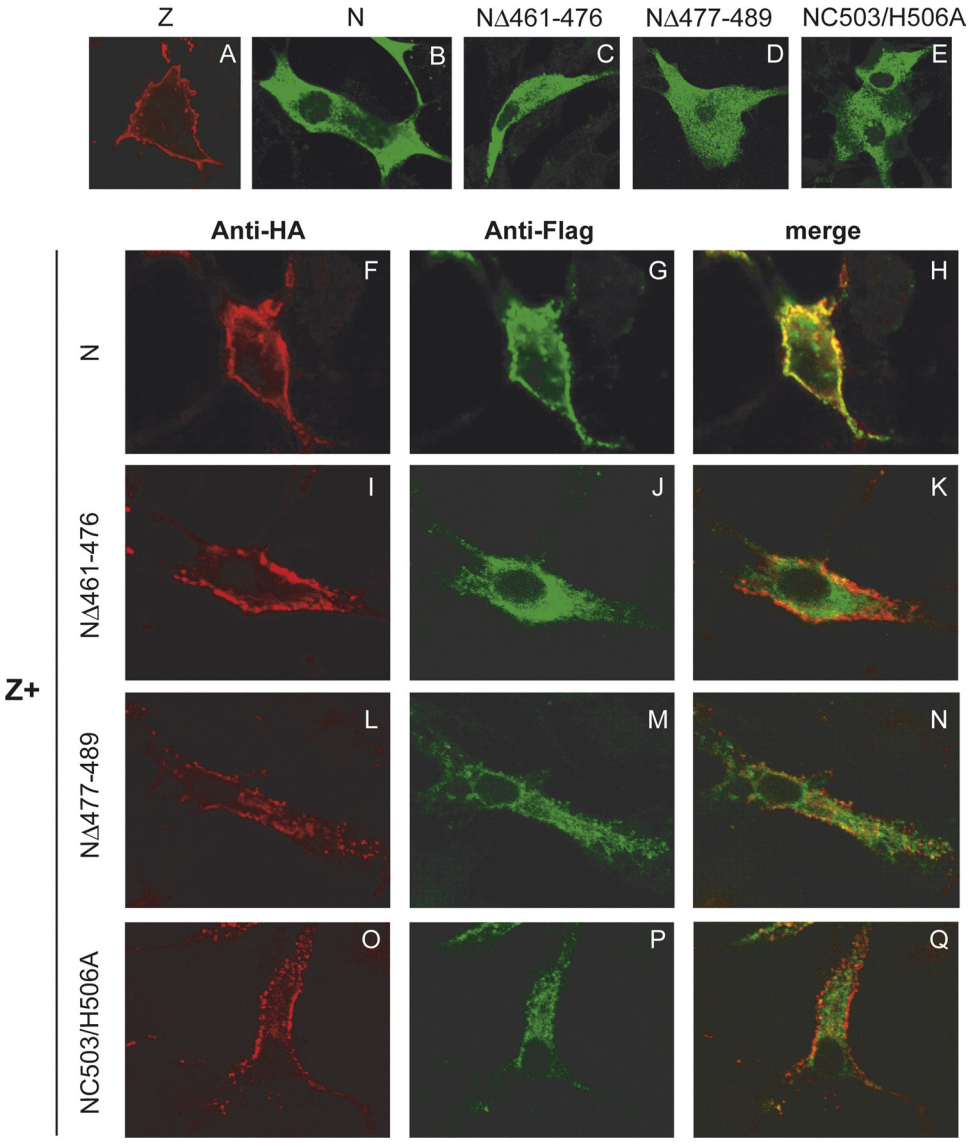

FIG. 4. Intracellular distribution of TCRV Z and wild-type or C-terminal mutant N proteins. BSR cells expressing TCRV Z-ha (A), TCRV N-Flag (B), or the indicated N mutants (C to E) individually or coexpressing TCRV Z-ha along with either wild-type TCRV N-Flag (F to H) or each of the Flag-tagged N mutants (I to Q) were fixed, permeabilized, and then probed with a mix containing mouse anti-Flag antibody (green) and rabbit anti-HA antibody (red). Images were collected using confocal microscopy, as indicated in Materials and Methods. (H, K, N, and Q) Merged images of the signals for N and Z proteins, as indicated.

to E). On the other hand, TCRV Z was mainly observed at the plasma membrane and, to a lesser extent, throughout the cytoplasm under all transfection conditions (Fig. 4A, F, I, L, and O). Coexpression with TCRV Z caused wild-type N to localize at the plasma membrane, where both proteins colocalized (Fig. 4G and H). In contrast, upon coexpression with Z, mutant NC503/H506A as well as mutants NΔ461-476 and NΔ477-489, failed to associate with the cell membrane (Fig. 4P, J, and M). Moreover, no colocalization between these N mutants and Z was observed (panels Fig. 4Q, K, and N). The same results were obtained when N-Flag or each of these C-terminal N mutants were coexpressed with JUNV Z protein (data not shown). These results provided new evidence demonstrating the requirement of the integrity of the CHCC motif and its adjacent sequences on TCRV N for binding Z.

Next, we examined the incorporation of N mutants into Z-induced VLPs (Fig. 5). In accordance with our results (Fig. 3), the replacement of residue C531 with alanine caused a reduction in the amounts of N mutant recruited into VLPs directed by either TCRV Z or JUNV Z, with VLP incorporation values estimated to be about 50% and 75%, respectively, those of wild-type N (Fig. 5, compare lane 6 to 4 and lane 12 to 10). Furthermore, mutation of amino acids C503 and H506 (lanes 5 and 11) or deletion of the sequence spanning residues 461 to 476 or 477 to 489 (lanes 2, 3, 8, and 9) resulted in undetectable levels of N incorporated into VLPs.

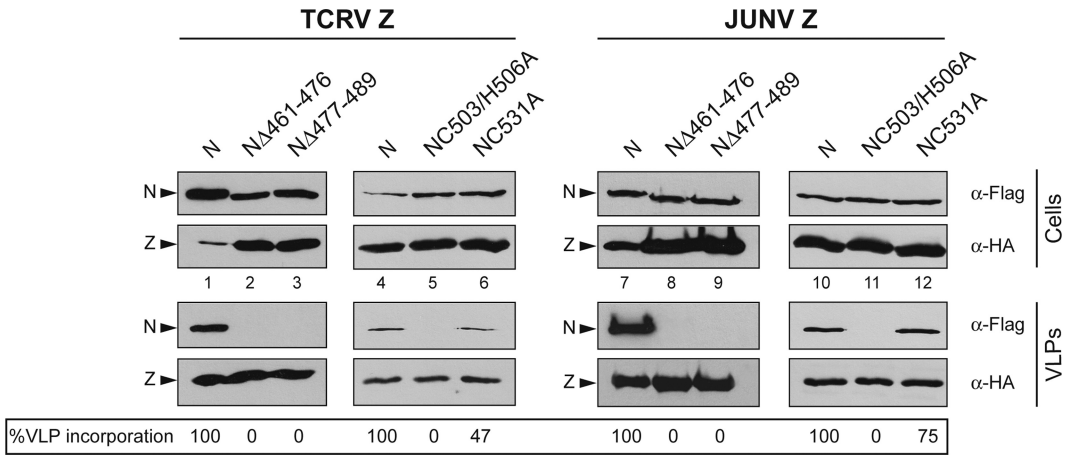

FIG. 5. Recruitment of N mutants into Z-containing VLPs. BSR cells were transfected to express either TCRV Z-ha or JUNV Z-ha (indicated TCRV Z and JUNV Z, respectively) along with TCRV N-Flag or each of the indicated N mutants. Cell extracts were prepared and VLPs were purified from the culture supernatants as described in Materials and Methods. Aliquots from both cell extracts and VLPs were analyzed by Western blotting, using either anti-Flag (α-Flag) or anti-HA (α-HA) antibody. Protein bands were quantified by densitometry, and percent VLP incorporation was estimated as indicated in the legend to Fig. 2. Mean values from two independent experiments are shown. The positions of N-Flag (N) and Z-ha (Z) are indicated by arrowheads.

Altogether, these results further supported the suggestion that the conserved Cys and His residues within the putative zinc-finger motif, as well as its adjacent region, comprising amino acids 461 to 489, are required for intracellular N-Z interactions and the incorporation of N into Z-directed VLPs.

Nucleoprotein oligomerization. Homo-oligomerization of capsid proteins is thought to play a major role in RNA encapsidation and the nucleocapsid structure of negative-stranded RNA viruses. Since, to our knowledge, no data showing homo-oligomerization of the N protein of any arenavirus has been reported, we investigated whether TCRV N oligomerizes within mammalian cells. To this end, CV1 cells were transfected to express wild-type N-Flag, and detergent-soluble cell extracts were obtained at 24 h posttransfection. To eliminate possible interactions between N protein and cellular RNA, cell lysates were incubated with RNase A. Then, cell lysates were divided into aliquots that were heated at increasing temperatures, with or without a reducing agent. Samples were resolved by SDS-PAGE and analyzed by Western blotting using anti-Flag antibody to detect N-Flag (Fig. 6). N protein was predominantly found as a monomeric form of ca. 70 kDa after sample incubation at room temperature in the absence of reducing agent (Fig. 6, lane 1). In addition, a well-defined band of nearly 125 kDa, consistent with the migration of an N dimeric form, was also observed (lane 1). Overexposure of the membranes allowed the detection of an additional band which might be assigned to N trimers (lane 1). Treatment of the lysates with reducing agent at temperatures up to 40°C did not affect the proportion of these bands (lanes 2 and 3), suggesting that disulfide bonds were not involved in oligomer stabilization. Incubation of the cell extracts at temperatures higher than 40°C caused the disappearance of the oligomeric forms, as well as that of a faster-migrating band, which might be a conformational variant of the monomer (lanes 4 to 6). These results suggest that N protein forms oligomers which could be stabilized by noncovalent bonds.

The N-terminal region of N protein is required for N-N interactions. To define the region of TCRV N involved in homotypic interactions, the ability of the previously generated N mutants (Fig. 1A) to self-associate was evaluated by using coimmunoprecipitation assays (Fig. 7). Briefly, a c-Myc epitope-tagged version of N (N-myc; see Materials and Methods) was coexpressed in CV1 cells along with either N-Flag or each of the N-Flag-truncated mutants. Western blot analysis of cytoplasmic extracts showed that each of the N mutant proteins accumulated to levels similar to those of N-Flag (Fig. 7, panel Input, Wb α-Flag). Likewise, N-myc expression levels were similar under all transfection conditions (panel Input, Wb α-cMyc). Immunoprecipitation of the cell lysates with anti-c-

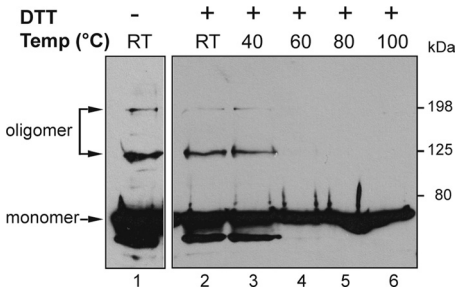

FIG. 6. N protein self-associates. BSR cells were transfected with 1 μg of pTCRV N-Flag, as indicated in Materials and Methods. At 48 h posttransfection, cells were lysed in buffer TNE-N containing protease inhibitors, and cell lysates were clarified by centrifugation at 16,000 × *g* for 10 min at 4°C. Aliquots of the cytoplasmic extracts corresponding to about 0.5×10^5 cells were treated with RNase A at a final concentration of 0.1 mg/ml for 20 min at room temperature. Then, samples were adjusted to 1× SDS-PAGE sample buffer. After addition (+) or not (−) of dithiothreitol (DTT), samples were incubated at the indicated temperatures for 5 min prior to being subjected to SDS-PAGE. Resolved proteins were analyzed by Western blotting using anti-Flag antibody. The molecular masses of the markers (in kDa) are indicated on the right. RT, room temperature.

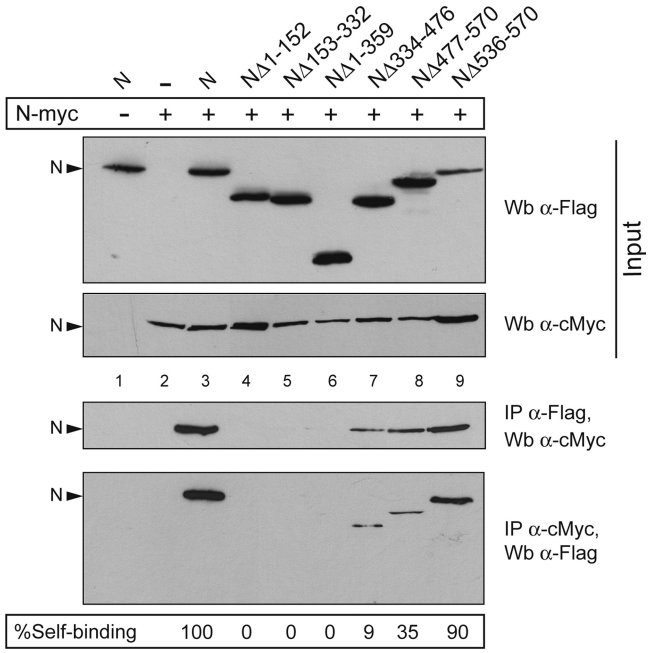

FIG. 7. The N-terminal region of N protein is essential for N-N interactions. CV1 cells were transfected to express TCRV N-myc along with either TCRV N-Flag (lane 3) or each of the indicated Flag-tagged N mutants (lanes 4 to 9). As controls, TCRV N-myc (lane 2) or TCRV N-Flag (lane 1) was expressed alone. Cell extracts, obtained at 24 h posttransfection as indicated in Materials and Methods, were analyzed by Western blotting (Input) using serum against either Flag (Wb α-Flag) or c-Myc (Wb α-cMyc). Additional aliquots of the cell lysates were immunoprecipitated using either anti-Flag (IP α-Flag) or anti-c-Myc (IP α-cMyc) antibody, followed by Western blot analysis of the precipitated proteins, as indicated. Percent self-binding refers to the ratio between N protein detected in immunoprecipitates (IP) to expressed N protein, quantified by densitometry. Mean values from at least three independent experiments are shown; standard deviations ranged from 4% (NΔ477-570) to 25% (NΔ536-570). Arrowheads indicate the positions of either N-myc or N-Flag (N).

Myc antibody, followed by Western blotting using anti-Flag antibody, led to the detection of N-Flag only in cells coexpressing N-myc and N-Flag (panel IP α-cMyc, lane 3), whereas neither N-Flag nor any cellular protein was detected in cells expressing N-Flag alone (panel IP α-cMyc, lane 1). Reciprocally, Western blot analysis using the anti-c-Myc antibody of proteins previously immunoprecipitated with anti-Flag antibody revealed N-myc only in lysates from cells coexpresing both proteins (panel IP α-Flag, lane 3), indicating that N-myc and N-Flag specifically coimmunoprecipitated. Similar results were obtained after pretreatment of the cell extracts with RNase A (data not shown). These data confirmed our observations (Fig. 6) that TCRV N displayed the ability to self-associate. In addition, the data indicated that deletion of 34 residues at the C terminus of N had little effect on N-N interaction, as mutant NΔ536-570 coimmunoprecipitated with N-myc at levels comparable to those exhibited by N-Flag, using either serum (panels IP, lane 9). Extension of the C-terminal deletion to position 477 or deletion of the sequence encompassing residues 334 to 476 caused mutant proteins to display self-interaction capacities that, albeit reduced, were readily detectable (panels IP, lanes 7 and 8). In these experiments, C-terminal mutants were specifically precipitated, as in the absence of N-myc they failed to immunoprecipitate with anti-c-Myc antibody (data not shown). These results suggested that the C-terminal region would be dispensable for N self-association, which was further supported by the fact that mutants carrying short C-terminal deletions (NΔ461-476 and NΔ477-489) or mutant NC503/H506A, with changes predicted to disrupt the putative zinc finger, also exhibited wild-type abilities of self-association (data not shown). In contrast, deletion of the N-terminal 359 residues abolished N-N interaction (panels IP, lane 6), and both mutants NΔ1-152 and NΔ153-332, which carry N-terminal truncations, were unable to coimmunoprecipitate with N-myc (panels IP, lanes 4 and 5), indicating that the N-terminal 332-amino-acid region of N is necessary for N to interact with itself.

In order to identify possible elements involved in N self-interaction, we performed a comparative analysis of nucleoprotein sequences from a representative set of arenaviruses. We searched for conserved motifs that could be associated with homo-oligomerization within the region spanning residues 1 to 332. While no obvious motifs were found between amino acids 153 and 332, we identified a well-conserved 28-amino-acid region near the N terminus of N, where a coiled-coil motif was predicted (Fig. 8A). The coiled-coil motif contains a characteristic repeating heptad pattern of amino acids (usually denoted abcdefg), with the occurrence of nonpolar residues preferentially in the first (a) and, in most cases, the fourth (d) positions of the heptads (38). Within TCRV N protein, this motif extends over positions 92 to 119, comprising four consecutive heptads, in which the first positions are occupied by the hydrophobic residues G92, L99, L106, and I113 (Fig. 8A). Remarkably, three of these residues (G92, L99, and L106) as well as two of those occupying fourth positions within the predicted motif (L102 and L109) are strictly conserved among 99 of 100 arenavirus N sequences accessible through the NCBI server (data not shown).

To analyze whether the predicted coiled-coil motif is important for TCRV N self-interaction, the sequence comprising the whole motif was deleted from the N-Flag-expressing plasmid. The resulting plasmid, designated pNΔ93-119, was cotransfected along with pTCRV N-myc in CV1 cells and N mutant homotypic interactions were investigated by coimmunoprecipitation. As shown in Fig. 8B, the level of expression of mutant NΔ93-119 was similar to that of wild type N-Flag (Fig. 8B, panel Input, Wb α-Flag; compare lane 4 to lane 3). However, the truncated protein did not coimmunoprecipitate with N-myc (panels IP, lane 4), implying that deletion of residues 93 to 119 completely abolished the ability of N to interact with itself. These results indicate that the 28-residue region harboring the predicted coiled-coil motif is essential for maintaining the ability of N to self-interact. We next examined the contribution of key residues within the coiled-coil motif to N self-assembly. To this aim, site-directed mutagenesis was used to replace residues occupying first and fourth positions in the heptad repeats with glutamine (Q), a neutral polar amino acid which displays a high frequency of occurrence in α helices. Single or double glutamine substitutions at these positions were predicted not to disrupt the overall folding of N protein but were expected to interfere with intermonomer interaction. Then, N mutants were evaluated for their ability to self-interact in coimmuno-

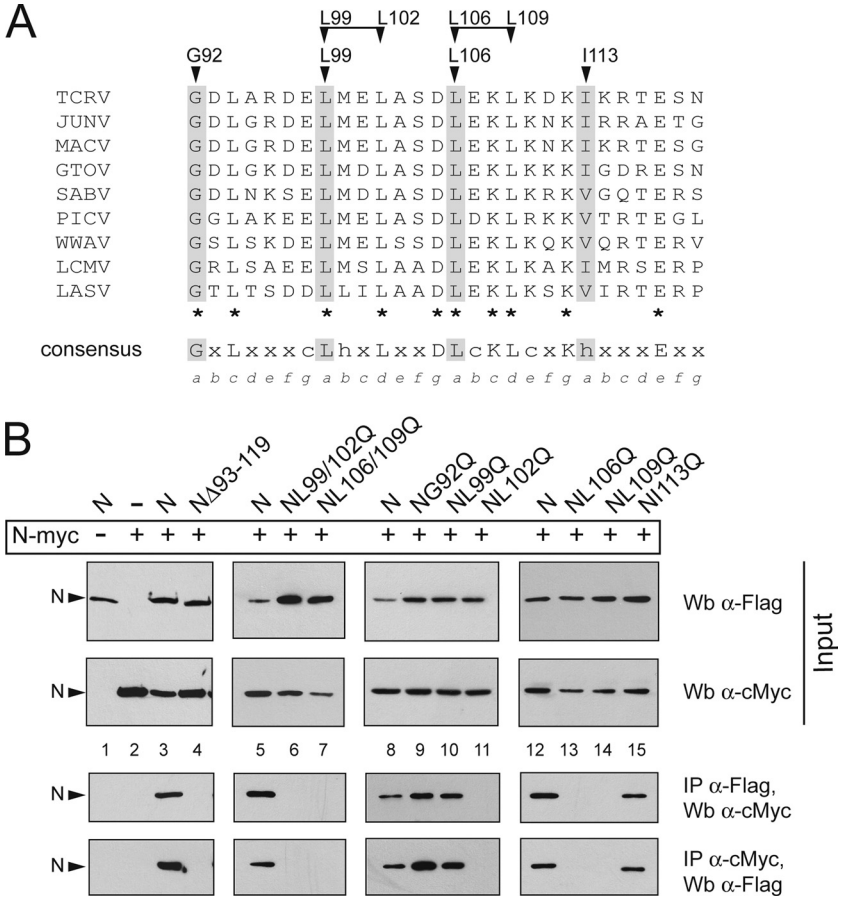

FIG. 8. Contribution of key residues within the predicted coiled-coil motif to N-N interactions. (A) Multiple-sequence alignment of arenavirus nucleoprotein sequences. The region shown, corresponding to residues 92 to 119 of TCRV N protein, was predicted to form an α-helical coiled-coil structure by using the algorithm of Lupas et al. (38), as indicated in Materials and Methods. Residues occupying first positions within the heptad repeats are shadowed. The GenBank accession numbers of the nucleoprotein sequences used are as follows: NP_694850 (TCRV), AAB65465 (JUNV), AAX99340 (MACV), NP_899211 (GTOV), YP_089666 (SABV), YP_138544 (Pichinde virus [PICV]), YP_001911114 (Whitewater Arroyo virus [WWAV]), AAX49342 (LCMV), and NCAP_LASSJ (LASV). Strictly conserved amino acids are marked with asterisks. Numbers on top indicate the positions within the TCRV nucleoprotein sequence which were replaced with glutamine. The consensus sequence is shown below; h, any hydrophobic residue, c, any charged amino acid; x, any residue. Letters a through g at the bottom identify amino acid positions within the coiled-coil motif. (B) CV1 cells were cotransfected with plasmids expressing TCRV N-myc and either TCRV N-Flag (lanes 3, 5, 8, and 12) or each of the indicated Flag-tagged N mutant proteins (lanes 4, 6, 7, 9 to 11, and 13 to 15). As controls, pTCRV N-myc (lane 2) or pTCRV N-Flag (lane 1) was solitarily transfected. Cells were lysed at 24 posttransfection, and cell extracts were immunoprecipitated using either anti-Flag (IP α-Flag) or anti-c-Myc (IP α-cMyc) antibody. Precipitated proteins were analyzed by Western blotting, as indicated on the right. Expression levels of wild-type N-Flag, Flag-tagged N mutants, or N-myc were examined by Western blotting (Input), using anti-Flag (Wb α-Flag) or anti-c-Myc (Wb α-cMyc) antibody. Arrowheads indicate the positions of N-myc or either wild-type or mutant N-Flag proteins (N).

precipitation experiments (Fig. 8B, lanes 5 to 15). Upon coexpression with N-myc in CV1 cells, all Flag-tagged N mutants were detected by Western blotting and reached expression levels comparable to or above those of wild-type N-Flag (panel Input, Wb α-Flag). Nevertheless, double point mutations L99/102Q and L106/109Q resulted in the failure of N protein to self-associate (panels IP, lanes 6 and 7). Moreover, whereas mutation of residue G92, L99, or I113 caused little or no effect (panels IP, lanes 9, 10, and 15), change of amino acid L102, L106, or L109 to Q completely abolished N-N interaction (panels IP, lanes 11, 13, and 14). These results indicated that residues L102, L106, and L109, which occupy key positions within the predicted coiled-coil motif, play a critical role in N self-interaction.

DISCUSSION

The N protein of arenaviruses is the major protein component of viral nucleocapsids. Although the primary function of the nucleoprotein is assumed to depend on its association with the viral RNA, its ability to take part in multiple protein-protein interactions is likely to be important in several steps of the viral life cycle. Nevertheless, relatively few molecular data about the functional domains of N are available so far.

Here, we show an essential role of the C-terminal region of N in N-Z interaction and VLP incorporation. Moreover, we demonstrate for the first time that in order for these interactions to take place, the integrity of a putative zinc-finger motif, as well as its flanking sequences, is required. We also provide

biochemical evidence that TCRV N interacts with itself and define a newly recognized molecular determinant of N homotypic interactions.

Sequences within the C-terminal region of N involved in binding Z. We used mutational analysis to identify regions and specific sequences in TCRV N relevant for N-Z interaction. The data presented here show that the C-terminal region spanning residues 360 to 570 of N is required for both the interaction between N and Z and the recruitment of N into Z-induced VLPs (Fig. 1 and 2). Our findings are consistent with those of previous studies on the Mopeia virus N protein, which concluded that the C-terminal half of N is involved in its incorporation into VLPs directed by Z protein (55). We went further and demonstrated that deletion of short sequences in the region spanning positions 461 to 489 abolishes the ability of N to interact with Z, as determined by coimmunoprecipitation assays and confocal microscopy (Fig. 3 and 4). Moreover, we showed that these mutations abrogate N incorporation into Z-directed VLPs (Fig. 5).

There are precedents for the involvement of zinc fingers in protein function for other viral proteins, such as the nucleocapsid domain of HIV-1 Gag, the human respiratory syncytial virus M2-1 protein, and Ebola virus transcription activator VP30 (14, 20, 44). Remarkably, a putative zinc-finger motif (CHCC) was previously described within the C-terminal region of arenavirus nucleoproteins (47). Moreover, evidence that the region of JUNV N harboring the putative zinc-finger motif displays zinc-binding activity has been provided (60). However, the requirement of this motif for arenavirus nucleoprotein functionality had not been investigated. Our findings indicate that the integrity of the CHCC motif and its N-terminal flanking sequences are essential for N to retain the ability to bind Z (Fig. 3 and 4) and necessary for the recruitment of N into VLPs directed by Z from either TCRV or JUNV (Fig. 5). Considering that deletion of the sequence spanning residues 536 to 570 of N also abrogated N-Z interactions and the incorporation of N into Z-VLPs (Fig. 1 and 2), it seems possible that residue C536, predicted as being included in the CHCC motif (47) (Fig. 3A), and/or other C-terminal sequences may also contribute to Z binding. Although further studies will be necessary to fully establish the identity of the residues directly involved in the N-Z interface, it is conceivable that the CHCC motif may be necessary for the proper folding of the Z-binding domain of N protein. Importantly, the fact that similar results were obtained by comparing the ability of N mutants to interact with either TCRV Z or JUNV Z (Fig. 1 to 3 and 5) suggests that N-Z interactions are likely to occur via a molecular mechanism common (at least) to the New World arenaviruses.

In addition to its role in maintaining N-Z interactions, the CHCC motif might be necessary for other functions of N protein. Interestingly, the double substitution C503/H506A abrogated the ability of TCRV N to support minigenome expression in our TCRV minireplicon system (data not shown). This observation suggests that the disruption of the CHCC motif may affect the ability of N to interact with the L polymerase, to bind viral RNA, or both. It is noteworthy that the analysis of nucleoprotein sequences from representative arenaviruses, using the server BindN (http://bioinfo.ggc.org/bindn), showed a cluster of conserved basic amino acids that are predicted to bind RNA. These residues are located between positions 513 and 524 of TCRV N, which overlap the region comprising the CHCC motif (data not shown).

The N-terminal domain of N mediates N-N interactions. Here we provide the first evidence that TCRV N has the ability to associate with itself into oligomers, of which the putative dimer appears to be the prevalent form (Fig. 6 and 7). In addition, functional characterization of deletion mutants revealed that both the N-terminal 152 residues and the region spanning amino acids 153 to 332 of TCRV N play a critical role in N homotypic interactions (Fig. 7). The ability of TCRV N to self-interact appeared to be independent of its association with RNA, as N oligomers were detected in the absence of viral or cellular RNA (Fig. 6). On the basis of precedent data from other negative-stranded RNA viruses (2, 5, 13, 16, 26, 27, 32, 33, 45, 61, 64), the capacity of TCRV N to self-associate is expected to be displayed by all arenavirus N proteins.

By using secondary structure prediction algorithms, the region encompassing amino acids 92 to 119 of TCRV N was predicted to fold as a coiled-coil structure (Fig. 8A), a prediction that can be extended to nucleoprotein sequences from other representative Old World and New World arenaviruses (data not shown). Coiled coils, known as protein-protein interaction domains, consist of two or more amphipathic α-helical peptides that are packed together to form a supercoil. The characteristic feature of this domain is a heptad periodicity of amino acids, with key hydrophobic residues defining the oligomer interface (7, 38). Our observation that deletion of the sequence spanning residues 93 to 119 abolished N-N interaction (Fig. 8B) raises the possibility that the predicted coiled-coil motif may be essential for TCRV N homo-oligomerization. More importantly, mutation of residues occupying key positions (positions a and/or d) within the heptad repeats completely abrogated N self-interaction (Fig. 8B), further supporting the notion that the amino-terminal coiled-coil motif is an important determinant for N self-association.

Coiled coils have been shown to be important for self-interaction of hantavirus nucleoprotein (2–4, 63). Nevertheless, the contribution of additional sequences that stabilize homotypic interactions has also been reported (28, 29). Similarly, the requirement of the region spanning residues 153 to 332 for TCRV N to self-interact (Fig. 7) indicates that this region may also contribute to maintain N-N interactions. Alternatively, modifications within this region may prevent the coiled coil from being accessible for intermonomer interaction.

Studies on influenza virus N protein have demonstrated the importance of homotypic interactions on RNA binding and transcriptional activities of ribonucleoprotein complexes (9, 12, 16, 65). Likewise, self-association of the N protein of hantaviruses as trimers has been shown to confer specificity for binding the panhandle structure formed by the viral RNA termini (42, 43). We found that deletion of the sequence spanning residues 93 to 119 or mutation of residue L102, L106, or L109 to glutamine resulted in failure of N to promote the amplification of a TCRV minireplicon (data not shown). This suggests that N homotypic interactions may be important for the role of N in viral transcription and replication. In contrast, homo-oligomerization was not necessary for N to bind Z or for the recruitment of N into Z-directed VLPs, as demonstrated by the observation that deletion of the N-terminal 152 residues of N (Fig. 1 and 2) did not substantially affect these processes. In

addition, the fact that C-terminal mutants NΔ461-476 and NΔ477-489 were also unable to support minigenome amplification in our TCRV minireplicon system (data not shown) suggests that, consistent with previous observations on lymphocytic choriomeningitis virus (LCMV) N protein (40), the overall structural integrity of N may be required for its involvement in viral RNA synthesis.

Several studies have shown that the nucleoprotein of viruses belonging to the *Paramyxoviridae* family can generally be divided into two defined regions: an N-terminal moiety, necessary for self-assembly, and a C-terminal domain, implicated in binding to other viral proteins, such as the phosphoprotein (P) and the matrix protein (M) (24, 30, 35). Functional similarities to these nucleoproteins have been reported for the nucleoprotein of filoviruses (13, 34, 46, 64). The three-dimensional structures of the nucleoprotein from viruses belonging to the *Paramyxoviridae*, *Rhabdoviridae*, and *Bornaviridae* families indicate that these proteins adopt a two-domain (N-terminal and C-terminal) structure (1, 18, 52, 57). From our results, a similar overall organization may be envisaged for the N protein of arenaviruses, with an N-terminal domain being responsible for self-association into oligomers and a C-terminal domain being engaged in heterotypic interactions with Z.

Of note, a recent report has shown that the C-terminal region (residues 382 to 386) of the prototypic LCMV N protein plays a critical role in counteracting the host type I interferon (IFN) response (40). Thus, in addition to being involved in the interaction with Z protein, the C-terminal domain of the arenavirus N protein might participate in heterotypic interactions with several different cellular partners.

In summary, in this report we provide new information about the functional organization of the arenavirus N protein. Our mutational analysis led us to define two different functional domains that mediate either N homotypic or N heterotypic interactions. Moreover, we report the requirement of key sequence motifs that appear to be crucial for maintaining these specific N-N and N-Z interactions and for protein functionality. Further investigations into these essential molecular interactions might provide important insights into the molecular aspects of arenavirus replication and assembly and may help to develop new antiviral strategies.

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ADDENDUM IN PROOF

The organization of arenavirus nucleoprotein in two main (N-terminal and C-terminal) domains is further supported by the recently solved X-ray crystal structure of Lassa virus nucleoprotein (X. Qi et al., Nature **468:**779–783, 2010).

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