Viral evolution identifies a regulatory interface between paramyxovirus polymerase complex and nucleocapsid that controls replication dynamics

Julien Sourimant, Vidhi D. Thakkar, Robert M. Cox, Richard K. Plemper*

Paramyxoviruses are negative-polarity RNA viruses of major clinical importance. The dynamic interaction of the RNA-dependent RNA polymerase (RdRP) complex with the encapsidated RNA genome is mechanistically and structurally poorly understood. Having generated recombinant measles (MeV) and canine distemper (CDV) viruses with truncated nucleocapsid (N) protein showing defects in replication kinetics, we have applied a viral evolution approach to the problem. Passage of recombinants resulted in long-range compensatory mutations that restored RdRP bioactivity in minigenome assays and efficient replication of engineered viruses. Compensatory mutations clustered at an electronically compatible acidic loop in N-core and a basic face of the phosphoprotein X domain (P-XD). Co-affinity precipitations, biolayer interferometry, and molecular docking revealed an electrostatic-driven transiently forming interface between these domains. The compensatory mutations reduced electrostatic compatibility of these microdomains and lowered coprecipitation efficiency, consistent with a molecular checkpoint function that regulates paramyxovirus polymerase mobility through modulation of conformational stability of the P-XD assembly.

INTRODUCTION

The order Mononegavirales encompasses nonsegmented RNA virus families with negative-polarity genomes. Several of these families contain major human and animal pathogens, such as Ebola virus, rabies virus, measles virus (MeV), and respiratory syncytial virus (RSV) (1). MeV and several additional members of the paramyxovirus family such as mumps virus (MuV) and the parainfluenza viruses are the etiologic agents of some of the most severe pediatric viral diseases.

Although the family is large and expanding, central features of genome architecture and RNA-dependent RNA polymerase (RdRP) complex organization are well conserved (2). The viral genomes are N-encapsidated, and the resulting ribonucleoprotein (RNP) complexes are organized in a helical configuration. The viral polymerase (L) protein contains all enzymatic activities necessary for RNA synthesis, which includes phosphodiester bond formation, polyadenylation, mRNA capping, and cap methylation, and is recruited to the RNP by the P protein (Fig. 1A). P is a homotetramer with a modular organization in which short, structured functional domains are interspersed by large structurally disordered segments. Two interacting domains between the P and N proteins have been identified: (i) a C-terminal three-helix bundle X domain (P-XD) that interacts with a molecular recognition element (MoRE) located in the large, unstructured C-terminal N-tail that is present in morbilliviruses, henipaviruses, and potentially respiroviruses, and (ii) an N-terminal molecular response element in P that has been shown to interact with the structured core domain of free N monomers, preventing premature N multimerization and preserving an RNA-free state of N (3–5).

Mononegavirales nucleocapsid high-resolution structures are available for a number of representative viruses, including MeV (6, 7), human parainfluenza virus 5 (HPIV-5) (8), and RSV (9). Unstructured N-tail–like domains were found to be conserved among the paramyxoviruses and filoviruses but absent from pneumovirus and rhabdovirus capsids. Furthermore, high-resolution structures of MoRE assemblies with P-XD were solved for MeV and Hendra virus (HeV), which showed a four-helix arrangement consisting of three P-XD–derived helices forming a triangular prism that interacts with a single MoRE-derived α-helix. Although MoRE binding to P-XD is not required for initial recruitment of the polymerase to the RNP template (10), N-MoRE:P-XD interactions are important to prevent premature RdRP termination and correct negotiation of intergenic junctions (11, 12).

The KD (dissociation constant) of the isolated MeV, HeV, and NiV (Nipah virus) N-MoRE interaction with P-XD is 2 to 20 μM (13). However, the tetrameric nature of P and the high density of N-tails in close proximity to the polymerase complex loaded onto the helical RNP template generate high avidity. It therefore remains elusive how advance of MeV—and in fact any mononegavirus—RdRP complexes along the encapsidated template is achieved and regulated, which requires repetitive dissolution and reformation of P interaction not only with N but also with P-XD and L, as recently shown (14). It was suggested that the unstructured central MeV N-tail section located upstream of MoRE may contribute to MoRE separation from P-XD (13). However, we have successfully relocated MoRE into N-core in recombinant, replication-competent MeV (12) and removed all of the central N-tail section (approximately 60 amino acids) without negatively affecting RdRP bioactivity in minigenome assays (15). We also successfully recovered closely related canine distemper virus (CDV) recombinants with corresponding N-tail truncations, which replicated efficiently in cell culture and remained partially pathogenic in the ferret model of CDV infection (15). These findings demonstrated that paramyxovirus polymerase bioactivity does not depend on the physical presence of the unstructured N-tail section. However, genetic evaluation of the N-tail truncated CDV revealed that robust virus growth coincided with the appearance of a mutation at residue 156 in N-core (15), implying that the deleted N-tail section may function as a long-range regulator of RdRP activity.
In the present study, we implemented an in vitro virus evolution approach to elucidate this mechanistic role of unstructured N-tail domains in virus replication. Having identified and validated a panel of compensatory mutations that emerged independently in N-core or P-XD in distinct selection lineages, we found that experimentally confirmed substitutions restoring efficient virus replication clustered in electronically compatible N-core and P-XD microdomains. Direct interactions between these domains were tested biochemically using purified proteins and through biolayer interferometry, confirming the identification of a previously unidentified, intermediate-affinity interface between RNP-embedded N-core and P-XD that is critical for RdRP bioactivity. The spatial location and electronic properties of the microdomains are conserved across diverse paramyxoviruses. Our results suggest that unstructured mononegavirus N-tail domains evolved as major checkpoints for viral polymerase dynamics.

RESULTS

The N-tail–modified CDV that we previously recovered harbored a large internal tail truncation spanning residues 425 to 479 (Fig. 1B). This virus remained partially pathogenic in the ferret animal model of CDV infection (15). Deep sequencing revealed two point mutations in the N-core domain and at the origin of N-tail, E156Q and A410D, respectively, raising the question of whether these changes were instrumental for the efficient growth of the recombinant through a compensatory effect.

Fitness penalty of N-tail truncation is compensated through point mutations in N or P

To test this hypothesis, we first validated bioactivity of the original tail-truncated N lacking these mutations in plasmid-based minigenome assays (Fig. 1C). Because RdRP bioactivity is critically affected by the relative ratio of nucleocapsid and polymerase complexes, we generated activity profiles of mutant and standard N over a wide ratio range. In the presence of standard N, RdRP activity adhered to a log-normal model with increasing relative P-L amounts, reaching a peak at a 4.9:1 (N:L-P) ratio. The tail-truncated N mutant likewise followed a ratio-dependent activity optimum curve, but peak signal was reached at a lower 3:1 (N:L-P) ratio and peak height was reduced to 43% compared to standard N.

Activity profiling thus confirmed that the tail-truncated N is bioactive but revealed a statistically significant activity penalty. To explore whether distinct avenues to compensate tail truncations are available to the protein complex, we sought to expand the portfolio of compensatory mutation candidates through multiple independent virus recovery transfections from cloned complementary DNA (cDNA), containing either standard N (recCDV N wt; three independent transfections) (16) or tail-truncated N [recCDV NΔ[425–479]; 11 independent transfections (15)]. We transferred three individual infectious centers of each recovery transfection reaction to CDV-permissive Vero cells stably expressing canine SLAM (Vero-cSLAM) when multinucleated giant cells or syncytia, which represent the predominant cytopathic effect (CPE) associated with...
CDV infection, became detectable microscopically. Within 48 hours of transfer, cultures having received standard recCDV N wt infectious centers showed extensive CPE, producing a mean of 52 progeny syncytia each (Fig. 1D). By comparison, recovery of recCDV NΔ[425–479] was likewise successful, but initial virus spread was drastically reduced with a mean of only 0.89 (0 to 4) syncytia observed 48 hours after transfer. To assess virus growth kinetics at this very early stage after recovery [passage 1 (p1)], we quantified the enlargement of this inaugural round of progeny syncytia. Standard recCDV N wt replicated without appreciable time delay after recovery, reflected by rapid expansion of syncytia (Fig. 1, E and F). In contrast, recCDV NΔ[425–479] followed a biphasic initial replication pattern. First, infectious centers expanded slowly, requiring passaging of infected cells to maintain cultures in the most permissive exponential cell growth phase. After approximately 60 hours, however, syncytia growth rates increased drastically to a level equivalent to that of standard recCDV N wt. All subsequent infections of cells with these recCDV NΔ[425–479] populations resulted in rapid, recCDV N wt-like CPE formation.

Having subjected 10 independently recovered recCDV NΔ[425–479] populations to 10 consecutive passages each on Vero-cSLAM cells, we Sanger-sequenced the viral N and P genes (Fig. 1G). Seven of these p10 virus populations contained allele-dominant point mutations in N, which were located in N-tail in the two candidates and in N-core in the other five. Two virus populations featured mutations exclusively in the P protein, and only one contained changes in both N and P. Whenever mutations in P emerged, at least one substitution was located in P-XD. By comparison, none of the three independently recovered and passaged standard recCDV N wt populations acquired changes in the N or P protein.

The biphasic initial replication kinetic of all newly recovered recCDV NΔ[425–479] and the identification of allele-dominant substitutions in N and/or P exclusively in these populations are consistent with rapid in cellula evolution of the N-tail truncated recCDV after initial recovery. These observations suggest that the individual mutations restore viral fitness through compensatory effects. Most of the substitutions were located outside of N-tail—only one candidate contained a change (R424S) in immediate proximity of the truncation site—indicating that compensation is achieved mechanistically through long-range effects.

**Compensatory mutation candidates cluster in distinct N-core and P-XD microdomains**

Sequence alignment of different morbillivirus N proteins revealed that the substitutions affect residues that are well conserved within the paramyxovirus genus (fig. S1). To test for compensatory effects, we rebuilt all candidate mutations in the context of both N wt and NΔ[425–479] and determined activity profiles over the broad P-L to N ratio in minigenome assays (three selected candidates with mutations in different areas are shown in Fig. 2A; all others are shown in figs. S2 and S3A). Comparing informative peak RdRP activities, we identified three distinct mutations in N and five in P that statistically significantly restored bioactivity of NΔ[425–479] in the minigenome assays (Fig. 2, B and C), including the N-E156Q A410D double mutation that had first emerged in our previous study (15). CDV strains S804PheH (used for adaptation) and Onderstepoort (the basis for the minigenome system) contain a natural A/T allele variation of N residue 410. However, insertion of a T410A substitution in the Onderstepoort minigenome system did not affect polymerase activity, indicating that this variation has no functional impact (fig. S3B).

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**Fig. 2. Identification of specific mutations compensating for N-tail truncation.** (A) Minigenome activity profiles of full-length (blue) and tail-truncated (red) N. Normalized activities of selected mutations in full-length (blue line and symbols) and tail-truncated (red line and symbols) N background (n = 3) are overlaid. A natural allele A/T allele variation exists at residue 410 between CDV strains S804PheH and Onderstepoort. Statistical analysis and regression modeling as in Fig. 1C. (B and C) Peak minigenome activities after rebuilding of candidate substitutions, analyzed as in (A). Each set of peak activity in the context of either N or Δ[425–479] was compared with one-way ANOVA with Dunnett’s multiple comparisons post hoc test. Mutations associated with statistically significant polymerase activity differences in either N background or showing the greatest increase in the presence of tail-truncated N are highlighted with yellow shading. (D) Localization of compensatory mutation candidates in N-core. Surface representation (left) and ribbon model enlargements (right) of mutation sites are shown [Protein Data Bank (PDB): 4UFT]. Substituted side chains as sticks, color-coded by positive (green), neutral (blue), or negative (red) effect on RdRP activity in the distinct N-tail background. (E) Localization of compensatory mutation candidates in P-XD. Ribbon models of P-XD (PDB: 1T6O) are shown, color coding as in (D).
When we located the different N-core substitutions in a homology model of CDV nucleocapsid that was generated on the basis of the cryo–electron microscopy reconstruction reported for MeV (6), D151E, D154G, E156Q [associated with statistically unchanged or significantly higher minigenome activity in the NA[425–479] background], and F209C were predicted to reside in close proximity to each other in a defined microdomain located on the outer surface of the RNP helix (Fig. 2D). The remaining mutation, E103K, was posited between consecutive rungs of the helical RNP assembly, possibly in proximity to the origin of N-tail protruding from the RNP at the intersections between neighboring rings.

While most of N-tail is intrinsically disordered, three substitutions appeared one each in the three conserved tail subdomains: box1 (S415P), box2 (L503F), and box3 (R519G) (Fig. 1A). Minigenome activity testing revealed three distinct phenotypes associated with these changes: S415P statistically significantly increased RdRP bioactivity in both truncated and full-length N-tail background (113 and 165%, respectively), L503F had no major effect on bioactivity (66 and 72%, respectively), and R519G caused activity losses in either N background (12 and 32%, respectively) (Fig. 2B). These findings underscore the importance of box1 residues in regulating polymerase bioactivity.

Although morbillivirus P proteins are more sequence diverse than the N proteins, P mutations A465V, R469G, H479N, and K486N are all located in the conserved C-terminal P-XD (Fig. 2E). All of these substitutions statistically significantly boosted RdRP bioactivity in minigenome assays in the context of tail-truncated NA[425–479], while combination with standard N wt had either no statistically signficant effect or reduced (R469G) bioactivity (Fig. 2C). A crystal structure has been released for MeV P-XD, revealing folding into a triangular prism conformation (17). When located in a corresponding CDV P-XD model, we found all four mutations to populate a single side of the prism created by α-helices 1 and 2 (Fig. 2E). This α1α2 face is distinct from the known P-XD interfaces α2α3 with the N-MoRE domain (18, 19) and α1α3 with the L protein (14) and has not previously been implicated in engaging in protein-protein interactions. Only one P protein mutation, P42L, was located outside of P-XD in a more variable zone near the N terminus of the protein.

**Recombinant, tail-truncated CDVs with candidate compensation mutations regain full fitness and are genetically stable**

On the basis of the performance in minigenome activity assays and location in diverse areas of the N and P proteins, we selected the N-E156Q A410D double mutation, N-S415P, and P-R469G in P-XD for reconstruction in the context of standard and N-tail–truncated recombinant CDVs (Fig. 3A) and determined recovery profiles and growth kinetics. All recCDVs’s carrying either of these mutations in the NA[425–479] background were recovered as efficient as standard recCDV, but each of the substitutions negatively affected virus recovery when combined with N wt (Fig. 3B). Initial replication kinetics of the three recCDV NA[425–479] with the reconstructed substitutions were likewise indistinguishable from that of standard recCDV (Fig. 3C), while recCDVs with full-length N showed initial growth delays (N-E156Q/A410D mutation) or a severe and lasting growth block (N-S415P).

**Fig. 3. Recovery of recCDV with confirmed compensatory mutations.** (A) Schematic of recCDV genome organization [red star: N-E156Q + A410D (CDV 5804PeH background); green star: N-S415P; blue star: P-R469G; mutant color coding is maintained in all subpanels of this figure]. (B) Enumeration of progeny syncytia after transfer of initial infectious centers after recovery. (C) Mean syncytium extension of three independently recovered recombinants each, analyzed at 14, 22, and 35 hours post-infection. (D) Genetic stability of recCDV with full-length (n = 3) or Δ[425–479] (n = 10) N and engineered substitutions as specified after five passages. N and P genes were analyzed. (E) Multicycle growth profiles of recCDV as specified. Symbols represent mean viral titers (n = 3), and error bars denote SD to the mean. Two-way ANOVA with Dunnett’s multiple comparisons post hoc test.

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To assess genetic stability of the different CDVs, we subjected all recombinants to five consecutive passages in three independent replicates each, followed by sequence analysis of the N and P open reading frames (ORFs) (Fig. 3D). While most candidates were genetically stable, the N-S415P substitution triggered, in all cases, additional substitutions when present in recCDVs with full-length N. One replicate of recCDV with N-tail truncation and N-S415P developed a change in P (P-L148R), although this substitution was not allele dominant after five passages (fig. S4). Multicycle viral growth curves confirmed that standard recCDV-like replication rates and peak titers were restored in all three recCDV NΔ[425–479] populations carrying the reconstructed mutations (Fig. 3E). Maximal progeny titers of recCDV NΔ[425–479] N-S415P slightly exceeded those of recCDV N wt.

These observations demonstrate that the tested mutations in N and P fully compensate for the NΔ[425–479] tail truncation under physiologically relevant conditions of virus replication in cultured cells, establishing a functional link between the microdomain identified on N-core and the α1α2 face of P-XD.

Compensatory mutations are located in microdomains with complementary electronic properties

Inspection of the nature of the compensatory substitutions improving RdRP activity in the minigenome assays revealed that the majority affected negatively charged residues in N-core (D151, D154, and E156) and positively charged amino acids in P-XD (R469, H479, and K486) (Fig. 4, A to D). To directly probe the importance of charge in these microdomains for bioactivity, we generated a series of charge-neutralization and/or charge-reversal mutations covering both areas and assessed the effect on RdRP bioactivity in the context of standard and tail-truncated N (Fig. 4, E to H, and figs. S5 and S6). All charge reversals in the acidic loop in N-core abrogated or statistically significantly reduced polymerase activity in the context of both full-length and tail-truncated N (illustrated for residues D156 and D158 in Fig. 4E, summarized for all substitutions tested in Fig. 4F). Charge neutralization was better tolerated, returning peak activities of 52 and 39% residual activities for N-E148N in the context of standard and tail-truncated N proteins, respectively, and 134 and 20% for D158N. Both E161N and E208N eliminated polymerase activity.

Altering the positively charged α1α2 P-XD face of the P-XD helix bundle was more agreeable with polymerase bioactivity overall (Fig. 4, G and H). The activity was unchanged by mutation P-R469E and drastically increased by P-R472E charge reversal in the context of tail-truncated NΔ[425–479] (163% of reference activity; Fig. 4H). Noteworthy, the P-R472E substitution had no effect on RdRP activity in the context of full-length N. Double and triple combinations of charge-reversal mutations moderately to drastically reduced polymerase activity in the context of both standard and truncated N. Whereas introduction of either a positive or negative charge at position 465 reduced activity with tail-truncated N, only a negative charge had a clear impact on full-length N (~51%) (Fig. 4H and fig. S6).

These results reveal that maintaining a high local density of negative charge in the N-core microdomain is required for polymerase functionality. Greater resilience of the α1α2 P-XD face to charge reversal suggests more redundancy in this system. However, the differential effect of P-R472E and A465D or A465R on RdRP bioactivity in the context of NΔ[425–479] versus full-length N emphasizes the functional link between the presence of the unstructured central N-tail section and the α1α2 face of P-XD.

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Fig. 4. Electronic properties of compensatory microdomains in N-core and P-XD. (A to D) Ribbon (A and C) and electrostatic surface (B and D) representations of compensatory microdomains in CDV N-core (A and B) and P-XD (C and D). Residues subjected to mutagenesis are labeled, and negative (red) and positive (blue) charges are indicated. (E to H) Peak minigenome activities in the distinct N-tail backgrounds after charge-reversal or charge-neutralization mutagenesis as specified in N (E and F) or P (G and H). Statistical analysis and regression modeling as in Fig. 2 (B and C). One-way ANOVA with Holm-Sidak’s multiple comparisons post hoc test.
Purified P-XD:N-MoRE complex specifically interacts with N-core

To biochemically test whether the acidic N loop and P-XD micro-domains defined by the compensatory mutations interact with each other, we purified glutathione S-transferase (GST)–tagged CDV P-XD and P-XD:N-MoRE complexes after prokaryotic expression (Fig. 5A), the latter synthesized as single polypeptides as described for MeV (18). Independently, we purified hexahistidine-tagged N-core fragments truncated after tail residue 419, which reportedly prevents compaction of the corresponding nucleocapsids (20). Two mutated versions of these proteins, P-R469E and N-E156K in P-XD and N-core, respectively, were generated and purified in parallel to determine specificity of candidate interactions.

With an electrophoretically authenticated set of purified proteins at hand (fig. S7), we analyzed the direct interaction of N-core and P-XD:N-MoRE through affinity coprecipitation followed by gel fractionation (Fig. 5B). Approximately 25% (±17%) of the N[1–419] input material coprecipitated with P-XD:N-MoRE, demonstrating efficient interaction of the proteins (Fig. 5C). In contrast, free P-XD was unable to bind to N[1–419]. GST alone also did not interact with N[1–419], and introduction of the E156K charge-reversal mutation into N-core abrogated binding. For orthogonal validation of these interactions, we performed a photocoupling assay (Fig. 5D) and biolayer interferometry (Fig. 5E–G).}

Fig. 5. Biochemical interaction of compensatory microdomains in N-core and P-XD. (A) Top: Ribbon representation of P-XD (pink)/N-MoRE (orange) (PDB: 1T6O). Bottom: Triangular prism configuration of P-XD. (B) Affinity coprecipitation with Glutathione Sepharose beads. Coomassie blue–stained gels (left) and anti-CDV N immunoblots (right) of eluted protein fractions after SDS–polyacrylamide gel electrophoresis are shown. *Copurified cellular contaminants. (C) Densitometry of coprecipitations. Values denote precipitation efficiencies relative to input material (n ≥ 3 ± SD). One-way ANOVA with Holm-Sidak’s multiple comparisons post hoc test. (D) Biolayer interferometry showing association and dissociation of N[1–419] ± E156K substitution with biosensors loaded with indicated GST fusions. (E) Affinity coprecipitation as in (B) with natural P-XD compensatory mutations A465V and A469G and engineered A465R. Coomassie blue–stained gels. (F) Densitometry of coprecipitations as in (C) (n = 5 ± SD). One-way ANOVA with Holm-Sidak’s multiple comparisons post hoc test. (G) Phenotypic effects of compensatory and engineered mutations at P positions 465 and 469. (H) Molecular docking of P-XD:N-MoRE complexes (PDB: 1T6O) into N-core (PDB: 4UFT). Top: Surface of N protomers (blue, purple, and green) and P-XD (pink) with N-MoRE (orange). Bottom: Predicted contacts between P-XD and N-core acidic loop and N-MoRE with N-core loop (residues 133 to 142; yellow). Right: Docking scores and PRODIGY predictions (36).
copper precipitation results, we used biolayer interferometry for label-free quantitative assessment of protein binding using GST-specific biosensors coated with the different GST-tagged P-XD polypeptides or, for reference, GST alone. All sensors were tested against standard or mutant N[1–419] polypeptides at a concentration range of 0.5 to 4 μM (Fig. 5D). Concentration-dependent interactions in the micromolar range were detected between P-XD:N-MoRE complexes and standard N[1–419], although precise kinetics could not reasonably be extracted because of the large number of binding sites present in the multimeric N assemblies and the GST-fusion protein dimers. Binding of free P-XD lacking MoRE to N[1–419] was also detected, albeit substantially weaker. Each of the engineered point mutations in N-core and P-XD abolished binding, and interaction of N[1–419] with biosensors coated with free GST remained negligible, confirming specificity of the assay. This panel of biochemical assays therefore revealed consistently that P-XD:N-MoRE complexes interact specifically with N-core and implicate positively charged residues located in the α1α2 face of P-XD prism as instrumental for efficient binding.

Having validated the co-affinity precipitation assay, we explored the effect of specific P-XD mutations that emerged from viral evolution, A465V and A469G, on the P-XD:N-MoRE interaction with N-core (Fig. 5, E and F). Both substitutions decreased precipitation efficiency, whereas further increasing the positive charge of the P-XD α1α2 face through an A465R enhanced the interaction, consistent with the reduced bioactivity of this mutant in the minireplicon assays. These results indicate that compensatory mutations restore virus replication in the presence of tail-truncated N by reducing binding affinity of P-XD:N-MoRE for N-core. Engineered mutations at P residues 465 and 469 showed a similar inverse correlation between biochemical interaction and polymerase activity in the context of tail-truncated N (Fig. 5G).

To assess candidate docking poses of P-XD:N-MoRE complexes to the nucleocapsid assembly, we used the data-driven HADDOCK webserver in conjunction with available structures of morbillivirus N-core [Protein Data Bank (PDB): 4UFT] (6) and P-XD-MoRE (PDB: 1T6O) (18). Because the virus evolution approach had highlighted the α1α2 face of P-XD and acidic N loop microdomains, we included orientation of residues within these domains toward each other as a mandatory spatial restraint. After two refinement steps, semiflexible minimization and molecular dynamics with explicit water layer, we obtained several top-scoring interaction poses with similar orientation. The complex represented in Fig. 5H postulates a total buried surface area of approximately 1008 Å² and brings, as requested, residues in the acidic N loop including D154, E156, and D158 and the α1α2 P-XD face (among others, R469 and K478) in close proximity. The predicted favorable contacts are predominantly based on electrostatic interactions (−332.3 kcal/mol). Unexpectedly, this configuration postulates a second interface between solvent-exposed residues of N-MoRE associated with the docked P-XD and distinct N-core residues located in the preceding N protomer that include the flexible N-core loop spanning residues 133 to 142 (fig. S8) (6). A predicted binding affinity of 160 μM is consistent with the moderate interaction strengths detected biochemically through bi-layer interferometry and affinity coprecipitation.

**DISCUSSION**

The C-terminal XD of paramyxovirus P protein interacts with a microdomain in the structurally largely disordered N-tail domain (13) and the L protein (14), respectively, and little was known about how these interactions could affect N-core organization. In this study, we have established a molecular link between paramyxovirus P-XD binding to MoRE located in the N-tail and subsequent interaction of P-XD with N-core, which is substantiated through physiologically relevant directed viral evolution interfaced with functional and in vitro biochemical analyses.

Quite unexpectedly, the viral evolution–based study has identified not just one, but three distinct structural domains, two located in the N protein and one in P, that can harbor point mutations restoring efficient replication of recombinant viruses lacking large sections of N-tail. Of these compensation hotspot domains, changes of residues located at the tail origin (positions 401 to 419) were the least surprising considering their immediate linear proximity to the truncation site. However, effective compensatory mutations mapped predominantly to an exposed acidic N loop on the surface of N-core (residues 146 to 161) or to a basic face of the P-XD triangular prism, formed by helices α1 and α2.

Cryo–electron microscopy models of MeV and parainfluenza virus type 5 (PIV-5) RNP showed strong structural homologies (6, 8), indicating an overall comparable architecture. Alignment of 72 paramyxovirus N protein sequences underscored strict conservation of the acidic N loop highlighted by the CDV compensation mutations (fig. S1). Because of fuzzy appearance in MeV RNP electron density maps, this loop had been discussed as a hypothetical interaction site (5, 6), but no potential binding partner was proposed. However, anecdotal evidence implicates this section of N-core in ensuring proper polymerase function: Sendai virus (SeV) harboring an N-D153Y point mutation produced an abnormally high amount of copy-back defective interfering genomes (21), substitutions of NiV N residues 142 to 146 for alanines decreased N interaction with P (22), and we have shown that MeV N-MoRE relocation into N-core increased the relative frequency of nonproductive polycistronic viral mRNAs in infected cells (12). In each of these reports, residues targeted for substitution or N-MoRE relocation were located within, or in close proximity to, N-core residues homologous to the compensatory acidic loop identified in CDV N (fig. S9).

Transient backfolding of MeV N-MoRE onto the RNP has been proposed previously based on nuclear magnetic resonance studies of purified nucleocapsids (23). However, the nature of possible MoRE binding sites on N-core and the physiological importance of this weak interaction remained unclear. Our results demonstrate a biochemically appreciable interaction of N-MoRE:P-XD complexes with N-core, which indicates that the stable four-helix bundle configuration of MoRE complexed with P-XD is the N-core binding–competent form. Our identification of this interaction through viral evolution confirms physiological relevance for viral fitness, and five lines of evidence support the idea that P-XD forms a direct, transient interface with N-core: (i) Compensatory mutations mapped to P-XD or N-core, but not to N-MoRE; (ii) the microdomains in P-XD and N-core defined by these compensatory mutations are electronically compatible (fig. S10); (iii) engineered and natural compensatory mutations introduced into the two opposing microdomains disrupted binding in biochemical assays; (iv) purified N-MoRE in isolation was unable to efficiently interact with N; and (v) an energetically favorable docking pose could be found in silico.

Top-scoring docking poses of the P-XD interaction with the acidic loop in N-core placed P-XD–associated MoRE into contact with the flexible loop around N-core residues 133 to 142 in the preceding
N monomer. We have previously physically relocated MoRE of closely related MeV into this loop, downstream of residue 138, and recovered replication-competent recombinant viruses (12). The docking poses raise the intriguing possibility that successful MoRE relocation in the previous study was achievable precisely because MoRE naturally relocates spatially into the proximity of the target site selected for transfer (Fig. 6A). Thus, the previous N engineering may have placed the relocated MoRE and, subsequently, MoRE-associated P-XD in a spatial position relative to the RNP helix trunk that mimicked the physiological arrangement.

The interaction of C-terminal CDV P residues with N-core in the RNP assembly is reminiscent of the well-studied contacts between pneumovirus P and N. In the case of RSV specifically, the C-terminal residues of P engage in interactions with a tail-less nucleocapsid core.
that are modulated by electrostatic contacts (24). This N binding
domain is also, in both cases, partially overlapping with the L binding
domain (14, 25, 26), which brings L in close proximity to the RNA.
Despite a remarkable lack of sequence conservation between paramyo-
virus and pneumovirus nucleocapsids, structural alignments of RSV
and CDV RNP models demonstrate that the P binding pockets in RSV
RNPs are positioned in close proximity to the compensatory
acidic N loop identified in the CDV RNP N-core. This strong struc-
tural mimicry implies that P-XD association with N-core identified
here may reflect an ancestral step in mononegavirus negotiation of
the RNP template.

To better understand the importance of this interaction for poly-
merase function, we wondered what the mutations identified through
viral evolution may actually compensate for. Shortening of the un-
structured N-tail section could, in principle, disturb the interaction
through reduced positional flexibility of N-tail lacking more than
50 structurally disordered residues. In this scenario, compensatory
mutations may act through increasing binding affinity. However,
all substitutions emerging from virus evolution reduced electronic
compatibility of the P-XD and N-core microdomains, and those
tested biochemically impaired coprecipitation efficiency. These
results support the alternative model that the compensatory muta-
tions reduced affinity of the N and P domains to each other. Wild-type
N-tails add considerable electron density to the outside of the RNP
helix and are thought to refold upon binding of P-XD to MoRE to
allow access of the polymerase complex to the template (10). This
tail-ordering step modulates polymerase performance (12) and
should culminate in the interaction of MoRE-bound P-XD with
N-core (Fig. 6B). If the disordered N-tail residues are eliminated,
reordering naturally becomes obsolete, presumably shortening the
time interval between MoRE interaction with P-XD and MoRE:P-XD
complex binding to N-core. We propose that substitutions reducing
affinity of MoRE:P-XD for N-core compensate for this accelerated
rate of MoRE:P-XD collision with N-core. In this model, N-tail re-
ordering constitutes a regulatory element controlling polymerase dy-
namics. In the absence of a disordered N-tail domain, the recCDVs
evolved to restore proper polymerase processivity by down-modulating
affinity of the interface between P-XD and N-core, effectively re-
placing kinetic control of interface formation with thermodynamic
regulation (Fig. 6B).

Ultimately, the formation of the three-way N-MoRE:P-XD:N-core
interaction may directly contribute to allowing polymerase advance
along the encapsidated template. In addition to the P-XD interac-
tion with N-MoRE, our laboratory has recently demonstrated the interaction of a second distinct face of the P-XD trimeric prism
with the polymerase L. We have shown that this interaction involves
specifically one P-XD of the P tetramer, while the other three XDs
are competent for N-MoRE binding (14). However, the molecular
mechanism triggering iterative dissociation of the N-MoRE:P-XD
and L:P-XD interaction, which emerged as a prerequisite for poly-
merase mobility, remained elusive. Here, we show the interaction of
the third P-XD prism face with N-core, placing P-XD at a pivotal
position in between the RNP, N-tails, and the polymerase protein.
Residue R465 in MeV P-XD has been shown to act as a determinant
for the stability of the P-XD triple helix assembly through the for-
mation of salt bridges between helices α1 and α2 (27). On the basis
of the placement of P-XD at the center of multiple transiently form-
ing interfaces and this demonstrated effect of electronic interactions
on P-XD conformational stability, we propose that the assembly of
the electrostatic complex with N-core identified in this study triggers
a switch from the stable triple helix P-XD configuration to a less-
ordered intermediate. This structural reorganization is anticipated
to alter binding affinities to N-MoRE and/or L, enabling temporary
separation of P-XD from its binding partners as proposed (14)
and advance of the polymerase complex along the template by one
N protomer. Spontaneous restoring of the stable triple helix confor-
mation once P-XD is removed from the destabilizing electronic in-
fluence of the N-core binding site sets the stage for re-binding to L
(14) and N-MoRE of a downstream N protomer followed by repeat
of the cycle. In this mechanistic model, the N-core to P-XD inter-
face thus functions as a molecular checkpoint that governs poly-
merase mobility through structural modulation of the P-XD triple
helix configuration.

MATERIALS AND METHODS

Cell culture
African green monkey kidney epithelial cells [CCK-81; American
Type Culture Collection (ATCC)] stably expressing canine signal-
ing lymphocytic activation molecule (Vero-cSLAM) (28) and baby
hamster kidney cells (C-13; American Type Culture Collection) sta-
bilized expressing T7 polymerase (BSR-T7/5) (29) were maintained
at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium
supplemented with 7.5% fetal bovine serum and were reselected
with genetin (100 μg/ml) at every fifth passage. Transient trans-
fections were performed with GeneJuice (Novagen) according to the
manufacturer’s protocol.

Molecular biology
Plasmids encoding expression constructs of CDV strain Onderstepoort
N, P, and L (16), morbillivirus minigenome (10), and plasmids har-
boring full-length cDNA copies of the CDV strain 5804PEH genome
(16) were previously described. Construction of the nucleoprotein
truncation [425–479] without the extra residues resulting from the
original cloning strategy was removed by site-directed mutagenesis.
For protein production, the sequence of CDV N strain 5804 encom-
passing residues 1 to 419 was amplified by polymerase chain rea-
tion (PCR) with a C-terminal hexahistidine tag and an enterokinase
cleavage site (QDGGNDDDDKLEHHHHHH) and cloned onto plas-
mid pET30a+. To express GST-XD, the residues of P strain 5804
[457–507] were amplified by PCR and cloned in pGEX 6P1 down-
stream of the GST and thrombin cleavage sequence between Bam
HI and Eco RI. Extra C-terminal residues from the cloning strategy
resulted in the added C-terminal residues GSGSQL. To express the
fusion GST-XD-box2 containing N-MoRE, residues 486 to 505 of N
strain 5804 were directly fused downstream the GST-XD sequence,
with residues GS and QL. All substitutions were engineered by site-
directed mutagenesis, and sequences were verified by Sanger
sequencing (Genewiz).

Minigenome luciferase reporter assay
BSR-T7/5 cells (5000) were seeded in a clear bottom white wall
96-well plate and transfected upon reaching 60% confluence with
plasmids encoding the morbillivirus luciferase replicon reporter
(0.044 μg), CDV-N (0.016 μg), and a twofold serial dilution of CDV-L
and CDV-P from 0.02 μg each. The total amount of DNA transfect-
d was adjusted with a pCDNA3.1 plasmid (Invitrogen). Specifically,
for each experiment, plasmids were combined in master mixes
containing all components, but the plasmid encoding the mutation-bearing protein was investigated. These master mixes were then serially diluted five times in twofold steps with a mix of pCDNA3.1 and replicon reporter, keeping the total DNA amount and the amount of reporter plasmid equal in every dilution step. Each dilution was finally mixed with the plasmid encoding the mutation-bearing protein investigated. In the case of P mutations, master mRNAs were further prepared with either CDV-N or CDV-NA[425–479]. For each experiment, plates were seeded in parallel, and transfections were carried out with a master mix of GeneJuice transfection reagent (MilliporeSigma) according to the manufacturer’s instructions. Each experiment included a reference plate containing a serial dilution of the unmutated plasmid for normalization. Firefly luciferase activities were determined 24 hours after transfection in a Synergy H1 microplate reader (BioTek) using ONE-Glo luciferase substrate (Promega). Relative RdRP activities (relA) were determined using a 12-well plate of Vero-cSLAM cells infected at a multiplicity of infection of 0.01, followed by incubation at 37°C for 40 to 48 hours. When microscopically observed virus-induced CPE reached approximately 90%, cell-associated viral particles were released through two consecutive freeze/thaw cycles, followed by a clearance spin (1000g, 5 min), and storage of single-use aliquots at −80°C. Viral titers were determined by TCID50 (median tissue culture infectious dose) titration on Vero-cSLAM cells as described (30).

**Multistep virus growth curves**
A 12-well plate of Vero-cSLAM cells was infected at a multiplicity of infection of 0.001, followed by incubation at 37°C for 60 hours. At 12, 18, 24, 36, 48, and 60 hours post-infection, one well of infected cells was harvested and titrated by end-point dilution assay. Each time point consisted of three independently infected cell monolayers.

**Sequence alignments**
Alignments were conducted using T-Coffee PSI-Coffee server and formatted with ESPript (31).

**Protein homology modeling**
Modeling of CDV nucleoprotein (5804P strain) assembly was conducted using SWISS-MODEL server on expasy.org and MeV nucleoprotein assembly structure (PDB: 4UF1) (5). Modeling of SeV Cantell strain clone cCdi (AB855654.1) and CDV (5804P strain) RNA-free nucleoprotein monomers was conducted using SWISS-MODEL server on expasy.org using MeV RNA-free nucleoprotein structure (PDB: 5E4V) (5). Modeling of CDV, HPIV-3, NiV, and PIV-5 phosphoprotein XDs was conducted using SWISS-MODEL server on expasy.org using MeV (PDB: 1OKS) (17), SeV (PDB: 1R4G) (32), HeV (PDB: 4HEO) (33), and MuV (PDB: 3BBZ) (34) P-XD crystal or solution structure. Modeling of MeV nucleoprotein with internal N-MoRE (12) was performed on I-TASSER server (35) using additional restraints with α-helix secondary structure specified for residues of MoRE (QPQDSSRSADALLRQAMAGI) and using the docked pose of P-XD-MoRE (PDB: 1T6O) on MeV nucleocapsid (PDB: 4UF1) as additional template (see the molecular docking method).

**Molecular docking**
N-core (PDB: 4UF1) (6) and P-XD-MoRE (PDB: 1T6O) (18) complexes were generated using HADDOCK webserver (36). HADDOCK uses ambiguous interaction restraints to guide the first steps in the docking process. Active and passive residues were defined on the basis of recCDV N[425–479] compensatory mutations and surrounding residues, respectively, using their equivalent residues after sequence alignment with MeV sequences (N active residues: D154, E156, and Q158; N passive residues: E148 and F209; P active residues: R465, K469, K478, and M482; P passive residues: R472 and D486). Initially, 1000 docking poses were generated with rigid body docking, and the best 200 poses were then refined by a semiflexible simulated annealing in torsion angle space stage. This is a three-step molecular dynamics–based refinement. This stage was followed by an explicit water refinement stage with short (2-fs steps) molecular dynamics simulation at 300 K. The final scoring combined electrostatics, desolvation, and van der Waals energy terms in combination with the energetic contribution of the restraint used to drive
the docking. Binding affinity prediction was performed using the PRODIGY server (37).

**Recombinant protein expression and purification**

GST fusion proteins were expressed in competent One Shot BL21 pLysS chemically competent *Escherichia coli* (Thermo Fisher Scientific). Cells were grown at 37°C for 8 hours or until saturation, and protein expression was induced for 16 hours at 28°C by adding one volume of LB broth with ampicillin (100 μg/ml) and 0.33 mM final isopropyl-β-d-thiogalactopyranoside (IPTG). Cells were pelleted and lysed in GST lysis buffer containing 50 mM tris-HCl (pH 7.4) (room temperature), 150 mM NaCl, 0.2% Triton X-100, a cocktail of protease inhibitor (Pierce, Thermo Fisher Scientific), and chicken egg white lysozyme (1 mg/ml) for 1 hour at room temperature. Lysates were treated with Pierce universal nuclease (Thermo Fisher Scientific), clarified by 30-min centrifugation at 15,000 g and 4°C, and incubated overnight at 4°C with Glutathione Sepharose 4B beads (GE Healthcare). Beads were washed three times with 20 volumes of wash buffer containing 50 mM tris-HCl (pH 7.4) (room temperature) and 150 mM NaCl and stored at 4°C.

For recombinant nucleoprotein purification, BL21 *E. coli* transformed with pET N[1–419] or pET N[1–419]-E156K were grown at 37°C for 8 hours or until saturation and protein expression was induced for 16 hours at 20°C by adding one volume of LB broth with kanamycin (50 μg/ml) and 0.33 mM (final concentration) IPTG. Cells were pelleted, frozen, and lysed in N lysis buffer containing 50 mM tris-HCl (pH 7.4) (room temperature), 450 mM NaCl, 1 mM MgSO₄, a cocktail of protease inhibitor (Pierce, Thermo Fisher Scientific), and chicken egg white lysozyme (1 mg/ml) for 1 hour at room temperature. Lysates were treated with Pierce universal nuclease (Thermo Fisher Scientific) and clarified by centrifugation twice at 15,000 g and 4°C for 30 min. Clear lysates were mixed with Ni-NTA Superflow resin (Qiagen) for 1 hour with 20 mM imidazole and washed three times with 20-bed volumes of 50 mM tris-HCl (pH 7.4) (room temperature) and 450 mM NaCl with 60 mM imidazole. Proteins were eluted twice with one volume of wash buffer containing 500 mM imidazole, followed by buffer exchange for phosphate-buffered saline (PBS) using PD-10 desalting columns with Sephadex G-25 resin (GE Healthcare).

**Affinity coprecipitations**

To allow protein interactions, ~200 ng of purified N[1–419] or N[1–419]-E156K was mixed for 1 hour at room temperature and 1000 rpm shaking, with ~100 μg of purified GST, GST-MoRE, GST-XD, GST-XD R469E, or GST-XD-MoRE (with or without substitutions A465V, A465R, R469G, or R469E) immobilized on 20 μl of Glutathione Sepharose 4B beads (GE Healthcare). Beads were washed three times with 10-bed volumes of PBS. Proteins were eluted with two-bed volume of 1× Laemmli buffer, and 10 μl was analyzed through SDS–polyacrylamide gel electrophoresis on 12% gels, followed by staining with R250 Coomassie blue. Densitometric analysis was performed using scanned gels with Fiji software (38). For Western blot analysis, gels were blotted on polyvinylidene difluoride membranes (Millipore) with semi-dry transfer and subjected to chemiluminescence detection using monoclonal antibodies directed against the CDV nucleoprotein (clone DV2-12, Invitrogen). Immunoblots were developed using a ChemiDoc digital imaging system (Bio-Rad) for image visualization.

**Biolayer interferometry**

GST (± XD or XD-MoRE, ± R469E)–coupled Biosensors (Fortebio) were dipped into increasing concentrations (0.5 to 4 μM) of recombinant nucleoproteins for 600 s in PBS–0.01% bovine serum albumin–0.002% Tween 20 and then dipped back into kinetic buffer in the absence of recombinant nucleoproteins for 600 s to follow dissociation. Reference sensors with GST were used to control for nonspecific binding. Real-time binding kinetics were analyzed and calculated using the Octet Red software package.

**Statistical analyses**

Experimental variation and statistical significance of differences between sample means were assayed using either one- or two-way analysis of variance (ANOVA) or nonparametric Kruskal–Wallis test, with appropriate post hoc tests as specified in the figure legends, using the Prism 8 (GraphPad) software package. In all experiments, the significance level α was set to <0.05. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. For initial setup of the minireplicon ratio test and virus spread quantitation, datasets were pretested for normal or log-normal distribution using Anderson-Darling, D’Agostino-Pearson, and Shapiro–Wilk and Kolmogorov-Smirnov tests. For clarity, data variation from independent repeats was represented either directly when possible by a symbol for each data point or with error bars representing geometric SD to the mean.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/10/eaa21590/DC1

**Fig. S1.** Multisequence alignments of N and P proteins of representative members of the paramyxovirus family.

**Fig. S2.** Minigenome activity profiles of full-length (blue) and tail-truncated (red) N.

**Fig. S3.** Minigenome activity profiles of full-length (blue) and tail-truncated (red) N.

**Fig. S4.** Minigenome activity profiles of full-length (blue) and tail-truncated (red) N.

**Fig. S5.** Minigenome activity profiles of full-length (blue) and tail-truncated (red) N.

**Fig. S6.** Minigenome activity profiles of full-length (blue) and tail-truncated (red) N.

**Fig. S7.** Expression and purification of recombinant N and P protein subsets.

**Fig. S8.** Molecular docking of P-XD:N-MoRE complexes into N-core.

**Fig. S9.** Position of the acidic N-core loop in distinct paramyxovirus N proteins and activity associations.

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4 March 2020

SCIENCE ADVANCES | RESEARCH ARTICLE

1. Influenza virus nucleocapsid (N) protein interacts with the L polymerase to control transcription and genome replication.

2. The L gene is essential for the generation of functional hemagglutinin.

3. The L gene is involved in the induction of the L intermediate.

4. The L gene is required for the generation of functional BRSV genome promoter.

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Viral evolution identifies a regulatory interface between paramyxovirus polymerase complex and nucleocapsid that controls replication dynamics

Julien Sourimant, Vidhi D. Thakkar, Robert M. Cox and Richard K. Plemper

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