Structure and Functional Analysis of the RNA- and Viral Phosphoprotein-Binding Domain of Respiratory Syncytial Virus M2-1 Protein

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Introduction

Human respiratory syncytial virus (RSV), a pneumovirus of the Paramyxoviridae family in the Mononegavirales order, is an important respiratory pathogen and the major cause of bronchiolitis and pneumonia in children [1]. Bovine RSV on the other hand represents an important economic issue due to the high morbidity and mortality of infected calves [2]. Whereas current efforts are mainly focused on the development of safe RSV vaccines for infants, the development of antiviral drugs specifically targeting viral-specific functions such as the RSV RNA-dependent RNA polymerase (RdRp) complex by increasing polymerase processivity. M2-1 is a modular RNA binding protein that also interacts with the viral phosphoprotein P, another component of the RdRp complex. These binding properties are related to the core region of M2-1 encompassing residues S58 to K177. Here we report the NMR structure of the RSV M2-1S8–177 core domain, which is structurally homologous to the C-terminal domain of Ebola virus VP30, a transcription co-factor sharing functional similarity with M2-1. The partial overlap of RNA and P interaction surfaces on M2-1S8–177, as determined by NMR, rationalizes the previously observed competitive behavior of RNA versus P. Using site-directed mutagenesis, we identified eight residues located on these surfaces that are critical for an efficient transcription activity of the RdRp complex. Single mutations of these residues disrupted specifically either P or RNA binding to M2-1 in vitro. M2-1 recruitment to cytoplasmic inclusion bodies, which are regarded as sites of viral RNA synthesis, was impaired by mutations affecting only binding to P, but not to RNA, suggesting that M2-1 is associated to the holonucleocapsid by interacting with P. These results reveal that RNA and P binding to M2-1 can be uncoupled and that both are critical for the transcriptional antitermination function of M2-1.

Abstract

Respiratory syncytial virus (RSV) protein M2-1 functions as an essential transcriptional cofactor of the viral RNA-dependent RNA polymerase (RdRp) complex by increasing polymerase processivity. M2-1 is a modular RNA binding protein that also interacts with the viral phosphoprotein P, another component of the RdRp complex. These binding properties are related to the core region of M2-1 encompassing residues S58 to K177. Here we report the NMR structure of the RSV M2-1S8–177 core domain, which is structurally homologous to the C-terminal domain of Ebola virus VP30, a transcription co-factor sharing functional similarity with M2-1. The partial overlap of RNA and P interaction surfaces on M2-1S8–177, as determined by NMR, rationalizes the previously observed competitive behavior of RNA versus P. Using site-directed mutagenesis, we identified eight residues located on these surfaces that are critical for an efficient transcription activity of the RdRp complex. Single mutations of these residues disrupted specifically either P or RNA binding to M2-1 in vitro. M2-1 recruitment to cytoplasmic inclusion bodies, which are regarded as sites of viral RNA synthesis, was impaired by mutations affecting only binding to P, but not to RNA, suggesting that M2-1 is associated to the holonucleocapsid by interacting with P. These results reveal that RNA and P binding to M2-1 can be uncoupled and that both are critical for the transcriptional antitermination function of M2-1.

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subgenomic capped and polyadenylated mRNAs. Transcription proceeds by a sequential stop-and-restart mechanism in which the polymerase responds to cis-acting signals present in intergenic regions [4]. Transcription is (re)initiated at a highly conserved 9–10 nucleotide transcription promoter (gene start, GS) signal. Semi-conserved 12–13 nucleotide gene ends (GE) signal for polyadenylation and release of the nascent mRNA [5]. The polymerase has a propensity to dissociate from the N-RNA template, but cannot reinitiate at a downstream gene in case of premature termination [6], which leads to a decreasing transcription gradient from the 3’ to the 5’ end of the genome. For all known pneumoviruses, RdRp driven transcription depends on M2-1. RSV M2-1 is a transcription antitermination factor that is important for the efficient synthesis of full-length mRNAs [7] as well as for the synthesis of polycistronic read-through mRNAs [4,6,8,9]. The latter activity is thought to facilitate polymerase access to promoter-distal regions of the genome, and hence transcription of all genes [3]. It was shown that the M2-1 protein reduces termination at all gene junctions, but that the efficiency in the presence of M2-1 varied at the different gene junctions [6,9]. However, mechanisms by which M2-1 prevents the polymerase from terminating transcription remain to be clarified. There are at least three different scenarios. (i) M2-1

in vitro
could bind to the nascent mRNA transcript to facilitate transcription elongation, perhaps by preventing the mRNA from re-hybridizing to the template, or forming secondary structures that might destabilize the transcription complex. This hypothesis is sustained by the finding that RSV mRNAs are co-precipitated with M2-1 from RSV infected cells [10]. (ii) The polymerase processivity enhancing effect of M2-1 could be due to an increase of the affinity of the polymerase for the genomic RNA template in a sequence non-specific manner. (iii) M2-1 could recognize GE sequences either on the nascent mRNA or on the RNA template and prevent the release of the polymerase complex from its template, favoring transcription re-initiation at the downstream GS sequences.

In RSV-infected cells, M2-1 co-localizes with the other RdRp components in inclusion bodies (IBs) [11], which are regarded as centers of RNA synthesis [12]. The basic 194-residue RSV M2-1 has been shown to be an RNA binding protein, but specificity of M2-1 RNA binding has been debated. It was reported that M2-1 was able to bind to long RNAs without sequence specificity and with an apparent K_d of 30 nM, and that it bound specifically to short (80 nucleotides) but not long (700 nucleotides) RNAs [13]. Elsewhere it was demonstrated that the polymerase activity of M2-1 was independent of RNA binding ability [14]. Line broadening due to RNA binding was observed in the 1H-15N HSQC spectrum (see Figure S1). Treatment with RNAse A reversed these effects. This result confirmed the RNA binding ability of M2-1 by NMR spectroscopy and report the solution structure of RSV M2-158–177, which shows structural homology with the C-terminal domain (CTD) of the Ebola virus (EBOV) VP30 protein. In this α-helical domain we have identified residues that contribute to two adjacent, partially overlapping contact surfaces, with P and RNA respectively. We show that mutations of several of these residues specifically disrupt the M2-1:P or M2-1:RNA interaction in vitro and have a drastic effect on intracellular co-localization of full-length M2-1 with P as well as on the function of M2-1 as a transcription co-factor.

Results

Solution NMR structure of RSV M2-158–177

The boundaries of the protein fragment M2-158–177 were chosen to focus on the binding regions of RNA and RSV phosphoprotein determined previously, but also to exclude the oligomerization domain and the disordered C-terminus, which are not necessary for the interactions with RNA and P [15]. Line widths of the solution NMR spectra were compatible with a monomeric state, and M2-158–177 was amenable to structure determination by NMR, in contrast to tetrameric full-length M2-1. The resonance assignments were reported elsewhere [20]. M2-158–177 contains a single globular domain spanning residues G73-I171 and comprising six helices: α1 (G75-G85), α2 (K92-E105), α3 (S108-D117), α4 (K124-K140), α5 (K143-R151) and α6 (D155-I171). The N-terminus (S38-L74), which corresponds to the linker to the upstream oligomerization domain of M2-1, is disordered. The α-helix bundle consists of a scaffold, formed by α1, α2, α5 and α6, and an α3–α4 hairpin stacked on α6 (Figure 1A). M2-158–177 displays two oppositely charged faces (Figure 1B). The positively charged face contains a large basic cluster along a groove delimited by helices α2 (K92, α2 (K92-R126), on α3 (K112, K113 and R115), and between α4 and α5 (R139, K140 and K143) as shown in Figure 1B. The putative overall tetrameric domain organization of full-length M2-1 is schemed in Figure 1C.

RNA binds to the main basic cluster of M2-158–177

Incubation of M2-158–177 with yeast RNA in a ~1:1 molar ratio resulted in simultaneous shifting and broadening of several 1H-15N cross peaks in the 1H-15N HSQC spectrum of M2-158–177 (see Figure S1). Treatment with RNase A reversed these effects. This experiment confirmed the RNA binding ability of M2-158–177 in vitro. The observed fast to intermediate exchange regime was an indication for a weak interaction. To get rid of the broadening contribution by transversal relaxation of large RNAs twice the molecular weight of M2-158–177, we investigated the RNA:M2-158–177 interaction by NMR by using short synthetic 10–15 nucleotide negative-sense (genomic) RNAs containing selected transcriptional signals [3, 21] as well as their complementary positive-sense sequences. We tested the 3′ polymericase entry site (leader), the U-rich region upstream of the first GS signal, the GS, and the F and SH gene ends (GE_F and GE_SH). Sequences are detailed in Table 1. The oligonucleotides were designed to minimize self-association or formation of secondary structure. Only the leader-neg (5′-CGCAUUUUUUCGGGU-3′) and long U-rich (5′-CGCAUUUUUUCGGGU-3′) sequences were predicted to form hairpins with a poly-U loop and a stem of two or three G-C and A-U pairs with negative free energies. Calculations were carried out on the nfold web server [22]. The absence of self-association was assessed by 1H NMR using 200 μM oligonucleotide solutions in water, except for leader-pos and GE_F-pos, for
which two broad imino proton signals were observed in the guanosine and uridine regions respectively, corresponding to partial formation of two G-C and U-A base pairs. Finally formation of an RNA duplex with the two complementary strands GE_F-pos and GE_F-neg was observed by NMR with sharp signals corresponding to base-paired uridine imino protons.

Oligonucleotide binding was followed by chemical shift perturbation experiments in 1H-15N HSQC and 1H-13C HSQC experiments, as exemplified for the short polyA sequence in Figure 2 (panels A to C). The chemical shift variation profile in the 1H-15N HSQC spectrum of short polyA is shown in Figure 2D. Chemical shift perturbation profiles were similar for all RNAs and consistent with those observed for yeast RNA. A complete set of profiles for all oligonucleotides is given in Figure S2. For all RNAs the largest chemical shift changes were observed for residues belonging to the main basic cluster, i.e. K92-V97 (a2), L149-L152 (a5) and D155-K159 (a6), as illustrated in Figure 2E. These residues contribute to forming a continuous positively charged surface, which is consistent with an RNA binding surface (Figure 2F). Chemical shift variations in 1H-13C HSQC experiments were observed for residues in the same region, solvent exposed methyl side chains in the hinge between helices a5 and a6 as well as R151-H6 protons being affected by RNA binding (see Figure 2B and 2C).

Short A-rich RSV RNA sequences preferentially bind to M2-158–177

The fast to moderately fast exchange regime in both 1H-15N and 1H-13C HSQCs allowed to extract apparent dissociation constants determined from amide (1H and 15N), methyl (1H and 13C) and R151-Hd chemical shift variations (at 14.1 T and 293 K).

| Designation | Oligonucleotide sequence | \( K_d \) (\( \mu \text{M} \)) |
|-------------|--------------------------|-----------------|
| leader-neg  | 3'-UGCGCUUUUUUACGC-5'    | 75 ± 15         |
| leader-pos  | 5'-ACGCGAAAAAAAU-3'      | 2.5 ± 1.5       |
| long U-rich | 3'-UUGGCUUUUUCUACC-5'    | 125 ± 35        |
| short U-rich| 3'-GGUUUUUUUA-5'         | >600            |
| short A-rich| 5'-CCAAAAAAAU-3'         | 22 ± 6          |
| GS-neg      | 3'-CCCCGGUUUAU-5'        | 110 ± 15        |
| GE_SH-neg   | 3'-UCAAUUUAUUUUU-5'      | 75 ± 15         |
| GE_SH-pos   | 5'-AGUUAUUAAA-3'         | 13 ± 5          |
| GE_F-neg    | 3'-UCAAUUUAUUUU-5'       | 85 ± 25         |
| GE_F-pos    | 5'-AGUUAUUAAA-3'         | 11 ± 5          |
| GE_F-ds2    |                           | 4.5 ± 2.5       |
| GE_F-DNA    | 3'-TCAATATATTTT-5'       | 250 ± 70        |
| UGA2        | 3'-CGCGAUAUUUGCGG-5'     | 60 ± 10         |

Table 1. Apparent M2-158–177:oligonucleotide dissociation constants determined from amide (1H and 15N), methyl (1H and 13C) and R151-Hd chemical shift variations (at 14.1 T and 293 K).

\( K_d \) errors were estimated from standard deviations of \( K_d \) values determined for individual residues. Double-stranded GE_F RNA was formed with equal amounts of the complementary GE_F-neg and GE_F-pos strands.

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constants for a binding model with a 1:1 stoichiometry. The fitted chemical shift variation curves for individual residues in a fast exchange regime are given in Figure S3. The values of the apparent dissociation constants (Kd) are recapitulated in Table 1. They range from 2.5 \(\mu\)M to 600 \(\mu\)M and fall into two groups. Except for a short U-rich RNA sequence, Kds for U-rich negative-sense RNAs are in the 75–125 \(\mu\)M range. UGA2, an RSV-unrelated stem-loop RNA comprising a U-stretch and five base pairs, used as a control, binds with similar affinity (Kd = 60 \(\mu\)M). A second control was carried out with a short single stranded DNA equivalent to the GE_F-neg RNA, showing that the same DNA sequence (250 \(\mu\)M) binds with slightly less affinity than the RNA sequence (85 \(\mu\)M). Apparent Kd for A-rich positive-sense RNA sequences (leader-pos, GE_SH-pos, GE_F-pos and short A-rich) are in the 2.5–22 \(\mu\)M range. A last experiment with the double-stranded F gene end (GE_F-ds, 4.5 \(\mu\)M) shows that the affinity is further increased as compared to the affinities of single-stranded RNAs. Taken together, these results indicate that M2-1 58–177 binds to RNA with lower affinity than reported for full-length M2-1 [10,13]. Importantly they show that the presence of A-rich stretches increases binding affinity as compared to U-rich stretches and that RNA base-pairing might also play a role.

As shown above, helices \(\alpha_2\), \(\alpha_5\) and \(\alpha_6\) form a uniform RNA binding surface. However, residues L74 and G75 in helix \(\alpha_1\), which are located on the opposite negatively charged side of the protein, are also affected by RNA binding. For all RNAs, Kd determined for residues in helix \(\alpha_1\) were always the same as those measured for residues of the main binding site. This strongly suggests that perturbations in \(\alpha_1\) on the one hand and in \(\alpha_2\), \(\alpha_5\) and \(\alpha_6\) on the other hand are related to the same binding event.
Moreover as the hypothetical second contact surface with RNA would be very distant from the main binding site, we exclude the possibility of a second binding site at α1. However RNA binding to α2 could be transmitted to α1 by slight alterations of helix-helix packing.

The P binding region of M2-158–177 is adjacent to the RNA binding site

To compare RNA and P affinities for M2-158–177, we performed isothermal titration calorimetry (ITC) experiments using this truncated form of M2-1. Results showed that the phosphoprotein P binds to M2-158–177 with a stoichiometry of 1:1 and a K_d of ~3 μM (Figure S4). We investigated this interaction further by NMR for atomic details. Perturbations induced by tetrameric full-length P in 1H-15N and 1H-13C HSQC spectra of M2-158–177 were monitored. Due to the size of the complex and the unfavorable exchange regime, addition of 0.5 molar equivalents of P was accompanied by extensive overall line broadening in the 1H-15N HSQC spectra, except for the unstructured N- and C-termini (see Figure S5A). At lower P concentrations, the majority of cross-peaks remained detectable. Residues T130-L165 exhibited larger line broadening, suggesting that P binds to the α4/α5/α6 region. Transferred cross-saturation (TCS) experiments [23] were carried out with 1H-15N-M2-158–177 to more specifically identify residues involved in P binding (Figure 3, panels A to D). Saturation of the methyl protons of P resulted in reduction of M2-1 cross peak intensities in the regions V127-S137 and L152-T164 (helices α4

Figure 3. Probing phosphoprotein P binding to M2-158–177 by NMR perturbation experiments. (A, B, C and D) Results of transferred cross-saturation (TCS) experiments carried out with 150 μM 1H-15N-M2-158–177 in the presence of P (15 μM, 91% D₂O, 14.1 T, 293 K). (A) Close-up of the 1H-15N HSQC spectra with methyl proton saturation of P (blue) and without (orange). (B) Per residue plot of reduced 1H-15N HSQC cross-peak intensities, calculated as a ratio between intensities with (I_sat) and without (I_0) methyl proton saturation and corrected by the reduced cross-peak intensities measured in the absence of P. (C) and (D) Mapping of the TCS effect on the structure. 15N atoms of residues with ΔI/I_0 > mean+1sd are shown as purple spheres. The surface formed by these residues is colored in purple. (E, F, G and H) 1H-15N HSQC cross-peak intensity perturbation experiments of 15N13C-M2-158–177 (50 μM) in the presence of P 100–166 (100 μM, 14.1 T, 298 K). (E) Close-up of the 1H-15N HSQC spectra with P 100–166 (blue) and without (red). (F) Per residue plot of the resulting cross-peak intensity reduction relative to the reference intensity. (G) and (H) Mapping of the intensity variations on the structure. 15N atoms are shown as spheres for residues with ΔI/I_0 > 75% (blue) and >70% (cyan). The same color code is used for the surface representation.

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and α6, and α5/α6 hinge). They form a nearly uniform surface on M2-1 (Figure 3D). Since P residues 100–120 were reported to be critical for M2-1 binding [14,24], we also monitored intensity variations in 1H-15N HSQC spectra in the presence of a truncated form of P, P100-166, comprising this region as well as the oligomerization domain of P [25,26]. The results are shown in Figure 3 (panels E to H). Line broadening was enhanced for helices α4 and α6 (Figures 3F and 3G), which is consistent with the results obtained with full-length P. In summary, P binds to a region proximal to the RNA anchoring surface. Helices α6 and α5 were sensitive to both P and RNA binding in our NMR experiments, and could thus contribute to both interaction sites, while α4 and α2 appear to be involved specifically in P binding and RNA binding, respectively.

Effects of surface mutations in the P and RNA binding regions on M2-1 controlled transcription probed by a minigenome

Based on these results, we designed single residue mutations of the full-length M2-1 protein targeting the binding sites of P and RNA. Solvent-exposed residues were first substituted by Ala. The effect of these mutations on transcription antitermination by M2-1, which was previously shown to be the same for RSV-specific and heterologous sequences [6], was assessed using an RSV dicistronic subgenomic replicon, pM/Luc. It contains the authentic M/SH gene junction, and the Luc reporter gene downstream of the gene start sequence present in this gene junction. The expression of the Luc gene in this system is absolutely dependent on the presence of a functional M2-1 [15]. The pM/Luc plasmid was co-transfected in BHK-21 BSRT7/5 cells expressing T7 RNA polymerase together with p-β-gal, pL, pP, pN, and pM2-1. Luciferase activities were determined and normalized based on β-galactosidase expression [5,8,15,27,28]. Except for L148A and N163A, most of the Ala substitutions had only little effect on transcription, as assessed by Luc expression (Figure 4A). As the P- and RNA-binding surface identified by NMR is highly positively charged, residues were substituted by Asp to emphasize the electrostatic effect of mutations. When substituted by Asp, K92 (in helix α2), a residue involved in RNA but not in P binding according to the NMR results, and R126 and T130 (in helix α4), two residues involved in P but not in RNA binding, appeared to be critical for transcription. A similar effect on transcription was observed for four other residues (K150, R151, T160 and N163, in helices α5 and α6), that belong to the dual RNA/P binding surface. In contrast, Ala and Asp mutants of K158, which is also

Figure 4. Effect of M2-1 mutations on RSV transcription and association with the nucleocapsid. (A) Analysis of RSV specific M2-1-controlled transcription with WT and M2-1 substitution mutants. BSRT7/5 cells were transfected with RSV pP, pN, pL, and pM2-1 plasmids and an RSV specific minigenome containing the firefly luciferase reporter gene, together with p-β-gal constitutively expressing β-galactosidase. Luciferase activity, measured 24 h after transfection, was normalized by β-galactosidase activity, and the luciferase activity gained with WT M2-1 set to 100%. The mean value and confidence intervals (error bars) result from 3 separate experiments performed in duplicate. A control was run without M2-1. (B) Expression of M2-1 mutant proteins in BSRT7 cells. Cells were co-transfected with plasmids encoding M2-1 mutants and N. Cell extracts were analyzed by Western blotting with rabbit polyclonal antibodies against M2-1 and N. Expression levels of M2-1 were normalized against N expression and compared to tubulin. (C, D) Colocalization studies of M2-1 with N-P complexes. Plasmids encoding N, P, and M2-1 mutants were transfected into BSRT7/5 cells. Immunofluorescence analysis was performed on cells fixed 24 h after transfection, by using rabbit polyclonal anti-N (1:100) or anti-P (1:500) and Alexa Fluor 594 goat anti-rabbit (1:1000) antibodies, and mouse monoclonal anti-M2-1 (1:140 dilution) and Alexa Fluor 488 goat anti-mouse (1:1000) antibodies. Horizontal bars correspond to 10 μm. doi:10.1371/journal.ppat.1002734.g004
located in the RNA/P binding region, still displayed more than 90% activity as compared to the WT (Figure 4A). As a control, we further verified by NMR that the deactivating mutations did not disrupt the protein fold (Figure S6).

**Interaction of M2-1 surface mutants with P-N complexes in cellulo**

Co-expression of P and N proteins (in the absence of other viral proteins) in cells induces the formation of cytoplasmic inclusion bodies containing P-N complexes, as observed in RSV infected cells [11]. When co-expressed with P and N, WT M2-1 also localizes preferentially in these IBs as seen in Figure 4D [11]. We analyzed the intracellular localization of the M2-1 mutants in cells co-transfected with expression vectors for P, N and M2-1 by fluorescence microscopy (Figure 4C and 4D). Contrary to WT M2-1, the mutants R126D, T130D, L148A, T160D and N163D were excluded from the IBs, but spread all throughout the cytoplasm. In contrast K92D, K150D and R151D retained their localization to cytoplasmic IBs. The mutants were expressed at comparable levels, as determined by Western blotting (Figure 4B), and were correctly folded when purified from E. coli and analyzed by NMR (Figure S6).

**In vitro binding of M2-1 mutants to P and RNA**

To verify that the residues identified using NMR and the minigenome assay are critical for RNA- and/or P-binding, the in vitro RNA and P binding capacities of eight M2-1 mutants selected by the Luc assay were investigated. As M2-1 did not migrate in native agarose gel, it was not possible to obtain electrophoretic mobility shift assays (EMSA) with the GST-free forms. We thus used M2-1 fused to GST for the in vitro binding assays with RNA and P. For RNA binding assays, we used either full-length (tetrameric) or truncated 58–177 (monomeric) forms of M2-1 fused to GST. GST-M2-1, incubated with tRNA, was analyzed by EMSA. Formation of GST-M2-1 58–177:RNA complexes was only impaired by the K92D, K150D and R151D mutations, which did not prevent M2-1 association with IBs (Figure 5A and 5B). The effect of single mutations of the full-length form on in vitro RNA-binding was smaller than with truncated M2-1 58–177, probably owing to the higher avidity of tetramers for RNA compared to monomers, and the Lys/Arg repetitions that limit the effect of single substitutions (data not shown). In vitro M2-1-P interactions were assessed by GST pull-down assays. As shown in Figure 5C and 5D, a ≥50% decrease in P binding was observed for the mutants R126D, T130D, L148A, T160D and N163D, which were excluded from IBs, but could still bind RNA. The two separate binding surfaces for RNA and P, determined from these 8 residues, are illustrated in Figure 5E. Together with the cellular localization experiments, these results show that there is a strong correlation between the reduced capacity of M2-1 to pull-down P in vitro and its exclusion from IBs. Conversely, since residues specifically involved in RNA binding appear not to be crucial for recruitment of M2-1 to IBs, the M2-1:RNA interaction is not required for this process.

**Discussion**

**Similarity between pneumovirus M2-1 and filovirus VP30 transcription co-factors**

In contrast to other members of the *Mononegavirales* order for which transcription is driven by only three proteins (N/NP, P and L), pneumoviruses and filoviruses encode a fourth transcription co-factor (M2-1 and VP30 respectively). Parallels have been drawn between VP30 and M2-1 on the basis of their functions and domain organization [17,29]. Both were shown to be necessary for efficient transcription in reconstituted minigenome systems [7,30]. In addition, although VP30 and M2-1 were shown to be dispensable for RNA replication [30,31,32], both are required to rescue recombinant EBOV or Marburg virus and RSV [33,34,35]. M2-1 and VP30 contain an N-terminal Cys3His1 motif that does not bind RNA directly, but that is essential for VP30 RNA binding [15,17,36,37]. In the case of M2-1 this motif is indispensable for function, but its exact role is still unknown [17,18,19]. Like M2-1, VP30 also contains an oligomerization domain downstream of the Cys3His1 motif, which is necessary for its function during transcription [13,36]. But, despite functional similarities, the sequence identity between the two proteins is very low and it was not possible to know that they were evolutionary related.

Here we show that the globular domains M2-1 58–177 and EBOV VP30CTD [38] are structurally homologous and that they display the same α-helix bundle fold. The structures were aligned on the DALI server [39] which yielded a Z-score of 5.7 and an rmsd of 3.9 Å for 92 aligned residues and 9% sequence identity (see Figure 6). The core helices α1, α2, α5 and α6 align well, with a slight shift of α1M2-1 with respect to α1VP30. The α3–α4 hairpins are skewed relative to each other. Moreover M2-1 58–177 and VP30CTD contain several identical hydrophobic residues in helices α5 and α6 (indicated in Figure 6), which are involved in stabilizing inter-helical contacts and appear to be semi-conserved among pneumovirus/metapneumovirus M2-1 and filovirus VP30 protein sequences (Figure S7). Although they mainly contribute to inter-helix packing, it is noteworthy that one of them (L148) is also critical for M2-1 transcription antitermination. VP30CTD contains an additional 7th C-terminal helix, which has no counterpart in M2-1. It stabilizes the crystallographic VP30CTD dimer by interaction with α1. Since full-length M2-1 forms tetramers with the oligomerization domain just upstream of the core domain, its monomeric state in isolation suggests that the core domains may at best be loosely associated with each other in the tetramer, in the absence of partner molecules, as schemed in Figure 1C.

The overall structural match rationalizes the relationship between M2-1 and VP30. Importantly both proteins associate with the nucleocapsid by means of their globular core domains [10,14,38,40]. Whereas the VP30CTD:nucleocapsid interaction is mediated by the EBOV nucleoprotein but not by RNA [41], the M2-1:nucleocapsid interaction has been proposed to be mediated by RNA [10] and by P [14]. RNA binding to VP30CTD has not been observed [38], in contrast to what we report for M2-1 58–177. As no direct M2-1:N complex was evidenced by NMR and the deactivating mutations did not prevent M2-1 association with IBs (Figure 5A and 5B). The overall structural match rationalizes the relationship between M2-1 and VP30. Importantly both proteins associate with the nucleocapsid by means of their globular core domains [10,14,38,40]. Whereas the VP30CTD:nucleocapsid interaction is mediated by the EBOV nucleoprotein but not by RNA [41], the M2-1:nucleocapsid interaction has been proposed to be mediated by RNA [10] and by P [14]. RNA binding to VP30CTD has not been observed [38], in contrast to what we report for M2-1 58–177. As no direct M2-1:N complex was evidenced by NMR and the deactivating mutations did not prevent M2-1 association with IBs (Figure 5A and 5B). The overall structural match rationalizes the relationship between M2-1 and VP30. Importantly both proteins associate with the nucleocapsid by means of their globular core domains [10,14,38,40]. Whereas the VP30CTD:nucleocapsid interaction is mediated by the EBOV nucleoprotein but not by RNA [41], the M2-1:nucleocapsid interaction has been proposed to be mediated by RNA [10] and by P [14]. RNA binding to VP30CTD has not been observed [38], in contrast to what we report for M2-1 58–177. As no direct M2-1:N complex was evidenced by NMR and the deactivating mutations did not prevent M2-1 association with IBs (Figure 5A and 5B).

**M2-1 58–177 binds to mRNA transcripts rather than to genomic RNA**

M2-1 was first described as an antitermination factor, preventing cessation of chain elongation and release of the nascent mRNA [7]. Later M2-1 was reported to inhibit transcription termination at the GE signals to produce polycistronic readthrough mRNAs [6,8,9], depending on GE sequences. The semi-conserved 12–13 nucleotide GE segments fall into three groups with respect to this property: the first (NS1/NS2, NS2/N, M2/L, and L/trailer) contains sequences inefficient for transcription termination and is insensitive to M2-1; the second (N/P, P/M, M/SH, SH/G, G/F) is very efficient in transcription termination but not sensitive to M2-1; finally (F/M2) is highly sensitive to M2-1 [9]. M2-1 did not direct
readthrough at the leader-NS1 junction [6]. Thus, it was suggested that M2-1 would not only prevent inappropriate intragenic termination, but also allow the polymerase to access promoter-distal regions of the genome and to transcribe downstream genes [9]. Cuesta et al. reported that renatured M2-1 bound preferentially to a short positive-sense leader RNA in vitro [13], while Cartee and Wertz co-immunoprecipitated RSV mRNA with M2-1 from infected cells treated with Actinomycin D [10]. These observations suggested that M2-1 could modulate transcription termination by recognizing specific viral RNA sequences, either on transcribed mRNAs or on the genomic RNA template. Contrary to transcribed viral mRNA, genomic RNA is encapsidated. However if the model of RNA synthesis by the vesicular stomatitis virus, a prototype of nonsegmented negative-strand RNA viruses, could be transposed to RSV, genomic RNA would become accessible as the nucleocapsid is being locally disassembled by the transcription complex [42,43]. By comparing the affinities of M2-158–177 for short 10–15 nucleotide RSV RNA sequences, we found

Figure 5. Effect of mutations affecting M2-1-controlled transcription on M2-158–177:RNA and M2-1:P complex formation in vitro. (A and B) Electrophoretic mobility shift assay (EMSA) of M2-1:RNA complex formation. Eluted GST-M2-158–177 (WT and mutants selected using the minigenome assay, 100 μM final concentration) were incubated with yeast tRNA (~50 μM final concentration) for 1 h at room temperature. (A) Complexes were resolved by agarose gel electrophoresis stained with ethidium bromide. (B) Proteins were revealed by amido black staining. M2-1 mutations are indicated above each lane. (C and D) GST pull-down of purified P by GST-M2-1 (WT and the same mutants as in A and B). (C) GST-M2-1 or GST were incubated alone (+) or in the presence of P (+), washed, and analyzed by SDS-PAGE. P was also run alone (lane P). (D) Coomassie blue-stained gels were scanned and M2-1:P binding was quantified using ImageJ software and corrected for nonspecific binding to GST. Errors were estimated to ±5%. (E) The surfaces formed by the 8 residues, for which mutants were analyzed, are indicated on the M2-1 structure according to their binding partner: in red (RNA binding) and blue (P binding).

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that M2-158–177 bound preferentially to positive-sense RNA sequences such as the 12 first bases of the positive-sense leader (Kd = 2.5 μM), positive-sense GEss (11 and 13 μM) and an A-rich sequence located on the positive-sense leader (22 μM). Affinities of the negative-sense signal sequences were systematically lower by one order of magnitude or more than those of their complementary sequences. But there was no difference between the SH and F gene ends, which are respectively insensitive and highly sensitive to M2-1. The 3′ and 5′ extremities of the leader and the GS displayed a similar behavior to that of GEss of same polarity, indicating that M2-158–177 does not discriminate between transcription signals of same polarity. Sequence specificity of RSV transcription antitermination thus appears not to be linked to the M2-1 core domain. These results are also consistent with the observation that M2-1 is not required for initiation of RNA transcription [7]. Our results show that M2-158–177 displays a preference for purine-rich and especially A-rich RNAs found in positive-sense RSV RNAs over pyrimidine-rich sequences containing U-stretches found in negative-sense RNAs. Thus, they reinforce the hypothesis that M2-1 binds preferentially to positive-sense RNA transcripts rather than to the template, in agreement with previous reports [10,13]. An additional finding is that M2-1 binds to the double-stranded F gene end with similar or better affinity (4.5 μM) than to the positive-sense sequence alone (11 μM). This implies that M2-1 could bind to the nascent mRNA transcript, either still bound to the template or released from it. In both cases, in order to facilitate transcription elongation, M2-1 could prevent formation of mRNA secondary structures that might destabilize the transcription complex, in analogy to the function of N protein that binds to the nascent RNA during replication.

Functional implications of the interaction of M2-1 with P

Using in vitro experiments, we have previously shown that RNA and P bind to M2-158–177 in a competitive way [15]. Here we have characterized two distinct binding surfaces for P and RNA by NMR (Figure 5E). Their edges are partially overlapping. The P binding epitope, which is buried between helix α1 and the hinge between α5 and α6 (Figure 3D and 3H), might be occluded by RNA binding in the vicinity, and conversely RNA binding might be hampered by bound P due to steric hindrance. Both binding sites are located on the positively charged face of M2-158–177, underlining the importance of electrostatic interactions for association with RNA and with P. This hypothesis is also in agreement with the predicted negatively charged surface in computer models of the M2-1 binding region of the P tetramer, spanning residues 100–120 of P [44]. Redundancy of positive charges in the P and RNA binding region certainly accounts for the weak effect on Luc expression observed for Ala mutants of residues in this region in the minigenome assay, as compared to Asp substitution, which introduced opposite charges. Electrostatic interactions could be further emphasized in the M2-1 tetramer if the core domains were arranged to provide an extended binding surface consisting of two adjacent positively charged clusters. In addition to charge effects, specific hydrophobic interactions may also contribute, as suggested for the interaction between P and M2-1 in [14]. This could apply to RNA as well. Although the range of RNA affinities for M2-158–177 (micromolar to millimolar) is an indicator for non-specific binding mediated by the RNA phosphate backbone, corroborated by binding of a DNA sequence equivalent to GE_F-neg, the difference between A-rich and U-rich RNA affinities indicates that the nature of the bases also comes into play.

The large M2-158–177:RNA binding surface determined by NMR coincides with the main positively charged cluster of M2-158–177 (Figure 1B and 2F), which is well conserved among Pneumovirinae (Figure S7). We confirmed by mutagenesis that the three basic residues K92, K150 and R151, included in this epitope, are crucial for in vitro RNA binding to M2-158–177 and for transcription enhancement in vitro by full-length M2-1. However they did not prevent association with cytoplasmic IBs or P binding. In contrast, mutants R126D, T130D, L148A, T160D and N163D, for which M2-1-controlled transcription was impaired and which still bound RNA in vitro, had lost their ability to bind P in vitro and did not co-localize with N-P complexes in cytoplasmic IBs. These residues are not well conserved in Pneumovirinae M2-1 proteins (Figure S7A), but this would be consistent with the sequence variability of the M2-1 binding region of P (100–120), which has co-evolved with M2-1. Altogether the NMR and mutagenesis results provide a rationale for the competitive binding previously observed between P and RNA [15]. They highlight the role of P for the recruitment of M2-1 to cytoplasmic IBs that contain N and P and where viral RNA synthesis takes place, by analogy to Rhabdoviridae [12].

Possible cooperative effects in full-length tetrameric M2-1, involving the N-terminal Cys8His motif and interactions between core domains, may induce an increased affinity for both P and RNA as well as sequence specific recognition of RNA, with respect to the core domain of M2-1. However the overall lower affinities of RNA for M2-158–177, as compared to P, suggest that M2-1 binds P...
preferentially in the absence of viral RNA so that M2-1 would be recruited as an RNA-free M2-1:P complex to the IBs. This hypothesis is in agreement with our fluorescence microscopy observations, since WT M2-1 and mutants that do not bind RNA are found likewise in IBs containing N and P (Figure 4C and 4D). Indeed, higher affinity to RNA would result in sequestration of M2-1 in the cytoplasm, where cellular RNA is highly accessible, and M2-1 would not be recruited to the IBs. The relatively low $K_d$ are in the same range as those measured for the Measles virus P-N interaction [45], this intrinsically weak association being probably required for movement of the viral polymerase during RNA transcription.

In summary, our results indicate that not only P:M2-1, but also RNA:M2-1 interactions are required for efficient transcription activation by M2-1. Association with P is strictly required for recruitment to the viral RNA synthesis site. As suggested by the proximity of the binding sites, it is likely that the P:M2-1 interaction is displaced in favor of other interactions, RNA:M2-1 interactions in particular, in the context of the holonucleocapsid. The higher affinity of M2-1$_{38-177}$ for the 5’ end of the positive-sense Leader RNA (2.5 μM), which is in the same range as the affinity for P (3 μM), suggests that M2-1 could be loaded onto the polymerase during transcription initiation. It can further be speculated that an L:M2-1 interaction also takes place when the P:M2-1 interaction breaks down, thus altering the sensitivity of the polymerase to transcription termination signals. A similar L:VP30 interaction was recently reported for EBOV [46]. This hypothesis is currently under investigation for RSV.

Materials and Methods

Protein expression and purification

Recombinant P, P$_{100-166}$, M2-1 and M2-1$_{38-177}$ (WT and mutants) as well as $^{15}$N, $^{13}$C- and/or $^2$H-labeled M2-1$_{38-177}$ were expressed in E. coli BL21(DE3) strain. Full-length M2-1 and M2-1$_{38-177}$ were produced as GST fusion proteins as described in [15] and [25] with a thrombin cleavage site. M2-1 amino acid substitution mutants were obtained with the Quickchange site-directed mutagenesis kit (Stratagene) by using pGEX-M2-1, pGEX-M2-1$_{38-177}$ and pM2-1 as templates. Isotopically labeled proteins for NMR were produced in minimal M9 medium supplemented with 1 g/L $^{15}$NH$_4$Cl and 4 g/L $^{13}$C- or unlabeled yeast tRNA (Sigma, final concentration 50 μM) and then incubated for an additional hour with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (1:1000) IgG (Invitrogen). Cells were observed with a Nikon TE2000 inverted microscope equipped with a Photometrics CoolSNAP ES2 camera. Images were processed using MetaVue software (Molecular Devices).

Minigenome assay

BSRT7/5 cells stably expressing T7 RNA polymerase [47] were maintained in EMEM supplemented with 10% FCS/1-glutamine/penicillin-streptomycin under 5% CO$_2$ at 37°C. The cells were grown in an incubator at 37°C under 5% CO$_2$, pN, P, pM2-1 and pL plasmids coding for HRSV (strain Long) N, P, M2-1 and L proteins respectively, under the control of the T7 promoter, have been described previously [15,48]. An encephalomyocarditis virus internal ribosome entry site (IRES) sequence was placed between the T7 promoter and the inserted ORF to enhance protein expression in BSRT7/5 cells, as previously described [48,49]. The pM/Luc subgenomic replicon was derived from the pM/SH subgenomic replicon [8] and has been described previously [15]. It contains two transcription units, the second encoding the firefly luciferase (Luc) gene under the control of the M/SH intragenic sequence. The expression of the Luc gene in this system is absolutely dependent on the presence of M2-1 [15]. BSRT7/5 cells were transfected with pN, pP, pL, pM2-1, pP-Gal coding for beta-galactosidase under the control of the Rous sarcoma virus promoter (Promega) and pM/Luc, where luciferase expression is controlled by the RSV M/SH intergenic region [15]. Luciferase activity was determined in triplicate 24 h post-transfection as previously described [15]. Cells were lysed in luciferase lysis buffer (30 mM Tris, pH 7.9, 10 mM MgCl$_2$, 1 mM dithiothreitol [DTT], 1% [vol/vol] Triton X-100, and 15% [vol/vol] glycerol), and luciferase activities were evaluated twice for each cell lysate with an Anthos Luc 3 luminometer (Bio Advance).

Transfections and indirect immunofluorescence analysis

BSRT7/5 cells were transfected with pP (0.4 μg), pN (0.4 μg) and pM2-1 (0.2 μg) containing either WT or mutant M2-1 by using Lipofectamine2000 (Invitrogen). Samples were fixed after 24 h in 4% paraformaldehyde, and permeabilized in PBS containing 0.1% Triton X-100 and 3% BSA. Each coverslip was incubated with primary antibodies: anti-N (1:100 dilution) and anti-P (1:500 dilution) rabbit polyclonal sera, and anti-2M2 and 22K4 anti-M2-1 monoclonal antibodies (1:40 dilution) [50]. These samples were incubated for 1 h at room temperature, washed, and then incubated for an additional hour with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (1:1000) IgG (Invitrogen). Cells were observed with a Nikon TE2000 inverted microscope equipped with a Photometrics CoolSNAP ES2 camera. Images were processed using MetaVue software (Molecular Devices).

Analysis of in vitro RNA and P binding by M2-1 and M2-1 mutants

GST-M2-1$_{38-177}$ fusion proteins (WT or mutants, final concentration 100 μM) were eluted by GSH and incubated with yeast RNA (Sigma, final concentration 50 μM) in a final volume of 10 μL. Complexes were resolved by 15% agrose gel electrophoresis in 1× Tris-Glycine buffer at 4°C, stained with ethidium bromide and amido black. GST pull-down of purified recombinant P by full-length GST-M2-1 fusion proteins (WT and mutants) was performed by incubating 10 μL aliquots of a 50% slurry of Glutathione-Sepharose 4B beads (GE Healthcare) containing ~25 μM GST-M2-1 in PBS with a 3-fold molar excess of P for 1 h at 20°C under agitation. Beads were washed extensively with PBS, boiled in 25 μL Laemmli buffer and analyzed by SDS-PAGE and Coomassie blue staining. Bands were quantified using ImageJ software. Affinity between P and M2-1 was determined by isothermal titration calorimetry.

Isothermal titration calorimetry assays

Raw ITC data were processed and quantitative analysis of the P-M2-1$_{38-177}$ interaction was performed on a MicroCal ITC200 microcalorimeter (Microcal, Northampton, MA). Samples were dialyzed against 1× PBS for 15 h. The experiments were carried out at 20°C. The P concentration in the microcalorimeter cell (1.4 mL) was of 33 μM. In total, 20 injections of 2 μL of M2-1$_{38-177}$ solution (concentration 335 μM) were carried out at 180 s intervals, with stirring at 1000 rpm. The experimental data were fitted to a theoretical titration curve with software supplied by MicroCal (ORIGIN). This software generates titration curves based on the relationship between the heat generated by each injection and $\Delta H$ (enthalpy change in kcal/mol), $K_d$ (dissociation constant), n (number of binding sites), the total protein concentration and free and total ligand concentrations.

RNA oligonucleotide synthesis

10–15 nucleotide RNAs corresponding to viral RNA sequences of negative and positive polarity were synthesized on a Pharmacia
Gene Assembler Plus using phenoxacyetyl B-RNA phosphorimidites and Universal Support resin (Glen Research) with a protocol adapted for RNA from DNA solid-phase synthesis [51]. The products were HPLC-purified (Beckman) by anionic chromatography on a DEAE-Sepharose column (Pharmacia) in 10 mM phosphate buffer pH 6.9 and eluted with a linear gradient from 0.55 to 1 M NaCl in 80 min. The oligonucleotides were extensively dialyzed in water. If necessary the pH was adjusted to 7 with 0.5 M NaOH and the concentration determined using the A260 value. Aliquots were then lyophilized and stored at −20°C. The oligonucleotide sequences are given in Table 1. Folding properties of RNAs were estimated by using the mfold web server [22].

NMR spectroscopy

NMR measurements were carried out at 293 K (or 298 K) on Bruker Avance 600, 800 and 950 MHz spectrometers equipped with triple resonance cryoprobes. All samples were in 30 mM sodium phosphate buffer pH 6.8, 150 mM NaCl, 1 mM DTT and 7% D2O. Resonance assignment was reported elsewhere [52]. 15N-NOESY-HSQC (800 MHz) and 13C-NOESY-HSQC (950 MHz) experiments, simultaneously edited for aliphatic and aromatic 13C, with 80 and 100 ms mixing times, were recorded using 1DHN M15N-M2-158–177 and 100 mM 15N-M2-158–177 samples respectively. Data were processed with Topspin 2.1 or NMRPipe [53]. Spectra were analyzed with Sparky [54]. Residual dipolar couplings (RDCs) were collected on 15N-labeled M2-158–177 in two alignment media. The first sample contained 120 mM 15N-M2-158–177 and 100 mM 13C-M2-158–177 in 200 mM Tris (pH 7.4) and 7% D2O. Resonance assignment was reported elsewhere [52]. RDCs were extracted from spin-state selective InPhase-AntiPhase 1H-15N HSQC experiments acquired in interleaved fashion. Residual dipolar couplings (RDCs) were collected on 15N-labeled M2-158–177 in a stretched 6% acrylamide/bisacrylamide (37.5:1:1) gel (deuteron splitting ΔH = 3 Hz). The second sample contained 200 mM 15N-M2-158–177 in a 5% Tris-HCl/C12E5 phase with r = 0.96 molar ratio (ΔH = 26.7 Hz). D2O experiments were recorded in interleaved fashion with and without saturation, with 1.5 s recycling delay, at 14.1 T and 293 K. Intensity ratios were determined based on experiments with and without saturation. Control experiments were carried out without P to account for spin diffusion and effects of residual aliphatic protons in M2-158–177.

NMR structure calculation

M2-158–177 structures were calculated with CYANA 2.1 [55], using distance restraints obtained from 15N- and 13C-NOESY-HSQC spectra and backbone torsion angles generated with TALOS. They were refined in Xplor-NIH [56] using 1DHN M15N- M2-158–177 chemical shift and cross-peak intensity perturbations in 1H-15N and 1H-13C HSQC experiments acquired in interleaved fashion. Measurements were carried out at 293 K at each step, at magnetic fields of 14.1 T and 298 K. The reference spectrum of M2 solution of P1–241 centrifuged at 15 krpm in a TLA55 rotor (Beckman) for 15 min and exchanged into D2O buffer with biopin columns according to the manufacturer’s instructions (Biorad). Transferred cross-saturation experiments [23] were conducted with 130 mM 2H15N labeled M2-158–177 in 91% D2O in the presence of 15 mM (10%) unlabeled P1–241 protein. Broadband proton saturation (2 s) was achieved with a 1.8 kHz Wurst pulse centered at 0.8 ppm. Spectra were recorded in interleaved fashion with and without saturation, with 1.5 s recycling delay, at 14.1 T and 293 K. Intensity ratios were determined based on experiments with and without saturation. Control experiments were carried out without P to account for spin diffusion and effects of residual aliphatic protons in M2-158–177.

Data deposition

Atomic coordinates and structural constraints have been deposited in the Protein Data Bank (PDB accession code 2L9J).

Accession numbers

Swiss-Prot P04545.1
UniProt P28887.1

Supporting Information

Figure S1 Binding of yeast RNA to 15N-M2-158–177 followed by 1H,15N HSQC spectra. Measurements were done at 298K and a field of 14.1 T. The reference spectrum of 15N-M2-158–177 (50 μM) is shown in black, the spectrum after addition of 4 mg/mL yeast RNA (~1:1 molar ratio) in red and after treatment by RNAse A in green. (EPS)

Figure S2 1H,15N chemical shift perturbations measured for 15N-M2-158–177 in the presence of RNA. 1H,15N HSQC spectra were recorded at 14.1 T and 293 K with 50 or 60 mM protein. Per-residue plots of combined 1H,15N chemical shifts in absolute values are represented for (a) Yeast RNA (Roche) (4 mg/mL), for (b) an RSV unrelated hairpin UG2A hairpin (20:1 RNA:protein molar ratio), for single-stranded RSV genomic negative-sense (neg) and positive-sense (pos) RNA sequences, (c) neg leader (6:1), (d) neg gene start (2.5:1), (e) neg SH gene end (2.5:1),
Figure S3 Determination of apparent RNA dissociation constants by titration experiments with 1H-15N HSQC experiments of 15N-M2-158–177. (A) 1H and 15N chemical shift differences of backbone amides or Q93 side chain (denoted H, N, He21, He22, and Nc2 respectively) are displayed versus the ratio of RNA concentration relative to protein for chosen residues showing the largest chemical shift amplitudes. Measurements were done at 14.1 T and 293 K. Protein concentration was 50 or 60 μM. 15N chemical shifts were scaled with a factor 1/10 to be of comparable magnitude to 1H. Plots are shown for following RNA sequences, "pos" and "neg" denoting RSV genomic positive-sense and negative-sense sequences: (a) UGA2 hairpin, (b) neg leader, (c) neg gene start, (d) neg SH gene end, (e) neg F gene end, (f) neg long U-rich, (g) pos SH gene end, (h) pos short A-rich, (i) pos F gene end and (j) double-stranded F gene end. The data were fitted to a binding model with 1:1 stoichiometry in Origin software. The mean values of apparent Kd and the statistical error are indicated for each RNA.

EPS

Figure S4 ITC binding isotherms for P binding to M2-158–177. (A) Raw binding data obtained for 20 automatic injections of M2-158–177 (2 μl for each injection, 353 μM protein concentration) into a cell containing P (200 μl initial volume; 35 μM initial protein concentration). Proteins were suspended in 1× PBS. (B) Integrated titration curve obtained from the raw data in panel A after baseline subtraction. The solid squares represent the experimental data, while the solid line corresponds to the standard multiple independent binding-site model that was fitted to the data. The corresponding average Kd value is 3 μM with a stoichiometry of 1:1.

TIF

Figure S5 Intensity variations of M2-158–177HSQC spectra in the presence of phosphoprotein. (A) Superposition of 1H-15N HSQC spectra of 15N-labeled M2-158–177 without (black) and with 0.5 equivalent of full-length RSV phosphoprotein P (blue) shows overall line broadening on addition of P. (B) Arginine 13C4-1H2 and lysine 13C2-1H2 region of 1H-15N HSQC spectra of 15N-labeled M2-158–177 in the presence of 0.25 (red) and 0.5 (blue) equivalents of full-length P (22.3 T, 298 K) overlaid on the reference spectrum (black). Broadening is observed for K92 and K158.

EPS

Figure S6 Changes in 1H-15N HSQC spectra of M2-158–177 single mutants K92D, R126D, T130D, L148A, K150D, R151D and T160D and N163D. K92D, K150D and R151D strongly reduced in vitro RNA binding to M2-1 whereas R126D, T130D, L148A, T160D and N163D were excluded from cytoplasmic inclusion bodies containing RSV phosphoprotein. All resulted in a nearly total loss of distribution in the Lac minigenome assay. (A) 1H-15N HSQC spectra of 15N-labeled M2-158–177 single mutants (125 μM, 18.7 T, 293 K) have a similar pattern to that of wild type and show that the mutant proteins are well folded. (B) Chemical shift difference plots relative to WT M2-158–177 indicate that chemical shift changes occur only for residues close to the mutation in the sequence, as expected if no conformational rearrangement takes place. In the case of K92D (in helix α2), the spatially close region around K150 (in helix α2) is also affected, and vice versa. A 15N NOESY-HSQC experiment with 80 ms mixing time was recorded with K150D to verify the presence of HNε-HNε correlations, characteristic of 7-helices.

EPS

Figure S7 Sequence alignment of M2-1 and VP30 proteins. (A) Alignment of primary sequences of M2-1 proteins of human Respiratory Syncytial Virus (hRSV, strain Long, UniProt accession number P28307.1), bovine RSV (bRSV, strain ATU31908, NC_001989.1), Pneumonia virus of mouse (PVM, NP_173333), human Metapneumovirus (HMPV, AAM12941.1), and avian Metapneumovirus (AMPV, NP_443984). Strictly identical residues are highlighted in red. Residues with more than 70% similarity are boxed in blue. Secondary structures extracted from the M2-158–177 structure are indicated above each block. Single mutated M2-158–177 residues which severely impair RSV transcription are indicated by a red star if the residues are strictly conserved and a blue star if they diverge in more than 2 sequences. (B) The primary protein sequences of M2-1 proteins were manually aligned with VP30 protein sequences of Zaire Ebola virus (EBOV_Z, UNP Q05323), Reston EBOV (EBOV_R, NP_690585.1) and Lake Victoria Marburg virus (MARV_LV, NP_001531157.1). Strictly identical residues are highlighted in red and located in the Cys3His putative zinc finger and in the oligomerization domains. Residues with more than 70% similarity are boxed in blue. (C) Primary sequences of VP30 proteins were aligned separately from M2-1 and secondary structures extracted for EBOV_Z VP30 CTD (pdb 2BBB). Alignments were edited with ESPript 2.2 [60].

EPS

Text S1 Supplemental Materials and Methods.

DOC

Table S1 NMR structure statistics of RSV M2-158–177.

DOC

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Author Contributions

Conceived and designed the experiments: CS JFE. Performed the experiments: CS JFE MLB. Written the paper: CS JFE.

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