The feet of the measles virus polymerase bind the viral nucleocapsid protein at a single site

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Abstract: Measles virus has a single-stranded RNA genome that is organized into a helical complex by the viral N protein. The resulting structure is termed the nucleocapsid and is traversed by the viral polymerase during RNA synthesis. The P protein, the noncatalytic subunit of the polymerase, provides the “legs and feet” that allow the polymerase to walk along its protein-RNA template. The polymerase feet are very simple three-helix bundles, only 50 amino acids in size. Previously, we have shown that these feet grasp the viral N protein during movement by attaching to a short sequence (amino acids 487–503) within the disordered and surface-exposed tail of N, causing it to fold into a helix. The result is a weak-affinity complex with a short lifetime, which would allow the polymerase to take rapid steps forward. The structure of the complex was determined using X-ray crystallography. This simple model of binding was challenged by a paper in this journal, claiming that a downstream sequence in the tail of N (amino acids 517–525) was also critical for the association. Its presence was reported to enhance the overall affinity of the polymerase feet for N by three orders of magnitude. We have, therefore, examined binding of the polymerase foot domain to amino acids 477–525 of N using quantitative biophysical techniques, and compared the results to our previous binding studies, performed using amino acids 477–505 of N. We find no evidence that the sequence downstream of amino acid 505 influences binding, validating the original single-site binding model.

Keywords: isothermal titration calorimetry; surface plasmon resonance; reversible molecular interactions; binding; paramyxoviruses

Introduction

The RNA polymerase of measles virus uses a helical protein-nucleic acid complex as its template. This

complex, termed the nucleocapsid, protects and organizes the single-stranded RNA genome of the virus. Interaction between the polymerase and nucleocapsid is mediated, at least in part, by a cluster of small helical domains found at the end of one of polymerase subunits (the viral P protein). Four of these helical “feet” are connected by flexible and largely unstructured “legs” to an upstream coiled-coil, which serves to oligomerize P, and anchor it to the catalytic subunit of the polymerase (the viral L protein). Although the mechanism of polymerase

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translocation remains poorly characterized, it is clear that the feet of the polymerase are critical for attachment. The feet must repeatedly bind and release the nucleocapsid as the replication machinery moves, requiring specific yet reversible interactions. This general model of polymerase organization has resulted from numerous studies on measles and closely related viruses (reviewed in Refs. 1–6). The purpose of this note is to address a specific disagreement in the literature concerning the regions of measles N involved in binding the feet of the polymerase.

Using pulldown experiments, we previously mapped the binding site for the measles polymerase foot domain to a short and contiguous sequence (amino acids 477–505) within the unstructured tail of the N protein.⁷ Binding of the foot domain to this sequence was studied quantitatively using two different physical techniques: solution NMR spectroscopy and isothermal titration calorimetry. The results of these studies were consistent, and analyzable in terms of a simple 1:1 binding scheme. The equilibrium dissociation constant (K_D) was determined to be 13 μM at 20°C, by both NMR spectroscopy and ITC.^{7,8} Binding is kinetically rapid, with an estimated k_{on} of $0.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, which approaches the diffusion-controlled limit. We determined an X-ray crystal structure of the N tail peptide in complex with the polymerase foot domain, which rationalized the binding data⁸ [(Fig. 1(A)). It was confirmed that amino acids 483–503 from N bind to a hydrophobic face of the foot in an α -helical conformation with binding and folding of N largely coupled.⁸ The polymerase-nucleocapsid interaction in measles virus was, thereby, shown to depend on a weak-affinity protein complex with a short lifetime; a finding that appears compatible with translocation of the polymerase along the nucleocapsid during RNA synthesis.

A paper published by Bourhis *et al.*⁹ subsequently challenged this model. They studied association of the polymerase foot domain with the unstructured tail of N (amino acids 401–525) and with various deletion mutants, using Surface Plasmon Resonance (SPR). Based on these studies, it was concluded that a second site (amino acids 517–525 of N) was involved in binding the polymerase foot domain, in addition to the site previously identified (amino acids 483–503 of N). The apparent dissociation constant (K_D) governing binding of the foot to the tail of N was determined to be 81 nM, three orders of magnitude larger than our previous estimate. Although binding of amino acids 517–525 to the foot domain was not correlated with any spectroscopically detectable structural change, deletion of this region reduced K_D to 12 μM , a value in agreement with previous estimates. Subsequent studies have confirmed that there is no direct interaction between the region 517–

Figure 1. A: The measles polymerase foot domain in complex with amino acids 486–504 of N, as visualized by X-ray crystallography.⁸ A ribbon diagram is displayed, together with the associated molecular surface (semitransparent). B: Schematic diagram showing the organization of the measles virus N protein. The assembly domain, responsible for RNA packaging and nucleocapsid formation, is followed by a 125 amino acid constitutively disordered “tail.” The binding site for the polymerase foot domain (amino acids 487–503), and the downstream sequence which has been reported to enhance the affinity of the interaction (amino acids 517–525) are both indicated.

525 and the foot domain.^{10,11} This work led to a “two-site” model of attachment in which a sequence downstream of the helical binding determinant enhances the overall binding affinity of this system by an unknown physical mechanism [Fig. 1(B)].

However, this two-site model is in conflict with some prior experimental observations. In qualitative pull-down experiments, we observed no reduction in binding affinity when the putative affinity-enhancing sequence (amino acids 515–525) was deleted. [Fig. 1(B) in Ref. 7]. Additionally, in the study proposing the two-site model, a construct that lacks amino acids 489–525 (NTAIL Δ 2,3) reportedly still binds to the polymerase foot domain with measurable affinity ($K_D = 41 \mu M$). Our own data indicate that this construct is binding incompetent [Fig. 1(B) in Ref. 7], something that is also suggested by the X-ray crystal structure of the N/P complex [Fig. 1(A)]. The NTAIL Δ 2,3 polypeptide retains only 5 of the 21 the residues from N that are directly involved in complex formation. This

Figure 2. Binding of the measles polymerase foot domain to N₄₇₇₋₅₂₅ characterized by isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). (A) Integrated and corrected ITC data fit to a single set of sites model (all sites identical and equivalent). The filled circles represent the experimental data, whereas the solid line corresponds to the model. The binding curve results from 28 injections of the polymerase foot domain (each injection 10 μ L, protein concentration 549 μ M) into a calorimeter cell containing N₄₇₇₋₅₂₅ (cell volume 1.41 mL, initial protein concentration 47 μ M). (B) SPR sensorgrams resulting from transient injections of the polymerase foot domain (5–35 μ M), over immobilized N₄₇₇₋₅₂₅ at two different densities. (C) Mean equilibrium response for the data in (B) fitted to a 1:1 binding model. The hollow circles represent the experimental data, whereas the solid line corresponds to the fitted model.

calls into question the validity of the SPR-derived affinity measurements on which the two-site attachment model is based.

To resolve this question, we have evaluated binding of the measles polymerase foot domain to amino acids 477–525 of N, a peptide which encompasses both the crystallographically-identified binding site, and the sequence reported by Bourhis *et al.* to dramatically enhance the binding affinity [Fig. 1(B)]. Through comparison with our previous binding studies, these experiments directly test the validity of the “two-site” binding model.

Results

The binding of the measles polymerase foot domain (P₄₅₇₋₅₀₇) to measles N₄₇₇₋₅₂₅ was characterized using

isothermal titration calorimetry (ITC).¹²⁻¹⁶ N₄₇₇₋₅₂₅ was loaded into the calorimeter sample cell, and titrated with P₄₅₇₋₅₀₇, achieving a molar ratio of 1:2.6 at the end of the titration. The data, following integration and correction for the heats of dilution, were fit with a standard model allowing for a set of independent and equivalent binding sites [Fig. 2(A)]. The estimates for the model parameters are shown in Table I, together with those obtained previously from an ITC study of foot binding to N₄₇₇₋₅₀₅.⁷ It should be noted that estimates for the stoichiometry, *n*, are very sensitive to small errors in the measured protein concentrations.^{14,17} Overall, the presence or absence of the sequence 506–525 makes no significant difference to the binding process. This experiment, therefore, invalidates the “two-site” model of foot attachment.

Table I. Binding of the Measles Polymerase Foot Domain to the Measles N protein: Model Parameters Derived from ITC Studies (20°C)

Region of N studied	Stoichiometry, n	Dissociation constant, K_D (μM)	Binding enthalpy, ΔH (kJ/mol)	Binding entropy, ΔS (J/K.mol)
477–505 ^a	0.93	13	–46	–59
477–525	0.80	7.4	–45	–56

^a Data taken from Ref. 7.

To help understand the discrepancy with the SPR-based affinity measurements which gave rise to the model, we also characterized binding using SPR.^{18–21} The constitutively disordered N_{477–525} was immobilized on the surface of the sensor chip. To achieve efficient coupling of this peptide to the carboxylated surface using carbodiimide chemistry, we appended a non-native N-terminal tag to the peptide, incorporating three lysine residues. Subsequently, we transiently injected the polymerase foot domain over the immobilized peptide and followed the change in optical signal in real time. The resulting SPR sensorgrams [Fig. 2(B)] have a square-wave appearance, characteristic of low-affinity binding with rapid on- and off-rates (see e.g. Refs. 22,23). This precludes any attempt at kinetic analysis of the data. The equilibrium (steady-state) SPR response, as a function of mobile reactant concentration, was fitted to the equation appropriate for a simple 1:1 binding event. The fit of the data to the model was adequate, with some indications of non-random departures from the model [Fig. 2(C)]. The analysis yielded estimates for K_D (at 25°C) of 19 μM (low density of N_{477–525} on the chip) and 12 μM (high density of N_{477–525} on the chip), consistent with the ITC measurements.

Discussion and Conclusion

Our results, obtained using both ITC and SPR, are not compatible with the two-site model for attachment of the foot domain to the N protein. This model rests upon a single set of experimental observations; the measurement of binding kinetics and affinities by SPR.⁹ Our own SPR data demonstrate how this discrepancy has likely arisen.

In the method of SPR (reviewed in Refs. 18–21), one of the interacting molecules is immobilized on the sensor surface, and its binding partner is transiently introduced into the buffer flowing over the surface. The formation and breakdown of the complex is then followed optically in real time. In principle, both the binding affinities and the kinetic parameters of reversible interactions can be measured in this fashion.

The buildup and decay of the SPR signal following transient introduction of the mobile binding partner can be described by simple differential rate equations, the form of which depends on the assumed reaction scheme (see e.g., Refs. 24,25).

Direct fitting of the relevant mathematical expressions to SPR sensorgrams obtained at differing concentrations of the mobile binding partner (“global analysis”) can yield the on and off rates for complex formation and dissociation, and hence equilibrium dissociation constants.^{25,26} This is the method that Bourhis *et al.* used to analyze their binding data, assuming a simple 1:1 binding scheme between N and the polymerase foot domain.

However, the ability to measure a SPR signal does not imply that kinetic parameters can be sensibly derived from that signal. Because the rate at which the mobile reactant can be transported across the sensor surface is finite, fast reactions may become transport-limited, which complicates the interpretation of the experimental data.^{27,28} In extreme cases, very rapid on- and off-rates give rise to “square wave” sensorgrams at all applied reactant concentrations, exemplified by the interaction studied here [Fig. 2(B)]. This makes kinetic analysis impossible, as binding proceeds too quickly to be observed experimentally. Fundamental limits are imposed by the time resolution of the instrument (0.1s for the BIAcore 2000 used in this study), and the presence of injection noise in the sensorgrams. The rectangular appearance of the sensorgrams in Figure 2(B) is consistent with estimates of the kinetic parameters governing the interaction obtained using solution NMR spectroscopy.⁸ Association and dissociation are extremely rapid ($k_{on} = 0.5 \times 10^8 M^{-1} s^{-1}$, $k_{off} = 600 s^{-1}$). We conclude that kinetic analysis of SPR data cannot be routinely applied to this system and consequently, that the binding parameters reported by Bourhis *et al.* are not reliable.

In cases such as this, the steady-state SPR signal can still be examined as a function of the mobile reactant concentration. This type of analysis yields the equilibrium constant(s) governing the interaction. In general, this provides a good experimental check on the validity of parameters obtained from the kinetic approach.²⁹ Analyzed in this fashion, our SPR data yield estimates of the dissociation constant in the low micromolar range [Fig. 2(C)].

This study re-emphasizes some of the common principles which govern operation of the replication machinery in the *Paramyxovirinae*; a family that includes measles, mumps, and Sendai viruses. Although the intermolecular forces which drive the polymerase feet to interact with the nucleocapsid

differ in these viruses,^{7,8,30–33} in all three cases, the interaction involves the coupled binding and folding of protein domains. For measles and Sendai viruses, a weak-affinity protein complex with a short lifetime is known to result.^{8,31} This is consistent with rapid movement of the polymerase along its template during RNA synthesis.

Materials and Methods

Protein preparation

All proteins (measles virus Moraten vaccine strain; Genbank AF266287) were produced by heterologous expression in *Escherichia coli*. An expression plasmid encoding the measles polymerase foot domain (P_{457–507}), fused to the C-terminus of Glutathione-S-Transferase (GST) has been described previously.⁷ Using standard DNA manipulation techniques, two additional expression plasmids were constructed, encoding the measles N tail peptide (N_{477–525}) fused to the C-terminus of GST (Detail in Supporting Information Table S1). All of the GST fusion proteins can be specifically cleaved with Tobacco Etch Virus (TEV) protease to release the polypeptides of interest. In the case of N_{477–525}, the liberated proteins carry either a non-native dipeptide (TS) or pentapeptide (TSKKK) at their N-terminus. The former was used for ITC measurements, whereas the latter facilitated efficient coupling of N_{477–525} to the sensor chip for SPR studies.

Expression plasmids were transformed into BL21 (DE3) (Stratagene). Transformed bacteria were grown in Luria broth supplemented with 1%(v/v) glycerol and 50 µg/mL kanamycin. Cultures were shaken in flasks at 37°C, until the optical density at 600 nm was 0.8–1.0. Protein expression was then induced by addition of isopropylthiogalactopyranoside, to a final concentration of 0.5 mM, and the cultures maintained for 5 h at 28°C before harvest. Affinity purification of the GST fusion proteins; on-column cleavage with TEV protease, and elution of N_{477–525} and P_{457–507} was carried out as previously described.⁷ P_{457–507} was further purified using cation exchange chromatography; binding the protein to SP Sepharose HP resin (GE HealthCare) buffered with 12.5 mM MOPS/KOH pH 7.0/50 mM NaCl, and eluting it with a linear salt gradient. N_{477–525} was further purified by anion exchange chromatography; binding the protein to Q Sepharose HP resin (GE HealthCare), buffered with 12.5 mM Tris/HCl pH 8.5/50 mM NaCl, and eluting it with a linear salt gradient.

Protein concentrations were determined from UV absorption measurements at 280 nm,^{34–36} made using a Shimadzu UV-2501PC spectrophotometer. Both proteins under study contain a single tyrosine residue and no tryptophan or cysteine residues. Accordingly the molar extinction coefficients for the

proteins, in their native state, were taken to be 1490 M^{–1}cm^{–1}.³⁵ The likely error in protein concentrations determined in this fashion is ±10%.^{34–36}

Isothermal titration calorimetry

ITC experiments were performed using a VP-ITC microcalorimeter (MicroCal) operated at 20°C. N_{477–525} and P_{457–507} were exhaustively dialyzed into 10 mM NaHPO₄–NaH₂PO₄ pH 7.0/100 mM NaCl/0.5 mM sodium azide. Before performing titrations, the protein samples were outgassed at 1/3 atm for 8 min. P_{457–507} (concentration 549 µM) was loaded into the injection syringe, whereas N_{477–525} (concentration 47 µM) was loaded into the calorimeter sample cell. The titration comprised 28 injections of 10 µL each, spaced at intervals of 360 s (Supporting Information Fig. S1). Protein concentrations in the calorimeter cell following each injection were calculated as previously described.³⁷ Heats of dilution were determined from control titrations, where P_{457–507} was injected into buffer or buffer injected into N_{477–525} (Supporting Information Fig. S1). The baseline around each heat pulse was approximated with a low-order polynomial, and the heat generated per injection obtained by numerical integration of the raw data. Heats of dilution were subtracted from the observed binding heats before model fitting. The data were fit to a single set of sites model (all sites independent and equivalent)^{38–40} using nonlinear least squares.

Surface plasmon resonance

SPR experiments were carried out using a BIAcore 2000 system (GE Healthcare) operated at 25°C. Purified N_{477–525} (carrying the TSKKK N-terminal pentapeptide) was immobilized on the carboxylated surface of a CM5 sensor chip using amine-coupling chemistry. N_{477–525} contains no lysine residues; hence, the peptide must be immobilized through its non-native N-terminus. Briefly, the chip surface was activated using a 1:1 mixture of 0.4 M 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride and 0.1 M N-hydroxysuccinimide. N_{477–525} (600 µg/mL in 10 mM sodium acetate buffer pH 3.5) was then injected over the activated surface at a flow rate of 5 µL/min. The level of immobilization was controlled by varying the contact time of the peptide with the activated surface. Unreacted esters were subsequently blocked with an injection of 1 M ethanolamine. N_{477–525} was immobilized at high and low densities, corresponding to 57 and 281 response units (RU) respectively (1 RU corresponds to a change in mass of ~1 pg protein/mm² on the sensor surface).^{41,42} Interaction studies were carried out by injecting P_{457–507} (5–35 µM in 20 mM MOPS/KOH pH 7.0/100 mM NaCl) over the immobilized peptide at a flow rate of 15 µL/min, and following real-time changes in the refractive index at the sensor surface.

Measurements were made in triplicate at each concentration. The signal from a reference cell, that had been activated and blocked without addition of N_{477–525}, was subtracted from all sensorgrams to correct for artifacts such as bulk refractive index changes and nonspecific binding. Repeat buffer injections, interspersed with the sample injections, were used to correct for any slow drift in the baseline signal. The equilibrium response (R_{eq}) as a function of mobile analyte concentration ($[A]$) was then fit to the equation expected for a simple 1:1 binding model^{21,43}:

$$R_{eq} = R_{max}/(1 + K_D/[A])$$

using nonlinear least squares. In the expression above, R_{max} is the SPR response when binding is saturated, and K_D is the equilibrium dissociation constant governing binding.

Figure 1 was prepared with the assistance of MacPymol (<http://www.pymol.org/>).

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