Cooperative assembly and dynamic disassembly of MDA5 filaments for viral dsRNA recognition

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Results

MDA5 Binding to dsRNA is Cooperative. Antiviral signaling through MDA5 is stimulated by dsRNA but not by ssRNA or dsDNA (8, 19). To examine whether the differential binding affinities of MDA5 are responsible for dsRNA specificity, we purified recombinant human MDA5 from Escherichia coli (Fig. S1A) and examined its interaction with 112 bp dsRNA and dsDNA, and 112 nt ssRNA using an EMSA. These model nucleic acids were derived from MDA5 cDNA (Table S1), and no significant secondary structure was predicted for the ssRNA (mFold).

EMSA revealed that MDA5 is capable of high-affinity binding to dsRNA, dsDNA, and ssRNA with dissociation constants (Kd) of 22, 33, and 57 nM, respectively (Fig. 1). Despite the comparable binding affinities between the nucleic acid types, only the interaction between MDA5 and dsRNA is highly cooperative. By EMSA, MDA5 addition to dsRNA resulted in a bimodal distribution of mostly either free (RNA-only) or saturated (fully complexed) products (Fig. 1A). By contrast, MDA5 complexed with ssRNA or dsDNA exhibits a broad distribution of intermediate sizes, indicative of less cooperative binding. In a quantitative analysis of the EMSA results (Fig. 1B), the dsRNA binding curve is sharply sigmoidal with a Hill coefficient of 4.0, whereas the binding curves for ssRNA and dsDNA are more hyperbolic with Hill coefficients of 1.5 and 2.7, respectively. EMSA analysis


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of another 112 bp dsRNA with different sequence (dsRNA2, Table S1) reveals a similar bimodal distribution of RNA upon MDAS binding and sigmoidal binding curve (Fig. S1B), suggesting that highly cooperative binding of MDAS is dependent on dsRNA structure but not sequence.

**Only dsRNA Stimulates ATP Hydrolysis by MDAS.** We next asked whether the ATP hydrolysis activity of MDAS is dependent on the type of bound nucleic acid. We measured the ATP hydrolysis rate of MDAS using two sets of 112 bp/nt dsRNAs, ssRNAs, or dsDNAs derived from two different sequences (Table S1) at an equivalent nucleotide concentration (4.8 μg/mL, i.e., 71.4 nM 112 bp dsRNA). The ATP hydrolysis activity of MDAS was strongly stimulated by dsRNAs, but not by ssRNAs or dsDNAs (Fig. 1C), consistent with previous observations (20). A 112 bp RNA–DNA hybrid and 7 kb genomic ssRNAs derived from two picornaviruses, encephalomyocarditis virus (EMCV) and Mengo virus (MV), which contain highly structured internal ribosome entry sites (IRES) (Fig. 1C), were also unable to stimulate ATP hydrolysis (Fig. 1C). This result suggests that dsRNA is the only type of nucleic acid that supports MDAS ATP hydrolysis.

**MDAS Forms Filamentous Oligomers Along the Length of dsRNA.** To better understand the nature of the interaction of MDAS with dsRNA in comparison with ssRNA and dsDNA, we visualized complexes of MDAS with nucleic acids using negative-stain EM. We included the cross-linker reagent, bismaleimide (BMH), and ATP analogue, ADP–aluminum fluoride (ADP•AlF₄⁻) in the sample preparation to improve complex homogeneity and stability (Fig. S2A). The migration of the BMH-cross-linked MDAS: dsRNA complex was identical to that observed for the native complex by EMSA.

The electron micrographs of the MDAS: dsRNA: ADP•AlF₄⁻ complex revealed that MDAS forms a filamentous oligomer upon binding to 112 bp dsRNA (Fig. 2A). Rod-like oligomers were rarely observed with ssRNA and dsDNA, (<3% of dsRNA), and were more heterogeneous in size (Fig. S2C). Each nucleoprotein filament of MDAS with 112 bp dsRNA is 32–37 nm long. RNA is not directly visible in the negatively stained samples, but the agreement between the observed filament length and predicted length of 112 bp dsRNA (∼33 nm, ∼2.8 Å helical rise per base pair) supports the idea that the filament is arranged along the length of dsRNA. To test whether the filament extends with RNA length, we examined the complex formed with 512 bp dsRNA. Longer filaments of 150–170 nm were observed, consistent with the expected length of a 512 bp dsRNA molecule (Fig. 2A). Thus, dsRNA promotes MDAS filament assembly along the length of dsRNA.

To obtain a more detailed understanding of the filament assembly, we calculated class averages of the 112 bp complex. Each filament consists of seven or eight segments (Fig. 2B). This result is in agreement with the number of intermediate complex bands in EMSA (Fig. 2C), suggesting that each segment corresponds to

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**Fig. 1.** Only binding of MDAS to dsRNA is cooperative and stimulates ATP hydrolysis. (A) EMSA with 112 bp/nt dsRNA, ssRNA, and dsDNA. A lane in which approximately 50% of MDAS is bound to the respective nucleic acid (red box) was chosen for each sample, and the distribution of the complexes is displayed in the left-hand panel. (B) The EMSA results in A were fit to the Hill equation to obtain dissociation constant ($K_d$) and Hill coefficient (Nh). Estimated $K_d$ and Nh are 22 nM and 1.5 for dsRNA, and 57 nM and 2.7 for dsDNA, respectively. Plotted values are mean ± SD (n = 2). (C) ATP hydrolysis rates (mean ± SD, n = 4) of MDAS (0.3 μM) free of nucleic acids, MDAS bound to 112 bp/nt dsRNA, ssRNA, dsDNA, DNA–RNA hybrid (sequence 1, □ sequence 2, see Table S1), IRES (1 kb), and genomic RNA (gRNA) (∼7 kb) of EMCV and MV. Equivalent mass concentration (4.8 μg/mL) was used for all nucleic acids.

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**Fig. 2.** MDAS forms filamentous oligomers along the length of dsRNA. (A) Representative electron micrographs of negatively stained MDAS in complex with 112 and 512 bp dsRNA in the presence of ADP•AlF₄⁻. Representative particles used for averaging are circled in red. See Fig. S2C for electron micrographs of MDAS in complex with ssRNA and dsDNA. (B) Representative class averages of MDAS in complex with 112 bp dsRNA. The red box in the magnified view corresponds to a minimum binding unit of MDAS in EMSA (C). The number of binding units per complex ($n_b$) is displayed at the bottom left for each average. (See also Fig. S2D) (C) EMSA of 62, 112, and 162 bp dsRNAs with MDAS (0.3 μM) visualized with SybrGold stain to enhance the intermediate complexes that are otherwise difficult to detect. Enumeration of intermediate complexes reveals that the full complex contains 4, 7–8, and 11 binding units of MDAS for 62, 112, and 162 bp dsRNAs, respectively. (D) Multi-angle light scattering (−OD₄₈₅ and $M_w$ estimation) suggests that the molecular mass of the full complexes formed on 62 and 112 bp dsRNAs are 854 (±43) kDa, and 1,878 (±13) kDa, respectively.
the minimal binding unit. The tight packing of these segments along the filament is consistent with cooperative binding observed from EMSA. Each unit occupies approximately 1.3 helical turns of the RNA (15 bp), but no clear indications of helical symmetry could be detected in the averages or their Fourier transforms. Occasionally segments in the filaments slightly deviate from the central axis, causing a modest level of curvature in the filament; this curvature may result from heterogeneity in the ADP+AlF₄⁻ occupancy or in the dimer-dimer interface. A more detailed analysis of the structure was not feasible because of the low resolution of the averages.

To determine the stoichiometry of the MDA5:dsRNA complex, we measured the molecular mass of the BMH-cross-linked filament using multilight scattering (MALS) (Fig. 2D). We found that MDA5 forms complexes of 854 kDa with 62 bp and 1,878 kDa with 112 bp dsRNA, which corresponds to 6.7 and 15.5 MDA5 monomers per RNA molecule, respectively. From EMSA, we observed approximately 4 and 7–8 complex bands for 62 and 112 bp dsRNAs, respectively (Fig. 2C). This result suggests that each segment in EM averages and each binding unit in EMSA represents an MDA5 dimer. The stoichiometry of one MDA5 dimer per 15 bp is also evident from two other supporting experiments involving MDA5 titration and EMSA examination of mixed populations of MDA5 with different sizes (Fig. S3). Interestingly, our MALS analysis (Fig. S1A) and a previous report (13) suggest that MDA5 is monomeric in solution, indicating that MDA5 dimerizes upon dsRNA binding. Although a complex of a single MDA5 monomer bound to dsRNA is often undetectable by EMSA with dsRNA longer than 62 bp, it is apparent with 15 bp dsRNA (Fig. S3B). This finding suggests that dimerization is cooperative only in the context of filament.

ATP Hydrolysis Occurs by Monomers of MDA5 Throughout the Filament. The unique property of dsRNA in promoting both filament assembly and ATP hydrolysis by MDA5 raised the question of whether filament formation is required for ATP hydrolysis, and whether ATP hydrolysis of an MDA5 molecule depends on its position within a filament. To address these questions, we examined ATP hydrolysis activity of MDA5 with 15, 112, and 512 bp dsRNAs. To account for the low binding affinity of MDA5 to 15 bp dsRNA, we titrated MDA5 to excess (Fig. S3C) and measured intrinsic ATP hydrolysis activity of MDA5 (instead of binding or dissociation kinetics which often dominate the steady-state reaction rate at low enzyme or substrate concentration). We measured ATP hydrolysis rates using high concentration of MDA5 (4 μM) or dsRNA (40 μg/mL, i.e., 10.7 μM MDA5 binding site) that would saturate 0.6 μg/mL dsRNA or 0.3 μM MDA5, respectively. Use of the same total base-pair (i.e., mass) concentration also ensures the same amount of MDA5 binding sites for all reactions. Note of the same total base-pair (i.e., mass) concentration also ensures that an individual dimer can hydrolyze ATP with little positional preference within a filament. Thus, the ATP hydrolysis dependence of the ATP hydrolysis activity of the two MDA5 monomers in a dimeric binding unit or MDA5 molecules within a filament, we measured the ATP hydrolysis activity of heterodimers or heterofilaments formed on 15 bp or 512 bp dsRNA by a mixture of WT and catalytically inactive mutants. We used alanine mutants of K335 and D443, the two conserved residues important for ATP binding and hydrolysis (21), because these mutants do not hydrolyze ATP but retain high affinities to dsRNA (Fig. S4 B–C). We confirmed that WT and K335A or D443A form heterodimers and heterofilaments using EMSA (Fig. S4 D–E). We premixed WT and the mutants at various ratios with a fixed total MDA5 concentration, and formed a complex with a 15 or 512 bp dsRNA with the same two conditions used in Fig. 3A (Fig. 3C and Fig. S4C). In all cases, the ATP hydrolysis rate per WT (derived from the relative rate in Fig. 3B and Fig. S4F divided by the WT concentration) remained constant. These results suggest that the intrinsic ATP hydrolysis activity of a WT subunit is unaffected by the catalytic activity of its dimeric partner or neighboring molecules within a filament.

ATP Hydrolysis by MDA5 Promotes Dissociation from dsRNA. Previous studies with several helicases suggested that ATP hydrolysis can trigger their dissociation from RNA (21, 22). To test the effect of ATP hydrolysis on the interaction between MDA5 and dsRNA, we performed a time-dependent EMSA, in which the time evolution of the level of MDA5:dsRNA complex was monitored in the presence of a protein trap, heparin, which binds MDA5 but does not stimulate ATP hydrolysis. MDA5 was preincubated with 512 bp RNA, and the reaction was initiated by adding a mixture of ATP and a 166-fold excess of heparin relative to RNA. The ability of heparin to trap MDA5 at this ratio was confirmed from EMSA in which a premixture of MDA5 with heparin prevents MDA5 binding to dsRNA (Fig. S5C). In the absence of ATP (Fig. 4A), MDA5:dsRNA complex is stable with less than 30% of 15 or 512 bp dsRNA. In contrast to WT MDA5 with ATP where approximately 50% of dsRNA was free of MDA5 within 15 s (Fig. 4A), we examined whether the rapid dissociation of WT is due to hydrolysis or binding of ATP, we replaced ATP with a nonhydrolyzable ATP analog, β,γ-methyleneadenosine 5′-triphosphate (ADPCP) in the dissociation reaction. ADPCP binds to MDA5 as evidenced by its inhibition of ATP-hydrolysis activity but does not interfere with electrophoresis which is the case for ADP+AlF₄⁻ (Fig. S3 A–B). In the presence of ADPCP, approximately 50% of total dsRNA was converted to free RNA in 5 min, as opposed to 15 s for ATP (Fig. 4A). Thus, the ATP-dependent rapid dissociation WT MDA5 is primarily due to ATP hydrolysis.

Consistent with the above result, a single-round ATP hydrolysis assay also revealed similar dissociation kinetics during ATP hydrolysis. We monitored ATP hydrolysis by MDA5 in complex with 512 bp dsRNA in the presence and absence of heparin. The initial rates for both reactions were comparable. However, the rate with heparin declined to a halt within 1 min, whereas the rate without heparin remained constant over 3 min (Fig. 4B). The overlapping time interval for ATP hydrolysis and dissociation kinetics suggests that the rapid decrease in the ATP hydrolysis rate in the heparin reaction is due to MDA5 dissociation from dsRNA.
WT complex and dissociation of individual MDA5 molecules is copurified with dsRNA. Thus, the catalytic mutants stabilize the effect of K335A and D443A on ATP-dependent dissociation of individual MDA5 monomers hydrolyzing ATP at a time reaches a certain threshold independent of the initial filament length. This prediction was also be true if a filament disassembles cooperatively, either as an entire filament or in smaller fragments, when a fraction of MDA5 monomers hydrolyzing ATP at a time reaches a certain threshold that is independent of filament length (i.e., linearly increasing stability). Alternatively, if filament disassembly occurs from an end as individual monomers or dimers, or disassembles cooperatively with a higher threshold for longer filaments (i.e., nonlinearly increasing stability), MDA5 would dissociate more slowly from longer dsRNA. We compared the dissociation rate of WT MDA5 using the single-round kinetic assay with 512, 1,012, and 2,012 bp dsRNAs. The time derivative of the single-round kinetics (Fig. S5F) showed a more rapid reduction of the ATP hydrolysis rate for shorter dsRNAs with half-lives of 0.2, 0.6, and 1.3 min for 512, 1,012, and 2,012 bp dsRNAs, respectively (Fig. 4C). The more rapid decline in the ATP hydrolysis rate with shorter dsRNAs suggests a more rapid dissociation of MDA5 from shorter dsRNAs, which is inconsistent with models in which MDA5 dissociates from the filament interiors or cooperatively with its threshold independent of filament length.

To further validate the length-dependent dissociation mechanisms, we utilized the K335A/D443A mutants to stall the dissociation reaction, and compared the level of WT that remained bound to dsRNAs of different lengths using the single-round kinetic assay. WT was premixed with an equal amount of K335A/D443A, and complexes were formed with dsRNAs of 112, 512, and 2,012 bp at the equivalent total base-pair concentration. To ensure equivalent binding of WT to dsRNA at the beginning of the reaction, we used 0.2 μM MDA5, which is 10-fold higher than the value of Kd for 112 bp (Fig. 1B). We measured ATP hydrolysis progression between 4 and 10 min during which no ATP hydrolysis was observed for the WT alone reaction. In the presence of K335A or D443A, however, ATP hydrolysis continued during this time frame and the rate correlated with dsRNA length; the fold increase in the rate from 112 to 512 bp and 2,012 bp is 4.3 and 15.4 for K335A and 2.5 and 10.7 for D443A, in comparison to a 4.6- and 18-fold increase in length (Fig. 4D).

In principle, if disassembly occurs only from a filament end, the rate ratios would be equal to or greater than the length ratios. The closely matching of rate and length ratios suggests that end disassembly could be the primary mechanism with some level of interior dissociation, or that the filament disassembles cooperatively with nonlinearly increasing stability with filament length.

Catalytic Mutants Inhibit ATP-Dependent Dissociation of Wild-Type MDA5 from dsRNA. To test interdependence of dissociation among individual MDA5 molecules within a filament, we examined the effect of K335A and D443A on ATP-dependent dissociation of WT MDA5. We first mixed WT MDA5 with the mutants, and performed the single round kinetic assays using 512 bp dsRNA. Unlike the reaction of WT alone with heparin, which stops within 1 min, the reactions with K335A and D443A progressed over 4 min at a rate 70% of that of WT alone without heparin. This result suggests that 70% of WT MDA5 remained bound to dsRNA over 4 min in the presence of K335A or D443A. This interpretation is supported by a pull-down assay with biotinylated RNA, which showed that WT copurifies with dsRNA after 3 min incubation with ADPCP but not with ATP (Fig. S5E). Addition of K335A/D443A to the ATP reaction restores the level of WT copurified with dsRNA. Thus, the catalytic mutants stabilize the WT complex and dissociation of individual MDA5 molecules is interdependent.

ATP-Driven MDA5 Dissociation is Filament Length-Dependent. We next asked whether ATP-dependent MDA5 dissociation depends on the length of the filament. If every ATP hydrolysis event were equally capable of dissociating an individual MDA5 molecule from both filament interiors and ends, the dissociation rate would be independent of the initial filament length. This prediction would also be true if a filament disassembles cooperatively, either as an entire filament or in smaller fragments, when a fraction of MDA5 monomers hydrolyzing ATP at a time reaches a certain threshold that is independent of filament length (i.e., linearly increasing stability). Alternatively, if filament disassembly occurs from an end as individual monomers or dimers, or disassembles cooperatively with a higher threshold for longer filaments (i.e., nonlinearly increasing stability), MDA5 would dissociate more slowly from longer dsRNA. We compared the dissociation rate of WT MDA5 using the single-round kinetic assay with 512, 1,012, and 2,012 bp dsRNAs. The time derivative of the single-round kinetics (Fig. S5F) showed a more rapid reduction of the ATP hydrolysis rate for shorter dsRNAs with half-lives of 0.2, 0.6, and 1.3 min for 512, 1,012, and 2,012 bp dsRNAs, respectively (Fig. 4C). The more rapid decline in the ATP hydrolysis rate with shorter dsRNAs suggests a more rapid dissociation of MDA5 from shorter dsRNAs, which is inconsistent with models in which MDA5 dissociates from the filament interiors or cooperatively with its threshold independent of filament length.

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A Partial Loss-of-Function Mutant, I923V, Exhibits Impaired Filament Assembly and Reduced Kinetic Stability. A partial loss-of-function SNP variant I923V (Fig. 5A), which has been associated with a reduced risk for type 1 diabetes, was reported to have reduced signaling activity but retain similar affinity to a dsRNA mimic, poly I:C, as WT MDA5 (23, 24). We asked whether the reduced signaling by I923V is due to impaired filament assembly and stability. We found that I923V binds to 112 bp dsRNA with similar affinity but with reduced cooperativity (Kd of 18 nM vs. 22 nM, Hill coefficient of 1.9 vs. 4.0) (Fig. 5B).

Consistent with low cooperative binding, EM revealed that I923V is less efficient in filament formation with 112 bp dsRNA (Fig. 5C). The class averages revealed that the overall RNA binding modes of I923V and WT are similar, but the I923V complex contains 4–7 dimers rather than the 7–8 dimers seen in the WT complex. The histogram analysis of filament length distribution also indicates that 45% of the WT complexes are approximately 36-nm long, whereas only 15% of the I923V complexes are 36 nm and 60% are 27–31.5 nm (Fig. 5D). Consistent with this finding, the molecular mass of the I923V:112 bp dsRNA complex estimated from MALD is 209 kDa (~1 dimer) less than that of the WT complex (Fig. 5C).

We next examined the kinetic stability of I923V using the single-round ATP hydrolysis assay. Both WT and I923V were complexed with 2,012 bp dsRNA, and the progression of the ATP hydrolysis reaction in the presence of heparin was monitored (Fig. 5E). Although both reactions initiated with similar efficiencies, the I923V reaction reached a plateau faster than the WT reaction, resulting in a lower level of ATP hydrolyzed during the single-binding event. Rate analysis suggests that I923V dissociates from dsRNA approximately four times more rapidly than WT (Fig. 5F). This finding suggests that I923V has a reduced kinetic stability compared with WT, and supports our hypothesis...
that extended filament formation is important for maintaining kinetic stability of the MDA5:dsRNA complex.

Discussion

The molecular mechanism for recognition of long (>1–2 kb) viral dsRNA by MDA5 remains poorly understood, and our limited understanding has been largely inferred from knowledge of a paralogous receptor, RIG-I, despite its divergent RNA and viral specificity. We report here the previously uncharacterized architecture of a filamentous assembly of MDA5 on dsRNA, and its dynamic ATP-dependent disassembly to provide a mechanism of how MDA5 both recognizes dsRNA and auto-regulates its antiviral signaling activity in a length-dependent manner. We demonstrate here that binding affinity alone is insufficient to confer dsRNA specificity to MDA5 with high-affinity binding observed for multiple types of nucleic acids. Despite low stringency nucleic acid discrimination on the basis of affinity, MDA5 is able to distinguish between dsRNA and other types of nucleic acids through cooperative filamentous assembly along the length of dsRNA. Analogous filament structures have been observed with RecA which forms filamentous oligomers along DNA and promotes strand exchange during homologous recombination (25). MDA5 is structurally related to RecA in that its central helicase domain consists of two RecA-like subdomains. However, unlike the RecA filament whose formation requires ATP binding (26), MDA5 filament formation does not require ATP or its analogues (Figs. S24 and S5A). We speculate that the C-terminal domain (CTD) of MDA5, which is absent in RecA, plays an important role in the cooperative assembly process of the filament. A partial loss-of-function mutation, I923V, within the CTD lowers both the cooperativity and the extent of filament assembly without affecting the dsRNA affinity of MDA5 (Fig. 5). In addition, replacement of the MDA5 CTD by the RIG-I CTD abolishes the cooperativity while retaining high-affinity interaction with dsRNA (Fig. S6E). Filament formation was recently proposed for RIG-I based on atomic force microscopy (16); however, we did not observe either filament formation of RIG-I by EM or cooperative binding by EMSA (Fig. S2B).

We demonstrated that the MDA5:dsRNA filament is a highly dynamic structure, the stability of which is tightly regulated by ATP hydrolysis. Using quantitative single- and multiround kinetic analysis and catalytic mutant interference studies, we provide evidence supporting that ATP hydrolysis occurs throughout the filament with little coordination between neighboring MDA5 molecules (Fig. 3). ATP hydrolysis-driven MDA5 dissociation, however, occurs in a coordinated manner as evidenced by a markedly increased stability of a filament upon incorporation of catalytically inactive mutants (Fig. 4B). This coordinated dissociation occurs at a rate inversely proportional to the filament length (Fig. 4C), suggesting that not every ATP hydrolysis event leads to MDA5 dissociation. We propose two mechanisms to explain this length-dependent dissociation. First, MDA5 may dissociate from dsRNA as monomers or dimer pairs from filament extremities. This end dissociation could be because MDA5 molecules in the filament ends are less stable than those in the interiors due to the lack of stabilizing contacts with neighboring molecules. Based on this end-disassembly model, the positive effect of the catalytic mutants on the filament stability could be explained by the mutants capping the filament ends and preventing progression of filament disassembly. Second, MDA5 may cooperatively dissociate from dsRNA as an entire filament or smaller fragments whose stability increases nonlinearly with filament length. In this model, the catalytic mutants may increase filament stability by increasing the threshold required for filament disassembly by ATP hydrolysis.

The dual and seemingly opposing roles of ATP hydrolysis as both a consequence of filament formation (or dsRNA binding) and as a trigger for filament disassembly is reminiscent of the roles of nucleotide hydrolysis in other filamentous proteins, such as RecA, actin, and tubulin. These proteins utilize either repetitive or single-round of nucleotide hydrolysis as a counterbalance between filament growth and turnover (25, 27). Competition between the filament assembly and disassembly processes was also evident for MDA5 in EMSA during the ATP hydrolysis steady state, which revealed reduced apparent affinity in comparison without ATP (Fig. S5A). Although comprehensive understanding of the MDA5 filament dynamics would require more detailed knowledge on both assembly and disassembly processes, the observed length-dependent dissociation provides a basis for how MDA5 could self-regulate its interaction with dsRNA and thus antiviral signaling potential according to the underlying dsRNA length. This notion is in agreement with the known requirement of long (>1–2 kb) dsRNA to efficiently elicit interferon signaling activity of MDA5 in the cell (8). Thus, our observations suggest a previously unrecognized role of ATP hydrolysis in autoregulation of MDA5 filament growth and turnover.

The highly cooperative MDA5 filament formation raises intriguing possibilities for other antiviral functions of MDA5. As many receptors in the immune system cluster for effective signal...
transmission (17, 18), clustering of MDA5 on dsRNA may facilitate recruitment of MAVS or subsequent oligomerization of MAVS, which itself was recently shown to form prion-like fibrillar structures during antiviral signaling (10). It has been proposed that recruitment of MAVS by MDA5 depends on a conformational change of MDA5 induced by ATP hydrolysis (1, 10). It would be interesting to test whether ATP hydrolysis by individual MDA5 molecules within a filament recruits MAVS and promote their oligomerization through induced proximity. In addition, MDA5 filament formation may also perform a unique antiviral effector function by limiting access of viral proteins to RNAs, thereby interfering with viral replication or packaging. Interestingly, a recent study identified MDA5, along with RIG-I, as one of the most broadly acting antiviral effectors (28). Our present studies provide a basis for future investigations on more detailed mechanisms and functions of MDA5 in antiviral defense.

**Experimental Procedures**

**Electron Microscopy.** Methods for protein and RNA preparation are described in SI Text. MDA5 was incubated with a 20:1 molar ratio of MDA5 to 112 bp dsRNA (ssRNA or dsDNA) in 20 mM Hepes, pH 7.0, 150 mM NaCl, 5 mM ADP·AlF₄⁻, and cross-linked using 0.4 mM BMH at room temperature (RT) for 30 min. Negatively stained specimens were prepared as described in ref. 29. Images were collected with a Tecnai T12 electron microscope (FEI) and processed using the SPIDER software package (see SI Text).

**Multilangle Light Scattering.** The absolute molecular mass of the complexes formed by WT MDA5 or I923V with dsRNA was determined using a Superose 6 column (GE) attached to a miniDAWN TREOS detector (Wyatt Technology) in 20 mM Hepes, pH 7.0, 75 mM NaCl. MDA5:dsRNA complexes were prepared as described.

**Electrophoretic Mobility Shift Assay.** EMSA was performed using 5'-32P-, 3'-fluorescein- or Cy3-labeled RNAs. Unless mentioned otherwise, 0.2 nM of 32P-labeled RNA was mixed with the indicated amount of MDA5 in buffer A (20 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, and 2 mM DTT) with 2 mM ADPCP. The mixture was incubated at RT for 10 min and were analyzed on BisTris native gels (Life). For time-dependent EMSA, reactions were incubated at 37 °C for indicated time periods.

**ATP Hydrolysis Assay.** The ATP hydrolysis rate (initial velocity) was determined using Green Reagent (Enzo) (30). Unless mentioned otherwise, MDA5 was preincubated with RNA in buffer A at 37 °C, and the reaction was initiated by addition of 2 mM ATP. For single-round kinetic assays, a mixture of 200 μg/ml heparin and 2 mM ATP was added to the reaction. For initial velocity measurement, aliquots were withdrawn at five time points between 15 s and 30 min, and were quenched with 50 mM EDTA on ice. The Green Reagent was added to the quenched reaction at a ratio of 9:1, and OD₅₅₀ was measured using a Synergy2 plate reader (BioTek).

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