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Mechanistic insights into RNase L through use of an MDMX-derived multi-functional protein domain

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ABSTRACT

RNase L is part of the innate immune response to viral infection. It is activated by a small oligonucleotide (2–5A) whose synthesis is initiated as part of the interferon response. Binding of 2–5A to the N-terminal regulatory region, the ANK domain, of RNase L activates its ribonuclease activity and results in cleavage of RNA in the cell, which ultimately leads to apoptosis of the infected cell. The mechanism by which 2–5A activates the ribonuclease activity of RNase L is currently unclear but 2–5A has been shown to induce dimerization of RNase L. To investigate the importance of dimerization of RNase L, we developed a 15 kDa dimerization-inducing protein domain that was fused to the N-terminus of RNase L. From these studies we provide direct evidence that dimerization of RNase L occurs at physiologically relevant protein concentrations and correlates with activation of ribonuclease activity. We also show that the binding of 2–5A to RNase L promotes dimerization of the ANK domain and suggest how this could transmit a signal to the rest of the protein to activate ribonuclease activity. Finally, we show that the dimerization-inducing domain can be used as a general fusion partner to aid in protein expression and purification.

1. Introduction

A key step in the response to viral infection is the interferon-induced up-regulation of a wide variety of genes that are involved in the innate adaptive immune response [1]. One set of interferon-inducible genes encodes the 2′–5′oligoadenylate synthetase (OAS) proteins, which act as a sensor for double-stranded RNA in the cell [2]. Upon binding to double strand RNA, the OAS proteins synthesize short polyadenylate molecules that are connected by unique 2′–5′ linkages, collectively referred to as 2–5A. The ribonuclease RNase L is activated by the binding to 2–5A and it proceeds to cleave single-stranded RNA molecules, ultimately leading to apoptosis of the virally infected cell [3].

RNase L is a 741 amino acid protein that is comprised of two regions: the N-terminal activation domain that binds 2–5A (residues 1 to 333) and the C-terminal region that encompasses the ribonuclease activity (residues 334 to 741) [4]. The 2–5A binding domain, or ANK domain, is comprised of 8 ankyrin repeats followed by a 9th incomplete ankyrin repeat, and binds to 2–5A with a K_d in the picomolar to low nanomolar range [5,6]. The region between residues 334 and 741 is proposed to contain two sub-regions, the kinase-like domain and the ribonuclease domain [3]. The kinase-like domain derives its name from the presence of several sequence motifs that are common in kinases, although it does not exhibit any kinase activity and does not hydrolyze ATP [7]. There is evidence, however, that ATP may be required for ribonuclease activity of RNase L [8,9]. Mutations within the kinase-like domain have been shown to directly affect the ribonuclease activity suggesting the kinase-like domain and ribonuclease domain may form a continuous functional unit [7].

A range of biophysical studies have shown that 2–5A promotes dimerization of RNase L, although it is not clear if the dimerization results in activation of ribonuclease activity [10,11]. Analysis of 2–5A-induced ribonuclease activity at physiologically relevant sub-nanomolar protein concentrations showed a concentration-dependent lag phase that was proposed to reflect dimerization prior to activation of ribonuclease activity [5]. How the dimerization of RNase L is induced by 2–5A has also been the subject of debate. The complex between the ANK domain and 2–5A originally crystallized as a monomer, suggesting that the ANK domain was not responsible for dimerization [12]. A recent report, however, has shown that the ANK domain bound to 2–5A crystallized as a dimer, with the 2–5A from each monomer making additional contacts to the other monomer [13]. To investigate the role of dimerization in activation of RNase L, we placed a unique dimerization inducing domain, termed the "ZMP" domain, at the N-terminus of RNase L. On its own the ZMP domain is a monomer, but can be induced to form a dimer by the addition of a small organic molecule, RO-2443 [14]. We show that dimerization of RNase L induced by RO-2443 is not sufficient for activation of ribonuclease activity but requires a conformational rearrangement induced by

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2–5A that is distinct from the dimer induced by RO-2443. Our studies provide evidence that 2–5A induces dimerization via the ANK domain and that this dimerization correlates with activation of ribonuclease activity. We also show that the region encompassing the 9th incomplete ankyrin repeat is key in transmitting a signal from the ANK domain to the ribonuclease domain. Finally, we show that the ZMP/dimerizing domain can be used as a general fusion protein expression partner that can improve protein expression and be used for affinity purification of the attached protein.

2. Materials and methods

2.1. Vector construction (RNaseL, ZMP-RNase L, ZMP, and GST-PMI)

The target genes for all constructs were synthesized at Genscript (Piscataway, NJ). Specifically the following genes were synthesized: His(6)-RNase L, His(6)-TEV-RNase L(1–333), His(6)-TEV-RNase L(432–741), His(6)-RNase L(1–333, TEV, 334–741), His(6)-ZMP, His(6)-ZMP-RNase L, and GST-PMI. "His(6)" refers to a stretch of 6 histidines that were used in affinity purification. "TEV" refers to a TEV protease cleavage. The amino acid sequence of ZMP comprised residues 15 to 129 of zebrafish MDMX with two mutations, L46V and V95L. For the proteins produced in insect cells, BamHI and NotI restriction sites were engineered at either end of each gene to facilitate cloning into the pFastBac vector (Invitrogen). For GST-PMI, which was expressed in bacteria, NcoI and XhoI sites were introduced at the ends of the gene sequence to facilitate cloning into the pET15b expression vector (Novagen). The amino acid sequence of the linker sequence following the last amino acid of GST was GASTDAGS, and the PMI sequence following on from the linker was TSFAEYWNLLSP.

2.2. Insect and bacterial cell expression

The bacterial expression vectors were transformed into BL21(DE3) cells (Invitrogen) and grown in TB media at 37 °C until an OD (600 nm) of 0.6 was reached. The cells were cooled down to 18 °C, induced with 0.5 mM IPTG, and left to grow overnight at 18 °C. The cells were pelleted by centrifugation and stored at −80 °C until use. The vectors encoding proteins to be expressed in insect cells were transformed into MAX Efficiency® DH10Bac™ *E. coli* competent cells (Invitrogen) for transposition into the bacmid DNA. Blue/white selection was utilized to identify colonies containing the recombinant bacmid and the DNA was prepared as instructed in the competent cells user manual. The recombinant bacmid DNA was transfected into Sf-9 cells to produce recombinant baculovirus using Cellfectin (Invitrogen). The viruses were subjected to two rounds of amplification, and then titered. Sf-9 cells were grown in Sf-900 II SFM media (Invitrogen) with 2% FBS at a cell density of 2.5×10^6 and were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 2. The cells were cultured at 27 °C in a shaking vessel for 48 h before harvesting by centrifugation. The cell pellets were stored at −80 °C until use.

2.3. Protein purification

The His(6)-RNase L, His(6)-RNase L(1–333, TEV, 334–741), and His(6)-ZMP-RNase L constructs were purified in essentially the same manner. The cell pellets were resuspended in 50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 1 mM DTT, 5 mM $MgCl_2$, 100 μM ATP, 20 mM imidazole, and complete EDTA-free protease inhibitors (Roche) at 4 °C. The cells were lysed by passing through a microfluidizer twice and then centrifuged at 100,000 *g* for 1 h using a Beckman ultracentrifuge. After filtering the supernatant was passed over two 5 mL Ni-charged HisTrap HP columns connected in series. Buffer solutions were as follows: buffer A contained 50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and 100 μM ATP, and buffer B was identical to buffer A but contained 1 M imidazole. The column was washed with 2% buffer B before eluting the protein with a gradient from 2 to 40% buffer B over 8 column volumes. DTT was added to the combined fractions to give 1 mM and the protein passed over a Superdex 200 26/60 equilibrated with the same solution as buffer A described previously. The fractions were combined and dialyzed overnight into 50 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 100 μM ATP, and 1 mM DTT. The protein was further purified over a 5 mL Hitrap Q (GE Healthcare). Running buffers were as follows: buffer A contained 50 mM Tris pH 8.0, 10% glycerol, 5 mM $MgCl_2$, and 100 μM ATP, and buffer B contained the same as buffer A but with 1 M NaCl. The protein was loaded onto the Hitrap Q at 5% buffer B, the resin washed, and the protein eluted with a gradient of 5 to 55% buffer B over 5 column volumes. The protein was dialyzed overnight into 20 mM Hepes pH 7, 150 mM NaCl, 10% glycerol, 1 mM DTT, 5 mM $MgCl_2$, and 100 μM ATP, concentrated to around 2 mg/mL, and stored at −80 °C until use. RNase L(334–741) was generated from purified His(6)-RNase L(1–333, TEV, 334–741) by cleaving with excess TEV protease (Accelagen) overnight at 4 °C, and then passing over a Ni-charged HisTrap HP column. The HisTrap column removed both the His(6)-RNase L(1–333, TEV) and the hexahistidine-tagged TEV protease. The RNase L(334–741) was then concentrated to around 2 mg/mL and stored at −80 °C until use. To remove Mg^{2+} and ATP from stock solutions of RNase L and RNase L (334–741) the proteins were passed over a Superdex 200 26/60 column equilibrated with 20 mM Hepes pH 7, 150 mM NaCl, 10% glycerol. DTT was added to give 1 mM, and the proteins were concentrated to around 2 mg/mL and stored at −80 °C until use. The cell pellets containing His(6)-ZMP were resuspended in 50 mM Tris pH 8, 500 mM NaCl, 20 mM imidazole, 1 mM DTT, and complete-EDTA free protease inhibitors (Roche). The cells were lysed by two passes through a microfluidizer (Microfluidics Inc.) then centrifuged at 100,000 *g* in a Beckmann centrifuge for 1 h. The supernatant was filtered and loaded onto two 5 mL Ni-charged HisTrap HP columns (GE Healthcare). The buffers for the column were as follows: buffer A contained 50 mM Tris pH 8 and 500 mM NaCl, and buffer B contained 50 mM Tris pH 8, 500 mM NaCl, and 1 M imidazole. The resin was washed with 2% buffer B and the protein eluted with a gradient of 2 to 40% buffer B over 8 column volumes. The fractions were combined and DTT added to give 1 mM. The protein was then passed over a Superdex 75 26/60 column equilibrated in buffer A described for the HisTrap column. The fractions were combined, DTT added to give 1 mM, and the protein concentrated to 8 mg/mL, before storing at −80 °C until use. Purification of His(6)-RNase L(432–741) was performed in a similar manner, the only difference being the inclusion of 10% glycerol in the chromatography and dialysis buffers. The cell pellets for the GST-PMI fusion protein were resuspended in 20 mM Hepes pH 7, 150 mM NaCl, benzonase (Novagen), and complete protease inhibitors (Roche), and lysed by two passes through a microfluidizer (Microfluidics Inc.). After centrifugation at 14,000 rpm, the supernatant was filtered and loaded onto two 5 mL GSTrap columns (GE Healthcare), equilibrated in 20 mM Hepes pH 7 and 150 mM NaCl. After washing the resin the protein was eluted with equilibration buffer with 20 mM glutathione. After affinity purification the protein was passed over a Superdex 200 26/60 column equilibrated with 20 mM Hepes pH 7 and 150 mM NaCl, and then concentrated to between 5 and 10 mg/mL and stored at −80 °C until use.

2.4. Purification of the ZMP domain and ZMP-RNaseL by GST-PMI

The ZMP domain purifications were performed in 20 mM Hepes pH 7, 150 mM NaCl, and 1 mM DTT. The ZMP-RNaseL purifications were performed in the same buffer but included 10% glycerol, 5 mM $MgCl_2$ and 100 μM ATP. For the purification of the ZMP domain with GST-PMI, 20 mg of GST-PMI at 1 mg/mL were loaded onto a 5 mL GSTrap HP column (GE Healthcare) at 2 mL/min. 5 mg of the ZMP domain at 0.2 mg/mL were loaded onto the GST-PMI charged GSTrap

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