Step-by-Step Progress Toward Understanding the Hepatitis C Virus RNA Helicase

Dumont S, Cheng W, Serebrov V, Beran RK, Tinoco IJ, Pyle AM, Bustamante C. RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. Nature 2006;439:105-108. (Reprinted by permission from Macmillan Publishers Ltd: www.nature.com)

Abstract

Helicases are a ubiquitous class of enzymes involved in nearly all aspects of DNA and RNA metabolism. Despite recent progress in understanding their mechanism of action, limited resolution has left inaccessible the detailed mechanisms by which these enzymes couple the rearrangement of nucleic acid structures to the binding and hydrolysis of ATP. Observing individual mechanistic cycles of these motor proteins is central to understanding their cellular functions. Here we follow in real time, at a resolution of two base pairs and 20 ms, the RNA translocation and unwinding cycles of a hepatitis C virus helicase (NS3) monomer. NS3 is a representative superfamily-2 helicase essential for viral replication, and therefore a potentially important drug target. We show that the cyclic movement of NS3 is coordinated by ATP in discrete steps of 11 +/- 3 base pairs, and that actual unwinding occurs in rapid smaller substeps of 3.6 +/- 1.3 base pairs, also triggered by ATP binding, indicating that NS3 might move like an inchworm. This ATP-coupling mechanism is likely to be applicable to other non-hexameric helicases involved in many essential cellular functions. The assay developed here should be useful in investigating a broad range of nucleic acid translocation motors.

Comments

Most antiviral drugs inhibit viral enzymes and numerous groups have been racing to develop enzyme inhibitors as hepatitis C virus (HCV) therapeutics. The HCV genome encodes four enzymes, and several HCV enzyme inhibitors are finally in clinical trials, the most promising of which inhibit a protease that is part of an HCV protein called nonstructural protein 3 (NS3). HCV enzyme inhibitors have been so difficult to develop because HCV enzymes must be synthesized using recombinant DNA technology, and three of the four HCV enzymes behave poorly as purified recombinant proteins. The one HCV enzyme that can be synthesized in a relatively active state was the subject of a recent study published in *Nature,* which used one of the most advanced biophysical techniques available to today's enzymologist.[1] The study was led by Carlos Bustamante's laboratory at the University of California, Berkeley, in collaboration with Berkeley's Ignacio Tinoco and Anna Marie Pyle's lab at Yale. The team used "optical tweezers" to examine the movements of single molecules on RNA. Their data verify the results of numerous prior studies that employed more traditional enzymology and open new possibilities for understanding precisely how HCV replicates its genome in human cells.

Dumont et al.[1] studied NS3, but they did not analyze its protease function. Rather, they examined the ability of NS3 to separate an RNA duplex. This reaction occurs after the single-stranded HCV RNA genome enters a cell and the HCV RNA-dependent RNA polymerase (the NS5B protein) synthesizes an RNA complement. NS3 helicase separates the two complementary HCV RNA strands so that new HCV genomes can be synthesized and so that the virus can evade double stranded RNA-triggered antiviral responses. ATP provides the fuel for NS3 to separate duplexes. As evidence for the critical need for this "helicase" activity, a functional NS3 helicase is needed both for replication of subgenomic HCV replicons[2] and for infection of chimpanzees.[3]

Over 10 years ago, the C-terminal two-thirds of HCV NS3 was shown to be a nucleic acid-stimulated ATPase[4] capable of unwinding RNA and DNA.[5,6] Shortly thereafter, three groups crystallized this portion of NS3 and determined its structure at atomic resolution.[7-9] Later structural studies examined HCV helicase using NMR,[10] examined the full-length NS3 protein with both its helicase and protease intact,[11] and analyzed multiple helicase protomers bound to a single DNA strand.[12] There are few other helicases, and none that unwind RNA, with such detailed atomic structures available. As a consequence, HCV helicase has become a hot topic not only for researchers with a desire to develop antiviral drugs, but also for those interested in understanding helicases in general.

The above structural studies reveal that three domains comprise the NS3 helicase. The first two (N-terminal) domains are similar to a domain first seen in the *E. coli* RecA protein. All helicases possess RecA-like domains, which form a motor that transports the protein along a nucleic acid tract. ATP normally binds helicases at the interface between two RecA like domains (Fig. 1A). The third domain is unique and differs even in structures of closely related helicases. One strand of DNA (and pre-

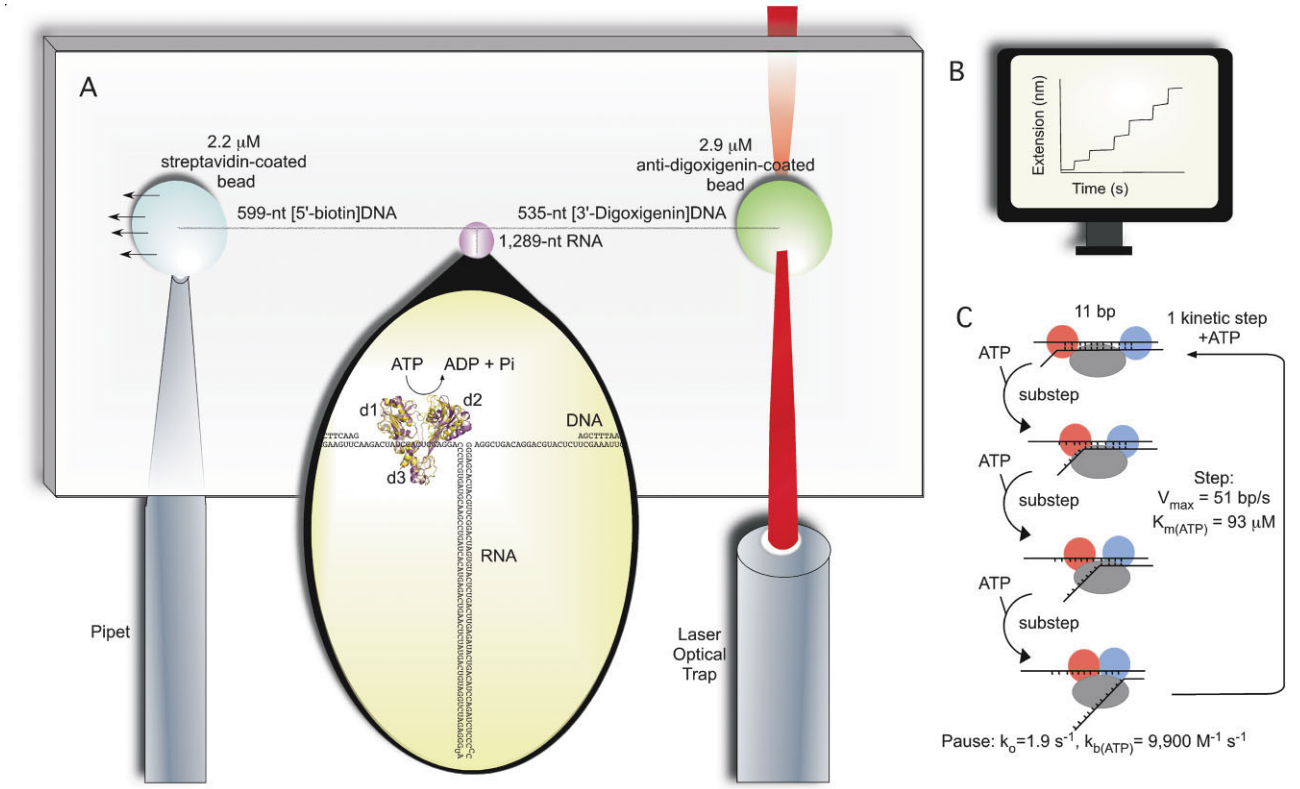

Fig. 1. Movement of HCV helicase as monitored with optical tweezers. (A) Apparatus designed by Dumont et al.[1] A RNA substrate that forms a hairpin is tethered to beads held in optical tweezers. Two HCV helicase structures (PDB files 8OHM and 1HEI) are superimposed on the loading region of the substrate. ATP likely binds between domain 1 (d1) and domain 2 (d2) and RNA binds the cleft separating domain 3 (d3) from domains 1 & 2. Note the rotation of domain 2 relative to the other domains. (B) Ideal schematic of data reported in the study by Dumont et al. showing that helicase allows the substrate to be extended in a series of 11 base pair steps. (3) Kinetic model for HCV helicase action presented by Dumont et al.[1]

sumably RNA) binds HCV helicase in a cleft that separates domain 3 from the first two domains.[9,12]

The numerous HCV helicase structures only reveal the conformation of the protein in the presence and absence of ssDNA. To understand how the protein moves, we need to also view structures of nucleic acid-bound HCV helicase in the presence of ATP and the product ADP. Presently, such structures are only available for one other helicase that resembles HCV (called PcrA). PcrA structures reveal that ATP binding and hydrolysis causes rotation of the RecA-like domains so that the protein can crawl along DNA like an inchworm and simultaneously twist the double helix to actively disrupt base pairs.[13,14] Alignments of different HCV helicase structures reveal that a similar domain rotation occurs in NS3 (Fig. 1A).[7,12] Kim et al.[9] proposed that this rotation of domain 2 relative to domains 1 & 3 could lead to translocation of the protein by one or two bases as the protein ratchets like an inchworm along the strand bound below domains 1 & 2 (Fig. 1A).

Testing ideas explaining how HCV helicase travels along RNA is difficult because directly visualizing HCV helicase movements is not yet possible. Dumont et al.[1] have taken one step closer to this elusive measurement by applying techniques first used to study classical molecular motors like myosin and kinesin. Dumont et al.[1] measured helicase movements indirectly by measuring the amount of RNA that is unwound as the protein steps through a double helix. In their protocol (Fig. 1A), an RNA molecule over 1,200 nucleotides is hybridized to two DNA strands, each of which is attached to a bead that is held with optical tweezers. The RNA portion of this substrate forms a hairpin that serves as the helicase substrate. The optical tweezers then gently pull the beads apart, at a force that will not separate the base pairs holding the hairpin together or the hydrogen bonds holding the RNA to the DNA. When the helicase loads onto a single-stranded region directly upstream from the hairpin and disrupts one of the base pairs in the hairpin, the same force is then able to extend the DNA/RNA complex slightly further. The distance extended is measured with the tweezers and plotted versus time.

The resulting data reveal that HCV helicase unwinds the 60 base pair hairpin in 5-6 steps each averaging about

11 base pairs (Fig. 1B). Strand separation during each step is rapid, averaging 51 base pairs per second. Between each step, the protein usually pauses for less than a second. When ATP is not available to immediately bind after hydrolysis, each step is sometimes broken down into about three sub-steps. When one considers both the steps and the pauses between them, the helicase takes about 8 seconds to unwind 60 base pairs, meaning that a single HCV helicase can traverse the entire (≈9,600 nucleotide) HCV genome in about 21 minutes.

The data obtained using optical tweezers provide the clearest support for the "n-step" kinetic model that has been used for many years to fit data obtained using assays that only detect whether HCV helicase completely unwinds a substrate.[15] The n-step model states that a helicase unwinds a substrate in a series of steps and it defines the number of bases unwound between two rate-limiting steps as the "step size." Step size can be diagnostic of how a helicase unwinds DNA (or RNA). The ratcheting inchworm model predicts a small step size (1-3 base pairs) because the inchworm model contends that HCV helicase sequentially contacts neighboring nucleotides.[9] Although initial measurements of the number of base pairs unwound in a single step by HCV helicase supported such a small step size,[16] the present work confirms later measurements that noted larger step sizes.[17, 18] Dumont et al.[1] focus discussion mainly on the difference between their 11 base pair step size and that measured by Serebrov and Pyle[17] as 18 base pairs. Dumont et al.[1] claim that the difference between their study and that of Serebrov and Pyle[18] is that HCV helicase acts as a monomer when studied with the tweezers while in bulk solution the active form is a dimer. The only evidence for the fact that a monomer acts in the present study is that no trace differences were seen when NS3 concentrations were lowered to 1 nM. In contrast, Serebrov and Pyle[18] found that bulk solution unwinding rates slowed significantly in this concentration range.[18]

Since the results obtained with optical tweezers are clearly not in accord with earlier molecular models for HCV helicase action, Dumont et al.[1] present a new model in which the protein stretches over 11 base pairs and separates chunks of RNA 3-4 base pairs at a time (Fig. 1C). How the new model relates to current structures is not clear considering that only five nucleotides are seen bound in the known RNA binding cleft, and considering that domain 2 cannot rotate over distances spanning 11 base pairs. One can only assume that the two domains shown in their model (Fig. 1C) are not intended to relate to the RecA-like domains (d1 & d2, Fig. 1A), but rather to two hypothetical RNA binding sites, one acting as what the authors call a "helix opener" and one that acts as a "translocator." One way to relate these hypothetical binding sites to the current atomic structures would be to use optical tweezers to examine some of the over 50 site-directed HCV helicase mutants that have been described in the literature.

It is tempting to speculate on how examining the motions of the HCV helicase at an atomic level will impact future HCV drug development. A few potent HCV helicase inhibitors have been recently reported,[19,20] and the assay developed by Dumont et al.[1] could explain precisely how these inhibitors function and could guide the design of better compounds. NS3 protease substrates or inhibitors might also impact unwinding rates. In addition, other HCV enzymes could also be examined, and in theory, the action of the entire HCV replication complex could be analyzed using single-molecule techniques. The assay developed by Dumont et al.[1] clearly opens exciting new possibilities in HCV research, the potential of which will be fully realized once more laboratories gain access to specialized equipment needed for single-molecule studies.

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Hepatitis C Vaccines: Inducing and Challenging Memory T Cells

Folgori A, Capone S, Ruggeri L, Meola A, Sporeno E, Bruni Ercole B, Pezzanera M, et al. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. Nat Med 2006;12:190-197. (Reprinted by permission from Macmillan Publishers Ltd: www.nature.com.)

Abstract

Three percent of the world's population is chronically infected with the hepatitis C virus (HCV) and at risk of developing liver cancer. Effective cellular immune responses are deemed essential for spontaneous resolution of acute hepatitis C and long-term protection. Here we describe a new T-cell HCV genetic vaccine capable of protecting chimpanzees from acute hepatitis induced by challenge with heterologous virus. Suppression of acute viremia in vaccinated chimpanzees occurred as a result of massive expansion of peripheral and intrahepatic HCV-specific CD8+ T lymphocytes that cross-reacted with vaccine and virus epitopes. These findings show that it is possible to elicit effective immunity against heterologous HCV strains by stimulating only the cellular arm of the immune system, and suggest a path for new immunotherapy against highly variable human pathogens like HCV, HIV or malaria, which can evade humoral responses.

Comments

Hepatitis C virus (HCV) infection is a disease of high prevalence around the world, and continues to spread rapidly in countries with untested blood supplies. Why is no effective vaccine available so far?

A key feature of most vaccines is the induction of neutralizing antibodies. In many cases, infusion of neutralizing antibodies is also used as passive post-exposure prophylaxis. Although antibody-based neutralization has already been described years ago in the chimpanzee model of HCV infection,[1] efficient screening for neutralizing antibodies, that inhibit HCV entry into hepatocytes, has only recently become feasible when appropriate *in vitro* infection systems were developed.[2]

In the natural course of HCV infection, however, the humoral immune response seems to fail because high titers of *in vitro*-neutralizing antibodies coexist with high HCV titers in persistently infected patients.[3] Thus, these antibodies, albeit neutralizing *in vitro*, appear to be insufficient to completely block HCV entry into cells *in vivo* and to clear the infection. Moreover, HCV antibody titers have been shown to decline and are often undetectable in long-term recovered persons,[4] raising the question of the longevity of an antibody-based vaccine response against HCV.

Although it remains possible that a panel of antibodies can be engineered *in vitro* and used as post-exposure prophylaxis,[5] recent studies have emphasized the role of the cellular immune response in protection against HCV. In particular, a series of rechallenge studies of spontaneously HCV-recovered chimpanzees demonstrated a crucial role of CD4 and CD8 T cells in protective immunity.[6-10] Thus, most preclinical vaccination trials are now focused on the induction of robust T cell responses, which via induction of CD4 T cell help for B cells, may ultimately also aid the humoral immune response.

In a recent study,[11] Folgori et al. explored a T cell-based vaccine in the chimpanzee model of HCV infection. Five chimpanzees were vaccinated at weeks 0 and 4 with a replication-deficient serotype 6 adenoviral vector encoding the NS3-NS5B region of the HCV BK strain (genotype 1b), followed by vaccination at week 25 with a replication-deficient serotype 24 adenoviral vector encoding the same HCV antigens. At weeks 35, 37 and 39, the immune response was boosted by intramuscular injection of a recombinant DNA plasmid. Five control chimpan-