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Transcription Termination Factor Rho Can Displace Streptavidin from Biotinylated RNA*

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In Escherichia coli, binding of the hexameric Rho protein to naked C-rich Rut (Rho utilization) regions of nascent RNA transcripts initiates Rho-dependent termination of transcription. Although the ring-shaped Rho factor exhibits in vitro RNA-dependent ATPase and directional RNA-DNA helicase activities, the actual molecular mechanisms used by Rho to disrupt the intricate network of interactions that cement the ternary transcription complex remain elusive. Here, we show that Rho is a molecular motor that can apply significant disruptive forces on heterologous nucleoprotein assemblies such as streptavidin bound to biotinylated RNA molecules. ATP-dependent disruption of the biotin-streptavidin interaction demonstrates that Rho is not mechanistically limited to the melting of nucleic acid base pairs within molecular complexes and confirms that specific interactions with the roadblock target are not required for Rho to operate properly. We also show that Rho-induced streptavidin displacement depends significantly on the identity of the biotinylated transcript as well as on the position, nature, and length of the biotin link to the RNA chain. Altogether, our data are consistent with a “snow plough” type of mechanism of action whereby an early rearrangement of the Rho-substrate complex (activation) is rate-limiting, physical force (pulling) is exerted on the RNA chain by residues of the central Rho channel, and removal of structural obstacles from the RNA track stems from their nonspecific steric exclusion from the hexamer central hole. In this context, a simple model for the regulation of Rho-dependent termination based on the modulation of disruptive dynamic loading by secondary factors is proposed.

In Escherichia coli, a large fraction of transcription termination events require the participation of the endogenous Rho protein (1, 2). The Rho factor is a ring-shaped homohexamer (3–5) that possesses in vitro RNA-dependent ATPase (6) and ATP-dependent RNA and RNA-DNA helicase activities (7, 8). To induce transcription termination, Rho hexamers usually bind to characteristic C-rich/G-poor segments of the nascent transcript called Rut² (for Rho Utilization) sites from which they move directionally along the RNA chain to catch up with and ultimately dissociate the transcription elongation complex (TEC) located at the transcript 3’-end (9–18). At present, the exact mechanism by which Rho dissociates the TEC remains unknown. It has been believed for some time that the Rho helicase could directly unwind the RNA-DNA hybrid that the 3’-end of the nascent transcript forms with the DNA template within the TEC. However, recent structural data indicate that this short ∼9-base pair-long RNA-DNA hybrid is too deeply buried within the TEC interior for direct helicase action (19, 20). This observation has prompted alternative models whereby the Rho enzyme, once positioned on the polymerase surface, applies NTPase-driven physical force to pull the RNA from the TEC and/or drive forward the polymerase into an hypertranslocated and unstable state (21–23). Importantly, these models imply that the RNA and RNA-DNA unwinding activities of Rho are essentially nonspecific in vitro byproducts of the enzyme progression along the RNA track. This proposal is reminiscent of the “snow plough”-type of mechanisms proposed for various helicases and is consistent with the emerging view of helicases being able to perform a larger set of remodeling tasks on nucleic acid (NA) and nucleoproteic assemblies than simply separating NA strands (24–26).

Rho function can be tightly regulated by transcription factors, such as NusA and NusG (27, 28), that alter the conformation of the TEC (29, 30). Although these alterations are likely to modulate the strength and/or elongation rate of the TEC, Rho may also respond differentially to specific, factor-induced, structural features of the TEC isoforms. Similarly, defects in Rho-dependent termination induced by single-point mutations of the α-C-terminal domain of the RNA polymerase (RNAP) may stem from altered Rho-RNAP contacts (31). However, the presence of specific Rho-RNAP interactions would support a mechanism slightly more elaborate than simple snow plough displacement of structural obstacles from the NA track.

Although the TEC is a macromolecular assembly that is much more complex than regular helicase substrates (i.e. bare NA helices), it derives a large part of its thermodynamic stability from NA base pairings (see Ref. 32 and references therein). This raises the possibility that Rho mecanochemical work may

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2 The abbreviations used are: Rut, Rho utilization; TEC, transcription elongation complex; NA, nucleic acid; nt, nucleotide; BtN, biotinylated nucleotide residue; RNAP, RNA polymerase; MOPS, 4-morpholinepropanesulfonic acid.
Motor Mechanisms of Transcription Factor Rho

Experimental Procedures

Materials—Chemicals and enzymes were obtained from Sigma-Aldrich and New England Biolabs, respectively. Oligonucleotides were purchased from Biomers.net and Dharmacon and were systematically purified by polyacrylamide gel electrophoresis (PAGE). Core streptavidin (13.3 kDa/monomer) was obtained from Promega. The Rho protein (concentrations expressed in units of hexamers throughout this report) was prepared and characterized as described previously (37, 41).

The DNA templates encoding the R107 and R132 transcripts were obtained by PCR amplification of specific regions of the pAS02 plasmid that contains the R107 sequence downstream from a T7 promoter (36). These templates, or the pAS02 plasmid linearized with SmaI (for the preparation of the R132 transscripts), were transcribed in vitro with T7 RNA polymerase as described previously (42) using larger reaction volumes (500 μl) and longer incubation times (2–3 h). The resulting transcripts were purified by 5–7% denaturing PAGE and stored at ~20 °C in MOPS buffer (10 mM MOPS, pH 6.5, 1 mM EDTA).

Preparation of the Biotinylated Transcripts—We used two distinct synthesis routes to attach biotin-derivatized linkers to various positions of aRut (artificial Rut; see Refs. 9, 36, 37) containing RNA molecules. On the one hand, the transcripts were biotinylated specifically at their 3′-ends by controlled oxidation with sodium periodate followed by coupling with EZ-link biotin hydrazides (Pierce), as described (43). On the other hand, synthetic RNA oligonucleotides containing primary amine linkers at specific positions were reacted with EZ-link biotin succinimidyl esters (Pierce). Biotinylated R132 molecules were then obtained by ligating these modified oligonucleotides to the 3′-ends of R107 transcripts using T4 RNA ligase and DNA splints, as described (44). Because neither synthesis route ensured 100% biotinylation of the RNA molecules, the correct products were selected through their ability to bind streptavidin: 10 pmol ^32P-labeled molecules were incubated with streptavidin (1 μmol) in hybridization buffer (150 mM potassium acetate (KAc), 20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin) for 15 min at 30 °C. Streptavidin-RNA complexes were then purified by 7% native PAGE (gels contained 0.3% SDS to maximize the fractions of specific complexes), eluted from the gel bands in streptavidin buffer (0.3 M KAc, 100 mM streptavidin) for 1.5 h at 30 °C, and precipitated with 3 volumes of ethanol before being stored at ~20 °C in helicase buffer (150 mM KAc, 20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol). The amounts of byproducts (such as complexes containing more than one RNA strand/streptavidin tetramer or biotinylated RNA strands bound to streptavidin dimers) were substantially reduced (~10%) with this procedure. However, it is possible that a small fraction of the biotinylated RNA-streptavidin complexes evolve into an unstable population (see “Results”) during the elution step. The RNA concentrations were determined by UV measurements with a μL-spectrophotometer (Nanodrop) and/or from specific activities of the samples. The DINA melt server (45) was used to predict the secondary structures of the RNA constructs.

Streptavidin Displacement Reactions—Mixed streptavidin-RNA complexes (~1 nm, final concentration) were purified with Rho hexamers (20 nm) in helicase buffer and incubated for 3 min at 30 °C. The displacement reaction was initiated with a mixture of ATP, MgCl2 (1 mM, final concentrations), and free biotin (10 μM, final concentration). Reaction aliquots were taken at various times, mixed with 3 volumes of quench buffer (150 mM KAc, 20 mM HEPES, pH 7.5, 26 mM EDTA, 0.4% SDS, 5% Ficoll-400), and immediately loaded on a 6% polyacrylamide gel containing 0.3% SDS to selectively denature Rho-RNA complexes. All displacement reactions were performed at least in triplicate but could not be repeated under single-turnover conditions because poly[rC], which is used as the Rho trap (35–37), had a dose-dependent inhibitory effect on Rho-induced dissociation of the RNA-streptavidin complexes (data not shown; peculiar poly[rC] effects have also been observed in other circumstances; see Refs. 37, 46). Detection and quantification of gel bands were performed with a Storm-860 imager and related software (GE Healthcare). The fractions of individual products present in the reaction at a given time were determined with the following formula, \( F_t = (I - F_0 \times \Sigma I_0) / (1 - F_0) \times \Sigma I_0 \) (with \( F_0 = I_0 / \Sigma I_0 \)), where \( I_0 \) and \( I \) are the intensities of the product.
The biotin-streptavidin complex with the formation of a productive Rho-RNA complex (36), a large distance (>60 nt) was set initially between the aRut site and a 3'-terminal H3N residue (R132-3Bt construct; Fig. 1A). In a first series of experiments, 32P-labeled R132-3Bt constructs were mixed with core streptavidin (this mature form lacks the C-terminal polypeptide domain that can compete for the biotin binding site) (47), and the resulting complexes were purified by PAGE (see “Experimental Procedures”). The RNA-streptavidin complexes were then incubated with Rho for 3 min at 30 °C before addition of an initiation mixture containing ATP, MgCl2, and an excess of biotin (to trap free streptavidin molecules). Reaction aliquots were removed at various times and immediately loaded on a native (with respect to NA species) gel containing 0.3% SDS (in these electrophoretic conditions, Rho-RNA complexes are selectively denatured; data not shown). The spontaneous release of core streptavidin from the biotinylated transcripts was also monitored in control experiments containing ADP instead of ATP. As shown in Fig. 1B (ADP lanes and graph), this reaction is very slow with the R132-3Bt-streptavidin complexes (koff ~ 0.6 × 10−3/min at 30 °C), which is in good agreement with published work on the biotin-streptavidin interaction (koff ~ 3 × 10−3/min at 37 °C) (33, 34). However, dissociation of the R132-3Bt-streptavidin complexes was dramatically accelerated under conditions promoting Rho helicase activity (Fig. 1B, ATP lanes). This effect was strictly dependent on the presence of Rho, MgCl2, and excess biotin in the reaction mixture and was inhibited by pairing of a complementary oligonucleotide to the aRut sequence (data not shown). Altogether, these data are consistent with the active displacement of streptavidin from the R132-3Bt transcript promoted by the Rho enzyme.

The kinetic profile of the streptavidin displacement reaction is complex, probably reflecting distinct reaction phases and pathways (Fig. 1B, graph). On the one hand, a small fraction (Afast < 5%) of the RNA-streptavidin complexes were quickly dissociated (kfast ~ 10 min−1) in an ATP-independent fashion. This minor reaction was unaffected by the presence of nonspecific competitors (bovine serum albumin or tRNA) in the mix-
The Efficiency of Streptavidin Displacement Depends on the Position of Biotin Attachment to the RNA Substrate—In this case, however, Rho could not actively displace streptavidin from the biotinylated RNA substrate (Fig. 2). Thus, the displacement efficiency of the Rho enzyme depends on the position of biotin attachment to the RNA chain (note, however, that Rho can displace a DNA oligonucleotide hybridized to the 110–132 region of the R132-U8Bt substrate with normal helicase efficiency; data not shown).

The formation of a productive Rho-RNA complex, the likely crucial rate-limiting step during in vitro RNA-DNA helicase reactions, is sensitive to the local steric environment (36). We therefore wondered whether structural hindrance due to the binding of a bulky streptavidin tetramer closer to the Rho loading site could account for the loss of streptavidin displacement activity with the R132-U8Bt substrate. To test this hypothesis, we introduced a biotin moiety at the 3’-end of the R107 transcript (R107-3oxBt substrate; see “Experimental Procedures”), thereby generating an 8-nt shorter distance between the 34N residue and the aRut site than in R132-U8Bt. Interestingly, Rho actively displaced streptavidin from R107-3oxBt (Fig. 3A), which rules out a simple distance effect to explain the absence of Rho activity with R132-U8Bt (Fig. 2). Because the displacement reaction was moderately efficient with the R107-3oxBt substrate, we also tested longer RNA substrates bearing the same biotin linker at their 3’-end (R132-3oxBt and R157-3oxBt substrates). Although Rho-induced displacement of streptavidin was improved significantly with R132-3oxBt (with an efficiency per enzyme run now comparable with NA unwinding reactions), it was not better with the longer R157-3oxBt substrate (Fig. 3B). From these data, it appears that the position of the 34N residue within the RNA chain rather than its mere distance to the aRut site (a minimal spacer of single-stranded RNA is likely required, however; see Ref. 36) affects the streptavidin displacement reaction triggered by Rho. Variations in the reaction efficiency upon small changes of the RNA sequence that might affect the secondary structure in the vicinity of the 34N residue (data not shown) suggest that the RNA fold may be an important factor. However, factors such as the length of the RNA chain or how biotin is attached to the RNA (i.e. internally or at the 3’-end) may also contribute significantly to the differences in reactivity.
Moreover, we note that the amplitude of the ATP-independent side reaction \((A_{\text{fast}})\) and the rate of spontaneous dissociation \((k_{\text{off}})\), two Rho-independent features, somewhat vary with the biotinylated substrate (Figs. 1–3). This suggests that subtle variations in the structure and/or strength of the biotin-streptavidin complex may also contribute to the observed differences in Rho efficiency.

**Spatial Limitations on Rho Dissociation Activity**—We have shown that Rho can actively dissociate streptavidin molecules bound to the 3'-end of biotinylated RNA substrates (Figs. 1B and 3). This indicates that, as for NA unwinding (36), all of the Rho contacts to the RNA chain that are necessary for productive interaction and dissociation activity are made upstream from the location of the roadblock target. This property in turn offers a good opportunity to estimate the spatial boundary for Rho action. In effect, by altering the length of the biotin linkage, one can easily change the spacing between the location of productive Rho-RNA interactions \((i.e.\) where pulling on the RNA chain occurs) and the point where Rho eventually applies force on streptavidin. We thus prepared the R132-3'-ox:LCBt substrate, which is identical to R132-3'-oxBt excepted for the biotin spacer arm, which is \(\sim 10\ \text{Å}\) longer (Fig. 4A, inset). This first modification had little effect on the ATP-dependent streptavidin displacement reaction (compare Fig. 3B, left, with Fig. 4A). When the spacer length was increased by another \(\sim 10\ \text{Å}\) (R132-3'-ox:PEG4Bt substrate), however, Rho could no longer displace streptavidin (Fig. 4B). Thus, the spatial register for productive Rho action probably does not exceed \(\sim 20\ \text{Å}\) from the point of downstream Rho interaction with cognate RNA residues. This is significantly less than the minimal distance (\(\sim 50\ \text{Å}\)) between the Rho primary interaction sites and the solvent-exposed surface on the opposite (downstream) side of the hexamer ring (Fig. 4C) (5). If one assumes that the RNA substrate traverses the central Rho channel (48) and that streptavidin dissociation occurs on the downstream side of the hexamer (streptavidin being too bulky to enter the central Rho channel), then the data imply that productive Rho-RNA contacts are formed inside the central channel of the enzyme (Fig. 4C). This agrees well with current biochemical and structural information and models whereby mechanoochemical work (pulling) of Rho on the RNA strand is mediated by mobile side chains within the central channel of the enzyme (5, 21, 38, 39, 49).

**DISCUSSION**

Previous studies have shown that transcription termination factor Rho from *E. coli* can remove NA strands annealed to a transcript substrate using ATP hydrolysis as an energy source (helicase activity) (7, 8, 35–37, 50–52). We now show that mechanochemical work of Rho is not limited to the melting of NA pairings, as the enzyme can also actively dissociate streptavidin from a biotinylated RNA substrate in an ATPase-dependent fashion. This process is mediated by productive Rho-RNA contacts that form within the central channel of the Rho hexamer less than 20 Å from the point where disruptive force is applied on the streptavidin-biotin complex (Fig. 4C). Furthermore, streptavidin dissociation occurs with an efficiency (up to \(\sim 50\%/\text{enzyme run}\)) and at a rate \((k_{\exp} \sim 0.1\ \text{min}^{-1} \text{ at } 30^\circ\text{C})\) that are comparable with the ones of Rho-mediated unwinding of aRut-containing RNA-DNA constructs (36, 37) or dissocia-
tion of TECs stalled on various DNA templates (22, 53, 54). Overall, this model “RNPase” activity is a direct demonstration of the general capacity of the Rho enzyme to dissociate components that are not covalently linked to the 3’-end of the RNA (1). Similar displacement activities have been previously evidenced for DNA and RNA helicases having various biological roles and functional oligomeric states (25, 26). Thus, Rho is not unique in its ability to displace roadblocks of various kinds from an NA track nor is this ability specific to the quaternary structure, substrate preference (i.e. RNA versus DNA), or biological function of the enzyme. On the other hand, the fact that Rho can break various kinds of interactions (NA pairings, biotin-streptavidin) with similar efficiencies supports a snow plough-type of mechanism of action in which no specific interactions with the obstacle to be displaced are required. In this class of mechanisms, the enzyme interacts specifically with and tracks directionally on a single NA strand and in the process can disrupt nonspecifically intervening obstacles such as hybridized NA strands or bound proteins (55). Substrate requirements are therefore intrinsically limited to the tracking strand and usually include the presence of a small portion of single-stranded NA that serves as the initial helicase binding site as do Rut loading sites for Rho hexamers (48).

One way to regulate a snow plough molecular machine is through interference with (or facilitation of) the formation of a competent enzyme-NA complex. In the case of Rho, such a scenario has been recently highlighted in vivo with substrate variants of the θ terminator (56). Regulation of Rho function at the level of the formation of a productive enzyme-NA complex may be also facilitated by kinetic control at this early stage (36). Interestingly, similar kinetic profiles for the ATP-dependent streptavidin displacement and NA unwinding reactions (see “Results” and Refs. 36, 37) suggest that, at least in our standard in vitro conditions, the kinetic regimen (with a rate-limiting activation step) does not change with the nature of the object to be displaced. It follows that a maximal $k_{\text{exp}}/k_{\text{off}}$ ratio of ~150 (Fig. 1B) is probably much less than the actual rate enhancement of streptavidin displacement by the Rho enzyme (if activation of the Rho-substrate complex is rate-limiting, then $k_{\text{exp}} = k_{\text{activation}} \ll k_{\text{strept dissociation}[ATP]}$). This uncertainty, in turn, precludes a direct estimation of the disruptive force exerted by Rho on the biotin-streptavidin interaction. Early atomic force microscopy measurements yielded a bond rupture (dissociation) force of ~200 pN for the biotin-streptavidin pair (57, 58). The potential ability of Rho to develop high dissociation forces appears consistent with its biological role if one considers that forces up to 30 pN were not sufficient to mechanically pull RNA from the TEC during single-molecule experiments with laser optical tweezers (59). However, one should also note that, under non-equilibrium conditions, dissociation forces (and interaction lifetimes) can greatly vary with the force loading rate (60, 61). This parameter may therefore contribute to regulate Rho-dependent termination late on the termination pathway. Indeed, the inherently distinct energy landscapes of the Rho and TEC dissociation pathways should generate different dissociation force versus loading rate relationships (Fig. 5) (60, 61). It follows that, depending on dynamic loading, one nucleoproteic complex may be easier to brake than the other, which could in turn modulate termination efficiency (Fig. 5). Although probably too simplistic, this scenario may nonetheless contribute to explain NusG effects at certain Rho-dependent terminators (27, 28). Indeed, by physically connect-
ing Rho and the RNAP (62, 63), a “spring-like” NusG (64) may cushion their intermolecular encounter (thus, reducing dynamic loading), increase the timeframe of Rho action, and thus favor RNA dissociation from the TEC at smaller dissociation forces (Fig. 5). Conversely, NusA-induced slowing of the TEC (28) may trigger a more violent intermolecular collision (and higher loading rate) and favor Rho release over TEC dissociation (Fig. 5). Of course, other factors such as the lateral stability of the TEC at pause sites (22, 65) or the probability of Rho-TEC encounter (classical kinetic competition model) (66) may also contribute significantly to the termination efficiency and are likely to become predominant in some cases (as, for instance, when NusA favors termination) (28). Further work is in progress to examine these various aspects in detail.

In summary, our results support that Rho-dependent termination stems from a snow plough-type of mechanism of steric exclusion of the TEC from the central channel of the Rho hexamer. Basal termination efficiency, however, may be significantly altered early on the termination pathway by factors impacting a productive Rho-RNA interaction (RNA conformation, steric hindrance) or at later stages by factors modulating the intermolecular collision and/or the respective strengths of the Rho-RNA and TEC complexes (dissociation probability, RNAP and Rho translocation rates, force distributions, and loading rates). Whether the specific RNAP mutations that alter Rho-dependent termination (31) fall in the later category awaits further work.

Motor Mechanisms of Transcription Factor Rho

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Motor Mechanisms of Transcription Factor Rho

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