doi:10.1016/j.jmb.2011.04.026 J. Mol. Biol. (2011) 410, 959–971

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Single-Molecule Studies Reveal that DEAD Box Protein DDX1 Promotes Oligomerization of HIV-1 Rev on the Rev Response Element

Rae M. Robertson-Anderson, Jun Wang, Stephen P. Edgcomb, Andrew B. Carmel, James R. Williamson and David P. Millar*

Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Received 29 January 2011; received in revised form 10 April 2011; accepted 11 April 2011

Edited by M. F. Summers

Keywords:
viral RNA transport; cellular cofactors of HIV; ribonucleoprotein assembly; RNA–protein interactions; single-molecule fluorescence microscopy

Oligomeric assembly of Rev on the Rev response element (RRE) is essential for the nuclear export of unspliced and singly spliced human immunodeficiency virus type 1 viral mRNA transcripts. Several host factors, including the human DEAD box protein DDX1, are also known to be required for efficient Rev function. In this study, spontaneous assembly and dissociation of individual Rev–RRE complexes in the presence or absence of DDX1 were observed in real time via single-molecule total internal reflection fluorescence microscopy. Binding of up to eight fluorescently labeled Rev monomers to a single RRE molecule was visualized, and the event frequencies and corresponding binding and dissociation rates for the different Rev–RRE stoichiometries were determined. The presence of DDX1 eliminated a second kinetic phase present during the initial Rev binding step, attributed to nonproductive nucleation events, resulting in increased occurrence of higher-order Rev–RRE stoichiometries. This effect was further enhanced upon the addition of a non-hydrolyzable ATP analog (adenylyl-imidophosphate), whereas ADP had no effect beyond that of DDX1 alone. Notably, the first three Rev monomer binding events were accelerated in the presence of DDX1 and adenylyl-imidophosphate, while the dissociation rates remained unchanged. Measurements performed across a range of DDX1 concentrations suggest that DDX1 targets Rev rather than the RRE to promote oligomeric assembly. Moreover, DDX1 is able to restore the oligomerization activity of a Rev mutant that is otherwise unable to assemble on the RRE beyond a monomeric complex. Taken together, these results suggest that DDX1 acts as a cellular cofactor by promoting oligomerization of Rev on the RRE.

Introduction

Rev, a key regulatory protein of human immunodeficiency virus (HIV) type 1, activates nuclear export of unspliced and partially spliced viral mRNAs, which encode the RNA genome and the genes for the structural proteins Gag, Pol and Env, respectively (reviewed in Ref. 1). Rev binds to the Rev response element (RRE), a highly conserved region of the viral mRNA, which contains a single

*Corresponding author. E-mail address: millar@scripps.edu.

Abbreviations used: AMP-PNP, adenylyl-imidophosphate; ARM, arginine-rich motif; A555, Alexa Fluor 555; RRE, Rev response element; TIRF, total internal reflection fluorescence; HIV, human immunodeficiency virus; wt, wild type.

high-affinity binding site for Rev, although as many as eight Rev molecules can bind to a single RRE.[2–4] In fact, binding of a single Rev to the RRE is incapable of activating mRNA export, indicating that oligomerization of Rev on the RRE is essential for Rev function.[5,6] Further, while Rev is the central player, a number of cellular proteins also contribute to nucleocytoplasmic export of viral mRNAs and enhance Rev function.[7] Because Rev-mediated RNA export is essential for viral replication, the Rev–RRE complex is a potential therapeutic target for treatment of HIV/AIDS. However, owing to the complexity of the Rev pathway and the many macromolecular interactions in which it participates, effective therapies that target Rev have yet to be realized.

The human DEAD box protein DDX1 has been implicated as a cellular cofactor of Rev.[8,9] DEAD box proteins typically function as ATP-dependent RNA helicases and are involved in many aspects of RNA metabolism, including ribosome biogenesis, RNA splicing, translation and RNA degradation.[10,11] DDX1 is known to be required for efficient Rev function and proper nuclear localization of Rev in mammalian cells[8] and human astrocytes.[9] DDX1 has also been shown to interact with the N-terminus of Rev in yeast and mammalian two-hybrid systems.[8] Direct physical interactions between DDX1 and Rev and between DDX1 and the RRE were subsequently confirmed by *in vitro* binding studies.[12] In addition, silencing of DDX1 in HIV-1-infected HeLa cells drastically reduces virus particle production.[12] Taken together, these results establish that DDX1 is a key cellular cofactor of Rev, essential for Rev function and virus replication. However, the mechanism by which DDX1 acts to promote Rev function is not understood. More generally, the role that DEAD box proteins play in ribonucleoprotein assembly and nucleocytoplasmic transport of retroviral RNA is largely unexplored.

Since oligomeric assembly of Rev on the RRE is required for nuclear export of HIV mRNAs, we hypothesized that DDX1 may act as a cellular cofactor by assisting in the oligomerization process. Here, we test this hypothesis by using a single-molecule fluorescence spectroscopic method to observe individual steps in Rev–RRE assembly[13] in the presence of DDX1. Binding of up to eight Rev monomers on a single RRE was observed, and the presence of DDX1 significantly enhanced this assembly. The effect of DDX1 on Rev–RRE assembly was also examined in the presence of ADP or the non-hydrolyzable ATP analog adenylyl-imidophosphate (AMP-PNP). The rate constants for Rev monomer binding and dissociation were determined for each step of assembly, revealing the effect of DDX1 and nucleotide cofactors at specific points on the assembly pathway. These measurements were performed across a range of DDX1 concentrations to determine whether DDX1 promotes oligomeric assembly by interacting with Rev or the RRE, which have different affinities for DDX1. In addition, we tested the effect of DDX1 on an oligomerization-deficient Rev mutant. Our results demonstrate that DDX1 strongly promotes oligomerization of Rev on the RRE, explaining why DDX1 is required for efficient Rev function.

Results

Experimental system

Previously, we used total internal reflection fluorescence (TIRF) microscopy to monitor the assembly of fluorescently labeled Rev on single RRE molecules immobilized on a quartz surface. Individual Rev binding and dissociation steps were directly observed in real time as discrete jumps in fluorescence intensity from the surface-bound complexes. Statistical analysis of many fluorescence intensity trajectories recorded during individual assembly reactions provided detailed information on the stoichiometry and kinetics of the Rev–RRE interaction.[13] Here, we use the same method to investigate binding of Rev to the RRE in the presence of DDX1 and nucleotide cofactors (Fig. 1). One modification of the present study was to use the full 351-nt RRE instead of the truncated RRE construct used previously. The truncated RRE was only capable of binding up to four Rev monomers, whereas the full RRE binds up to eight monomers (see below), allowing us to examine higher-order binding events during Rev–RRE assembly. Full-length RRE was generated by *in vitro* transcription, biotinyated at the 5′ end and immobilized on a polyethylene-glycol-treated quartz surface coated with streptavidin. The fluorophore labeling site within the Rev protein was also different than that in the previous study. For this study, both native cysteines of Rev were mutated to serine, and a single cysteine residue used for labeling was introduced into an N-terminal extension, which also contained the His_6 tag used for affinity purification. We chose to label the Rev construct at this position to ensure that the labeling would not affect the native function of the protein. Apart from these changes, Rev was labeled with Alexa Fluor 555 (A555) and purified as described previously.[13] Similarly, the single-molecule TIRF data were acquired and processed as in the previous study.

To validate the new reagents, we first examined a binary system consisting of labeled Rev and full-length RRE (no DDX1 present). Typical fluorescence intensity trajectories (time traces) reveal discrete and abrupt transitions between states exhibiting different fluorescence intensities, reflecting spontaneous