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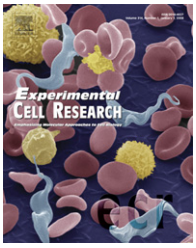

Research Article

Transcription-dependent nucleolar cap localization and possible nuclear function of DExH RNA helicase RHAU

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ABSTRACT

RHAU (RNA helicase associated with AU-rich element) is a DExH protein originally identified as a factor accelerating AU-rich element-mediated mRNA degradation. The discovery that RHAU is predominantly localized in the nucleus, despite mRNA degradation occurring in the cytoplasm, prompted us to consider the nuclear functions of RHAU. In HeLa cells, RHAU was found to be localized throughout the nucleoplasm with some concentrated in nuclear speckles. Transcriptional arrest altered the localization to nucleolar caps, where RHAU is closely localized with RNA helicases p68 and p72, suggesting that RHAU is involved in transcription-related RNA metabolism in the nucleus. To see whether RHAU affects global gene expression transcriptionally or posttranscriptionally, we performed microarray analysis using total RNA from RHAU-depleted HeLa cell lines, measuring both steady-state mRNA levels and mRNA half-lives by actinomycin D chase. There was no change in the half-lives of most transcripts whose steady-state levels were affected by RHAU knockdown, suggesting that these transcripts are subjected to transcriptional regulation. We propose that RHAU has a dual function, being involved in both the synthesis and degradation of mRNA in different subcellular compartments.
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Introduction

The stability of many mRNAs is dynamically regulated either positively or negatively by a variety of signals through the interaction of specific sequences in the RNA molecule and proteins that recognize them [1]. The AU-rich element (ARE) is one of the best-characterized *cis*-acting instability elements located in the 3′ untranslated region of various mRNAs with short half-lives, including those encoding cytokines, growth factors, proto-oncogenes, and transcription factors [2]. In a previous work searching for regulatory proteins binding to the ARE of unstable urokinase mRNA, we identified a putative RNA helicase (alias: DHX36) termed RHAU (RNA helicase associated with AU-rich element), together with two further known ARE-binding proteins, HuR and NF90 (NFAR1) [3]. In vivo and in vitro studies have shown that RHAU enhances poly(A) shortening and decay of urokinase mRNA in a manner dependent on ARE in the message and ATPase activity of RHAU. We also found that RHAU interacts with the poly(A) ribonuclease PARN and the exosome, indicating that urokinase mRNA decay is triggered by RHAU recruitment of the RNA-degrading machinery to the ARE in the mRNA [3].

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DExH/D RNA helicases are ATP-hydrolytic enzymes that unwind duplex RNA molecules or remodel protein–RNA complexes [4,5]. Based on the conserved sequence motif II required for the ATPase activity, DExH/D proteins are classified into three subfamilies, termed DEAD, DEAH and DExH (in single letter amino acid code); RHAU belongs to the DExH subfamily. The DExH/D protein family is the largest group of enzymes in eukaryotic RNA metabolism and is involved in virtually all aspects of RNA metabolism. These proteins act as ATP-driven motors or switches at specific points in processes such as pre-mRNA splicing or ribosome biogenesis [4]. Some are also involved in the regulation of gene expression and in many cases they have multiple functions, acting at different steps from transcription and splicing to RNA export [6]. For example, DEAD box proteins p68 (DDX5) and p72 (DDX17) regulate transcription via interaction with transcription regulators such as β-catenin [7], MyoD [8], Smads [9], HDAC1 [10], and p53 [11], thus playing a role in epithelial mesenchymal transition, myogenesis, regulation of apoptosis, general transcriptional repression, and tumorigenesis, respectively. They are also required for pre-mRNA splicing [12,13] and alternative splicing [14] as well as the processing of rRNA and microRNAs [15]. RNA helicase A (RHA), a DExH helicase closely related to RHAU, also plays many roles in the regulation of gene expression. In the nucleus, RHA interacts with RNA polymerase II and transcriptional regulators such as CBP/p300 [16], BRCA1 [17], and NF-κB [18], as well as promoters of the p16INK4a and MDR1 genes [19,20], and activates their transcription. RHA is also involved in RNA export mediated by the constitutive transport element (CTE) [21,22], in RNA splicing by interacting with SMN (survival motor neuron complex), and in the translation of selected mRNAs [23,24]. Most recently, RHA was also identified in the RNA-induced silencing complex (RISC) in HeLa cells, functioning as an siRNA-loading factor [25]. These observations indicate that a single RNA helicase can play many different roles depending on its interaction with different molecules in various cellular environments.

We identified RHAU originally as a regulator of urokinase mRNA stability acting in the cytoplasm [3]. However, analysis of endogenous RHAU protein levels after cell fractionation, and of the exogenous RHAU level after transient transfection, showed higher expression in the nucleus than the cytoplasm ([3] and in this study), suggesting that RHAU has additional functions in the nucleus. In the present work, we investigated the nuclear distribution and potential nuclear function of RHAU. Here we show that RHAU is actively translocated into the nucleus and that its localization is regulated by its ATPase activity. Using microarray analysis of HeLa cells in which RHAU was efficiently knocked down by inducible RHAU-specific short hairpin RNA (shRNA), we investigated RHAU-regulated genes and mRNAs.

Materials and methods

Plasmids

Oligonucleotides used in this paper are presented in Supplementary Table 1.

Plasmid pTER was kindly provided by Hans Clevers [26]. To construct pTER-shRHAU, annealed oligonucleotides of shRHAU-s and shRHAU-as were inserted into BglII/HindIII sites of the pTER vector to target RHAU mRNA at the site 1344–1364 nt. pTER-shLuc was kindly provided by A. Hergovich and B.A. Hemmings [27].

To derive the N-terminal fusion plasmid pEGFP-RHAU, full-length RHAU was cut out from pcDNA3-HA-RHAU [3] using BamHI/XhoI and inserted into the BglII/SalI sites of pEGFP-C1 (Clontech laboratories, Inc., Mountain View, CA). To introduce the ATPase-deficient mutation, pEGFP-RHAU-E335A was made using site-directed mutagenesis with oligonucleotides (E335A-s and E335A-as) that mutate the Glu335 of RHAU to Ala. To derive the C-terminal fusion plasmids pRHAU-EGFP and pRHAU-E335A-EGFP, full-length RHAU was amplified by PCR using the primers RHAU-fw and RHAU-rv with the plasmids pcDNA3-HA-RHAU and EGFP-RHAU-E335A, respectively, and inserted into the EcoRI/BamHI sites of pEGFP-N1 (Clontech). pcDNA3-Flag-RHAUsm was made by replacing the HA tag of pcDNA3-HA-RHAU in HindIII/BamHI sites with annealed oligonucleotides coding the Flag sequence. To introduce silent mutations into the RHAU expression vector at the shRHAU-targeting site, we did site-directed mutagenesis using oligonucleotides RHAUsm-s and RHAUsm-as to amplify, using PCR, a mutated vector that contained two point mutations, G1350A and A1353G. To derive pcDNA3-HA-p68 and pcDNA3-HA-p72, full-length cDNAs of p68 and p72 were amplified with the primers p68-fw/p68-rv and p72-fw/p72-rv, respectively, using cDNA derived by reverse transcription of purified HeLa total RNA, and inserted into BamHI/XhoI sites of the pcDNA3.1(+)-HA vector. pcDNA3.1(+)-HA was made by inserting the annealed oligonucleotide fragment coding the HA sequence into the HindIII/BamHI sites of the pcDNA3.1(+) vector (Invitrogen Corporation, Carlsbad, CA). The sequences of all plasmids made by PCR-cloning were confirmed. pcDNA3.1-HDAC1^{FLAG} and pcDNA3.1-HDAC3^{FLAG} were kindly provided by from P. Matthias (FMI).

Cell culture and stable/transient transfection

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum at 37 °C in the presence of 5% CO_2. T-REx-HeLa cells (Invitrogen) were maintained as above with the additional supplement of 3 μg/ml blasticidine (Invitrogen). T-REx-HeLa cells were stably transfected with pTER-shRHAU or pTER-shLuc vectors using FuGENE6 (Roche Applied Science, Rotkreuz, Switzerland) and selected with zeocin (InvinoGen, San Diego, Calif.) at a final concentration of 450 μg/ml. Zeocin-resistant colonies were picked up as independent clones. To induce shRNA expression, cells were treated with doxycycline (Sigma-Aldrich Co.) at a final concentration of 1 μg/ml. Transient transfection of plasmid DNA using FuGENE6 was performed according to the instructions of the manufacturer, using 1 μg plasmid DNA and 3 μl FuGENE6 per 35-mm dish.

Antibodies

Mouse anti-RHAU monoclonal antibody was generated against a peptide sequence that corresponds to the C-terminal of RHAU, 991–1007 aa, as reported previously [28]. Rabbit anti-H3-K9 trimethylation and rabbit anti-NDH II (RNA helicase A) antibodies were kindly provided by A. Peters [29] and S. Zhang [30], respectively. Commercially obtained antibodies were:

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