Kinetics of rat CSD-C2 binding to H3.3 RNA

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Abstract

Cold-shock domain containing protein C2 (CSD-C2; also known as PIPPin) is an RNA-binding protein conserved in the evolution that interacts with the 3'-untranslated region (3'-UTR) of rat H1.0 and H3.3 histone messengers. Biolayer interferometry (BLI) is a technique that measures changes in an interference pattern generated from visible light, reflected from an optical layer, and a biolayer which contains molecules of interest. In this study, we used the BLI methodology in order to analyze and describe the binding properties of CSD-C2 and the mRNA encoding the rat brain histone protein H3.3. Recombinant CSD-C2 was incubated with in vitro transcribed, and biotinylated H3.3 RNA fragments bound to streptavidin-conjugated Octet optical biosensors. In order to define the RNA region involved in binding, we used RNA probes corresponding to different portions of H3.3 RNA 3'-UTR.

In this study, we showed that CSD-C2 binds to the last 199 nucleotides of the H3.3 RNA 3'-UTR, and that the apparent affinity constant of the interaction is in the nanomolar range. In addition, this study confirmed that BLI can be a very efficient and reliable method for studying RNA-protein interactions.

Abbreviations: CSD-C2: Cold Shock Domain Containing Protein 2; IL-18: Interleukin-18; IL-33: Interleukin-33; RBP: RNA-Binding Protein; 3'-UTR: 3'-Untranslated Region; BLI: Biolayer Interferometry; hnRNP: Heterogeneous nuclear ribonucleoprotein

Introduction

Post-transcriptional regulation of RNA metabolism depends on a series of regulatory proteins (RNA-binding proteins, RBPs), which contain different RNA-binding motifs as well as domains involved in protein-protein interactions [1]. RNA-RBP association can be modulated by interactions with further proteins, non-coding RNAs, and post-translational modifications of RBPs, all of which can be induced by extracellular signals [2].

During development, mammalian brain accumulates the core histone H3.3, and the linker histone H1.0 [3-5]. The concentration of the corresponding mRNAs decreases around birth, while the corresponding proteins accumulate: these differences depend on post-transcriptional regulatory events [6]. We previously identified a set of H1.0 RNA-binding proteins [7], among which abundant RBPs, such as heterogeneous nuclear ribonucleoprotein (hnRNP) K and hnRNP A1, and molecular chaperones (heat shock cognate 70, Hsc70) [8]. Based on the screening of expression cDNA libraries with labeled, in vitro transcribed histone RNAs, we also cloned the H3.3/H1.0 RNA-binding protein CSD-C2, which contains a cold-shock domain [9]. Moreover, we demonstrated that CSD-C2 protein binds H1.0 and H3.3 RNAs at the end of their 3'-UTRs, in a region encompassing the polyadenylation signal possibly forming a hairpin structure [9]. Co-immunoprecipitation assays showed that some of the identified proteins interact with each other and with other nuclear proteins. For example, we found that CSD-C2 interacts with hnRNP A1, Y-box-binding protein 1 (YB-1), hnRNP K, and probably Hsc70 [8].

Herein, we report kinetics and specificity of CSD-C2 binding to H3.3 mRNA, as well as identification of the H3.3 RNA region more directly involved in binding.

Experimental procedures

In vitro transcription and 3'-end biotinylation of RNA

pR4 and pM4 plasmids [7,9] were linearized by restriction with Hind III and used as templates for in vitro transcription (Riboprobe system, Promega, WI). RNA was precipitated with ethanol, collected by centrifugation at 10,000 x g for 15 min, air dried and resuspended in distilled water.

All the RNA fragments produced by in vitro transcription were subsequently biotinylated using the "RNA 3'-end biotinylation kit" from Pierce (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s instructions.

Recombinant proteins

Recombinant (6-his-tagged) CSD-C2 was purified as previously reported [10].

Human recombinant (6-his-tagged) IL-18 and IL-33 were a kind gift from Dr. Ronald Tynes of the Northwestern University, Muttenz, Switzerland.

Biolayer interferometry (BLI)

Kinetics of CSD-C2 binding to its RNA substrates was studied...
In order to better analyze the binding ability of CSD-C2, we decided to adopt BLI as an approach for confirming, on one hand, ability of CSD-C2 to bind H3.3 mRNA, while measuring, on the other hand, more precisely the binding constants for this interaction.

Preliminary experiments with whole H3.3 RNA showed that BLI experiments cannot be performed with large RNAs, probably because of irregular folding of the molecules when bound to sensors. As mentioned above, we previously showed that rat brain CSD-C2 protein binds to H3.3 mRNA at the level of its 3′ end [9]. In order to confirm the ability of the 3′-UTR of the H3.3 RNA to bind CSD-C2, and to calculate the affinity of this interaction, we used two RNA probes (Figure 1): R4, which corresponds to the whole 3′-UTR of the messenger, and M4, which corresponds to the last 199 nucleotides of the 3′-UTR.

After biotinylation, the probes were used in the BLI binding assays, as described in Experimental Procedures.

Figure 2 and 3 show graphic representations of typical binding experiments, using, respectively, R4 and M4 RNA and increasing amounts (10-40 ng/μl, i.e., 0.3-1.3 μM) of the CSD-C2 protein.

Binding curve fitting were obtained using the 1:1 fitting model and were then processed to calculate the affinity constants. Experiments were repeated 4 times for each fragment: Table 1 summarizes mean values of the apparent association and dissociation constants of
the reactions for R4 and M4 fragments, and the mean values of the estimated affinity constants.

The results obtained confirmed that CSD-C2 binds to the last 199 nucleotides of H3.3 mRNA (M4 region) and also allowed to estimate the interaction constants (apparent KD: $4.53 \times 10^{-8}$ M). In a distinct set of BLI experiments, we analyzed the interaction between a biotinylated fragment spanning from nt 1400 to 1712 of rat H1.0 RNA and CSD-C2 protein, and the estimated KD of the reaction was also in the nanomolar range (unpublished results). As already mentioned, for both RNAs the binding region to CSD-C2 is localized in the last part of the 3'-UTR, encompassing the polyadenylation site, suggesting that in vivo regulation of polyadenylation of these RNAs by CSD-C2 could be involved in their expression.

To demonstrate the H3.3 messenger binding specificity to CSD-C2, a set of control experiments was carried out incubating biotinylated R4 and M4 RNAs with recombinant IL-18 and IL-33 proteins as control analytes. Upper panel of Figure 4 shows, as an example, graphic representation of the binding between R4 RNA and CSD-C2 at different concentrations of CSD-C2. Graphic shows values obtained at 10 ng/μl (a), 20 ng/μl (b), and 40 ng/μl (c).

To ascertain that the differences in the binding responses were not due to a higher unspecific binding of the CSD-C2 to the sensors, compared to IL-18 or IL-33, binding values were also calculated subtracting the binding response of each protein to the sensor, in the absence of RNA, from the whole binding responses; subtracted values obtained for each protein are reported in the individual lower panels, and demonstrate that the higher binding response of CSD-C2 is actually due to RNA binding.

Table 1. Apparent KD values calculated for the interaction between CSD-C2 protein (analyte) and the two fragments of the 3'-UTR of H3.3 histone RNA R4 and M4 (ligands), shown in Figure 1.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Analyte</th>
<th>Apparent K on (M's⁻¹)</th>
<th>Apparent K off (s⁻¹)</th>
<th>Apparent KD (nM)</th>
</tr>
</thead>
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<tr>
<td>M4 RNA</td>
<td>CSD-C2</td>
<td>$7.29 \pm 1.68 \times 10^4$</td>
<td>$5.57 \pm 1.97 \times 10^4$</td>
<td>$74.0 \pm 18.2$</td>
</tr>
<tr>
<td>R4 RNA</td>
<td>CSD-C2</td>
<td>$2.27 \pm 1.70 \times 10^4$</td>
<td>$6.89 \pm 2.16 \times 10^4$</td>
<td>$45.3 \pm 29.9$</td>
</tr>
</tbody>
</table>

Figure 4. Graphical representation of the absolute values obtained from the analysis of the interactions between R4 fragment and CSDC2 (black line), IL-18 (dark grey line), or IL-33 (light grey line). Only association and dissociation steps are shown. Responses are plotted against time of reaction. All proteins have been loaded at a concentration of 660 nM. Lower inserts are graphic representation of the values for R4/CSDC2, R4/IL-18, or R4/IL-33 interactions obtained subtracting binding values of the corresponding protein to the sensors (unspecific binding) from the values represented in the upper panel.

Binding analyses using M4 RNA and IL-18 or IL-33 produced similar results (data not shown).

**Conclusions**

The results obtained confirmed that CSD-C2 binds to last 199 nucleotides of H3.3 mRNA (M4 region) and also allowed to estimate the interaction constants (apparent KD: $4.53 \times 10^{-8}$ M).

In addition, this study confirmed that BLI can be a very efficient and reliable method for studying RNA-protein interactions, and could be of great help in modelling the pathways at work in the cells to build up RNA-protein complexes formed by multiple proteins, with different affinities for RNA.

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References


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