Biomedical Data Mining

Extended Abstract

Regulation of the activity of the promoter of RNA-induced silencing, C3PO

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Abstract

RNA-induced silencing is an interaction which permits cells to direct the union of explicit proteins. RNA quieting is advanced by the protein C3PO (part 3 of RISC). We have recently discovered that phospholipase Cβ, which increments intracellular calcium levels in light of explicit G protein signals, hinders C3PO action towards specific qualities. Understanding the boundaries that control C3PO movement and which qualities are affected by G protein actuation would help foresee which qualities are more powerless against downregulation. Here, utilizing a library of 1018 oligonucleotides, we show that C3PO ties oligonucleotides with underlying particularity however little arrangement explicitness. Then again, C3PO hydrolyzes oligonucleotides with a rate that is delicate to substrate solidness. Critically, we find that oligonucleotides with higher Tm esteems are hindered by bound PLCβ. This finding is upheld by microarray examination in cells over-expressing PLCβ1. Taken together, this examination permits forecasts of the qualities whose post-transcriptional guideline is receptive to the G protein/phospholipase Cβ/calcium flagging pathway.

Introduction

The RNA-induced silencing complex (RISC) has been estimated to regulate 30% of mammalian genes. The regulation of RISC is not completely clear, but in drosophila it has been found that RISC activity is promoted by C3PO2 and in humans, C3PO may be required for RISC activity. C3PO promotes silencing by degrading the passenger strand of the siRNA leaving the guide strand free to hybridize to its specific mRNA and become degraded. Besides its role in RNA silencing, C3PO has been shown to function in diverse cellular processes such as chromosomal translocations, neuronal development, mRNA transport, and tRNA processing. C3PO consists of 6 translin subunits that are thought to confer silencing RNA binding specificity, and 2 TRAX subunits that have nuclease activity. There is a wealth of structural information about C3PO, but the factors that regulate its activity, and its regulation in cells are unknown. Understanding the specificity of C3PO for different oligonucleotides and its ability to cleave these oligonucleotides would help us identify which genes are most susceptible for downregulation through RNA-induced silencing.

Recently, we found that C3PO is a binding partner of PLCβ.11 PLCβ mediates calcium signals generated through receptors coupled to Gαq family of heterotrimeric G proteins whose ligands include acetylcholine, dopamine, bradykinin, angiotensin II, and others. In cells, PLCβ and C3PO interact in the cytosol and studies using purified proteins show that PLCβ binds to an external site on one or both of the TRAX subunits of C3PO. C3PO binds to the same region of PLCβ as its activator, Gαq, and high levels of C3PO will quench Ca2+ signals generated by Gαq-coupled GPCRs in cells. Similarly, over-production of PLCβ can reverse siRNA downregulation of genes such as GAPDH and LDH presumably through its interaction with TRAX. However, PLCβ does not reverse knockdown of other genes such as Hsp90 and cyclophilin A. The basis for this selection is intriguing and it appears that the activity of C3PO changes when PLCβ is bound.

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Result

We established the binding specificity of C3PO by SELEX. In this method, a DNA library is transcribed into an RNA pool, which is incubated with His-tagged protein pre-bound to Ni-NTA resin. The protein-bound RNAs are separated by phenol-chloroform extraction, reverse–transcribed to DNA, and amplified by PCR. This whole process makes up one round of SELEX and the resulting amplified DNA from PCR is the starting point of the subsequent selection round. The initial 6 rounds of SELEX were performed using translin which forms an inactive C3PO-like octamer at elevated concentrations, and the 4 final rounds were performed using catalytically inactive (D193A) C3PO. The library consisted of ∼1 × 1018 different oligonucleotides having a randomized 30 nt region sandwiched between two constant regions at the ends to allow for PCR amplification (see Methods). This length was chosen to be slightly greater than the average length of silencing RNAs (22 nt). The binding conditions were made more stringent with subsequent rounds by decreasing the incubation time from 5 hours to less than 5 minutes, and by increasing the RNA:protein stoichiometry from 1:80 to 1:1. At the end of round 6 and round 10, we sequenced 25 and 90 sequences from the amplified DNA out of the many remaining. These sequences were subjected to multiple sequence alignment (MSA) by Multalign and motif search by MEME suite. This analysis showed that the sequences were not similar and only indicated a consensus of G at the 3′ end of the randomized region. Additionally, when we checked the nucleotide composition of the sequences, we find a decrease in C% from round 6 to round 10 which was compensated by an increase in G% indicating a lack of preference for A/T or G/C base pairs. This lack of specificity suggests that selection may be based on structural stability rather than specificity. Comparison of the selected sequences against the human genome database using BLAT online server could not be related to any specific gene families.

We predicted the structures of the selected RNA sequences using the RNAfold server and found that the predicted minimum free energy structures for all the sequences had similar features showing a high potential to fold into double stranded structures with intermediate loops for mismatched nucleotides. Since these sequences favored the formation of double strands, we used structural information from duplexes to further characterize the sequences. In particular, we used known structural data for basepair step parameters24, 25 (shift, slide, rise, tilt, roll, and twist) and flexibility along those directions to characterize the sequences. We noticed a marked tendency for bending along the tilt axis and stiffness regarding slide in the oligonucleotides selected from the SELEX experiment as compared to those coming from sets of randomly generated RNA sequences.

Discussion

siRNAs are being considered as a natural, epigenetic method to treat cancer and other diseases. Before these therapies become available, it is important to understand how gene silencing by miRs is regulated and how regulation changes under different cellular conditions. C3PO, which is thought to be required for efficient RNA silencing is an ancient enzyme found in both primitive and complex organisms. Structural studies have provided speculative information about the mechanism of C3PO catalysis. Here, we have focused on how C3PO may be regulated in cells through its interaction with PLCβ.

Cells contain many different miRs that compete for C3PO binding and the levels and populations of these miRs may range from a few copies to many thousands depend on the type and state of the cell. We determined C3PO binding specificity by screening a library of ∼1018 oligonucleotides. Although we found little indication of sequence specificity, we did find strong preference for mixed stem-loop structures. This preference correlates well with the observation that in cells C3PO degrades the passenger strand only after a nick from Ago2 to yield a silencing miR with mixed single and double stranded character. The optimal number of ∼14 paired bases also correlates well with the ∼10 base pair between the passenger and guide strands of silencing RNA. SELEX studies also suggested that C3PO prefers sequences a high slide

force and low tilt force, i.e. structures that are easier to bend into the nucleotide backbone, and ones whose bases do not move along the backbone axis. However, the 90 oligonucleotides sequenced from the last SELEX pool did not correlate with any known genes, nor did they correlate with consensus sequences at breakpoint junctions of some chromosomal translocations where TRAX/translin are thought to impact.

We tested the effect of PLCβ on C3PO catalysis of different oligonucleotides and find that PLCβ only affects the hydrolysis rates that have a relatively high Tm. The reduction in rate of more stable oligonucleotides by bound PLCβ appears to be independent of substrate structure. The ability of PLCβ to inhibit slower reactions can be explained by the simple rate equations that stem from the coupled equilibria shown in. Because our data show that oligonucleotide binding is much stronger to isolated C3PO as opposed to C3PO-PLCβ, the oligonucleotide will prefer to bind to C3PO during the time that is has dissociated PLCβ. If the hydrolysis rate is fast, as in the case for miRs with low values of Tm, the C3PO-RNA complex rapidly returns to free C3PO. However, if the Tm is high, the C3PO-RNA complex is longer lived and available for PLCβ binding which inhibits the conformational changes needed for efficient C3PO-oligonucleotide interactions. Even though the effect of PLCβ is seen in the rate rather than binding, we find that its impact on rate with the Tm of the oligonucleotide falls into line with the behavior seen for the blunt-ended oligonucleotides. We note that an alternate model may be that the off-rate of RNA from C3PO-PLCβ complex is faster than the rate of hydrolysis of more stable oligonucleotides and that high Tm oligonucleotides would dissociate from the complex before they are hydrolyzed. This idea would be consistent with the data in showing that DNA has a lower affinity, and possibly a faster off rate from the complex as compared to free C3PO.

Conclusion

The cellular activity of C3PO impacts RNA silencing, tRNA processing and other processes that are important for normal cell function. While substrate selection of C3PO is nonspecific, its activity is highly dependent on substrate stability. We find that this stability directly relates to the impact PLCβ has on C3PO function which in turn may relates to the ability of G proteins to impact RNA-induced silencing. Specifically, our studies describe a subset of genes that have the potential to be regulated by GPCRs coupled to the Gαq/PLCβ pathway, and which genes will function independently of these signals.

Materials and Methods

Materials

The DNA library for SELEX was a generous gift from Dr. Steven Lodmell (University of Montana, Missoula, MT). The Ambion MEGAshortscript T7 transcription kit was procured from Life Technologies. AMV reverse transcriptase kit was ordered from Promega (Madison, WI). The restriction enzymes EcoRI and XbaI and the dNTPs were obtained from NEB (Ipswich, MA). The recombinant human C3PO-pET DUET1 plasmid was a generous gift from Dr. Hong Zhang (University of Texas Southwestern, Dallas, TX). Fluorescent probes were purchased from Invitrogen.

Protein purification and preparation of inactive C3PO

Translin and C3PO proteins were expressed and purified by Ni-NTA affinity chromatography following the protocol as described earlier. Briefly, plasmids for translin (pET15b with an N-terminal 6XHis tag) and human C3PO (pET DUET1 vector containing TRAX with an N-terminal 6X His tag and translin) were transformed into Rosetta 2 DE3 bacterial competent cells. C3PO was rendered catalytically inactive by mutating the active site Asp193 of TRAX to alanine. The transformed bacterial cells were grown and protein synthesis was induced by isopropyl β-D-1-thiogalactopyranoside treatment at a final concentration of 1mM for 3–4 hours. After protein induction period, cells were harvested and sonicated. The lysed cells were centrifuged at 10,000g for 25 minutes at 4°C and supernatant was added to pre-washed Ni-NTA beads (Qiagen) and rotated for 1.5 hours for translin and overnight for C3PO. The Ni-NTA beads were washed, and the proteins were eluted by an imidazole gradient of

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up to ~200 mM imidazole. The eluted fractions were analyzed for translin and C3PO proteins by SDS-PAGE followed by Coomassie Blue staining. The concentrations of purified proteins were measured using a Bradford assay and measuring the absorbance at 280 nm by Nanodrop.

DNA library

The DNA library was comprised of oligonucleotides that consisted of a random 30 pairs (bps) region flanked by constant regions on each side to give a total length of 59 base pairs:

5′-GGCAUUACGGCCGGG-
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-
GCGUCUUCUAGAGC-3′

Theoretically, the library should contain 430 (1.15 × 1018) different oligonucleotides. The constant regions were designed to contain restriction sites for EcoRI and XbaI, a transcription initiation site at 5′ end and complementary sites for primers which are required for polymerase chain reaction (PCR).

Selection of RNAs that bind to translin and C3PO by SELEX

The experimental steps for SELEX were performed as described earlier19 with some modifications to eliminate the use of radioactive material. Briefly, the DNA library was transcribed into an RNA library using the MEGAshortscript kit from Ambion, Life Technologies. After the transcription reaction, the mixture was treated with DNase leaving an RNA library, called Pool 0 RNA. Pool 0 RNA was purified by phenol/chloroform extraction and ethanol precipitation, and then excised from the correct length band on a 10% denaturing gel (with 8M urea). The purified Pool 0 RNA was then incubated with Ni-NTA resin for 1 hour at room temperature (RT) in a rotator in buffer containing 0.1M NaCl, 20 mM Tris HCl, 5 mM MgCl2 (binding buffer) to discard RNA that bound to Ni-NTA resin instead of the target protein (translin/C3PO) as a pre-selection round. Pool 0 RNA that did not bind to the resin was incubated with Ni-NTA resin pre-bound to translin for 5 hours at RT in binding buffer in a rotator. The resin were washed (4X) to remove RNA that did not bind to translin along with those that bind weakly. The RNA that binds to translin/C3PO was phenol/chloroform extracted (4X) and precipitated by ethanol which was then reverse transcribed to cDNA and amplified by RT-PCR. Reverse transcription (RT) was done using the AMV reverse transcriptase (Promega). Prior to PCR, a gradient PCR was done to gauge the melting point Tm for the cDNA. Then, using the Tm from gradient PCR, PCR reaction was performed using Taq polymerase (Invitrogen) to amplify the cDNA into double stranded DNA which was further digested using the restriction enzymes EcoRI and XbaI. This whole cycle consists of one SELEX round and the resultant DNA is the starting material for the subsequent SELEX round.

The incubation time of the RNA pool and target protein was decreased from 5 hours in Round 1 to a few minutes in Round 10 to increase the probability of selecting strong binders. The first six rounds were done using translin followed by four rounds with inactive C3PO. In the last three rounds, imidazole was first used to elute the His tagged translin/C3PO from Ni-NTA resin followed by purification of the RNA that bound to the target protein by phenol/chloroform extraction to improve the selection efficiency.

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