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

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Abstract: RNA-induced silencing is a process which allows cells to regulate the synthesis of specific proteins. RNA silencing is promoted by the protein C3PO (component 3 of RISC). We have previously found that phospholipase C β , which increases intracellular calcium levels in response to specific G protein signals, inhibits C3PO activity towards certain genes. Understanding the parameters that control C3PO activity and which genes are impacted by G protein activation would help predict which genes are more vulnerable to downregulation. Here, using a library of 10¹⁸ oligonucleotides, we show that C3PO binds oligonucleotides with structural specificity but little sequence specificity. Alternately, C3PO hydrolyzes oligonucleotides with a rate that is sensitive to substrate stability. Importantly, we find that oligonucleotides with higher T_m values are inhibited by bound PLC β . This finding is supported by microarray analysis in cells over-expressing PLC β 1. Taken together, this study allows predictions of the genes whose post-transcriptional regulation is responsive to the G protein/phospholipase C β /calcium signaling pathway.

Keywords: phospholipase C β ; RNA interference; G protein signaling; calcium signaling; component 3 of RNA-induced silencing

Introduction

The RNA-induced silencing complex (RISC) has been estimated to regulate 30% of mammalian genes.¹ 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.³ 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

Abbreviations: C3PO, component-3 promoter of RISC; FRET, Förster resonance energy transfer; GPCRs, G protein coupled receptors; miR, microRNA; PLC β , mammalian inositol-specific phospholipase C β ; RISC, RNA induced silencing complex; siRNA, small-interfering RNA; TRAX, translin associated factor X

Additional Supporting Information may be found in the online version of this article.

We have found that G protein signals are linked to RNA-induced silencing through phospholipase C β and the nuclease C3PO. Here, we describe the specificity of C3PO—oligonucleotide associations and how they are perturbed by PLC β . These studies may ultimately allow us to predict which genes are impacted by G protein signals.

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been shown to function in diverse cellular processes such as chromosomal translocations,^{4–6} neuronal development,⁷ mRNA transport,⁸ and tRNA processing.^{9,10} 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 PLC β .¹¹ PLC β mediates calcium signals (see Refs. 12,13) generated through receptors coupled to G α_q family of heterotrimeric G proteins whose ligands include acetylcholine, dopamine, bradykinin, angiotensin II, and others.^{14,15} In cells, PLC β and C3PO interact in the cytosol and studies using purified proteins show that PLC β binds to an external site on one or both of the TRAX subunits of C3PO.^{11,16} C3PO binds to the same region of PLC β as its activator, G α_q , and high levels of C3PO will quench Ca²⁺ signals generated by G α_q -coupled GPCRs in cells.¹⁷ Similarly, over-production of PLC β can reverse siRNA downregulation of genes such as GAPDH and LDH presumably through its interaction with TRAX.¹⁶ However, PLC β 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 PLC β is bound.¹⁸

RNA-induced silencing is increasingly being considered as a therapeutic strategy for a variety of diseases (e.g. <http://rnaitherapeutics.blogspot.com>). However, many questions remain about the regulation of RNA silencing in cells, and the connection between gene silencing and G protein signaling is just beginning to be uncovered. In this study, we have characterized binding and nuclease activity of C3PO and its regulation by PLC β . These studies allow for predictions about which miRs are regulated by C3PO and which are affected by G protein signals.

Results

Characterization of the binding specificity of C3PO

We established the binding specificity of C3PO by SELEX.¹⁹ 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.^{3,20} The library consisted of $\sim 1 \times 10^{18}$ 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 [Fig. 1(A,B)].^{21,22} 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.

Predicted structures for the selected RNA sequences

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 (Fig. 2).²³ 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 parameters^{24,25} (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.

We next tested whether we could predict sequences that contribute to specific binding to C3PO. We started from 5 million random sequences of 30 bps, flanked with the same regions as in the SELEX experiment, bringing the oligonucleotides to a size of 59 bps. We then filtered those sequences that exhibited an average slide force constant greater than 2.8 kcal/(mol Å²) and those with a tilt force constant lower than 0.031 kcal/(mol deg²). These sequences are easy to bend into the backbone and it is hard to displace bases along the backbone when in a

Figure 2. *Top* Comparison of histograms showing distribution of number of “duplex” pairings in the 90 sequences generated from SELEX to 11,000 random sequences from *in silico* studies. *Bottom* Predicted structures of some of the RNA sequences identified by SELEX

A A [6’FAM]GGCATTACGGCCGGGTGAATGAATTATAATTATAGTAATTAGAGCGTCTTCTAGAGC
 B [6’FAM]GGCATTACGGCCGGGAGGATATATTGTATGTTAGGATGCAGTTGAGCGTCTTCTAGAGC
 C [6’FAM] GGCATTACGGCCGGGTTGTTTGTTGATAAATGTATGTAATGAGAGCGTCTTCTAGAGC

Figure 3. (A) Sequences of the oligonucleotides used for experimental binding studies where the nucleotides shown in red have been varied as described in the text. (B) Binding of 6’FAM labelled ssDNA sequences to inactive C3PO alone and (C) with bound PLCβ. Studies were performed at room temperature in 20 mM HEPES, 0.16M NaCl, pH 7.4 and the data are a compilation of three independent experiments where the titrations were done in triplicate. The concentration of DNA was held constant through the titration. Data are shown for 15 nM and identical Kd values were obtained at 30 nM FAM-ssDNA

Figure 4. (A) FRET efficiency of 25 nM C3PO labeled with both Alexa 488 and Alexa 555, was measured at room temperature and in 20 mM Hepes, 0.16M NaCl, pH 7.4 in the absence and presence of 25 nM DNA [sequence B shown in Fig. 3(A)]. Also shown is the change in FRET when 25 nM PLCβ was added to the C3PO-DNA complex. FRET was monitored by the % decrease in donor intensity. Identical results were obtained with the C ssDNA sequence, where $n = 6$. (B) FRET efficiency when ssDNA (either sequence B or C) was added to the C3PO-PLCβ complex. (C) Schematic of the FRET results

and lower (C)). The DNAs were labeled on their 5' terminus with a fluorescent probe (FAM). Binding of the oligonucleotide to C3PO was monitored by the increase in fluorescence anisotropy of the FAM probe. Using a longer DNA-RNA construct, we verified that the inactive mutant bound oligonucleotide with an affinity identical to the native complex¹⁸ and additionally found that binding is substantially weakened (at least 20 fold) when the salt concentration is raised to 0.5M NaCl. Figure 3(B) shows the binding curve of C3PO to the three different oligonucleotides at two initial DNA concentrations (30 and 15 nM) and shows the dissociation constants derived from these experiments. The K_d values were within error for both 15 nM and 30 nM DNA experiments supporting the idea that we are observing equilibrium binding. These results show that all 3 sequences bind strongly and similarly to C3PO. Since the sequences differed in their ability to bend along the short axis and the ability of the bases to slide, these data show that these characteristics play little, if any role in C3PO binding.

We then determined the effect of PLCβ on oligonucleotide binding to C3PO. We note that there are 4 known isoforms of PLCβ that all have the characteristic ~400 amino acid C-terminal tail which is required for strong C3PO binding.¹¹ The studies here used either PLCβ1 or β3 and it is probable that the PLCβ2 and here 4 isoforms behave similarly. Unexpectedly, we find that when we add oligonucleotides to the pre-

formed C3PO-PLCβ complex, oligonucleotide binding is weakened by approximately factor of 20 [Fig. 3(C)].

The reduced oligonucleotide binding of C3PO with bound PLCβ suggests that PLCβ may affect conformational changes in C3PO required for DNA/RNA binding (see Ref. 26). We investigated this possibility using Förster resonance energy transfer (FRET). These studies were performed by engineering an inactive C3PO mutant containing two Cys residues: one on each of the TRAX subunits, whose approximate distance as estimated from the crystal structure of hC3PO, is ~70Å (Supporting Information Fig. S1). The results are shown in Figure 4(A,B) and summarized in the schematic in 4(C). For C3PO, the average FRET efficiency was approximately 31%. For the pre-formed C3PO-PLCβ complex, the FRET efficiency increased to 38%. After nucleotide was added to free C3PO there was a marked increase in FRET efficiency to 43%. After PLCβ was added to the C3PO-oligonucleotides, the FRET efficiency increased to ~50%. In contrast, we could not detect a change in FRET efficiency when the nucleotides were added to the pre-formed C3PO-PLCβ complex even at concentrations where the oligonucleotide should be completely bound according to the K_d value measured above. These studies show that the binding of either PLCβ, or the binding of oligonucleotides to C3PO result in a clamping of the TRAX subunits. Importantly, the binding of PLCβ to C3PO prevents the conformational changes required for strong oligonucleotide binding.

Hydrolytic activity of C3PO varies greatly with oligonucleotides

Even though oligonucleotides bind to C3PO with little specificity, our previous studies suggested that the hydrolytic activity of C3PO is sensitive to oligonucleotide sequence.²⁷ Additionally, we note that nucleases involved in gene-silencing, especially Ago-2, have been reported to prefer sequences enriched in U or A as compared to C or G at the 5' end.²⁸ To determine whether the activity of C3PO was sequence dependent, we determined the variation in catalytic rates of cleavage of different oligonucleotide sequences and structures. In an initial screen, we prepared ssDNAs with varying A/T and G/C content and placed a fluorophore on the 5' end and a quencher on the 3' end. Thus, degradation of the oligonucleotide as catalyzed by C3PO results in a de-quenching of the probe and an increase in fluorescence which can then be fit to a first order exponential to yield a rate constant. We find that oligonucleotides composed only of A/T bases are hydrolyzed 100 fold faster than ones composed of G/C [Fig. 5(A)]. The addition of PLC β did not greatly change the rates of hydrolysis of each of the oligonucleotides although a delay in hydrolysis seen for the in the A/T sample, which is consistent with a reduction in oligonucleotide binding due to PLC β (see Discussion). These results show that C3PO catalysis depends on the A/T content, and in turn, the stability and T_m of the oligonucleotide sequence

We extended these studies by measuring C3PO activity towards several stem-loop structures in the form of RNA beacons. RNA beacons are designed to detect specific mRNAs by having a loop region that will hybridize to a target mRNA sequence (see Ref. 29). This hybridization results in opening of the stem region and dequenching of the fluorophore on the 5' end as it moves away from the quencher on the 3' end.

Unlike our previous work where we quantified the effect of PLC β on C3PO–oligonucleotide binding at very limiting substrate concentrations,¹⁸ here, we measured binding under conditions where the concentrations of all 3 components are similar and competitive to better understand situations that might occur in the cell. We find that the apparent dependence of hydrolysis rates on T_m appears to carry over to stem-loop structures that varied in sequence and stability [Fig. 5(B), Table II]. Importantly, we find that the rates of hydrolysis for these structures directly correlate with the T_m of the loop region. However, when PLC β is bound to C3PO, the rates of hydrolysis of the slowly degraded, high T_m oligonucleotides are uniformly decreased, but the presence of PLC β has little effect on the ones in the faster, low T_m group. The effect of PLC β on the hydrolytic rates seem to fall into two regimes that depend on the T_m of the oligonucleotide [Fig. 5(C)]. As explained in the discussion, these

results are consistent with the idea that by blocking oligonucleotide binding, PLC β primarily affects hydrolysis that requires oligonucleotide turnover.

Microarray studies show that expression of PLC β increases the populations of lower T_m miRs

The results presented above predict that in cells, the binding of PLC β to C3PO will reduce the hydrolysis of miRs with higher T_m values and changing the distribution of miRs. To determine whether this is the case, we performed a microarray study that compared the miR distribution in HEK293 cells with low endogenous PLC β and HEK293 cells over-expressing PLC β . When we calculated the T_m values of the miR that are upregulated by PLC β by a factor of ~ 2 [Fig. 6(A)], and compared these to the T_m values for the 13 miRs that are most strongly downregulated by PLC β [Fig. 6(A)], we find that the T_m of upregulated group appears to be lower ($p = 0.016$) [Fig. 6(B)]. These results suggest under cellular conditions where PLC β is bound to C3PO such as those that occur during differentiation,³⁰ the hydrolysis of more stable miRs are inhibited and miRs with lower T_m values are preferentially produced.

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.²¹ Structural studies have provided speculative information about the mechanism of C3PO catalysis.^{2,31} Here, we have focused on how C3PO may be regulated in cells through its interaction with PLC β .

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 (see Ref. 32). We determined C3PO binding specificity by screening a library of $\sim 10^{18}$ 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

Figure 5. Hydrolysis of different oligonucleotides at 25 nM that were labeled with a fluorophore on the 5' end and a quencher on the 3' end as catalyzed by 5 nM C3PO. Cleavage was followed by an increase in fluorescence as the fluorophore is dequenched monitored at room temperature in 20 mM Hepes, 0.16 M NaCl, pH. 7.4. (A) model ssDNAs, where the sequence, T_m and cleavage rate is given in the legend. (B) examples of the effect of PLC β on C3PO hydrolysis rate of a stem-loop structure with a higher T_m value (left), and one with a lower T_m value (right) where $n = 3$. (C) Graph showing the effect of PLC β on the rates of hydrolysis with T_m where the stem-loop structures are given in black circles, siRNAs are in blue, and model DNAs are in red. The slashes signify discontinuities on the x-axis to include all points on an expanded scale

Table I. Estimated Distances Between TRAX 257 Side Chains for C3PO Alone, When the Two DNA Sequences Given in Fig. 3A are Added, and When PLCβ is Added to the C3PO-DNA Complex

	Sequence A	Sequence B
C3PO	80 Å	80 Å
+ DNA	73 Å	74 Å
+ PLCβ	70 Å	71 Å

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.⁹

C3PO clearly hydrolyzes sequences and structures at very different rates. Comparing linear oligonucleotides rich in A/T to ones rich in G/C reduces the rate of hydrolysis 100 fold. Using RNA beacons, we find that the hydrolysis rates are directly linked to the T_m of the loop or the stem regions, and thus hydrolysis depends on the stability of the nucleotide in a predictable way. Given that binding is nonspecific, then the primary indicator of which miRs will prevail in a given situation depends on the stability of the miR and the number of copies in the cell.

We have shown that PLCβ binds to an external site on TRAX or between the two TRAX subunits of C3PO and this binding inhibits activity.¹⁸ Oligonucleotides bind in a central cavity between a translin tetramer and a translin-TRAX dimer that may involve a partial opening of the octamer with reassembly around the substrate.³³ Our FRET studies show a reduced distance between the TRAX subunits with oligonucleotide binding consistent with a clamping of the back end of the octamer. PLCβ reduces the distance between the two TRAX subunits, and inhibits oligonucleotide binding suggesting that PLCβ inhibits the necessary conformational changes in C3PO needed for productive binding. The studies here use blunt-ended linear and stem-loop oligonucleotides. siRNA have overhanging single stranded dinucleotides on the 5' and 3' ends and our previous studies show their hydrolysis is influenced by PLCβ.¹⁸ We propose that PLCβ restricts the same conformational movements in C3PO used to process siRNAs as it uses to bind the blunt-ended stem loop structures. We speculate inhibition is due

to PLCβ blocking substrate going into or out of the central C3PO cavity. While this model explains PLCβ's ability to inhibit C3PO in cells, it does not explain the specificity of PLCβ inhibition towards select genes.

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 T_m. The reduction in rate of more stable oligonucleotides by bound PLCβ appears to be independent of substrate structure [Fig. 5(C)]. The ability of PLCβ to inhibit slower reactions can be explained by the simple rate equations that stem from the coupled equilibria shown in Figure 7. 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 it has dissociated PLCβ. If the hydrolysis rate is fast, as in the case for miRs with low values of T_m, the C3PO-RNA complex rapidly returns to free C3PO. However, if the T_m 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 T_m of the oligonucleotide falls into line with the behavior seen for the blunt-ended oligonucleotides [red points, Fig. 5(C)]. 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 T_m oligonucleotides would dissociate from the complex before they are hydrolyzed. This idea would be consistent with the data in Figure 3 showing that DNA has a lower affinity, and possibly a faster off rate from the complex as compared to free C3PO.

We determined whether this simple coupled equilibria model occurs in cells by monitoring changes in miR populations with PLCβ over-expression. We find that over-expression produced large changes in many miRs that are associated with a variety of pathologies. In accord with our solution results showing that PLCβ only affects the activity of C3PO towards more stable oligonucleotides, we find that the 13 miRs that are upregulated by PLCβ over-expression have a compiled lower T_m as compared to the 13 miRs that are downregulated

Table II. Rates of Hydrolysis of Stem-loop Structures

Sequence (all with 5' FAM and 3'BHQ)	T _m °C	k(C3PO)/s	k(C3PO-PLCβ)/s
GGAGCAAGTTGGACGAGAGGCGGGCTCC	65	95 ± 4	38 ± 7
GAGACGGAGGGGGCGAGAAGGGTCTC	60	20 ± 6	10 ± 7
CACGCCCCCGAAGTTTGCTGCGTG	68	500 ± 10	200 ± 6
CGGCACAGTGTCTTCTCCCTTCCCTGCCG	56	1200 ± 40	1400 ± 10
GGACCTCACAAGTTGGACGAGAGGGTCC	54	2500 ± 10	2500 ± 60
GGACCTCACAAGTTGGACGAGAGGGTCC	51	1100 ± 6	1300 ± 12

Figure 6. (A) Results of microarray data showing the top miRs that are upregulated and downregulated when PLC β is over-expressed. (B) Box plots showing the values of T_m compiled for the most upregulated and downregulated miRs given in panel A

(Fig. 6). We speculate that we may ultimately be able to predict which miR families are impacted by the presence of PLC β . We note that the cellular level of PLC β can be quite variable depending on the cell line as well as the growth and culture conditions. For example, we have found that neuronal cells exhibit a large increase in PLC β concentrations during the early stages of differentiation, resulting in a substantial increase in PLC β -C3PO complexes, and that these complexes are required for cells to move into the differentiated state.³⁰ Importantly, the levels and distributions of different miR populations are expected to vary widely depending on the cell type and local conditions. These miRs all compete

for C3PO binding, which is not specific, and thus it is only the subset of the stable miRs that will be affected by PLC β .

In summary, the cellular activity of C3PO impacts RNA silencing, tRNA processing and other critical cell functions. While the substrate selection of C3PO is nonspecific, its activity is highly dependent on substrate stability. Oligonucleotide stability drives the impact of the enzyme PLC β , which connects C3PO function to G protein signaling, on C3PO function. Our studies describe a subset of genes that have the potential to be regulated by GPCRs coupled to the G α q/PLC β pathway and which will function independently of these signals. These studies show that predictive models of RNA silencing should include the ubiquitous PLC β enzymes.

Figure 7. Diagram showing the competitive reactions between C3PO, PLC β and RNA where the dissociation constants are for the DNAs measured here (Fig. 3) and previous values for the association of PLC β to C3PO and C3PO-DNA have been reported.¹⁸

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 PLC β 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

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).

The DNA obtained after the RT-PCR step of SELEX rounds 6 and 10 was ligated into pUC8 vector (1:3 and

1:5 plasmid:insert ratio) after cutting both the DNA and the plasmid with the restriction enzymes EcoRI and XbaI, and treating with shrimp alkaline phosphatase to prevent self-ligation. The ligated pUC8 plasmids were cloned into bacterial competent cells by transformation. The successfully ligated clones were selected by ampicillin resistance and the plasmid was isolated by Miniprep kit (Qiagen). The plasmids were sequenced with primers for pU8 vector to get the inserted DNA sequence which provides the information about the selected RNA for comparative analysis.

Preparation of inactive C3PO

C3PO was rendered inactive by mutating Asp193 to Ala of both TRAX subunits using Quikchange II mutagenesis kit (Stratagene) and confirming by sequencing and gel based DNA cleavage assays.

Computational analysis of the sequences selected from SELEX

Multalign program was used to perform multiple sequence alignment for selected sequences.²¹ The MEME program from the online motif study portal MEME suite was used to predict the presence of common motifs in the selected sequences.²² BLAT (BLAST Like Alignment Tool) online tool from UCSC genome server was used to see if the sequences matched any known genes or parts of genome.³⁶ RNAfold server was used for the prediction of the RNA structure for the selected RNAs.²³

Sequence results from SELEX studies were evaluated by comparing them with the properties of random RNAs generated by in house python scripts and were analyzed and filtered on the basis of structural and flexibility base pair step parameters.²⁴ A standalone copy of RNAfold was used to analyze the secondary structure of filtered sequences. To check the structural/flexibility profile, we used moving average values of the sequences (step of 10 base pairs), rather than individual values at each base pair step which would have been too noisy.^{36,37}

Protein labelling

C3PO was labeled with Alexa 488 and/or Alexa 555 (Life Technologies) by adding a 1.5 stoichiometric amount of purified protein, gently mixed, and left on ice overnight for 12 hours. The labeled protein was separated from free probe by loading the solution into a PD-MiniTrap column containing freshly swollen and washed Sephadex-200 beads at 4°C, and eluting with buffer (20 mM Hepes, 0.16 M NaCl, pH 7.4).

Anisotropy and FRET experiments

Measurements were performed on an ISS PCH spectrofluorometer (ISS, Urbana, IL). All studies were done at room temperature. Anisotropy studies were used to follow the binding of 6'FAM-oligonucleotides to C3PO in 20 mM Hepes, 0.16 M NaCl, pH 7.4. For

FRET measurements, we compared singly labelled C3PO (either Alexa 488 or Alexa555) and doubly labelled C3PO along with controls of PLCβ and three different unlabeled DNA strands were analyzed. The unlabeled controls were shown not to influence the intensity at the given wavelengths and were therefore negated. In some studies, DNA was added to C3PO prior to PLCβ, and in others, PLCβ was added to C3PO before DNA, allowing the C3PO-PLCβ complex to form. FRET was measured by exciting Alexa 488 donors at 490 nm and measuring the increase in Alexa 555 acceptor emission at 580 nm, and the efficiency was calculated using: $1 - \frac{F_d'}{F_d}$, where F_d' is the donor fluorescence in the presence of acceptor, and F_d is the donor fluorescence without acceptor. For F_d , the single labelled Alexa 488 C3PO intensity at 525 nm was used, and for F_d' the double labelled Alexa 488 and 555 C3PO intensity at 525 nm was used. These values were then graphed into a box-plot after 6 trials. Distances between the probes were calculated using:

$$E = \frac{1}{1 + (r/r_0)^6}$$

Hydrolysis of ssDNA and stem-loop DNA

The cleavage rates for FAM and BHQ1 labeled ssDNA with varying AT and GC contents, and that for the FAM-BHQ1 labeled stem-loop DNAs were determined by measuring the increase in the labeled oligonucleotide fluorescence as the FAM separated from BHQ1 due to cleavage catalyzed by the addition of C3PO. This increase in fluorescence was monitored at room temperature over time until a plateau was reached. The rates were calculated by fitting the curves to a first order exponential rise using Sigmaplot.

Microarray studies

Total cell RNA was extracted by using a miRNeasy Kit (Qiagen, Valencia, CA, USA) following manufacturer's protocol. After assessing RNA quality and quantity, miRNA was profiled using Affymetrix GeneChip 3.0 miRNA arrays (Santa Clara, CA, USA). Briefly, total RNA was labeled with the Affymetrix FlashTag Biotin HSR RNA Labeling kit and then hybridized overnight with the array, according to the manufacturer's specifications. Following hybridization, the arrays were washed, stained and scanned in an Affymetrix GeneChip Scanner 3000 7G. The scans were performed and analyzed using Affymetrix Command Console and Expression Console software.

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