An RNA aptamer that interferes with the DNA binding of the HSF transcription activator

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ABSTRACT

Heat shock factor (HSF) is a conserved and highly potent transcription activator. It is involved in a wide variety of important biological processes including the stress response and specific steps in normal development. Reagents that interfere with HSF function would be useful for both basic studies and practical applications. We selected an RNA aptamer that binds to HSF with high specificity. Deletion analysis defined the minimal binding motif of this aptamer to be two stems and one stem–loop joined by a three-way junction. This RNA aptamer interferes with normal interaction of HSF with its DNA element, which is a key regulatory step for HSF function. The DNA-binding domain plus a flanking linker region on the HSF (DL) is essential for the RNA binding. Additionally, this aptamer inhibits HSF-induced transcription in vitro in the complex milieu of a whole cell extract. In contrast to the previously characterized NF-κB aptamer, the HSF aptamer does not simply mimic DNA binding, but rather binds to HSF in a manner distinct from DNA binding to HSF.

INTRODUCTION

Heat shock factor (HSF) is a potent transcription activator that is highly conserved from yeast to humans. HSF plays a central role in activating gene expression in response to environmental stresses including heat shock, and regulates a wide range of downstream target genes in the genome (1). A genome-wide study showed that ~3% of Saccharomyces cerevisiae genes are functional targets of HSF. Many are involved in a wide variety of important cellular functions such as signal transduction, energy generation, vesicular transport and chaperone function (2). HSF function is essential for the stress response, for viability in yeast (3) and for early development in Drosophila (4). HSF is also involved in the aging process in Caenorhabditis elegans (5), as well as in extra-embryonic development in mammals (6). In addition, downregulating HSF activity sensitizes cancer cells to some anti-cancer drugs (7).

HSF, which functions during heat shock as a homo-trimer, has a highly conserved DNA-binding domain and trimerization domain, and a less conserved activation domain. Trimerized HSF binds tightly to a conserved heat shock element (HSE) that is composed of the basic unit, ‘AGAAn’, arranged as inverted repeats; e.g. a 15 bp sequence containing three such units, called HSE3 (AGAAGTTCTAGAAG), is a good binding target for an HSF trimer (8). In between the DNA-binding domain and trimerization domain, there is a flexible linker region that is essential for positioning the DNA-binding domain in a HSF homotrimer (9). Upon heat shock or other stresses, the trimerization domain, which contains leucine zipper repeats become available for multimerization, and the resulting HSF trimers bind tightly to HSEs of heat shock genes (1). HSF activates transcription by further recruitment of other important transcription factors or complexes such as mediator complex to the heat shock promoters (10).

A major goal of our laboratory is to identify specific reagents that can interfere with particular macromolecular interactions in order to dissect transcriptional mechanisms in vitro and in vivo (11,12). Heat shock genes provide an attractive model system for these studies. Because the HSF/DNA interaction is a key regulatory step in heat shock gene activation, generating reagents that can specifically disrupt this interaction is critical. RNA aptamers are reagents that can be selected from a random RNA sequence pool for their ability to bind tightly to a protein target. Once isolated, such aptamers can be used to interfere with specific macromolecular interactions for evaluating mechanistic questions both by simply adding the aptamers to in vitro transcription
systems or by expressing aptamer-encoding genes at high levels in cells and organisms (11,13).

Only a few RNA aptamers have been selected against transcription factors that recognize specific DNA sequences. The best-characterized example is an NF-xB aptamer. This RNA aptamer has a structure that mimics the structure of normal DNA element binding to NF-xB, when the aptamer is bound to the protein (14). This example raises the possibility that transcription factors might have a common nucleic acid-binding surface for both endogenous and selected nucleic acid molecules (14).

We characterized an HSF aptamer and show here that it can interfere with the normal interaction of HSF and DNA. However, this aptamer binds to HSF in a manner mechanistically distinct from that of DNA binding to HSF, demonstrating that such selected RNA aptamers can bind transcription factors by mechanisms that do not simply mimic the DNA element. The elaborate structural features of this HSF aptamer, namely a three-way junction structure might account for some of its surprising properties. Furthermore, the ability to mechanistically inhibit HSF function also makes this aptamer a molecular tool with potential significance in clinical applications where diseases are influenced by HSF activity.

MATERIALS AND METHODS

Proteins and SELEX

Baculovirus expressed dHSF was purified as described elsewhere (15). MBP-fused dHSF and His-tagged full-length yHSF were expressed in Escherichia coli and purified with conventional affinity column chromatography. Partial yHSF proteins and point mutation yHSFs were expressed and purified using previously described protocols (9). The linker peptide (underlined) and extra residues for dimerization (WQFENFIRGREDLLEKIIRQKGSSNACLIN) was synthesized on a continuous flow PerSeptive Biosystems (Framingham, MA) peptide synthesizer and purified to homogeneity by reversed-phase C18-high-performance liquid chromatography.

The selection of RA1-HSF aptamer was performed using MBP-fused dHSF and the SELEX method based on nitrocellulose filter partitioning with final selection by electrophoretic mobility shift assay (EMSA) (11). We preformed 14 cycles of selection and selected 5 identical sequences (named as ‘RA1-HSF’), from a total of 20 sequences cloned from the final stage pool. The remaining 15 sequences showed no detectable HSF-binding activity.

EMSA

The general scheme of EMSA was adapted and modified from previous work (16). RNA probes were internally labeled with [α-32P]UTP by using a T7 in vitro transcription kit (MAXScript Kit; Ambion, Austin, TX). DNA is end-labeled with [α-32P]ATP with T4 polynucleotide kinase. The 10 μl binding solution contains 1x binding buffer (10 mM Tris, 40 mM KOAc and 1 mM MgCl2, pH 7.6), 1 μg carrier yeast RNA, 4 μg carrier BSA, 5 mM DTT, 10% glycerol, 6 U of Superase-In (Ambion), plus protein and labeled RNA. The concentration of the labeled RNA probe was below 1 nM in most experiments to ensure an excess protein concentration. Protein and RNA were incubated at room temperature for 30 min, and 10 min at 4°C before loading on a 6 or 9% native polyacrylamide gel or a 2% agarose gel. The polyacrylamide gels contained 1/4 TBE buffer and 1 mM MgCl2, and the agarose gels contained 1× TAE buffer. Gels were run at 100–150 V at 4°C for 1–2 h. They were then dried and exposed over a phosphorimager plate, and scanned after 4 h overnight exposure using a STORM image scanner.

Competition assay

Competition assays were performed in the same binding solution as described for EMSA. For DNA and RNA competition assays, RNA aptamer probes were labeled with [α-32P]UTP as described above. The HSE3 DNAs and the HSE3 RNA were end-labeled with [γ-32P]ATP with T4 polynucleotide kinase. An excess of a particular ice-cold DNA or RNA was co-incubated with the labeled RNA or DNA and HSF protein for 30 min at room temperature, and examined by EMSA. In the protein–protein competition assay (Figure 4B), the RA1-HSF aptamer was labeled as above, and different amounts of each protein construct were incubated together with the RNA for 30 min at room temperature to allow competition for the binding to the RNA. Samples were submitted to gel electrophoresis and exposed as described above for EMSA.

Double-strand annealing experiment

Annealing of the two RNA strands was performed by incubating the labeled RNA strand A and unlabeled RNA strand B at 70°C in 1x binding buffer for 10 min, and the temperature was reduced to room temperature gradually. Both the annealed RNA mixture and labeled single strand of RNA alone were incubated with 40 nM dHSF at room temperature for 30 min before loading on to an agarose gel for the EMS assay.

In vitro transcription assay

Yeast Strain BJ1991 (prb1 pep4 gal2 leu2 trp1 ura3) was grown in yeast extract/peptone/dextrose (YPD) to an OD600 of 2.0. Cells were harvested, and whole cell extracts were prepared by using a mortar and pestle as described previously (17). Protein concentration was determined by Bradford assay. In vitro transcription was performed based on a protocol adapted from Ref. (12). Briefly, transcription reactions were carried out at room temperature in a 25 μl final lume using a plasmid template pJJ461 (200 ng) that contains an upstream HSE (CTTCTAGAAGCTTCTAGAAG) and the yeast CYC1 promoter fused to a 290 nt G-less cassette. Yeast whole cell extract (120 μg) was incubated for 2 min in transcription buffer [20 mM HEPEs, pH 7.6, 100 mM potassium glutamate, 10 mM MgOAc, 5 mM EGTA, 2.5 mM DTT, 10 μM ZnSO4, 10% glycerol, 20 U of RNase Inhibitor (SUPERase-In; Ambion) plus an ATP regeneration system (3 mM ATP, 30 mM creatine phosphate and 150 ng of creatine kinase). Aptamers and recombinant proteins were added to the extract mixture at the concentrations indicated, together with the addition of DNA template. Transcription was initiated with NTPs (10 μCi of [α-32P]UTP, 50 μM UTP, 250 μM CTP and ATP, final concentrations) and terminated with stop solution (10 mM Tris, 20 mM EDTA, 0.2 M NaCl,
1 μg of glycogen and 25 U of RNase T1, pH 7.6). The samples were incubated at 37°C for 30 min, digested with proteinase K in the presence of SDS (2%) for 20 min before being phenol/chloroform extracted and ethanol precipitated. RNA products were separated on a 6% polyacrylamide sequencing gel.

RESULTS
Defining the critical sequences of the RA1-HSF aptamer required for HSF binding

An RNA aptamer against HSF was selected from a pool of $10^{14}$ RNA molecules that can bind to bacterially expressed dHSF. The mfold program (18) predicted that the most stable RNA secondary structure is composed of a three-way junction radiating three different stem–loops, which we defined as stem–loops 1, 2 and 3 as shown in Figure 1A. The predicted stem–loop 1 is essential for the aptamer function and could not be shortened (data not shown). We defined the minimal functional motif by trimming both stem–loops 2 and 3 in a distal-to-central manner and testing the resulting RNAs for HSF-binding activity by EMSA. A 45 nt sequence was finally defined as the minimal structure that still carries detectable binding activity: 1 bp less from either stem–loop 2 or 3 resulted in sharp decrease of binding activity (Figure 1B and C). This minimal aptamer structure, which we refer to as the CORE (Figure 1D), is relatively large and complex compared to most other minimal functional motifs from other identified RNA aptamers, indicating that its interaction with HSF is likely to be extensive. The apparent binding $K_d$ for full-length aptamer binding to the full-length dHSF is 20–40 nM and 40–80 nM for the CORE aptamer. However, we cannot rule out that certain bases in the middle of the CORE sequence may be deleted or replaced without compromising aptamer-binding activity.

Figure 1. Characterization of the HSF-binding aptamer RA1-HSF. (A) The secondary structure of RA1-HSF predicted by the mfold program. (The arrow points to a region on stem–loop 3 that has an ‘AGAAU’ sequence, which is similar to a repeating unit of an HSE.) (B) A sketch of a deletion series of constructs that were designed and used to define the minimized binding motif of the aptamer. Each deletion construct trims one more base pair from either stem–loop 2 or 3, keeping the other stem–loop end connected with either the original loop or an extra tetraloop (CUUCGG, represented by a small square). The constructs marked with a star mark were chosen for analysis by EMSA shown in (C). (C) The HSF binding of labeled constructs examined by EMSA, where Core is the minimized RNA-binding construct shown in (D); A3t4 is the Core shortened by 1 bp on stem–loop 3; A2t2 is the Core shortened by 1 bp on stem–loop 2; RA1-HSF is the original full-length aptamer shown in (A). (The shifted positions for the different RNA–protein complexes are similar because the size difference between RNA constructs is relatively small compared to the full-length dHSF trimer/RNA complex). (D) The predicted secondary structure of the ‘Core’ is the same as that shown in the squared region of (B). (Note that there is a tetraloop holding the trimmed stem–loop 2 end in the real construct.) (E) The binding curve for RA1-HSF aptamer to HSF protein. The percentage of bound fraction of RA1-HSF to the protein versus total RNA (y-axis). The concentration of HSF protein in nM (x-axis). The error bars represent standard errors from three independent EMSA experiments (except for the 10 nM point, which shows the range from two experiments).
Confirming the three-way junction structure of the aptamer by a double-strand annealing experiment

To test whether the three-way junction structure that was predicted by mfold is the active conformation, we sought to assemble this structure by an independent method, where extra base pairing on stem–loops 2 and 3 ensures the formation of the three-way junction. We designed a double-stranded RNA annealing experiment where the CORE aptamer sequence was divided between two complementary RNA molecules (Figure 2A and B). We tested whether annealing of these two RNA molecules could reconstruct the aptamer-binding activity. If the real secondary structure was different from the predicted structure, this reconstruction of activity would very likely fail. The additional base pairs lock in two of the predicted stems and minimize the potential for additional structures involving bases in the third stem–loop. The annealing of these two RNAs produced strong HSF-binding activity as tested by EMSA, whereas individual RNAs alone had no activity (Figure 2C). These results provided an independent test of the three-way junction nature of the aptamer structure. Furthermore, this experiment suggested another level for modulating the activity of an aptamer by ‘heterodimer design’. The fact that the function of an aptamer depends on the presence of two separate RNA molecules provides a strategy for tightly controlling its activity.

RA1-HSF binds specifically to the HSF protein

Because numerous nucleic acid-binding proteins exist in a cell, it is important to show the HSF aptamer binds with specificity to its proposed target, HSF. First, we examined

Figure 2. Test of the three-way junction structure of the aptamer by a double-strand annealing experiment. (A) A schematic illustration of the double-strand annealing experiment. (B) The secondary structures for strand A and strand B alone as predicted by the mfold program; and strand A annealed with strand B. (C) EMSA showing the binding Hsf to strand A, strand B alone or annealed strand A and strand B.

Figure 3. Testing the specificity of the aptamer RNA–protein interaction. (A) The interactions of labeled RA1-HSF RNA with different transcription factors, TBP, GAGA and Gal4-VP16 (G4-VP) were tested by EMSA. (B) EMSA performed by using labeled RA1-HSF in SF9 cell lysate with vector expressing dHSF (+) or with empty vector (−). (C) Competition assay done by using 100-fold unlabeled yeast RNA (yR), HSE3 dsRNA (HSE3R), HSE3 dsDNA with a single point mutation (HSE3m), and HSE3 dsDNA (HSE3) to compete with labeled RA1-HSFaptamer binding to HSF protein.

the specificity of this aptamer by testing the interaction of this aptamer to several other transcription factors (TBP, GAGA factor, Gal4-VP16) that bind to DNA. None of them showed any binding activity to the aptamer even at a protein concentration of 250 nM (Figure 3A). Second, the specificity of the aptamer/HSF interaction was tested in the background of whole, insect-cell lysate proteins. Insect culture cells (SF9 cells) that do or do not express Drosophila
HSF protein were lysed, followed quickly by EMSA using $^{32}$P-labeled RA1-HSF. The aptamer RNA could form a single RNA–protein complex band only with the cell lysate that expresses dHSF, which indicated that this aptamer specifically recognizes dHSF at least in the background of whole insect cell lysates (Figure 3B).

Interestingly, a double-stranded region on the stem–loop 3 of the aptamer has the sequence ‘AGAAU’, which corresponds to the 5 bp repeating unit of the HSE DNA sequence (Figure 1A). However, a dsRNA containing the sequence resembling HSE3 dsDNA failed to compete with the labeled aptamer for binding to HSF (Figure 3C). This result ruled out the possibility that this aptamer binds to HSF simply through the part of double-stranded RNA that carries the corresponding sequence of HSE DNA, though this is not out of expectation given the difference of helix structure between DNA and RNA (19).

This aptamer binds both bacterially expressed dHSF and insect-expressed dHSF (Baculovirus expression system) with almost the same affinity. This implies that the binding is not significantly influenced by the post-translational modification of HSF (data not shown). Interestingly this aptamer also binds to yeast HSF1 protein with affinity similar to Drosophila HSF. Therefore, we used a yHSF deletion series to define the minimal region on HSF for aptamer binding in the following experiments.

The DNA-binding domain plus its flanking linker region (DL) of the HSF protein is essential for binding aptamer RNA

Given that the aptamer RNA and HSE3 DNA are competitive in their binding to HSF, we anticipated that the RNA was likely to bind to the DNA-binding surface of the HSF protein, perhaps by structurally simulating HSE DNA. However, we observed that the interactions of HSF with the RNA aptamer and with HSE3 DNA show important differences. A previous study has shown that DNA-binding domain alone is sufficient for HSE3 DNA binding (9). Surprisingly, the DNA-binding domain alone is not sufficient for the RNA binding even at a protein concentration as high as 5 μM (data not shown). In order to define the region required for RNA binding, we used a deletion series of yHSF protein, starting with a peptide that contained the DNA-binding domain, the conserved 21 amino acid linker, the non-conserved 52 amino acid linker and the trimerization domain. This construct, which we refer to as DLT, binds to the aptamer at approximately the same affinity as the full-length protein. The non-conserved 52 amino acid linker was not required for RNA binding (compare lanes A and B in Figure 4B), and this part of Hsf1 is known not to be essential for structural integrity or in vivo function (9). However, the 21 amino acid conserved linker was absolutely required for RNA binding (compare...
lanes B, C, D and E in Figure 4B). Surprisingly, the trimerization domain was not required, as long as the peptide containing the DNA-binding domain and linker was dimerized through an added cysteine near the C-terminus (lane F in Figure 4B). Because EMSA could fail to detect weak interactions, we also performed a competition experiment. We used the different HSF domains to compete with the RNA binding to the DLT construct. The competition results show that the DL, but not the DNA-binding domain, monomeric or dimeric linker peptide (Lm, Ld), competes with the DLT for binding aptamer RNA (Figure 4D). This indicates that the DL peptide contains the minimal set of domains required for the aptamer binding.

The RNA aptamer binds to HSF in a manner distinct from HSE DNA binding to HSF. Though the DNA-binding domain alone is sufficient for HSE3 DNA binding, some mutations within the conserved protein linker region can dramatically decrease the DNA-binding activity presumably by changing the positional relationships of the DNA-binding domains in the trimer HSF (20). To test whether the linker requirements for the HSF binding to RNA are similar to those for HSF binding to DNA, we used six different point mutation versions of the DLT construct that varied at five conserved residues within the 21 amino acid conserved linker region (Figure 5). EMSA results showed no correlation between DNA binding and RNA binding to the proteins containing these point mutations. Some mutations diminished the DNA binding but not RNA binding, whereas some mutations diminished RNA binding but not the DNA binding (Figure 5).

We conclude that the binding pattern of the RNA aptamer to HSF is distinct from that of DNA binding to HSF. The results also further confirm that the conserved linker region is critical for the HSF interaction with the RNA aptamer.

The HSF RNA aptamer inhibits HS transcription in yeast cell extracts, and this inhibition activity is reversed by the addition of DL

The result that this aptamer could compete with DNA binding to HSF indicates that this aptamer could downregulate HSF transcriptional activity. We tested the effects of this aptamer on heat shock (HS) genes by using a yeast cell extract in vitro transcription system. RA1-HSF RNA was added into the yeast whole cell extract, which contains necessary components for HS transcription and a reporter yeast gene whose promoter has an HSE3 element (Materials and Methods). This yeast transcription system has been described and applied successfully to determine inhibitory effects of other aptamers against other transcription factors previously (12). The results in Figure 6 show that the RA1-HSF RNA aptamer inhibits the transcription on HS promoter at a concentration as low as 10 nM. Moreover, adding purified recombinant DL protein reversed this transcription inhibition (Figure 6). This result not only confirms the inhibitory activity of this aptamer on HS genes at least in a yeast cell extract transcription system, but also demonstrates that the inhibitory activity of this aptamer is specifically through the HSF interaction with DNA, since the presence of extra DL could reverse the inhibitory effects completely. In contrast, adding DL alone caused insignificant change to the overall transcription, which ruled out the possibility that DL reversed the inhibition by stimulating transcription through an independent activation pathway. By using DL instead of full-length yHSF, we avoided the possibility that the additional recombinant yHSF may squelch transcription by binding other proteins that interact with other domains of yHSF. Thus, the RNA aptamer appears to inhibit transcription through a specific interaction with the DL domain of HSF and, moreover, these results demonstrated the potential utility of aptamers in dissecting of transcriptional mechanisms.

Figure 5. The RNA aptamer binds to the HSF in a distinct manner with DNA HSE3 binding to HSF. (A) Comparing the binding of the RNA aptamer and the DNA HSE3 to the DLT HSF and to two of the mutated DLT HSF derivatives, LM2 (R274M) and LM5 (F261A). (B) A summary of binding activity of the RNA aptamer and the DNA HSE3 to DLT-yHSF and six point-mutant derivatives where ‘+++’ shows binding activity similar to wild-type HSF; ‘+’ shows lower than WT but detectable binding activity at a protein concentration of 1 μM; ‘−’ shows non-detectable binding activity at a protein concentration of 1 μM.

Figure 6. The HSF RNA aptamer inhibits transcription in yeast cell lysate. The major transcription products identified with arrows. Lane 1 shows the no-template control while all the remaining lanes show transcription from the plasmid template pJ461 which contains an upstream HSE; lanes 3 and 4 are transcription in the presence of RA1-HSF; lane 5 is the transcription in the presence of DL; lane 6 is in the presence of both RA1-HSF and DL. Concentrations of each component added are indicated in the figure.
DISCUSSION

The HSF aptamer we have selected and characterized here has an unusually complicated secondary structure. The predicted secondary structure has three stem–loops connected by a three-way junction. Serial deletions of the aptamer defined a minimized aptamer-binding motif. This secondary structure has been further confirmed by independently assembling a homologous three-way junction using two separate RNAs, whose annealing produced full HSF-binding activity. Because complicated RNA structures with one or more branches, account for only <1% of the secondary structures in a 40mer random sequence pool as used here (21), the functional domain of a selected RNA aptamer is often a single stem–loop structure. Why did we not select simpler HSF aptamers? Perhaps the starting pool does not contain a simple structured RNA that binds tightly to HSF. Also, a more complicated RNA structure may be favored in the selection of an RNA that binds to the complicated and flexible structure of HSF protein. For example, the linker region of the HSF, which is essential for the aptamer binding, is a highly flexible unstructured region (9).

Most previously characterized RNA aptamers to DNA-binding proteins were found to bind to the DNA-binding surface of the targeted protein (12,22). In a structural study of an NF-kappaB/aptamer complex, Huang et al. (14) found that the NF-kappaB RNA aptamer is a DNA mimic, and matches perfectly the DNA-binding surface of NF-kappaB. In contrast to the NF-kappaB example, our HSF aptamer provides the first example of an RNA aptamer selected to a DNA-binding factor that can compete with DNA but binds to the protein in a manner that is distinct from DNA binding to the protein. Moreover, even though the linker region of HSF has been proven to be essential for the aptamer binding, this does not rule out the possibility that the DNA-binding domain can provide a direct contribution to the binding.

We have previously generated RNA aptamers as inhibitors of particular macromolecular interactions of the general transcription factor TATA-binding protein (TBP) (12). Here we have generated and characterized an RNA aptamer that is a highly effective inhibitor of a key upstream transcription activating factor. The fact that this RA1-HSF aptamer can inhibit HSF-induced transcription in vitro in the complex milieu of a whole cell extract demonstrates the potential usefulness of this aptamer for both in vitro and in vivo studies of HSF function. To our knowledge, there is no drug that targets this DNA-binding function of HSF. We envision that the information derived from this and future studies with this aptamer will prove useful in the diagnosis and treatment of diseases that are influenced by HSF function.

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