RNA Molecules That Bind to and Inhibit the Active Site of a Tyrosine Phosphatase*

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Protein tyrosine phosphatases (PTPases) are essential proteins in many cellular processes. In vitro selection was used to evolve high affinity RNA aptamers to the Yersinia PTPase from two random pools varying in length. Selected aptamers from the two different pools share a 21-residue conserved sequence. They bind to their target with dissociation constants of 18 and 28 nM and inhibit the enzyme with IC₅₀ values of 10 and 35 nM, but do not bind a related PTPase. Modification of the PTPase’s active site cysteine with the alkylating agent iodoacetate results in a loss of binding affinity. These experiments suggest that the selected aptamers act by binding at or near the active site and might therefore be useful in defining the interactions between PTPases and their targets.

Approximately 30% of intracellular proteins are phosphoproteins. The phosphorylation and dephosphorylation of these phosphoproteins regulate a variety of metabolic processes, including signal transduction, growth, differentiation, and viral infection (1–3). The phosphorylation states of proteins are in turn controlled by a variety of protein kinases and phosphatases. Together, protein kinases and phosphatases are estimated to encode for as much as 4% of the eukaryotic genome (4).

While the number and complexity of protein kinases have been appreciated for many years, the importance of protein phosphatases has emerged more recently. In particular, protein tyrosine phosphatases (PTPases) seem to play a critical role in regulating cellular and viral replication. Over 75 PTPases have been identified to date, and based on genome sequencing estimates, there are expected to be over 500 in humans alone (5). The PTPase catalytic domain consists of roughly 250 amino acids and is the only region of sequence similarity among the multiple, different PTPases (3, 6). The PTPase active site contains a catalytically essential cysteine (7–10) embedded within a highly conserved, 11-residue sequence motif ((Ile/Val)-His-Cys-X-Ala-Gly-X-Gly-Arg-X-Gly). A subclass of protein phosphatases that can dephosphorylate serine, threonine, and tyrosine residues has also been identified. These so-called dual-specificity phosphatases are marked by the active site sequence His-Cys-X-Gly-X-Arg-Ser-(Thr).

The crystal structures of the Yersinia PTPase, Yop51Δ162, the human PTPase, PTP1B, and the dual-specificity phosphatase, VHR, have all been solved (11–13). All three enzymes contain a phosphate-binding loop with the catalytic cysteine residue at its center. The crystal structures of two receptor tyrosine phosphatases, RPTPα D1 and RPTPμ D1, have also recently been determined (14, 15). The secondary and tertiary structures of the catalytic domains of the receptor tyrosine phosphatases are largely similar to those of Yop51Δ162 and PTP1B, and they also appear to contain phosphate-binding loops at their active sites.

Since PTPases are emerging as important proteins in oncogenesis and pathogenesis, they may prove to be good targets for drug discovery. Many of the PTPases show excellent substrate specificity; interactions with substrates are determined by the amino acids flanking the phosphorylated tyrosine target (16–19). Unfortunately, the development of specific inhibitors has proven to be difficult. Most of the known inhibitors to PTPases (vanadate, iodoacetate) act by general modification of common active site residues. Even peptide-based inhibitors that take advantage of PTPase recognition “codes” do not always possess specificity toward only one PTPase.

In vitro selection can be used to generate nucleic acid binding species (aptamers) that bind tightly to protein targets, including those that are not normally thought to bind nucleic acids (reviewed in Osborne and Ellington (20)). Anti-protein aptamers have been shown to bind their targets with extremely high specificity. For example, aptamers that target the β1 isozyme of protein kinase C do not recognize the α isozyme, which is 80% identical (21). Similarly, aptamers that recognize basic fibroblast growth factor do not recognize acidic fibroblast growth factor (22, 23), despite the fact that the two proteins are 55% identical (24).

Since PTPases present a phosphate-binding loop to their substrates, we hypothesized that this class of enzymes might productively interact with other phosphate-laden compounds, such as nucleic acids, and thus might prove to be excellent targets for in vitro selection experiments. If anti-PTPase aptamers could be selected, they might be expected to localize to the protein active site and be highly specific inhibitors of enzymatic activity. To test this hypothesis, we focused on one of the best studied of the PTPases, the bacterial enzyme Yop51. The gene encoding Yop51 has been found to be a virulence determinant in the genus Yersinia (25, 26), which includes the causative agent of the bubonic plague (7). The kinetic parameters (27–31), substrate specificity (17, 19, 32), and structure (11, 33, 34) of the Yop51 enzyme have been determined. We have selected aptamers that bind tightly and specifically to Yop51. As predicted, the aptamers localize to the protein active site and inhibit enzymatic activity. The aptamers selected to
Selection of Anti-tyrosine Phosphatase Aptamers

 bind Yop51 or other PTPase targets should prove useful for studying protein-protein interactions, dissecting the complex web of cellular signal transduction pathways, and developing novel pharmaceuticals.

EXPERIMENTAL PROCEDURES

Materials—Yop51Δ162 was prepared in the laboratory of Dr. Dixon at the University of Michigan according to previously published protocols (27). Yop51 was purchased from New England Biolabs (Beverly, MA). [γ-32P]ATP and [α-32P]UTP were from NEN Life Science Products. All other chemicals were reagent grade or molecular biology grade.

Methods—The selection of aptamers that bind Yop51Δ162 was initiated with two different RNA pools, N30 and N71, whose synthesis and purification has previously been described (35, 36). In short, both pools contained randomized regions of either 30 or 71 nucleotides, flanked by constant regions that were required for enzymatic amplification. The 3′-constant region allowed cDNAs to be synthesized from selected RNAs. Both constant regions were utilized during polymerase chain reaction amplification, and the 5′-constant region contained a T7 promoter sequence which allowed RNA molecules to be regenerated from double-stranded DNA templates. The double-stranded DNA pools (1 μg; 2 × 10^13 sequences) served as templates for in vitro transcription; transcripts were synthesized using an Ampliscribe kit (Epicient Technologies, Madison, WI) according to the manufacturer’s instructions. Following transcription, the RNA pools were purified on 10% denaturing polyacrylamide gels, and the amount of RNA isolated was quantitated based on an extinction coefficient of 0.025 mg ml^-1 cm^-1.

In the first round of selection approximately 9 × 10^13 N30 RNA molecules were used, and approximately 6.5 × 10^13 N71 RNA molecules were used. In other words, almost all sequences in the original double-stranded DNA pool should have been represented several times over.

In Vitro Selection—The concentration of Yop51Δ162 in each round was 0.05 μM, while the concentration of the N30 RNA pool was 0.76 μM, and the concentration of the N71 RNA pool was 0.54 μM. RNA pools in selection buffer (20 μM Tris pH 7.6, 150 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol; 100 μl final volume) were thermally equilibrated by heating to 65°C for 3 min and cooling to room temperature over a period of 10 min. To exclude filter-binding sequences from the selection, the thermally equilibrated pools were first passed over a 0.45-μm filter. To exclude filter-binding sequences from the selection, the reactions were filtered on a vacuum manifold (Schleicher & Schuell) containing a piece of pure nitrocellulose (Midwest Scientific, St. Louis, MO) over a piece of Hybond filter paper (Amer sham Corp.) as described in Weeks and Cech (38). In this method, the nitrocellulose filter captures protein-bound radiolabeled RNA, while the Hybond paper captures unbound radiolabeled RNA. For all binding curves, the amount of radioactivity retained at each protein concentration was quantified on a PhosphorImager. To calculate equilibrium dissociation constants, the program Kaleidagraph (Abelbeck Software, Reading, PA) was used to fit the data to the equation:

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f = C + \frac{[L]}{K_d} + [L]
\]

where f is the fraction of the aptamer bound, C is the binding capacity of the aptamer, and [L] is the concentration of protein, by the least squares method.

Cloning and Sequencing—Each selected pool was ligated into a TA cloning vector (Invitrogen, Carlsbad, CA) and cloned into Escherichia coli Invac® (Invitrogen). Plasmid DNA was isolated and sequenced using Sequenase 2.0 (U. S. Biochemical Corp., Cleveland, OH) according to the protocol provided with the enzyme. Secondary structures for individual aptamers were predicted using the program Mulfold (39).

Inhibition Studies—Aptamer inhibition of Yop51 dephosphorylation of peptide substrate was measured using a tyrosine phosphatase assay system (Promega, Madison, WI). The kit provides a fully phosphorylated peptide substrate (DADEpYvLIPQQG) and measures the release of phosphate by following the formation of a molybdate-malachite green-phosphate complex. Individual aptamers, ranging in concentration from 0 to 2 μM, were incubated in 25 μM bis-tris propane and 5 mM MgCl2 (50 μl final volume) with 120 μM peptide and 0.2 nM Yop51*. The reaction was terminated at ambient temperature by addition of 50 μM of a peptide substrate. Phosphate release was measured spectrophotometrically at 405 nm (10 min) and converted to pmol using a standard molybdate-malachite green-phosphate complex. The data points that were obtained were within the linear range of the assay. Following the complex formation, the amount of phosphate produced was determined by measuring the absorbance of the solution at 600 nm.

Active Site Modification—To specifically modify the active site cysteine of Yop51*, approximately 5 μM of protein were incubated in 15 μl of selection buffer with 2 mMiodoacetate for 30 min at 37°C (40). Varying amounts of modified Yop51* were added directly to radiolabeled RNA in selection buffer. The phosphatase reaction was then incubated at ambient temperature for 1 h prior to filtration on the vacuum manifold. As a control, unlabeled RNA was incubated with iodoacetate under identical conditions, repurified on a 10% acrylamide gel, labeled, and incubated with modified and unmodified protein in identical reactions.

RESULTS AND DISCUSSION

Selection of Anti-Yop51* Aptamers—To determine whether aptamers that recognized the active site of a protein tyrosine phosphatase could be isolated, we utilized the tyrosine phosphatase (Yop51) from Yersinia enterocolitica as a selection target. The structure of Yop51Δ162 with a tungstate anion had been determined and revealed that phosphate was likely bound in a relatively open, positively charged pocket. We hypothesized that the phosphodiester backbone of a RNA molecule might interact with the PTPase in a manner similar to that of phosphate, and that particular RNA sequences or struc-
A recombinant Yop51 (Yop51*Δ162) that lacked the N-terminal 162 amino acids was used as the selection target. Yop51*Δ162 does not contain the protein's export signal sequence but does include the entire catalytic domain of the protein. Yop51*Δ162 has been shown to be fully active toward peptide and protein substrates (27). The recombinant Yop51 also contains a single amino acid substitution, cysteine 235 to arginine, that dramatically increases its yield when expressed in E. coli (27); this sequence change is designated as Yop51*. While in vitro selection experiments were carried out with the truncated tyrosine phosphatase (Yop51*Δ162), binding experiments were carried out with both Yop51*Δ162 and the full-length protein (Yop51*). Experiments with truncated and full-length protein did not yield significantly different results, and therefore the aptamers in this report are generally referred to as anti-Yop51* aptamers, rather than as anti-Yop51*Δ162 aptamers.

RNA aptamers that could bind tightly and specifically to Yop51*Δ162 were isolated from two pools that contained random regions of either 30 (N30) or 71 (N71) nucleotides in length. The two different pools were used in order to allow the isolation of aptamers that might have different sequence or structural complexities. As an example, while it might be difficult to isolate a structure as complex as a tRNA cloverleaf from a pool that spanned only 30 random sequence positions, such a structure could conceivably be isolated from a pool that spanned 71 random sequence positions.

In each round of selection, the protein was mixed with the RNA pool, and bound species were separated from unbound by passing the mixture over a modified cellulose filter. Moderate binding activity was observed with the N30 pool following the fifth round of selection (Table I), and a nonspecific competitor, tRNA, was added to the binding reaction to increase the stringency of the selection and to promote the isolation of high affinity aptamers. Overall, our strategy appeared to be successful; by the eighth round a large portion of the selected population (23% of the selected N30 pool, and 53% of the selected N71 pool; Table I) could bind to Yop51*.

At this point, the selection was terminated, and individual aptamers were cloned and sequenced. The in vitro selection protocol had narrowed the N30 pool from 2.3 × 10^13 sequences at the start to just a single sequence at the conclusion, with some minor variants (Fig. 1). Similarly, the N71 pool had been winnowed from 1.6 × 10^13 sequences to only two major two-sequence classes (Fig. 1). Initial binding experiments indicated that the sequence class that contained aptamer N71yc2 interacted with both the protein and the modified cellulose filter, and this class was not further examined.

A Sequence and Structural Motif That Binds Yop51*—Surprisingly, the aptamers derived from the N30 pool shared extensive sequence similarity with aptamers derived from the N71 pool. Twenty-one residues in the consensus anti-Yop51* aptamer derived from the N30 pool were identical to the sequence of the consensus anti-Yop51* aptamer derived from the N71 pool (Fig. 1). These results are similar to results of selections carried out against both the human immunodeficiency virus type 1 Rev protein and vascular endothelial growth factor. Anti-Rev aptamers isolated from RNA libraries that spanned either 18 or 32 random sequence positions contained a similar sequence and secondary structural motif (41–43). Similarly, anti-vascular endothelial growth factor aptamers isolated from modified RNA libraries that spanned either 30 or 50 random sequence positions were similar to one another (44).

The selection of similar sequence motifs from pools of different lengths can potentially be attributed to what has been called "the tyranny of short motifs." In other words, a shorter motif has a greater initial chance of survival because it is present in a random sequence pool at a higher frequency than is a longer motif. A longer motif will be preferentially selected only if its affinity for a target is much higher than that of the shorter motif. However, in the current selection it seems unlikely that the commonality of the selected, 21-residue motif was an artifact of prevalence. A particular 21-residue motif will be found roughly once in every 4^21 or 4 × 10^{12} sequences. Given that the pools used in the selections that targeted Yop51* contained from 1 to 2 × 10^{13} unique nucleic acid species, the sequence motif that was eventually returned was almost as complex as possible. Moreover, given that the same motif was derived from different pools with different numbers of random sequence positions and different constant regions, it is more likely that the common anti-Yop51* binding motif is optimal, at least for sequences of this size.

The program Mulfold was used to generate possible secondary structures for the aptamers. Interestingly, the aptamers from the N30 selection folded into a simple stem-loop structure (Fig. 2), while the aptamers from the N71 selection appeared to fold into a convoluted structure with many loops (not shown). Given that the same, long sequence motif was identified in both pools, it is unlikely that this motif was being presented in different structural contexts. To examine whether the identified sequence motif might be similarly presented in both sets of aptamers, suboptimal foldings of the aptamers derived from the N71 pool were examined. In several of the suboptimal folds, a stem-loop structure that was very similar to the stem-loop structure predicted for the N30 pool was observed (Fig. 2). It is interesting to note that the N30 aptamers fortuitously use three residues from their constant region to form the paired stem that contributes to the common sequence and structural motif, while in the N71 aptamers the same three residues are derived from the randomized core.

While these initial structural predictions were based solely on sequence analysis and modeling, they have been further supported by experimental analysis. The functional boundaries of N71yc2 were mapped by alkaline hydrolysis (45), and the minimal sequence that can interact with Yop51* is shown in Fig. 2e. This secondary structure of the minimal aptamer is predicted to be a RNA stem-loop similar to the stem-loop fold of N71yc2 and to the stem-loop structure of N30yc5.

**Anti-Yop51* Aptamers Bind to Yop51* with High Affinity and Specificity—**Since the degenerate pools had been winnowed to predominant aptamer families, single members of each selected population (N30yc5, N71yc2, and N71yc16) were further characterized. Interactions between these anti-Yop51* aptamers and Yop51*Δ162 were probed using a filter-binding assay similar to that employed for selection. When binding was
examined as a function of protein concentration, the dissociation constants of the aptamer-protein complexes were found to be 28 nM for N30yc5 and 18 nM for N71yc16 (Fig. 3). Similar experiments carried out with N30yc5 and the full-length protein, Yop51*, gave a dissociation constant of 50 nM (data not shown).

The specificity of binding was examined using a homologous tyrosine phosphatase, rat PTP1 (sequence identity of 20%) (46). X-ray structural analysis has revealed that the overall tertiary structures and active sites of Yop51 and PTP1 are extremely similar (11, 12, 46). Nonetheless, in our standard assay the anti-Yop51* aptamers showed only background binding (less than 1% of applied counts) to PTP1. The ability of anti-Yop51* aptamers to discriminate between different tyrosine phosphatases is comparable to the ability of anti-basic fibroblast growth factor aptamers to discriminate between different members of the fibroblast growth factor family (47).

Anti-Yop51* Aptamers Inhibit Yop51*—To determine if the anti-Yop51* aptamers might be useful as reagents for probing the function of Yop51 in vitro or in vivo, we examined whether anti-Yop51* aptamers could inhibit tyrosine phosphatase activity. These studies used a phosphorylated peptide based on an autophosphorylation site in the epidermal growth factor receptor (19) as a physiologically relevant substrate. When aptamers N30yc5 and N71yc2 were assayed for their ability to inhibit PTPase activity, the observed IC50 values were 35 and 10 nM, respectively (Fig. 4). These values were consistent with the dissociation constants calculated for aptamers complexed with the truncated protein. No inhibition of enzymatic activity was observed with the unselected RNA pools.

Anti-Yop51* Aptamers Bind to the Active Site of Yop51*—To further test the hypothesis that anti-Yop51* aptamers were binding and blocking access to the active site of the protein, we specifically modified the active site of Yop51* and assayed whether or not the modifications blocked interactions with anti-Yop51* aptamers. It has previously been shown that iodoacetate labels only the active site cysteine of Yop51* (40). Following treatment with iodoacetate the enzyme’s PTPase activity was completely inhibited, as assayed by para-nitrophenyl phosphate cleavage. Unmodified and modified proteins were then mixed with anti-Yop51* aptamers and complexes were isolated by filtration over modified cellulose, as before. As can be seen in Fig. 5, alkylation of the Yop51* active site completely inhibits not only the activity of the protein but also binding of the RNA aptamers to the protein. To control for the possibility that residual iodoacetate might modify the structure or the function of the RNA in some way, the aptamers were incubated with iodoacetate alone, purified, and incubated

**Fig. 1. Sequences of anti-Yop51* aptamers.** The names of the aptamers can be found on the right (e.g., N30yc5). The number in parentheses is the number of independent clones that corresponded to a given aptamer sequence. The 21-residue motif that is held in common between anti-Yop aptamers derived from the N30 and N71 pools is underlined. Constant sequence regions are separated from the random sequence region by an asterisk (*). Similarities between aptamers in a given class are represented as a dash (–), while differences between aptamers in a given class are represented as changes relative to the first sequence listed.

**Fig. 2. Predicted secondary structural models of anti-Yop aptamers.** The program Mulfold was used to derive secondary structural models for aptamers N30yc5 and N71yc2. The secondary structural model for N30yc5 is predicted to be uniquely stable; similarly stable suboptimal folds were not found. The secondary structural model shown for N71yc2 is not uniquely stable; other folds can be generated. However, this secondary structural model presents the 21-residue motif common to the two classes of aptamers (bold) in a similar structural context.
with unmodified Yop51\(^*\). Modification of the RNA resulted in little or no loss of the binding activity (data not shown).

Taken together, these results suggest that the anti-Yop51\(^*\) aptamers bind at or near the protein active site. We originally hypothesized that the aptamers would “seek out” the protein active site because it is positively charged and can bind phosphate. The ability of negatively charged aptamers to competitively bind to proteins in place of other negatively charged substrates or ligands appears to be general. For example, aptamers selected to bind to thrombin, vascular endothelial growth factor, and basic fibroblast growth factor all appear to compete with the negatively charged ligand heparin for binding (23, 45, 48). Our new results suggest that phosphodiester-laden aptamers may also be able to occupy some of the same sites on proteins such as dehydrogenases or kinases that are otherwise occupied by phosphates, pyrophosphates, phosphodiester, or other phosphate derivatives.

Moreover, it is interesting to note that aptamers that have been selected solely for their ability to bind to proteins also seem to generally inhibit the function of these proteins. For example, aptamers selected to bind protein kinase C also inhibit catalytic function (although in this instance it is unknown whether the aptamers interact with the protein active site or a distal regulatory site) (21). Similarly, aptamers selected to bind the human T cell leukemia virus I regulatory protein Tax can inhibit interactions with other transcription factors (49). We have dubbed the general inhibition of protein function by nucleic acid binding species the “homing principle,” and believe...
that it may be a consequence of how aptamers form functional structures. The homing principle can best be appreciated by contrasting aptamers with other biopolymer binding species derived from random (or partially random) sequences: antibodies. While antibodies have preorganized binding cusps, aptamers do not. The presence of preorganized binding cusps on antibodies allows them to bind to protruding surface epitopes on proteins. Aptamers that formed both structural platforms and binding cusps similar to those found in antibodies would require a great deal of nucleic acid sequence information. In contrast, aptamers that formed globular structures that could be surrounded by proteins, rather than vice versa, might form a similar number of protein:nucleic acid interactions with much less nucleic acid sequence information. Thus, the simplest and most robust binding species that can be formed from random sequences are those that seek out binding sites and surface crevices on proteins. Such binding sites and surface crevices are by and large functional domains on proteins. For example, in the current study the anti-Yop51* aptamers folded to form structures that could fit into the active site of an enzyme. Thus, by selecting for binding function from a random sequence nucleic acid library, there is frequently a concomitant inhibition of protein function.

The study of signal transduction pathways typically involves the manipulation of the activities of individual signal transduction proteins. While PTPases play an important role in the life cycle of cells, it has been difficult to elucidate the biological function of these enzymes because few specific antimetabolites have been found that can be used to specifically modulate PTPase activities. The development of anti-PTPase aptamers may therefore aid in delineating the intracellular targets of PTPases and understanding the metabolic functions of individual PTPases.

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