Tenascin-C Aptamers Are Generated Using Tumor Cells and Purified Protein*

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Tenascin-C (TN-C) is an extracellular matrix protein that is overexpressed during tissue remodeling processes, including tumor growth. To identify an aptamer for testing as a tumor-selective ligand, SELEX (systematic evolution of ligands by exponential enrichment) procedures were performed using both TN-C and TN-C expressing U251 glioblastoma cells. The different selection techniques yielded TN-C aptamers that are related in sequence. In addition, a crossover procedure that switched from tumor cell to purified protein selection was effective in isolating two high-affinity TN-C aptamers. When targeting tumor cells in vitro, the observed propensity of naive oligonucleotide pools to evolve TN-C aptamers may be due to the abundance of this protein. In vivo, TN-C abundance may also be well suited for aptamer accumulation in the tumor milieu. A size-minimized and nuclease-stabilized aptamer, TTA1, binds to the fibrinogen-like domain of TN-C with an equilibrium dissociation constant \(K_d\) of \(5 \times 10^{-9}\) M. At 13 kDa, this aptamer is intermediate in size between peptides and single chain antibody fragments, both of which are superior to antibodies for tumor targeting because of their smaller size. TTA1 defines a new class of ligands that are intended for targeted delivery of radioisotopes or chemical agents to diseased tissues.

Tenascin-C is a very large (>1 \(\times\) 10^6 Da) hexameric glycoprotein that is located primarily in the extracellular matrix (ECM). TN-C is expressed during fetal development, wound healing, tumor growth, atherosclerosis and psoriasis, suggesting a role for this protein in tissue remodeling processes (reviewed in Refs. 1 and 2; see also Refs. 3–5). As judged by Western blotting and immunohistochemical staining (6–16), TN-C levels in tumors are significantly higher than in normal tissue. Further, TN-C levels are predictive of local tumor recurrence and are correlated with invasiveness and distant metastasis (17–19), although these findings remain controversial. Tumor metastases can also express TN-C (10, 20). In addition to localization in tumor stroma, TN-C can be associated with tumor vascular structures (21–24) and may promote angiogenesis through interaction with the integrin \(\alpha_v\beta_3\) (25). Because of the abundance of TN-C in tumor stroma and its association with angiogenesis, high-affinity TN-C ligands may be clinically useful tumor-targeting agents. In fact, radiolabeled antibodies to TN-C are currently being evaluated in glioblastoma patients (26, 27) with significant responses to treatment in a phase II study (28).

Aptamers are typified by high affinity and specificity for their cognate proteins (reviewed in Refs. 29–31) and can be considered as oligonucleotide analogs of antibodies. However, as nucleic acids, aptamers are fundamentally distinct from antibodies. In having small size (8–15 kDa) relative to antibodies (150 kDa), aptamers are candidates for rapid tumor penetration and blood clearance. These are useful attributes for noninvasive diagnosis of disease (32) and may provide advantages over antibodies and fragments thereof, which demonstrate slower tissue penetration and clearance rates. To identify an aptamer for investigation of tumor-targeting and blood clearance properties, we describe herein a SELEX process to identify TN-C aptamers and then focus attention on a single aptamer, TTA1.

The SELEX process uses large \((10^{14}–10^{15})\) oligonucleotide pools to identify binding species, i.e. aptamers, to a variety of purified molecular targets. In addition to generating aptamers against purified proteins/small molecules, SELEX technology can generate aptamers to cells (33) and tissues. The advantages of complex targets include freedom from the need to define and purify a molecular target, and presentation of proteins in native folding and glycosylation states. For complex SELEX experiments, identifying optimal selection conditions is theoretically possible (34) but remains a challenging task. In contrast, selection against purified protein allows ready experimental manipulation to achieve optimal enrichment of high-affinity aptamers (35) and requires no deconvolution to identify the cognate protein (or lipid, oligosaccharide, nucleic acid, etc.). Relative to cells and tissues, purified proteins often exhibit lower nonspecific binding of nucleic acids, and therefore selections proceed more rapidly. Because each has advantages, we elected to use both purified protein and cells as target sources to obtain TN-C ligands.

A previous SELEX experiment targeting U251 glioblastoma cells identified a DNA aptamer that binds to tenascin-C, demonstrating that TN-C is a selectable target on U251 cells. The ssDNA aptamer displays greatly reduced binding affinity at
physiological temperatures,4 perhaps because these initial cell SELEX experiments were performed at 4 °C. Thus the aptamer has relatively low affinity (Kd ~100 nm) at 37 °C and, being composed of DNA, is susceptible to nuclelease activity in vivo. These features render the DNA aptamer unsuitable for in vivo applications. To identify aptamers for use as tumor-targeting agents, we undertook SELEX experiments at 37 °C using a nuclease-stabilized 2'-F pyrimidine oligonucleotide library.

**EXPERIMENTAL PROCEDURES**

**Cells, Proteins, and Oligonucleotides—**U251 cells, derived from a human glioblastoma, were obtained from the National Cancer Institute (Research Facility, Frederick, Frederick, Maryland Heights, MA) and were coated for 2 h at room temperature with 200 µl of Dulbecco’s phosphate-buffered saline containing tenascin-C. Control wells lacked tenascin-C in this initial coating step. After being coated, the wells were blocked using HBSSM buffer containing 1 g/liter casein (1-blot, Tropix). This switch of blocking agent was performed to decrease background binding of aptamer pools to the plate surface. Binding and wash buffer consisted of HBSSM buffer containing 0.1% Tween 20. The aptamer pool was diluted into 100 µl of binding buffer and incubated for 30 min at 37 °C in the protein-coated wells. After binding, six washes of 200 µl each were performed. The wells were then emptied and placed on top of a 95 °C heat block for 5 min (“heat elution”). Standard avian myeloblastosis virus (AMV)-reverse transcriptase reactions (50 °C) were performed as described (38). Two synthetic primers, 5N7 (see above) and 3'-biotinylated (described above) and 2'-OH purine nucleotides. For cell selections, U251 cells were grown to 90% confluence in 12-well tissue culture plates (Falcon 3047, Becton Dickinson). After the cells were washed with binding buffer, 32P body-labeled aptamer pools were incubated with cells in binding buffer for 18 h at 4 °C. Binding activity was determined using standard methods (41, 42). Nitrocellulose filter partitioning assays were performed as described (43). Briefly, 32P end-labeled oligonucleotides at 0.5 × 10^{10} cmol were incubated with increasing concentrations of TN-C in HBSMC buffer + 0.01% (w/v) human serum albumin at 37 °C for 15 min. Reactions were then filtered over nitrocellulose, and bound cpm were quantitated. Data were fit to binding constants as described (44).

**To measure binding of aptamers to protein immobilized on plates, anti-tenasin-C monoclonal antibodies (mTN12, mouse TN-specific, Sigma; Hx306, human TN-specific, Harold Erickson, Duke University) were adsorbed to MicroLite-2 96-well plates (Dynex Technologies) in HBSSM buffer for 18 h at 4 °C. Wells were washed four times with HBSSM buffer and blocked with 200 µl of HBSSM buffer containing 0.1% (v/v) 1-blot (Tropix) + 0.5% (w/v) BSA. Tenascin-C (150 µg) was added to each well and incubated in the dark, chemiluminescence was detected using a luminometer (LB 96P, Berthold, Nasau, NH).

4 B. Hickey, data not shown.
5 United States Patent No. 60/034,651 filed January 8, 1997.
no protein control wells. Progress was quantitated by measuring the background binding of the RNA pools to polystyrene without protein and RT-PCR amplification of the bound oligonucleotides. The cell selections were monitored by measuring the amount of protein with each well in a 96-well plate and the dissociation constant ($K_d$) of the protein-oligonucleotide complexes.

At round 6, the blocking agent was switched from human serum albumin to casein, which improved the subsequent round, selection plate diameter pmol RNA/plate

| Round | Plate diameter | pmol RNA/plate |
|-------|---------------|----------------|
| 1     | 2 × 150       | 1500           |
| 2     | 150           | 1500           |
| 3     | 150           | 1500           |
| 4     | 150           | 1500           |
| 5     | 150           | 1500           |
| 6     | 150           | 1500           |
| 7     | 150           | 1500           |
| 8     | 150           | 1500           |

**RESULTS**

To identify aptamers to TN-C, a tripartite SELEX experiment was carried out as diagrammed in Fig. 1. In the first arm, purified TN-C was used. The second arm consisted of selection against a TN-C-expressing glioblastoma cell line, U251. This arm was subdivided into EDTA and Trizol “elutions” to recover bound aptamers. A third arm was a crossover from cell selections onto purified protein selections.

A SELEX Experiment Using Purified TN-C—Human TN-C was adsorbed to polystyrene 96-well microtiter plates. To initiate selections, a random aptamer pool consisting of 10^14 oligonucleotides was generated using RNA polymerase. The oligonucleotides contained 2'-F pyrimidines and 2'-OH purines with a 40-nucleotide random sequence region flanked by fixed sequences for RT-PCR. Selections were performed according to Drolet et al. (40), essentially consisting of protein-oligonucleotide incubations, washes to remove unbound oligonucleotides, and RT-PCR amplification of the bound oligonucleotides. The amounts of protein with each well in a 96-well plate and the amount of input RNA are indicated in Table I.

A qualitative assessment of PCR amplification indicated that background binding of the RNA pools to polystyrene without associated tenasin-C (“no protein” control) was increasing through the initial five rounds. At round 6, the blocking agent was switched from human serum albumin to casein, which resulted in dramatically decreased aptamer pool binding to the no protein control wells. Progress was quantitated by measuring the affinity of 32P-labeled aptamer pools for TN-C using a nitrocellulose filter capture assay (45). After five rounds, a slight improvement in binding was evident. Coincident with the switch in blocking agent, the amount of TN-C binding in the aptamer pool rose dramatically in round 6. By round 8, affinity had increased at least 1000-fold to an equilibrium dissociation constant ($K_d$) of 3 × 10^{-9} M. As no further affinity improvement was evident in the subsequent round, selection was deemed complete at round 8.

**A SELEX Experiment Using Tumor Cells**—A second experiment used human U251 glioblastoma cells as the target source. These cells construct an ECM containing abundant TN-C [46]. Cells were grown to confluence and incubated with 10^{14} sequences of a random oligonucleotide pool identical to that described above) at 37°C for 1 h. After extensive washing, a final wash buffer containing 10 mM EDTA was applied to elute EDTA-sensitive aptamers. Because nucleic acid structures and nucleic acid-protein interactions often utilize divalent cations, it was expected that EDTA would elute a subset of cell-bound aptamers. These cells were solubilized, nucleic acids were extracted using Trizol™, a reagent that combines chaotropic denaturation of proteins with organic extraction of nucleic acids, and then the remaining aptamers were collected. Thus the EDTA served to elute a subset of bound aptamers, and the subsequent Trizol elution collected all remaining aptamers along with cellular RNAs. Aptamers from both EDTA and Trizol elutions were amplified by RT-PCR and transcribed, closing the first round of this SELEX experiment. Unlike the purified protein SELEX experiment, cell and input RNA concentrations remained constant throughout nine rounds of selection (Table I).

The progress of the cell selections was monitored by measuring the binding of radiolabeled aptamer pools to U251 cells. To analyze the EDTA elution SELEX experiment, Fig. 2A compares binding of a control aptamer pool to rounds 3, 5, and 9. The control aptamer pool bound the cells detectably, and binding was saturable. Relative to this nonspecific binding, rounds 3, 5, and 9 showed progressively increasing binding.

Similar to the EDTA elution pools, the Trizol pools showed increased binding compared with a random aptamer pool (Fig. 2B). The T9 (Trizol round 9) pool showed less apparent binding than the T5 pool. This was due to increased binding to the polystyrene surface (data not shown). This outcome suggests

| Round | pmol protein/well | pmol RNA/well |
|-------|-------------------|---------------|
| 1     | 12 (6 wells)      | 200 (6 wells) |
| 2     | 12                | 200           |
| 3     | 12                | 200           |
| 4     | 12                | 200           |
| 5     | 2                 | 33            |
| 6     | 2                 | 33            |
| 7     | 2                 | 33            |
| 8     | 0.2               | 3.3           |

**TABLE I**

Tenascin-C SELEX experiment

| Round | pmol protein/well | pmol RNA/well |
|-------|-------------------|---------------|
| 1     | 12 (6 wells)      | 200 (6 wells) |
| 2     | 12                | 200           |
| 3     | 12                | 200           |
| 4     | 12                | 200           |
| 5     | 2                 | 33            |
| 6     | 2                 | 33            |
| 7     | 2                 | 33            |
| 8     | 0.2               | 3.3           |

**TABLE II**

Crossover SELEX experiment: cells to Tenascin-C

| Round | pmol protein/well | pmol RNA/well |
|-------|-------------------|---------------|
| E9P1/T9P1 | 2 | 33 |
| E9P2/T9P2 | 2 | 33 |
A faint signal was detected in the T9 pool; a low signal was also evident, with P8 showing significantly higher binding (Fig. 3). The SPR assay using soluble TN-C therefore served a qualitative role; we observed low binding to the tumor cell Trizol arm, increased binding to the tumor cell EDTA arm, and the highest binding to the purified protein arm.

A Crossover SELEX Experiment Using Tumor Cells and Puriﬁed TN-C—To enrich TN-C aptamer representation in the tumor cell aptamer pools, a crossover SELEX experiment was performed as diagrammed in Fig. 1. The two cell-selected pools were subjected to two rounds of protein selection (Table I), generating pools E9P2 (E9P2 = 9 rounds of EDTA elution from cells and 2 rounds of protein selection) and T9P2. Affinities for TN-C were then determined using a nitrocellulose ﬁlter binding assay (Fig. 4). For comparison, the P8 pool was included. This analysis indicated that two rounds of crossover selection on TN-C improved the afﬁnity of E9 by 50-fold. For the Trizol arm, afﬁnity rose from undetectable to $2 \times 10^{-9}$ m in two rounds. Remarkably, just two rounds of crossover selection were required to enrich the high-afﬁnity aptamers that were rare in each tumor cell aptamer pool.

To determine whether the U251 aptamer pools contain TN-C aptamers, binding was investigated using a surface plasmon resonance (SPR) assay. Aptamer pools were biotinylated at the 5’ terminus and immobilized, via streptavidin, onto the surface of a biosensor chip. TN-C binding was then measured by SPR. Specific binding of TN-C to aptamer pools P8 and E9 was evident, with P8 showing signiﬁcantly higher binding (Fig. 3). A faint signal was detected in the T9 pool; a low signal was also detected using the ﬁlter binding assay described below. Quantitative measurements were not possible because of the large mass of TN-C (> 1 mDa), which slows diffusion through the surface matrix. A further hindrance to quantitation is the hexameric structure of TN-C, which likely causes slow dissociation from the surface because of multivalent interactions. In addition, it was not possible to couple active TN-C to the matrix. The SPR assay using soluble TN-C therefore served a qualitative role; we observed low binding to the tumor cell Trizol arm, increased binding to the tumor cell EDTA arm, and the highest binding to the purified protein arm.

Isolation and Sequencing of High-afﬁnity TN-C Ligands—To analyze the content of selected pools, ﬁve aptamer pools were cloned and sequenced. Aptamers could be grouped into the three families shown in Fig. 5. Family I members were found in the P8, E9, and T9 pools. These sequences are related through the consensus sequence GACNYUCNCN_{5}GCAYC and have afﬁnities for TN-C ranging from 20 to $100 \times 10^{-9}$ m. The T9 pool contains many different family I sequences, consistent with the high sequence complexity predicted by C_{f} analysis (data not shown). Family II members are related through the consensus sequence CGU(C/G)CC(G/A). Consistent with their overrepresentation in the P8 and E9P2 pools, family II aptamers have the highest afﬁnities for TN-C. Although family II aptamers from the crossover SELEX procedure (E9P2 clones) are highly related to family II aptamers obtained using purified TN-C, they are highly related to family II aptamers obtained using purified TN-C.
Aptamer Binding Specificity and Species Cross-reactivity—We next examined the cross-reactivity of a human TN-C aptamer toward mouse TN-C. Because mouse TN-C was not available in purified form, a modified enzyme-linked immunosorbent assay was developed in which TN-C was captured onto a surface and incubated with an aptamer. First, anti-tenascin antibodies were immobilized onto polystyrene 96-well microtiter plates. The wells were then incubated with the tissue culture supernatant of mouse (3t12) or human (U251) cell lines, capturing TN-C onto the surface. Capture was confirmed using a second monoclonal antibody. Aptamer TTA1 (described below), a truncated version of aptamer TN-9, was chosen to test species cross-reactivity. TTA1 was 5'-biotinylated and incubated with captured TN-C at increasing concentrations. After rapid washing, bound TTA1 was quantified. Fig. 7 indicates that half-maximal binding to human TN-C occurred at $20 \times 10^{-9}$ m, similar to the measured $K_d$ of $5 \times 10^{-9}$ m. In contrast, half-maximal binding to mouse TN-C occurred at $400 \times 10^{-9}$ m, a 20-fold reduction relative to the human protein. Binding of the control aptamer, TTA1NB, to the captured TN-C was significantly decreased relative to the binding of TTA1 (Fig. 7). These data indicate that, despite 93% sequence identity between the human and mouse TNfbg domains, aptamer TTA1 has high specificity for human TN-C. Further tests of specificity were conducted by incubating the biotinylated aptamer with adherent cells that express either human or mouse TN-C. We again observed greatly diminished binding of TTA1 to cells expressing mouse TN-C (data not shown). In this case, the cells have developed an ECM, indicating the specificity of TTA1 for a single protein within the native ECM. In general, aptamers selected for binding to a particular protein exhibit low cross-reactivity toward unrelated proteins and even have low cross-reactivity toward highly related proteins. For example, a P-selectin aptamer displays 10,000–100,000-fold selectivity for P-versus L- and E-selectin (48).

A final measure of specificity indicates that TTA1 does not bind appreciably to a wide range of extracellular matrix proteins. The radiolabeled aptamer binds to tumor tissue that expresses TN-C but does not bind to tissue that lacks TN-C or to tumors that do not express human TN-C.6

**Aptamer TTA1: Size Minimization and Further Nuclease Stabilization**—To prepare an aptamer for *in vivo* experimentation, we made three alterations: size minimization; further stabilization against nuclease activity; and incorporation of a bioconjugation handle for addition of biotin, fluorescent tags, and protein conjugates. We used the biotinylated aptamer TTA1 to assess the size effects of nuclease stabilization. We compared the aptamer TTA1 with TTA1M, a 20-fold reduction relative to the human protein. Binding of TTA1 to TN-C. We again observed greatly diminished binding of TTA1 to cells expressing mouse TN-C (data not shown). In this case, the cells have developed an ECM, indicating the specificity of TTA1 for a single protein within the native ECM. In general, aptamers selected for binding to a particular protein exhibit low cross-reactivity toward unrelated proteins and even have low cross-reactivity toward highly related proteins. For example, a P-selectin aptamer displays 10,000–100,000-fold selectivity for P-versus L- and E-selectin (48).

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6 B. Hicke, unpublished observations.
radiometal chelators, etc. Ideally, such changes maintain the affinity and specificity of the aptamer. A cloned aptamer sequence is typically 70–80 nucleotides in length. For efficient chemical synthesis, it is desirable to identify the minimal high-affinity aptamer sequence. This can be initiated by determining the maximum permissible truncations at the 5’ and 3’ termini. Using described techniques (49) on aptamer TN-9, we found that no nucleotides could be removed from the 5’ terminus, and 16 nucleotides could be removed from the 3’ terminus. This exercise produced aptamer TN-9.4, which has $K_d = 10^9$ M for TN-C (Fig. 8).

To identify extraneous nucleotides that reside within the 5’ and 3’ termini required for high-affinity binding to TN-C (e.g. internal loops that do not contribute to the protein-oligonucleotide binding interaction), an RNA secondary structure prediction algorithm (50, 51) was utilized. TN-9.4 and its analogous family II sequences, TN-7.4 and TN-21.4, were each subjected to the algorithm. A predicted structure common to all three aptamers was a three-way junction that places the conserved CGUCGCC element at the center of the junction. Of the predicted stems, the distal portion of the second stem did not appear conserved in sequence or length, suggesting that it is dispensable for high affinity binding to TN-C. By chemically synthesizing a series of deletions in this stem, we found that 17 nucleotides could be replaced by a non-nucleotide spacer, (CH$_2$CH$_2$O)$_6$, with no affinity loss (TN-9.6, Fig. 8). Although consistent with the three-way junction predicted by the algorithm, this internal deletion does not rule out other potential structures. Together, the truncation and internal deletion analyses enabled trimming of a 55-nucleotide sequence to 39 nucleotides and a small spacer. Such a size reduction is critical for efficient chemical synthesis of the aptamer. As shown in Fig. 8B, the size-minimized species has $K_d = 5 	imes 10^{-9}$ M, a 5-fold loss in affinity relative to the full-length aptamer.

Pyrimidine positions, as opposed to purines, are the primary source of nucleolytic instability in plasma. Therefore the pyrimidines are protected from nuclease activity by 2’-F groups incorporated during the SELEX procedure (52). Further stabilization can be achieved by converting purines to 2’-OCH$_3$ purines (52). This occurs after selections, because the addition of 2’-OCH$_3$-modified purines to the existing SELEX procedure causes inefficient transcription. To identify purines that could be converted to 2’-OCH$_3$ without loss of function, aptamer TN-9.4 was divided into five sectors based on the putative three-stem junction structure (Fig. 9). In general, sectors were chosen so that 2’-OCH$_3$ substitutions occurred on one strand of a putative helix, to reduce any helical distortion caused by the substitutions. All purines in each sector were synthetically

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$^7$ Found on the Web at bioinfo.math.rpi.edu/~zukerm/.

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Fig. 5. Sequences and affinities of YN-C aptamers. Aptamers from three SELEX procedures (protein, cell, cell/protein crossover) were grouped into families based on sequence similarity. For simplicity, the fixed sequences at the 5’ and 3’ ends have been omitted. Affinities for TN-C were measured by a nitrocellulose filter binding assay. % of pool, aptamer representation in the pool from which it was cloned; P8, purified protein SELEX procedure eighth round; E9, U251 A EDTA elution ninth round; T9, Trizol elution ninth round.

Table II

| Family I |
| --- |
| TN-4 | Protein | P8 | 4 | CAACUGCAGAAA GACUCAUCUC CGAUCAUCUGUCACCC 45 |
| TN-32 | Protein | P8 | 4 | CAACUGCAGACA GCUGACCCGA ACAGCAUCUGUCACCC 15 |
| TN-29 | Protein | P8 | 4 | CAACUGCAGACA GCUGACCCGA ACAGCAUCUGUCACCC 25 |
| TN-14 | Protein | P8 | <2 | CAACUGCAGACA GCUGACCCGA ACAGCAUCUGUCACCC 20 |
| E9-3 | Cells | E9 | 24 | ACAGCAUCUGUCACCCGAACAAACCCGCCC 100 |

Consensus:

| CACC | GAUCAC | GCAYG | CCCC |

Family II

| E9P2-1 | Cells/Protein | E9P2 | 21 | CCCCCACUACGCGCUGGCCGUAACCCAGCCC CCCC 8 |
| E9P2-2 | Cells/Protein | E9P2 | 35 | CCCCCACUACGCGCUGGCCGUAACCCAGCCC CCCC 4 |
| TN-7 | Protein | P8 | 7 | AACACCGACCGCUGGCCGUAACCCAGCCC CCCC 7 |
| TN-21 | Protein | P8 | 9 | ACACCGACCGCUGGCCGUAACCCAGCCC CCCC 10 |
| TN-9 | Protein | P8 | 22 | AACACCGACCGCUGGCCGUAACCCAGCCC CCCC 2 |

Consensus:

| CNYA | CGCUCCAG | GAAU | CCCC |

Family III

| TN-27 | Protein | P8 | <2 | AAACCGACCGCUGGCCGUAACCCAGCCC CCCC 110 |
| TN-44 | Protein | P8 | 7 | AAACCGACCGCUGGCCGUAACCCAGCCC CCCC 10 |
| TN-39 | Protein | P8 | <2 | AAACCGACCGCUGGCCGUAACCCAGCCC CCCC ND |
| TN-24 | Protein | P8 | 13 | AAACCGACCGCUGGCCGUAACCCAGCCC CCCC 18 |

Consensus:

| GACC | UCUGGCC |

Table II: Family frequencies in aptamer pools.

Five sequence sets are tabulated: selection using protein (P6 = protein sixth round, etc; P8), U251 EDTA elution (E9) and Trizol elution (T9) arms, and cells/protein crossover (E9P2; E9 + 2 rounds protein). By visual inspection, sequences were grouped into one of four families or were considered as unrelated ("Others"). Elution, method used for eluting bound aptamer during the SELEX protocol. "$K_d$ for TN-C" refers to the affinity of the aptamer pool.
substituted with 2'-OCH₃ purines, and affinity for TN-C was measured (Table III). In sectors 2 and 4, binding affinity decreased 20-fold and 1000-fold, respectively, because of the 2'-OCH₃ substitutions. We then identified which of the nine purines in sectors 2 and 4 were responsible for affinity loss upon 2'-OCH₃ substitution; in the context of complete substitution in sectors 1, 3, and 5, individual purines were substituted and the aptamers tested for affinity (Table III). In so doing, we found that substitution at four of the nine Gs caused loss of affinity: G₉, G₂₈, G₃₁, and G₃₄.

Finally, the size minimization and 2'-OCH₃-substitution data were combined to synthesize aptamer TTA1 (Fig. 10), a 39-mer. A nonbinding control aptamer, TTA1.NB, was also synthesized. TTA1.NB has a 5-nucleotide deletion; it does not bind TN-C at concentrations up to 10⁻⁶ M (data not shown).

FIG. 6. SPR assay: binding of tenascin-C fragments to aptamer pools. 5'-Biotinylated aptamer pools and ssDNA aptamer GB41 were immobilized onto a streptavidin surface. TN-C or bacterially expressed TN-C domains were introduced by flow across the surface at 200 × 10⁻⁹ M, and binding was measured at 25 °C by SPR (BIACORE 2000). Values for binding to a random aptamer pools were subtracted from each data set. The beginning of the dissociation phase (buffer only) is marked by an arrowhead. A, binding of full-length tenascin-C. B, binding of a tenascin region consisting of fibronectin type III domains A–D (TNfnA–D). C, binding of fibronectin type III repeats 3–5 (TNfn3–5). D, binding of the fibrinogen-like domain (TNfbg). ---, random aptamer pool; ----, DNA aptamer GB41; --, protein round 4 (P4) pool; ----, P8 pool.

In this work, our goal was to identify a physiologically active, nuclease-stabilized aptamer that could be tested for tumor targeting capability in vivo. A previous tumor cell SELEX experiment yielded a ssDNA aptamer that binds to a major extracellular matrix component, tenascin-C. However, selection at 4 °C resulted in an aptamer that binds moderately well at 4 °C (K_d ≈ 100 × 10⁻⁹ M) but poorly at physiological temperatures (K_d > 1 × 10⁻⁶ M). In addition, DNA is not sufficiently stabilized against nuclease activity to which blood-borne nucleic acids are exposed. Nevertheless, the cell selections had identified a protein that is of significant interest as a marker for tissue remodeling processes including tumor formation. We therefore performed new cell selections and protein selections using a 2'-F pyrimidine library at 37 °C.

In the tumor cell SELEX experiment, EDTA elution was superior to Trizol elution for enrichment of cell-binding aptamers. Because nucleic acid structures and nucleic acid-protein interactions often require divalent cations, we reasoned that low EDTA concentrations could selectively elute a subset of cell- or ECM-bound aptamers. In contrast, the chaotropic agent contained in Trizol removes oligonucleotides indiscriminately from cells, ECM, polystyrene, etc. We found that Trizol elution enriched a population of polystyrene binding sequences that hindered the enrichment of cell-binding aptamers. C_f analysis predicted far higher sequence complexity in the ninth round Trizol pool than in the ninth round EDTA pool. This prediction was borne out by sequence analysis that showed that the E9
pool had repeated sequences, whereas the Trizol pool had no duplicates in 50 analyzed clones. Thus, EDTA elution was superior to Trizol in reducing background binding to polystyrene and in creating an aptamer pool that had converged on a set of cell-binding aptamers.

In the tumor cell SELEX experiment, TN-C was a dominant protein for aptamer selection. There are at least two explanations for this result. First, aptamers bound to membrane targets are candidates for internalization and subsequent degradation by nucleases, and would not be recovered during selections. A second more likely explanation for preferential selection of TN-C aptamers is target abundance. TN-C is present at high (1–10 μM) concentrations in the ECM (16, 46). In a random oligonucleotide pool, any single aptamer is present at a vanishingly small concentration (~10–20 nM). Therefore, concentration of the aptamer target determines the rate and extent of aptamer binding in initial rounds of selection, favoring abundant proteins as targets. The TN-C μM concentration in the ECM is sufficient to drive the binding of single-copy aptamers in an initial round of the SELEX process and is perhaps sufficient to serve as a dominant target over other less abundant proteins. Furthermore, extended washes were used to remove unbound oligonucleotides. High-affinity aptamers, including the TN-C aptamer described here, typically have $k_{\text{off}}$ values of $\sim 10^{-3}$ s$^{-1}$ (44, 48, 53), or $t_{1/2}$ values of 2–10 min. After 50 min of washing, ~97% of an aptamer with $t_{1/2} = 10$ min would be washed away unless the target protein is at sufficiently high concentration for rebinding to occur. Therefore, two factors, extensive washing and the rarity of individual aptamers in a random pool, may each bias cell selections toward abundant proteins. These factors may account for the propensity of two different U251 cell SELEX experiments to isolate tenasin-C aptamers.

For the identification of tumor-targeting aptamers, tumor cells have strengths and weakness relative to purified protein. Using cells enables the presentation of epitopes in their native state and also requires no knowledge of a potential target. The cell SELEX procedure can lead to the identification of new target proteins or the new appreciation of a known protein as an aptamer target. Indeed, this occurred with the initial U251 cell selections that identified an ssDNA aptamer against TN-C, as well as the identification of a new 42-kDa trypanosome protein (54). A disadvantage of cell selections is that slow convergence of cell SELEX pools is caused by higher background binding of nucleic acids to cells (55) than to purified proteins. Furthermore, selection for high affinity may be compromised because concentration of the target protein(s) is unknown. This is illustrated by the experiments in which TN-C ligands dominated the cell selections at round 9, but pool affinity was poor at $K_d = 100 \times 10^{-9} \text{ M}$. This pool was then exposed to purified protein in the crossover procedure. By applying selection pressure for affinity, the $K_d$ for TN-C improved 50-fold in two rounds and isolated two dominant sequences. Therefore high-affinity aptamers were present, but not evident, in the E9 tumor cell pool. By extension, the E9 pool may contain a variety of low abundance/high-affinity aptamers to additional tumor cell proteins of interest. To summarize, cell selections may guide new target identification in unbiased fashion and can be complemented by crossover procedures (or de novo SELEX experiments) to select for high-affinity binding to a protein of interest.

The repeated emergence of TN-C as a target suggests that abundant ECM proteins may be generally well suited as targets for cell-based aptamer selections. It is possible that abundant ECM proteins may also be well suited for aptamer-based tissue targeting in vivo. However, this hypothesis is currently difficult to test; a key difference between in vitro and in vivo selection is that after intravenous injection, oligonucleotides must run a gauntlet of nucleases and blood clearance mechanisms before gaining access to a target tissue, decreasing library complexity significantly. Further experiments using in vivo aptamer selection are needed to address these issues. A truncated aptamer that can be nuclease-stabilized and modified at will has considerable advantages in vivo over the aptamer pools typically used for selections. Therefore aptamers designed for in vivo tissue targeting can be readily derived using protein- and cell-based selection techniques, with appropriate post-selection modifications. As tissue- and organism-based SELEX procedures mature, we suggest that abundant ECM proteins may be generally well suited as targets of tissue targeting can be readily derived using protein- and cell-based selection techniques, with appropriate post-selection modifications. As tissue- and organism-based SELEX procedures mature, we suggest that abundant ECM proteins may be generally well suited as targets for tissue targeting.

To characterize the binding and epitopes of family I, II, and III tenasin-C aptamers, we used an SPR assay. Each ligand family binds to the C-terminal fibrinogen-like domain, TNfbg. Although experimental conditions were not optimized to determine the kinetics of aptamer binding (41), the $k_{\text{on}}$ and $k_{\text{off}}$ for aptamer TN-9 were $-10^8$ M$^{-1}$ s$^{-1}$ and $10^{-3}$ s$^{-1}$, respectively. This corresponds to a $K_d$ of $1 \times 10^{-9} \text{ M}$ as compared with our equilibrium measurement of $K_d = 2 \times 10^{-9} \text{ M}$ using a nitrocellulose filter partitioning assay. TNfbg is one of two basic domains of an otherwise acidic protein that has an overall pI predicted to be ~5. Notably, a previous U251 cell SELEX experiment isolated a TN-C aptamer that binds the other basic region, which consists of fibronectin type III repeats 5–5. The two SELEX experiments differed in selection temperature (37 versus 4°C) and oligonucleotide library (2'-F pyrimidine/2'-OH purine versus 2'-H), but it is not clear how these differences caused the current SELEX experiments to preferentially identify aptamers for the fibrinogen-like domain. It is known that distinct aptamers can bind different epitopes on a single-domain protein (56), and these data extend the observation by

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8 C. K. Lynott, data not shown.
FIG. 8. Aptamer size minimization. A predicted secondary structure of TN-9 is the three-stem junction shown (A). Removal of 16 nucleotides from the 3' end results in TN-9.4. An internal deletion of TN-9.4 replaced 17 nucleotides, G^{30}-C^{16}, with a single (CH\textsubscript{2}CH\textsubscript{2}O)\textsubscript{6} that was incorporated synthetically, producing TN-9.6. Aptamers were 5' end-labeled with \textsuperscript{32}P, and affinity for tenascin-C was measured using a nitrocellulose filter partitioning assay. Data fits give $K_d = 1 \times 10^{-9}$ M (TN-9, circles), $2 \times 10^{-9}$ M (TN-9.4, squares), and $5 \times 10^{-9}$ M (TN-9.6, diamonds). No binding of tenascin-C to a random aptamer pool is evident (triangles).
TTA1 is a size-minimized and nuclease-stabilized aptamer that binds with high affinity to tenasin-C, an abundant extracellular matrix protein that is overexpressed during tissue remodeling processes. The potential clinical advantages of aptamers for tissue targeting have been discussed (32). These advantages include high affinity and specificity, small size, amenity to chemical modification to alter biodistribution, and pharmacokinetics, and rapid tissue penetration. TTA1 can be conjugated to a variety of radioisotope chelators, fluorescent dyes, and imaging agents for in vivo targeting.

### TABLE III

**TN-9.4: affinity of 2'-OCH<sub>3</sub> purine-substituted species**

To identify purines that cannot be substituted with 2'-OCH<sub>3</sub> purines, that aptamer was sectored into five areas as shown in FIG. 9. Aptamers were synthesized in which all purines in a sector were substituted with 2'-OCH<sub>3</sub> purines. Each aptamer was tested for tenascin-C affinity using a nitrocellulose filter partitioning assay. Having identified sectors showing affinity loss upon substitution, individual purines within the affected sectors were studied within the context of complete substitution in sectors 1, 3, and 5. The sum of these data was incorporated into a maximally 2'-OCH<sub>3</sub>-substituted aptamer (in the context of the size-minimized 39-mer) to form TTA1, shown in FIG. 10.

| Sector | Nucleotide | K<sub>d</sub> for TN-C |
|--------|------------|---------------------|
| 1      |            |                     |
| 2      |            |                     |
| 3      |            |                     |
| 4      |            |                     |
| 5      |            |                     |

*See FIG. 9.*

indicating that conditions can also alter the dominant aptamer-binding domain on a large, multidomain protein.

Because reduced size may lead to increased tissue penetration rates (32) and will lead to more efficient chemical synthesis, we focused on size reduction of TN-9. Notably, an internal deletion was identified with the aid of an RNA structure prediction algorithm. This algorithm predicted that TN-9.4 could form a three-stem junction, among other possibilities, as a secondary structure. In comparing four family II aptamers, the algorithm revealed a potentially variant stem. We found that a deletion within this stem removed 17 nucleotides but left high-affinity binding intact. The resulting aptamer, TN-9.6, has a 5-fold reduced affinity relative to the full-length aptamer. Importantly, TN-9.6 is 17 nucleotides, or ~6 kDa, smaller than TN-9.4. The reduced size of TN-9.6 could increase tissue penetration rates and cause it to clear from the blood more rapidly than TN-9.4, which is an advantage for in vivo imaging.

To further stabilize the aptamer against nuclease activity, purines were substituted with 2'-OCH<sub>3</sub> groups. A combinatorial approach for identifying purine positions that tolerate 2'-OCH<sub>3</sub> substitution has been described (49). In that study (49), a library of partially 2-OCH<sub>3</sub>-substituted aptamer molecules was synthesized. A selection experiment then separated binding from nonbinding species. The 2'-OCH<sub>3</sub> substitution pattern of the binding species was then identified by base hydrolysis, indicating purines that tolerate the substitution with retention of aptamer function. In our present work, the aptamer was sectored into five parts, and all purines in each sector were substituted, followed by affinity analysis. We found affinity reductions in two of five sectors and analyzed the contribution of individual nucleotides to affinity within these two sectors. In the end, 15 of the 19 purines could be substituted with 2'-OCH<sub>3</sub> without significant effect on affinity. Compared with the combinatorial 2'-OCH<sub>3</sub> substitution technique, a disadvantage of this sectoring approach is in the quantity of oligonucleotides that must be synthesized. The advantages are found in 1) obtaining direct binding data on a substituted species, rather than inferring effects on affinity from the selection experiment; and in 2) simpler oligonucleotide syntheses and binding experiments. Neither method requires knowledge of aptamer structure. After size reduction, 2'-OCH<sub>3</sub> substitution, 3' capping, and incorporation of a 5' amine, the synthetic aptamer TTA1 has a K<sub>d</sub> of ~5 × 10<sup>-9</sup> M for binding to human TN-C, which is only a 5-fold reduction in affinity from the parent aptamer.

Taken together, the expression pattern of TN-C, demonstrated binding to integrins, and demonstrated adhesive activities (1) suggest that TN-C may play an active role in tissue remodeling processes. Such processes include tumorigenesis, angiogenesis, atherosclerosis, and wound healing. In particular, recent data implicate the fibrinogen-like domain in binding to the integrin α<sub>5</sub>β<sub>1</sub> (25), a critical protein for angiogenesis. The aptamer described here binds tightly to the fibrinogen-like domain of TN-C and therefore has potential application in investigating the role of TN-C in tissue remodeling processes.

FIG. 10. Aptamer TTA1: modifications and putative secondary structure. All pyrimidines are 2'-F and all purines are 2'-OCH<sub>3</sub> except the Gs, marked by arrowheads, which are 2'-OH. (CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub> is a phosphoramidite linker joining G<sup>9</sup> with C<sup>37</sup>. Nucleotides 10–26 have been deleted. The oligonucleotide is synthesized with a 3'-3' linkage and a 5' primary amine incorporated by phosphoramidite coupling. TTA1.NB is a nonbinding sequence that is identical to TTA1 except that it contains a deletion in which CCCUG-3'-3' T is replaced by 3'-3'. K<sub>d</sub> for tenasin-C = 5 × 10<sup>-9</sup> M.
biologically active moieties. Thus modified, TTA1 can be tested for targeted delivery to the extracellular matrix of tumors and/or atherosclerotic lesions.

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