ARTICLE

Discrimination of Recombinant from Natural Human Growth Hormone Using DNA Aptamers

John G. Bruno, Maria P. Carrillo, Taylor Phillips, and Allison Edge*

Operational Technologies Corporation, San Antonio, Texas 78229, USA

Detection of athletes who use synthetic human growth hormone (hGH; or somatotropin) to enhance physical strength and obtain an advantage in competitive sports is a formidable problem, as rhGH is virtually identical to the natural pituitary hormone. However, some post-translational and other modifications have been documented by chromatographic separation and mass spectrometry (MS) in a small percentage of rhGH. In the present work, development of DNA aptamers against research-grade rhGH and natural hGH with adsorption of the rhGH aptamers against natural hGH was shown to produce a small family of aptamer sequences that bound consistently with greater affinity to rhGH over a low nanogram-to-microgram range in ELISA-like microplate assays. This collection of rhGH discriminatory aptamer sequences shared some short sequence segments and secondary structural features. The top rhGH discriminatory aptamers also appeared to cross-react with human myoglobin and BSA but not with bone collagen peptides and an unrelated viral envelope peptide. The cross-reactivity results suggested several strings of up to five consecutive amino acids that might serve as common epitopes for aptamer binding. SDS-PAGE revealed that the rhGH existed largely as a 45-kDa dimer, and the natural hGH was almost exclusively monomeric. The existence of the rhGH dimer suggests that a discontinuous "bridge" epitope may exist on the rhGH, which spans the subunits, thereby accounting somewhat for the difference in detection. Overall, these results suggest that aptamers might be useful for routine, presumptive laboratory screening to identify athletes who are potentially cheating by administration of rhGH.

KEY WORDS: doping, ELISA, post-translational modification, SELEX, somatotropin

INTRODUCTION

Detection of exogenous recombinant human growth hormone (rhGH) in athletes is a problem complicated by virtually identical amino acid sequences between the vast majority of natural and recombinant polypeptides and the short lifetime of rhGH in circulation.[1–5] Several indirect strategies exist for detection of hGH-induced biomarkers, such as insulin-dependent growth factors and the recently discovered hemoglobin α chain.[1,6] Although these strategies are promising, the ultimate solution may be addition of a taggant that will emerge in serum or urine to mark exogenous use of rhGH.[1] Until or unless a marker is added to rhGH, the research and drug testing communities will continue to be faced with the daunting problem of discriminating natural endogenous hGH from exogenous hGH (almost exclusively rhGH). Direct markers of rhGH exist in the form of post-translational modifications of the host cell but at quite low levels. Hepner et al.[2,3] have shown that ~2% of rhGH (even pharmaceutical-grade rhGH) becomes modified at various amino acids by the host bacteria (*Escherichia coli* strain K12), which produce the recombinant polypeptide. The use of mass spectrometry (MS) to detect these markers is expensive and not pragmatic for routine detection in athletes. Additionally, MS may not be sensitive enough to detect trace levels of modified rhGH in body fluids.[2,3]

Development of mAb to all of the potentially altered "epitopes" and modified forms of rhGH produced in other strains or species of bacteria or yeasts would be expensive and labor-intensive and may still not produce assays capable of discriminating rhGH from the natural hormone. Therefore, we set out to determine if highly rhGH discriminatory DNA aptamers could be developed with affinity great enough to detect low levels of modified rhGH. This rationale is supported by the literature, as some aptamer sequences have been documented to be potentially more specific[7–9] and possess higher affinity[10,11] than many comparable antibodies, while obviating animal hosts to suppress production costs. Recently, Calik et al.[12] reported aptamers that bind and detect hGH, but no hGH DNA aptamer sequences were given.

*ADDRESS CORRESPONDENCE TO: John G. Bruno, Operational Technologies Corp., 4100 N.W. Loop 410, Suite 230, San Antonio, TX 78229, USA (Phone: 210-731-0015, ext. 2228; E-mail: john.bruno@otcorp.com)

There are at least 11 modifications of rhGH known from the work of Hepner et al.[2] for *E. coli* strain K12, which applies to 2% of medically available Genotropin® (Pfizer, Groton, CT, USA). For initial proof-of-principle, we elected to obtain a more readily available research grade of rhGH, which is also produced in *E. coli* for aptamer development, and compare ELISA-like plate assay binding of aptamers developed against purified, natural hGH and rhGH with those resulting from adsorption of the rhGH aptamer pool with natural hGH. It was hypothesized that some of the aptamers remaining in the adsorbed pool might be highly specific for rhGH after subtraction of any natural hGH-specific sequences. If successful, other aptamer sequences might be developed to detect the minor percentage of post-translational modifications in pharmaceutical grade Genotropin®, Humatrope®, Norditropin®, Nutropin®, Saizen®, and Serostim® or other brands of rhGH for rapid, presumptive tests to screen suspected athletes for further confirmatory tests.

MATERIALS AND METHODS

Targets, DNA Aptamer Development, Adsorption, Cloning, and Sequencing

rhGH and natural hGH targets with >95% purity after SDS-PAGE were purchased from GenWay Biotech (San Diego, CA, USA; Cat. Nos. 10-002-38017 and 11-511-248592). The natural hGH and rhGH proteins were Type 1 growth hormone with the National Center for Biotechnology Information Accession Number NP_000506.2 and Swiss Protein Accession Number P01241. According to correspondence with GenWay Biotech, the rhGH had no additional reporter or capture tags attached to it. The bone collagen C-telopeptide (CTx) and N-telopeptide (NTx) and viral (Crimean-Congo Gn611) peptides were synthesized at GenScript (Piscataway, NJ, USA), and their amino acid sequences are given in the main text. Human cardiac myoglobin was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and BSA (Fraction V) was from Fisher Scientific (Pittsburgh, PA, USA).

hGh and rhGH (20 μg; targets) hGh and rhGH (targets) from GenWay Biotech were immobilized onto 2.8 μ Dynal tosyl-coated magnetic beads (MBs; 2×10^9 MBs, Invitrogen, Carlsbad, CA, USA) for 2 h at 37°C. Target-conjugated MBs were collected for 2–3 min on a Dynal MPC-S® magnetic collection device, and the supernate was withdrawn carefully with a pipette tip. Target-MBs were then resuspended by vortexing briefly in 1 mL 1× binding buffer (BB; 1×, 0.5 M NaCl, 10 mM Tris-HCl, and 1 mM $MgCl_2$, pH 7.5–7.6) and washed by agitation for 5 min. Target-coated MBs were collected and washed three times in this manner and then resuspended in 1 mL 1× BB for storage at 4°C.

All oligonucleotides were obtained from Integrated DNA Technologies (IDT; Coralville, IA, USA). MB-based aptamer selection and development by systematic evolution of ligands by exponential enrichment (SELEX) were performed essentially as reported previously.[7,8,13–17] The randomized SELEX template library sequence was: 5′-ATCCGTCACACCT-GCTCT-N_{36}-TGGTGTTGGCTCCCGTAT-3′, where N_{36} represents the fully randomized, 36-base region of the DNA library. Primer sequences were: 5′-ATACGGGAGC-CAACACCA-3′ (designated forward) and 5′-ATCCGTCA-CACCTGCTCT-3′ (designated reverse) to prime the template and nascent strands, respectively. The random library was reconstituted in 500 μL sterile, nuclease-free water and heated to 95°C for 5 min to ensure that the DNA library was single-stranded (ss) and linear. The hot template solution was added to 100 μL target-MBs (2×10^8 beads) with 600 μL sterile 2× BB. The DNA library and target-MB suspension (1.2 mL) were mixed at room temperature (RT) for 1 h. Target-MBs with bound DNA (round one aptamers) were magnetically collected with the Dynal MPC-S® unit. The DNA-target-MB complexes were washed three times in 400 μL sterile 1× BB.

Following the third wash, the DNA-target-MB pellet (about 75 μL) was used in a PCR reaction to amplify the bound DNA as follows. The MB pellet was split into 15 μL aliquots and added to five Easy Start™ Micro 50 tubes (Molecular BioProducts, Inc., San Diego, CA, USA), which contained most of the nonperishable components of a PCR reaction beneath a wax seal. A total of 3 μL 1:10 primer mix (10% forward primer plus 10% reverse primer) in nuclease-free deionized water or ~20 nmol each primer/mL plus 1 μL (5 U) Taq DNA polymerase (Fisher Scientific) and 5 μL 2 mM $MgCl_2$ was added to each of the five tubes. PCR reactions were supplemented with 0.5 μL Perfect Match™ *E. coli* ss binding protein (Stratagene, La Jolla, CA, USA) to inhibit high MW concatamer formation. PCR was carried out as follows: an initial 95°C phase for 5 min, followed by 20 cycles of 1 min at 95°C, 1 min at 53°C, and 1 min at 72°C, followed by a 72°C completion stage for 7 min and refrigeration at 4°C. This constituted the first of five rounds of MB-SELEX.

To begin the second round and all subsequent rounds, four of the five original PCR tubes were heated to 95°C for 5 min to release bound DNA from the target-MBs. The fifth tube was always retained and refrigerated as a back-up for that round of the SELEX process. All available DNA (25 μL/tube) was siphoned out of the hot tubes without removing the target-MBs before the tubes cooled significantly, and the DNA was pooled. The 100-μL hot DNA was added to 100 μL fresh target-MBs in 200 μL 2× BB and allowed to mix for 1 h at RT. Thereafter, the selection and amplification process was repeated for four more

rounds of MB-SELEX with checking for 72 bp aptamer PCR product by ethidium bromide-stained 2% agarose electrophoresis after each round.

Following round five, the rhGH aptamers were adsorbed with hGH-MBs and reamplified by 20 rounds of PCR. Aptamers were cloned into chemically competent *E. coli* using a GC cloning kit (Lucigen, Middleton, WI, USA), and clones were sent to Sequetech (Mountain View, CA, USA) for proprietary GC-rich DNA sequencing. Sequence names are coded throughout, indicating that particular sequences derived from selection against natural hGH (H), rhGH (rH), or absorbed rhGH aptamers (Ar), along with F for forward and R for reverse clones, as numbered.

ELISA-Like Enzyme-Linked Aptamer Sorbent Assay (ELASA)

To evaluate relative affinity rankings for each of the candidate hGH and rhGH aptamers, an ELASA[7,8,13–17] was conducted by first immobilizing 1 μg hGH or rhGH targets in 100 μL 0.1 M $NaHCO_3$ (pH 8.5) overnight at 4°C in covered, flat-bottom, polystyrene 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany; Cat. No. 655101). The plates were decanted and washed three times in 200 μl 1× BB. Wells were then blocked with 150 μL 2% ethanolamine in 0.1 M $NaHCO_3$ for 1 h at 37°C, followed by three more washes with 200 μL 1× BB as before. The 106 total 5′-biotinylated hGH and rhGH aptamers from IDT were rehydrated in 100 μL 1× BB for 1 h with gentle mixing on a rotary mixer and applied to their corresponding microplate wells at 1 nmol/well for 1 h at room temperature (~25°C) with gentle mixing. The plates were decanted and washed three times in 200 μL 1× BB for at least 5 min/wash with gentle mixing. A 1:5000 dilution of streptavidin-peroxidase (100 μl) from a 1-mg/ml stock solution (Thermo Scientific, Waltham, MA, USA; Product No. 21126) in 1× BB was added per well for 30 min at RT with gentle mixing. The plates were decanted and washed three times with 200 μL 1× BB/well as before. One-Component® ABTS substrate (100 μl; Kirkegaard Perry Laboratories, Gaithersburg, MD, USA), which had been equilibrated to RT, was added per well for 15 min at RT, and reactions were halted by addition of 100 μL 1% SDS. Absorbance was quantified using a microplate reader at λ = 405 nm.

SDS-PAGE and Staining

Natural hGH and rhGH (10 μL; 10 μg) from GenWay Biotech were mixed 1:1 with Novex® Tris-glycine SDS loading buffer (Invitrogen) and loaded into separate wells of a 4–20% gradient Precise™ SDS-polyacrylamide minigel (Pierce, Rockford, IL, USA) without heating, along with 5 μL 8–220 kD Color Burst™ MW markers (Sigma Chemical Co.). The gel was run in an Invitrogen Xcell SureLock™ tank with cold Tris-SDS-HEPES (BupH™) running buffer at 100 V for 45 min. The gel was rinsed briefly in deionized water and then stained with Invitrogen's SimplyBlue™ Coomassie blue stain and destained in deionized water, according to Invitrogen's instructions, and photographed. The same gel was destained further and fixed in 100 mL 40% ethanol and 10% acetic acid overnight at RT with gentle mixing. Thereafter, the gel was

TABLE 1

Comparison of Top rhGH Discriminatory Aptamer Sequences

H43R
ATCCGTCACACCTGCTCTGGGGTGTATCCTTTTATGGATCTGCGAGAATAAGTCTGGTGTTGGCTCCCGTAT
H49R
ATCCGTCACACCTGCTCTTGGGTGAATCAATTGTTCGTACTCCACGTGATTCTGTGGTGTTGGCTCCCGTAT
H55F (71 bases)
ATACGGGAGCCAACACCATAAGCAGAGAACTACAAGACGATCCCATATTCCAAAGAGCAGGTGTGACGGAT
H56F (71 bases)
ATACGGGAGCCAACACCAGGTTGGCGTCAAAAGAAATAAGGGATATACATTAGAGAGCAGGTGTGACGGAT
H57R
ATCCGTCACACCTGCTCTAGGATTTTTGTTATGTC**GGCCA**CGATGGGGACCCCGTGGTGTTGGCTCCCGTAT
RH50F
ATACGGGAGCCAACACCACATGACGGGTGACTAAAAGACGAAATCTCCCAATTAAGAGCAGGTGTGACGGAT
RH54F
ATACGGGAGCCAACACCACCTGAGCGCCGGCAGCACTCACTATTTGTCCCCGCAAGAGCAGGTGTGACGGAT
AR31R
ATCCGTCACACCTGCTCTAAAACATG***GGCCCA***ATTAGGCGTGATCCAACCCGTCTGGTGTTGGCTCCCGTAT

All sequences are written 5′–3′, from left to right, and flanking 18 base primer regions are underlined. Identical sequence strings or segments are color-coded.

TABLE 2

ELASA Screening Results (Averages of Three Trials with $A_{405\ nm}$ Differences)

hGH aptamer ELASA plate layout

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	rH-31F	rH-31R	rH-34F	rH-34R	rH-35Fa	rH-35Ra	rH-35Fb	rH-35Rb	rH-36Fa	rH-36Ra	rH-36Fb	rH-36Rb
B	rH-37F	rH-37R	rH-38F	rH-38R	rH-39F	rH-39R	rH-45/60F	rH-45/60R	rH-48F	rH-48R	rH-49F	rH-49R
C	rH-50F	rH-50R	rH-52F	rH-52R	rH-54F	rH-54R	rH-55F	rH-55R	rH-57F	rH-57R	rH-58F	rH-58R
D	Ar-31F	Ar-31R	Ar-32F	Ar-32R	Ar-33F	Ar-33R	Ar-34F	Ar-34R	Ar-36F	Ar-36R	Ar-38F	Ar-38R
E	Ar-39/57F	Ar-39/57R	Ar-41F	Ar-41R	Ar-42F	Ar-42R	Ar-45F	Ar-45R	Ar-47F	Ar-47R	Ar-48F	Ar-48R
F	Ar-49F	Ar-49R	Ar-50F	Ar-50R	Ar-51F	Ar-51R	Ar-54F	Ar-54R	Ar-55F	Ar-55R	Ar-56F	Ar-56R
G	Ar-58F	Ar-58R	Ar-59F	Ar-59R	Ar-60F	Ar-60R						
H												

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	H-33F	H-33R	H-37F	H-37R	H-39F	H-39R	H-41F	H-41R	H-42F	H-42R	H-43F	H-43R
B	H-49F	H-49R	H-52F	H-52R	H-54F	H-54R	H-55F	H-55R	H-56F	H-56R	H-57F	H-57R
C	H-58F	H-58R	H-60F	H-60R								

Δ Plate result averages

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.278	0.392	−0.103	−0.078	−0.403	−0.012	0.269	−0.339	−0.036	0.131	0.164	−0.282
B	−0.008	−0.093	0.132	0.037	−0.180	0.030	0.152	−0.016	−0.008	−0.150	−0.079	−0.141
C	0.520	0.125	−0.033	−0.033	0.467	−0.039	−0.333	0.033	−0.428	−0.318	0.247	0.292
D	0.043	0.647	0.061	0.080	0.244	0.025	−0.037	0.052	−0.078	0.425	−0.192	0.186
E	0.462	−0.025	0.026	0.145	−0.213	−0.033	0.087	−0.020	−0.037	0.019	−0.142	−0.256
F	0.217	−0.372	−0.191	0.167	0.409	0.232	0.004	0.091	−0.103	0.002	0.056	−0.037
G	0.076	−0.106	−0.330	−0.024	0.049	−0.039						
H												

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.281	0.271	0.127	0.196	0.027	0.223	0.418	0.437	0.322	0.343	0.337	0.512
B	0.278	0.482	0.343	0.227	0.382	0.356	0.486	0.360	0.513	0.462	0.467	0.501
C	0.273	0.282	0.161	0.259								

Continued

TABLE 2.—*Continued*

Versus rhGH plate result averages

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.796	1.825	0.668	0.727	0.974	0.796	1.066	0.831	0.947	1.133	1.423	1.356
B	1.344	1.292	1.060	0.701	1.019	0.704	1.322	1.379	1.119	0.904	1.283	0.719
C	1.584	1.168	0.814	0.649	1.559	0.829	0.820	0.924	0.686	1.116	1.524	1.582
D	1.342	1.562	1.391	0.749	1.310	0.745	0.774	0.687	1.071	1.483	1.319	1.563
E	1.536	1.087	1.040	0.804	1.120	0.789	0.927	0.864	0.648	0.948	1.133	0.884
F	1.412	0.825	0.995	1.239	1.097	0.838	0.920	1.000	0.833	0.828	1.207	0.860
G	1.322	0.946	1.231	0.796	0.649	0.784						
H												

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.542	1.191	1.484	1.327	1.587	1.542	1.532	1.416	1.371	1.231	1.374	1.372
B	1.686	1.624	1.705	1.113	1.355	1.075	1.258	1.274	1.465	1.350	1.430	1.354
C	1.107	1.398	1.178	1.348								

Versus natural hGH plate result averages

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.518	1.433	0.771	0.805	1.377	0.808	0.797	1.169	0.983	1.001	1.259	1.637
B	1.351	1.385	0.928	0.664	1.199	0.674	1.170	1.395	1.127	1.054	1.362	0.860
C	1.064	1.044	0.847	0.682	1.092	0.868	1.153	0.891	1.114	1.434	1.277	1.290
D	1.299	0.915	1.330	0.669	1.066	0.720	0.811	0.634	1.148	1.058	1.511	1.377
E	1.074	1.111	1.014	0.659	1.333	0.822	0.840	0.884	0.685	0.930	1.275	1.140
F	1.195	1.196	1.185	1.072	0.688	0.606	0.916	0.909	0.936	0.826	1.151	0.897
G	1.246	1.053	1.560	0.820	0.600	0.823						
H												

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.261	0.920	1.357	1.131	1.560	1.319	1.115	0.980	1.049	0.888	1.037	0.860
B	1.408	1.142	1.362	0.886	0.973	0.719	0.772	0.914	0.951	0.888	0.963	0.853
C	0.834	1.117	1.017	1.090								

$A_{405\ nm}$, Absorbance at 405 nm.

silver-stained using an Invitrogen SilverQuest™ staining kit, according to the manufacturer's instructions, and re-photographed.

RESULTS

A total of 106 candidate DNA aptamer sequences was obtained against the hGH targets. For brevity, only the top eight discriminatory aptamer sequences are reported in Table 1, but the entire list of proprietary aptamer candidate sequences will be reported in a pending patent application. It is of interest to note that several of the clones produced identical sequences (namely, RH45F and 45R were identical to RH60F and 60R, and AR39F and 39R were identical to AR57F and 57R; sequences not shown). A number of other much shorter segments were found to be common in the aptamer sequence data, and some of these are highlighted in Table 1.

Table 2 summarizes the ELISA-like aptamer-based (ELASA) plate assay results for three separate trials of all 106 aptamers versus 1 μg of natural or rhGH. Typical ELASA results appeared like the plates shown in Fig. 1 with several obvious differences in absorbance patterns of the various aptamer sequences versus the hGH and rhGH targets. Difference values between the rhGH- and hGH-coated plates were obtained by subtracting the absorbance of a given aptamer bound to rhGH versus its absorbance when bound to natural hGH and averaged over the three trials, as shown in Table 2. The top eight aptamer differences in absorbance (i.e., $A_{405\ nm}$ against rhGH$-A_{405\ nm}$ against hGH$=\Delta$ absorbance), as highlighted in Table 2, were selected for further titrations against serial dilutions of hGH and rhGH to see if the differences were reliable over a broad range of target concentrations (5 μg–5 ng). The differences in affinity (Δ absorbance) were generally true for all eight discriminatory aptamer sequences across this analyte range, as demonstrated by data in Figs. 2 and 3.

Cross-reactivity of the top eight discriminatory aptamer sequences was also tested by ELASA against proteins or peptides that might be expected in human body fluids and some that would not be expected. Among the expected or possible proteins were human myoglobin, which could be present in serum and other body fluids, as a result of skeletal muscle damage induced by strenuous exercise. Also in the possible group of analytes were two human type I bone collagen peptides: CTx (SAGFDFSFLPQPPQEKAHDGGRYYRA) and type I bone collagen NTx (DEKSTGG), which might be expected from bone remodeling or degradation. Among the unrelated analytes were BSA and a Crimean-Congo viral peptide designated Gn611 (TQEGRGHVKLSRGSE). Fig. 4 shows the average ELASA absorbances at $\lambda = 405$ nm of the eight different rhGH discriminatory aptamers after assay against 1 μg each applied target. Clearly, there was substantial cross-reactivity of the rhGH discriminatory aptamers with BSA and myoglobin.

Although BSA is not much of a concern due to its bovine origin, myoglobin could give a falsely high ELASA value, especially in athletes after heavy exercise. The cross-reactivity is not too surprising when one examines the amino acid sequences of natural hGH and rhGH versus human myoglobin, as in Fig. 5. Comparative analyses of these protein sequences with the Swiss Institute of Bioinformatics or SIM alignment tool, known as the Expert Protein Analysis System or the ExPASy Proteomics Server (http://expasy.org/tools/sim-prot.html), reveal several short amino acid segments or strings with 75–80% homology. Most notable was the sequence "FRKDM" (highlighted in yellow) in Fig. 5.

We also analyzed the secondary stem-loop structures of the top eight discriminatory aptamers, as shown in Fig. 6, using free-energy minimization calculated by internet-based Vienna RNA software[18,19] using DNA parameters[20] at 25°C. These structures revealed several identical or highly similar sequence segments (highlighted by color-coded boxes and circles in Fig. 6) in or near loop structures, suggesting binding pockets or areas for analyte-induced fit. The most common segment to emerge from this analysis was GGGTG, which occurred three times in the top eight

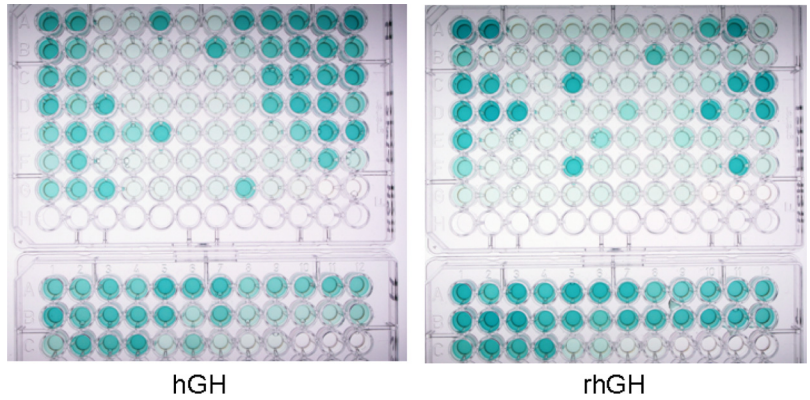

FIGURE 1

Representative photographs of actual ELASA screening plates with results for the 106 candidate aptamer sequences against 1 μg of immobilized natural hGH or rhGH, along with negative controls.

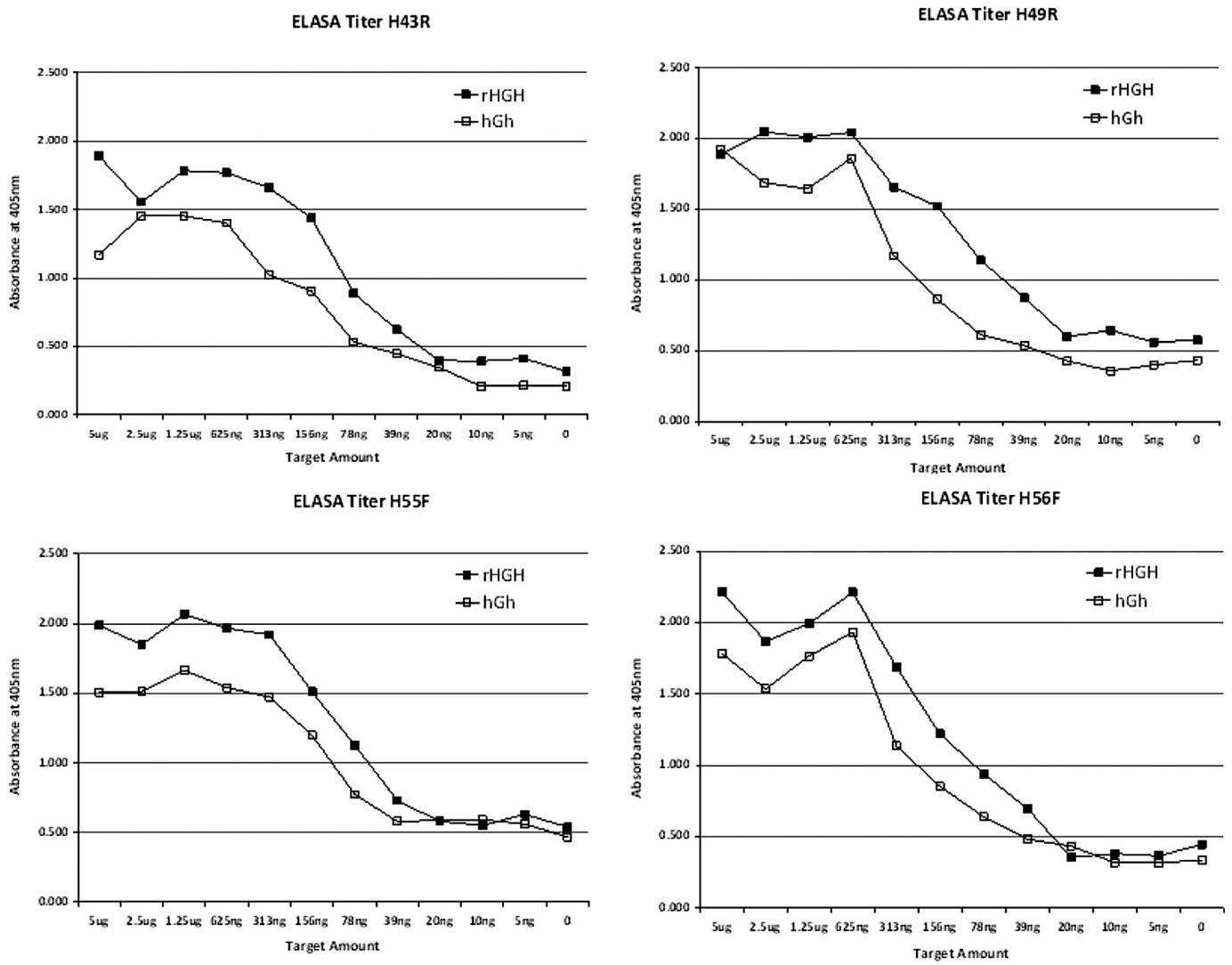

FIGURE 2

Broad-range ELASA titrations (5 μg–5 ng) for top discriminatory aptamers.

discriminatory aptamers and nine times overall in the 106 aptamer sequence population. Also notable were CCCC (occurred twice in the top discriminatory aptamers and 24 times in the total population) and runs of AAAA (with or without GA appended), which occurred twice in the best discriminatory aptamers and 18 times in the overall population.

It was assumed that the natural and rhGH would exist as monomers of ~22 kD based on the literature.[4,21–23] However, as Fig. 7 illustrates, the recombinant hGH existed largely as a dimer at 45 kD, and both monomeric forms migrated ahead of the 20-kD marker. Some other minor differences in polypeptide-banding patterns may exist in the Coomassie blue and silver-stained gels shown in Fig. 7, but it is impossible to draw any conclusions about differences between the natural and rhGH, beyond the obvious difference between monomeric- and dimeric-banding patterns from the figure.

DISCUSSION

The data presented here illustrate the principle that DNA aptamers can discriminate similar forms of the same target polypeptide in an ELISA-like microplate format. The rhGH used in these studies is not a pharmaceutical grade but served to provide proof-of-concept, suggesting that pharmaceutical targets such as Genotropin® might be detectable by an aptamer-based, ELISA-like plate assay. Detection could be based on minor post-translational differences (glycosylations, etc.) in the recombinant form of the growth hormone, which are added by bacterial or fungal host cells.[2,3]

According to GenWay Biotech, the rhGH used in

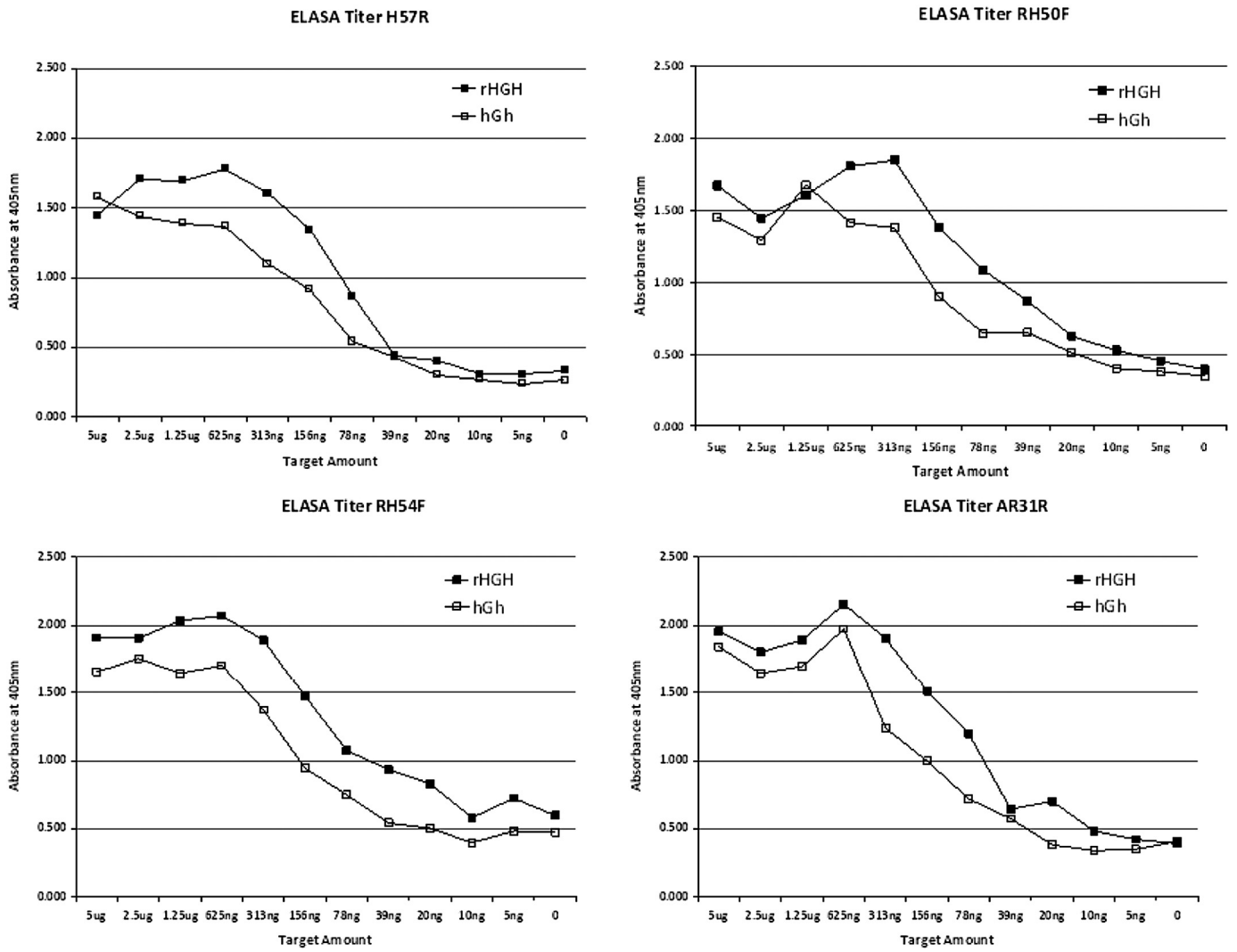

FIGURE 3

Broad-range ELASA titrations for top discriminatory aptamers (continued).

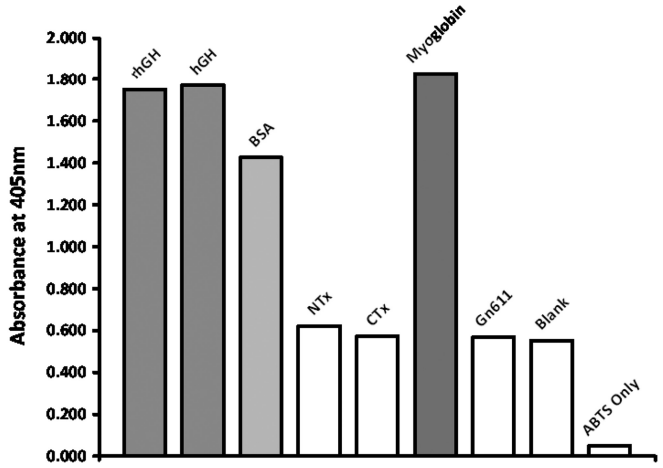

FIGURE 4

Cross-reactivity $A_{405\ nm}$ means of the top eight rhGH discriminatory aptamers versus related and unrelated proteins or peptides.

these studies did not contain any additional reporter or immobilization tags (e.g., no 6× His) that could serve as majorly different epitopes between hGH and rhGH for aptamer-based discernment. Although we cannot currently identify the exact epitope or epitopes that differ between the natural and rhGH, it appears that they do exist and can be identified consistently by some of our aptamers.

The cross-reactivity studies seem to provide some tantalizing clues about potential epitopes, but a more systematic epitope map must be developed for the research grade and pharmaceutical grade rhGH targets to truly identify aptamer-binding sites that discriminate various types of rhGH. We intend to undertake such studies and perhaps develop rapid, presumptive aptamer-based assays to initially screen athletes who may be illegally using rhGH products. Of course, more sophisticated HPLC and mass spectral analyses would still be required for confirmation of illegal rhGH use.

Recombinant and Natural hGH (GenWay Biotech)
MFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFS
ESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYD
LLKDLEEGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFR
KDMDKVETFLRIVQCRSVEGSCGF

Myoglobin
MGLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSE
DEMKASEDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFI
SECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG

Bovine Serum Albumin
MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYL
QQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYG
DMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYL
YEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLTSS
ARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCH
GDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLP
PLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATL
EECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRY
TRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEK
TPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQ
IKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKL
VVSTQTALA

FIGURE 5

Amino acid sequence comparison of the GenWay Biotech natural hGH and rhGH with human myoglobin and BSA sequences from the PubMed protein database after sequence alignment analyses. Color-coded amino acid segments are identical or highly homologous.

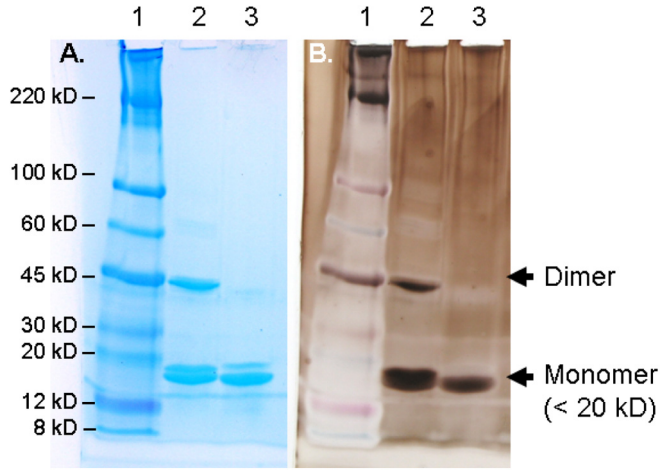

FIGURE 7

Coomassie blue (*A*) and silver-stained (*B*) SDS-HEPES 4–20% gradient PAGE results comparing rhGH (largely dimeric form in Lane 2) with natural pituitary hGH (monomeric subunit <20 kD in Lane 3). Lane 1 contains MW standard markers.

The other interesting aspect of the present work involves the observation that the rhGH existed largely in the dimeric form. Grigorian et al.[23] purport that covalent disulfide bonds bind the subunits together to form a 45-kD homodimer as opposed to the divalent cation (especially Zn^{2+}) subunit-subunit association hypothesis.[22] It is of interest to note that one cysteine (C) exists adjacent to the suspected FRKDM aptamer epitope in hGH, and another cysteine exists downstream in the vicinity of this segment.

This observation would actually suggest that the disulfide bonds could at least partially block aptamer access to the suspected epitope, making it less accessible in the rhGH dimer. However, this does not seem to be the case, as binding to rhGH appears to be stronger (greater $A_{405\ nm}$ for rhGH vs. hGH) across the range of hGH and rhGH amounts examined (Figs. 2 and 3). Therefore, the data better fit a "bridge" epitope model, in which somewhat distal amino acids are brought in close proximity in the dimer, leading to a discontinuous but preferred epitope for

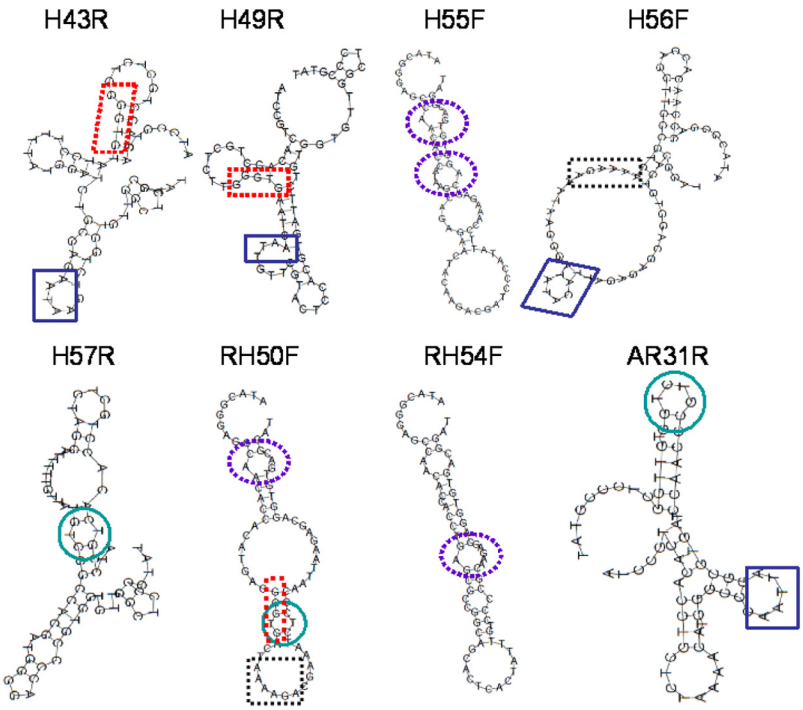

FIGURE 6

Comparison of secondary structures for the top eight rhGH discriminatory aptamers, as determined by Vienna RNA software using DNA parameters at 25°C. Identical or similar segments in or near loops are shown by color-matched boxes or circles.

aptamer binding to rhGH. The natural and rhGH were examined in the same calcium-free PBS for electrophoresis. So, the divalent cation association hypothesis seems unlikely as a cause for dimer formation, making the disulfide linkage more likely.

Grigorian et al.[23] and other authors[5,21] also emphasize the heterogeneity amongst natural forms of hGH, including a 20-kDa, and lighter forms of the hormone. Regardless of the natural heterogeneity, the present work suggests that aptamers may be able to lock onto one or more consistent post-translational (e.g., glycosylation or amino acid modification) differences in recombinant forms of hGH to enable rapid screening of suspected rhGH misuse by athletes. Although further work and computer modeling will be necessary to draw any real conclusions, the biomolecular technique of using aptamers to differentiate isoforms of hGH and rhGH appears promising.

ACKNOWLEDGMENT

Work was funded by a 2009 grant from the World Anti-Doping Agency (WADA).

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