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Insulin Capture by an Insulin-Linked Polymorphic Region G-Quadruplex DNA Oligonucleotide

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Abstract: Insulin capture by a G-quadruplex DNA oligonucleotide containing a two-repeat sequence of the insulin-linked polymorphic region (ILPR) of the human insulin gene promoter region is reported. The immobilized oligonucleotide was demonstrated to capture human insulin from standard solutions and from nuclear extracts of pancreatic cells with high selectivity, using affinity MALDI mass spectrometry and affinity capillary chromatography. Insulin was preferentially captured by the two-repeat ILPR oligonucleotide over another G-quadruplex-forming oligonucleotide, the thrombin-binding aptamer, as well as over a single repeat of the ILPR sequence that is not capable of forming the G-quadruplex architecture. Binding was shown to involve the β chain of insulin. The discovery raises the possibility that insulin may bind to G-quadruplex DNA formed in the ILPR in vivo and thereby play a role in modulation of insulin gene expression, and it provides a basis for design of insulin analogues to probe this hypothesis. The availability of a DNA ligand to human insulin has analytical importance as well, offering an alternative to antibodies for in vitro or in vivo detection and sensing of insulin as well as its isolation and purification from biological samples.

Introduction

The insulin-linked polymorphic region (ILPR) is a noncoding minisatellite in the human insulin gene promoter region located 363 base pairs upstream of the insulin gene.[1−7] It is one of several genetic loci associated with genetic susceptibility to insulin-dependent diabetes mellitus.[8] The ILPR contains tandem repeats of a G-rich repeat unit of the consensus sequence 5′-$ACAG_4TGTG_4$-3′. Oligonucleotides containing at least two tandem repeats can form intramolecular G-quadruplex structures in vitro.[1] Polymorphism in the ILPR includes variability in the number of tandem repeats and variability in the repeat sequence arising from single base pair substitutions. Variants of the ILPR sequence that destabilize or preclude G-quadruplex formation are also associated with significantly lower transcription rates,[1,8,9] suggesting a possible role of G-quadruplex formation in the regulation of transcription the insulin gene. Supporting evidence is found in the case of the transcription factor Pur-1, which preferentially binds to ILPR tandem repeats that form inter- or intramolecular G-quadruplex structures. This has raised the hypothesis that Pur-1 modulates insulin transcription through recognition of G-quadruplex structures in the ILPR;[8] however, G-quadruplex recognition did not in all cases correlate with transcriptional activity, suggesting that there may be alternate regulatory mechanisms that involve other protein interactions with the "transcription machinery" or modifications of the ILPR tertiary structure.[8]

We report here the discovery that insulin exhibits binding affinity toward G-quadruplex DNA formed by a two-repeat length of the ILPR consensus sequence (hereinafter referred to as "$ILPR_2$"). This intriguing finding raises the possibility that insulin itself may play a role in regulation of its own gene through association with G-quadruplexes formed in the ILPR region. Affinity capture of insulin from commercial protein samples, including purified human insulin and nuclear extracts from human pancreatic cells, by the $ILPR_2$ oligonucleotide was demonstrated using previously described platforms for affinity matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[10] and affinity capillary chromatography.[11] The DNA oligonucleotides were immobilized through a covalent

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linker to fused silica surfaces. Immobilization is intrinsic to both capture schemes and provides a realistic model for protein binding to G-quadruplexes formed in the ILPR that would be anchored in the chromosomal strand. G-quadruplex formation by similarly immobilized DNA has been confirmed in separate studies of the thrombin-binding DNA aptamer,[10,11] which forms a two-plane G-quadruplex structure.[12,13]

Results for $ILPR_2$ were compared with those for bare (unmodified) fused silica surfaces to account for any nonspecific surface adsorption, surfaces coated with a single ILPR sequence ("$ILPR_1$") that cannot form an intramolecular G-quadruplex to see if binding is due to sequence rather than conformation, surfaces coated with the thrombin-binding aptamer ("TBA") to test for specificity among different G-quadruplex DNA oligonucleotides, and surfaces coated with an oligonucleotide derived by scrambling the TBA sequence ("Scrambled") that cannot form a G-quadruplex[11] to test for nonspecific DNA binding. Cross-reactivity among G-quadruplex DNA-binding proteins and G-quadruplex DNA was investigated by comparing capture of insulin and thrombin at $ILPR_2$- and TBA-coated surfaces.

Experimental Section

Materials. The 5′-thiol-modified oligonucleotides, including the G-quadruplex-forming, double-repeat ILPR sequence ($ILPR_2$) (5′-$(ACAG_4TGTG_4)_2$-3′), the ILPR single-repeat sequence ($ILPR_1$) (5′-$(ACAG_4TGTG_4$-3′), the thrombin-binding aptamer (TBA) (5′-GGTTGGTGTGGTTGG-3′), and the scrambled TBA sequence (Scrambled) (5′-GGTGGTGGTTGTGGT-3′), were custom-synthesized by Eurogentech North America (San Diego, CA). The oligonucleotides were brought to a concentration of 2.5 mM in deionized water and stored at −4 °C. Potassium monohydrogen phosphate, potassium dihydrogen phosphate, Trizma buffer, and (3-aminopropyl)triethoxysilane (3-APTES) were from Sigma−Aldrich (St. Louis, MO). Sulfosuccinimidyl 4-(*N*-maleimidomethyl)cylcohexane-1-carboxylate (SMCC) and tris-(2-carboxyethyl)phosphine (TCEP) were from either Pierce Chemical (Rockford, IL) or Sigma−Aldrich. The MALDI matrix was either sinnapinic acid (SA), 20 mg/mL in 50:50 acetonitrile:water containing 0.1% trifluoroacetic acid, or 2-(4-hydroxyphenylazo)benzoic acid (HABA), 2.5 mg/mL in 50:50 acetonitrile:water, both from Sigma−Aldrich. Human insulin (10 mg/mL in 25 mM HEPES, pH 8.2) and human serum albumin (HSA), lyophilized powders from Sigma, were diluted to the appropriate concentration with buffer. Thrombin (156 μM in 25 mM HEPES) from Haematologic Technologies (Essex Junction, VT), human pancreatic nuclear extract (HPNE) from Active Motif (Carlsbad, CA), and DL-1,4-dithriothreitol 99% (DTT) from Acros (Morris Plains, NJ) were used as received without further purification.

Preparation of DNA-Coated MALDI Surfaces and Capillaries. Oligonucleotide-coated spots were prepared on fused silica slides of 1 mm thickness (Valley Design Co., Westford, MA) as previously described.[10] Briefly, this entailed activation of the fused silica surface by rinsing with methanol, water, and 1 M sodium hydroxide, followed by attachment of 3-APTES and coating of the silated surface with the heterobifunctional linker to form spots of approximately 2 mm diameter. The 5′-thiol-modified oligonucleotides were treated with TCEP to cleave the disulfide bonds and then reacted with the linker on the slide surface overnight at 4 °C to create the oligonucleotide-coated spots. The slides were then rinsed with pH 8 potassium phosphate buffer to remove any unbound oligonucleotides, dried under nitrogen, sealed, and stored at room temperature.

For the affinity capillary chromatography experiments, the inner surfaces of bare fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 47 cm total length and 100 μm i.d. were coated with DNA using the same procedure described above for the MALDI plates.[10,11] Following the attachment process, the capillaries were flushed with Tris buffer (20 mM Tris, 5 mM KCl, pH 7.3), sealed with Parafilm, and stored at 4 °C.

Protein Capture Experiments. For the affinity MALDI experiments, 1 μL of sample was incubated on each oligonucleotide-coated spot at room temperature for 30 min. The slide was then rinsed with water to remove any unbound or weakly associated species and dried. A MALDI matrix was then added to the spots and allowed to crystallize. The slide was then mounted directly onto a conventional stainless steel MALDI target plate using double-sided tape, and the spots were analyzed by MALDI-TOF-MS using either a Voyager DE (Applied Biosystems, Foster City, CA) or an AutoFlex II (Bruker, Billerica, MA) system.

The affinity capillary chromatography experiments were performed using either a P/ACE 5000 or a P/ACE MDQ CE system (Beckman Coulter, Fullerton, CA) according to the following procedure. One capillary volume of protein was loaded onto the capillary under low pressure (0.5 psi) and incubated overnight. This volume was followed by a capillary volume of wash buffer (20 mM Tris, 5 mM KCl, pH 7.3), both of which were collected. This served as the "load" collection. Three more capillary volumes of the wash buffer were passed through the capillary under low pressure to remove loosely associated proteins from the surface and collected. This served as the "wash" collection. A single capillary volume of elute buffer (20 mM Tris, 8 M urea) was then introduced, and the capillary temperature, which was maintained at 25 °C up to this point, was raised to 50 °C. After 30 min, the capillary volume and two additional capillary volumes of the elute buffer were passed through the capillary, collected, and combined to form the "elute" collection. Each of the collections, load, wash, and elute, was diluted to a constant volume of 25 μL using its corresponding wash or elute buffer and stored at 4 °C.

In previous work, we showed that capillaries coated with TBA have a binding capacity of approximately 0.08 thrombin molecules per nm^2 surface area.[11] Extending these results to the present work as a first approximation, we could predict a total capacity of 60 pmol of protein for the capillaries of length and internal diameter that were used. This corresponds to an insulin concentration of 20 μM in a single capillary volume of 3.7 μL. To ensure an excess of protein to maximize protein capture, a solution of 10 times this concentration, 200 μM, of protein was incubated in the various capillaries.

Total protein in each collected fraction was determined using fluorescence emission spectroscopy. Fluorescence emission spectra of collections from the capillaries and of human insulin standard solutions were collected using an SLM-AMINCO model 8100 Series 2 (HORIBA Jobin Yvon, Edison, NJ) fluorescence spectrometer with an excitation wavelength of 280 nm. Samples were contained in 0.1 cm path length quartz cuvettes from Starna Cells (Atascadero, CA). Concentrations were determined from calibration curves constructed using the standard solutions.

The identities of the proteins in the elute collections were investigated by conventional MALDI-TOF-MS. Prior to the analysis, the 25 μL collection solutions were desalted and preconcentrated by passing the solution through a C-18 Zip-Tip reverse-phase chromatography tip (Millipore, Billerica, MA). The retained proteins were eluted from the Zip-Tip with 5 μL of either 50:50 acetonitrile/H_2O with 0.1% trifluoroacetic acid or sinnapinic acid matrix. In cases where HABA matrix was used, 1 μL of the eluent was mixed with 1 μL of HABA matrix on a stainless steel MALDI plate. Because SA matrix is recommended for masses up to 40 000 Da by the instrument manufacturer and HABA is most efficient for larger masses, both matrices were needed for complete protein analysis.

Results and Discussion

Affinity Capture Studies: Insulin Standard. The first set of experiments compared insulin capture at $ILPR_2$-coated

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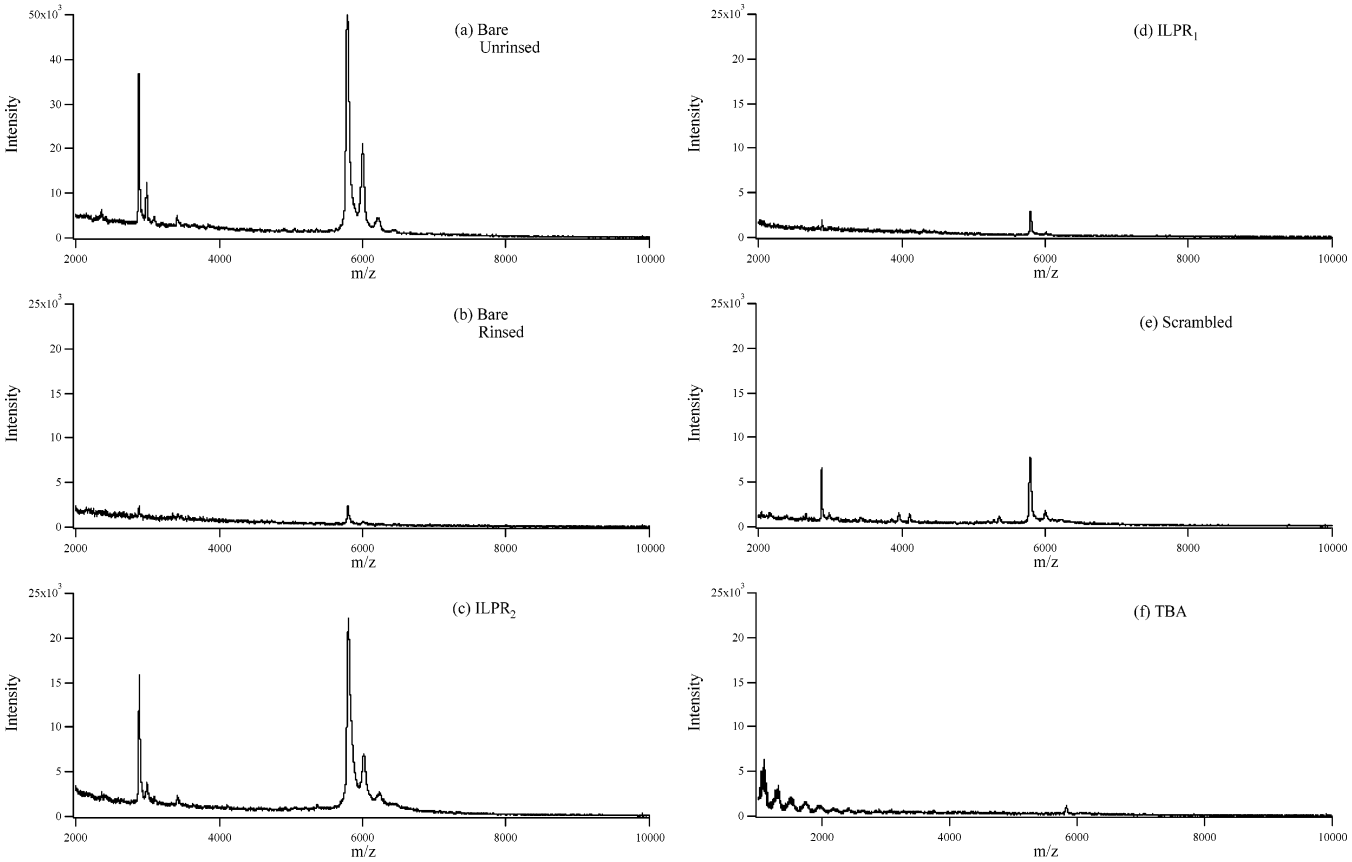

Figure 1. MALDI-MS of 10 μM insulin on various surfaces: (a) bare fused silica, unrinsed; (b) bare fused silica, rinsed; (c) $ILPR_2$; (d) $ILPR_1$; (e) Scrambled; and (f) TBA.

Table 1. Results for Insulin Capture by Affinity MALDI-MS at Various Surfaces, Averaged over Several Spots and Normalized to $ILPR_2$ (in Bold), with % Relative Standard Deviation (%RSD)

			peak intensity		peak area	
sample	surface	# spots	mean	%RSD	mean	%RSD
insulin (10 μM)	bare unrinsed	3	110	50	110	70
	$ILPR_2$	**3**	**100**	**20**	**100**	**40**
	$ILPR_1$	6	8	80	4	90
	TBA	4	2	80	1	130
	scrambled	3	10	100	8	120
	bare rinsed	3	5	60	2	100
insulin (1 μM)	**$ILPR_2$**	**3**	**100**	**9**	**100**	**20**
	$ILPR_1$	6	7	80	4	80
	scrambled	3	5	30	3	70

surfaces with capture at other surfaces. Figure 1a shows the spectrum of insulin on a bare (uncoated) spot that was not rinsed prior to application of MALDI matrix. This spectrum exhibits the expected insulin peak at 5808 *m*/*z* and serves as a standard for comparison with spectra of the other surfaces. Figure 1b shows the spectrum for insulin on a bare spot that was rinsed prior to application of matrix. The absence of any significant peaks confirms that protein is effectively removed from the bare surface in the rinsing step. Figure 1c shows the spectrum of insulin captured at the $ILPR_2$ surface according to the procedure described in the Experimental Section. Insulin capture is clearly indicated by the presence of peaks at 5808 and 2900 *m*/*z*, corresponding to singly and doubly charged insulin ions, respectively. By comparison, very little insulin capture is indicated at the $ILPR_1$ surface (Figure 1d) or the scrambled TBA surface (Figure 1e), neither of which can form an intramolecular

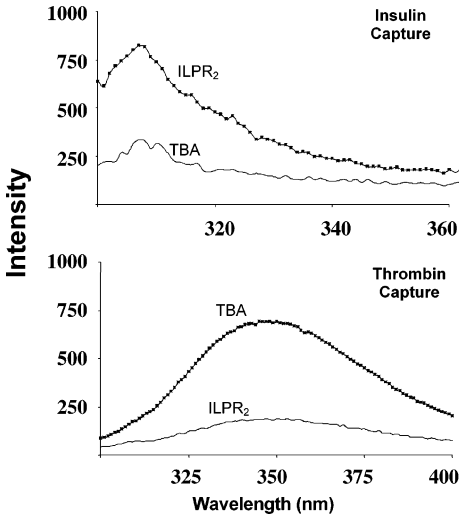

Figure 2. Fluorescence emission spectra of collections of captured proteins. Top: Insulin captured from one capillary volume of 200 μM insulin and eluted from $ILPR_2$- and TBA-coated capillaries. Bottom: Thrombin captured from one capillary volume of 156 μM thrombin and eluted from TBA- and $ILPR_2$-coated capillaries.

G-quadruplex structure. Specificity of insulin for the ILPR G-quadruplex over the TBA G-quadruplex is indicated by the much smaller degree of capture at the TBA surface (Figure 1f).

Table 1 shows quantitative results for insulin capture experiments from 10 and 1 μM insulin standard solutions. At both concentrations, insulin capture was at least 10-fold greater for $ILPR_2$ than for any of the other surfaces (discounting the bare, unrinsed surface). The standard deviations in all cases are much

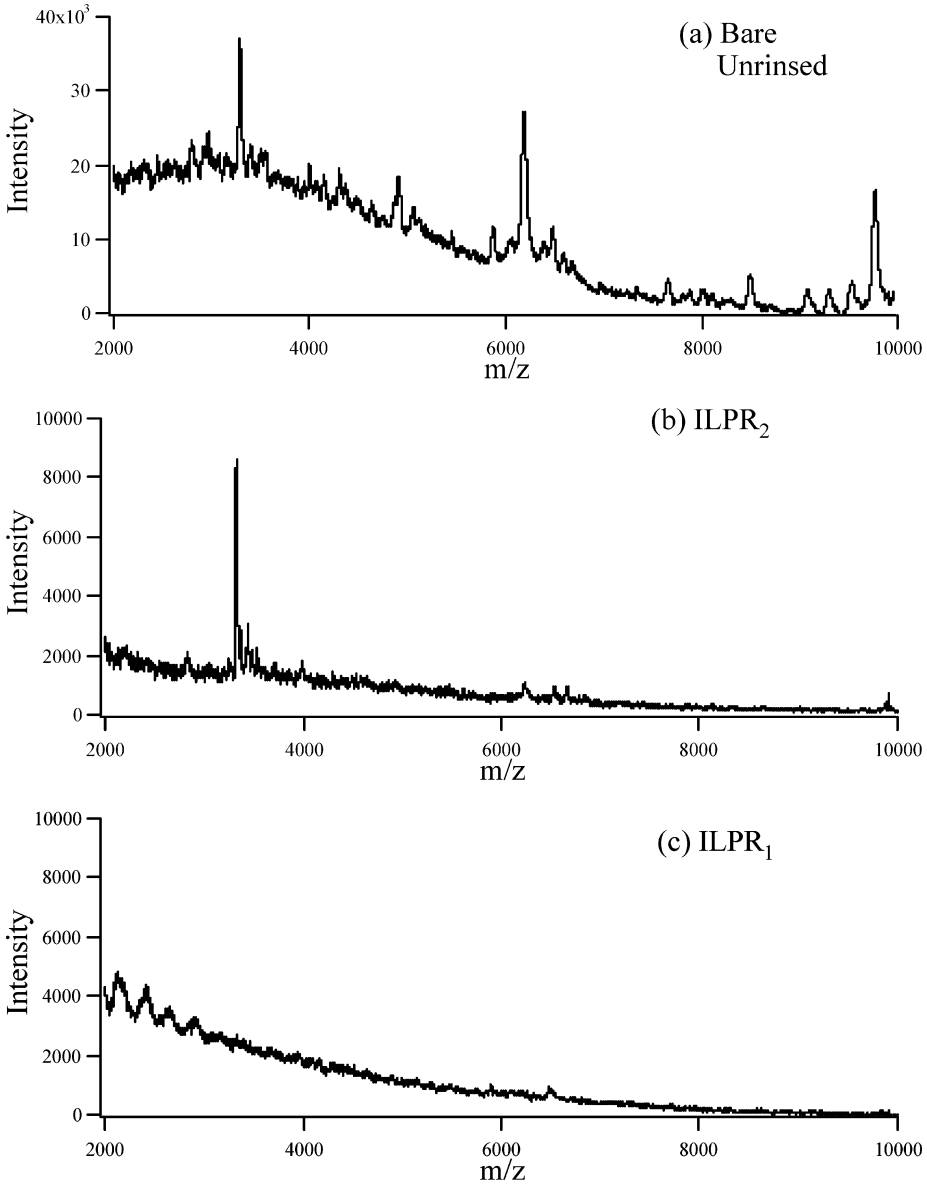

Figure 3. Affinity MALDI-MS of HPNE on (a) bare fused silica, unrinsed; (b) $ILPR_2$; and (c) $ILPR_1$ surfaces.

lower for the $ILPR_2$ surface than for the other surfaces, indicating that retention of insulin due to affinity capture at the $ILPR_2$ surface is much more reproducible than retention due to nonspecific adsorption events at the other surfaces.

Cross-reactivity between G-quadruplex-binding proteins and G-quadruplex DNA was further investigated using the affinity capillary chromatography method to compare capture of insulin with capture of thrombin on $ILPR_2$-coated and TBA-coated capillaries. Protein identities were confirmed using MALDI-TOF-MS. Circular dichroism spectra (shown in the Supporting Information for insulin and $ILPR_2$) confirmed unfolding of the protein and DNA structures under the conditions used for release and elution of the captured protein. Fluorescence spectra of the elute collections, shown in Figure 2, were used to compare protein capture.

On the basis of the fluorescence spectral peak areas, we found that 5 times more insulin is captured on the $ILPR_2$ capillary than on the TBA capillary, and at least 3 times more thrombin is captured on the TBA capillary than on the $ILPR_2$ capillary. These results confirm that there is selectivity among the different G-quadruplex structures toward their respective protein targets.

Interestingly, the TBA and Scrambled capillaries gave similar results for insulin capture, indicating that the G-quadruplex aptamer is no better at capturing insulin than the scrambled, non-G-quadruplex oligonucleotide. The affinity of insulin for $ILPR_2$ but not for TBA is attributed to differences between the TBA structure[13] and the proposed $ILPR_2$ intramolecular G-quadruplex structure.[1] The presence of four G-tetrads in $ILPR_2$ vs two in TBA is unlikely to be responsible, since proteins generally interact with the connecting loops rather than the G-tetrads themselves, which merely provide a scaffold for arrangement of the loops. It is known that the parallel loops of sequence -TT- in TBA are the site of interaction with thrombin.[14] Further work is needed to determine the binding site of insulin in the $ILPR_2$ G-quadruplex structures.

Insulin and Human Serum Albumin. Nonspecific protein interference was evaluated using human serum albumin (HSA).

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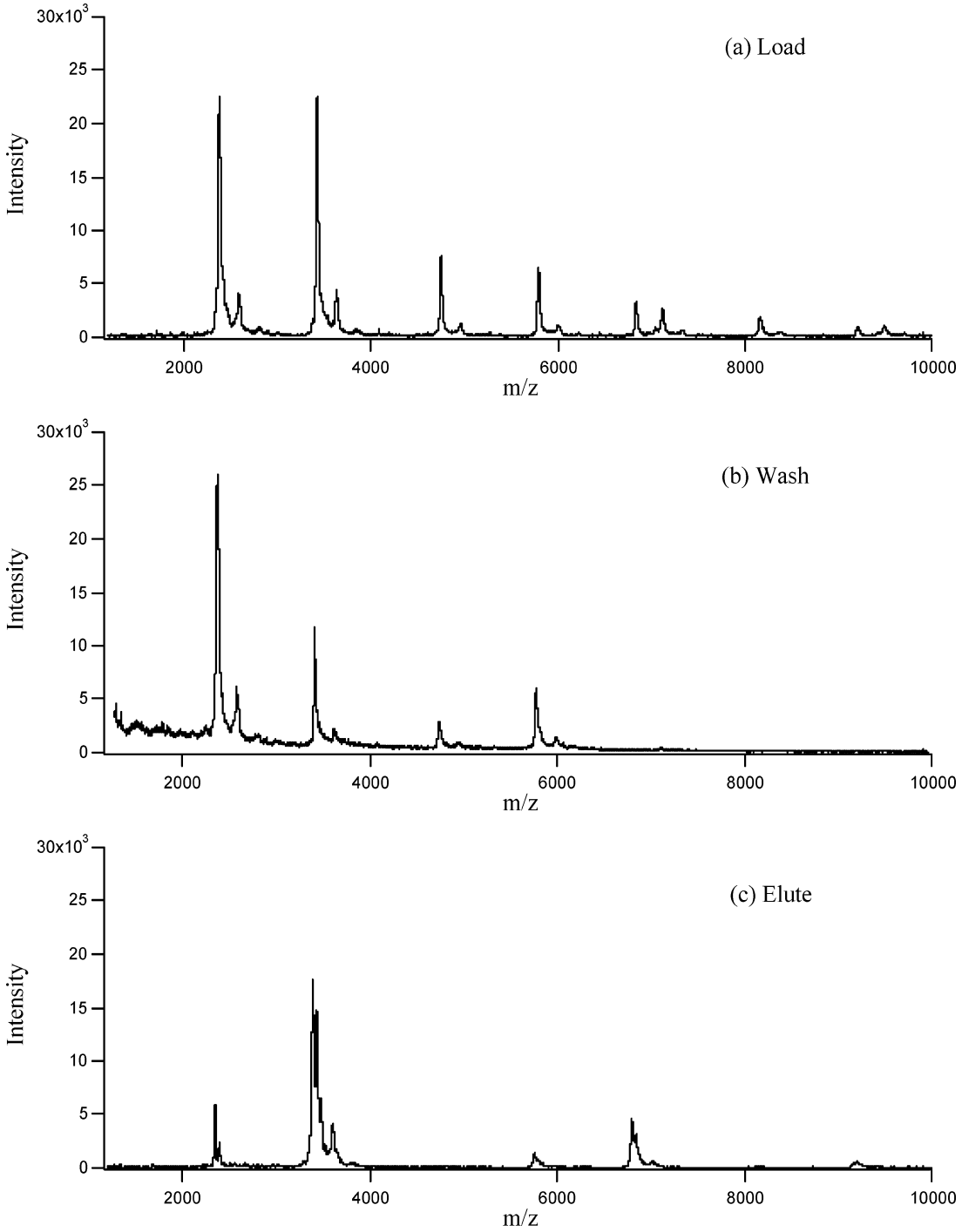

Figure 4. MALDI mass spectra of collections from affinity capillary chromatography capture experiment of 500 μM insulin incubated for 30 min with 500 μM DTT before loading onto the $ILPR_2$ capillary.

In the affinity MALDI experiments, both pure HSA and equimolar mixtures of HSA and insulin were loaded onto ILPR-coated plates. There was no evidence of significant capture of HSA at the ILPR spot, nor did HSA interfere with capture of insulin from an equimolar mixture of the two. Similarly, in the affinity capillary chromatography experiments, there was no evidence of HSA capture in the $ILPR_2$-coated capillary, nor did the presence of an equimolar concentration of HSA interfere with the recovery of insulin.

Human Pancreatic Nuclear Extract. Insulin capture from commercial human pancreatic nuclear extract (HPNE) was investigated using both affinity MALDI-MS and affinity capillary chromatography. The pancreas was chosen as the source because it is the site of human insulin synthesis, and a nuclear extract was chosen instead of a total cell lysate because the nucleus is the site of genomic DNA localization within the cell.

Representative affinity MALDI-MS results are shown in Figure 3 both for HPNE diluted 1:10 in SA matrix at a bare surface that was not rinsed prior to MALDI-MS analysis and for capture at $ILPR_2$ and $ILPR_1$ surfaces. The spectrum of HPNE at the bare, unrinsed surface (Figure 3a) shows a number of peaks resulting from multiple proteins, as expected for a nuclear extract. The spectrum for $ILPR_2$ (Figure 3b) shows a large peak near 3400 *m*/*z*, tentatively assigned to the β chain of insulin.

Human insulin consists of a 21 amino acid α chain (2382 Da) and a 30 amino acid β chain (3430 Da) that are linked by two disulfide bonds. The presence of the individual chain is attributed to the presence in the commercial HPNE preparation of 500 μM DTT, which can break the disulfide bonds between the α and β chains in the insulin molecule. No peaks are evident in the spectrum for the $ILPR_1$ surface (Figure 3c), indicating no protein capture.

Insulin Treated with DTT. To further investigate our hypothesis that the β chain of insulin is the site for interaction with the $ILPR_2$ oligonucleotide, as indicated by the HPNE capture results, we conducted both affinity capillary chromatography and affinity MALDI capture experiments on insulin standard that was treated with DTT before capture. Figure 4 shows the mass spectra of the load, wash, and elute collections for the affinity capillary chromatography capture experiment on an $ILPR_2$-coated capillary.

In the load collection, which contains unretained proteins in the sample that was incubated in the capillary overnight, we see approximately equal signals from both the α chain around 2380 *m*/*z* and the β chain around 3430 *m*/*z*. Peaks at higher *m*/*z* correspond to multimers of the α and β chains. In the wash collection, which contains loosely associated compounds removed during the subsequent washings of the capillary with three capillary volumes of buffer, a much larger peak is observed for the α chain than for the β chain. Finally, the elute collection, which contains affinity-captured proteins that are released under the denaturing conditions of the elute buffer, exhibits a much larger peak for the β chain than for the α chain. This indicates that there is preferential retention of the β chain over the α chain due to affinity interactions between the $ILPR_2$ G-quadruplex and the β chain.

Affinity MALDI experiments of insulin treated with DTT provide additional support of our hypothesis that the β chain is the site of interaction of insulin with the $ILPR_2$ G-quadruplex. Eleven different $ILPR_2$ spots incubated with DTT-treated insulin all exhibited capture of the β chain, while only four spots showed evidence of α chain capture. Averaged over the 11 spots, the intensity for the β chain was 20 000 ± 10 000 (RSD = 50%), while that for the α chain was 4000 ± 5000 (RSD = 125%), which is insignificant. When the same sample was incubated at six $ILPR_1$-coated spots, less than a tenth as much β chain (mean intensity = 1500 ± 700, RSD = 47%), and no α chain, was captured. These results show highly preferential capture of β chain over α chain by $ILPR_2$ and much higher affinity of β chain for $ILPR_2$ over $ILPR_1$.

The implication of the insulin β chain in the interaction between insulin and the $ILPR_2$ is particularly important because it opens the door to consideration of other insulin proteins, such as the insulin-like growth factors (IGFs), that contain the same α and β chains as insulin in a contiguous peptide. The three-dimensional structures of the insulin proteins are similar, with identical hydrophobic cores.[15,16] Due to their structural similarity, the receptors of one protein can also bind to the others. Accumulation of insulin and IGFs in the nucleus, where they can participate directly in regulation of nuclear events, has been well described.[17−27] Like insulin, IGFs are circulating hormones that participate in many functions, including cell growth and proliferation, and have been implicated directly or indirectly in diabetes.

Conclusion

Affinity capture of insulin by the G-quadruplex formed by a two-repeat ILPR sequence is demonstrated. Only a small fraction as much insulin is captured by surfaces coated with the G-quadruplex thrombin-binding aptamer or with non-G-quadruplex-forming oligonucleotides, including a single repeat of the ILPR consensus sequence or the scrambled sequence of the thrombin-binding aptamer. Cross-reactivity studies between the thrombin-binding aptamer and the two-repeat ILPR demonstrated specificity among G-quadruplex structures and their protein targets. Nonspecific binding of human serum albumin by the two-repeat ILPR was negligible, and albumin did not interfere with insulin capture. Binding of insulin to the two-repeat ILPR occurs through the β chain of insulin.

The results of this work suggest that insulin proteins may participate in regulation of the insulin gene by association through the β chain with intramolecular G-quadruplex structures in the ILPR region. Further studies are needed to test this hypothesis in vivo using insulin β chain analogues and using molecular modeling to probe the nature of the molecular interaction. In the analytical arena, the availability of a DNA-binding ligand to human insulin offers an alternative to antibodies for in vitro and in vivo detection and sensing of insulin as well as its isolation and purification from biological samples. Although the genome-inspired $ILPR_2$-binding ligand to insulin was not derived combinatorially and is not therefore an "aptamer", it offers the same advantages, such as ease of production and manipulation, stability, relatively small size, and reusability.[28,29]

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Supporting Information Available: Circular dichroism spectra of insulin and $ILPR_2$ in solution, demonstrating loss of conformation in denaturing buffer and at elevated temperature. This material is available free of charge via the Internet at http://pubs.acs.org.

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