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Association of Insulin-Like Growth Factor 2 with the Insulin-Linked Polymorphic Region in Cultured Fetal Thymus Cells

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Abstract

The Insulin-Linked Polymorphic Region (ILPR) is a regulatory sequence in the promoter region upstream of the human insulin gene and is widely recognized as a locus of type 1 diabetes susceptibility. Polymorphism of the ILPR sequence can affect expression of both insulin and the adjacent insulin-like growth factor 2 (IGF-2) gene. Several ILPR variants form G-quadruplex DNA structures in vitro that exhibit affinity binding to insulin and IGF-2. It has been suggested that the ILPR may form G-quadruplexes in vivo as well, raising the possibility that insulin and IGF-2 may bind to these structures in the ILPR in chromatin of live cells. The present work establishes the presence of IGF-2 in the nucleus of cells cultured from human fetal thymus and its association with the ILPR in the chromatin of these cells. In vitro experiments support the involvement of G-quadruplex DNA in the binding interaction.

The Insulin-Linked Polymorphic Region (ILPR) is a minisatellite in the promoter region of the insulin gene (INS) [1,2]. It is part of the IDDM2 locus of genetic susceptibility to type 1 diabetes (also known as insulin dependent diabetes mellitus, or IDDM) and its role in the genetics of type 1 diabetes has been the subject of numerous studies [3-15]. The ILPR has been shown to influence expression of both INS and the nearby insulin-like growth factor 2 (IGF-2) genes [16-18]. Allelic variations in the ILPR that are associated with high risk for type 1 diabetes are also associated with decreased expression of the insulin gene in the thymus during early development [12,14,16,19-22]. Thymic expression of pancreatic islet beta-cell molecules including insulin is critical for development of self-tolerance; if expression is compromised, the body will produce T-lymphocytes that will attack pancreatic beta cells, leading to type 1 diabetes.

The ILPR is found only in primates and is highly polymorphic in humans [1,2,4,18,23,24]. Polymorphism arises both from variability in the number of tandem repeats and variability in the frequency and distribution of the different variants of the repeat sequence that are 14-15 bases in length. It has been suggested that the ILPR may exert control over insulin and IGF-2 expression as part of a nuclear matrix attachment region that modulates accessibility of transcription factors to the corresponding genes [16,17]. In this scheme, length polymorphism would tend to affect the ability of the ILPR to attach to the nuclear matrix while sequence polymorphism would tend to affect the ability of the ILPR to bind to regulatory proteins in the nuclear matrix.

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Oligonucleotides containing two or more repeats of some variants including the most prevalent “variant a” (5'-ACAG₄TGTG₄-3') have been shown to form intramolecular G-quadruplex structures *in vitro* and it has been suggested that G-quadruplex formation may occur in the ILPR *in vivo* and may be involved in its regulatory roles [25-32]. For example, transcription factor Pur-1 binds to ILPR tandem repeats that form G-quadruplex structures *in vitro* and insulin gene transcription rates are significantly lowered by mutations in the ILPR that destabilize the G-quadruplex structures [32].

Previous work has established that insulin and IGF-2 bind with high affinity *in vitro* to oligonucleotides comprising two-repeat sequences of ILPR variant a (ILPRa) or of ILPR variant h (ILPRh), but not of ILPR variant i (ILPRi) [33-35]. The CD spectra of ILPRa and ILPRh exhibit peaks at both 260 nm and 295 nm while the spectrum of ILPRi exhibits only the peak at 260 nm [34]. While CD spectra do not unambiguously reveal specific G-quadruplex structures [36], these results indicate that ILPRi lacks the conformation available to ILPRa and ILPRh that results in a peak around 295 nm and is consistent with certain intramolecular G-quadruplexes such as antiparallel or propeller structures [36]. This suggests that G-quadruplex structures of ILPRa and ILPRh are involved in their binding interactions with IGF-2 and insulin and raises the possibility that these proteins may interact with transient G-quadruplex structures in the ILPR *in vivo* and affect regulation of the insulin and/or IGF-2 genes.

The present work focuses on establishing the presence of IGF-2 in the nuclei of cultured human fetal thymus cells (HFTCs) and determining if IGF-2 is associated with the ILPR in chromatin purified from these cells. Involvement of G-quadruplex structures in the association was investigated using an *in vitro* affinity capture and detection technique in which nuclear extract (NE) from the HFTCs was incubated with ILPR oligonucleotides that were covalently immobilized on fused silica probe surfaces as previously described [37,38]. Captured proteins were analyzed directly on the probe surface using Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS).

Experimental Procedures

Cell culture

A cell line derived from human fetal thymus tissue was obtained from American Type Culture Collection (ATCC, Manassas, VA) (Cat. No. CRL-10936). The human fetal thymus cells (HFTCs) were grown in Iscove's Modified Dulbecco's Medium (ATCC) with 10% fetal bovine serum (HyClone, Logan, UT), 5% human male AB serum (Fisher Scientific, Pittsburgh, PA), and 100 units/mL Penicillin-Streptomycin (HyClone) at 37 °C and 5% CO₂.

Whole cell lysate and subcellular fractionation

Whole cell lysate (WCL) was prepared by washing confluent plates of HFTCs two times with ice cold phosphate buffered saline (PBS, pH 7.4) and then scraping the cells with a plastic cell scraper into 500 µL (per plate) ice cold RadioImmunoPrecipitation Assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 6 mM sodium deoxycholate, 1% Triton-X 100, 0.1% SDS, 2 mM EDTA, pH 8.0) with 5 µL protease inhibitor cocktail III (PIC) (Research Products International Corp, Illinois, Chicago). The lysate was then transferred to a microcentrifuge tube, and spun at maximum speed (17,000×g) for 5 min at 4 °C (adapted from Hu *et al.*) [39]. Supernatants were removed and stored at -20°C.

Nuclear extracts (NE) were prepared by differential centrifugation. All centrifuge steps were conducted at 4 °C unless otherwise stated. Confluent plates of HFTCs were first washed

with 1 mL ice-cold Dulbecco's PBS (Sigma, St. Louis, MO). Another 1 mL ice-cold Dulbecco's PBS was added to the plate. Cells were then scraped, transferred to a microcentrifuge tube and spun at 850×g for 2 min. The cell pellet was washed with one volume of solution A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, pH 7.9 with 0.1% NP-40, 1 mM dithiothreitol and 10 µL PIC per mL solution) and centrifuged at 850×g for 2 min. The pellet was then incubated in 1.5 volumes of solution A for 10 min on ice, followed by centrifugation at 3300×g for 30 s. The resultant supernatant was stored at -20 °C as cytoplasmic extract. The remaining pellet was washed twice with one volume of solution B (20 mM HEPES, 1.5 mM MgCl₂, 20 mM KCl, 1 mM EGTA, 1 mM EDTA, pH 7.9 with 0.1% NP-40, 1 mM dithiothreitol and 10 µL PIC per mL solution) and centrifuged at 13500×g for 10 min. The pellet was then re-suspended in 0.5 volume of solution B and added drop-wise while vortexing to 0.5 volume of solution C (50 mM HEPES, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, pH 7.9 with 0.1% NP-40, 1 mM dithiothreitol and 10 µL PIC per mL solution). The mixture was then incubated at 4 °C for 30 min and centrifuged at maximum speed for 30 min. The resultant supernatant was stored at -80 °C as NE [40].

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed according to the ChIP-IT™ Express protocol (Active Motif, Carlsbad, CA). Briefly, live HFTCs were first fixed using formaldehyde to crosslink and preserve protein/DNA interactions. The chromatin was then isolated and sheared into 100-500 bp fragments by either sonication or digestion using ChIP-IT™ Enzymatic Shearing Cocktail (Active Motif). Specific protein/DNA complexes were immunoprecipitated using the IGF-2 antibodies. The protein/DNA crosslinking was then reversed through incubation at 65 °C, and proteins were further removed by treatment with proteinase K. The DNA fragments of interest were recovered and analyzed using PCR.

The PCR was conducted on a MyCycler™ PCR machine (BioRad, Hercules, CA) using the following protocol. Initial melt step was held at 94 °C for 5 min, followed by 40 cycles of 94 °C denaturing for 20 s, 50 °C annealing for 30 s, and 72 °C extension for 2 min, and then held at 4 °C. The PCR primer set 1 includes 5'-ACA GGG GTG TGG GGA C-3' as the forward and 5'-TTT CCA CAT TAG ACC AGG AG-3' as the reverse, and the primer set 2 includes 5'-ATG GCG GCA TCT TGG GCC ATC-3' as the forward and 5'-TGT CCC CAC ACC CCT G-3' as the reverse. All primers were procured from Integrated DNA Technologies (IDT) (San Diego, CA). PCR products were then run on a 2% agarose gel containing 1 µg/mL ethidium bromide and were imaged on the ChemiImager™ 4400 (Alpha Innotech, San Leandro, CA).

Total DNA after fragmentation, but before precipitation, was utilized as a positive control, while the DNA bound nonspecifically to beads (with no antibody) was used as a negative control.

Western blotting

Protein samples were first denatured in Laemmli 2× loading buffer (4% SDS, 0.1 M DTT, 20% glycerol, 0.004% bromophenol blue in 0.125 M Tris) at 1:1 ratio at 95 °C for 5 min. The samples were then run on a SDS-polyacrylamide gel (15%) in a running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, pH 8.3) at 120v for 45 min. The proteins were transferred to an Immobilon™ PVDF membrane (Millipore, Billerica, MA) at 50v for 50 min in an ice bath using a transfer buffer (25 mM Tris, 190 mM glycine, 30% methanol, 0.005% SDS).

Membranes were then blocked in a 5% milk solution (Tris buffered saline, pH 7.4, with 0.05% NP-40, 5% dry milk) for 1 h at ambient temperature (Ta), rinsed with HRP buffer

(100 mM Tris, 300 mM NaCl, pH 8.0 with 0.05% NP-40, 0.1% bovine serum albumin (BSA)), and incubated with a primary antibody diluted in a mixture of 5% milk solution/HRP buffer at 1:1 ratio overnight at 4 °C. Membranes were then rinsed with HRP buffer and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in PBS containing 0.2% Tween-20 (PBS-T) for 1 h at Ta, followed by additional rinses with PBS-T and PBS. The blots were then developed using SuperSignal® West Femto substrate (Pierce Biotechnology, Rockford, IL), and chemiluminescence images were taken on a ChemiImager™ 4400 (Alpha Innotech).

Primary antibodies used include rabbit anti human IGF-2 polyclonal antibody (AbD Serotec, Raleigh, NC), rat anti tubulin monoclonal antibody (Chemicon, Millipore, Billerica, MA), and rabbit anti fibrillarin polyclonal antibody (Abcam, Cambridge, MA). Secondary antibodies used include HRP-conjugated donkey anti-rabbit IgG and HRP-conjugated anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). The antibody concentrations used in western blotting were determined based upon manufacturers' recommendation.

Immunoprecipitation

IGF-2 antibodies (50 µL) were first immobilized onto protein A resin (150 µL of 50% suspension) (GenScript Corp, Piscataway, NJ) through overnight incubation at 4 °C. HFTC nuclear extracts were pre-cleared with protein A resin (GenScript Corp.) at 4 °C for 30 min at a concentration of 150 µL resin suspension per mL of NE to remove any non-specific binding to the resin. The pre-cleared NE (1 mL) was then incubated with antibody immobilized protein A resin (200 µL) overnight at 4 °C. The beads were washed 3× with ice-cold PBS buffer, suspended in 25 µL Laemmli 2× loading buffer, and heated at 95 °C for 5 min to elute bound proteins. The eluted proteins were then analyzed using western blotting as described above. For the control experiments, a secondary antibody rather than the primary antibody was used to precipitate proteins.

Immunocytochemistry

HFTCs were grown on glass coverslips to about 30% confluency, fixed in methanol containing 1mM EGTA at −20 °C, rinsed with PBS, air-dried, and then blocked with cell blocking solution (CBS, 5% goat serum, 1% BSA and 0.05% sodium azide in PBS) at 4 °C overnight. Coverslips were then incubated with a primary antibody cocktail (IGF-2 and lamin A antibodies in CBS) for 1 h at Ta or overnight at 4 °C, followed by incubation with a fluorescent secondary cocktails for 1 h at Ta. Labeled coverslips were then mounted onto slides using ProLong® Gold mounting media with antifade reagent (Invitrogen, Carlsbad, CA). Cell images were taken on a Leica inverted microscope equipped with a custom-built spinning disk confocal system (McBain Instruments, Chandler, AZ) using Volocity Software (Improvision, Waltham, MA).

Primary antibodies used include mouse anti-Lamin A monoclonal antibody (Abcam) and IGF-2 antibodies mentioned above. Fluorescent secondary antibodies include Alexa Fluor® 594 conjugated anti-rabbit IgGs, and Alexa Fluor® 488 conjugated goat anti-mouse (Molecular Probes/Invitrogen).

Affinity protein capture and detection *in vitro*

IGF-2 (Human, Recombinant, expressed in e.coli, MW 7.5 kDa) was from Sigma. The custom-synthesized, 5'-thiol-modified oligonucleotides, including two-repeat ILPR variant a (ILPRa) (5'-(ACAG₄TGTG₄)₂-3') and two-repeat ILPR variant i (ILPRi) (5'-ACAG₃TCCTG₄)₂-3' were from IDT. The oligonucleotides were attached to fused silica MALDI probe chips (20 mm × 20 mm × 0.75 mm) (Valley Design, Westford, MA) through a covalent linker as previously described [33,34,37,38]. Immediately prior to surface

attachment, oligonucleotides were heated to 95 °C for 5 min to melt any intermolecular and intramolecular structures and then cooled to room temperature in the presence of K⁺ ion to promote the intramolecular G-quadruplex formation, as confirmed by CD spectroscopy. For the affinity MALDI capture experiment, 1 µL of sample (1 µM IGF-2 standard solution or NE of HFTC) was incubated on each oligonucleotide modified spot at Ta for 30 min. Chips were then rinsed with deionized water for 30 s to remove any weakly bound or unbound peptides and dried under nitrogen gas. This incubation-rinse cycle was repeated two more times, each with a new 1 µL aliquot of sample, to concentrate captured proteins at the surface [33,34,37,38]. MALDI matrix sinapinic acid (SA) (Sigma-Aldrich) was then applied to the spot and allowed to crystallize. The chip was then mounted on a custom aluminum MALDI target and each spot was analyzed by MALDI-Time-of-Flight (TOF)-MS using a Bruker AutoFlex II instrument (Billerica, MA). After use the spots were rinsed with 50% acetonitrile in water to remove the MALDI matrix, proteins and any concomitants. PBS buffer was applied to the surface to reconstitute it prior to reuse.

Results

IGF-2 expression and localization in HFTC

If IGF-2 interacts with the ILPR in a physiologically relevant manner, it must enter into the nucleus. We used immunoprecipitation, immunoblotting and immunocytochemistry to determine if IGF-2 is present in the nucleus of the cultured human fetal thymus (HFTCs). Results show that IGF-2 is highly expressed and can be detected by immunoblotting with or without immunoprecipitation in both the whole cell lysate (WCL) and the nuclear extract (NE) (Figure 1).

To confirm the results seen by immunoblotting, we performed immunocytochemistry on cultured HFTCs (Figure 2). Three-dimensional confocal imaging was performed on HFTCs labeled with the nuclear envelope protein, Lamin A, to ensure that these puncta were indeed in the nucleus (Figure 2A). IGF-2 was typically localized to bright nuclear spots in all of the HFTCs examined (100/100 cells counted, Figure 2B).

Interactions of IGF-2 with the ILPR in HFTC

To determine if IGF-2 is associated with the ILPR region *in vivo*, we performed chromatin immunoprecipitation (ChIP) with antibodies to IGF-2. We probed the precipitate with PCR primer sets designed to amplify the ILPR region (Figure 3A). Therefore, if IGF-2 binds and precipitates the ILPR, we would expect to see amplification of fragments of varying lengths due the polymorphism in this region. We observed a ladder of multiple bands with two independent primer sets from total chromatin (positive control), which indicated that our primers were appropriate (Figure 3B). We then used both primer sets to amplify DNA from the IGF-2 antibody pull-down. We observed a similar ladder of multiple bands (Figure 3B). The intensity of the products amplified by primer set 1 was less than those amplified by primer set 2, which may represent differences in the efficiency of amplification by the two primers. Together, these data indicate that DNA containing the ILPR region is co-precipitated with IGF-2, which suggests that IGF-2 binds to the ILPR region of DNA in HFTC nuclei.

In vitro affinity capture studies

In order to establish a possible link between the association of IGF-2 with ILPR in the chromatin of the live HFTCs described above and the association of IGF-2 with G-quadruplex DNA formed by the ILPRa oligonucleotide *in vitro*, we used an affinity MALDI-MS technique that was developed to study *in vitro* protein capture at oligonucleotide-modified surfaces [37,38]. As described in the Experimental section, sample

is incubated with oligonucleotides that are immobilized at the MALDI probe surface through a covalent linker, followed by rinsing to remove unbound and weakly associated proteins and sample concomitants. Captured protein is then detected directly at the probe surface using MALDI-TOF-MS. Although intramolecular G-quadruplex formation by the immobilized oligonucleotides was not directly confirmed in this work, the precautions taken in the immobilization procedure to melt any pre-existing structures, remove any oligonucleotides that were not covalently attached, and allow the immobilized oligonucleotides to refold in the presence of K^+ , should promote the intramolecular G-quadruplex. We previously demonstrated intramolecular G-quadruplex formation for a thrombin-binding aptamer that was immobilized using the same procedure [37]; verification in that case was facilitated by the prior knowledge that thrombin binds to the aptamer only when the aptamer is in its intramolecular G-quadruplex conformation.

Figure 4 shows affinity capture results for IGF-2 standard solution and HFTC NE at ILPRa- and ILPRi-modified MALDI probe surfaces as well as conventional MALDI-MS spectra of the IGF-2 standard and the NE at unmodified probe surfaces. IGF-2 capture from the standard solution is much greater at the ILPRa surface (Figure 4A) than at the ILPRi surface (Figure 4B) and there is evidence of IGF-2 capture from the HFTC NE at the ILPRa surface (Figure 4C) but not the ILPRi surface (Figure 4D). The conventional MALDI-MS spectrum of the IGF-2 standard is shown in Figure 4E for comparison with the affinity capture spectra. It yields the same IGF-2 peak as the spectrum of the protein captured from the IGF-2 standard at the ILPRa surface (Figure 4A). The conventional MALDI-MS spectrum of HFTC NE is shown in Figure 4F. It shows several small peaks but, unlike the affinity capture spectrum of HFTC NE at the ILPRa surface (Figure 4C), it does not include the IGF-2 peak. This not surprising since there is a multitude of proteins at varying abundances and with different MALDI efficiencies in the total NE. Previous work has demonstrated the ability of the affinity MALDI-MS platform to selectively capture and concentrate specific, low abundance proteins from complex mixtures in order to facilitate their detection [37,38]. In the present work, detection of IGF-2 in HFTC NE is achieved through affinity capture at the ILPRa-modified surface (Figure 4C) even though IGF-2 is not detected by conventional MALDI-MS of the HFTC NE (Figure 4F). Capture of IGF-2 from HFTC NE that was spiked with IGF-2 standard on the ILPRa-modified surface (Figure 4G) confirms the ability to extract IGF-2 from the HFTC NE sample.

The results in Figure 4 confirm the presence of IGF-2 in the nucleus of HFTC and indicate that IGF-2 exhibits selective, affinity binding to ILPRa but not ILPRi. Since ILPRi lacks the CD peak exhibited by ILPRa at 295 nm that is indicative of certain intramolecular G-quadruplex structures, these results suggest that the ILPRa G-quadruplex is involved in the association with IGF-2. This is supported by previous work that showed in vitro, high affinity capture of IGF-2 by ILPRa and ILPRh, which exhibits a similar CD spectrum to ILPRa including the peak at 295 nm, but not by ILPRi [34].

Discussion

It is becoming increasingly clear that peptides and growth factors may localize to the nucleus and directly affect transcription, in addition to their well-established effects on cell signaling in the cytoplasm [41-45]. It is also clear, however, that the nuclear role of these peptides and growth factors is highly regulated and may only occur in specific tissues or circumstances [41,45]. The ILPR region is thought to be an important regulatory element for the expression of both insulin and IGF-2 [16-18,32]. The observation that IGF-2 is localized to the nucleus of HFTCs and can bind to the ILPR in HFTC chromatin is an important result with implications for insulin and perhaps IGF-2 regulation in thymus in early development. It has been suggested that insulin-mediated insulin expression may be important during early

development of human thymus to allow for the establishment of immunological self-tolerance [21,22,46-48]. It is worth noting that the same *in vivo* experiments were also performed for insulin in the HFTCs but the results were inconclusive and are therefore not included here. In light of the *in vitro* studies that established affinity binding interactions between ILPR G-quadruplexes and both insulin and IGF-2 [33-35], however, further exploration of possible roles of both insulin and IGF-2 in regulating transcription of the insulin and IGF-2 genes through association with the ILPR is warranted.

A central consideration in this work is the role of G-quadruplex formation in the interactions between IGF-2 and the ILPR in the chromatin of the HFTCs. It is well established that some of the ILPR sequences form intramolecular G-quadruplex structures *in vitro* [25-30,33,34]. Separate work has shown that ILPRa and ILPRh, either free in solution or immobilized at a surface, bind to both insulin and IGF-2 at a sequence (VCG(N)RGF) common to both proteins, where (N) is E in insulin and D in IGF-2 [35]. It was also established that ILPRi, which does not form the G-quadruplex that gives rise to a CD peak at 295 nm, does not exhibit affinity binding to insulin or IGF-2 [34,35]. It is therefore reasonable to suggest that intact IGF-2 and possibly insulin, and/or peptides derived from these proteins, may associate with the ILPR in chromatin through interactions with transient, intramolecular G-quadruplex structures similar to those formed *in vitro* by ILPRa and ILPRh.

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Abbreviations

BSA	Bovine Serum Albumin
CBS	Cell Blocking Solution
ChIP	Chromatin ImmunoPrecipitation
HFTC	Human Fetal Thymus Cells
HRP	Horseradish Peroxidase
IGF-2	Insulin-like Growth Factor 2
IDDM	Insulin-Dependent Diabetes Mellitus
ILPR	Insulin-Linked Polymorphic Region
INS	Insulin gene
IP	ImmunoPrecipitation
NE	Nuclear Extracts
PBS	Phosphate Buffered Saline
PBS-T	PBS containing 0.2% Tween-20
PIC	Protease Inhibitor Cocktail
RIPA	RadioImmunoPrecipitation Assay
Ta	Ambient Temperature
WCL	Whole Cell Lysate

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Figure 1.

Detection by immunoprecipitation of IGF-2 in the nucleus of human fetal thymus cells. WCL: whole cell lysate; NE: nuclear extract prepared by subcellular fractionation; NE-IP: immunoprecipitation of NE with IGF-2 antibody; IP-control: the NE precipitated using protein A beads. Blots were probed with an antibody to tubulin, which should only be found in the cytoplasm, to confirm efficient fractionation. Immunoprecipitated samples were probed with two or more IGF-2 antibodies to confirm results (data not shown).

IGF-2 in Nucleus

Figure 2. Detection of IGF-2 in HFTC nuclei in whole cells (IGF-2 found in 100/100 cells counted). Cells were labeled with antibodies to IGF-2 (red) and Lamin A (green, to identify the nuclear envelope). The 3-D image showed additional features on XZ and YZ planes in addition to those on the XY plane of the 2-D image.

Figure 3.

IGF-2 binding to ILPR as demonstrated by chromatin immunoprecipitation. (A) PCR primers were designed based on the ILPR sequence from the INS gene (GenBank accession # J00265). For primer set 2, PS2F is the forward primer and the ILPRa sequence (ILPRa with the C-rich strand) is the reverse primer. For primer set 1, the ILPRa (G-rich strand) is the forward primer and PS1R is the reverse primer. As there are many tandem repeats of ILPRa in the -2359/-307 region, the variant a primers should anneal to any one of these repeats, thereby resulting in a number of sequences amplified by PCR. (B) Amplified PCR products from ChIP samples and controls using primer set 1 (lane 1) and primer set 2 (lane 2). Lane 3 is standard DNA ladder. Non-specific DNA bound to ChIP beads was used as a negative control, while total HFTC DNA was used as a positive control. Both primer sets resulted in the amplification of a ladder of multiple bands, although the intensity of those amplified by primer set 2 was higher than these amplified by primer set 1.

Figure 4.

In vitro affinity MALDI-MS protein capture studies at oligonucleotide-modified MALDI probe surfaces and conventional MALDI-MS studies at unmodified MALDI probe surfaces for IGF-2 standard and HFTC NE. (A) Capture profile of IGF-2 standard on ILPRa-modified surface. (B) Capture profile of IGF-2 standard on ILPRi-modified surface. (C) Capture profile for HFTC NE on ILPRa-modified surface. (D) Capture profile for HFTC NE on ILPRi-modified surface. (E) IGF-2 standard profile on bare (unmodified) probe surface. (F) Total HFTC NE profile on bare (unmodified) probe surface. (G) Capture profile of HFTC NE spiked with IGF-2 standard on ILPRa-modified surface. Note that the relative intensity scales on the y-axes vary among the spectra. The arrows in some spectra indicate the m/z corresponding to the +1 ion of IGF-2 (7.5 kDa).