The Efficacy of Small Interfering RNAs Targeted to the Type 1 Insulin-like Growth Factor Receptor (IGF1R) Is Influenced by Secondary Structure in the IGF1R Transcript*

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The type 1 insulin-like growth factor receptor (IGF1R) is often overexpressed by tumors and mediates growth and apoptosis protection. We previously showed that antisense reagents complementary to the IGF1R translation start site enhance radio- and chemosensitivity and impair Atm function. However, these agents induce relatively modest IGF1R down-regulation and affect insulin receptor levels. To identify alternative sites for molecular targeting, we utilized scanning oligonucleotide arrays to probe the secondary structure of IGF1R mRNA. This strategy enabled selection of antisense oligonucleotides that generated high heteroduplex yield with IGF1R but not insulin receptor transcripts. Antisense oligonucleotides that hybridized strongly to IGF1R mRNA caused IGF1R down-regulation within intact tumor cells, whereas weakly hybridizing oligonucleotides were inactive. Furthermore, the ability of small interfering RNAs (siRNAs) to block IGF1R expression correlated with the accessibility of the target sequence within the transcript. Thus, siRNAs corresponding to weakly hybridizing oligonucleotides caused minor IGF1R down-regulation, whereas siRNAs homologous to accessible targets induced profound sequence-specific IGF1R gene silencing, blocked IGF signaling, and enhanced tumor cell radiosensitivity. This indicates that secondary structure in the target transcript has a major effect on siRNA efficacy. These findings have implications for siRNA design and suggest that IGF1R-targeting agents incorporating this mode of action have potential as anticancer therapy.

The type 1 insulin-like growth factor receptor (IGF1R)1 is often overexpressed by tumors (1–3), and IGF1R activation mediates tumor cell proliferation, motility, and protection from apoptosis (4). Tumor growth can be inhibited in vivo by blocking IGF1R expression using antisense agents targeting the IGF1R translation start site (TSS) (5). We previously showed that TSS antisense oligonucleotides (ASOs) and antisense RNA enhanced tumor cell sensitivity to cytotoxic drugs and ionizing radiation and impaired the function of Atm (6, 7). However, we could not suppress IGF1R expression by more than 80% and sought to identify alternative sites for molecular targeting.

Intramolecular folding of mRNAs renders all but 5–10% of most transcripts inaccessible to binding of complementary nucleic acids, but the complex secondary structure of long mRNAs is not amenable to accurate modeling (8–11). We used an array-based screen (12) to identify sites within the human IGF1R transcript that were accessible to RNase-H-mediated cleavage. This information on secondary structure allowed us to identify molecular agents that induced IGF1R down-regulation without affecting insulin receptor (IR) expression. Structural constraints were shown to govern the activity of ASOs and also of small interfering RNAs (siRNAs) that mediate RNA interference (RNAi) in mammalian cells (13).

MATERIALS AND METHODS

Oligonucleotides—A 12-mer deoxyribo-phosphodiester oligonucleotide (dN12) was synthesized as described (12). Phosphorothioate oligonucleotides were synthesized and HPLC-purified at the Cancer Research UK Oligonucleotide Laboratory, South Mimms, UK. RNA/DNA chimeric oligonucleotides were designed as described (13); the 5′ base of each sense strand was immediately downstream of an AA doublet, and each strand incorporated 19 bases of RNA with two 3′ deoxythymidines. An inverted sequence control duplex was made for each siRNA. These oligonucleotides were synthesized and HPLC-purified at Transgenomic Laboratories, Glasgow UK. Complementary strands were annealed in 100 mM potassium acetate, 30 mM HEPES KOH, pH 7.4, 2 mM magnesium acetate by incubating at 85 °C for 1 min followed by 37 °C for 1 h. Duplex formation was confirmed by electrophoresis through 5% low melting temperature agarose (NuSieve GTG, FMC BioProducts, Rockland ME).

RNase Mapping—The 1.6-kilobase HindIII-Asp718 fragment of human IGF1R cDNA was cloned into HindIII and Asp718-digested pBS KS- (Stratagene). This plasmid was digested with NotI, blunt-ended with Klenow enzyme, digested with EcoRV, and re-ligated to remove extraneous sequence between the T7 promoter and 5′ end of the insert. The resulting construct pIGF1R1551Δ55 was linearized with Asp718. End-labeled transcripts representing 1–1551 nt of IGF1R mRNA were made by in vitro transcription at 30 or 37 °C with 50 μCi of [γ-32P]GTP (>5000 Ci/mmol, Amersham Biosciences), 750 μM each ATP, CTP, and UTP, and 75 μM unlabeled GTP. Transcripts were purified using MicroSpin G-25 columns (Amersham Biosciences) and analyzed on 6% denaturing polyacrylamide gels. For RNase mapping reactions, 5 fmol of 5′ end-labeled transcript were incubated with 0.5 units of RNase H and 0–500 pmol of deoxyribo-phosphodiester oligonucleotide library (dN12), and the products were analyzed by gel electrophoresis.

Antisense Oligonucleotide Scanning Arrays—Arrays were made as
described (12) using amimated polypropylene (Beckman Instruments, Fullerton, CA) as substrate. The synthesis was carried out using standard nucleotide-cyanethyl-phosphoramidites on an adapted ABI 394 DNA synthesizer (Applied Biosystems). Reagents were delivered to the substrate surface using a diamond-shaped mask with a 30-mm diagonal. The mask was sealed against the polypropylene to create a cell into which reagents for first base synthesis were delivered and then removed. After each nucleotide coupling the mask was displaced by 1.5-mm steps to create arrays of 1–20-mer antisense oligonucleotides. Internally radiolabeled IGF1R1581 transcripts were obtained by in vitro transcription with T7 RNA polymerase, with 20 mM of [α-32P]UTP (~3000 Ci/mmol, Amersham Biosciences), 750 μM each ATP, CTP, and GTP, and 18.75 μM unlabeled UTP. A 2.5-kilobase SalI-PstI fragment of human IR cDNA was cloned into pBluescript SK and linearized with PstI, and internally radiolabeled transcripts representing bases 1–2499 were made as above. Array hybridizations were performed in 1 M NaCl, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.01% SDS at 25 or 37 °C using 50–60 fmol of transcript. Arrays were washed and imaged using the program xseq as described (14).

RNase H Assays for Analysis of Individual Oligonucleotides—End-labeled IGF1R transcripts were obtained by in vitro transcription at 37 °C (as above) and purified through Mini Quick Spin RNA Columns (Roche Molecular Biochemicals). RNase H assays used 0.5 fmol of end-labeled transcript incubated in 50 mM Tris-Cl, pH 7.4, 50 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 units of RNase H (Promega), 20 units of RNase inhibitor (Promega) with 0.5–50 fmol of ASO in 10 μl. After 30 min at 37 °C reactions were terminated by the addition of 10 μl of formamide gel loading buffer, and the products were analyzed by electrophoresis through 6% denaturing polyacrylamide gels.

Cell Culture and Transfection—Tumor cell lines MDA-MB-231 (human estrogen receptor negative breast cancer), ME (human melanoma (15), A549 (human non-small cell lung cancer), DU145 (human androgen-resistant prostate cancer), UC101 (human ovarian cancer), and B16.F1 (mouse melanoma) were cultured in RPMI 1640 medium with 10% fetal calf serum, and all were negative for mycoplasma infection. Cells were transfected with ASOs using Cytofectin (Glen GSV) or Oli-gofectamine (Invitrogen) according to the manufacturer’s instructions. All siRNA transfections were performed using Oligofectamine. Total RNA was made using RNAeasy columns (Qiagen) and 0.5 μg of each sample was analyzed for expression of IGF1R and actin using the Access reverse transcription-PCR kit (Promega) according to the manufacturer’s instructions. The sequences of PCR primers were: IGF1R forward, 5’-gaaatctgcgggccaggcatcg-3’; IGF1R reverse, 5’-gaaatctgcgggccaggcatcg-3’; actin forward, 5’-ctgaaataagcctgatgccatct-3’; actin reverse, 5’-ctgaaataagcctgatgccatct-3’. PCR cycles were 48 °C for 45 min, 94 °C for 2 min, followed by 20 cycles (shown in preliminary reactions to give quantitative yield of product) of 94 °C for 30 s, 54 °C for 45 s, 68 °C for 2 min, with a final 7 min at 68 °C. After 48 h some transfected cultures were serum-starved overnight and stimulated with 50 nM long-R3-IGF-I (GroPep). Cells were lysed and analyzed as described (6) by immunoblotting for IGF1R (Santa-Cruz), IR (Santa Cruz), phospho-Ser-473 Akt (Cell Signaling Technology), total Akt (Cell Signaling Technology), and β-tubulin (Sigma). Immunofluorescent detection of the IGF1R used antibody nIR3 (Ab1, Oncogene Science) as
described (16). Activated IGF1R was detected by immunoprecipitation for phosphorylserine (P-Tyr-100, Cell Signaling Technology) and immunoblotting for IGF1R/H9252-subunit. To measure survival, cells were disaggregated 24 h after transfection and re-seeded in 6-cm dishes at 2000 cells/dish. After 4–6 h to allow adherence, some dishes were irradiated in a 137Cs source at 3 gray/min. After 1–2 weeks of incubation at 37 °C, visible colonies were stained and counted (7). Statistical analysis was performed with GraphPad Prism/3.0c software using the paired t test for comparisons of two treatments and analysis of variance for multiple comparisons.

RESULTS

We used a two-step empirical screen (12, 14) to investigate binding accessibility in the IGF1R transcript and to identify structurally homologous regions within the insulin receptor transcript. First, RNase mapping identified broad regions of accessibility within the 5’ 1.6 kilobases of the IGF1R transcript (Fig. 1A). Based on the relative yield of cleavage products and the mixed nature of the sequence, we selected for further study a 150-nt region corresponding to bases 536–685 of the human IGF1R sequence (17). To locate more precisely the regions of high and low accessibility, we synthesized ASO-scanning arrays complementary to this region. The ASOs were covalently bound by their 3′ ends to the aminated polypropylene substrate and represented all possible complementary sequences of 1–20 residues within the selected region. Probing with IGF1R mRNA at 37 °C (Fig. 1B) showed negligible binding to monomers at the edges of the array. Along the center line 20-mers showed significant heteroduplex yield only in the accessible regions of transcript, with three peaks of hybridization in the region 590–668 nucleotides (peaks 1, 2, 4; Fig. 1B). Hybridizations at 25 °C resulted in higher heteroduplex yield (not shown), with a similar hybridization pattern and an additional focus of hybridization not present at 37 °C (region 3, Fig. 1B).

We also hybridized the array to human IR mRNA to identify ASOs that could affect expression of the IR, which has 60% sequence homology with the IGF1R (Fig. 1B). This analysis revealed two peaks of heteroduplex formation between...
regions of IR RNase H activity (18). The array screen had revealed two ASO, which may be sufficient (if accessible to binding) to induce B. This ASO hybridized with 1–transcript than to the IGF1R was no detectable IR down-regulation in cells transfected with scrambled controls and immunoblotted with 30 scrambled oligonucleotide. treated with equivalent concentrations of ASOs 1, 3, or scrambled sequence (Scr) controls. B, graph shows the mean ± S.E. of 3–5 independent evaluations of each ASO transfected at 30 or 300 nM. IGF1R levels were corrected for loading differences and expressed as % levels in cells treated with equivalent concentrations of scrambled oligonucleotide. C, MDA-MB-231 breast cancer cells were transfected with 30–300 nM ASO4, TSS ASO, or scrambled controls and immunoblotted for IGF1R and IR.

IR mRNA and the IGF1R ASOs on the array (lower panel, Fig. 1B).

We synthesized 20-mer phosphorothioate ASOs that showed intense (ASOs 1, 2, 4) or negligible (ASOs 3, 6) binding to IGF1R mRNA at 37 °C (Fig. 1B and Table I). In a cell-free assay, these ASOs induced RNase H-mediated cleavage of IGF1R mRNA that was proportional to the relative affinity of hybridization when tethered on the scanning array (Fig. 1C). Effects on IGF1R expression in human tumor cells were assessed using transfection conditions that resulted in uptake of fluorescently tagged ASO into 90–95% of cells 5–24 h after transfection (not shown). We observed significant IGF1R down-regulation in MDA-MB-231 breast cancer cells transfected with 300 nM ASOs 1 (p < 0.01), 2 (p < 0.01), and 4 (p < 0.05) that hybridized strongly to the transcript at 37 °C (Fig. 2, A and B). There was no effect on IGF1R expression in cells transfected with ASOs 3 and 6 that hybridized weakly at 37 °C (Fig. 2, A and B). The same patterns of relative efficacy were observed in ME human melanoma and B16.F1 murine melanoma (not shown).

We compared the novel ASOs with an IGF1R ASO complementary to the IGF1R TSS, the conventional site for antisense targeting (5). In cell-free RNase H assays, this sequence generated an indistinct 50-nt cleavage product (Fig. 1C). In transfected MDA-MB-231 cells ASOs 1, 2, and 4 caused significantly greater IGF1R down-regulation than the TSS ASO at 30 nM (p < 0.01 for each comparison), but there was no difference at 300 nM (Fig. 2B). In addition we saw evidence of modest but consistent down-regulation of the insulin receptor by 300 nM TSS ASO (69 ± 8% of levels in scrambled control-treated cells compared with 112 ± 11% for ASO4, p < 0.01; Fig. 2C). We did not investigate secondary structure in the region of the IGF1R translation start site, and the TSS ASO has negligible homology to the start site of the IR transcript. However, several regions of IR mRNA have 4–5 bases of homology with the TSS ASO, which may be sufficient (if accessible to binding) to induce RNase H activity (18). The array screen had revealed two regions of heteroduplex formation between IR mRNA and the IGF1R ASO array, one immediately upstream of ASO1 (Fig. 1B). This ASO hybridized with 30-fold greater intensity to the IGF1R transcript than to the IR and caused minimal effects on IR expression (85–90% of control levels at 150–300 nM). There was no detectable IR down-regulation in cells transfected with ASOs 2 or 4 (Fig. 2C and data not shown). Thus, the scanning array permitted identification of ASOs that induced sequence-specific IGF1R down-regulation without affecting expression of the IR.

This study was extended to evaluate the effects of secondary structure on IGF1R down-regulation induced by siRNAs. We synthesized a 21-mer RNA duplex R2 corresponding to ASO2, which was immediately downstream of the required AA motif (13), and that was the most intensely hybridizing ASO from the scanning array (Fig. 1B). Duplex R2 induced profound sequence-specific IGF1R gene silencing, to −1% of levels in cells treated with an inverted control duplex (Fig. 3). This suggests that the inability to suppress IGF1R expression by greater than 80% using antisense was unlikely to be due to poor transfection efficiency but was more likely a fundamental limitation of the AS approach. An 18-mer R2 duplex with a 3-bp 3′ deletion was less effective than the 21-bp R2, whereas comparable IGF1R down-regulation was induced by 24- and 27-mer duplexes representing 3- and 6-bp 3′ extensions of R2 (see Table I and Fig. 3D). This is in contrast to duplex length requirements for RNAi in Drosophila (19) but consistent with recently reported characteristics of RNAi in mammalian cytoplasmic lysate (20).

The effects of R2 were compared at the RNA and protein levels with R6, a duplex corresponding to ASO6 that failed to hybrideize to IGF1R mRNA on the array (Fig. 1B). Compared with R6, duplex R2 caused greater inhibition of expression of the IGF1R measured by reverse transcription-PCR (Fig. 4A) and induced more profound dose-dependent reduction in IGF1R protein levels than R6 (p < 0.05 at 0.5 and 50 nM, p < 0.01 at 5 nM; Fig. 4, B and C). Indeed the difference between these two duplexes was greater than that between R2 and a mutant R2 duplex (Fig. 4, B and C). It was notable that in this system a single base pair mutation reduced but did not abolish siRNA activity. R2 and R6 were also compared in a range of human and murine cell lines, and in all cases R2 caused significantly more profound IGF1R down-regulation (DU145 (p < 0.01), A549 (p < 0.05), ME (p < 0.01), B16 (p < 0.05); Fig. 4D). This indicates that secondary structure in the transcript has a major effect on siRNA efficacy and suggests that the structural features dictating access are robust and conserved between different cell lines and species.

We then assessed the efficacy of a second pair of duplexes
based on the sequence around peak 4 of hybridization between IGF1R mRNA and the IGF1R ASO scanning array (Fig. 5A). Duplexes R4 and R5 were designed to target 19-mer sequences immediately downstream of AA motifs at bases 636 and 639, respectively, of the IGF1R sequence (17). Data from the scanning array indicated that there was a 6.5-fold difference in hybridization intensity between the equivalent 19mer ASOs (Fig. 5B). Both duplexes induced IGF1R down-regulation in human ovarian and prostate cancer cells, but R4 was significantly more potent (p < 0.05) in both cell lines (Fig. 5, C and D).

To further investigate the effects of ASOs and siRNA on intracellular signaling, the relatively modest IGF1R down-regulation induced by ASOs 1–6 or siRNA R6 was not sufficient to abolish IGF-1 signaling to Akt (Fig. 6A and data not shown). In contrast transfection with R2 induced profound IGF1R gene silencing such that we could not detect IGF-1-induced phospho-Akt. Thus, a 3-bp shift had major effects on siRNA efficacy, paralleling differences in heteroduplex yield on the scanning array.

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FIG. 5. Small sequence shift has major effect on hybridization intensity and siRNA efficacy. A, phosphorimaging analysis of the peak 4 region of hybridization of IGF1R mRNA to 19-mer IGF1R ASOs on the scanning array. ASOs equivalent to duplexes R4 and R5 are marked by arrowheads and by black bars in the histogram. B, the table shows hybridization intensity (arbitrary units) of the scanning array of 19-mer ASOs equivalent to duplexes R2, R4, R5, and R6. C, representative immunoblot showing effects of 10 nm siRNAs or inverted controls (Inv2) on IGF1R levels in human DU145 prostate cancer cells. D, results (mean ± S.E.) of triplicate analyses of IGF1R protein levels in DU145 prostate cancer and UC101 ovarian cancer cells transfected with 10 nm R4 or R5.

FIG. 6. siRNAs corresponding to intensely hybridizing ASOs block IGF signaling and post-irradiation survival. A, MDA-MB-231 cells transfected with 100 nM RNA duplexes were serum-starved overnight and treated with 50 nM IGF-1 for 30 min. Comparable results were seen in two sets of independently prepared MDA-MB-231 cell lysates. PY, phosphotyrosine; IP, immunoprecipitation; IB, immunoblot. B, clonogenic survival in MDA-MB-231 cells after transfection with 100 nM siRNA, inverted control duplexes (Inv), ASO4, or scrambled control (Scr4). Results represent the mean ± S.E. of colony counts in triplicate dishes. Similar results were obtained in two further clonogenic assays. C, clonogenic survival of transfected MDA-MB-231 cells that were unirradiated or irradiated at 2 gray. The fraction surviving 2 gray irradiation (SF2) is shown as the mean ± S.E. of three independent assays.

DISCUSSION

We previously showed correlation between array hybridization and effects on gene expression, by assessing in vitro translation of cyclin B transcripts following ASO microinjection into Xenopus oocytes (12). Here, we have shown that intensity of array hybridization at 37 °C successfully predicted for target down-regulation within intact tumor cells. This is despite the fact that array conditions were non-physiological; ASOs were tethered at the 3’ ends, and the composition of the hybridization buffer did not reflect the protein-rich cytosolic microenvironment. Furthermore, hybridizations were performed in 1 M NaCl since there was insignificant heteroduplex formation at physiological (150 mM) salt concentration (not shown). In a search for effective IGF1R ASOs to combat epithelial hyperplasia in psoriasis, Wraith et al. (21) used a program designed to predict regions of the transcript lacking internal duplex and hairpin structure (21). One of the selected 15-mer ASOs fell within the region we screened, targeting nt 653–667 of the IGF1R transcript. This was found to be ineffective and indeed shown to hybridize weakly on our scanning array (relative hybridization intensity ~0.03 compared with 1.0 for ASO2; Fig. 1B). In accordance with our results, even the most effective ASOs suppressed IGF1R expression only to ~25% of control levels (21).

We found major differences in the activity of IGF1R siRNAs, paralleled by changes in the intensity of hybridization of the equivalent ASOs to IGF1R mRNA. There have been two recent reports of differential efficacy of siRNAs, against human immunodeficiency virus-1 rev (22) and Tissue Factor (23), attributed in the latter report to protein binding at/near the siRNA target site. This is an unlikely explanation for our findings since we observed parallel differences in ASO and siRNA efficacy within intact cells and in cell-free (and virtually protein-free) RNase H assays. The parallels we observed between array hybridization and siRNA efficacy suggest that secondary structure in the IGF1R transcript was a major factor in determining the efficacy of synthetic 21-mer siRNAs. Unlike phosphorothioate ASOs that induce RNase H activity, siRNAs act as “guide sequences” to direct the RNA-induced silencing complex to the homologous sequence in the target transcript (24). The precise nature of the molecular interaction between siRNAs and target mRNA is unclear. The need for access comparable with that required for ASO binding supports the concept, as originally proposed when RNAi was first recognized (24, 25), of direct interaction by base-pairing between the transcript and component(s) of the duplex. This is consistent with the recent demonstration that antisense strands can mediate RNAi in mammalian cytoplasmic lysate (20).

The success rate for effective siRNA design is clearly higher than the ~10% reported for ASOs (10, 26). However a requirement for access could explain why some siRNAs are ineffective. Elbashir et al. (13) were unable to down-regulate vimentin in HeLa cells, but in a later report vimentin expression was effectively silenced by siRNAs directed against different regions of the same transcript (26). We have demonstrated here that mRNA folding has major consequences for siRNA-induced gene silencing, with clear implications for the design of effective siRNAs. Furthermore, the potency of these siRNAs in blocking IGF signaling and tumor cell survival suggests that IGF1R-targeting agents incorporating this mode of action have potential as anticancer therapy.
Acknowledgments—We are grateful to Dr. Renato Baserga, Kimmel Cancer Centre, Thomas Jefferson University, Philadelphia for human IGF1R cDNA and to Dr. Joe Barr, Howard Hughes Medical Institute Research Laboratories, University of Chicago, Illinois for human insulin receptor cDNA.

Note Added in Proof—After this manuscript was submitted, Vickers et al. (27) reported a correlation between transcript sites effectively targeted by siRNAs and by optimized ASOs, supporting the conclusion that siRNA efficacy is affected by mRNA secondary structure.

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