Insulin-like growth factor-I receptor is suppressed through transcriptional repression and mRNA destabilization by a novel energy restriction-mimetic agent

Po-Chen Chu1, Samuel K. Kulp1 and Ching-Shih Chen1,2,∗

1Division of Medicinal Chemistry, College of Pharmacy and Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43221, USA and 2Institute of Basic Medical Sciences, National Cheng-Kung University, Tainan 70101, Taiwan

∗To whom correspondence should be addressed. Tel: +614 688 4008; Fax: +614 688 8556; Email: chen.544@osu.edu

Insulin-like growth factor-I receptor (IGF-IR) represents one of the major targets by which dietary or chemically induced energy restriction mediates chemopreventive effects in animal tumor models. However, the mechanism underlying this cellular response remains unclear. In the course of investigating the suppressive effect of the energy restriction-mimetic agent CG-5 on IGF-IR expression in prostate cancer cells, we identified a novel posttranscriptional mechanism by which the RNA-binding protein human antigen R (HuR) regulates IGF-IR expression through messenger RNA (mRNA) stabilization. Previously, we demonstrated that Sp1 and HuR proteins were concomitantly targeted for ubiquitin-dependent degradation by β-transducin repeat-containing protein in response to CG-5. Although this loss of Sp1 expression contributed to CG-5-mediated IGF-IR down-regulation, enforced specific protein 1 (Sp1) expression could only partially protect cells from the drug effect. The small interfering RNA-mediated silencing of HuR suppressed IGF-IR expression by reducing mRNA stability, whereas ectopic HuR expression increased IGF-IR mRNA stability and protein expression and, when coexpressed with Sp1, blocked CG-5-mediated IGF-IR ablation. RNA pull-down and immunoprecipitation analyses indicated that HuR selectively bound to the distal region of the IGF-IR 3′ untranslated region (UTR), whereas no interaction with the 5′UTR was noted. Evaluation of a series of truncated HuR mutants revealed that the RNA recognition motifs (RRM2 and RRM3) were involved in IGF-IR 3′ UTR binding and the consequent increase in IGF-IR mRNA stability. Although these data contrast with a previous report that HuR acted as a trans-translation repressor of IGF-IR mRNA through 5′ UTR binding, our finding is consistent with the reported oncogenic role of HuR in conferring stability to target mRNAs encoding tumor-promoting proteins.

Introduction

Substantial evidence indicates that the insulin-like growth factor (IGF)-I/IGF-I receptor (IGF-IR) signaling cascade plays a pivotal role in promoting carcinogenesis, tumor progression and metastasis in many types of cancer (1–3). In the course of malignant transformation, cancer cells upregulate IGF-I/IGF-IR signaling by overexpressing IGF-IR and/or acquiring autocrine/paracrine capacity for IGF-1-mediated signaling, thereby bypassing the dependency on circulating IGF-I (4). Stimulation of IGF-IR by IGF-I leads to the activation of the downstream Ras/mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt signaling networks, conferring a growth and survival advantage to tumor cells (1,2). Moreover, dysregulated IGF-I/IGF-IR signaling has been associated with the development of resistance against chemotherapeutic and radiation therapies (5,6). Therefore, IGF-IR represents a therapeutically relevant target for cancer treatment, which is reflected in the large number of IGF-IR-directed monoclonal antibodies (mAbs) and tyrosine kinase inhibitors currently in clinical trials in multiple types of cancer (6,7–9).

Expression of the IGF-IR gene is modulated by a number of transcription factors in response to different physiological or pathological stimuli (4). Although Sp1 is a potent transactivator of the IGF-IR gene (10), several transcription factors with tumor suppressor activities including p53, the breast cancer gene-1 (BRCA1) and the Wilms’ tumor suppressor (WT1) have been reported to negatively regulate IGF-IR expression through functional interactions with Sp1, p53 and estrogen receptor α, respectively, in different cell systems (4,11–14). Clinical evidence indicates that dysregulated expression of Sp1 or loss of function of any of these tumor suppressors results in constitutive upregulation of IGF-IR expression (15–18). In addition, IGF-IR expression is subject to posttranscriptional regulation through changes in the stability and/or translation of the encoding mRNA (19), among which the reported role of the mRNA-binding protein HuR in the translational repression of IGF-IR is noteworthy (20,21).

Data from this and other laboratories have demonstrated that IGF-I/IGF-IIR signaling represents a major target by which dietary or chemically induced energy restriction mediates chemopreventive effects in different rodent models of cancer (22–28). For example, our recent study indicated that CG-5, a novel glucose transporter inhibitor (29), suppressed prostate epithelial proliferation in TRAMP (transgenic adenocarcinoma of the mouse prostate) mice, in part, by downregulating IGF-IR expression (24). This finding is consistent with a recent report that genetic reduction of IGF-IR mimics the anti-cancer effects of caloric restriction in a pancreatic neoplasia model (23). In the course of investigating the suppressive effect of CG-5 on IGF-IR expression in prostate cancer cells, we identified a novel posttranscriptional mechanism for the regulation of IGF-IR expression involving HuR-mediated mRNA stabilization through binding to the 3′-untranslated region (UTR). Although this finding contrasts with the reported function of HuR as a translation initiation repressor of the IGF-IR transcript by binding to its 5′ UTR (20,21), it conforms to the tumor-promoting role of HuR in conferring stability and/or altering translation rates of target mRNAs encoding a plethora of anti-apoptotic and survival signaling effectors (30–33). In this study, we obtained evidence that HuR stabilizes IGF-IR mRNA through binding to the 3′ UTR. HuR contains three RNA recognition motifs (RRMs) and a hinge region between RRM2 and RRM3 encompassing the HuR nucleocytoplasmic shuttling (HNS) motif (31,32). Examination of various truncated forms of HuR indicates that this binding was mediated through the interaction of the RRM2-HNS-RRM3 domain with the HuR-binding site (ATCCATTTTTTTTTTTTTTAGG) (34) located in the distal region of the IGF-IR 3′ UTR. In light of the overexpression of HuR protein in many types of malignancies (35–38), this HuR-mediated mRNA stabilization might underlie the ability of cancer cells to upregulate IGF-IR expression.

Abbreviations: 2-DG, 2-deoxyglucose; AREs, AU-rich elements; β-TCP, β-transducin repeat-containing protein; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HNS, HuR nucleocytoplasmic shuttling; HuR, human antigen R; IGF-I, insulin-like growth factor-I; IGF-IR, insulin-like growth factor-I receptor; mAb, monoclonal antibody; mRNA, messenger RNA; ProT, prothymosin; qRT–PCR, quantitative real-time–PCR; RRM, RNA recognition motif; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; Sp1, specific protein 1; T1/2, half-life; UTR, untranslated region.

Materials and methods

Cell culture and reagents

LNCaP Pc-3 and DU-145 human prostate cancer cells and OVCAR-3 human ovarian cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained and treated with individual test agents in 10% fetal bovine serum (FBS)-supplemented RPMI 1640 medium.
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Primer sequences were as follows: IGF-IR: 5′-p-1135 Y-IGF-1R, CMV26 vector. The Myc-tagged full-length HuR plasmid as template followed by cloning into the p3XFLAG-CMV26 vector. The Myc-tagged full-length β-TrCP and AF-β-TrCP were generated from a β-Tcp1 clone as described previously (39). Primers expressing Sp1 short hairpin RNA and HuR small interfering RNA (siRNA) were obtained from Sigma–Aldrich (Irvine, CA). Antibodies against various proteins were purchased from Cell Signaling Technology (Beverly, MA); FLAG from Sigma–Aldrich and Santa Cruz, respectively. Transfection was performed by electroporation using the Nucleofector Kit of the Amaxa Nucleofector System (Lonza, Walkersville, MD) according to the manufacturer’s protocol. Immunoblotting was performed as described previously (29).

Semiquantitative PCR and quantitative real-time–PCR

Total RNA was isolated and reverse-transcribed to cDNA using TRizol reagent (Invitrogen) and the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s instructions. Quantitative real-time–PCR (qRT–PCR) was carried out using the Bio-Rad CFX96 Real-Time PCR Detection System with iQ SYBR Green Supermix (Bio-Rad) and the following primers: IGF-IR: 5′-CTCCTGCAACCTCCATCTCGT-3′ and 5′-TTTCTGCCTCGTGCCCAGAGA-3′ and 18s ribosomal RNA: 5′-ACCGTGTTGGACACACTTG-3′ and 5′-GTCCTAACAT-CTGCCAGGA-3′. The biotin-cytidine triphosphate-labeled RNAs were purified by NucAway spin columns (Ambion) according to the manufacturer’s instructions. RNA pull-down assays were carried out by incubating LNCaP cell lysates with purified 100 ng SIRT1 and 1 μg RNA) for 1 h at room temperature. Protein–biotinylated RNA complexes were isolated with streptavidin–Sepharose (Sigma–Aldrich) at 4°C for 2 h with rotation. After washing with binding buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl and 1% Triton X-100), the complexes were resuspended in 2× SDS sample buffer (100 mM Tris–HCl pH 6.8, 4% SDS, 5% beta-mercaptoethanol, 20% glycerol and 0.1% bromophenol blue), boiled for 10 min, and resolved on 10% SDS-polyacrylamide gel and subjected to immunoblotting with anti-HuR antibody.

Immunoprecipitation of RNA–protein complexes

Immunoprecipitation was used to assess the ability of endogenous HuR or various exogenous HuR mRNAs, LNCaP cells, or transfected or transiently transfected with plasmids encoding a series of Flag-tagged truncated forms of HuR, to be lysed in buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma–Aldrich) on ice for 30 min. Cell lysates were briefly sonicated (three times for 10 s) and then centrifuged at 13 000 g for 10 min, after which 10% and 10% by volume of supernatants was reserved as input, and the remainder was incubated with anti-HuR or anti-FLAG antibodies in the presence of protein A/G agarose (Santa Cruz) at 4°C for 12 h after a brief centrifugation, the immunoprecipitates were washed four times with 100 μl wash buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM MgCl2 and 0.05% NP-40), and RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The extracted RNA was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad), followed by RT–PCR to detect the presence of different fragments of the IGF-IR promoter region (900 to 287 nt). The sequenced primers were 5′-CGACGCCTGCACCGGTTCGAC-3′ and 5′-GAGCTCCGTAAGGCTCAGC-3′.
the glycolysis inhibitor 2-DG versus glucose deprivation on IGF-IR expression in prostate cancer cells. CG-5, a glucose transporter inhibitor (29), differentially suppressed the viability of LNCaP, DU-145 and PC-3 cells with the respective IC₅₀ values of 4, 6.5 and 9 µM (Supplementary Figure S1A, available at Carcinogenesis Online). As shown in Figure 1A, exposure of LNCaP cells to CG-5, 2-DG and glucose-depleted medium resulted in concentration- and time-dependent decreases in IGF-IR expression, at both protein and mRNA levels, accompanied by a parallel reductions in Ser473-Akt phosphorylation. Similarly, PC-3 and DU-145 cells, which differed in the abundance of IGF-IR protein, were comparably susceptible to CG-5-mediated ablation of IGF-IR (Figure 1B), indicating that this drug effect was not a cell line-specific cellular response. Moreover, when IGF-IR signaling was activated by exogenous IGF-I, as evidenced by IGF-IR hyperphosphorylation, CG-5 was effective in suppressing both phosphorylation and expression of IGF-IR in LNCaP cells (Figure 1C).

CG-5 suppresses IGF-IR expression at both transcriptional and posttranscriptional levels

The transcription of IGF-IR gene expression is transactivated by Sp1 (10), but repressed by WT1, p53 and BRCA1 (4). Consistent with our previous finding that β-TrCP-facilitated degradation of Sp1

![Fig. 1. Effects of the glycolysis inhibitors CG-5, 2-DG and glucose-depleted medium on IGF-IR expression in prostate cancer cells. (A) Upper panels, western blot and/or RT–PCR analyses of concentration-time-dependent effects of CG-5, 2-DG and glucose deprivation on IGF-IR expression and Ser473-Akt phosphorylation in LNCaP cells in 10% FBS-supplemented RPMI 1640 medium. Lower panels, relative expression levels of IGF-IR protein and p-Ser473-Akt. Amounts of immunoblotted proteins were analyzed by densitometry and expressed as a percentage of that in the respective control group (n = 3). (B) CG-5-mediated inhibition of IGF-IR expression in DU-145 and PC-3 cells. Upper panel, differential protein expression levels of IGF-IR in three prostate cancer cell lines, LNCaP, PC-3 and DU-145. Lower left and middle panels, western blot and RT–PCR analyses of the concentration-dependent effect of CG-5 on the protein and mRNA expression levels of IGF-IR in DU-145 and PC-3 cells. Lower right panel, relative protein expression levels of IGF-IR in response to CG-5 treatment in DU-145 and PC-3 cells. Protein abundance was analyzed by densitometry and expressed as a percentage of that in the control group (n = 3). (C) Western blot analysis of the concentration-dependent suppressive effects of CG-5 on the phosphorylation and expression levels of IGF-IR in LNCaP cells cotreated with IGF-I or vehicle control (left) and the corresponding densitometric analysis of relative expression levels (right; n = 3). All western blots shown are representative of three independent experiments. *P < 0.05.
represents a hallmark cellular response to energy restriction (41,42). CG-5-induced downregulation of IGF-IR expression was accompanied by a parallel decrease in the expression level of Sp1 in LNCaP cells (Figure 2A). This CG-5 treatment also reduced the expression of WT1, and, to a lesser extent, BRCA1, whereas no apparent change in p53 expression was noted (Figure 2A), thereby refuting the involvement of these transcriptional repressors in the CG-5-induced decrease of IGF-IR expression. Together, these findings suggest a role for Sp1 in mediating suppression of IGF-IR in response to CG-5, which was supported by several lines of evidence. First, siRNA-mediated knockdown of Sp1 diminished the abundance of IGF-IR (Figure 2B).

Second, ChIP analysis revealed a concentration-dependent inhibition of Sp1 binding to the IGF-IR promoter by CG-5 (Figure 2C). Third, ectopic expression of Sp1 significantly increased IGF-IR promoter activity in a luciferase reporter assay relative to the pCMV control in untreated cells (Figure 2D). Nevertheless, ectopically expressed Sp1 could not fully protect LNCaP cells from the suppressive effect of CG-5 on IGF-IR promoter activity (Figure 2D) or IGF-IR protein expression (Figure 2E), suggesting an additional, posttranscriptional mechanism by which CG-5 reduced IGF-IR protein expression. As CG-5 activates β-TrCP1-facilitated degradation of Sp1 and other target proteins through the downregulation of the oncogenic E3 ligase...
Fig. 3. Evidence that CG-5-mediated suppression of IGF-IR expression is, in part, mediated through decreased IGF-IR mRNA stability as a result of reduced HuR expression in LNCaP cells. (A) Upper panel, RT–PCR analysis of the effect of CG-5 relative to dimethyl sulfoxide control on IGF-IR mRNA stability. Cells were pretreated with CG-5 for 12 h, followed by cotreatment with 10 µM actinomycin D (Act. D) for the indicated periods of time in 10% FBS-supplemented RPMI 1640 medium. Lower panel, densitometric analysis of the change in IGF-IR versus GAPDH mRNA abundance in the cells treated as described in the upper panel. Amounts of mRNA are expressed as a percentage of that present at the 0 h time point on a log scale. The numbers listed represent the t1/2, which is defined as the time required for IGF-IR mRNA to decrease to 50% of its initial abundance. (B) Western blot analysis of the concentration- and time-dependent effects of CG-5 on the abundance of HuR and IGF-IR. (C) Western blot analysis of the effects of siRNA-mediated knockdown (left) and ectopic expression (right) of HuR, relative to the respective scrambled siRNA and pCMV controls, on the protein expression of IGF-IR and cyclin E, a HuR target for mRNA stabilization, in LNCaP cells. (D and E) Upper panel, RT–PCR analyses of the effects of siRNA-mediated knockdown (D) and ectopic expression (E) of HuR, relative to the respective scrambled siRNA and pCMV controls, on the stability of IGF-IR mRNA in LNCaP cells cotreated with 10 µM Act. D in 10% FBS-supplemented RPMI 1640 medium. Lower panel, densitometric analysis of the change in IGF-IR versus GAPDH mRNA abundance in cells at the indicated time intervals per the respective RT–PCR analyses. Amounts of mRNA are expressed as a percentage of that present at the 0 h time point on a log scale. The numbers listed in the graphs represent the t1/2 of IGF-IR mRNA. All western blot and RT–PCR analyses were performed three times with similar results.
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Skp2 (41), we examined the effect of ectopic expression of ΔF-β-TrCP1, an F-box-deleted, dominant-negative mutant form of β-TrCP1 (43), on CG-5-mediated suppression of IGF-IR protein expression. As shown in Figure 2F, dominant-negative inhibition of β-TrCP, which was confirmed by the prevention of drug-induced Sp1 degradation, completely abolished the suppressive effect of CG-5 on IGF-IR protein levels. These findings suggest the involvement of another β-TrCP-targeted protein in the CG-5-facilitated reduction of IGF-IR expression that acts through a posttranscriptional mechanism.

CG-5 suppresses IGF-IR expression, in part, by decreasing mRNA stability through HuR downregulation

Based on this premise, we assessed the effect CG-5 on the stability of IGF-IR mRNA in LNCaP cells following inhibition of transcription by the RNA synthesis inhibitor actinomycin D. Treatment with CG-5 shortened the half-life (t1/2) of IGF-IR mRNA relative to the dimethyl sulfoxide control (4.4 h versus 7.3 h, respectively), whereas stability of the mRNA encoding the housekeeping protein GAPDH was unaffected (Figure 3A). We rationalized that this decreased stability might be associated with the reported ability of CG-5 to reduce the expression of HuR, an mRNA-binding protein involved in regulating the stability of a broad range of mRNAs (44).

Previously, we demonstrated that inhibition of glycolysis by CG-5 or glucose deprivation facilitates the nuclear export of HuR to the cytoplasm, where it is targeted by β-TrCP for degradation via a protein kinase Cα-dependent mechanism (44). As shown in Figure 3B, this CG-5-mediated suppression of HuR was accompanied by decrease in IGF-IR levels in a concentration- and time-dependent manner. Furthermore, the role of HuR in mediating IGF-IR mRNA stability was supported by several lines of evidence. First, the ability of CG-5 to suppress IGF-IR expression was mimicked by siRNA-mediated knockdown of HuR. As shown, genetic silencing of HuR resulted in parallel decreases in the abundance of IGF-IR and cyclin E, a known HuR target for mRNA stabilization (45) (Figure 3C, left panel). Conversely, ectopic expression of HuR through transient transfection increased the expression levels of IGF-IR and cyclin E (right panel). Second, these changes in IGF-IR expression induced by the silencing or enforced expression of HuR were associated with corresponding effects on IGF-IR mRNA stability. Specifically, siRNA-mediated silencing of HuR decreased IGF-IR mRNA stability relative to that in the scrambled siRNA control (t1/2 2.7 h versus 5.1 h; Figure 3D), whereas ectopic HuR expression increased the t1/2 of IGF-IR mRNA as compared with the control (t1/2 16.9 h versus 5.3 h) (Figure 3E). In contrast, no change in the mRNA stability of GAPDH was noted in response to either condition.

![Fig. 4.](https://academic.oup.com/carcin/article-abstract/34/12/2694/2463973) HuR acts as a stabilizing protein for IGF-IR mRNA in OVCAR-3 ovarian cancer cells: effects of CG-5 on protein expression and mRNA stability. (A) Representative immunoblot of the concentration-dependent effects of CG-5 on the expression of IGF-IR, Sp1 and HuR in 10% FBS-supplemented RPMI 1640 medium after 24 h of treatment (left) and the corresponding densitometric analyses of relative expression levels (right; n = 3). (B) Western blot analyses of the time-dependent effect of 5 µM CG-5 (left) and the concentration-dependent effect of CG-5 after 12 h of treatment (right) on the expression of HuR versus IGF-IR in 10% FBS-supplemented RPMI 1640 medium. (C) Upper panel, RT–PCR analysis of the effect of CG-5 on IGF-IR mRNA stability. Cells were pretreated with CG-5 or dimethyl sulfoxide control for 12 h, followed by cotreatment with 10 µM actinomycin D (Act. D) for the indicated periods of time in 10% FBS-supplemented RPMI 1640 medium. Lower panel, densitometric analysis of the change in IGF-IR versus GAPDH mRNA abundance in cells at the indicated time intervals per the RT–PCR analysis. Amounts of mRNA are expressed as a percentage of that present at the 0 h time point on a log scale. The numbers listed in the graphs represent the t1/2 of IGF-IR mRNA.
These data supporting the role of HuR in promoting IGF-IR expression through mRNA stabilization in LNCaP cells, however, contrast with reports showing that HuR can act as a translational repressor of IGF-IR mRNA (20,21). Specifically, it was reported that siRNA-mediated knockdown of HuR increased IGF-IR expression in OVCAR-3 ovarian cancer cells (20). To address this discrepancy, we performed the aforementioned experiments to assess the role of HuR in regulating IGF-IR expression in OVCAR-3 cells.

As shown in Figure 4A, CG-5 dose-dependently reduced the expression of IGF-IR in OVCAR-3 cells, which was associated with parallel decreases in the expression levels of HuR and, to a lesser extent, Sp1 (Figure 4A). These CG-5-mediated reductions of HuR and Sp1 were also noted in the two other cancer cell lines examined, that is, PC-3 and DU-145 (Supplementary Figure S1B, available at Carcinogenesis Online), indicating that this drug effect was not a cell line-specific cellular response. Reminiscent of that noted in LNCaP cells (Figure 3B), CG-5 facilitated the inhibition of HuR and IGF-IR expression in a time- and concentration-dependent manner (Figure 4B). The suppressive effect of CG-5 on IGF-IR expression in OVCAR-3 cells was, in part, attributable to reduced mRNA stability.
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as the \( t_{1/2} \) of IGF-IR mRNA decreased from 10.8 to 5.2 h following CG-5 treatment (Figure 4C). Moreover, siRNA-mediated knockdown and enforced expression of HuR in OVCAR-3 cells resulted in decreased and increased IGF-IR protein expression, respectively, in a manner comparable with that of another HuR target for mRNA stabilization, cyclin E (Figure 5A). Moreover, as was the case in LNCaP cells, these changes were associated with corresponding effects on the \( t_{1/2} \) of IGF-IR mRNA. Specifically, HuR silencing decreased IGF-IR mRNA \( t_{1/2} \) relative to that in the scrambled siRNA control (12.6 h versus 6.2 h; Figure 5B), whereas ectopic expression of HuR increased the stability of IGF-IR mRNA over that in the empty vector-transfected control (\( t_{1/2} > 24 \) h versus 10.9 h; Figure 5C).

Together, these findings suggest that CG-5-induced suppression of IGF-IR expression was mediated at both transcriptional and posttranscriptional levels through effects on \( \beta \)-TrCP-facilitated degradation of Sp1 and HuR, respectively (Figure 6A). This premise was corroborated by comparing the protective effects of the ectopic expression of Sp1 and HuR, individually or in combination, on CG-5-mediated IGF-IR ablation in LNCaP cells. As shown in Figure 6B, CG-5-induced suppression of IGF-IR could be fully blocked only when both HuR and Sp1 were overexpressed, whereas ectopic expression of HuR alone, like that of Sp1 (Figure 2E), gave rise to a partial protective effect.

**Mode of regulation of IGF-IR mRNA stability by HuR**

It has been demonstrated that HuR interacts with target mRNAs through its three RRM, which recognize AU-rich elements (AREs) in the 5′ or 3′UTR (31–33). To discern the involvement of the IGF-IR 5′UTR versus 3′UTR in HuR binding, we performed RNA probe pull-down assays using the SIRT1 3′UTR (46) and DNMT3b 3′UTR (47) as positive controls and the GAPDH 3′UTR as a negative control. LNCaP cell lysates were exposed to individual biotinylated transcripts, followed by streptavidin-bead pull-down and western blot analyses. As shown in Figure 7A, HuR selectively bound the IGF-IR 3′UTR, whereas its 5′UTR counterpart was unable to pull-down HuR. The inability of the IGF-5′UTR to interact with HuR was verified by immunoprecipitation of endogenous HuR-containing ribonucleoprotein complexes in LNCaP cells, in which cell lysates were exposed to anti-HuR antibodies or IgG followed by protein A/G-conjugated agarose beads, and RNAs associated with the immunoprecipitates were analyzed by RT–PCR (48,49). As shown, the IGF-IR 5′UTR was undetectable in the HuR-containing and control immunoprecipitates, indicating the lack of endogenous HuR-IGF-IR 5′UTR complexes in LNCaP cells (Figure 7B).

By employing the photoactivatable ribonucleoside crosslinking and immunoprecipitation technique, a recent study identified seven clusters on the IGF-IR 3′UTR, with lengths ranging from 17 to 47 nt, as HuR-binding sites (34) (Figure 7C and Supplementary Figure S2, available at Carcinogenesis Online), which were characterized by the presence of multiple U-rich RNA recognition elements. Pursuant to this report, we investigated the HuR-binding site(s) within the IGF-IR 3′UTR that are involved in the interaction of HuR with IGF-IR mRNA in LNCaP cells. However, in light of the large size of the IGF-IR 3′UTR (7.1 kb in length), the IGF-IR 3′UTR was divided into five different segments (F1–F5; Figure 7C, upper panel and Supplementary Figure S2, available at Carcinogenesis Online) and the binding of endogenous HuR to each of these fragments was examined. Among these fragments, six of the reported HuR-binding sites were localized to F1 and F2, one resided in F5, whereas F3 and F4 contained none. Immunoprecipitation of endogenous HuR-containing ribonucleoprotein complexes in LNCaP cells, followed by PCR amplification of each of the five 3′UTR segments revealed that, among these five partial transcripts, HuR selectively bound to 3′UTR-F5, whereas no detectable binding of the other four fragments with HuR was determined (Figure 7C, lower panel). Moreover, this interaction between HuR and 3′UTR-F5 was abolished after ablation of HuR expression by CG-5 (Figure 7C, lower panel) or siRNA-mediated silencing (Figure 7D), refuting the involvement of non-specific interactions.

The role of the 3′UTR-F5 in interacting with HuR to mediate IGF-IR mRNA stabilization was further assessed by using a luciferase reporter assay, in which the 3′UTR-F5 and the 3′UTR of ProTα mRNA as a positive control (50) were inserted into the 3′-end
of the luciferase gene within the pMIR-REPORT Luciferase vector (Figure 7E). LNCaP cell transfectants expressing either the IGF-IR 3′ UTR-F5 or ProTα 3′ UTR exhibited significantly greater luciferase reporter activity ($P < 0.05$) relative to the empty vector-transfected controls, which, however, was abolished when endogenous HuR was silenced. The results of this functional assay are consistent with the proposed role of IGF-IR 3′ UTR-F5 in enhancing mRNA stability through interaction with HuR.

RRM2 and RRM3 are involved in mediating the interaction of HuR with the IGF-IR 3′ UTR

HuR contains three RRMs and a hinge region between RRM2 and RRM3 encompassing the HNS motif (Figure 8A, left panel) (31,32). Of these three RRMs, RRM1 and RRM2 bind AREs in the 3′ UTR of target mRNAs (51,52), whereas RRM3 was reported to bind long-chain poly(A) tails (52). To shed light onto the mode of recognition between HuR and the IGF-IR 3′ UTR-F5, we investigated the interaction of IGF-IR 3′ UTR-F5 with a series of Flag-tagged, truncated HuR mutants (Figure 8A, left panel), including RRM1-RRM2, RRM2-HNS-RRM3, HNS-RRM3, RRM2-HNS and RRM3, in comparison with the full-length HuR. Immunoprecipitation of the mutant or wild-type HuR using anti-Flag antibody, followed by PCR for detection of associated IGF-IR 3′ UTR-F5 in the presence and absence of 24 h of treatment with CG-5 (D) IP analysis showing that siRNA-mediated knockdown of HuR eliminates the formation of HuR-IGF-IR 3′ UTR-F5 complexes. (E) The IGF-IR 3′ UTR-F5 increases the stability of the luciferase reporter mRNA through interactions with endogenous HuR ($n = 3$). Luciferase reporter assays demonstrate the ability of the IGF-IR 3′ UTR-F5 and ProTα 3′ UTR (positive control) to significantly increase the luciferase activity ($*P < 0.05$) in transfected LNCaP cells, which, however, was abrogated by siRNA-mediated knockdown of endogenous HuR. All experiments were performed three times with similar results.
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The functional consequence of the above findings was validated by the ability of ectopically expressed RRM2-HNS-RRM3 mutant to increase the stability of IGF-IR mRNA. As shown, the levels of ectopically expressed RRM1-RRM2 and RRM2-HNS-RRM3 were substantially higher than that of endogenous HuR in LNCaP transfectants (Figure 8B). Overexpression of RRM2-HNS-RRM3 prolonged the t_{1/2} of IGF-IR mRNA relative to the control (9.9 h versus 4.5 h), whereas enforced expression of RRM1/2 had no appreciable effect (4.4 h).

Discussion

In this study, we demonstrate a functional interplay between Sp1 and HuR in regulating IGF-IR expression through transcriptional activation and mRNA stabilization, respectively. As HuR and Sp1 proteins were targeted for β-TrCP-mediated proteasomal degradation in response to glucose deprivation and CG-5 (41,42,44), the concurrent downregulation of these two proteins facilitated the suppression of IGF-IR expression at both transcriptional and posttranscriptional levels.

The present data contrast with a previous report that HuR acts as a translational repressor of IGF-IR mRNA by binding to the 5′ UTR (20). Although the reason for this discrepancy is unknown, the ability of HuR to enhance IGF-IR expression is consistent with its oncogenic role by conferring stability to target transcripts encoding a broad range of tumor-promoting and antiapoptotic proteins, such as ProTα, cyclooxygenase-2, HIF-1α, cyclins, Bcl-2, p21, XIAP and vascular endothelial growth factor (31–33). Our data indicate that alterations of the expression level of HuR in LNCaP and OVCAR-3 cells through ectopic expression or siRNA-mediated knockdown led to parallel changes in IGF-IR expression. Equally important, these changes were associated with corresponding effects on the t_{1/2} of IGF-IR mRNA, suggesting that HuR increases the stability of IGF-IR mRNA. Moreover, we obtained evidence that HuR facilitates this stabilization by binding to the 3′ UTR, in lieu of the reported 5′ UTR (20), through a region encompassing the RRM2-HNS-RRM3 domains.

Relative to RRM1 and RRM2, the role of RRM3 in regulating HuR binding to target mRNAs is not well defined as this motif, unlike RRM1 and RRM2, is not required for ARE recognition. However, we rationalize that RRM3 might be involved in mediating the stabilizing effect of HuR on certain target mRNAs through its putative function in maintaining the stability of the RNA–protein complex (32). This premise is supported by earlier reports that deletion of RRM3 from HuR resulted in the loss of HuR's ability to stabilize reporter constructs bearing the c-fos or β-globin ARE (53,54), which is reminiscent of our present finding.

Although the IGF-IR 3′ UTR was reported to contain seven HuR-binding sites distributed throughout its entire span of 7 kb (34), our data show that HuR selectively binds to the
recognition site located at the distal region (fragment 5) of the 3′UTR. Relative to other six binding sites (AAATTTTTACCTT TACCTTTTACCCCTATAC, TACCTTTTACCCCTATAC, TACCTTTTACCCCTATAC, TACCTTTTACCCCTATAC, TACCTTTTACCCCTATAC, TACCTTTTACCCCTATAC), this binding site (ATCCATTTTTTTTTTTTTTTTTATATG) contains the longest poly-U stretch of 18 nt. This difference might underlie the preferential binding of HuR to this distal binding site. Moreover, mutational analysis demonstrated the involvement of RRM2 and RRM3, but not RRM1, in HuR-mediated IGF-IR mRNA stabilization, which suggests the pivotal role of RRM3-mediated recognition of the poly(A) tail in this protein–mRNA interaction.

In summary, we have elucidated a mechanism by which CG-5 inhibits IGF-IR expression in cancer cells through transcriptional repression and mRNA destabilization downstream of β-TrCP-facilitated degradation of Sp1 and HuR, respectively. This unique mechanism might underlie the suppressive effect of dietary or chemically induced energy restriction on IGF-IR expression that has been reported in different animal models of carcinogenesis (24,26). Equally important, we obtained evidence that HuR acts as a stabilizer, as opposed to a translational repressor of IGF-IR mRNA, which is consistent with its role in promoting tumorigenesis.

Supplementary material
Supplementary Figures S1 and S2 can be found at http:// carcin.oxfordjournals.org/

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References

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