IMP-3, a member of the insulin-like growth factor-II (IGF-II) mRNA-binding protein (IMP) family, is expressed mainly during embryonic development and in some tumors. Thus, IMP-3 is considered to be an oncofetal protein. The functional significance of IMP-3 is not clear. To identify the functions of IMP-3 in target gene expression and cell proliferation, RNA interference was employed to knock down IMP-3 expression. Using human K562 leukemia cells as a model, we show that IMP-3 protein associates with IGF-II leader-3 and leader-4 mRNAs and H19 RNA but not c-myc and β-actin mRNAs in vivo by messenger ribonucleoprotein immunoprecipitation analyses. IMP-3 knock down significantly decreased levels of intracellular and secreted IGF-II without affecting IGF-II leader-3, leader-4, c-myc, or β-actin mRNA levels and H19 RNA levels compared with the negative control siRNA treatment. Moreover, IMP-3 knock down specifically suppressed translation of chimeric IGF-II leader-3/luciferase mRNA without altering reporter mRNA levels. Together, these results suggest that IMP-3 knock down reduced IGF-II expression by inhibiting translation of IGF-II mRNA. IMP-3 knock down also markedly inhibited cell proliferation. The addition of recombinant human IMP-II peptide to these cells restored cell proliferation rates to normal. IMP-3 and IMP-1, two members of the IMP family with significant structural similarity, appear to have some distinct RNA targets and functions in K562 cells. Thus, we have identified IMP-3 as a translational activator of IGF-II leader-3 mRNA. IMP-3 plays a critical role in regulation of cell proliferation via an IGF-II-dependent pathway in K562 leukemia cells.

The human insulin-like growth factor II (IGF-II) mRNA-binding protein (IMP) family consists of IMP-1, IMP-2, and IMP-3. The three closely related IMP family members contain six RNA binding motifs, including two RNA recognition motifs and four heterogeneous nuclear ribonucleoprotein K-homology (KH) domains (1). IMP-3 has amino acid identities of 65.7 and 59.7% with IMP-1 and IMP-2, respectively. The sequence similarities of the RNA binding domains among the IMP proteins, especially within the KH domains, are much higher (2). All three IMPs can bind to human IGF-II mRNA with high affinity in vitro (1, 3). IMP-1 is identical to the mouse c-myc coding region determinant-binding protein (CRD-BP) (4–6) and the chicken β-actin mRNA-binding protein-1 (ZBP-1) (7). IMP-3 is identical to the KH domain-containing protein overexpressed in cancer (KOC) (8) and the Xenopus laevis Vg1 mRNA-binding protein (Vg1-RBP/Vera) (9, 10). Whereas no orthologs for IMP-2 have been found, p62, a human hepatocellular carcinoma autoantigen, seems to be a splice variant (11).

So far, at least five RNA targets for IMP-1 have been reported, including IGF-II, c-myc, β-actin, tau, and H19. IMP-1 can affect stability, localization, and translation of its target RNAs (1, 3, 4, 7, 12, 13). IMP-1 binds to the 5′-UTR of IGF-II leader-3 mRNA and inhibits its translation. IMP-1 also binds to the 3′-terminus of the untranslated H19 RNA and regulates its subcellular location. CRD-BP/IMP-1 binds to the coding region instability determinant of c-myc mRNA and stabilizes c-myc mRNA in a cell-free mRNA decay system. Chicken ZBP-1/IMP-1 binds to the “zipcode” segment in the 3′-UTR of β-actin mRNA and localizes the mRNA to the lamellipodia of fibroblasts (1, 3, 4, 7, 12). Tau mRNA is an axonally localized mRNA in neurons (14–16). IMP-1 can bind to the 3′-UTR of tau mRNA, but the functional significance for the binding is unknown (13).

IMP-1 is thought to be an oncofetal protein since it is mainly expressed during embryogenesis and in some tumors; it is reduced or absent in adult tissues (6, 17–19). Recent investigations show diverse functions for IMP-1 in various cell types. For example, overexpression of IMP-1 in mammary epithelial cells of transgenic mice induces mammary tumors and increases IGF-II mRNA levels by 100-fold without affecting cellular IGF-II protein levels (20). IMP-1 knock-out mice exhibit dwarfism and translation inhibition of both leader-3 and leader-4 isoforms of IGF-II mRNA (21). Our previous studies showed that IMP-1 knockdown by RNA interference promotes cell proliferation via up-regulation of IGF-II mRNA and protein levels, which may involve a nuclear mechanism (22). Thus, saline; GADPH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; RIP, messenger ribonucleoprotein immunoprecipitation; qRT-PCR, real-time, quantitative, reverse transcription-PCR; KH, K-homology; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium].

This paper is available on line at http://www.jbc.org
IMP-3 Activates Translation of IGF-II mRNA

IMP-1 appears to be a phylogenetically conserved and multifunctional RNA-binding protein. The human KH domain-containing protein KOC was originally identified in human pancreatic cancer (8). *Xenopus* Vg1 mRNA-binding protein (Vg1-RBP) was identified by using human KOC as a probe, and it has an amino acid sequence identity of 84% with KOC. Thus, Vg1-RBP is thought to be a *Xenopus* homologue of human KOC. *X. laevis* Vegetal 1 (Vg1) mRNA encodes a transforming growth factor β-like protein that stimulates the formation of mesoderm at the vegetal cortex. Vg1-RBP binds to the 3'-UTR of Vg1 mRNA and localizes the mRNA to the vegetal pole of *X. laevis* oocytes at stages III and IV (for review, see Refs. 23 and 24). Vg1-RBP is also implicated in regulation of the migration of neural crest cells during early neural development (25). Human IMP-3 was isolated in RD rhabdomyosarcoma cells and found to be identical to KOC. IMP-3/KOC can bind to the 5'-UTR of IGF-II leader-3 mRNA and the 3'-UTRs of all the IGF-II mRNA isoforms (1, 3). However, the functional significance is not clear. Transgenic overexpression of IMP-3/KOC results in remodeling of the exocrine pancreas (26). Although IMP family members share high structural similarity, recombinant mouse IMP-3/KOC only binds to the 5'-UTR of IGF-II leader-3 mRNA and not to some IMP-1 target RNAs such as c-myc and β-actin by *in vitro* UV cross-linking assay. This suggests functional differences among the IMP members (2). IMP-3 seems to be involved in tumorigenesis and embryonic development. High levels of IMP-3 mRNA were detected in pancreatic cancer cell lines and tissues as well as other human tumors such as gastric cancer, soft tissue sarcoma, colon carcinoma, RD rhabdomyosarcoma cells, and K562 human leukemia cells (1, 8, 22, 27–29). The IMP-3 transcript has similar expression patterns with IMP-1 during mouse embryonic development. For example, they are expressed at early stages, peak around embryonic day 12.5, decrease until birth, and then are low or absent in adult tissues (1, 21). This fetal expression profile overlaps that of IGF-II leader-3 mRNA, which implies a regulatory effect of IMP-3 on IGF-II gene expression in combination with the binding of IMP-3 to IGF-II leader-3 mRNA (1, 21). Moreover, human and mouse IMP-3 and *Xenopus* Vg1-RBP have similar fetal expression patterns. For instance, high levels of the transcripts were seen in the gut, pancreas, kidney, skin, snout, placenta, and brain during mouse development (24). Together, these studies indicate that IMP-3 may play a pivotal role in tumorigenesis and development. However, the regulatory effects on expression of its target mRNAs and the functional significance remain to be elucidated.

In this study we knocked down IMP-3 expression by RNA interference (RNAi) in human K562 leukemia cells, which were utilized for the identification of IMP-1 function, to examine IMP-3 function (22). We found that IMP-3 knockdown inhibited translation of IGF-II leader-3 mRNA without affecting its mRNA levels. Furthermore, IMP-3 knockdown reduced cell proliferation through an IGF-II-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Preparation of siRNA**—The human IMP-3 and IMP-1 SMARTpool siRNA duplexes were designed and chemically synthesized by Dharmacon Research (Lafayette, CO). The SMARTpool siRNA is a mixture of four different siRNA duplexes targeting distinct coding region sequences of IMP-3 (GenBank™ accession number NM_006547) or IMP-1 (GenBank™ accession number AF117106). The sequences of the SMARTpool siRNAs are proprietary. The negative control siRNA contained nucleotides randomly arranged (5'-aac ugg gua agc ggc cag aag-3'). BLAST searches against human genome sequences in GenBank™ were performed by Dharmacon to ensure specificity of the siRNAs. The siRNA duplexes were dissolved in 1× universal RNA oligo buffer (20 mM KCl, 6 mM HEPES-KOH (pH 7.5), 0.2 mM MgCl₂).

**Cell Culture and siRNA Transfection**—K562 cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine (Invitrogen) at 37 °C in 5% CO₂. 5×10⁵ K562 cells were transfected with either 200 nM IMP-3 siRNA or both IMP-3 and IMP-1 siRNAs (double knockdown) or control siRNA (negative control) or an equal volume of 1× universal RNA oligo buffer (mock control) by electroporation using a Gene Pulser (Bio-Rad) as described previously (22). Transfected cells were maintained in regular culture medium without antibiotics for the times indicated in the figure legends.

**Western Blot Analysis**—Cytoplasmic and nuclear fractions were prepared using the CellLytic NuCLEAR™ extraction kit (Sigma). Total protein concentration of the extracts was quantified by Bradford assay using the protein assay reagent (Bio-Rad) following the manufacturer’s instructions. For Western blot analysis, cytoplasmic (40 μg) and nuclear (20 μg) lysates were size-fractionated by SDS-PAGE and transferred onto nitrocellulose membranes (Fisher). Antibodies used and their dilutions are as follows: c-Myc (Oncogene) 1:300; α-tubulin (Sigma) 1:10,000; lamin A/C (Upstate Biotechnology) 1:1,300; IMP-1, 2, 3 (as described previously (22)) 1:5,000; goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (Promega) 1:2,500; goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma) 1:3,000. Western blot analysis was carried out using the SuperSignal West chemiluminescent substrate kit (Pierce) according to the manufacturer’s instructions. The blots were stripped and reprobed with anti-α-tubulin or lamin A/C antibody. α-Tubulin and lamin A/C served as the loading controls for cytoplasmic and nuclear protein fractions, respectively. Western results were quantified by using a DC120 Kodak Digital Image system (Eastman Kodak Co.).

**Enzyme-Linked Immunosorbent Assay (ELISA)**—IGF-II concentrations in cytoplasmic lysates and culture media of K562 were examined by ELISA using the non-extraction IGF-II ELISA kit (Diagnostic Systems Laboratories, Inc.) according to the manufacturer’s instructions as described previously (22). Media samples were pretreated with buffers provided in the kit to dissociate IGF-II and IGF-binding proteins before the assay. For detection of intracellular IGF-II protein, cytoplasmic lysates were prepared using the Celllytic NuCLEAR extraction reagent (Sigma) (30). The cytoplasmic protein in the lysates was measured by the Bradford assay using the protein assay reagent (Bio-Rad) following the manufacturer’s instructions.

**Dual Luciferase Reporter Assay**—Luciferase activity was examined by a dual luciferase reporter assay using the dual luciferase reporter assay kit (Promega) on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) as described previously (22). The plasmids pcDNA-IGF-II leader-3/luciferase (pcDNA-IGF-II-L3-Luc) and pcDNA-IGF-II leader-4/luciferase (pcDNA-IGF-II-L4-Luc) were kindly provided by Dr. Jan Christiansen. These plasmids contain the firefly luciferase coding region and the complete leader 3 (1164 bp) or leader 4 (94 bp) exon, respectively, in the pcDNA3.1 basic vector (Invitrogen) (1). 1×10⁵ K562 cells were co-transfected with 1 μg of pRL-SV40 Renilla luciferase control (Promega), pericells, or negative control siRNA or both IMP-1 and IMP-3 siRNAs by electroporation as described above. pRL-SV40 served as internal control. pGL-Promotor vector containing the firefly luciferase coding region (Promega) was used as a control for the effects of 5'-UTR sequences on gene expression. Luciferase activity was measured at 48 h post-transfection. Firefly luciferase activity was normalized to Renilla luciferase activity in the same cell extract and expressed as a ratio of firefly/Renilla luciferase activity.

**Quantitative Real-time Reverse Transcription-PCR (qRT-PCR)**—H19 RNA and IGF-II, IGF-II leader-3, IGF-II leader-4, c-myc, IMP-1, IMP-3, β-actin, firefly luciferase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were examined by qRT-PCR. One-step qRT-PCR was performed using the QuantiTect Probe RT-PCR kit (Qiagen) on a MX-4000 Multiplex Quantitative PCR system (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The use of dual-fluorescence-labeled probes greatly increased the specificity of the qRT-PCR. The PCR primers and probes were designed using web-based Primer-3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and cloned into the pMAX™ vector (Invitrogen). The *Tₘ* values for the probes and primers are 70 and 60 °C, respectively. They are as follows: c-myc probe, 5'-6-FAM™-TGG ACA CCC TCC AGT TCG TCT-3'; c-myc forward primer, 5'-AGG AAA TCT TGC CCA TAG CA-3'; c-myc reverse primer, 5'-GGCA AGG AGA GCC GCTT AG-3'; IGF-II probe, 5'-6-FAM™-TGG ACA GCC TCC ATG TCT GTG TTA-3'; IGF-II forward primer, 5'-AAG TCT ATG GTC CCT CT-3'; IGF-II reverse primer, 5'-GGG AAA CAG CAC TCC.
IMP-3 Activates Translation of IGF-II mRNA

IMP-3 siRNA Specifically Inhibits IMP-3 Gene Expression—To examine the functions of IMP-3 in gene expression and cell proliferation, we utilized RNA interference to knock down its expression in K562 cells. Equal amounts of either IMP-3 siRNA or a negative control siRNA that includes nucleotides randomly arranged were transfected into K562 cells by electroporation. An equal volume of 1× universal RNA oligo buffer was used as a mock control. We employed Western blotting analyses and qRT-PCR to examine the relevant protein and mRNA levels, respectively. IMP-1, IMP-3, and c-Myc proteins distribute to both the nucleus and cytoplasm (22). Thus, both nuclear and cytoplasmic levels of the relevant proteins were examined after IMP-3 RNA interference. After IMP-3 siRNA transfection, cytoplasmic IMP-3 protein levels were reduced by 76% at 24 h (p < 0.01), 70% at 48 h (p < 0.01), 62% at 72 h (p < 0.01), and 64% at 96 h (p < 0.01) during the 4-day time course compared with the control siRNA treatment (Fig. 1, A and C). Nuclear IMP-3 protein levels exhibited similar changes to cytoplasmic levels after IMP-3 knockdown (Fig. 1, B and D). The RNA interference effect persisted 6 days, and then protein levels returned to normal (data not shown). IMP-2 expression is not detectable in K562 cells by Western blot analysis (22). We also examined IMP-1 levels to assess specificity of knockdown. IMP-3 siRNA did not significantly affect either nuclear or cytoplasmic IMP-1 levels (Fig. 1; p > 0.05). Our qRT-PCR analyses further demonstrated that transfection of IMP-3 siRNA decreased IMP-3 mRNA levels with similar magnitudes to the reductions in protein levels when compared with the control siRNA treatment; IMP-1 mRNA levels were not affected (data not shown). The negative control siRNA had no effect on IMP-3 mRNA or protein levels compared with the mock buffer control treatment (p > 0.05, data not shown). Also, IMP-3 knockdown did not influence either nuclear or cytoplasmic c-Myc protein levels, lamin A/C levels, or α-tubulin levels compared with the control siRNA treatment (Fig. 1; p > 0.05).

RESULTS

Treatment of K562 Cells with Recombinant Human IGF-II—K562 cells were transfected with negative control or IMP-3 siRNA as described above and seeded in a 96-well plate at a density of 2 × 10⁵ cells/well. Recombinant human IGF-II (Sigma), prepared in PBS following the manufacturer’s instructions, was added to cell cultures at concentrations of 10, 100, 500, 800 ng/ml, respectively. 20 μM/l PBS alone and 500 ng/ml BSA (Sigma) dissolved in PBS were used as controls. The cultures were maintained for 48 h. Proliferation of viable cells was examined by MTS assays as described above.

Statistical Analyses—The data are shown as the means ± S.E. Student’s t test and analysis of variance were performed. The Scheffé test was applied if a significant F value was obtained. A p value < 0.05 was considered statistically significant.

Cellular IMP-3 Associates with IGF-II mRNAs and H19 RNA—IMP family proteins contain very similar RNA binding domains, including two RNA recognition motifs and four KH domains (1). IMP-1/CRD-BP can bind to IGF-II, c-myc, β-actin, tau mRNAs, and H19 RNA and regulate localization, stability, and translation of target RNAs (1, 3, 4, 7, 12, 13). Tau mRNA is a neuronal mRNA (14–16). By contrast, IMP-3 was found to only bind IGF-II mRNA in vitro (1, 2). To identify the in vivo RNA targets of IMP-3 and determine whether IMP-3 can bind to the same targets of IMP-1 in vivo, RIP/qRT-PCR analyses were employed using anti-human IMP-3 antibody or control IgG and cytoplasmic lysates of K562 cells. As shown in Fig. 2,
IMP-3 Activates Translation of IGF-II mRNA

A–D, anti-human IMP-3 antibody precipitated IGF-II mRNAs, specifically leader-3 and -4 mRNAs, and H19 RNA. The control IgG did not precipitate these RNAs. IMP-3 antibody did not precipitate either c-myc or β-actin mRNAs (Fig. 2, E and F). The c-myc and β-actin PCR primers and probes are effective using total RNA as template, as shown in Fig. 3B. H19 RNA may represent a novel IMP-3-bound RNA. These qRT-PCR results were confirmed by electrophoresis of the PCR products (Fig. 2, G and H, and data not shown). Thus, both IMP-3 and IMP-1 associate with IGF-II mRNA and H19 RNA. Clearly, however, some of the RNA targets of IMP-1 are distinct from those of IMP-3 (see “Discussion”).

Effect of IMP-3 Knockdown on Target RNA Levels—Depending on the biological systems examined, IMP-1/CRD-BP levels can affect the levels of c-myc and IGF-II mRNAs (4, 20, 22). These observations, coupled with the high homology between IMP-3 and IMP-1 (1), prompted us to consider the possibility that IMP-3 might control the expression of its target RNAs, identified in Fig. 2, by controlling their abundance.

IMP-3 binds to IGF-II mRNA in vitro (1, 3), and it associates with the leader-3 and leader-4 isoforms of IGF-II mRNA and H19 RNA in vivo (Fig. 2). To determine whether IMP-3 controls the levels of its target RNAs, IMP-3 expression was knocked down by RNAi, and RNA target levels were assessed using qRT-PCR. IMP-3 knockdown had no significant effect on IGF-II leader-3 or leader-4 mRNAs or total IGF-II mRNA levels compared with the control siRNA treatment during the 4-day period (Fig. 3A, p > 0.05). By contrast, knockdown of IMP-1/CRD-BP results in elevated IGF-II mRNA levels (see “Discussion”) (22). Additionally, IMP-3 gene silencing by RNA interference did not significantly affect H19 RNA levels (Fig. 3B, p > 0.05). We also examined the effects of IMP-3 knockdown on c-myc and β-actin mRNA levels even though these mRNAs are not in vitro binding targets of IMP-3 in K562 cells. Our rationale was that Nielsen et al. (3) demonstrated that the IMPs can form heterodimers. Thus, we considered it possible that IMP-3 association with IMP-1 might impact the binding of IMP-1 to these mRNAs, and hence, their levels. Nonetheless, IMP-3 knockdown did not affect levels of either c-myc or β-actin mRNAs in comparison with the control siRNA treatment (Fig. 3B, p > 0.05). Based upon these results, we next considered that IMP-3 might control IGF-II protein levels and perhaps translation of IGF-II mRNAs as well; H19 RNA is not translated (32–34).

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**Fig. 1.** IMP-3 RNA interference specifically reduces cytoplasmic and nuclear IMP-3 protein levels in K562 cells. A and B, representative Western blots showing the effects of negative control and IMP-3 siRNAs on cytoplasmic (a) and nuclear (b) levels of the indicated proteins. C and D, quantitative analyses of the Western blot assays (n = 4) for the cytoplasmic (C) and nuclear (D) proteins. Intensity of each band was measured using a DC120 digital image system as described under “Experimental Procedures,” then normalized to α-tubulin (C) or lamin A/C (D) (loading controls). The normalized data from IMP-3 siRNA transfection were divided by that from control siRNA treatment, the single and double asterisks indicate p < 0.05 and p < 0.01, respectively.

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**Fig. 2.** Identification of endogenous IMP-3 target RNAs. Cellular IMP-3-associated RNAs in cytoplasmic lysates of K562 cells were isolated by RIP assay using anti-IMP-3 antibody. An equal amount of non-immune rabbit IgG was used as a negative control. The levels of the IMP-3 target RNAs in the immune precipitate were examined by qRT-PCR using the corresponding PCR primers and probes. PCR products were visualized on 2% agarose gels with ethidium bromide to ensure correct sizes, A–F, representative qRT-PCR amplification plots for the detected RNAs. A, IGF-II leader-4 mRNA. B, total IGF-II mRNA. C, IGF-II leader-3 mRNA. D, H19 RNA. E, c-myc mRNA. F, β-actin mRNA. G and H, representative agarose gels of the real-time PCR products, as described above. In panels G and H, tracks 1–4 indicate IGF-II, c-myc, β-actin, and H19 cDNAs, respectively. IMP-3 and IMP-3 Ab, precipitate using anti-human IMP-3 antibody; IgG, precipitate using non-immune rabbit IgG (n = 3).
**IMP-3 Activates Translation of IGF-II mRNA**

**IMP-3 Knockdown Inhibits Translation of IGF-II Leader-3 mRNA**—Because IMP-3 knockdown did not affect the levels of its RNA targets, IGF-II protein levels were examined by ELISA after IMP-3 gene silencing. After IMP-3 knockdown, intracellular IGF-II levels were decreased by 38–52% compared with the control siRNA treatment (*p* < 0.05–0.01) during the 4-day period post-transfection. The effect persisted at least 96 h with a maximum at 72 h after transfection (Fig. 4A). Likewise, IMP-3 gene silencing also significantly reduced IGF-II levels secreted into the culture medium in comparison with the control siRNA transfection (Fig. 4B, *p* < 0.01). These results indicated that IMP-3 knockdown decreased both intracellular and secreted levels of IGF-II.

Accordingly, to determine the molecular basis for decreased IGF-II levels after IMP-3 knockdown, translational regulation mediated by leader-3 and -4 of IGF-II mRNA was examined. IMP-3 or negative control siRNA, pcDNA-IGF-II-leader-3 or -4 firefly luciferase reporter construct, and pRL-SV40 Renilla luciferase control vector were co-transfected into K562 cells. Luciferase activity was determined by dual luciferase assay (Fig. 5A). Luciferase control vector levels were unchanged at the same time point after IMP-3 knockdown compared with the control siRNA treatment as assayed by qRT-PCR (Fig. 3B; *p* > 0.05). Surprisingly, IMP-3 knockdown had no effect on either leader-4/luciferase activity (Fig. 5B; *p* > 0.05) or mRNA levels (data not shown) even though leader-4 IGF-II mRNA is an *in vivo* binding target of IMP-3 (see "Discussion"). Nonetheless, these data demonstrated that IMP-3 knockdown by RNAi specifically inhibited translation of the IGF-II leader-3/luciferase reporter mRNA. This suggests that IMP-3 knockdown likely lowered IGF-II gene expression by reducing translation of the leader-3 mRNA. Thus, IMP-3 may normally promote translation of IGF-II leader-3 mRNA and up-regulate IGF-II protein expression.

**IMP-3 Knockdown Inhibits Cell Proliferation**—IGF-II is a secreted growth factor that promotes cell proliferation and inhibits apoptosis (35–39). Both endogenous and exogenous IGF-II promotes proliferation of K562 cells (40, 41). Our current data showed that IMP-3 knockdown down-regulated IGF-II gene expression (Fig. 4). Accordingly, we examined the effect of IMP-3 knockdown on cell proliferation. IMP-3 gene silencing by RNAi significantly decreased K562 cell number as assessed by trypan blue exclusion compared with the control siRNA treatment. IMP-3 knockdown decreased cell number by 32% at 24 h, 48% at 48 h, 60% at 72 h, and 50% at 96 h compared with the control siRNA treatment (Fig. 6A; *p* < 0.05–0.01). To confirm these results, cell proliferation was also examined by MTS assay. The MTS assay measures cellular dehydrogenase activity that is proportional to the number of viable cells in a culture. IMP-3 knockdown markedly inhibited cell proliferation compared with the control siRNA treatment (Fig. 6B; *p* < 0.05–0.01), consistent with the trypan blue exclusion assay data. The proliferation-inhibiting effect of IMP-3 knockdown was closely linked to both the kinetics of IMP-3 gene silencing and decreased IGF-II protein levels (Figs. 1, 4, and 6), suggesting that IMP-3 knockdown might regulate cell proliferation through down-regulation of IGF-II expression.

**Recombinant Human IGF-II Restores Cell Proliferation after IMP-3 Knockdown**—To determine whether reduced IGF-II levels in fact mediated the inhibitory effect of IMP-3 knockdown on cell proliferation, human IGF-II was added to cell cultures. K562 cells transfected with negative control or IMP-3 siRNA were maintained in culture medium containing either 500 ng/ml BSA, PBS alone (0 ng/ml IGF-II), 10, 100, 500, or 800 ng/ml recombinant human IGF-II, respectively, for 48 h. MTS assay was used to examine cell proliferation (Fig. 7). IMP-3
FIG. 5. IMP-3 knockdown specifically inhibits translation of IGF-II leader-3/luciferase reporter mRNA in K562 cells. K562 cells were co-transfected with siRNA, Renilla pRL-SV40 internal control construct, and either IGF-II leader-3/luciferase or IGF-II leader-4/luciferase reporter constructs. Plasmid pGL-Promoter, containing the coding region of firefly luciferase, was used as a control. Luciferase activity was examined by dual luciferase reporter assay and expressed as the ratio of firefly/Renilla luciferase (n = 4). A larger normalized ratio suggests higher firefly luciferase activity. A, IMP-3 knockdown had no effect on firefly luciferase activity from pGL-Promoter. B, IMP-3 knockdown had no significant effect on luciferase activity from the IGF-II leader-4/luciferase vector (p > 0.05). C, IMP-3 knockdown markedly inhibited luciferase activity from the IGF-II leader-3/luciferase vector (p < 0.01). Ratio, refers to the ratio of firefly to Renilla luciferase activity; con, control siRNA treatment; IMP-3, IMP-3 siRNA treatment; control, firefly luciferase activity from pGL-Promoter; leader-4/luc, luciferase activity from the IGF-II leader-4/luciferase vector; leader-3/luc, luciferase activity from the IGF-II leader-3/luciferase vector.

knockdown significantly inhibited cell proliferation by 51% (Fig. 7; p < 0.01) compared with the control siRNA treatment, similar to the results described above. Additionally, the BSA treatment had no influence on cell proliferation in comparison with the PBS blank control (Fig. 7; p > 0.05). The addition of recombinant human IGF-II at concentrations of 10, 100, 500 and 800 ng/ml to cells transfected with the control siRNA increased cell proliferation by 2% (p > 0.05), 38% (p < 0.01), 72% (p < 0.01), and 76% (shaded bars in Fig. 7; p < 0.01), respectively, compared with the BSA control. These results are consistent with previous observations that exogenous IGF-II promotes K562 cell proliferation (41). Moreover, in cells transfected with IMP-3 siRNA, exogenous IGF-II increased cell proliferation rates in a dose-dependent manner at concentrations from 100 to 800 ng/ml (Fig. 7, solid bars) compared with BSA-treated cells transfected with control siRNA (Fig. 7, gray-shaded bar left-most; p < 0.01). Thus, IGF-II at a concentration of 100 ng/ml was sufficient to reverse the inhibitory effect of IMP-3 knockdown on cell growth. These results suggest that IMP-3 may normally serve to maintain IGF-II at appropriate levels for cell proliferation.

DISCUSSION

Among the IMP family, IMP-1 and IMP-3 are highly expressed in K562 cells, whereas IMP-2 is undetectable by Western blot analysis (22). IMP-1/CRD-BP has been extensively studied. The RNA-binding protein IMP-3/KOC seems to play important roles in embryogenesis and tumorigenesis based upon its expression patterns (1, 2, 24, 28). However, its functional significance is unknown. To address the functions of IMP-3, we identified its RNA binding targets and examined the effects of IMP-3 knockdown on both their expression and cell proliferation. Knockdown of IMP-3 gene expression by RNAi revealed that IMP-3 normally promotes the translation of IGF-II leader-3 mRNA. Thus, IMP-3 promotes cell proliferation via an IGF-II-dependent pathway. IMP-3/KOC and IMP-1/CRD-BP appear to have both common and distinct RNA targets, and they appear to exert opposite effects on IGF-II expression and cell proliferation (see below).
IMP-3 Associates with IGF-II Leader-3 and Leader-4 mRNAs and H19 RNA in Vivo—There are four IGF-II mRNA isoforms, all with the same coding region and 3′-UTRs but distinct 5′-UTRs (designated leader-1 to leader-4) (42). Recombinant human and mouse IMP-3 bind to the 5′-UTR of IGF-II leader-3 mRNA, a major isoform in mammalian cells, in vitro UV cross-linking and mobility shift assays (1, 2). Our RIP analyses showed that cytoplasmic IMP-3 associates with IMP-1 leader-2 and leader-4 mRNAs in K562 cells (Fig. 2). The association of IMP-3 with IGF-II leader-3 mRNA likely occurs via its binding to the 5′-UTR of this mRNA isoform. Although IMP-3 does not bind to the 5′-UTR sequences of leader-4 mRNA (1, 2), IMP-3 does bind to the 3′-UTR of IGF-II mRNAs (3). As noted above, the 3′-UTR is common to all IGF-II mRNA isoforms (42). Our hypothesis is that it is the IMP-3-3′-UTR association that permits precipitation of IGF-II leader-4 mRNA in our RIP analyses.

Additionally, we found that IMP-3 associates with H19 RNA (Fig. 2D). However, cytoplasmic IMP-3 did not associate with either c-myc or β-actin mRNAs (Fig. 2, E and F), both of which are targets of IMP-1 in vitro. These data are in agreement with in vitro UV cross-linking data with recombinant mouse IMP-3, which binds to 5′-UTR sequences of IGF-II leader-3 but not c-myc or β-actin mRNAs (2). Mouse IMP-3 has 96.5% sequence identity to human IMP-3 (2). Additionally, although they are highly similar structurally, IMP-1 has several RNA targets that are distinct from those of IMP-3, indicating distinct functions between these two IMP members (see below).

IMP-3 Knockdown Reduces Translation of IGF-II Leader-3 mRNA—Because IGF-II leader-3 and leader-4 mRNAs and H19 RNA were found associated with IMP-3 in vivo (Fig. 2), we examined their levels after IMP-3 knockdown. Our results demonstrate that IMP-3 gene silencing had no effect on IGF-II leader-3, leader-4, c-myc, or β-actin mRNA and H19 RNA levels (Fig. 3). However, IMP-3 knockdown significantly decreased IGF-II protein, including both intracellular and secreted levels (Fig. 4). Knockdown also had no effect on either nuclear or cytoplasmic c-Myc protein levels (Fig. 1). The alterations in IGF-II protein levels were correlated closely with the kinetics of IMP-3 knockdown (Figs. 1 and 4). Our data further showed that IMP-3 knockdown specifically inhibited translation of chimeric IGF-II leader-3/luciferase mRNA but not IGF-II leader-4/luciferase mRNA (Fig. 5). Again, we would hypothesize that the absence of an effect on IGF-II leader-4/luciferase mRNA would be due to the observation that IMP-3 does not bind to 5′-UTR sequences of leader-4 mRNA, as noted above. Taken together, these results argue that IMP-3 knockdown reduced IGF-II levels by reducing translation of IGF-II leader-3 mRNA via its 5′-UTR. This indicates that, normally, IMP-3 should act as a translational activator of IGF-II leader-3 mRNA. Additional work will be required to assess the contribution of the 3′-UTRs, if any, to translational regulation of all the IGF-II mRNA isoforms.

IMP-3 Regulates Cell Proliferation via an IGF-II-Dependent Pathway—IGF-II, a secreted, fetal growth factor, plays a critical role in embryonic development (24). For example, IGF-II knock-out mice exhibit dwarfism and abnormal fetal development (43). IGF-II binds two types of cell surface receptors known as IGF-I receptor and IGF-II receptor (44). IGF-I receptor mediates most biological effects of IGF-II (36). IGF-II expression is reduced or absent in adult tissues but is highly expressed in some tumors and cell lines (for review, see Ref. 28). As a mitogen, IGF-II stimulates cell proliferation and inhibits apoptosis (35–39, 45). Human K562 leukemia cells express IGF-II, IGF-I receptor, and IGF-II receptor; exogenous IGF-II promotes cell proliferation, and anti-IGF-I receptor antibody inhibits basal growth of these cells (22, 40, 41, 46). IMP-3 binds to IGF-II leader-3 mRNA both in vitro (1) and in vivo (this work). Moreover, our data show that the kinetics of IMP-3 knockdown were closely linked to reduced intracellular and secreted IGF-II levels and slowed proliferation rates (Figs. 1, 4, and 6). Recombinant human IGF-II peptide completely restored cell growth in cells with knocked down expression of IMP-3 (Fig. 7). Together, these results strongly suggest that decreased IGF-II levels were responsible for the reduced cell proliferation resulting from IMP-3 knockdown.

IMP-3 and IMP-1 Possess Some Distinct Target RNAs and Opposing Functions—Although both IMP-1 and IMP-3 have similar structures and overlapped expression patterns (1), bind to IGF-II mRNA (1, 3), and regulate cell proliferation via an IGF-II pathway (22), they appear to have target RNAs unique to each protein. Moreover, they appear to have opposing functions in K562 cells in regard to regulation of IGF-II expression and cell proliferation. Our previous studies showed that IMP-1 knockdown by RNAi promotes cell proliferation and up-regulates IGF-II mRNA and protein levels through a possible nuclear mechanism (22). By contrast, our current data demonstrate that IMP-3 knockdown inhibited translation of IGF-II leader-3 without affecting mRNA levels (Figs. 3–5), and it inhibited cell proliferation (Fig. 6). Because the two IMPs seem to have opposite effects on cell proliferation and IGF-II expression in K562 cells, interplay between the two proteins may determine the level of IGF-II expression. Knockdown of both IMPs with similar efficiency by RNAi in K562 cells resulted in phenotypes similar to IMP-3 knockdown alone (data not shown). These phenotypes included slower cell proliferation rates and reduced IGF-II protein levels without concomitant changes in IGF-II mRNA levels (data not shown). The double knockdown also did not alter either c-myc mRNA or protein levels (data not shown). Thus, IMP-3 knockdown was dominant, which implies that, genetically, IMP-3 is epistatic to IMP-1. This indicates that IMP-1 function depends upon IMP-3. Therefore, observation of the effects of IMP-1 knockdown requires IMP-3 function. The biochemical basis for this comes from recent data showing that IMP-3 and IMP-1 (as well as IMP-2) can form heterodimers in RNA binding assays with IGF-II 3′-UTR sequences (3).

In conclusion, we have identified IMP-3 as a translational activator for IGF-II leader-3 mRNA. Our experiments revealed a novel function for IMP-3 in cell proliferation, such that IMP-3 promotes cell proliferation by inducing translation of IGF-II mRNA in K562 cells. IMP-3 and IMP-1 thus appear to have distinct functions in these cells.

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