Targeted Knockdown of the RNA-binding Protein CRD-BP Promotes Cell Proliferation via an Insulin-like Growth Factor II-dependent Pathway in Human K562 Leukemia Cells*

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The c-myc mRNA coding region determinant-binding protein (CRD-BP) was first identified as a masking protein that stabilizes c-myc mRNA in a cell-free mRNA degradation system. Thus, CRD-BP is thought to promote cell proliferation by maintaining c-Myc at critical levels. CRD-BP also appears to be an oncofetal protein, based upon its expression during mammalian development and in some tumors. By using K562 leukemia cells as a model, we show that CRD-BP gene silencing by RNA interference significantly promoted proliferation, indicating an inhibitory effect of CRD-BP on proliferation. Unexpectedly, CRD-BP knockdown had no discernible effect on c-myc mRNA levels. CRD-BP is also known as insulin-like growth factor II (IGF-II) mRNA-binding protein-1. It has been reported to repress translation of a luciferase reporter mRNA containing an IGF-II 5'-untranslated region known as leader 3 but not one containing IGF-II leader 4. CRD-BP knockdown markedly increased IGF-II mRNA and protein levels but did not alter translation of luciferase reporter mRNAs containing 5'-untranslated regions consisting of either IGF-II leader 3 or leader 4. Addition of antibody against IGF-II to cell cultures inhibited the proliferative effect of CRD-BP knockdown, suggesting that regulation of IGF-II gene expression, rather than c-myc mRNA levels, mediates the proliferative effect of CRD-BP knockdown. Thus, we have identified a dominant function for CRD-BP in cell proliferation of human K562 cells, involving a possible IGF-II-dependent mechanism that appears independent of its ability to serve as a c-myc mRNA masking protein.

The c-Myc and insulin-like growth factor II (IGF-II) gene promote cell proliferation (1). Post-transcriptional regulation of the c-myc proto-oncogene affects the levels and timing of c-Myc protein expression in mammalian cells. The levels of c-Myc protein, a transcription factor, establish a balance between proliferation, differentiation, and apoptosis (1–12). The c-myc mRNA coding region determinant-binding protein (CRD-BP) was first identified by its ability to mask c-myc mRNA from a polyribosome-associated endoribonuclease in a cell-free mRNA decay system (13). Thus, its function was postulated to maintain c-Myc expression at levels required to promote cell proliferation. CRD-BP is expressed in fetal and neonatal mammals and in some tumors. By comparison, its expression is reduced or absent in normal adult tissues (14–17). CRD-BP expression in mammary epithelial cells of adult transgenic mice induced mammary tumors (18). Thus, it is presumed to be an oncofetal protein. CRD-BP was later shown to be identical to IMP-1 (14, 19). CRD-BP/IMP-1 is highly related to IMP-2 and IMP-3, which together comprise a family of proteins that bind to the 5'-untranslated region of IGF-II leader 3 mRNA. As such, CRD-BP/IMP-1 can repress translation of an IGF-II-luciferase reporter mRNA without affecting levels of the reporter mRNA, and it may repress translation of IGF-II leader 3 mRNA during development (19). This property implies an inhibitory action for CRD-BP in cell proliferation. However, IMP-1 knockout mice are dwarfs, and their embryonic fibroblasts have a slower proliferation rate (20). This implies that CRD-BP can have a positive effect upon cell proliferation. Thus, based upon its proposed control of c-Myc and IGF-II levels, CRD-BP would appear to exert opposing effects on cell proliferation. Therefore, to explore post-transcriptional roles and mechanisms for CRD-BP in gene expression and cell proliferation, experiments were designed to knockdown its expression by using short interfering RNA (siRNA).

siRNA can induce sequence-specific gene silencing by promoting degradation of the homologous mRNA in organisms ranging from Caenorhabditis elegans to humans (21–30). This effect, known as RNAi, has become a powerful genetic tool for studying gene function in higher organisms. Here RNAi was employed to silence CRD-BP expression in K562 cells, which were utilized for the original identification of CRD-BP (13). K562 is a pluripotent, human leukemia cell line possessing erythroid and myeloid properties (31). Unexpectedly, CRD-BP knockdown up-regulated IGF-II mRNA and protein levels, had no effect on c-myc mRNA levels, and promoted cell proliferation via a possible IGF-II-dependent pathway.

EXPERIMENTAL PROCEDURES

siRNA Duplex Preparation—The CRD-BP/IMP-1 SMARTpool and single siRNA duplexes were chemically synthesized by Dharmacon Research (Lafayette, Co.). The SMARTpool siRNA is a mixture of four different siRNA duplexes targeting human CRD-BP/IMP-1 (GenBank™ accession number AF117106). The sequences of the SMARTpool siRNA are proprietary. The single siRNA sequence corresponded to...
the coding region 709–729 nucleotides after the start codon (5′-aag gag aac gca ggu gca gcu-3′). The negative control siRNA consisted of the same nucleotides randomly arranged (5′-aag gga gaa agg ggg cca aag-3′). siRNA duplexes containing dTUT 3′-overhangs. siRNA sequences were subjected to a BLAST search against human genome sequences in GenBank to ensure specificity. 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—Human K562 leukemia 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 × 106 cells were transfected with either 200 nM of SMARTpool, single CRD-BP, or negative control siRNA duplexes or an equal volume of 1× universal oligo buffer (mock control) by electroporation using a Gene Pulser (Bio-Rad). Electroporation parameters were 0.28 kV and 1050 microfarads. Cells were maintained for the times indicated in the figure legend. Specific RNA interference effects were confirmed by at least three independent experiments.

Western Blot Analyses—Nuclear and cytoplasmic extracts were prepared from K562 cells using the CelLytic NuCLEAR™ extraction kit (Sigma). Total protein in extracts was quantified by the Bradford assay by using the Protein Assay Reagent (Bio-Rad) according to the manufacturer’s protocol.

For body preparation, one CRD-BP/IMP-1-specific peptide (PEN-GRRGFSGESGQPRQ, residues 163–180), IMP-2-specific peptide (SPSPPPQRAQRG, residues 161–172), and IMP-3-specific peptide (TS-GMPPTSGPSPS, residues 379–391) was synthesized with an N-terminal cysteine (CyberSyn, Lenni, PA), coupled to keyhole limpet hemocyanin and used to immunize rabbits (Cocalico Biologicals, Inc., Reamstown, PA). Antisera were subjected to affinity purification by using the respective recombinant peptides immobilized on Sulfolink coupling gel (Pierce), elution with 100 mM glycine, pH 2.5, and immediate neutralization with Tris base. Other antibody sources and dilutions were as follows: IGF-II (Santa Cruz Biotechnology) 1:500, c-Myc (Oncogene) 1:300, α-tubulin (Sigma) 1:10,000, and heterogeneous nuclear ribonucleoprotein (hnRNP) A/B/C 1:300 (undiluted hybridoma supernatant) (32). The dilution for all of the IMP antibodies was 1:5,000. The antibodies were routinely diluted in 1× non-fat milk (Bio-Rad) with 0.1% Tween 20. Anti-mouse IgG (H + L) horseradish peroxidase conjugate (Promega) 1:2,500, and goat anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate (Sigma) 1:3,000.

For Western blot analyses, 50 μg of protein extract for each sample was fractionated in SDS-polyacrylamide gels and transferred to nitrocellulose (Fisher). Standard Western blotting assay was performed using SuperSignal West chemiluminescent reagent (Pierce) following the manufacturer’s instructions. α-Tubulin and hnRNP A/B/C proteins were used as the loading control for cytoplasmic and nuclear protein fractions, respectively. The relevant bands were scanned and quantified using a DC120 Zoom digital camera and Kodak Digital Science ID software (Eastman Kodak Co.).

Expression and Purification of Recombinant CRD-BP Protein—Native human recombinant CRD-BP protein was purified with the IMPACT kit (New England Biolabs) by using a fast protein liquid chromatography system (ISCO) following the manufacturer’s instructions.

The pCYB1-CRD-BP expression construct was kindly provided by Dr. Jan Christiansen (19). The complete coding sequence of CRD-BP was inserted into an Ndel + SapI-cleaved pCYB1 expression vector. CRD-BP was expressed by isopropyl 1-thio-b-D-galactopyranoside (IPTG) induction in Escherichia coli BL21/DE3 cells transformed with pRHI92 and a pCYB1-CRD-BP plasmid. pRHI92 promotes translation of open reading frames containing rare isoleucine and arginine codons by overexpressing the ileA and argU TRNAs (19). Human CRD-BP was then purified by using the IMPACT system and confirmed by Western blot analysis.

RNase Protection Assay—c-myc and γ-globin mRNA levels were examined by RNase protection assay in a single tube format as described previously (1). The c-myc antisense probe yields multiple protected fragments corresponding to mRNA molecules with closely spaced polyadenylation sites (1). γ-Globin mRNA served as the internal control.

Quantitative Reverse Transcription-PCR—H19 RNA and IGF-II, c-myc, Bmi-1, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were examined by quantitative real time reverse transcription-PCR (qRT-PCR). One-step qRT-PCR was performed using an MX-4000 Multiplex Quantitative PCR system (Stratagene, La Jolla, CA) and the Quantitect Probe RT-PCR kit (Qiagen) according to the manufacturer’s instructions. The PCR primers and probes were designed using web-based Primer-3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) and synthesized by Integrated DNA Technologies (Corvalle, IA). They are as follows: c-myc probe, 5′-FAM-CGG GCA CTT TCG ACT GAC ACT TAC A-TAMRA-3′, c-myc forward primer, 5′-ACG AAA CTT TGC CCA TAG CA-3′, and c-myc reverse primer, 5′-GAG GCA GCC TTT CAG AG-3′; 5′-FAM-TGG ACC CCA TTCCT AGC TTG-3′, IGF-II forward primer, 5′-AAG TCG ATG CTT GTG CTT CT-3′, and IGF-II reverse primer, 5′-CGG AAA CAC CAG CCC TCC TCA A-3′; CRD-BP probe, 5′-Cy5-GTT GCA GGG CCG AGC AGG AA-BHQ-3′, CRD-BP forward primer, 5′-AAC CTT GAG AGG ACC TTC ACT-3′, and CRD-BP reverse primer: 5′-ACG TGG GAA AAG ACC TAC AGC-3′; H19 probe, 5′-GAG TCA ACG GAT TTG AC-3′, and β-actin reverse primer, 5′-AGA GGA GTG TGG CTT TT-3′; and GAPDH probe, 5′-JOE-NHS-TCA AGT CTA AGA CCT AGC AGC AGC TTG-TTAMRA-3′; GAPDH forward primer, 5′-GAG TCA ACG GAT TGG GTC GT-3′, and GAPDH reverse primer, 5′-GAT CTC GCT CCT GGA AGA TG-3′. GAPDH was used as an internal quantitative control in the same tube (2-multiplex PCR). Cycler conditions for qRT-PCR are as follows: 50 °C, 30 min; and 95 °C, 15 min (94 °C, 20s, and 60 °C, 1 min) × 50 cycles. Total RNA was prepared with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, media samples were pretreated with buffers provided in the kit to dissociate IGF-II- and IGF-binding proteins prior to the assay. This eliminates the IGF-II-binding proteins from interfering with the ELISA. Samples of standards, controls, and pretreated growth medium were then incubated in microtitration wells pre-coated with IGF-II antibody, followed by treatment with horseradish peroxidase-conjugated IgG, development using the substrate tetramethylbenzidine, and stopped by sulfuric acid. The absorbance was measured at 450 nm with a reference of 620 nm using a Multiskan™ ELISA Reader (Lab System). The absorbance measured is directly proportional to the concentration of IGF-II present. A standard curve was plotted, and the concentration was calculated using Prism software (version 2.0, Graph Pad software).

Trypan Blue Exclusion Assay and MTS Assay—Cell counts were obtained by standard trypan blue (Sigma) exclusion according to the manufacturer’s protocol. MTS assay was performed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega).

Twenty microliters of CellTiter 96® Aqueous One Solution reagent (MTS tetrazolium compound) was added to each well of a 96-well culture plate containing K562 cells in 10 μl of culture medium. The plate was incubated for 2 h at 37 °C in 5% CO2. The absorbance at 490 nm was measured using an SLT Rainbow ELISA Reader (Tecan). The 490 nm absorbance was directly proportional to the number of viable cells in the culture (34).

Luciferase Reporter Assay—The plasmids pcDNA-IGF-II-L3-Luc and pcDNA-IGF-II-L4-Luc contain the firefly luciferase coding region inserted between the EcoRI and XbaI sites in the pcDNA1.1 basic vector (Invitrogen) and the complete leader 3 (1,164 bp) or leader 4 (94 bp) exon, respectively, in the HindIII site between the EcoRI and XbaI sites in the pcDNA1.1 basic vector (Invitrogen) and the complete leader 3 (1,164 bp) or leader 4 (94 bp) exon, respectively, in the HindIII site between the EcoRI and XbaI sites in the pcDNA1.1 basic vector (Invitrogen) and the complete leader 3 (1,164 bp) or leader 4 (94 bp) exon, respectively, in the HindIII site between the EcoRI and XbaI sites in the pcDNA1.1 basic vector (Invitrogen) and the complete leader 3 (1,164 bp) or leader 4 (94 bp) exon, respectively, in the HindIII site between the EcoRI and XbaI sites in the pcDNA1.1 basic vector (Invitrogen) and the complete leader 3 (1,164 bp) or leader 4 (94 bp) exon, respectively, in the HindIII site between the EcoRI and XbaI sites in the pcDNA1.1 basic vector (Invitrogen) and the complete leader 3 (1,164 bp) or leader 4 (94 bp) exon, respectively.
CRD-BP RNA Interference and Cell Proliferation

firefly luciferase activity. These were negligible. Firefly luciferase activity was normalized to *Renilla* luciferase activity in the same cell extract and is thus expressed as a ratio of firefly:*Renilla* luciferase activity.

Neutralization of IGF-II in Cell Culture Medium—K562 cells were transfected with negative control and CRD-BP siRNA duplex, respectively, as described above, and seeded in a 96-well plate at a density of 2 × 10^4 cells/well. To neutralize the IGF-II secreted into the growth medium by K562 cells, a polyclonal rabbit antibody against human IGF-II (200 μg IgG/ml in PBS, Santa Cruz Biotechnology) was added to cell cultures twice at concentrations of 2, 4, or 8 μg/ml at 0 and 24 h after transfection. 8 μg/ml nonimmune rabbit IgG (Sigma) and 40 μg/ml PBS buffer were used as negative controls. Cultures were maintained for 48 h post-transfection. The number of viable cells in each culture was determined by MTS assay as described earlier.

Statistical Analyses—Statistical analyses were performed using Student's *t* test and analysis of variance followed by Scheffe test, if a significant *F* ratio was obtained. The data were expressed as means ± S.E. A *p* value < 0.05 was considered to be statistically significant.

RESULTS

CRD-BP siRNA Specifically Inhibits CRD-BP Gene Expression—The effects of CRD-BP siRNA on CRD-BP levels were examined over a 4-day period. We transfected either a SMARTpool or an individual siRNA duplex targeted to human CRD-BP into human K562 leukemia cells by electroporation, as well as a negative control siRNA, which includes the nucleotides randomly rearranged. An equal volume of 1× universal RNA oligo buffer was used as a mock control. Western blotting analyses were performed to monitor protein levels. Cytoplasmic CRD-BP levels were reduced 40% at 24 h (p < 0.05) and reduced 92% at 48 h (p < 0.01) compared with the control siRNA (Fig. 1, A and B). The effect began to subside at 72 h (55% reduction, p > 0.01), and expression returned to control levels by 96 h (Fig. 1, A and B). These results indicated that the effect of CRD-BP RNAi is time-dependent. CRD-BP/IMP-1, IMP-2, and IMP-3 belong to a family of IGF-II mRNA-binding proteins (IMPs) with an overall sequence identity of 59% (19). IMP-3 expression exhibited no statistically significant change after CRD-BP/IMP-1 RNA interference (Fig. 1, A and B, and Fig. 2, A and B), demonstrating specificity of silencing. IMP-2 expression was undetectable by Western blotting analysis (data not shown). Nuclear levels of CRD-BP displayed kinetics similar to cytoplasmic CRD-BP (Fig. 1, C and D). The negative control siRNA duplex treatment did not alter CRD-BP expression levels in either the cytoplasm (Fig. 2, A and B) or nucleus (not shown) compared with the mock control treatment (p > 0.05). CRD-BP siRNA had similar inhibitory effects on CRD-BP mRNA levels as assessed by qRT-PCR (data not shown). This suggests that the siRNA promoted mRNA degradation. Similar effects were obtained by utilizing several CRD-BP siRNAs in a SMARTpool (see "Experimental Procedures"). This control served to minimize the possibility that the effects of the single siRNA for CRD-BP were because of off-target gene knockdown (35, 36). Transfection of a green fluorescent protein construct using the same electroporation conditions as those used for siRNAs revealed that transfection efficiencies were >90% (data not shown). Thus, we conclude that our procedures promote efficient and specific CRD-BP gene silencing.

Effects of CRD-BP Gene Silencing on c-myc mRNA Expression—Because CRD-BP was identified as a *c-myc* mRNA coding region determinant-binding protein and protects the mRNA from degradation by a polyribosome-associated endoribonuclease in *vitro*, *c-myc* mRNA levels were examined by RNase protection assay. CRD-BP knockdown did not affect *c-myc* mRNA levels in the cytoplasm when compared with the negative control siRNA treatment over the 4-day period (p > 0.05; Fig. 1, E and F). These results were confirmed by qRT-PCR (data not shown). This result was unexpected because CRD-BP is thought to stabilize *c-myc* mRNA (13, 37, 38).

To assess the effects of CRD-BP knockdown on *c-Myc* protein levels, Western blot analyses were performed using extracts prepared during the 4-day time course of siRNA treatment. Cytoplasmic levels of *c-Myc* exhibited no statistically significant change (Fig. 1, A and B). Cytoplasmic *c-Myc* likely represented protein synthesized but not yet transported to the nucleus. By contrast, CRD-BP gene silencing appeared to modestly alter nuclear *c-Myc* levels in a biphasic manner. Compared with the control siRNA, the CRD-BP siRNA led to increased *c-Myc* levels by 60% (p < 0.01) at 24 h in the early phase but led to decreased levels by 55% at 72 h (p < 0.01) in the later phase (Fig. 1, C and D). Thus, CRD-BP gene silencing might alter nuclear levels of *c-Myc*, but this effect appears minimal (see "Discussion").

CRD-BP Gene Silencing Promotes Cell Proliferation—Despite minimal effects on *c-myc* mRNA and protein levels, more robust proliferation of cells transfected with the CRD-BP siRNA was noticed compared with the control siRNA. Accordingly, cell numbers were examined during each day after transfection of siRNAs. As shown in Fig. 3, A–C, CRD-BP knockdown markedly increased cell number as assessed by trypan blue exclusion. Cell number increased by 23% at 24 h, increased by 40% at 48 h (p < 0.05), and reached a maximum at 72–96 h post-transfection (72–80%; p < 0.01). To confirm these results, cell growth was also examined by MTS assay (Fig. 3D). The MTS assay, a nonradioactive cell proliferation assay, measures cellular dehydrogenase activity, which is proportional to the number of viable cells in culture. Thus, the MTS assay can substitute for [3H]thymidine incorporation (34).

CRD-BP gene silencing significantly enhanced cell proliferation compared with the control siRNA treatment (Fig. 3D; p < 0.05–0.1), consistent with the differences in cell counts. In addition to the idea that CRD-BP may act as an oncogene, and promote mouse embryo fibroblast cell proliferation, these results suggested that CRD-BP could also act as an inhibitor of proliferation. Because *c-Myc* levels over the 4-day time course did not parallel the growth kinetics of K562 cells, it is unlikely that *c-Myc* is responsible for increased cell proliferation resulting from CRD-BP gene silencing. This prompted consideration of alternative mechanisms by which CRD-BP might affect cell proliferation.

CRD-BP Knockdown Up-regulates IGF-II Gene Expression—CRD-BP was identified previously as an IGF-II mRNA-binding protein that could suppress translation of a chimeric IGF-II/ luciferase reporter mRNA when ectopically overexpressed (19). Because IGF-II promotes proliferation of K562 cells, as well as other cell types (39–44), the effect of CRD-BP gene silencing on IGF-II protein levels was investigated. Cytoplasmic levels of IGF-II increased after transfection of CRD-BP siRNA compared with the control siRNA (p < 0.01; Fig. 1, A and B). The effect persisted to 72 h, peaking at 48 h post-transfection (>4-fold over control). In parallel, CRD-BP siRNA also led to significantly elevated IGF-II levels secreted into the culture medium compared with the control siRNA (p < 0.01; Fig. 4). The IGF-II concentration gradually increased following knockdown of CRD-BP protein expression and plateaued at 72 h post-transfection. These data demonstrated that CRD-BP knockdown increased both the intracellular and secreted levels of IGF-II protein. Thus, IGF-II induction was closely linked to both the kinetics of CRD-BP gene silencing and increased cell proliferation (Figs. 1B, 3C, and 4). Expression of the IGF-II gene is controlled by an array of mechanisms that create up to four mRNAs with identical coding regions and 3′-UTRs but distinct 5′-UTRs (reviewed in Ref. 45). A combination of alternative transcription initiation and alternative pre-mRNA splicing generates four distinct 5′-UTRs.
referred to as leaders 1 to 4 (L1–L4). Their translation is differentially regulated. For example, the leader 4 mRNA is constitutively translated, whereas the leader 3 mRNA is subject to translational suppression by CRD-BP/IMP-1 (19). This is based upon the observation that co-transfection of a CRD-BP expression construct and a chimeric IGF-II leader 3-luciferase reporter construct into NIH 3T3 mouse fibroblast cells suppressed luciferase expression without affecting levels of the reporter mRNA.

Accordingly, to explore the mechanism by which CRD-BP knockdown elevated IGF-II protein levels in our experiments, IGF-II translational regulation was examined. CRD-BP or control siRNA, pcDNA-IGF-II-leader-3 or firefly luciferase reporter construct, and pRL Renilla luciferase control vector were co-transfected into K562 cells. Luciferase activity was detected by the dual luciferase assay. CRD-BP knockdown had no significant effect on luciferase activity through 48 h post-transfection as compared with the control siRNA treatment (Fig. 5). These results indicated that CRD-BP knockdown had little effect on translation of the IGF-II leader 3 and leader 4 reporter mRNAs. As expected, translation of leader 4 luciferase mRNA was 15–20-fold higher than that of leader 3 reporter mRNA (Fig. 5), because leader 3 is 1,164 nucleotides long and possesses a high (48%) cytidine content (19). Due to the apparent absence of translational regulation, IGF-II mRNA levels were next examined by qRT-PCR with primers amplifying the coding region of IGF-II mRNA. CRD-BP knockdown significantly increased IGF-II mRNA levels over 4-fold by 48 h post-transfection as compared with the negative control siRNA (Fig. 6A). This result is consistent with the elevated IGF-II protein levels resulting from CRD-BP knockdown. As controls, the levels of H19 RNA and β-actin mRNA were assessed as well. Like IGF-II leader 3 mRNA, H19 RNA and β-actin mRNA are binding targets of CRD-BP (46, 47). H19 RNA is an untranslated RNA that is imprinted in conjunction with IGF-II (48). Its function is unclear. Previous observations showed that H19 RNA might be a negative regulator of IGF-II expression (49) and has tumor inhibiting or promoting properties, depending on the cell type (reviewed in Ref. 50). A lack of H19 RNA is linked to increased growth of an embryo (51). CRD-BP binds to
CRD-BP or IMP-3 protein bands was normalized to the intensity of buffer (mock control), respectively. In Western blot analyses, with CRD-BP siRNA, negative control siRNA, or 1 μg/ml IgG1 RNA levels (46). CRD-BP knockdown had no statistical localization in cultured cells but apparently does not control /H11032/H9251. Together, these results indicated that IGF-II about 92% compared with the control siRNA and mock control treated as the loading control for total cytoplasmic protein. CRD-BP knockdown specifically elevated IGF-II mRNA levels.

The 3′ terminus of H19 RNA and mediates its subcytoplasmic localization in cultured cells but apparently does not control H19 RNA levels (46). CRD-BP knockdown had no statistical effect on either H19 RNA (p > 0.05; Fig. 6B) or β-actin mRNA levels (p > 0.05; Fig. 6C). Together, these results indicated that CRD-BP knockdown specifically elevated IGF-II mRNA levels.

To ascertain whether elevated IGF-II mRNA levels were because of stabilization of the mRNA, decay of IGF-II mRNA was examined. Exponentially dividing cells were cultured with actinomycin D to inhibit transcription. RNA was purified at various time points and analyzed for IGF-II mRNA levels by using qRT-PCR. The primer pair was chosen in order to amplify a portion of the mRNA coding region. This was done for two reasons. First, the coding region is common to all four IGF-II mRNA variants (45). Thus, the primers would permit the combined assay of all IGF-II mRNAs. Second, decay of IGF-II mRNA is initiated by a slow endonucleolytic cleavage event in the 3′-UTR. In human cells examined so far, the 5′ decay product (containing the coding region) is rapidly degraded, whereas the 3′ fragment, consisting of 3′-UTR sequence and the poly(A) tail, is relatively stable (45). Therefore, the primers chosen should measure the levels of full-length IGF-II mRNA. The actinomycin D chase experiments showed that IGF-II mRNA did not decay at all in K562 cells during the 90-min treatment (Fig. 7). Similarly, 6 h of extended actinomycin D treatment and 24 h of 5,6-dichlorobenzimidazole riboside treatment did not reveal significant decay of IGF-II mRNA (data not shown). These results are consistent with those of Van Dijk et al. (45), who showed that IGF-II mRNA is relatively stable in a tetracycline-inducible mRNA decay system. As a control for the effectiveness of transcription inhibition in our assay, decay of c-myc mRNA was examined by using the same RNA samples. c-myc mRNA decays with rapid kinetics (half-life of 45 min) in K562 cells during actinomycin D treatment (33). As expected, c-myc mRNA decayed with rapid kinetics (data not shown). Together, these results indicated that IGF-II mRNA is relatively stable. Thus, the increase in IGF-II mRNA levels in response to CRD-BP knockdown may be due, at least in part, to transcriptional effects. However, due to the extremely low levels of IGF-II mRNA in K562 cells, coupled with its relatively long half-life, transcription by nuclear run-on assay was below the limit of detection (see “Discussion”).

Antibody against Human IGF-II Abates the Proliferative Effect of CRD-BP Knockdown—To determine whether IGF-II levels actually mediate the proliferative effect of CRD-BP gene silencing, a polyclonal antibody against human IGF-II was used to neutralize the IGF-II secreted by K562 cells into the culture medium. Cell cultures transfected with control or CRD-BP siRNA were incubated with PBS, 8 μg/ml nonimmune rabbit IgG, or either 2, 4, or 8 μg/ml rabbit polyclonal IgG against IGF-II for 48 h. Cell proliferation was then assessed by MTS assays. The nonimmune rabbit IgG had no obvious effect on cell proliferation compared with the PBS control (p > 0.05). The antibody to IGF-II significantly inhibited basal proliferation by 25, 41, and 44% (with 2, 4, and 8 μg/ml IgG, respectively) compared with the nonimmune rabbit IgG negative control at 48 h post-transfection of the control siRNA (p < 0.05–0.01; Fig. 8, shaded bars). These results were consistent with previous work reporting that IGF-II is required for proliferation of K562 cells and that IGF-II antibody inhibits K562 cell growth (52). Similarly, the antibody against human IGF-II at concentrations of 2, 4, and 8 μg/ml IgG, respectively, significantly repressed the proliferation of cells transfected with CRD-BP siRNA by 26, 45, and 64% compared with the nonimmune rabbit IgG control at 48 h post-transfection (p < 0.05–0.01; Fig. 8, solid bars). Thus, the effects were dose-dependent. Most importantly, at the highest concentration of antibody used, the effects of CRD-BP knockdown on proliferation were completely abrogated, i.e. proliferation was reduced to that of control cells. We conclude that IGF-II antibody is sufficient to block the proliferative effect induced by CRD-BP knockdown. These results strongly suggested that elevated IGF-II mediated the effect of CRD-BP knockdown on cell proliferation.

**DISCUSSION**

Specific CRD-BP RNA Interference Promotes Cell Proliferation—In this study, we have established a quantitative, efficient, and specific RNA interference system for CRD-BP by using human K562 leukemia cells as a model. For example, the efficiency of CRD-BP knockdown by RNAi was up to 92%, and the effects persisted up to 96 h (Figs. 1–4). The kinetics of RNA interference in human K562 cells were similar to previous observations with other mammalian cells (28). Because siRNAs can sometimes lead to off-target gene knockdown (35), we also performed parallel experiments using additional siRNAs targeted against different regions of CRD-BP. The similarity of results with the multiple siRNAs utilized increases the likelihood that the observed phenotypes were because of specific knockdown of CRD-BP and not an off-target gene(s) (36). By using this system, we have demonstrated that CRD-BP knockdown by RNAi significantly accelerated cell proliferation. Accordingly, CRD-BP may normally inhibit human K562 leukemia cell proliferation. This was unexpected based upon its suspected role as an oncoprotein and recent observations that CRD-BP-deficient mice are dwarfs and that their cultured embryonic fibroblasts exhibit reduced proliferation rates (20). Therefore, CRD-BP may regulate cell proliferation in a cell type-specific manner. CRD-BP binds to c-myc, IGF-II, β-actin mRNAs, and to H19 RNA, and it affects RNA localization, turnover, and translation (13, 19, 37, 38, 46, 47). Our results show that among those targets, neither c-myc, β-actin, nor H19 RNA appears to be responsible for the proliferative effect of CRD-BP knockdown. Instead, we identified an IGF-II-dependent pathway by which CRD-BP regulated cell proliferation (see below).

Effect of CRD-BP Gene Silencing on c-myc Gene Expression—As a transcription factor, the c-Myc oncoprotein regul-
lates growth, differentiation, and apoptosis of cells (1–6, 8–12). For example, transfection of antisense oligonucleotides targeting c-myc mRNA inhibits K562 cell proliferation, demonstrating the proliferative action of c-Myc in these cells (7). However, CRD-BP gene silencing promoted cell proliferation without affecting substantial changes in expression of c-Myc protein. For example, cytoplasmic c-Myc protein levels remained relatively constant (Fig. 1, A and B), whereas nuclear c-Myc protein levels were altered modestly as a result of CRD-BP knockdown (Fig. 1, C and D). Thus, CRD-BP knockdown may effect modest changes in either the translation or the transport and subsequent subcellular localization of c-Myc protein. Although the fluctuations in nuclear c-Myc protein levels resulting from CRD-BP knockdown might not have influenced K562 cell physiology in our study, they might nonetheless affect gene expression profiles within other cellular contexts. This possibility is based upon the broad range of regulatory roles of c-Myc in expression of genes involved in cellular processes other than proliferation, such as differentiation and apoptosis (1–6, 8–12). Additional experiments will be required to examine the effects of fluctuating nuclear c-Myc levels on these processes.

CRD-BP gene silencing also had little effect on c-myc mRNA levels, although CRD-BP expression was inhibited more than 90% (Fig. 1, E and F). This result was unexpected because...
CRD-BP is thought to be a c-myc mRNA-binding protein that stabilizes c-myc mRNA, based upon results with a cell-free mRNA decay system (13, 37). However, our estimates of molecule numbers revealed ~400,000 CRD-BP protein molecules and ~50 c-myc mRNA molecules per K562 cytoplasm. These numbers were estimated by Western blotting analysis of titrations of purified recombinant human CRD-BP and cytoplasmic extracts, and RNAse protection assays of titrations of c-myc mRNA transcribed in vitro (data not shown). Thus, unless knockdown is essentially 100%, we cannot exclude the possibility that sufficient CRD-BP molecules may remain after siRNA treatment to mask the relatively small number of c-myc mRNA molecules. Nonetheless, the level of CRD-BP knockdown achieved in our experiments is sufficient to produce phenotypes involving increased IGF-II expression and enhanced cell proliferation. Another possibility to account for no effect of CRD-BP knockdown on c-myc expression is that other transriptional and post-transcriptional regulatory pathways acted to maintain c-myc mRNA at a constant level in response to CRD-BP knockdown. This possibility is underscored by the observations that c-myc gene expression can be regulated at multiple levels (12, 53, 54). However, our observations are perhaps consistent with recent results showing that high level expression of CRD-BP in mammary tissues of transgenic mice and knockout of CRD-BP in mice had no effect on c-myc mRNA levels (18, 20).

Mechanism of the Proliferative Effect Resulting from CRD-BP Gene Silencing—IGF-II is a secreted, mitogenic polypeptide that promotes cell cycle progression and inhibits apoptosis (55–58). IGF-II is also highly expressed in various tumors, and it stimulates cell cycle progression and survival of tumor cells (39–44, 59–61). Taken together, the following three observations strongly suggest that increased cell proliferation induced by CRD-BP gene silencing was mediated by increased IGF-II expression. (i) The kinetics of CRD-BP knockdown were highly correlated with both elevated IGF-II levels and accelerated cell proliferation (Figs. 1B, 3, 4, and 6A). (ii) K562 cells express IGF-II and its receptor, and both endogenous and exogenous IGF-II can promote proliferation of these cells (40, 44, 52). (iii) Antibody against human IGF-II blocked the proliferative effect of CRD-BP RNA interference (Fig. 8).

Our study showed that CRD-BP knockdown increased both IGF-II mRNA and protein levels by similar magnitudes in K562 cells. Thus, the increased IGF-II mRNA levels can account for the increased protein levels. In support of this, CRD-BP knockdown had no apparent effect on translation of a chimeric IGF-II leader/luciferase reporter mRNA despite the observation that only IGF-II leader 3 mRNA was reported to be a CRD-BP-binding target among the four IGF-II mRNA isoforms (19). We conclude that CRD-BP may normally act to down-regulate IGF-II gene expression. Our results also demonstrated that IGF-II mRNA appears to be stable (Fig. 7). Therefore, it is unlikely that CRD-BP knockdown increased IGF-II mRNA levels by affecting its decay. IGF-II transcription might increase after CRD-BP knockdown. However, we estimate that there are, on average, less than three IGF-II mRNA molecules per K562 cell as determined by RNAase protection assay (data not shown). As a rare and stable mRNA, the transcription level of the IGF-II gene should be maintained at a very low level. Consistent with this, we were unable to detect CRD-BP expression in the RNA transcripts of K562 cells. Thus, additional studies will be required to determine how IGF-II mRNA levels were increased by CRD-BP knockdown.

The effects of CRD-BP on IGF-II gene expression may be cell type-specific as well. For example, in a transgenic mouse model...
of breast tumors, overexpression of CRD-BP in mammary tissues led to a 100-fold increase in IGF-II mRNA levels, whereas IGF-II protein levels were unchanged (18). By contrast, overexpression of CRD-BP/IMP-1 in mouse NIH 3T3 cells inhibited translation of a chimeric leader-3/luciferase mRNA without affecting reporter mRNA levels (19). In CRD-BP knockout mice, both leader 3 and leader 4 IGF-II mRNAs were redistributed from polysomes to the messenger ribonucleoprotein fraction, indicating that translation of those two transcripts was reduced (20). Our current data show that knockdown of CRD-BP expression increased both IGF-II mRNA and protein levels, without altering translation of IGF-II leader 3 and leader 4 luciferase reporter mRNAs in K562 cells. Taken together, these investigations indicate that the intracellular context may dictate the mode of IGF-II regulation by CRD-BP.

In summary, our results revealed a novel function for CRD-BP in cell proliferation involving an IGF-II-dependent mechanism that appears independent of its ability to serve as a c-myc mRNA masking protein. In addition to its postulated role as an oncofetal protein, CRD-BP can act to repress cell proliferation as well in some cell types.

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