The coding region determinant-binding protein/insulin-like growth factor II mRNA-binding protein (CRD-BP/IMP1) is an RNA-binding protein specifically recognizing c-myc, leader 3’ IGF-II and tau mRNAs, and the H19 RNA. CRD-BP/IMP1 is predominantly expressed in embryonal tissues but is de novo activated and/or overexpressed in various human neoplasias. To address the question of whether CRD-BP/IMP1 expression characterizes certain cell types displaying distinct proliferation and/or differentiation properties (i.e. stem cells), we isolated cell subpopulations from human bone marrow, mobilized peripheral blood, and cord blood, all sources known to contain stem cells, and monitored for its expression. CRD-BP/IMP1 was detected only in cord blood-derived CD34+ stem cells and not in any other cell type of either adult or cord blood origin. Adult BM CD34+ cells cultured in the presence of 5'-azacytidine expressed de novo CRD-BP/IMP1, suggesting that epigenetic modifications may be responsible for its silencing in adult non-expressing cells. Furthermore, by applying the short interfering RNA methodology in MCF-7 cells, we observed, subsequent to knocking down CRD-BP/IMP1, decreased c-myc expression, increased IGF-II mRNA levels, and reduced cell proliferation rates. These data 1) suggest a normal role for CRD-BP/IMP1 in pluripotent stem cells with high renewal capacity, like the data 1) suggest a normal role for CRD-BP/IMP1 in pluripotent stem cells with high renewal capacity, and the 2) indicate that altered methylation may affect the expression of c-myc and insulin-like growth factor II, and 4) indicate that the inhibition of CRD-BP/IMP1 expression might affect cancer cell proliferation.

The coding region determinant-binding protein/insulin-like growth factor II mRNA-binding protein (CRD-BP/IMP1) recognizes at least four RNAs. CRD-BP/IMP1 binds specifically to 1) one of the two independently cis-acting, c-myc mRNA instability elements (1), 2) the 5’-untranslated region of the leader 3’ IGF-II mRNA, which represents the major embryonic form of this message (2), 3) the H19 RNA, a gene product exhibiting an oncelfetal pattern of expression and whose role remains enigmatic (3), and 4) the neuron specific tau mRNA (4), which encodes for a microtubule-associated protein localized primarily in the cell body and axon of developing neurons.

CRD-BP/IMP1 is mostly expressed in embryonal tissues. Analysis of total RNA from mouse embryos indicated a peak of CRD-BP/IMP1 expression at embryonic day 12.5 followed by a decline toward birth and its disappearance in neonatal mice shortly after birth (3). In normal adult tissues, most studies failed to detect CRD-BP/IMP1 mRNA even with the highly sensitive technique of RT-PCR (3, 5–8). However, exceptions have been reported; CRD-BP/IMP1 expression has been detected by immunohistochemistry in the spermatogenic cells in human testis (5), whereas by RT-PCR a very faint CRD-BP/IMP1-specific band has been detected in material derived from adult mouse intestines (9). The expression levels detected in adult mouse intestines are substantially lower than those observed in the corresponding embryonal tissue, indicating perhaps its selective expression in certain rare intestinal cells in adulthood. The de novo expression or overexpression of CRD-BP/IMP1 has been reported in a variety of human neoplasias and in some of these tumor types characterizes the vast majority of the specimens studied (7, 8, 10–12). Furthermore, autoantibodies against CRD-BP/IMP1 have been recorded in the sera from 6 different cancer type patients, with frequencies ranging from 7.1 to 16.5% (13). Two recent animal studies clearly demonstrated a crucial role for CRD-BP/IMP1 in both development and cancer onset. CRD-BP/IMP1-deficient mice exhibit dwarfism, impaired gut development, and increased perinatal mortality (9), whereas transgenic mice exhibiting targeted expression of CRD-BP/IMP1 in adult mammary tissue develop tumors (14).

CRD-BP/IMP1 has been attributed various roles in determining the post-transcriptional fate of its RNA targets. CRD-BP/IMP1 acts as a nucleocytoplasmic shuttling protein (5) exhibiting a distinct pattern of localization in the cytoplasm (15). The protein is distributed along with microtubules and in motile cell transports toward the leading edge. Its nuclear export and cytoplasmic movement depend on RNA binding, implying

* This work was supported by a grant from the Regional Operational Program Attika 20 (MIS code 59605GR (to M. P.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Cancer Immunology Immunotherapy Center, Saint Savas Hospital, 171 Alexandras Avenue, 115 22 Athens, Greece. Tel.: 30-10-6409459; Fax: 30-10-6409516; E-mail: perez@cic.gr.

§ Contributed equally to this work.

The abbreviations used are: CRD-BP/IMP1, c-myc coding region determinant binding protein/insulin-like growth factor II mRNA-binding protein; siRNA, short interfering RNA; CB, cord blood; BM, bone marrow; MPB, mobilized peripheral blood; 5’-azacytidine; RT, reverse transcription; IGF, insulin-like growth factor; Ct, cycle threshold.

Panayotis Ioannidis‡§, Louisa G. Mahaira‡§, Sonia A. Perez‡¶, Angelos D. Gritzapis‡, Panagiota A. Sotiropoulou‡, Giannis J. Kavalakis§, Aris I. Antsaklis¶, Constantin N. Baxevanis‡, and Michael Papamichail‡

From the 3Cancer Immunology Immunotherapy Center, Saint Savas Hospital, 171, Alexandras Avenue, Athens 115 22, Greece and the 1st Obstetrics and Gynecology University Clinic, Alexandras Maternity Hospital, 80, Vassilissis Sofias Avenue, Athens 115 21, Greece

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that CRD-BP/IMP1 recognizes its targets into the nucleus and thereby defines their cytoplasmic fate. It has been postulated that CRD-BP/IMP1 may play a role in polarizing genetic information via cytoplasmic RNA localization, a critical mechanism especially in developmental systems for the generation of subcellular asymmetries in protein abundance. The co-association to the tau mRNA of CRD-BP/IMP1 with HuD (4), a protein involved in trafficking of this mRNA from the cell body to the distal parts of axon in developing neurons (16), supports a role in mRNA localization for CRD-BP/IMP1. The original studies implicated CRD-BP/IMP1 in the control of the translation of IGF-II leader 3’ mRNA (2) and of the stability of c-myc mRNA (1, 17). However, these latter functions may be secondary to the former, as the mRNA localization process may implicate its protection from decay as well as its translational repression while the message is transported along the cytoskeleton to the site where it will be translated. The two animal studies, previously mentioned, have not shed light but have rather perplexed the issue of the CRD-BP/IMP1 mode of action. Although the overexpression of CRD-BP/IMP in the NIH-3T3 cell line suppresses leader 3’ IGF-II mRNA translation (2) in the null mice, not only leader 3’ but leader 4’ IGF-II mRNA as well are redistributed from polysomes to RNP’s, indicating their reduced translational efficiency (9). On the other hand, in mice expressing de novo CRD-BP/IMP1 in mammary tissue, a 100-fold increase in the levels of IGF-II mRNA has been recorded, albeit without any increase in the IGF-II intracellular protein levels (14). Regarding the role of CRD-BP/IMP1 upon c-myc mRNA fate, the available data from a cell-free mRNA decay system indicate that CRD-BP/IMP1 binding protects c-myc mRNA from endonucleolysis, leading to a 4–8-fold increase in its half-life and steady state levels (1, 17). Nevertheless, no alterations of c-myc mRNA levels have been detected in the early stages of mammary transformation in transgenic mice with targeted CRD-BP/IMP1 expression in the tissues of CRD-BP/IMP1 null mice (9, 14). The above indicates that the action of CRD-BP/IMP1 upon its mRNA targets is more complex than initially thought and furthermore implies that in transformed adult cells CRD-BP/IMP1 may exert “illegitimate” roles due to its expression and/or overexpression in an inappropriate cellular context. Thus, the identification of the normal cell types that express CRD-BP/IMP1 could contribute toward the elucidation of its role in normal cellular processes as well as in tumorigenesis. In this study we show that among normal bone marrow (BM), mobilized peripheral blood (MPB), and cord blood (CB) subpopulations this gene is expressed exclusively within the CB CD34+ stem cell compartment and not in their adult counterparts. Moreover, we show that CRD-BP/IMP1 knock-down in the MCF-7 cancer cell line is followed by c-myc mRNA and protein down-regulation and by IGF-II mRNA up-regulation, providing evidence that CRD-BP/IMP1 may affect the expression of these two genes whose abnormal expression is associated with tumorigenesis. Finally, we observed a reduction in cell proliferation rates after CRD-BP/IMP1 knock-down. Although modest and reversible due to the transient action of the exogenously added siRNAs, this reduction implied that the sustained inhibition of CRD-BP/IMP1 expression may affect cancer cell physiology, and if so, CRD-BP/IMP1 may provide a putative therapeutic intervention target.

MATERIALS AND METHODS

CB, BM, and MPB Samples and Mononuclear Cell Purification and Phenotyping—Human CB (n = 29) samples from umbilical veins of normal preterm or full term (32–40 weeks) infants, granulocyte colony-stimulating factor-MPB (n = 10), and bone marrow aspirate samples (BM, n = 5) were obtained after informed consent and processed within 4 h of collection. Mononuclear cells were isolated by Ficoll–Hypaque centrifugation using standard procedures. The remaining red blood cells in CB samples were eliminated by negative selection with anti-glycophorin A microbeads (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. CD34+ cells were isolated with a CD34+ cell selection kit (Miltenyi Biotec). The CD34+ fraction was then serially positively selected for CD14+, CD56+, CD19+, and CD3+ using microbeads conjugated with the respective monocolonal antibodies (all purchased by Miltenyi Biotec). For the estimation of cell purity, isolated populations were washed twice with ice-cold phosphate-buffered saline, 1% bovine serum albumin, and fixed with 1% paraformaldehyde in phosphate-buffered saline. Samples were analyzed using FACS Calibur (BD Biosciences) and CellQuest analysis software. Isolated blood subpopulations had purity higher than 95%. The negative for all the above marker fractions (Lin− CD34+) was also obtained and tested in parallel with the other samples.

Ex Vivo Culture of BM—BM cells and 5′-Azacytidine (5azaC) Treatment—96-Well plates were seeded with 1–2 × 106 cells/well BM cells in StemSpan medium (StemCell Technologies) with or without 50 ng/ml stem cell factor, 20 ng/ml FLT-3 ligand, 25 ng/ml thrombopoietin, 10 ng/ml interleukin-6 in the presence or not of 10−6 M demethylating agent 5azaC (Sigma-Aldrich) and cultured for 15 days. Every 4 days half of the medium was discarded and replenished with fresh medium containing (or not) freshly added cytokines and 5azaC. At the end of the culture period separate cell samples were analyzed by fluorescence-activated cell sorter for the expression of CD34 and by RT-PCR for CRD-BP/IMP1 expression.

Cell Culture and siRNA Transfection—The human breast cancer cell line MCF-7 (ATCC, Manassas, VA) was cultured in minimum essential medium-α medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM l-glutamine (Invitrogen), 100 units/ml recombinant human insulin (Lilly), and 50 μg/ml gentamicin at 37 °C in a 5% CO2 atmosphere. Twenty-one nucleotide (nt) siRNAs targeted against the CRD-BP/IMP1 mRNA were chemically synthesized (Xeragon, Inc.). The sense and antisense sequences were: siCRD-BP-1, 5′-GCCGGCCUGCCUGUAGCUU-3′ and 3′-AGAUCUCAUGACGCGUGG-5′; siCRD-BP-2, 5′-GCGGAGCCACUAGGCUU-3′ and 3′-GGGGUUAGCACGCCGU-5′. The control non-silencing, fluorescein label siRNA was produced in parallel with the other samples. The control non-silencing fluorescein-labeled siRNA was used to optimize siRNA and siPORT lipid transfection of MCF-7 by Xeragon, Inc. (catalog number b4004). All siRNAs were diluted in 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate to a final concentration of 20 μM and stored in aliquots at −70 °C.

MCF-7 cells were regularly passaged to maintain exponential growth. The day before transfection 4.5 × 105 cells were plated per 60-mm plates. Transfection of siRNAs was carried out using the siPORT lipid transfection agent (Ambion, Inc.) following the manufacturer’s instructions. The control non-silencing fluorescein-labeled siRNA was used to optimize siRNA and siPORT lipid concentrations. Cellular siRNA uptake was estimated by fluorescence-activated cell sorter analysis 24 h after transfection. After optimization, each experiment was carried out using a final volume of 5 μl of siPORT lipid and a final concentration of 100 nM siRNA. Cells from duplicate wells in each experiment were pooled 48 h after transfection, and total RNA was isolated as described below. For the analysis of protein expression levels cells from three wells were pooled 48 h post-transfection, and total protein was isolated as described below. Each experiment was performed three times.
CRD-BP/IMP1 Expression in Bone Marrow, Mobilized Peripheral Blood, and Cord Blood Cell Subpopulations—In all organisms so far studied CRD-BP/IMP1 expression was found to be predominant in embryonal tissues. However, these studies have not addressed the question of whether CRD-BP/IMP1 expression is indiscriminate in all embryonal cell types or it characterizes certain cell populations with stem cell properties. BM, MBP, and CB are known to contain cells with stem cell characteristics. The best-defined hematopoietic stem cells (HSCs) are found within the CD34+ and the Lin-CD34- cell populations, although in the latter at very low frequencies (18, 19). We monitored for CRD-BP/IMP1 expression in cell subpopulations isolated from adult BM, adult MBP, and CB. Practically homogenous populations (purity >95%) were isolated after selection using immunomagnetic beads as described under “Materials and Methods.” We employed the nested RT-PCR approach to detect CRD-BP/IMP1 expression. This technique has been used previously to monitor for CRD-BP/IMP1 expression in tumor samples and normal tissues. Using this approach CRD-BP/IMP1 has been found to be specifically expressed in fetal and not in normal adult human tissues (7, 8, 10, 11).

The results of a typical experiment are shown in Fig. 1.
Among the different cell subpopulations tested, the CB CD34+ subpopulation was the only one found to express CRD-BP/IMP1. All 29 CB CD34+ samples studied were found positive, whereas none of the 5 BM CD34+ and 10 MPB CD34 cell subpopulations was isolated from 5 different CB, 3 MPB, and 3 BM expressed CRD-BP/IMP1. CD34+ cells were also found negative for CRD-BP/IMP1. This observation indicated that CRD-BP/IMP1 expression may not be an indiscriminate feature of embryonal cells but is rather a stem cell characteristic.

**5azaC Treatment Activates CRD-BP/IMP1 Expression in Adult BM CD34+ Cells**—It is noteworthy that the highly sensitive Nested-RT-PCR approach could not detect trace amounts of CRD-BP/IMP1 expression even in the enriched adult CD34+ cell subpopulations. The absence of leaky expression in the adult counterparts of CB CD34+ cells indicated very tight post-natal regulation of this gene. A common regulatory mechanism of gene expression during development and differentiation also involved in the permanent silencing of embryonal genes in adult tissues is the methylation of CpG dinucleotides in their regulatory regions (20). It has been shown previously that the CRD-BP/IMP1 proximal promoter region has a high CG content and is enriched in CpG dinucleotides (8). To investigate the putative involvement of CpG methylation in the epigenetic silencing of CRD-BP/IMP1 gene, we cultured adult BM CD34+ cells for a period of 15 days in the presence or absence of the demethylating agent 5azaC, quiescent or stimulated with a cytokine mixture that promotes the exit from G0/G1 of primitive HSC populations (21). We detected the de novo activation of CRD-BP/IMP1 expression in actively proliferating BM CD34+ cells after demethylating treatment (Fig. 2). On the contrary, no expression was detected in the non-5azaC-treated cells. These data indicated that CpG methylation directly or indirectly is involved in the tight regulation of CRD-BP/IMP1 in the adult BM CD34+ cells and furthermore imply that altered methylation, an epigenetic modification frequently encountered in malignancies (22, 23), may contribute to its de novo activation.

**Effects of CRD-BP/IMP1 Knock-down upon c-myc and IGF-II Expression**—We employed the siRNA methodology to knock down CRD-BP/IMP1 expression in the MCF-7 human breast cancer cell line to monitor for any putative effect upon c-myc and IGF-II expression. These cells, besides being easily transfectable, carry few extra c-myc gene copies (24), and thus, we anticipated that any effect of CRD-BP/IMP1 knock-down upon c-myc mRNA levels could be readily detectable. Small, synthetic, double-stranded RNAs can effectively mediate gene silencing through the RNA interference mechanism and have been successfully used to knock down the expression of a myriad of genes in cells from various organisms (25). After optimization of siRNA and siPORT lipid concentrations, ~85% of the cells were labeled positive with a control non-silencing fluorescent-labeled siRNA (Fig. 3A). Furthermore, a specific c-myc siRNA (MYC-si) from Ambion was used as a positive control and, the no-silencing (non-labeled) siRNA (Cont-si), provided in the same kit, was used as a negative control to monitor the efficiency of siRNA targeting. The usage of the MYC-si resulted in a reduction of 75% of c-myc mRNA levels compared with Cont-si at 48 h after transfection (p < 0.01, data not shown). Two chemically synthesized 21-nucleotide siRNAs targeting CRD-BP/IMP1 mRNA at positions 1114–1134 (CRD-si1) and 1468–1488 (CRD-si2) were used, and the effects upon CRD-BP/IMP1 mRNA levels were analyzed. Total RNA was isolated 48 h after transfection, and CRD-BP/IMP1 mRNA levels were estimated using the real-time quantitative RT-PCR approach. CRD-BP/IMP1 levels in each sample were normalized against β2-microglobulin levels monitored with the same methodological approach in a separate reaction. The results of a typical experiment are depicted in Fig. 3B. The mean values (±S.D.) of normalized CRD-BP/IMP1 mRNA levels in cells treated with the siRNAs targeting CRD-BP/IMP1 are depicted in Fig. 3C as a percentage relative to the normalized CRD-BP/IMP1 mRNA levels in cells treated with the Cont-siRNA. The results indicated that CRD-BP/IMP1 mRNA levels at 48 h after transfection were reduced by 80 and 75% upon using CRD-si1 and CRD-si2, respectively, compared with Cont-si (p < 0.01 for both siRNAs).

To monitor the effects of CRD-BP/IMP1 knock-down upon c-myc and IGF-II mRNA levels, MCF-7 cells were transfected with either the CRD-si1 or CRD-si2 and nucleotide siRNAs. Total RNA was isolated 48 h after transfection, and c-myc and IGF-II mRNA levels were estimated using the real-time quantitative RT-PCR approach and normalized against β2-microglobulin levels. The mean values (±S.D.) of normalized c-myc mRNA levels in cells treated with the siRNAs targeting CRD-BP/IMP1 are depicted in Fig. 4A as a percentage relative to the normalized c-myc mRNA levels in cells treated with the Cont-si RNA and show a 3-fold decrease in c-myc mRNA levels after CRD-BP/IMP1 knock-down (p < 0.01 for both siRNAs). To assess the effects of CRD-BP/IMP1 knock-down upon c-Myc protein levels, Western blot analysis was performed using whole cell lysates prepared at 48 h after siRNA transfection. The results depicted in Fig. 4, B and C, show that the c-myc mRNA reduction was accompanied by a reduction of c-Myc protein levels. These data provided for the first time direct evidence that the intracellular down-regulation of CRD-BP/IMP1 may affect the c-myc mRNA and protein levels, substantiating the in vitro findings of Bernstein et al. (1). Regarding the IGF-II, the estimated mean values (±S.D.) of normalized mRNA levels are depicted in Fig. 4D and show an approximately 15-fold increase in IGF-II mRNA levels after CRD-BP/IMP1 knock-down (p < 0.01, for both siRNAs). To assess whether the increase in IGF-II mRNA levels after CRD-BP/IMP1 knock-down was accompanied by an increase in the production of IGF-II, both Western blot analysis of whole cell lysates and enzyme-linked immunosorbent assay analysis of conditioned medium, collected at 48 h post-transfection, were performed. We were not able to detect the IGF-II protein by either approach due to the fact that the protein levels were below the detection limits of both methods. This is not surprising because, whereas in MCF-7 cells IGF-II mRNA is readily detected by conventional mRNA analysis methods, the levels of the corresponding protein are low in the conditioned medium, i.e. between 5 and 15 ng/ml per 107 cells (26, 27). Nevertheless, we cannot exclude the possibility that IGF-II production is...
increased after CRD-BP/IMP1 knock-down, as has been reported for K562 cells (28).

**CRD-BP/IMP1 Knock-down Reduces MCF-7 Proliferation Rates**—To assess any putative effect of CRD-BP/IMP1 knock-down and of the concomitant c-myc mRNA and protein down-regulation upon the cell proliferation rates, we measured

\[ \text{[H]Tdr} \] uptake at 24, 48, 72, and 96 h after transfection. As shown in Fig. 5A, a reduction of \(-22\%\) in \([H]Tdr\) incorporation rates was observed with both CRD-BP siRNAs at 24 and 48 h post-transfection, comparable with that obtained with the MYC-si. Furthermore, we detected the up-regulation of the cyclin kinase inhibitor p21WAF1/CIP1 in cells transfected with the specific CRD-BP/IMP1 siRNAs as presented as a percentage of the corresponding ratios of cells treated with the Cont-si. The values shown are the mean \(\pm\) S.D. from three independent experiments. B, Western blot analysis of c-Myc protein levels at 48 h after transfection with Cont-si, CRD-si1, CRD-si2, or MYC-si. C, c-Myc/GAPDH protein ratios presented as a percentage of the Cont-si transfected cells (mean of three independent experiments \(\pm\) S.D.). D, the IGF-II mRNA levels in transfected cells were analyzed by real-time RT-PCR and normalized to the corresponding \(\beta\)-microglobulin levels. The IGF-II/\(\beta\)-microglobulin ratios in cells transfected with the specific CRD-BP/IMP1 siRNAs are presented as a percentage to the corresponding ratios in the Cont-si treated cells.

**FIG. 3.** siRNA-mediated reduction of CRD-BP/IMP1 mRNA levels. A, efficiency of siRNA uptake by MCF-7 cells. MCF-7 cells were transfected with a control non-silencing fluorescein-labeled siRNA. 24 h after transfection the siRNA uptake was estimated by fluorescence-activated cell sorter analysis. Upon using the optimized siRNA and siPORT lipid concentrations described under "Materials and Methods," \(-85\%\) of the cells labeled positive. B and C, efficient siRNA targeting of CRD-BP/IMP1. MCF-7 cells were transfected with a control non-silencing siRNA (Cont-si) or the two siRNAs targeting CRD-BP/IMP1 mRNA (CRD-si1 and CRD-si2). 48 h after transfection total RNA was isolated, and CRD-BP/IMP1 and \(\beta\)-microglobulin mRNA levels were analyzed by real-time RT-PCR. B, real-time amplification plots from a typical experiment. The three curves on the left correspond to the \(\beta\)-microglobulin, whereas the three in the right correspond to the CRD-BP/IMP1 reactions. Samples from the different transfections are marked accordingly. C, the CRD-BP/IMP1/\(\beta\)-microglobulin mRNA ratios in cells transfected with the two specific CRD-BP/IMP1 siRNAs are presented as a percentage of the corresponding ratios of cells treated with the Cont-si. The values shown are mean \(\pm\) S.D. from three independent experiments.

**FIG. 4.** Effects of CRD-BP/IMP1 knock-down upon c-myc expression and IGF-II mRNA levels. A, MCF-7 cells were transfected with the Cont-si, CRD-si1, or CRD-si2. 48 h after transfection total RNA was isolated, and c-myc and \(\beta\)-microglobulin mRNA levels were analyzed by real-time RT-PCR. The c-myc/\(\beta\)-microglobulin mRNA ratios from cells transfected with the two specific CRD-BP/IMP1 siRNAs are presented as a percentage of the corresponding ratios of cells treated with the Cont-si. The values shown are mean \(\pm\) S.D. from three independent experiments. B, Western blot analysis of c-Myc protein levels at 48 h after transfection with Cont-si, CRD-si1, CRD-si2, or MYC-si. C, c-Myc/GAPDH protein ratios presented as a percentage of the Cont-si transfected cells (mean of three independent experiments \(\pm\) S.D.). D, the IGF-II mRNA levels in transfected cells were analyzed by real-time RT-PCR and normalized to the corresponding \(\beta\)-microglobulin levels. The IGF-II/\(\beta\)-microglobulin ratios in cells transfected with the specific CRD-BP/IMP1 siRNAs are presented as a percentage to the corresponding ratios in the Cont-si treated cells.
MCF-7 cells. 0.05). sate for the antiproliferative action of c-myfac-tion down-regulation in CRD-BP/IMP1 knock-down could not compen-
sations indicated that any putative augmented IGF-II targets whose recovery may be delayed. The above-mentioned
mRNAs, the IGF-II RNA, and the neuron-specific tau mRNA. The critical role for CRD-BP/IMP1 in development is revealed by the finding that CRD-BP/IMP1 null mice exhibited dwarfism, impaired gut development, and high perinatal lethality (9). Growth retardation in null mice increased progres-
sively from day E17.5, mainly due to tissue hypoplasia, and the spatiotemporal pattern of CRD-BP/IMP1 expression revealed increased expression in different embryonal days in various expanding and developing organs up to day E17.5, where its expression generally decreased. To address the question of
whether CRD-BP/IMP1 expression characterizes particular expanding and developing organs up to day E17.5, where its expression generally decreased. To address the question of
whether CRD-BP/IMP1 expression characterizes particular embryonic cell types, we analyzed isolated CB cell subpopulations and found its expression to be restricted exclusively to the CD34+ stem cell compartment. These cells are formed mainly in the fetal liver and spleen early in development and in the last trimester of pregnancy begin to migrate to BM through the blood stream, reaching the umbilical cord, where they are found at the time of birth. Thus, the above finding implies a
role for CRD-BP/IMP1 gene in fetal stem cell physiology. Inde-
pendent of the stage of human development, the ability to reconstitute the hematopoietic system upon transplantation in vivo resides mostly within the CD34+ population. However, various studies have shown that CB, BM, and PB CD34+ cells exhibit distinct functional characteristics regarding their self-
renewal, differentiation, and proliferative properties and their repopulating capacity in NOD/SCID mice (19, 30, 31). These differences are also reflected in their gene expression profile (19, 30–34), and our data show that CRD-BP/IMP1 is also differentially expressed in these cell populations.

It has been postulated that CRD-BP/IMP1 could affect the posttranscriptional fate of its RNA targets by determining their sub-cellular localization, by modulating their half-life, or by affecting their translatability. Herein we showed that target-
ing of CRD-BP/IMP1 expression in a human breast cancer cell line is followed by c-myc mRNA and protein down-regula-
tion and by IGF-II mRNA up-regulation. Previous studies have shown elevated, albeit not statistically significant, expression of c-myc in CD34+ fetal or CB cells compared with their adult BM counterparts (34). BM CD34+ cells are in a deep dormant state, and a marked increase of c-myc mRNA levels is observed upon cytokine and growth factor stimulation, probably due to the activation of various regulatory mechanisms acting upon c-myc expression. Quiescent adult CD34+ cells exposed to growth factors are rapidly committed and differentiate in vitro after a limited number of cell divisions. On the other hand, CD34+ cells in earlier ontogenetic stages possess distinct proliferation characteristics, and those isolated from fetal blood in the earlier stages of development are actively cycling. Until recently no data existed regarding a functional role of c-myc in HSC physiology. These cells, upon entrance to the cell cycle, are obliged to select self-renewal or differentiation, and it is well established that Notch signaling and HOXB4 expression pro-
more the former. Satoh et al. (35) have provided evidence indi-
cating that c-myc is a gene target and could be the downstream mediator of Notch and HOXB4 genes in HSC. The expression of a C-myc-inducible form in murine HSC not only enhanced the expression of the same genes as Notch and HOXB4 but also induced their growth without disrupting their biologic properties. The ectopic activation of c-Myc increases cell survival, and proliferation under all cytokine combinations studied does not compromise HSC immature characteristics and augments their reconstituting activities upon transfusion to lethally irradiated mice. On the other hand, Wilson et al. (36) have shown recently that although c-myc might be dispensable for mice BM HSC self-renewal, it plays a crucial role for promoting differentiation to committed progenitors. Their model proposes that fine tun-
ing of c-myc expression in the two daughter cells after HSC division generates asymmetries mainly regarding cell adhesion molecules, retaining one of the cells to the HSC niche and promoting leaving and differentiation of the other (36). Based on these observations it cannot be excluded that CRD-BP/IMP1 expression in fetal CD34+ cells could actually play a crucial role in their self-renewal, differentiation, and proliferation properties and their repopulating activity by affecting either c-myc expression or its mRNA intracellular distribution.

The abnormal expression of three of the thus far identified CRD-BP/IMP1 targets has been detected in human tumors and this together with the oncofetal pattern of CRD-BP/IMP1 expression led to the hypothesis that its de novo activation in tumors may be associated with the process of carcinogenesis. Conclusive evidence that CRD-BP/IMP1 acts as an oncogene comes from experiments with transgenic mice exhibiting targeted expression of the gene in their adult life in mammary glands (14). In this study quantitative analysis of CRD-BP/
IMP1 RNA target levels in mammary tissues, collected after the activation of CRD-BP/IMP1 expression but before the onset of preneoplastic or neoplastic lesions, has shown the elevated expression of IGF-II mRNA and H19 RNA but not the increase of c-myc mRNA or protein levels. This finding sticks because CRD-BP/IMP1 stabilizes c-myc mRNA levels in a cell-free mRNA decay system. To elucidate any putative effect of CRD-BP/IMP1 upon c-myc expression in intact cells, we employed the siRNA approach to knock-down CRD-BP/IMP1 expression, and we found that this was followed by a decrease in c-myc mRNA and protein levels. These data implied that c-myc mRNA could be an in vivo CRD-BP/IMP1 target and substantiated the notion that CRD-BP/IMP1 de novo expression in tumors might perturb c-myc normal regulation. Furthermore, the data indicated that targeting of CRD-BP/IMP1 could have pronounced effects upon c-myc expression.

This is of particular interest since c-myc is one of the most frequently deregulated genes in malignancies, and its transforming action has been linked to accelerated cell proliferation rates, cell growth, and metabolism and to the inhibition of cell differentiation (37–40). On the other hand, we detected a 15-fold increase in the IGF-II mRNA levels after CRD-BP/IMP1 knock-down. However, we were not able to detect whether this was followed by a concomitant increase in IGF-II produced or secreted levels. Controversial data regarding the CRD-BP/IMP1 mode of action upon IGF-II have been provided previously by the studies of Nielsen et al. (2), Hansen et al. (9), and Tessier et al. (14). Various hypotheses could be proposed to explain these differences, for example, cell type-specific modes of action or the execution of illegitimate roles by CRD-BP/IMP1 in adult tissues and/or in tumor cells. IGF-II can stimulate various cellular responses acting as a cell survival factor or mitogenic factor and can also modify metabolism. IGF-II augments in vitro both proliferation and differentiation of BM CD34+ cells, whereas it supports the maintenance of a great number of progenitor cells, thus suggesting a possible role in the expansion of committed progenitors (41). Furthermore, IGF-II functions as an autocrine factor in certain tumor types, including breast carcinomas (42–44). Herein we showed that CRD-BP/IMP1 knock-down and the concomitant c-myc down-regulation resulted to a reduction in cell proliferation rates in MCF-7 cells. The above finding implied that whatever the effect of CRD-BP/IMP1 knock-down upon IGF-II production in these cells, the down-regulation of c-myc expression suffices to reduce proliferation rates. Despite the fact that the antiproliferative effects observed were modest and transient, most likely due to the limitations of the methodology, these data indicated that a sustained reduction or inhibition of CRD-BP/IMP1 expression could have more pronounced effects upon cancer cell physiology, and if so, CRD-BP/IMP1 may provide a therapeutic intervention target. Recently, Liao et al. (28) reported that CRD-BP/IMP1 knock-down has no effect upon c-myc mRNA and protein levels, whereas it promotes cell proliferation via the up-regulation of secreted IGF-II in the K562 leukemic cell line. Conflicting with our data, these observations may be due to differences in the CRD-BP/IMP1 expression levels between the two cell lines. The presence of 50 c-myc mRNA copies and 400,000 CRD-BP/IMP1 protein molecules per K562 cytoplast has been estimated (28). Upon using the quantitative RT-PCR methodology, we calculated the CRD-BP/IMP1-glyceraldehyde-3-phosphate dehydrogenase mRNA ΔCt values for K562 and MCF-7 to be ~6 and ~14.5, respectively, indicating the substantially lower CRD-BP/IMP1 expression in MCF-7 cells. Thus, whereas we cannot exclude the possibility that CRD-BP/IMP1 may exert differential cell type-specific actions, a plausible explanation for the discrepancies observed in the two cell lines could be that CRD-BP/IMP1, being in vast excess, has not been depleted to the extent that can affect c-myc mRNA expression in K562 cells. Thus, with the c-myc levels unchanged, the increased IGF-II expression may result in the promotion of proliferation, as observed by Liao et al. (28).

The de novo CRD-BP/IMP1 expression has been detected in human tumors of different origins, and in some of these tumor types CRD-BP/IMP1 expression characterizes the vast majority of the samples studied (7, 8, 10–12). However, little is known about the mechanisms responsible for the activation of this gene in cancer. The amplification of CRD-BP/IMP1 gene locus has been detected in breast carcinomas (45); nevertheless, the high percentage of CRD-BP/IMP1-expressing samples found in tumor samples from this organ (10) cannot be explained solely by the presence of this genomic abnormality. The data presented herein indicate that this gene is tightly regulated in adult normal cells, at least in the hematopoietic tissue. However, our findings could not exclude the possibility that certain rare cells might exist in other adult tissues expressing CRD-BP/IMP1, as implied by the identification of low expression levels in mouse adult intestines (9). Upon culturing adult BM CD34+ cells in the presence of 5azaC, we identified the re-activation of CRD-BP/IMP1 expression, and this finding indicated that altered methylation may be directly or indirectly involved in the silencing of this gene in adult non-expressing cells. Our data implied that altered methylation, a common characteristic of cancer cells influencing the expression of oncogenes and oncosuppressor genes (22, 23), may affect the expression of CRD-BP/IMP1 in cancer. An extended study of the methylation status of CRD-BP/IMP1 promoter region in tumor samples would prove whether altered methylation is directly responsible for the de novo activation of CRD-BP/IMP1 in neoplasias.

In conclusion, our data point toward a normal role for CRD-BP/IMP1 in the biology of pluripotent stem cells with high renewal capacity, as with the CB CD34+ cells, and indicate that epigenetic mechanisms are involved in the regulation of its expression in adult non-expressing cells and imply that its de novo activation in cancer cells may affect the expression of c-myc and IGF-II, whose role in the control of cell growth is well established.

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CRD-BP/IMP1 Expression in HSC and Function in MCF-7 Cells
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Panayotis Ioannidis, Louisa G. Mahaira, Sonia A. Perez, Angelos D. Gritzapis, Panagiota A. Sotiropoulou, Giannis J. Kavalakis, Aris I. Antsaklis, Constantin N. Baxevanis and Michael Papamichail

J. Biol. Chem. 2005, 280:20086-20093.
doi: 10.1074/jbc.M410036200 originally published online March 14, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M410036200

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