A MAPK/HNRPK pathway controls BCR/ABL oncogenic potential by regulating MYC mRNA translation

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Altered mRNA translation is one of the effects exerted by the BCR/ABL oncoprotein in the blast crisis phase of chronic myelogenous leukemia (CML). Here, we report that in BCR/ABL+ cell lines and in patient-derived CML blast crisis mononuclear and CD34+ cells, p210BCR/ABL increases expression and activity of the transcriptional-inducer and translational-regulator heterogeneous nuclear ribonucleoprotein K (hnRNP K or HNRPK) in a dose- and kinase-dependent manner through the activation of the MAPK/ERK1/2 pathway. Furthermore, HNRPK down-regulation and interference with HNRPK translation - but not transcription-regulatory activity impairs cytokine-independent proliferation, clonogenic potential, and in vivo leukemogenic activity of BCR/ABL-expressing myeloid 32Dc13 and/or primary CD34+ CML-BC patient cells. Mechanistically, we demonstrate that decreased internal ribosome entry site (IRES)-dependent Myc mRNA translation accounts for the phenotypic changes induced by inhibition of the BCR/ABL-ERK1/2-dependent HNRPK translation-regulatory function. Accordingly, MYC protein but not mRNA levels are increased in the CD34+ fraction of patients with CML in accelerated and blastic phase but not in chronic phase CML patients and in the CD34+ fraction of marrow cells from healthy donors. Thus, BCR/ABL-dependent enhancement of HNRPK translation-regulation is important for BCR/ABL leukemogenesis and, perhaps, it might contribute to blast crisis transformation.

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Materials and methods

Cell cultures and primary cells

The IL-3–dependent 32Dc13 myeloid precursor, and 32D-BCR/ABL, K562, and TonB210.1 (G. Daley; Harvard University, Cambridge, MA) cells were maintained in culture in IMDM, 10% FBS, 2 mM l-glutamine (Gibco, Carlsbad, CA). Where indicated, WEHI-conditioned medium (10%) was used as source of mIL-3. 32D-BCR/ABL–derivative cell lines were generated by retroviral infection followed by antibiotic-mediated selection or fluorescence-activated cell sorter (FACS)–mediated sorting of the GFP+ (green fluorescent protein) cells. For cell starvation, cells were washed 4 times in PBS and incubated (12 hours) in IMDM/10% FBS. Samples of mononuclear hematopoietic cells from bone marrow of unidentifiable CML patients were kept overnight in IMDM, 50% FBS, 2 mM glutamine, and rhIL-3 (20 ng/mL), rhIL-6 (20 ng/mL), rhIg4-ligand (100 ng/mL), and rhKL (100 ng/mL) (Stem Cell Technologies, Vancouver, BC, Canada) and used for Western blot analysis or for CD34+ enrichment (CD34 MultiSort kit; Miltenyi Biotec, Auburn, CA). CD34+ normal bone marrow (NBm) cells from healthy donors were from AllCells (Berkeley, CA). Studies performed with human specimens from The Ohio State University Leukemia Tissue Bank (Columbus, OH), the Division of Hematology, Maisonneuve-Rosemont Hospital (Montreal, QC, Canada), and C. Gambacorti-Passerini (Division of Experimental Oncology, NCI, Milan, Italy) were approved by The Ohio State University Institutional Review Board. Survival of BCR/ABL-expressing cells was assessed by trypan-blue exclusion test. Methyccellulose-colony formation assays were carried out by plating 103 BCR/ABL-expressing cell lines or 104 CML-BC, Canada) and used for Western blot analysis or for CD34+ enrichment (CD34 MultiSort kit; Miltenyi Biotec, Auburn, CA). Colonies from cell lines and primary cells were scored 7 and 15 days later, respectively.

Plasmids

MigR1-HNRPK-HA, MigR1-(S284/353A)HNRPK-HA, and MigR1-(S284/353D)HNRPK-HA. The HA-tagged wild-type and S284/353A/HNRPK and S284/353D-HNRPK cDNAs were subcloned into the MigR1 retrovector vector. MigR1-HNRPK-C299-HA. The HA-tagged HNRPK-C299 fragment (amino acids 1-299) was polymerase chain reaction (PCR) generated using CMV-HA-C299 primers (P. Raychaudhuri, University of Illinois, Chicago, IL) and subcloned into the HpaI-digested MigR1 retrovector vector. MigR1-HNRPK-AS. HNRPK cDNA from pSP72-K (G. Dreyfuss, UPENN, Philadelphia, PA) was blunt-ended and subcloned in antisense orientation into MigR1.

pSuper.retro.Hnrpk shRNA. The shRNA Hnrpk was obtained by subcloning the mouse Hnrpk nucleotide sequence (5´-ggtagaatgtgagggggactg-3´) into the pSUPER.retro.neo+ GFP vector (OligoEngine, Seattle, WA).

MigR1-Flag-ERK1, MigR1-Flag-ERK1K71R, MigR1-Flag-ERK2, and MigR1-Flag-ERK2S283R. The wild-type and dominant-negative ERK1 and ERK2 were PCR generated using the pCEP4-ERK1-WT, pCEP4-K71R-ERK1, pCMV5-ERK2-WT, and pCMV5-K22ERK2 plasmids (M. Cobb, University of Texas South Western Medical Center, Dallas, TX) and 5’-primers containing a Xhol site, an ATG, the Flag-tag and the first 17 nucleotides of ERK1 or ERK2 cDNAs, and 3’-primers containing an EcoR1 site linked to the last 16 nucleotides of ERK1 or ERK2 cDNAs. The fragments were Xhol-EcoRI digested and subcloned into MigR1.

MSCV-puro-MYC. MSCV-based vector contains the MYC cDNA (G. Leone, Ohio State University, Columbus, OH).

pRF and the pRMF(IRES-MYC). pRF and the pRMF(IRES-MYC) were previously described.23

Northern blot analysis and RT-PCR

For Northern blot analysis, acid-phenol/guanidinium–extracted total RNA (5 μg) was hybridized to 32P-labeled mouse Hnrpk and MYC cDNAs. For reverse-transcriptase (RT)–PCR of Hnrpk pre-mRNA, 1 μg nucleolar RNA obtained from sucrose gradient-purified nuclei by acid-phenol/guanidinium–mediated extraction was DNaseI treated and reverse transcribed using 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase ( Gibco), 200 μM dNTPs, and 0.25 U/mL random hexamers (Pharmacia, Piscataway, NJ). cDNA was used as template for PCR. Hnrpk pre-mRNA primers were as follows: 5´-atgtagaatgtgagaggact-3´ and 5´-ggtggctactgtaaatggta-3´. As control, cDNA samples were adjusted to yield relatively equal amplification of GRB2. For RT-PCR amplification of MYC transcripts, cDNA was synthesized from total RNA of the CD34+ fraction of marrow from healthy donors and CML patients. hMYC mRNA was amplified using oligomers corresponding to nucleotides 1004 to 1023 and 1286 to 1306 of MYC cDNA. GRB2 mRNA levels were used to normalize levels of MYC mRNA among samples.

Western blot and metabolic labeling

Cells were lysed (107 cells/100 μL) in isotonic buffer (150 mM NaCl, 20 mM Hepes [pH 7.0], 1% NP-40, 10% glycerol) supplemented with protease and phosphatase inhibitors. For direct lysis, cells (2-3 × 107) were lysed in 20 μL Laemmli buffer. Antibodies used were as follows: monoclonal anti-HNRPK (G. Dreyfuss, UPENN, Philadelphia, PA), polyclonal anti- HNRPK (B. Dhillon, Santa Cruz Biotechnology, Santa Cruz, CA), anti-MYC (Ab-2) and antiphosphotyrosine (Calbiochem, San Diego, CA), anti-Crk (Transduction Laboratories, Lexington, KY), anti-HA (Covance, Princeton, NJ), anti-Flag (Sigma, St Louis, MO), and anti-pERK (Cell Signaling, Danvers, MA). For metabolic labeling, cells were PBS rinsed, cultured (2 × 106 cells/mL) for 60 minutes in methionine/cysteine-free RPMI-1640/10% dialyzed FBS (Gibco) and 2 ng/mL rmIL-3 (R&D Systems, Minneapolis, MN), and resuspended (5 × 106 cells/mL) in medium containing 200 μCi/mL (7.4 MBq) [35S]-methionine/cysteine (NEN Life Science Products, Boston, MA). After 90 minutes, cells were lysed in isotonic buffer and lysates were incubated (4°C, 2 hours) with protein G agarose–coupled anti-MYC antibody. Immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose, and visualized by autoradiography.

Immunofluorescence microscopy

Cytopsins were fixed in 3.7% formaldehyde, PBS rinsed, permeabilized (10 minutes) in PBS/0.05% Triton X-100 (Sigma), rinsed, and blocked (10 minutes) in PBS/4% goat serum. Incubation with the anti-HA and anti-HNRPK antibodies (1:250 dilution) with the fluorophore-labeled goat anti–mouse Texas Red and IgG Alexa 488 A-11001 (1:200 dilution; Molecular Probes, Carlsbad, CA), respectively, were carried out at room temperature (30 minutes). Slides were PBS rinsed, antifade treated (Molecular Probes), and analyzed by confocal microscopy (Zeiss LSM510, 40×/1.4 NA magnification, and Image software, both from Carl Zeiss, Thornwood, NY).

Luciferase assay

32D-BCR/ABL cells were transfected with the pRF or pRMF(IRES-MYC) plasmids by nucleofection using the manufacturer’s protocol (Amaza, Gaithersburg, MD). After 48 hours, firefly and renilla luciferase activities were assessed by the Dual Luciferase reporter system (Promega, Madison, WI) and detected with the Fluoroscan Ascent-FL luminometer (Thermo, Waltham, MA).

Myc mRNA association with HNRPK

Immunoprecipitation of the mRNAs associated with endogenous and exogenous HNRPK in BCR/ABL-expressing cells was performed as described25 using the monoclonal anti-HNRPK and anti-HA antibodies, respectively. Anti-Flag immunoprecipitation served as negative control. The presence of Myc transcripts was assessed by RT-PCR on equal amounts of RNA, extracted from RNP-enriched lysates (input) and from the anti-HNRPK and anti-HA immunoprecipitates. The mMYC primers used were as follows: 5´-ctgtgccttacctagcgt-3´ and 5´-ctgtaggttggtggactggt-3´. PCR products were gel fractionated and visualized by ethidium bromide staining.

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Results

Effect of BCR/ABL kinase activity on HNRPK expression

Using Affymetrix U74Av2 arrays (Affymetrix, Santa Clara, CA), we found that Hnrpk mRNA levels were down-regulated (~9-fold inhibition) in imatinib-treated (1 μM; 24 hours) compared with untreated BCR/ABL-expressing myeloid cells (not shown). Accordingly, Northern blot analysis revealed that Hnrpk mRNA levels were higher in IL-3–cultured 32D-BCR/ABL than parental 32Dc13 cells, and that HNRPK expression dropped off at levels similar to that of parental cells upon imatinib treatment (Figure 1Ai). To determine whether up-regulation of Hnrpk mRNA levels in 32D-BCR/ABL cells results from increased transcription and/or enhanced mRNA stability, we carried out RT-PCR on DNAse I–treated nuclear RNA using primers corresponding to the exon 2–intron 2 and intron 2–exon 3 junctions, and Northern blot on actinomycin D–treated (5 μg/mL; 24 hours) parental and BCR/ABL-expressing 32Dc13 cells. As shown, Hnrpk pre-mRNA transcripts are higher in 32D-BCR/ABL than IL-3–cultured 32Dc13 cells (Figure 1Aii). Furthermore, Hnrpk mRNA levels are only marginally affected by inhibition of RNA synthesis in 32D-BCR/ABL cells, whereas they progressively decrease in actinomycin D–treated 32Dc13 cells (Figure 1Aiii). As expected, HNRPK protein levels are up-regulated by BCR/ABL expression in 32Dc13 cells (Figure 1B, lanes 1-2), and in the doxycycline-treated (2 μg/mL; 3 days) TonB210.1 lymphoid precursors in which BCR/ABL is tetracycline inducible (Figure 1B, lanes 4-5). Accordingly, imatinib (48 hours, 1 μM) down-regulated HNRPK protein levels in IL-3–cultured 32D-BCR/ABL and Ph1K562 cells (Figure 1B, lanes 3, 7). However, HNRPK expression was similar in untreated and imatinib-treated 32D-BCR/ABL cells IL-3–cultured for 24 hours with the protein synthesis inhibitor cycloheximide (CHX, 20 μg/mL) (Figure 1C), suggesting that BCR/ABL does not affect HNRPK protein stability.

Increased HNRPK expression and its dependence on BCR/ABL kinase activity were also evident when we assessed HNRPK levels in mononuclear cells (MNCs) (Figure 1Dii) and in the CD34+ bone marrow fraction (Figure 1Dii) from CML patients and healthy donors (NBM). As shown, HNRPK expression was up-regulated more in CML-BC (nCD34+ = 3, nMNC = 7) and CML-AP (nCD34+ = 1) than in CML-CP (nCD34+ = 2, nMNC = 8) and NBM (nCD34+ = 2) samples. Moreover, imatinib treatment of CML-BCD34+ cells resulted in a marked decrease in HNRPK expression (Figure 1Dii). Note that the percentage of Ph1 cells in the CML-CP samples was 95% or more, and that BCR/ABL activity was higher in CML-BCD34+ than CML-CPD34+ cells (Figure 1Dii, inset).

HNRPK expression depends on the BCR/ABL-regulated MAPK pathway

To investigate the mechanism(s) whereby BCR/ABL enhances HNRPK expression, 32D-BCR/ABL and K562 cells were exposed to inhibitors of BCR/ABL-activated pathways.2,4 HNRPK protein
levels were significantly reduced by 8-hour treatment with the MAPK inhibitor PD098059 (50 μM) but not with inhibitors of PI-3K–dependent (25 μM LY204002), PLCγ1-dependent (1 μM U73122), mTOR/S6K-dependent (15 nM rapamycin), and PKCδ-dependent (5 μM bisindolylimidamide) signals (Figure 2A), suggesting that enhanced HNRPK gene expression may depend on BCR/ABL-induced activation of MAPK (ie, ERK1/2). Indeed, Hnrpk mRNA levels were inhibited by PD098059 treatment and expression of ERK1K71R and ERK2K52R dominant-negative mutants (Figure 2B). Conversely, Hnrpk mRNA and protein levels remained unchanged upon expression of wild-type ERK1 and ERK2 (Figure 2B).

To determine whether increased HNRPK expression requires high levels of BCR/ABL activity, 32Dcl3 cells were infected with MigR1-GFP-BCR/ABL11 and sorted for low, medium, and high GFP expression. As expected, GFP levels correlated with BCR/ABL expression in CML-BCCD34 and CML-CPCD34 patient samples. (E) Levels of pERK1/2, ERK1/2, and GRB2 in 32Dcl3 myeloid precursors transduced with a MigR1-HNRPK-C299-HA retrovirus (Figure 4A, lane 1) and CML-APCD34 cells with the MigR1-GFP-BCR/ABL bicistronic retrovirus and sorted for low, medium, and high GFP levels. (D) Levels of HNRPK, phospho-ERK1/2, total ERK1/2, BCR/ABL, and GRB2 in mononuclear marrow cells from paired chronic phase (CP) and blast crisis (BC) CML samples. (B) Effect of the MEK1/MAPK inhibitor PD098059 (lane 2) on HNRPK protein levels in 32D-BCR/ABL and K562 cells. Similarly, MAPK ERK1/2 activity was higher in CML-BCCD34 (n = 2) and CML-APCD34 (n = 1) than in CML-CPCD34 (n = 2) and NBMCD34 (n = 2) cells (Figure 2E). Conversely, ERK1/2 expression slightly increased in CML-CPCD34 and CML-BCCD34 compared with NBMCD34, but no significant differences were observed between CML-CPCD34 and CML-BCCD34 patient samples (Figure 2E).

In vitro and in vivo HNRPK requirement for BCR/ABL oncogenic activity

To investigate the requirement of HNRPK for BCR/ABL oncogenic potential, we knocked-down HNRPK in BCR/ABL-expressing cells and assessed their growth factor–independent proliferation, clonogenic potential, and ability to undergo granulocytic differentiation and induce tumors in SCID mice. Reduced HNRPK expression was achieved by infecting 32D-BCR/ABL cells with the MigR1-HNRPK-C299-HA retrovirus (Figure 4A, lane 1) and CML-APCD34 cells with the MigR1-GFP-BCR/ABL bicistronic retrovirus and sorted for low, medium, and high GFP levels. (D) Levels of HNRPK, phospho-ERK1/2, total ERK1/2, BCR/ABL, and GRB2 in mononuclear marrow cells from paired chronic phase (CP) and blast crisis (BC) CML patient samples. (E) Levels of pERK1/2, ERK1/2, and GRB2 in NBMCD34, CML-CPCD34, CML-APCD34, and CML-BCCD34 samples.

Figure 2. Role of MAPK in the regulation of HNRPK expression in BCR/ABL-expressing cells. (A) Effect of different chemical inhibitors of known BCR/ABL-activated pathways on HNRPK protein levels in 32D-BCR/ABL and K562 cells. (B) Effect of the MEK1/MAPK inhibitor PD098059 (lane 2) on Hnrpk mRNA (first row) and protein (third row) levels in parental 32Dcl3 cells (lane 3), vector-transduced 32D-BCR/ABL (lane 4), and 32D-BCR/ABL cells ectopically expressing wild-type MAPK ERK1 (lane 5), the K71R dominant-negative MAPK ERK1 mutant (lane 6), wild-type ERK2 (lane 7), and dominant-negative K52R ERK2 mutant (lane 8). mRNA and GRB2 are shown as controls for equal loading. (C) Effect of BCR/ABL tyrosine kinase on MAPK/ERK activity. pERK1/2 indicates phosphorylated (active) ERK1 and ERK2 mitogen-activated protein kinases. Western blots show levels of HNRPK, phospho-ERK1/2, total ERK1/2, BCR/ABL, and GRB2 in 32Dcl3 myeloid precursors transduced with a GFP/BCR/ABL bicistronic retrovirus and sorted for low, medium, and high GFP levels. (D) Levels of HNRPK, phospho-ERK1/2, BCR/ABL, and GRB2 in mononuclear marrow cells from paired chronic phase (CP) and blast crisis (BC) CML patient samples. (E) Levels of pERK1/2, ERK1/2, and GRB2 in NBMCD34, CML-CPCD34, CML-APCD34, and CML-BCCD34 samples.

Down-regulation of HNRPK significantly inhibited growth factor–dependent proliferation (Figure 3B) and colony formation (50%–65% inhibition) (Figure 3C). Seemingly, HNRPK knock-down resulted in decreased tumorigenic potential, as 32D-B/A-KAS cells formed tumors with an increased latency time and a 50% decreased weight (not shown). By contrast, HNRPK down-regulation only partially restored G-CSF–driven maturation of 32D-BCR/ABL cells, as cells at the intermediate stages of maturation but not neutrophil cells were detectable in G-CSF–cultured (9 days) 32D-B/A-K-shRNA cells (Figure 3D).

HNRPK transcription-regulatory activity is dispensable for BCR/ABL leukemogenesis

To investigate the importance of HNRPK transcriptional activity for BCR/ABL leukemogenesis, we transduced 32D-BCR/ABL cells with the MigR1-HNRPK-C299-HA retrovirus (Figure 4A, lane 3) containing a C-terminal truncated HNRPK that is retained in the nucleus (Figure 4B) where it binds DNA but is deficient in promoter transactivation.15 Cytokine-independent or -dependent proliferation in liquid culture (not shown) and colony formation (Figure 4C) was similar in vector-transduced and C299-HNRPK–transduced 32D-BCR/ABL cells. As expected, ectopic expression of HA-tagged wild-type HNRPK only slightly increased 32D-BCR/ABL clonogenic potential (Figure 4C). Similarly, C299-HNRPK expression did not impair the tumorigenic potential of 32D-BCR/ABL cells (not shown).

Because the C299-HNRPK mutant is still competent for DNA binding,15 we investigated whether its expression alters Myc mRNA expression, which is transcriptionally induced by the HNRPK binding to a CT-element within MYC promoter.16 Northern and Western blot analyses revealed that regulation of Myc expression in BCR/ABL-transformed cells does not involve HNRPK transcriptional activity, as C299-HNRPK expression did not
affect Myc mRNA and protein levels (Figure 4A, second and fourth panels).

**Requirement of HNRPK translation-regulatory activity for BCR/ABL leukemogenesis**

MAPK\textsuperscript{ERK1/2} are activated by BCR/ABL in a tyrosine kinase–dependent manner\textsuperscript{28,29} (Figure 5Aii), and MAPK\textsuperscript{ERK1/2}-dependent HNRPK phosphorylation drives HNRPK cytoplasmic accumulation in HeLa cells.\textsuperscript{21} Thus, inhibition of MAPK signaling might influence HNRPK subcellular localization also in BCR/ABL-transformed cells. Indeed, confocal microscopy on anti-HNRPK–stained cells showed a clear nuclear accumulation of HNRPK in PD098059- or imatinib-treated 32D-BCR/ABL cells (Figure 5A), suggesting that BCR/ABL kinase activity not only enhances HNRPK expression but also induces HNRPK cytoplasmic accumulation in a MAPK-dependent manner.

Reportedly, the HNRPK phosphomimetic S284/353D mutant, which carries the MAPK\textsuperscript{ERK1/2} phosphoacceptor sites (serines 284 and 353) mutated to aspartic acid, has cytoplasmic localization in HeLa cells and a constitutively enhanced translational-regulatory activity.\textsuperscript{21} By contrast, the nonphosphatable serine to alanine (S284/353A) mutant is nucleus localized and competes with the endogenous HNRPK for mRNA binding and nuclear export.\textsuperscript{21} Thus, to determine whether HNRPK-dependent regulation of mRNA translation is important for the phenotype of BCR/ABL-transformed myeloid progenitors, 32D-BCR/ABL cells were transduced with HA-tagged wild-type, S284/353A-HNRPK, or S284/353D-HNRPK cDNAs. 32D-BCR/ABL–derivative cell lines expressed the ectopic HNRPK proteins at similar levels (Figure 5Bii). Moreover, confocal microscopy on anti-HA–stained HA-HNRPK–expressing 32D-BCR/ABL cells revealed that the S284/353A-HNRPK and S284/353D-HNRPK mutants were exclusively localized in the nucleus and in the cytoplasm, respectively, while the wild-type HNRPK was prevalently cytoplasmic (Figure 5Bi).

In 32D-BCR/ABL cells, cytokine-independent growth (Figure 5Ci) and colony formation (Figure 5Cii) were impaired by S284/353A-HNRPK but not by wild-type or S284/353D-HNRPK expression. Note that the clonogenic activity of S284/353A-HNRPK–expressing 32D-BCR/ABL cells was only partially restored by IL-3 and G-CSF (Figure 5Cii). Consistent with their effects in vitro, wild-type (wt) and S284/353D-HNRPK-HA–expressing 32D-BCR/ABL cells revealed that the S284/353A-HNRPK and S284/353D-HNRPK mutants were exclusively localized in the nucleus and in the cytoplasm, respectively, while the wild-type HNRPK was prevalently cytoplasmic (Figure 5Bi).
received 5 or more weeks after cell injection (not shown). Infiltration of hematopoietic organs was observed in all mice that received 5Dii). However, modest to severe splenomegaly and leukemic transformation of wild-type K-HA–expressing 32D-BCR/ABL cells (Figure 5Bii). The survival of mice injected with S284/353A-K-HA–expressing 32D-BCR/ABL cells was significantly longer than that of mice injected with wild-type K-HA–expressing 32D-BCR/ABL cells (Figure 5Di). The morphology of spleens from mice injected with S284/353A-expressing 32D-BCR/ABL cells appeared normal (Figure 5D) and resembled that of control age-matched mice (not shown). Consistent with these findings, the survival of mice injected with S284/353A-K-HA–expressing 32D-BCR/ABL cells was significantly longer than that of mice injected with wild-type K-HA–expressing 32D-BCR/ABL cells (Figure 5Di). However, modest to severe splenomegaly and leukemic infiltration of hematopoietic organs was observed in all mice that died 5 or more weeks after cell injection (not shown).

HNRPK-dependent regulation of Myc expression is important for BCR/ABL oncogenic potential

By RT-PCR, we detected Myc mRNA in anti-HNRPK– and anti-HA immunoprecipitates from lysates of 32D-BCR/ABL cells expressing either the wild-type HNRPK or the HA-tagged serine 284 and 353 HNRPK mutants (Figure 6Ai, lanes 2-4), but not in anti-Flag immunoprecipitates used as a negative control (Figure 6Ai, lane 5). Accordingly, REMSA with extracts of parental 32Dc13, 32D-BCR/ABL, and K562 cells and 3P-labeled RNA oligonucleotide corresponding to nucleotides 406 to 431 of the IRES element present in the human MYC mRNA (HSMYC1; V00568), and containing the UCCCGA HNRPK binding site,21 showed that an RNA/protein complex is formed with lysates of BCR/ABL–expressing (Figure 6Aii, top panel, lanes 1-3) but not 32Dc13 (Figure 6Aii, top panel, lane 1) cells. Moreover, UV cross-linking showed that the protein present in 32D-BCR/ABL and K562 lysates and binding to the MYC IRES RNA-oligo has an apparent molecular weight identical to that of HNRPK (Figure 6Aii, bottom panel, lanes 1-3), suggesting that HNRPK up-regulates MYC expression upon binding to MYC IRES. Indeed, the presence of the MYC IRES element strongly enhanced expression of firefly luciferase in 32D-BCR/ABL transiently transduced with the pRMF (IRES-MYC)23 construct containing the renilla luciferase cDNA and the MYC IRES element fused in frame to the firefly luciferase reporter gene (wt-IRES) (Figure 6B).

Because decreased proliferation and leukemogenic potential of S284/353A-HNRPK–expressing 32D-BCR/ABL cells (Figure 5) may, in part, depend on inhibition of HNRPK-dependent MYC mRNA translation,23 S284/353A-expressing 32D-BCR/ABL cells were transduced with MSCV-puro-MYC and tested for cytokine-independent colony formation and tumorigenesis in SCID mice. Ectopic MYC expression (Figure 6C, inset) completely counteracted the effects of S284/353A-HNRPK (lane 2 in Figure 6Ci and 6Cii), as clonogenic potential and weight of tumors from S284/353A–HNRPK-HA-expressing 32D-BCR/ABL cells were transduced with the pRMF (IRES-MYC)23 construct containing the renilla luciferase cDNA and the MYC IRES element fused in frame to the firefly luciferase reporter gene (wt-IRES) (Figure 6B).
MYC expression countered the effect of S284/353A-HNRPK expression and restored CML-BC<sup>CD34</sup><sup>+</sup> clonogenicity (Figure 6D).

Thus, HNRPK-dependent regulation of MYC expression is important in vitro and in vivo for BCR/ABL oncogenic potential.

**BCR/ABL-induced MYC expression is translationally regulated by HNRPK in a MAPK-dependent manner**

In IL-3–cultured 32Dcl3 myeloid progenitors, p210<sup>BCR/ABL</sup> augmented MYC protein levels (Figure 7A, lanes 1-2) without significantly affecting MYC mRNA expression (Figure 7B, lanes 4-5). Accordingly, treatment of IL-3–cultured 32D-BCR/ABL cells with imatinib (1 μM, 24 hours) inhibited MYC protein expression without affecting MYC mRNA levels (Figure 7A and B, lanes 7 and 6, respectively), suggesting that BCR/ABL posttranscriptionally up-regulates MYC expression. Of note, MYC mRNA down-regulation was evident only upon IL-3 deprivation (12 hours) in 32Dcl3 and imatinib-treated 32D-BCR/ABL cells (Figure 7B, lanes 1-3) and in K562 cells growth arrested by imatinib (not shown).

Because MYC expression is required for proliferation and colony formation of CML-BC cells, and HNRPK knock-down induces MYC down-regulation (Figure 3), we assessed the effects of ectopic expression of wild-type, S284/353D–, and S284/353A-HNRPK on MYC protein and mRNA levels. MYC levels were markedly reduced in S284/353A-HNRPK–expressing 32D-BCR/ABL (Figure 7A, lane 5) and K562 (not shown) cells compared with vector-transduced cells (Figure 7A, lane 2). By contrast, MYC levels were increased in wild-type and S284/353D-HNRPK–expressing 32D-BCR/ABL (Figure 7A, lanes 3-4) and K562 (not shown) cells. Accordingly, inhibition of MAPK activity by PD098059 and ectopic expression of ERK1K2<sup>T202</sup> and ERK2K552R dominant-negative, but not wild-type, MAPKs also down-regulated MYC protein levels (Figure 7A, lanes 8-12). As expected, MYC mRNA levels remained unchanged in IL-3–cultured (Figure 7B, lanes 7-10) and IL-3–deprived (not shown) 32D-BCR/ABL cells expressing wild-type or mutant HNRPK proteins.

To assess whether the BCR/ABL-induced up-regulation of MYC expression reflects an HNRPK-dependent increase in MYC synthesis, we pulse labeled IL-3–cultured 32Dcl3 cells, and untreated and imatinib-treated 32D-BCR/ABL and S284/353A-HNRPK–expressing 32D-BCR/ABL cells with [35]<sup>S</sup>-methionine/cysteine and determined the levels of newly translated MYC after immunoprecipitation. Newly synthesized MYC is barely detected in 32Dcl3 cells, readily detected in BCR/ABL-expressing cells, and substantially inhibited in imatinib-treated or S284/353A-HNRPK–expressing 32D-BCR/ABL cells (Figure 7C). As expected, total MYC levels reflected its synthesis rate (Figure 7C, lower panel). In addition, in CHX-treated (20 μg/mL; 0-180 minutes) IL-3–cultured 32Dcl3, 32D-BCR/ABL, and derivative wild-type–expressing, S284/353D-expressing, and S284/353A-HNRPK–expressing cell lines, MYC protein stability is only slightly affected by expression of BCR/ABL or wild-type and mutant HNRPK proteins (Figure 7D).

Furthermore, MYC protein but not mRNA levels were higher in CD34<sup>+</sup> CML-BC and CML-AP than CD34<sup>+</sup> normal marrow cells and CD34<sup>+</sup> normal marrow cells (NBM) (Figure 7E). As expected, MYC levels correlated with those of HNRPK and with BCR/ABL kinase activity in CD34<sup>+</sup> CML patient-derived marrow cells and CD34<sup>+</sup> normal marrow cells (NBM) (Figure 7E). As expected, MYC levels correlated with those of HNRPK and with BCR/ABL kinase activity in CD34<sup>+</sup> CML patient-derived marrow cells and CD34<sup>+</sup> normal marrow cells (NBM) (Figure 7E). As expected, MYC levels correlated with those of HNRPK and with BCR/ABL kinase activity in CD34<sup>+</sup> CML patient-derived marrow cells and CD34<sup>+</sup> normal marrow cells (NBM) (Figure 7E).

**Discussion**

Cytogenetic and molecular changes occur in the vast majority of CML patients during transition to blast crisis; however, the mechanism(s) whereby each specific secondary genetic alteration contributes to disease progression is still largely unclear. Conversely, there is growing evidence attributing an important role to the BCR/ABL oncoprotein in determining the phenotype of
CML-BC cells.² In fact, increased BCR/ABL expression is a feature of CML-BC progenitors,³⁴⁻⁵⁰ and unrestrained BCR/ABL activity in CML-BC alters the expression of factors important for proliferation, survival, and maturation of myeloid progenitors.⁷ For example, in CML-BC, high BCR/ABL levels suppress C/EBPα-dependent differentiation, down-regulate G-CSFR, and enhance overexpression has also been associated with tumor progression and resistance to therapeutic drug-induced apoptosis in different types of cancer.³⁴⁻³⁷

Here we reported that in BCR/ABL-expressing myeloid and lymphoid progenitor cells and in CML-BC⁵¹²³⁴⁻⁵⁰ patient cells, BCR/ABL kinase activity induces HNRPK expression by enhancing HnRNP gene transcription and mRNA stability. Enhanced expression of this HnRNP is not restricted to CML-BC, as HNRPK overexpression has also been associated with tumor progression and resistance to therapeutic drug-induced apoptosis in different types of cancer.³⁴⁻³⁷

By assessing the mechanism whereby BCR/ABL augments HNRPK levels, we found that HnRpk mRNA expression depends on the BCR/ABL-regulated activity of MAPK⁴⁴⁻⁴⁵². In fact, BCR/ABL-graded expression activates MAPK⁴⁴⁻⁴⁵² and increases HNRPK levels in a dose-dependent manner. Consistent with the role of HNRPK as a target of MAPK activity, we showed that activation of MAPK⁴⁴⁻⁴⁵² is readily detectable in CML-BC⁵¹⁻⁵² and not in CML-BC-CD⁵¹⁻⁵² or NBM-CD⁵¹⁻⁵² primary cells, and that HNRPK knock-down inhibits growth factor–independent proliferation, colony formation, and tumorigenesis of BCR/ABL-expressing myeloid progenitor 32Dcl3 cells. The link between BCR/ABL, MAPK⁴⁴⁻⁴⁵² activation, and increased HNRPK expression is also supported by the evidence showing that BCR/ABL constitutively enhances proliferation of hematopoietic cells by circumventing cytokine-generated signals leading to MAPK activation, and that MAPK inhibition impairs CML progenitor growth.²⁸⁻³⁰,³⁸,³⁹ Moreover, HNRPK down-regulation resulted in reduced levels of Myc, which is not only essential for BCR/ABL transformation and proliferation of CML progenitors²⁷⁻³⁰ but is also transcriptionally and translationally induced by HNRPK.⁶⁻³⁹ Notably, growth factors (eg, EGF) that elicit mitogenic signals by potentiating HNRPK, which, in turn, increases MYC expression.⁴⁰

In BCR/ABL-transformed cells, HNRPK transcriptional activation function seems to be dispensable for BCR/ABL oncogenic potential, as the nuclear localized C299-HNRPK mutant, which binds DNA but lacks transactivation activity,⁵¹ affected neither Myc gene transcription nor the in vitro and in vivo growth of BCR/ABL-expressing myeloid cells. Accordingly, we found HNRPK prevalently localized in the cytoplasm of 32D-BCR/ABL cells. Although in other cell types, HNRPK, though capable of nucleocytoplasmic shuttling, is localized primarily within the nucleus,⁴¹ it also has been reported that sustained MAPK⁴⁴⁻⁴⁵² activity drives cytoplasmic accumulation of HNRPK in HeLa cells.²¹ Indeed, inhibition of MEK1 and short-term treatment of...
BCR/ABL cells with imatinib turned off BCR/ABL-dependent MAPKERK1/2 activity and induced HNRPK nuclear accumulation. Thus, HNRPK is not only transcriptionally but also posttranslationally regulated by BCR/ABL through the activity of MAPKERK1/2. Since MAPK activation is important for BCR/ABL leukemogenic potential,8,28,42,43 it was reasonable to speculate that in BCR/ABL-expressing cells, like in HeLa cells,21 HNRPK translation-regulatory activity depends on phosphorylation of HNRPK on serines 284 and 353 by the BCR/ABL-activated MAPKERK1/2, and that MAPKERK1/2-dependent HNRPK translation regulation may be important for BCR/ABL leukemogenesis. Indeed, in BCR/ABL-transformed myeloid progenitor 32Dc13 cells, expression of the nuclear-localized nonphosphatable S284/353A-HNRPK mutant recapitulated the effects of HNRPK down-regulation. In fact, marked inhibition of cytokine-independent proliferation and colony formation (~60% decrease) and suppression of in vivo BCR/ABL leukemogenic potential were the characteristics of a 32D-BCR/ABL cell line and primary CD34+ CML–BC cells transduced with the S284/353A-HNRPK mutant. Since this mutant still binds mRNAs containing a DICE element in their 3′UTR21 or an IRES in the 5′UTR (ie, MYC; Figure 6), it is plausible that its dominant-negative activity may derive from its ability to sequester mRNAs in the nucleus and, therefore, decrease the HNRPK-dependent nuclear export of the mRNA cargo available for translation. Indeed, we provided evidence showing that either wild-type or the nucleus-localized S284/353A mutant HNRPK binds Myc mRNA and that levels of an IRES-containing reporter gene were enhanced in myeloid progenitor cells by BCR/ABL expression. Note that, in reporter assays, the presence of 2 repeats of the 15-LOX-DICE element21 at the 3′-end of dsRED also altered the reporter gene expression in 32D-BCR/ABL cells (not shown). Consistent with our results, it has been shown that S284/353A-HNRPK expression in HeLa cells overrides HNRPK translation silencing of a DICE-bearing luciferase reporter construct.21 Although we cannot exclude the possibility that the detrimental effects of S284/353A-HNRPK expression on the in vitro and in vivo leukemogenic potential of BCR/ABL cells might, in part, depend on interference with the DICE-dependent HNRPK translation regulatory activity, our data suggest that inhibition of ERK-dependent phosphorylation of HNRPK on serines 284/353 impairs HNRPK-dependent translation regulation of IRES-bearing mRNAs (ie, MYC) in BCR/ABL-transformed cells. In fact, we showed that restoration of MYC expression is sufficient to rescue factor-independent colony formation and tumorigenic potential of 32D-BCR/ABL and primary CD34+ CML–BC cells from the inhibitory effects of S284/353A-HNRPK expression.

Steady-state and newly synthesized Myc protein levels were suppressed by S284/353A-HNRPK expression and inhibition of BCR/ABL or MAPKERK1/2 kinase activity. Mechanistically, it appears that BCR/ABL positively regulates MYC translation but not transcription by inducing ERK1/2-dependent phosphorylation of HNRPK. Further, expression of S284/353A-HNRPK mutant did not affect Myc protein stability. Consistent with the existence of a BCR/ABL-MAPK-HNRPK network positively regulating MYC mRNA translation in the advanced phase of CML, MYC protein but not mRNA expression was higher in the CD34+ fraction of CML–BC and CML–AP marrow cells than in the CD34+ fraction of normal and CML–CP patient marrow cells. These findings, which are consistent with the reported MYC overexpression found in CML–BC and with the requirement of MYC for BCR/ABL leukemogenesis,27,30,44-47 are only apparently in contrast with the reported ability of BCR/ABL to enhance MYC expression at transcriptional level,8,48 as most of the reported experiments showing BCR/ABL-dependent transcriptional induction of MYC mRNA have been performed either in growth factor–independent cell lines maintained in the absence of cytokines or by comparing total marrow cells from CML–CP and CML–BC patients.30,45 In these experiments, treatment of growth factor–independent cell lines (eg, K562) with imatinib results in proliferation arrest and apoptosis, 2 conditions in which MYC levels rapidly decline. Similarly, the differences in MYC mRNA levels detected in total CML–CP and CMP–BC marrow cells may reflect differences in the percentage of Ph1 and postmitotic cells within the CML samples. Indeed, increased MYC mRNA was not found in microarrays that used CD34+ or AC133a fraction of CML–CP and CML–BC marrow cells.52 Thus, one of the molecular mechanisms whereby BCR/ABL enhances MYC expression involves the MAPK-dependent regulation of HNRPK translation regulatory activity. However, increased MYC transcription can still be found in those CML–BC patients with amplification of the MYC gene,53-55 and we also cannot exclude the possibility that transcriptional, translational, and posttranslational mechanisms such as those involving the activity of Jak2 kinase46 may all participate in the regulation of MYC expression in primary CML–BC cells.

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