BCR/ABL activates mdm2 mRNA translation via the La antigen

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Summary

In a BCR/ABL-expressing myeloid precursor cell line, p53 levels were markedly downmodulated. Expression of MDM2, the negative regulator of p53, was upregulated in a tyrosine kinase-dependent manner in growth factor-independent BCR/ABL-expressing cells, and in accelerated phase and blast crisis CML samples. Increased MDM2 expression was associated with enhanced mdm2 mRNA translation, which required the interaction of the La antigen with mdm2 5′ UTR. Expression of MDM2 correlated with that of La and was suppressed by La siRNAs and by a dominant negative La mutant, which also enhanced the susceptibility to drug-induced apoptosis of BCR/ABL-transformed cells. By contrast, La overexpression led to increased MDM2 levels and enhanced resistance to apoptosis. Thus, La-dependent activation of mdm2 translation might represent an important molecular mechanism involved in BCR/ABL leukemogenesis.

Introduction

Chronic myelogenous leukemia (CML) is a clonal disorder arising from neoplastic transformation of the hematopoietic stem cells (Kantarjian et al., 1993). The typical course of CML involves progression from a protracted chronic phase, marked by accumulation of apparently normal neutrophils, to a rapidly fatal blast crisis characterized by clonal expansion of differentiation-arrested myeloid precursor cells. The BCR/ABL oncoproteins, the leukemia-specific gene products of the Philadelphia chromosome (Ph^1) translocation, are responsible for transformation of the hematopoietic stem cell through their deregulated tyrosine kinase activity; such activity is essential for recruitment and activation of multiple pathways that transduce signals leading to enhanced proliferative potential and survival of BCR/ABL-expressing hematopoietic precursor cells (Gordon, 1999; Osarogiagbon and McGlave, 1999; Sawyers, 1999). There is also evidence that BCR/ABL expression is required for maintenance of the leukemic phenotype. In mice, the disease process induced by BCR/ABL-expressing hematopoietic cells is suppressed upon downregulation of BCR/ABL levels or inhibition of protein function (Skorski et al., 1996a; Huettner et al., 2000; Druker et al., 1996); recent data in CML-blast crisis (BC) patients indicate that temporary clinical remission is achieved by treatment with the ABL kinase inhibitor STI571 (Druker et al., 2001). The reliance of CML-BC cells on BCR/ABL activity for their proliferation and survival is consistent with the reported enhanced expression of BCR/ABL in these cells (Gaiger et al., 1995; Elmaagacli et al., 2000; Wetzler et al., 1993). In some cases, such an increase in BCR/ABL levels might be caused by a double $Philadelphia^1$ chromosome, which is a relatively common secondary genetic change during disease progression (Kantarjian et al., 1987).

Inactivating mutations of the p53 gene are rarely found in chronic phase, but are relatively common in the blast crisis disease stage of CML (Feinstein et al., 1991), suggesting that loss of function of p53 plays an important role in disease progression. Indeed, loss of wild-type p53 potentiates the leuke-

SIGNIFICANCE

BCR/ABL oncoproteins enhance survival of hematopoietic cells by regulating the expression and/or the activity of anti- or proapoptotic proteins. In ~30% of CML-blast crisis, p53 is genetically inactivated, and $p53^{-/-}$/BCR/ABL^+ marrow cells induce aggressive leukemia in mice, suggesting that genetic or functional p53 inactivation is important in CML disease progression. Here we show that BCR/ABL enhances expression of MDM2, the negative regulator of p53, by inducing the expression of the La antigen that, in turn, activates mdm2 translation upon binding to its mRNA. In BCR/ABL cells, interference with La activity resulted in decreased MDM2 and increased adriamycin sensitivity. The importance of La in regulation of MDM2 emphasizes the relevance of translation regulatory pathways in the activity of BCR/ABL and, possibly, of other oncogenic tyrosine kinases.

mia-inducing effects of BCR/ABL, as indicated by the enhanced leukemogenic potential of BCR/ABL-expressing p53-deficient marrow cells (Skorski et al., 1996b; Honda et al., 2000). Since genetic inactivation of p53 is detected in approximately 25% of CML-BC patients (Feinstein et al., 1991), p53 might be functionally inactivated in some of the patients with a wild-type gene. Studies of p53 expression in CML have been complicated (Lubbert et al., 1988; Kastan et al., 1991; Bi et al., 1994), in part by the fact that the nonfunctional mutant p53 is more stable than wild-type p53, and in part because marrow samples of CML patients often include normal cells with wild-type p53. Nonetheless, some evidence suggests that BCR/ABL may negatively regulate p53 in CML, since expression of wild-type p53 is undetectable in certain Philadelphia[1] cell lines (Lubbert et al., 1988), and p53 levels become undetectable upon expression of BCR/ABL in a mouse hematopoietic progenitor cell line (Pierce et al., 2000). It has been well established that MDM2 negatively regulates p53 (Haupt et al., 1997; Kubbutat et al., 1997), in part by interacting with p53 in the nucleus (Momand et al., 1992) to inhibit its transcription activation function, and also by promoting p53 export and proteasome-dependent degradation in the cytoplasm (Tao and Levine, 1999; Fang et al., 2000b; Geyer et al., 2000; Boyd et al., 2000).

Since mdm2 is overexpressed in approximately 50% of patients with hematological malignancies and is associated with poor prognosis and chemotherapeutic drug resistance (Seliger et al., 1996; Faderl et al., 2000; Zhou et al., 1995, 2000), we asked whether BCR/ABL might induce an increase in MDM2 expression that would lead to functional inactivation of p53. While this work was in progress, a study reported that: (1) MDM2 levels were increased in a BCR/ABL-expressing cell line, and (2) MDM2 downmodulation by antisense oligonucleotides promoted growth factor deprivation-induced apoptosis (Goetz et al., 2001). However, the mechanisms underlying the enhanced MDM2 expression were not analyzed in detail (Goetz et al., 2001). We report here that BCR/ABL induces an increase of MDM2 expression by enhancing mdm2 mRNA translation via an RNA binding protein, identified by mass spectrometry and microsequencing as the La antigen, which recognizes a 27 nucleotide segment in the 5′ untranslated region of mdm2 mRNA. In BCR/ABL-expressing 32Dcl3 cells as well as in primary mononuclear marrow cells of CML-BC patients, La expression correlated with that of MDM2. A dominant negative La antigen mutant inhibited mdm2 mRNA translation in vitro and suppressed MDM2 expression in BCR/ABL-expressing cells. Likewise, La downmodulation by RNA interference led to markedly decreased MDM2 levels. By contrast, overexpression of wild-type La increased MDM2 levels in 32D-BCR/ABL cells. Together, these data suggest that the BCR/ABL oncoproteins can functionally inactivate p53 by enhancing MDM2 levels via La-dependent upregulation of mdm2 mRNA translation.

Results

p53 expression in BCR/ABL-expressing 32Dcl3

Myeloid precursor 32Dcl3 cells express wild-type p53 (Blandino et al., 1995). p53 protein levels were detectable in nuclear extracts of parental 32Dcl3 cells, while newly derived BCR/ABL-expressing 32Dcl3 cells showed markedly less expression, despite nearly identical levels of p53 mRNA (Figure 1A). Since the primary mechanism regulating p53 levels is proteasome-

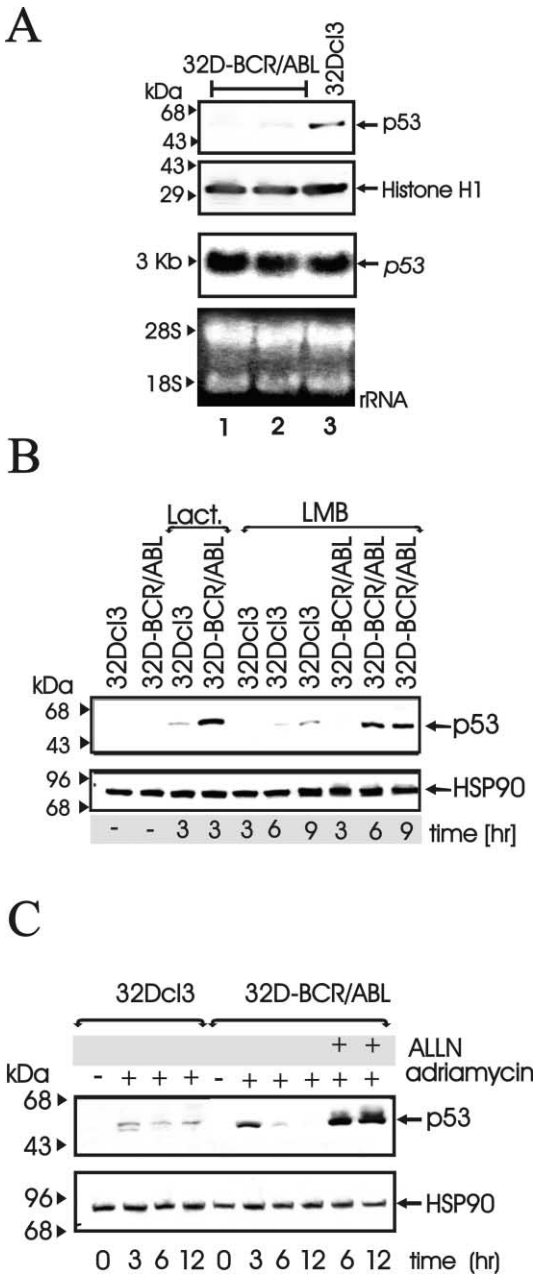

Figure 1. Effects of BCR/ABL on p53 expression

A: p53 nuclear protein (upper panel) and mRNA (3rd panel) levels were detected by Western and Northern blot, respectively, in parental (lane 3) and newly established BCR/ABL clones (lanes 1 and 2). Histone H1 and rRNA levels were monitored as controls for loading.

B: Effect of the proteasome inhibitor lactacystin (Lact.) and nuclear export inhibitor Leptomycin B (LMB) on endogenous p53 expression in parental and BCR/ABL-expressing 32Dcl3 cells.

C: Effect of adriamycin (alone or in combination with the proteasome inhibitor ALLN) on p53 expression in parental and BCR/ABL-expressing 32Dcl3 cells.

In **B** and **C**, HSP90 was monitored as a loading control, and treatment times are indicated below the lanes.

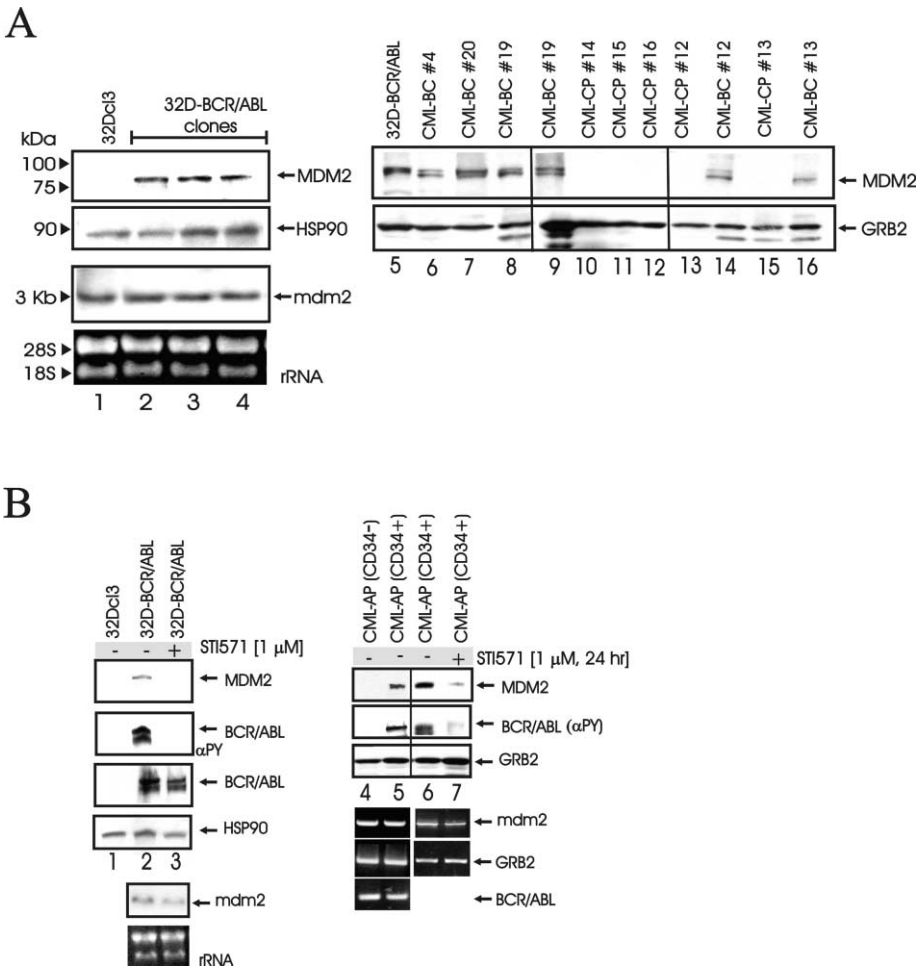

Figure 2. Expression of MDM2 in parental and BCR/ABL-expressing 32Dcl3 cells, and in mononuclear cells from patients with CML in chronic phase (CML-CP), accelerated phase (CML-AP), and blast crisis (CML-BC)

A: left, mdm2 expression (protein and mRNA) in parental and BCR/ABL-expressing 32Dcl3 cells (lanes 1–4); right, MDM2 levels in mononuclear marrow cells from multiple patients with CML-CP (lanes 10–12), CML-BC (lanes 6–9), and during disease progression from CML-CP to CML-BC in two patients (#12 and 13) (lanes 13–16) as indicated; 32D-BCR/ABL cells (lane 5) served as positive control; HSP90, GRB2, and ethidium bromide-stained total RNA served as loading controls.

B: MDM2 (protein and mRNA) and BCR/ABL expression in untreated and STI571-treated (24 hr) 32D-BCR/ABL cells (lanes 2 and 3), and in the $CD34^-$ and $CD34^+$ fraction of mononuclear cells from a CML-AP patient (lanes 4 and 5). Lanes 6 and 7 show MDM2 levels in untreated and STI571-treated (24 hr) CML-AP $CD34^+$ cells. GRB2 and HSP90 levels served as loading control. Levels of mdm2, GRB2, and BCR/ABL transcripts in the $CD34^-$ and $CD34^+$ fractions were revealed by RT-PCR.

dependent degradation in the cytoplasm (Haupt et al., 1997; Kubbutat et al., 1997; Freedman and Levine, 1998), p53 levels were assessed in parental and BCR/ABL-expressing 32Dcl3 cells treated with the proteasome inhibitor lactacystin and with leptomycin B (LMB), a specific inhibitor of nuclear export previously shown to prevent MDM2-dependent p53 degradation (Freedman and Levine, 1998). Western blots of whole cell extracts revealed low levels of p53 in untreated parental and BCR/ABL-expressing cells, but markedly higher levels in cells treated with lactacystin or LMB (Figure 1B). Expression of p53 was also tested upon treatment with adriamycin, a cytotoxic drug known to promote a transient increase in p53 levels by suppressing its degradation (Lakin and Jackson, 1999). As expected, adriamycin induced an increase in p53 levels in both parental and BCR/ABL-expressing cells (Figure 1C). In BCR/ABL-expressing cells, the increased p53 expression was more pronounced and more transient, as p53 levels were markedly downmodulated 6 and 12 hr after adriamycin treatment (Figure 1C); by contrast, in parental 32Dcl3 cells, p53 levels remained constant after adriamycin treatment for 3–12 hr (Figure 1C) and were downmodulated only after 24 hr (not shown). Treatment with the proteasome/calpain inhibitor ALLN prevented the downmodulation of p53 detected in BCR/ABL-expressing cells 6 and 12 hr after adriamycin treatment (Figure 1C). Adriamycin treatment induced the expression of a functional p53, as indicated by the increased levels of p53-regulated p21, BAX, and MDM2 in parental and BCR/ABL-expressing cells (not shown). These results suggest that an enhanced p53 degradation pathway counteracts the potential for high p53 expression in BCR/ABL-expressing cells.

Effects of BCR/ABL on MDM2 expression

p53 levels are primarily regulated via MDM2-dependent degradation (Haupt et al., 1997; Kubbutat et al., 1997). To assess whether MDM2 expression is regulated by BCR/ABL, Western blot analysis was performed using total extracts from parental and BCR/ABL-expressing 32Dcl3 cells. Expression of MDM2 was barely detectable in parental cells (Figure 2A, lane 1), but markedly increased in 32D-BCR/ABL newly derived cell clones (Figure 2A, lanes 2–4) and in a 32D-BCR/ABL established cell line (Figure 2A, lane 5). By contrast, mdm2 mRNA levels were similar in parental and BCR/ABL-expressing cells (Figure 2A). Thus, overexpression of MDM2 in BCR/ABL-expressing cells is not due to increased transcription or enhanced mdm2 mRNA stability. Two forms of mdm2 mRNA have been described (Brown et al., 1999): a long form (L-mdm2) that contains a 5′ leader with two upstream open reading frames (uORFs) derived from exon 1, and a short form (S-mdm2) that has a 5′ leader sequence derived from exon 2. Thus, we performed RT-PCR on DNase I-treated RNA using the appropriate set of primers to detect both forms. S- and L-mdm2 transcripts were detected

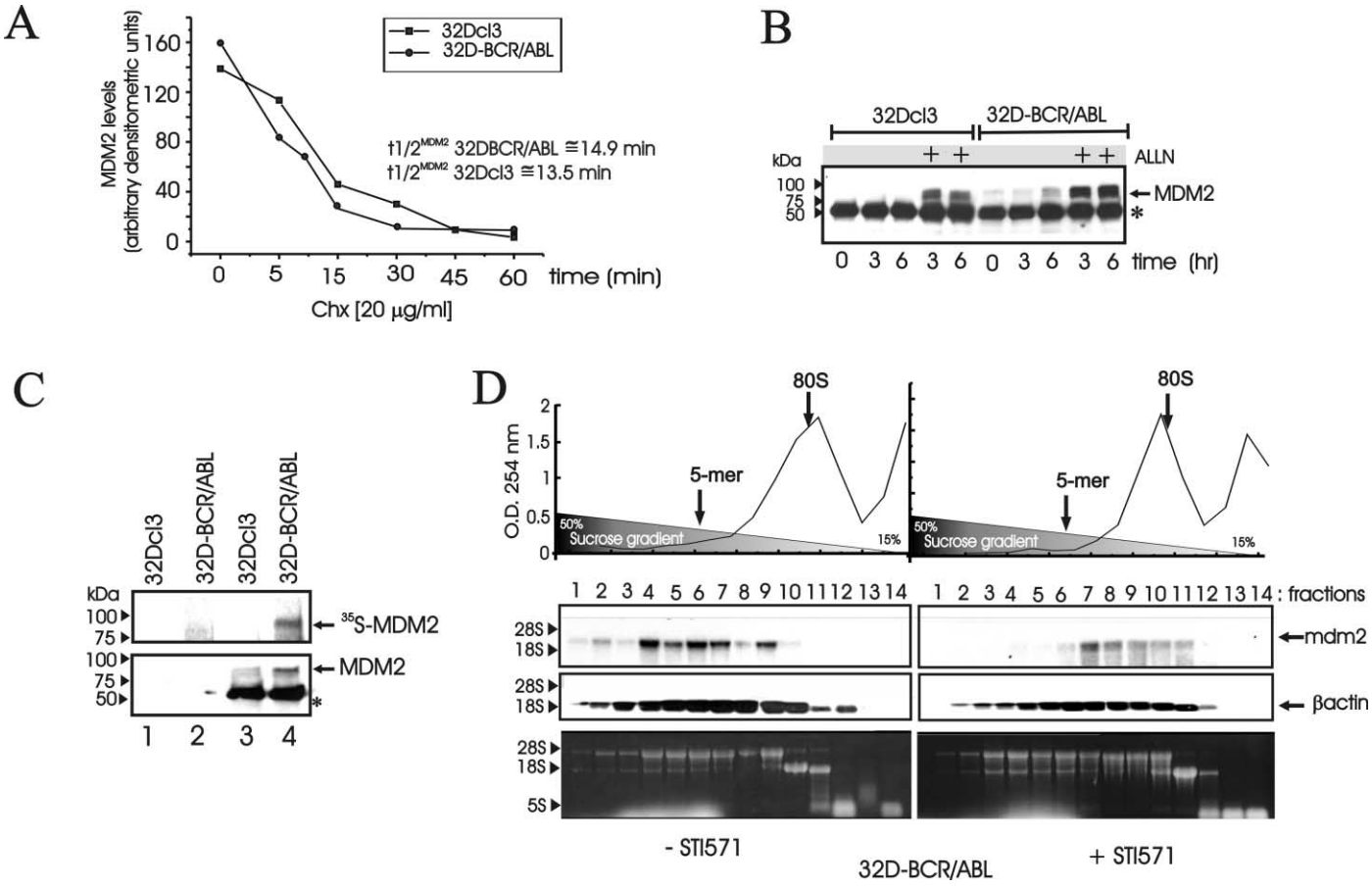

Figure 3. MDM2 half-life, synthesis, and polysome distribution in parental and BCR/ABL-expressing cells

A: MDM2 levels in cycloheximide-treated parental and 32D-BCR/ABL cells. MDM2 half-life was calculated using a described algorithm (Luscher and Eisenman, 1988).

B: MDM2 levels in anti-MDM2 immunoprecipitates of ALLN-treated parental and BCR/ABL-expressing cells. (*) Asterisk indicates Ig-G heavy chains.

C: Upper panel, levels of newly synthesized MDM2 in anti-MDM2 immunoprecipitates of ^{35}S methionine-labeled parental and BCR/ABL-expressing cells (lanes 3 and 4). Lanes 1 and 2 show control preclearings. Lower panel, levels of MDM2 by anti-MDM2 Western blotting of preclearings and anti-MDM2 immunoprecipitates.

D: Polysome/monosome distribution of mdm2 and β-actin mRNA. Extracts were prepared from untreated and STI571-treated 32D-BCR/ABL cells and separated by sucrose gradient centrifugation. The relative amount of mdm2 and β-actin mRNA in each fraction was determined by Northern blot (middle panel). The A_{254} profile and the position of the fractions corresponding to the peak of monosomes and polysomes indicated as 80S and 5-mer, respectively, are shown. Ethidium bromide staining of gel-fractionated RNA fractions (lower panel).

at similar levels in RNA from parental and BCR/ABL-expressing cells (not shown), further indicating that BCR/ABL-dependent MDM2 overexpression is not due to increased mRNA levels.

MDM2 levels were also analyzed in mononuclear CML marrow samples: in CML-BC samples, MDM2 expression was abundant, while in CML-chronic phase (CP) samples, it was virtually undetectable (Figure 2, lanes 6–12). Of note, MDM2 expression increased during CML disease progression (Figure 2, lanes 13–16) and correlated with that of BCR/ABL, which was also more abundant in CML-BC samples (not shown). The increased expression of MDM2 in BCR/ABL-expressing cells was dependent on BCR/ABL kinase activity, as indicated by the rapid downmodulation of MDM2 protein, but not mdm2 mRNA, in cells treated with the ABL kinase inhibitor STI571 (1 μM; 24 hr) (Figure 2B, compare lanes 2 and 3). At this concentration, the inhibitor suppressed the autophosphorylation activity of BCR/ABL without significant effect on its protein levels. STI571 also inhibited MDM2 protein, but not mRNA, expression in $CD34^+$ cells from a patient with CML in accelerated phase (CML-AP) (Figure 2B, lanes 6 and 7). Of note, expression of MDM2 was detected in mononuclear $CD34^+$ but not in mononuclear $CD34^-$ cells and correlated with that of BCR/ABL activity (Figure 2B, lanes 4 and 5). By contrast, mdm2 and BCR/ABL transcripts were detected at similar levels in the $CD34^+$ and $CD34^-$ fraction (Figure 2B, lanes 4 and 5, lower panels).

Translational regulation of mdm2 expression in BCR/ABL-expressing cells

The MDM2 protein exhibits a short half-life of approximately 15–30 min (Olson et al., 1993). To determine whether an extended half-life may account for the BCR/ABL-dependent overexpression of MDM2, we monitored MDM2 levels in parental and BCR/ABL-expressing 32Dcl3 cells treated for different times with the protein synthesis inhibitor cycloheximide (20

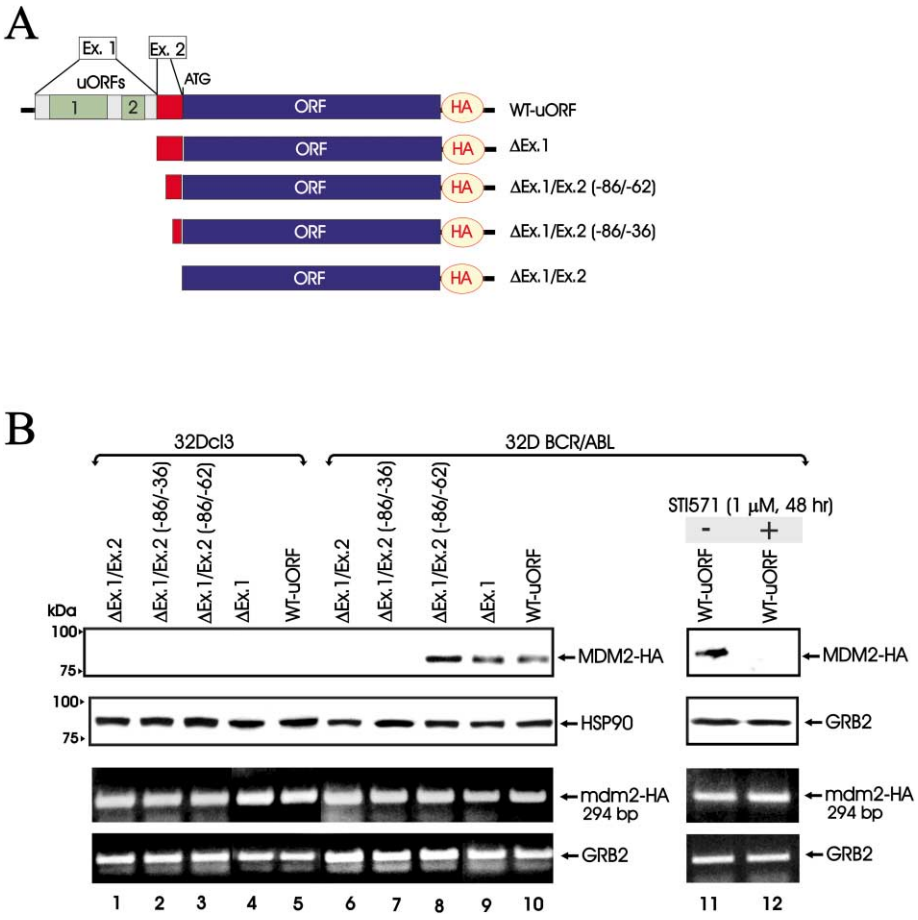

Figure 4. Translational regulation of mdm2 expression in parental and BCR/ABL-expressing cells

A: Schematic diagram of mdm2 cDNA plasmids carrying segments of decreasing length of the 5′ untranslated region and an HA-epitope at the 3′ end of mdm2 cDNA.

B: Anti-HA Western blots of MDM2 levels (upper panel) in GFP-positive parental and BCR/ABL-expressing 32Dcl3 cells retrovirally transduced with the mdm2 cDNA plasmid (lanes 1–10) shown in **A**. Western blot shows the effect of STI571 on ectopic MDM2 expression (lanes 11 and 12). RNA levels of ectopic mdm2-HA and GRB2 transcripts (lower panels) were measured by RT-PCR.

μg/ml). Analysis of MDM2 expression in untreated and cycloheximide-treated cells indicates that MDM2 has similar stability in both cell lines (Figure 3A). Indeed, the half-life of MDM2 was approximately 13.5 and 15 min in parental and BCR/ABL-expressing cells, respectively. In parental and BCR/ABL-expressing cells, MDM2 levels markedly increased upon treatment with the proteasome inhibitor ALLN, consistent with an active process of degradation in both cell lines (Figure 3B). Thus, BCR/ABL-induced MDM2 overexpression does not seem to depend on increased protein stability. Enhanced translation of mdm2 mRNA has been shown to be a mechanism leading to MDM2 overexpression (Landers et al., 1994; Capoulade et al., 1998). To assess whether the BCR/ABL-induced increase of MDM2 levels reflects an enhanced rate of synthesis, parental and BCR/ABL-expressing cells were pulse-labeled with ^{35}S-methionine, and levels of newly synthesized MDM2 were detected by immunoprecipitation. Newly synthesized MDM2 was readily detectable in BCR/ABL-expressing, but not in parental, 32Dcl3 cells (Figure 3C). To further assess the translation regulation of MDM2 expression, we assessed polysome- (efficiently translated) and monosome- (poorly translated) associated mdm2 mRNA in untreated and STI571-treated (2 μM; 12 h) BCR/ABL-expressing cells. Upon Northern blot hybridization of polysome- and monosome-associated RNA separated by linear sucrose gradient centrifugation, mdm2 mRNA was predominant in the polysome-associated fractions of BCR/ABL-expressing cells, whereas it was clearly shifted toward the monosome fractions after STI571 treatment (Figure 3D). By contrast, the polysome/monosome distribution of β-actin was not affected (Figure 3D). Thus, BCR/ABL-dependent signals appear to induce increased MDM2 expression by enhancing mdm2 mRNA translation. In choriocarcinoma cells overexpressing MDM2, L-mdm2 mRNA, which contain two upstream open reading frames (uORFs), were found in the monosome fractions, while the S-mdm2 transcripts (which lack the uORFs) were preferentially detected on polysomes (Brown et al., 1999). To examine the roles of the two uORFs (exon 1) and the intercistronic region (exon 2) for mdm2 translation, MDM2 levels were assessed in green fluorescent protein (GFP)-sorted parental and BCR/ABL-expressing 32Dcl3 cells after infection with a GFP-expressing retrovirus carrying the HA-tagged full-length mdm2 cDNA (WT-uORF) or the mdm2 cDNA deleted of: the uORFs and intercistronic region (ΔEx.1/ΔEx.2); the exon 1 and the initial 51 (ΔEx.1/ΔEx.2 -86/-36) or 25 (ΔEx.1/ΔEx.2-86/-62) nucleotides of exon 2; or only exon 1 (ΔEx.1) (Figure 4A). In three different experiments, expression of HA-tagged MDM2 was undetectable in parental 32Dcl3 cells, but readily detectable in BCR/ABL-expressing cells (Figure 4B). In BCR/ABL-expressing cells, ectopic expression of MDM2 was similar in cells transduced with WT uORF, ΔEx.1, or ΔEx.1/ ΔEx.2(-86/-62), but undetectable in cells transduced with either ΔEx.1/ΔEx.2 (-86/-36) or ΔEx.1/ΔEx.2 mdm2-HA construct (Figure 4B). In 32D-BCR/ABL cells transduced with the WT uORF mdm2-HA retrovirus, expression of HA-tagged MDM2 was suppressed by STI571 treatment (1 μM;

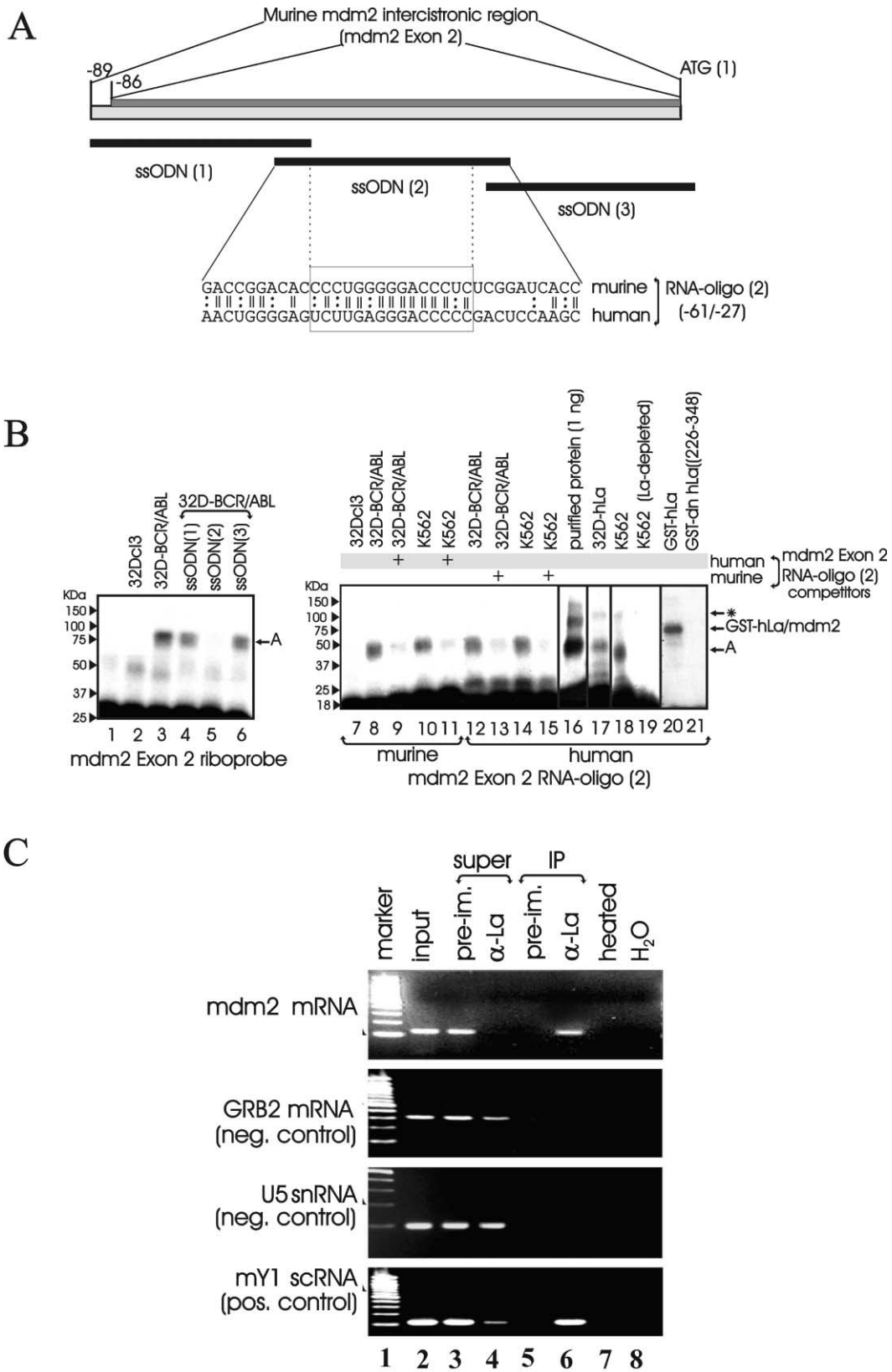

Figure 5. Characterization of the mdm2 mRNA-protein complex in BCR/ABL-expressing cells

A: Schematic diagram of murine mdm2 intercistronic region (exon 2) and of the ssODNs and RNA-oligomers used either as competitors or as probes in UV crosslinking assays. Sequence alignment shows homology between mouse and human mdm2 exon 2 RNA oligoribonucleotides.
B: Left, UV crosslinking with the murine mdm2 intercistronic riboprobe and cytoplasmic extracts from parental or BCR/ABL-expressing cells in the absence

48 hr) (Figure 4B, lanes 11 and 12), further supporting the essential role of BCR/ABL in enhancing MDM2 expression by a translational mechanism.

Transcripts of ectopically expressed HA-tagged mdm2 were detected at similar levels in 32Dcl3 and 32D-BCR/ABL-expressing cells (Figure 4B, lower panels), indicating that the differences in MDM2-HA levels in retrovirus-transduced cells depend on posttranscriptional mechanisms.

To further validate the use of the mdm2-HA constructs, 32D BCR/ABL cells transduced with WT-uORF mdm2-HA were treated with adriamycin (1 μg/ml, 3 hr) and ALLN (25 μM, 3 hr) to induce p53 expression and prevent its degradation, respectively. As expected, whole-cell lysates of these cells showed high levels of HA-tagged MDM2 and p53 expression (not shown), and anti-HA Western blotting of anti-p53 immunoprecipitates revealed that HA-tagged MDM2 is in complex with endogenous p53 (not shown), indicating that ectopic MDM2 contains an intact p53 binding domain.

A ~50 kDa RNA binding protein interacts with the intercistronic region of mdm2 mRNA

The experiments shown above are consistent with the possibility that BCR/ABL regulates an RNA binding protein that interacts with the intercistronic region of mdm2 mRNA to stimulate mdm2 mRNA translation. Thus, a riboprobe containing the intercistronic region (89 bases) of mdm2 mRNA (mdm2 exon 2) (Figure 5A) was tested in an UV-crosslinking assay for the ability to bind protein(s) in cytosolic extracts from parental and BCR/ABL-expressing 32Dcl3 cells, as well as from the Ph[1] K562 erythroleukemia cell line. An RNA-protein complex (mdm2 complex "A") was readily detected in lysates from murine BCR/ABL-expressing cells (Figure 5B, lane 3), but was barely detectable in lysates from parental 32Dcl3 cells (Figure 5B, lane 2). In UV crosslinking assays, a 1000-fold excess of 35-base single-stranded oligodeoxynucleotides ssODN1, ssODN2, and ssODN3 (corresponding to the 5′, middle, and 3′ portion of mdm2 exon 2, respectively) were used to examine the specificity of the binding complex. Formation of mdm2 complex A was specifically competed by ssODN2, but not by ssODN1 or ssODN3 (Figure 5B, lane 5). Thus, UV-crosslinking experiments were carried out using the ^{32}P-labeled mouse oligoribonucleotide (RNA-oligo 2) corresponding to ssODN2; once again, mdm2 complex A was detected in mouse and human BCR/ABL-expressing cells, but not in 32Dcl3 cells (Figure 5B, lanes 8 and 10). Sequence comparison of mouse and human mdm2 intercistronic regions revealed identical size (89 bases), but wide divergence except in their central segment (RNA-oligo 2) (Figure 5A). By using as probe the human RNA-oligo 2, identical complexes were detected in BCR/ABL-expressing mouse and human cells (Figure 5B, lanes 12–14) and, as expected, unlabeled murine or human RNA-oligo 2 inhibited formation of both human and mouse mdm2 complex A, respectively (Figure 5B, lanes 9, 11, 13, and 15). These data indicate that an RNA binding protein, conserved in mouse and human cells, recognizes a conserved central segment of the mdm2 intercistronic region that was shown above to be required for efficient translation of mdm2 mRNA in BCR/ABL-transformed cells.

The La antigen interacts with mdm2 mRNA and mediates MDM2 expression in vitro and in vivo

The mdm2 RNA binding protein responsible for complex A (Figure 5B) was purified from K562 cytoplasmic extracts by RNA affinity chromatography using a 5′ biotinylated mdm2 RNA-oligo-2 coupled to streptavidin-sepharose. The purified protein, examined by Coomassie blue staining, exhibited efficient binding to ^{32}P-RNA-oligo 2; 1 ng of the purified protein was equivalent to ~15 μg of K562 cytoplasmic extracts in its RNA binding activity (Figure 5B, lane 16). Mass spectrometry and microsequencing of the mdm2 mRNA binding protein identified several peptide sequences identical to the human La antigen. The La antigen is an RNA binding protein involved in the biogenesis and processing of RNA polymerase III transcripts and has also been shown to stimulate mRNA translation (Maraia and Intine, 2001; Wolin and Cedervall, 2002).

To determine whether the La antigen is the BCR/ABL-regulated protein interacting with RNA-oligo 2, cytoplasmic extracts from 32Dcl3 cells ectopically expressing the full-length human La cDNA were subjected to RNA UV crosslinking. As expected, complex A was detectable in La-expressing 32Dcl3 cells (Figure 5B, lane 17). Importantly, complex A was no longer detectable using La-immunodepleted K562 cell extracts (compare lanes 18 and 19), confirming that La is required for complex A formation. Bacterially expressed GST-La protein also interacted with the ^{32}P-labeled mdm2 RNA-oligo 2 (Figure 5B, lane 20); by contrast, an N- and C-terminal truncated La protein (aa 226–348) which lacks the RNA binding domain did not interact with the mdm2 oligoribonucleotide (Figure 5B, lane 21), as expected.

A specific physical association between La and mdm-2 mRNA was also demonstrated in 32D-BCR/ABL cell lysate by RT-PCR after immunoprecipitation (IP) with anti-La, but not preimmune, serum (Figure 5C). mdm-2 mRNA was readily detected in the anti-La IP (lane 6) but not in the preimmune IP (lane 5). As expected, mdm2 mRNA was also detected in total RNA prepared from the 32D-BCR/ABL cell extracts used for IPs (input, lane 1). Moreover, preimmune serum did not deplete mdm-2 mRNA from the extract (upper panel, lane 3), while anti-La did (lane 4). It is noteworthy that the pattern observed for mdm-2 mRNA was similar to that for mY1 scRNA (Figure 5C, bottom

(lanes 2 and 3) or presence (lanes 4–6) of the competitor ssODNs shown in **A**; right, UV crosslinking with mouse (lanes 7–11) or human (lanes 12–15) ^{32}P-labeled *RNA-oligo 2* and cytoplasmic extracts of BCR/ABL-expressing cells (32D-BCR/ABL and Ph[1] K562), in the absence or presence of cold competitor (mouse or human, as indicated) *mdm2 exon 2 RNA-oligo 2*. UV crosslinking of human *RNA-oligo 2* with purified mdm2 RNA binding protein (lane 16), cytoplasmic extracts of exogenous La-expressing 32Dcl3 cells (lane 17), La-immunodepleted and nondepleted K562 cytoplasmic extract (lanes 19 and 18), and bacterially produced GST-La (lane 20) or GST-dn-hLa (226-348) (lane 21). *, the high molecular weight protein interacting with RNA-oligo 2 may correspond to La homodimers.

C: Association of La with mdm2 mRNA. Equal aliquots of purified 32D-BCR/ABL total RNA isolated from the input (lane 2), anti-La, or preimmune serum immunoprecipitates (lanes 5 and 6), and from the supernatants (lanes 3 and 4) of the immunoprecipitates, were assayed by RT-PCR to detect mdm2, GRB2, U5 snRNA, and mY1 scRNA transcripts. The reaction in lane 7 contained anti-La immunoprecipitated RNA, but the RT enzyme mix was heat inactivated prior to use; the reaction in lane 8 contained water instead of RNA.

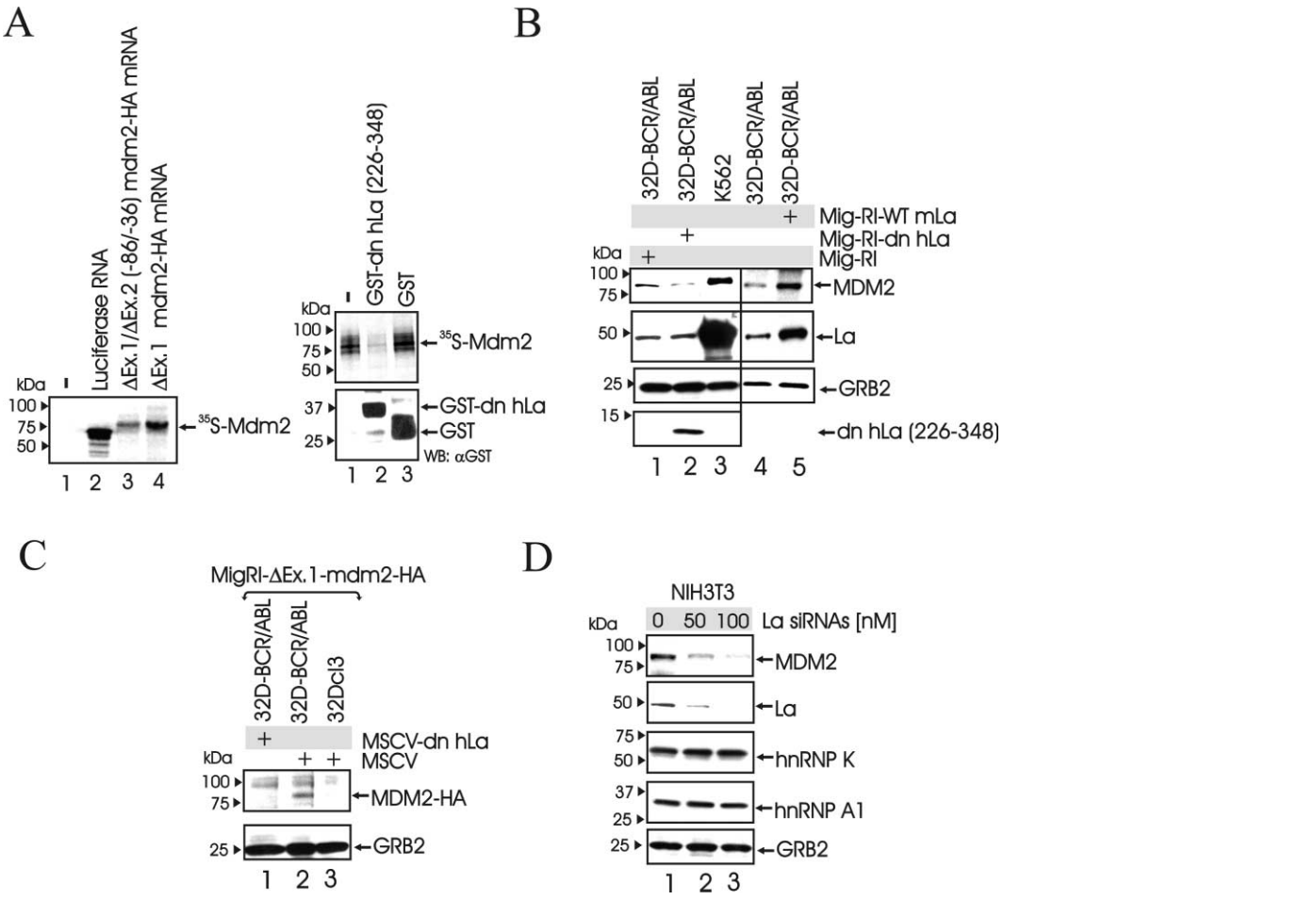

Figure 6. Effect of La antigen on MDM2 expression

A: Left: products of in vitro translation reactions programmed with no RNA (lane 1), luciferase control mRNA (lane 2), and equal amount of ΔEx.1/ΔEx.2-(-86/-36) mdm2-HA mRNA lacking the La binding site (lane 3) and ΔEx.1 mdm2-HA mRNA (containing the La binding site) (lane 4). Right: In vitro translation of full-length mdm2 mRNA (upper panel) in the absence (lane 1) or presence of bacterially purified GST-dn-hLa (lane 2) or GST only (lane 3). Lower panel shows anti-GST Western blotting of in vitro translation reactions.

B and C: Expression of endogenous MDM2 (B) and ectopic HA-tagged MDM2 (**C**) in 32D-BCR/ABL cells retrovirally transduced with dn-hLa (226-348) or full-length La cDNA as described in the text. As indicated, K562 cell extract was used as control for La expression.

D: Effect of La siRNAs on La and MDM2 expression in NIH3T3 cells. Untransfected cells (lane 1) and cells transfected with 50 nM (lane 2) or 100 nM (lane 3) La siRNAs. hnRNP-K, hnRNP-A1, and GRB2 levels were monitored as control for possible nonspecific effects on other RNA binding proteins and for loading.

panel), which is known to be stably associated with La (Hendrick et al., 1981). Grb2 mRNA and U5 snRNA, the latter of which is known not to be stably associated with La, served as negative controls (Figure 5C, middle panels). These results provide convincing evidence that mdm-2 mRNA is specifically associated with La in BCR/ABL-expressing myeloid precursor cells.

In vitro translation revealed that the 27-nucleotide sequence that interacts with La protein was required for efficient translation in rabbit reticulocyte lysate (RRL), since the mdm2 mRNA lacking the La binding site (ΔEx.1/ΔEx.2 [-86/-36] mdm2-HA mRNA) was much less efficiently translated than a mdm2 mRNA deletion mutant containing it (ΔEx.1 mdm2-HA mRNA) (Figure 6A, left panel). Since addition of purified hLa protein to this reaction had little stimulatory activity, we tested the effect of La antigen on mdm2 mRNA translation by using a truncated La (amino acids 226–348) protein that has been previously characterized as a useful tool to study La-dependent translation (Holcik and Korneluk, 2000; Craig et al., 1997). Although this mutant (dn-La) does not bind RNA (Figure 5B, lane 21), it retains the La dimerization domain and has been used as a dominant-negative mutant of La translation regulatory activity (Craig et al., 1997). As shown in Figure 6A (right panel), synthesis of ^{35}S-labeled MDM2 was suppressed by this mutant (GST-dn-hLa) but not by GST alone.

The effects of dn-hLa on MDM2 levels were also tested in 32D-BCR/ABL cells retrovirally transduced with the MigR1-dn-hLa retrovirus. Levels of endogenous MDM2 were approximately 75% lower in GFP-positive cells expressing dn-hLa than in cells transduced with the empty vector (Figure 6B). The dominant-negative La also suppressed expression of HA-tagged MDM2 in 32D-BCR/ABL cells transduced with ΔEx.1-mdm2-HA (Figure 6C). In complementary experiments, expression of endogenous MDM2 was upregulated in 32D-BCR/ABL cells transduced with the full-length mouse La cDNA (Figure 6B).

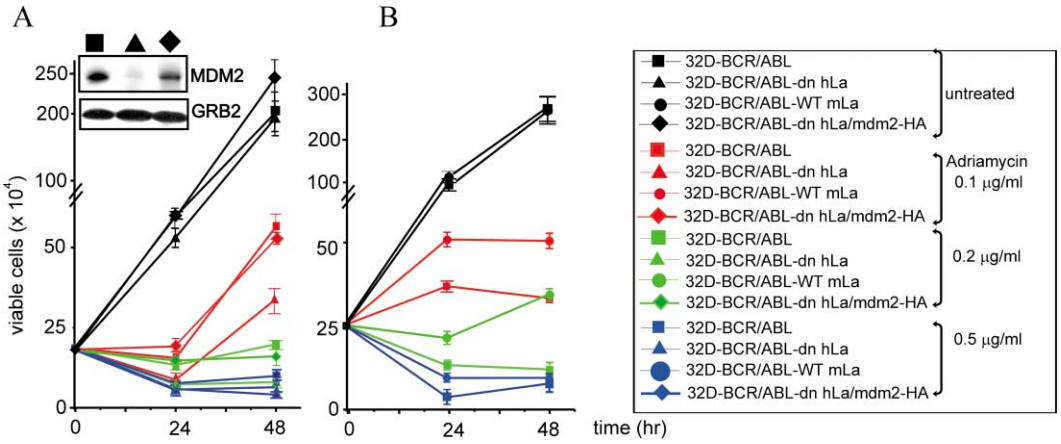

Figure 7. Effect of La antigen on adriamycin sensitivity of 32D-BCR/ABL cells

A: Number of viable 32D-BCR/ABL, dn-La-expressing 32D-BCR/ABL (32D-BCR/ABL dn-hLa), and dn-La/mdm2-HA coexpressing 32D-BCR/ABL cells (32D-BCR/ABL dn-hLa/mdm2-HA) after adriamycin treatment.

B: Number of viable 32D-BCR/ABL and wild-type La-expressing 32D-BCR/ABL (32D-BCR/ABL WT-mLa) cells after adriamycin treatment. Error bars reflect three different experiments; inset shows MDM2 protein levels in 32D-BCR/ABL, 32D-BCR/ABL dn-hLa, and 32D-BCR/ABL dn-hLa/mdm2-HA.

Since the La binding site is required for efficient mdm2 mRNA translation in vitro (Figure 6A) and for BCR/ABL-dependent MDM2 expression in vivo (Figure 4B), the results shown in Figure 6B and 6C establish a functional link between the La binding site in mdm2 RNA and the positive effects of La on MDM2 expression. The functional link between La expression and MDM2 levels was further strengthened by RNA interference in NIH3T3 cells, in which the uptake of small interfering RNAs (siRNAs) is several folds higher than in myeloid cells. As shown in Figure 6D, La expression was specifically downmodulated upon NIH3T3 transfection with a mix of siRNAs corresponding to nucleotides 310–610 of human La mRNA. Levels of two additional RNA binding proteins, hnRNP-K and hnRNP-A1, which are involved in the regulation of different aspects of mRNA metabolism including mRNA translation (Iervolino et al., 2002; Habelhah et al., 2001), were not affected (Figure 6D). The downmodulation of La expression was associated with a marked and specific decrease in MDM2 levels (Figure 6D).

32D-BCR/ABL cells expressing the dn-hLa were more sensitive to adriamycin than parental cells (Figure 7A). At three different drug concentrations, there was approximately a 50% difference in the number of viable cells between 32D-BCR/ABL and 32D-BCR/ABL-dn-hLa cells. The reduced cell number reflected an increased frequency of apoptotic cells and a reduced proportion of cycling cells (not shown). Ectopic expression of HA-tagged MDM2 rendered these dn-hLa-expressing cells similar to parental 32D-BCR/ABL cells in their susceptibility to adriamycin-induced apoptosis (Figure 7A), consistent with an MDM2-dependent effect of the dominant-negative La expression. By contrast, 32D-BCR/ABL cells overexpressing wild-type mouse La cDNA and exhibiting increased levels of MDM2 were more resistant than parental cells to adriamycin-induced apoptosis (approximately 50% increase in viable cell numbers) (Figure 7B).

Effect of BCR/ABL on La expression

To assess whether expression of the La antigen correlates with that of MDM2 and is regulated in a BCR/ABL-dependent manner, Western blots were performed on total cell lysate of primary mononuclear CML and BCR/ABL-expressing cells treated with STI571 (1 μM; 24 hr) (Figure 8). STI571 treatment suppressed La antigen expression in 32D-BCR/ABL cells (Figure 8A, lanes 1–3), in the Philadelphia[1] myeloid blast crisis cell lines EM-3 (Figure 8B) and K562 (not shown), and in $CD34^+$ cells from a CML-AP patient (Figure 8, lanes 12 and 13). In addition, La mRNA levels were similar in parental, untreated, and STI571-treated (1 μM; 48 h) BCR/ABL-expressing myeloid precursor cells (not shown), while the stability of newly synthesized ^{35}S-labeled hLa protein was markedly diminished in myeloid blast crisis EM-3 cells treated with STI571 ($t1/2^{hLa}$ EM-3 = 19.8 hr; $t1/2^{hLa}$ EM-3+STI571 = 11 hr) (Supplementary Figure S2 at http://www.cancercell.org/cgi/content/full/3/2/145/DC1), suggesting that BCR/ABL enhances La expression by activating signaling pathways leading to increased La protein stability. Indeed, in EM-3 (Figure 8B) and in 32D-BCR/ABL (not shown) cells treated with inhibitors of known BCR/ABL-activated pathways, La levels were markedly downmodulated by the specific phospholipase C-γ (PLC-γ) inhibitor U73122, but not by its inactive U73343 derivative and, to a lesser degree, by staurosporin and by the PI-3K inhibitor LY294002 (Figure 8B). By contrast, treatment of EM-3 cells with inhibitors of MAPK, S6K and PKCβ kinases did not affect La expression (Figure 8B). Expression of the La antigen was more abundant in mononuclear CML-BC and CML-AP^{CD34+} than in mononuclear CML-CP samples (Figure 8C, lanes 5–11), and increased during CML disease progression (Figure 8C, compare lanes 1–2 and lanes 3–4). Indeed, the pattern of La antigen expression in CML samples correlates with that of MDM2 (see Figure 2) and of BCR/ABL that is also more abundant in patient-derived CML-BC samples (not shown). Thus, levels of BCR/ABL, La antigen, and MDM2 appear to be functionally linked.

Discussion

During disease progression, CML progenitor cells acquire a number of genetic alterations that may explain the aggressive phenotype, chemotherapeutic drug resistance, and poor prog-

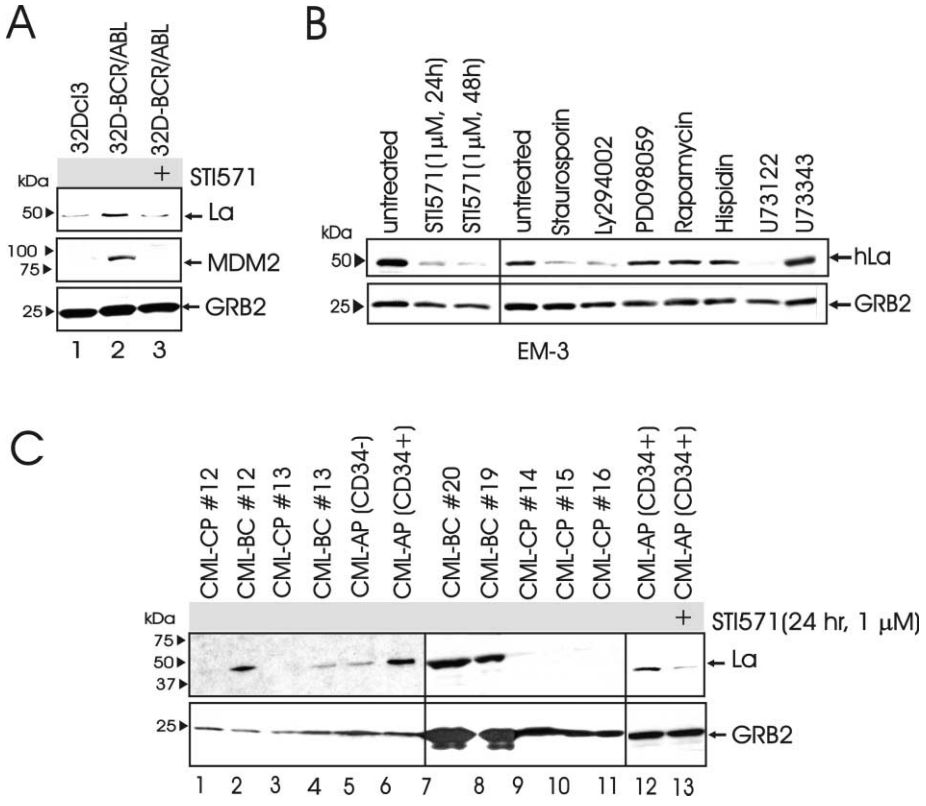

Figure 8. Effect of BCR/ABL on La expression

Effect of STI571 on La protein levels in 32D-BCR/ABL (**A**, lane 3), EM-3 (**B**) and CML-AP (**C**, lane 13) cells. Effect of different inhibitors of BCR/ABL activated pathways on La protein levels (**B**). Expression of La in patient-derived CML-CP, CML-AP ($CD34^-$ and $CD34^+$ fractions) and CML-BC primary mononuclear cells (**C**).

nosis of CML-BC. The *p53* gene is genetically inactivated in a cohort of CML-BC patients (Feinstein et al., 1991), and the p53 null background promotes a rapidly fatal disease process in mouse models of BCR/ABL-dependent leukemogenesis (Skorski et al., 1996b; Honda et al., 2000). Although other genetic abnormalities (e.g., chromosome 8 trisomy) (Kantarjian et al., 1993; Osarogiagbon and McGlave, 1999; Sawyers, 1999) may act independently of p53, p53 may be functionally inactivated in some CML-BC patients carrying a wild-type *p53* gene. Since a double Philadelphia chromosome is also a common secondary genetic alteration in CML-BC (Kantarjian et al., 1987) and levels of BCR/ABL often increase during disease progression (Gaiger et al., 1995; Elmaagacli et al., 2000), we assessed whether increased levels of BCR/ABL leads to downregulation of p53 expression, and if so, by which mechanism(s).

In 32Dcl3 myeloid progenitor cells p53 levels were downregulated by expression of BCR/ABL. This did not reflect altered expression of $p19^{ARF}$ (not shown), but instead correlated with increased MDM2 expression, suggesting that p53 is functionally inactivated by its negative regulator MDM2. Indeed, proteasome and nuclear export inhibitors raised p53 levels in these cells, consistent with an enhanced pathway of MDM2-mediated p53 degradation. MDM2 is overexpressed in more than 50% of myeloid and lymphoid leukemias and correlates with poor prognosis (Seliger et al., 1996; Faderl et al., 2000; Zhou et al., 1995, 2000), but p53 expression is not always downregulated in these samples. Since expression of a more stable, mutant form of p53 is only detected in some of these cases (Faderl et al., 2000; Zhou et al., 1995), the increased levels of MDM2 in leukemic cells may not be sufficient to induce degradation of wild-type p53. Thus, MDM2 may antagonize p53 by functionally suppressing its transactivating function (Momand et al., 1992; Thut et al., 1997; Wadgaonkar and Collins, 1999). According to a previous study (Ries et al., 2000), BCR/ABL might have led to increased transcription of mdm2 due to RAS-dependent effects (Ries et al., 2000; Cortez et al., 1995; Skorski et al., 1994); however, we found that mdm2 mRNA levels were comparable in parental and BCR/ABL-expressing cells and were only slightly downregulated after STI571 treatment. This suggested that the increased MDM2 levels in BCR/ABL-expressing cells are primarily dependent on posttranscriptional mechanisms, consistent with a previous report (Goetz et al., 2001), although in that case it was unclear whether this reflected enhanced MDM2 stability, synthesis, or both (Goetz et al., 2001). BCR/ABL has been shown to regulate protein levels by promoting or suppressing proteasome-dependent degradation (Dai et al., 1998; Perrotti et al., 2000). Our data demonstrated that the half-life of MDM2 was essentially identical in parental and BCR/ABL-expressing cells, indicating that inhibition of proteasome-dependent degradation does not account for increased MDM2 expression in BCR/ABL-expressing cells. Newly synthesized MDM2 was higher in BCR/ABL-expressing than in parental cells, suggesting that translational regulation is a critical mechanism contributing to the increased MDM2 levels in these cells.

Translational regulation of MDM2 expression was first suggested on the basis of in vitro and in vivo assays demonstrating different usage of in-frame translation initiation codons (Barak et al., 1994). Subsequently, Brown et al. (1999) demonstrated that the two uORFs included in the 5′ UTR of mdm2 mRNA inhibit translation when linked to reporter genes. Moreover, certain tumor cell lines overexpressing MDM2 exhibit enhanced translation and efficient polysome loading of mdm2 mRNA

(Brown et al., 1999; Landers et al., 1994; Capoulade et al., 1998), consistent with the existence of translation-regulatory mechanisms leading to MDM2 overexpression. Our studies suggest that in BCR/ABL-expressing cells, translation of mdm2 mRNA is not differentially influenced by the uORFs, but rather is dependent on a 27-nucleotide sequence located in the central part of the mdm2 exon 2 (intercistronic region). Mouse and human mdm2 exons 2 are conserved in size but are widely divergent in sequence except for a short region in its central portion (Figure 5). The importance of this conserved region was revealed by its requirement for efficient mdm2 expression both in vitro and in vivo. RNA probes and UV crosslinking identified a ~50 kDa RNA binding protein present in mouse and human BCR/ABL-expressing cells that interacts specifically with the conserved part of mdm2 exon 2 mRNA. Since the results suggested that the identified RNA binding protein might reflect the action of an mdm2 mRNA translation-stimulatory factor, this protein was purified on the basis of its ability to interact with the conserved sequence tract in mdm2 mRNA and was identified as the La antigen. Indeed, mdm-2 mRNA appears to be stably associated with La in BCR/ABL-transformed cells, since La immunodepletion abolished binding to mdm2 mRNA in REMSA (Figure 5B) and mdm2 mRNA recovery from the anti-La immunoprecipitate was comparable to that of mY1 scRNA (Figure 5C), a small cytoplasmic RNA that is stably associated with La (Hendrick et al., 1981).

This is not the first example of an RNA binding protein whose translation-regulatory activity is modulated by BCR/ABL. In fact, we recently reported that BCR/ABL induces, in a tyrosine kinase-dependent manner, the expression of the RNA binding protein hnRNP E2 that is directly responsible for translational suppression of C/EBPα, the major regulator of granulocytic differentiation (Perrotti et al., 2002).

Although the La antigen is mostly nuclear, where it is involved in the biogenesis of RNA polymerase III transcripts and processing of tRNA precursors, it is also present in the cytoplasm, and increases there under certain conditions (Wolin and Cedervall, 2002; Fan et al., 1998). For example, in poliovirus-infected cells, La is redirected to the cytoplasm, where it is believed to interact with the 5′ UTR of poliovirus mRNA to positively influence its translation (Maraia and Intine, 2001; Wolin and Cedervall, 2002, and references therein). In addition to multiple virus-derived mRNAs (Maraia and Intine, 2001; Wolin and Cedervall, 2002), La has also been reported to interact with cellular mRNAs. Recently, the La antigen was reported to activate IRES-dependent translation of the immunoglobulin heavy chain binding protein (BiP) mRNA upon interaction with its 5′ UTR (Kim et al., 2001). Remarkably, the only significant homology (~70% identity) between mdm2 and BiP mRNA 5′ UTRs is in the core sequence conserved in human and mouse mdm2 5′ UTR reported here, and this includes the region of BiP mRNA that has been shown to interact with La. La antigen was also identified as part of a multiprotein complex interacting with the IRES region of XIAP mRNA that is required for its translation in vitro and in vivo (Holcik and Korneluk, 2000). The segment of the mdm2 5′ UTR bound by the La protein did not function as IRES when driving GFP expression in BCR/ABL-expressing cells transduced with a bicistronic retrovirus (not shown; see Supplementary Figure S1 at http://www.cancercell.org/cgi/content/full/3/2/145/DC1), consistent with the possibility that the La protein has a more general role in the regulation of mRNA translation (Wolin and Cedervall, 2002). Interestingly, expression of XIAP was downmodulated in STI571-treated BCR/ABL-expressing cells (Fang et al., 2000a), suggesting that, in these cells, MDM2 and XIAP levels might be in part coregulated by La. Consistent with this, La is abundant in CML-BC primary samples, and its levels appear to correlate with BCR/ABL levels and tyrosine kinase activity. The observation that treatment with STI571 downregulates La protein expression by decreasing its stability is noteworthy, because it was thought that La levels are constitutive (Maraia and Intine, 2001; Wolin and Cedervall, 2002). Moreover, La expression was downmodulated by a specific PLC-γ inhibitor and, to a lesser degree, by the serine/threonine kinase (PKA, PKC, PKG, and CaM kinase) inhibitor staurosporin and by a PI-3K inhibitor. Since PLC-γ is activated by BCR/ABL (Gotoh et al., 1994) and is required for the activation of several PKC isoforms (Newton, 1995), it is possible that BCR/ABL-dependent activation of a specific PKC isoform (PKCβI/βII excluded) plays a role in regulating La levels. Of interest, the La protein is phosphorylated by PKC in HeLa cells (Broekhuis et al., 2000). However, it is unknown whether PKC phosphorylation of La protein also occurs in BCR/ABL-expressing cells and whether it is involved in the upregulation of La levels.

That the PI-3K inhibitor LY294002 also downmodulates La protein levels suggests the involvement of an additional pathway(s), although it cannot be excluded that interference with PDK1 plasma membrane translocation (which is regulated in a PI-3K-dependent manner) prevents PKC activation (Le Good et al., 1998).

The data described in this report indicate that La has a direct and positive effect on MDM2 expression because: (1) La recognizes a specific conserved sequence tract in mdm2 mRNA that is required for efficient MDM2 expression in vitro and in vivo; (2) a dominant-negative (dn) La mutant inhibited mdm2 mRNA translation in vitro and suppressed MDM2 levels in BCR/ABL-expressing cells; (3) downregulation of La expression by siRNAs led to a marked decrease in MDM2 levels; and (4) overexpression of wild-type La led to an increase in MDM2 expression.

Purified hLa protein had little stimulatory affect on in vitro translation of mdm2 mRNA; this may be explained by the presence of endogenous rabbit La in the reticulocyte lysate (Holcik and Korneluk, 2000, and data not shown), the low affinity of GST-La for the mdm2 5′ UTR, or the need for posttranslational modifications possibly occurring in BCR/ABL-transformed cells.

With regard to the latter possibility, we note that while serine 366, the major phosphorylation site in human La, has been shown to affect binding to precursor tRNAs (Intine et al., 2000), phosphorylation that occurs on three other sites has not been associated with any known activity of La (Broekhuis et al., 2000), leaving open the possibility that La phosphorylation may promote a stimulation of mdm2 mRNA translation.

That a La-mediated effect on MDM2 expression is functionally relevant for BCR/ABL leukemogenesis is indicated by the changes in susceptibility of BCR/ABL-expressing cells to adriamycin-induced apoptosis, as wild-type La-overexpressing cells were more resistant than parental cells, whereas cells expressing dn-La were more sensitive. Although MDM2 levels were markedly downmodulated in 32D BCR/ABL cells expressing dn-La, these cells neither exhibited spontaneous apoptosis nor altered cell cycle activity (not shown). This is consistent with the primary role of MDM2 as a regulator of p53 and suggests

that the proposed p53-independent effects of MDM2 (Sigalas et al., 1996) might not be important in BCR/ABL-expressing cells.

In summary, we have demonstrated that the expression of MDM2 is upregulated in BCR/ABL-expressing cells via enhanced translation that is dependent on increased levels of the RNA binding protein La and its direct interaction with a short segment of mdm2 5′ UTR. While a role for the La antigen in mRNA metabolism has previously been suggested (Maraia and Intine, 2001), our studies establish a link between La expression and the phenotype of hematopoietic cells transformed by an oncogenic tyrosine kinase, and suggest that La-dependent MDM2 upregulation may contribute to progression of CML into blast crisis.

Experimental procedures

Cell cultures and primary CML cells

Murine IL-3-dependent 32Dcl3 myeloid precursor cells and derivative cell lines were maintained in culture in IMDM medium supplemented with 10% heat-inactivated FBS (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine (Gibco, BRL, Gaithersburg, MD), and 10% WEHI-conditioned medium as a source of IL-3.

The EM-3 myeloid blast crisis cell line (Lubbert et al., 1988) was kindly provided by Dr. A. Keating (Ontario Cancer Center, Toronto, Canada).

Ficoll-separated mononuclear marrow (or peripheral) hematopoietic cells of patients with CML in chronic phase (CML-CP), accelerated phase (CML-AP), and myeloid blast crisis (CML-BC) were cultured overnight in IMDM with 20% FBS, 2 mM L-glutamine supplemented with human recombinant IL-3 (20 ng/ml), IL-6 (20 ng/ml), Flt-3 Ligand (100 ng/ml), and SCF (100 ng/ml) (StemCell Technologies Inc., Vancouver, Canada). CML-CP #12–13 and CML-BC #12–13 were from two patients with CML in chronic phase and after progression into blast crisis, respectively. $CD34^+$ cell separation was performed using a MACS CD34 Kit (Miltenyi Biotec., Auburn, CA). Stable phase CML samples were provided by Dr. A.M. Gewirtz (Dept. of Medicine, UPenn, Philadelphia, PA) and by the Cancer and Leukemia Group B Leukemia Tissue Bank (OSU, Columbus, OH). Accelerated and blastic phase CML samples were provided by Drs. N. Flomenberg (Bone Marrow Transplant Unit, TJU, Philadelphia, PA) and A.M. Gewirtz. Sample from patients at different stages of CML were provided by Dr. C. Gambacorti-Passerini (Experimental Oncology, NCI, Milano, and Hematology Section, S. Gerardo Hospital, Monza, Italy).

Where indicated, parental and BCR/ABL-expressing 32Dcl3 cells were treated for the indicated times with 10 μM lactacystin (Calbiochem, San Diego, CA), 25 μM ALLN (Calbiochem), 5 ng/ml Leptomycin B (kindly provided by Novartis Pharma AG, Basel, Switzerland), 20 μg/ml cycloheximide (Sigma) or 1 μg/ml adriamycin (APP, Inc.). To inhibit BCR/ABL tyrosine kinase activity, 32D-BCR/ABL, EM-3 cells or $CD34^+$ CML-AP cells were cultured for the indicated time in medium supplemented with the ABL-kinase inhibitor STI571 (1–2 μM) (kindly provided by Novartis Oncology).

Where indicated, EM-3 cells were treated with kinase inhibitors (16 to 24 hr) used at the following concentrations: rapamycin, 15 nM (Calbiochem, San Diego, CA); PD098059, 50 μM (Calbiochem); LY294002, 25 μM (Calbiochem); staurosporin, 1 μM (Calbiochem); hispidin (PKCβ inhibitor), 5 μM (Calbiochem); U73122, 1 μM (Biomol); U73343, 1 μM (Biomol).

The amphotropic-packaging cell line Phoenix E (Dr. G. P. Nolan, Stanford University School of Medicine) was maintained in culture in DMEM/10% FBS medium and grown for 16–18 hr to 80% confluence prior to transfection by calcium phosphate-DNA precipitation (ProFection system, Promega, Madison, WI).

Retroviral infection of parental and BCR/ABL-expressing 32Dcl3 cells

32Dcl3-derivative cell lines expressing pSRαMSVtKneo-$p210^{BCR/ABL}$, the MSCVpuro-, and/or the MigRI-based plasmids were generated by retroviral infections of parental and BCR/ABL-expressing 32Dcl3 cells as described (Pear et al., 1998). Briefly, infectious supernatants from transiently transfected Phoenix cells were collected 48 hr after transfection and used to infect parental and BCR/ABL-expressing cells; 24 hr later, infected cells were cultured in the presence of neomycin (1 mg/ml) or puromycin (2 μg/ml) for clonal selection, or sorted (EPICS Profile Analyzer, Coulter Inc., Hialeah, FL) for green fluorescent protein (GFP) expression.

Plasmids

WT-uORF-, *ΔEx.1-*, *ΔEx.1/ΔEx.2(-86/-36)-*, *ΔEx.1/ΔEx.2(-86/-62)-*, and *ΔEx.1/ ΔEx.2-mdm2-HA*: The 5′ UTR region of mdm2 cDNA, containing the nucleotides corresponding to exons 1 and 2 or deletions of these sequences, and part of the *mdm2* coding sequence (cds) were obtained by RT-PCR amplification using an upstream primer containing a XhoI site at the 5′-end and a downstream primer spanning the BamHI site of the mdm2 cds. The remaining *mdm2* cds was PCR-generated from the full-length murine mdm2 cDNA (kind gift of Donna L. George, University of Pennsylvania, School of Medicine, Philadelphia, PA) using an upstream primer spanning the BamHI site of mdm2 cds, and a downstream primer containing the last 23 nucleotides of the cds, a hemagglutinin (HA)-tag preceding the *mdm2* stop codon, and an EcoRI site. PCR products were XhoI/BamHI or BamHI/EcoRI-digested and subcloned directionally into the XhoI/EcoRI sites of the bicistronic IRES-GFP MigRI retroviral vector (Pear et al., 1998).

MSCV ΔEx.1 mdm2-HA: MIG RI ΔEx.1 mdm2-HA was digested with XhoI-EcoRI and the released fragment was cloned into the XhoI-EcoRI digested MSCVpuro retroviral vector.

pcDNA3 ΔEx.1 mdm2-HA: MigRI ΔEx.1 mdm2-HA was digested with BglII-EcoRI-and the released fragment was cloned into the BamHI-EcoRI-digested pcDNA3 plasmid.

pcDNA3 ΔEx.1/ΔEx.2(-86/-36) mdm2-HA: MigRI ΔEx.1/ΔEx.2 (-86/-36) mdm2-HA was digested with BglII-EcoRI-and the released fragment was cloned into the BamHI-EcoRI-digested pcDNA3 plasmid.

p*GEX-2T-hLa (1-408)* and *pGEX-2T-dn-hLa* (226-348) were the kind gift of N. Sonenberg (McGill University, Montreal, Canada).

MigR1 hLa: the coding sequence of *hLa* was obtained by BamHI/EcoRI digestion of the pGEX-2T-hLa plasmid and directionally subcloned into the BglII-EcoRI-digested MigR1 vector.

MigR1 mLa: full-length mouse La cDNA (Topfer et al., 1993) (kind gift of James McCluskey, Flinders Medical Center, Bedford Park, South Australia) was cloned into the EcoRI site of the MigR1 retrovirus.

MigR1 dn-hLa (226–348) and *MSCVdn-hLa (226–348)*: the La cDNA fragment encoding amino acids 226–348 was PCR amplified from the pGEX-2T-dn-hLa plasmid using a 5′ primer that includes a BglII restriction site, the ATG codon, and 18 nucleotides corresponding to amino acids 226–231, and a 3′ primer that includes 18 nucleotides corresponding to amino acids 343–348, the TAA stop codon, and the EcoRI restriction site. After BglII/EcoRI digestion, the product was cloned directionally in the BglII/EcoRI-digested MigR1 or MSCV-puro vector.

SK Ex.2 mdm2: mdm2 exon 2 was amplified by PCR from MigR1 WT-uORF mdm2-HA using a 5′ primer which includes the XhoI restriction site and a 3′ primer which includes the EcoRV restriction site. After XhoI/EcoRV digestion, the product was cloned into the XhoI/EcoRV-digested SK bluescript plasmid.

Western blot analysis, immunoprecipitation, and metabolic labeling

Cells were harvested, washed once with ice-cold PBS, and lysed (10^7 cells/100 μl lysis buffer) in hypertonic buffer (1% NP-40, 0.5 M NaCl, 10 mM Hepes [pH 7.5], 10% glycerol supplemented with protease and phosphatase inhibitors [1 mM phenylmethylsulfonylfluoride {PMSF}, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na_3VO_4, 50 mM NaFl, 10 mM β-glycerol-phosphate]). For nuclear fractions preparation, cells were lysed in buffer A (20 mM Hepes [pH 7], 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40) supplemented with the protease inhibitors described above, homogenized, and nuclei were sedimented at 1500 × g for 2 min. The nuclear pellet was washed three times in buffer A, resuspended in the same buffer containing 0.5 M NaCl, rocked gently for 30 min at 4°C, and clarified by centrifugation (15,000 × g, 10 min). For direct lysis, cells ($2–3 \times 10^5$) were washed once with ice-cold PBS and directly lysed in 20 μl of Laemmli buffer. Immunoprecipitation and Western blotting were performed as described (Perrotti et al., 2000).

Antibodies used were: monoclonal anti-p53 (Ab-1, Calbiochem); monoclonal anti-MDM2 2A10 (kind gift of Dr. A. J. Levine, The Rockefeller Univer-

sity, New York, NY); monoclonal anti-HSP90 (Transduction Laboratories); monoclonal anti-phosphotyrosine PY20 (Transduction Laboratories); monoclonal anti-c-Abl (Ab-3, Calbiochem); monoclonal anti-HA (Babco, Richmond, CA); monoclonal anti-GRB2 (Transduction Laboratories); monoclonal anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-Histone H1 (Santa Cruz Biotechnology); monoclonal anti-hLa (Transduction Laboratories), and anti-human La (Go) serum (Intine et al., 2002). anti-hnRNP A1 and anti-hnRNP K antibodies were kindly provided by Dr. G. Dreyfuss (University of Pennsylvania, Philadelphia, PA).

For metabolic labeling, 32Dcl3 and 32D-BCR/ABL cells were washed four times with PBS and cultured (2×10^6 cells/ml) for 60 min in methionine-free RPMI 1640 supplemented with 10% dialyzed FBS (Gibco BRL, NY) and 2 ng/ml recombinant murine IL-3 (R&D Systems Inc., Minneapolis, MN). Cells were washed and resuspended (5×10^6 cells/ml) in medium containing 200 μCi/ml of [^{35}S]-methionine (NEN™, Life Science Products, MA). After 90 min, cells were washed twice with cold Hank's solution and lysed in hypertonic buffer. Precleared lysates were incubated at 4°C for 2 hr with protein G-agarose (Oncogene Research Products, MA)-coupled anti-MDM2 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE under reducing conditions, transferred onto nitrocellulose and visualized after exposure to X-AR films (Eastman Kodak Co., Rochester, NY).

Isolation of polysome/monosome-associated RNA from untreated and STI571-treated BCR/ABL-expressing cells

RNA was prepared as described (Fu et al., 1996), with minor modifications. Briefly, untreated or STI571 (2 μM, 12 hr)-treated 32D-BCR/ABL cells (10^8) were washed twice in ice-cold Tris-saline solution (25 mM Tris-HCl [pH 7.5], 25 mM NaCl) containing 10 mM $MgCl_2$ and 10 μg/ml cycloheximide and lysed on ice in 1 ml of extraction buffer (1 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 665 μg/ml heparin, 1 mM PMSF, 150 μg/ml cycloheximide, 20 mM dithiothreitol, 500 U/ml RNasin). Lysates were nuclei-depleted, clarified by centrifugation (14,000 rpm, 5 min at 4°C) and layered onto a 15%–50% linear sucrose gradient (10 ml) prepared in extraction buffer (without NP-40 and PMSF). The gradients were centrifuged in an SW41 Beckman rotor at 38,000 rpm for 120 min at 4°C. Fourteen fractions of equal volume (750 μl) were collected from the bottom of the tubes. During fractionation, polysome profiles were monitored with a simultaneous A_{254} trace. RNA was prepared from each fraction by phenol/chloroform extraction and isopropanol precipitation. RNA was washed with ethanol and resuspended in 30 μl of DEPC-treated water.

Northern blot analysis and RT-PCR

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc., OH). For Northern blot analysis, total RNA (10 μg) or polysome-/monosome-associated RNA (5 μg/fraction) was fractionated onto denaturing 1% agarose/6.6% formaldehyde gels, transferred onto Hybond-nylon membrane (Amersham Pharmacia Biotech, Inc.), and hybridized to ^{32}P-labeled cDNAs probes specific for murine *mdm2*, murine *p53*, or *hLa*. RT-PCR amplification of mdm2 mRNA was carried from total RNA using mdm2 (mouse or human) specific primers. For detection of ectopic HA-tagged mdm2 mRNA in retrovirus-transduced cells, we used a 5′ primer (5′-TTGAAGGAGGAAACGCA GCA-3′) corresponding to nucleotides 1438–1457 of mouse mdm2 cDNA and a 3′ primer (5′-TCTTAAGCGTAATCTGGAACAT-3′) complementary to part of the HA sequence. BCR/ABL levels were determined by PCR as described (Perrotti et al., 2002). GRB2 mRNA levels were monitored as control for equal loading.

RNA UV crosslinking

Cytoplasmic extracts were prepared and used for UV crosslinking as described (Perrotti et al., 2002). Briefly, 10^7 cells were lysed in 100 μl of binding buffer [10 mM HEPES-KOH {pH 7.5}, 14 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.2% NP-40, 1 mM DTT, 1 mM PMSF, 25 μg/ ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na_3VO_4, 50 mM NaF and 10 mM β-glycerolphosphate] and the cytoplasmic fraction purified by centrifugation (2 min at 8500 × g at 4°C). Cytoplasmic proteins (15 μg) were incubated (30 min at room temperature) with 6.25×10^3 cpm of a 89 bp riboprobe corresponding to mdm2 exon 2 (made by T7-directed in vitro transcription from the EcoRV-linearized SK-Ex.2 mdm2 plasmid) or 35 mer oligoribonucleotide (murine RNA-oligo 2) corresponding to nucleotides −61 to −27 (relative to the ORF) (5′-GACCGGACACCCCUGGGGGACCCU-CU CGGAUCACC-3′) of mouse mdm2 mRNA, or 35 mer oligoribonucleotide corresponding to the human homolog of *mouse RNA-oligo 2*. Reactions were incubated for additional 20 min with 10 mg/ml heparin (Calbiochem) to reduce nonspecific binding. After UV crosslinking (3,500 μJ × 100, 30 min at room temperature), RNA-protein complex(es) were resolved by a 4%–15% or a 7.5% SDS-PAGE. In competition assays, RNA-protein binding reactions were performed in the presence of a 1000-fold molar excess of the (murine or human) RNA-oligo 2 or of single-stranded oligodeoxynucleotides corresponding to different segments of mdm2 exon 2: ssODN1 (5′-AGCCGT CTGCTGGGCGAGCGGGAGACCGACCGGAC-3′), ssODN2 (5′-GACCGGA CACCCCTGGGGGACCCTCTCGGATCACC-3′), ssODN3 (5′-GGATCACC-GCGCTTCTCCTGCGGCCTCCAGGCCAA-3′). La-depleted K562 cytoplasmic extracts were obtained by immunodepletion using anti-La (Go) human IgG coupled to protein G sepharose (Intine et al., 2002).

Purification of mdm2 mRNA binding protein

The mdm2 5′ UTR RNA binding protein was purified by RNA affinity chromatography using approximately 500 mg of cytoplasmic extracts from K562 cells (Perrotti et al., 2002). After concentration by Centriprep-10 (Millipore), cytoplasmic extracts (~70 mg/ml) in binding buffer were size-fractionated using a Sephacryl S-200 column (0.4 ml/min; bed volume 120 ml) equilibrated in the same buffer. Fractions retaining the ability to bind the mdm2 ^{32}P-RNA-oligo 2 in UV-crosslinking were loaded on a Hi-Trap heparin-sepharose column (Amersham Pharmacia Biotech), and the eluted fractions retaining the RNA binding activity were further purified by RNA affinity chromatography.

Briefly, 100 μg of the 5′-biotynilated mdm2 RNA-oligo 2 was coupled to a streptavidin-sepharose resin according to the manufacturer's instructions (Amersham Pharmacia Biotech). Fractions containing the mdm2 RNA binding protein were passed through the RNA-affinity column equilibrated in binding buffer at a flow rate of 0.1 ml/min. After collecting the flow-through, the column was washed with 10 volumes of binding buffer supplemented with 1 mg/ml of heparin followed by 10 volumes of binding buffer, and purified proteins eluted with a salt gradient (0.5–2 M NaCl). After dialysis in binding buffer, the affinity-purified fractions were tested by UV crosslinking, gel-fractionated, and stained with Coomassie blue. A gel slice from a preparative SDS-PAGE containing the purified protein was digested and sequenced by microcapillary reverse-phase high-performance liquid chromatography nanoelectrospray tandem mass spectrometry (mLC/MS/MS) on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer (Microchemistry Facility of Harvard University, Cambridge).

RT-PCR of coimmunoprecipitated RNA

1 ml of whole cell extract was made from 30 ml of 32D-BCR/ABL cells grown to a density of 8.0×10^5/ml. Immunoprecipitations (IPs) were performed using 50 μl of anti-La (Go) or nonimmune sera and 25 μl of protein A sepharose (PAS) as described (Steitz, 1989). Briefly, after conjugation, the IgG-PAS beads were washed four times with 1 ml of NET-2 and incubated with 50 μl of precleared extract in a final volume of 200 μl NET-2 for 3 hr at 4°C with rotation. The IgG-PAS beads were spun out and the supernatant was collected. The beads were then washed six times with 1 ml of cold NET-2. The beads were extracted twice with 100 μl of 50 mM Tris-Cl (pH 7.5), 2% SDS followed by 200 μl of H_2O. Total RNA was purified from equivalent amounts of the supernatants and input, as well as from all of the immunoprecipitated material using guanidinium thiocyanate, phenol, and chloroform, followed by ethanol precipitation such that the final volume of precipitated slurry for each sample was 750 μl. RT-PCR was performed using the Titan One tube RT-PCR System (Roche). For each of the RNAs assayed, equal aliquot of purified RNA samples were dissolved in 25 μl of 0.2 mM dNTPs, 5 mM DDT, 20 μM each primer, and 5 U RNasin and added to a reaction tube with 25 μl of 1X RT-PCR buffer (with Magnesium) and 1 μl of enzyme mix. For the reactions containing heat inactivated AMV reverse transcriptase, the enzyme mix was heated to 95°C for 2 min before addition. Reaction conditions were as follows: for GRB2 and mY1; 30 min at 50°C, 2 min at 95°C, followed by 22 cycles of 10 s at 95°C, 30 s at 58°C, and 45 s at 68°C; for mdm2 and U5, 25 cycles were used. Equal aliquots of the products were electrophoresed on a 2.5% agarose gel for ~45 min at 50 V. Primers (5′ to 3′): mdm2: TTCTCCTGCGGCCTCCAGGC and AGCAATGG TTTTGGTCTAACCAGA; mY1: GGCTGGTCCGAAGGTAGTG and AAAGAC TAGTCAAGTGCAGTAGTGAGA; U5: GATACTCTGGTTTCTCTTCAGATCG

and CCTCAAAAAATTGGTTTAAGACTCAG; Grb2: TCAGAATGGAAGCCAT CGCCA and TTCCAAACTTGACGGACAGGG.

In vitro translation assay

10 μg of plasmid pcDNA3-ΔEx.1 mdm2-HA (containing the La binding sequence) or plasmid pcDNA3 ΔEx.1/ΔEx.2(-86/-36) mdm2-HA (deleted of the La binding sequence) were linearized with EcoRI. Proteinase K (1 mg/ml, 30 min. at 55°C) was used to digest residual nuclease activity and the samples were extracted and precipitated with EtOH. The linearized DNAs served as templates for mdm2 mRNA synthesis by T7 RNA polymerase using the Ribomax large scale system (Promega, Madison) in 100 μl reactions containing 5 μg linearized DNA, T7 transcription buffer, 25 mM ATP, CTP, and UTP, 4 mM GTP, and 4 mM ribo m^7G cap analog at 37°C for 4 hr. RNA products were phenol/chloroform, ethanol precipitated, and stored at −20°C. Portions of the mRNA preparations were quantitated and calibrated by denaturing gel electrophoresis and ethidium bromide staining. For in vitro translation, equal amounts of mRNAs were translated using the Flexi-rabbit reticulocyte lysate (RRL) system (Promega, Madison,) following the protocol suggested by the manufacturer. Reactions were resolved by SDS-PAGE, and the gel was dried and then exposed to phosphorimager screen. The in vitro translation inhibition assay was instead carried out as described (Perrotti et al., 2002) using the EcoRI-linearized pcDNA3-ΔEx.1 mdm2-HA plasmid and the transcription/translation-coupled rabbit reticulocyte lysate system (TnT system; Promega) according to manufacturer's instructions in the absence or in the presence of 1 μg of GST-dn-hLa (226–348) fusion protein or GST alone. We fractionated 5 μl of each reaction on 4%–15% SDS-PAGE, transferred it onto a nitrocellulose membrane, and detected the ^{35}S-labeled translation products by autoradiography. Levels of GST or GST-dn-hLa (226–348) in the reaction mixes were monitored by anti-GST Western blotting.

Inhibition of La expression by RNA interference

A 300 base dsRNA corresponding to the human La mRNA in a region highly homologous to mouse La mRNA was PCR-generated using 50 pmoles of each primer (5′-aatttaatacgactcactataggaagcattgagcaaatcc-3′ and 5′-aatt taatacgactcactataggcttctggccaggggtctc-3) and 100 ng of HeLaS3 cDNA. The 300 bp PCR product was gel-purified, phenol/chloroform extracted, ethanol precipitated, and resuspended in nuclease-free water. The PCR products containing T7 promoters were used for transcription using the MEGAscript™ RNAi Kit (Ambion Inc.). The siRNAs were generated as follows: 15 μg of the 300 base long La dsRNA was digested (37°C; 2 hr) with recombinant bacterial RNase III (2.5 μg), in a 20 μl of 10 mM Tris 7.5, 2.5 mM $MgCl_2$, 0.1mM $CaCl_2$. Thereafter, H_2O (80 μl) was added and the sample phenol/chloroform-extracted, loaded onto a Microcon YM-100 spin column (500 × g; 12 min), ethanol precipitated, and resuspended in nuclease-free H_2O.

For in vivo siRNAs transfection, NIH3T3 cells were plated (200,000/well) in 6-well tissue culture plates. After 24 hr, cells were transfected with RNase III-digested RNA (50 nM and 100 nM) using the siPORT™ Lipid transfection agent (Ambion Inc.) according to the manufacturer's protocol. 72 hr after, transfection cells were harvested and analyzed by Western blot for La, MDM2, hnRNP A1, hnRNP K, and GRB2 expression.

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