

# Bcr-Abl Kinase Modulates the Translation Regulators Ribosomal Protein S6 and 4E-BP1 in Chronic Myelogenous Leukemia Cells via the Mammalian Target of Rapamycin<sup>1</sup>

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## Abstract

**Identification of signaling pathways downstream of Abl tyrosine kinase may increase our understanding of the pathogenesis of chronic myelogenous leukemia (CML) and suggest strategies to improve clinical treatment of the disease. By combining the use of a phosphospecific antibody recognizing a substrate motif of serine/threonine kinases with bioinformatics, we found that the translational regulators ribosomal protein S6 and 4E-BP1 are constitutively phosphorylated in CML cells. Experiments with specific inhibitors indicated the phosphorylation is downstream of Bcr-Abl kinase and the mammalian target of rapamycin (mTOR). These results suggest that Bcr-Abl may regulate translation of critical targets in CML cells via mTOR. They also provide a rationale for testing the combination of mTOR inhibitors with the Abl kinase inhibitor imatinib in patients with CML. The mTOR inhibitor rapamycin enhanced imatinib-mediated killing of CML cell lines *in vitro*, and it overcame imatinib resistance in cells with *Bcr-Abl* gene amplification.**

## Introduction

CML<sup>3</sup> is a pluripotent stem cell disorder characterized by the presence of the Philadelphia chromosome. The Philadelphia chromosome results in the production of a fusion gene, *Bcr-Abl*, that encodes a cytoplasmic protein with constitutive tyrosine kinase activity. Dysregulated Abl kinase activity leads to the activation of multiple distinct signaling cascades, including signaling molecules such as Ras, Raf, Myc, Stat, Jun, PI3K, and AKT (1). Inhibition of Abl kinase by the small molecule inhibitor imatinib has led to remarkable clinical responses, even in patients with late stage disease (2). However, the responses in this latter group of patients have been short-lived and are characterized by imatinib-resistant disease recurrence within a few months. The emergence of resistance has led to a search for downstream targets of Bcr-Abl kinase signaling that may mediate the altered growth properties of Bcr-Abl-transformed cells. Some of these targets might be involved in imatinib resistance, and they may provide targets for therapeutic intervention in imatinib-resistant disease. Signaling pathways that have been shown to be necessary for Bcr-Abl transformation in CML include the Ras and PI3K/AKT pathways. However, for both these pathways, the critical downstream intermediates have not been clearly established. For example, whereas it is

clear the PI3K activation is essential for Bcr-Abl-mediated transformation (1), it is unclear which of the potential downstream effectors, which include BCL2/BCL-X<sub>L</sub>-associated death promoter (BAD), FOXO1a, nuclear factor κB, glycogen synthase kinase-3β, mTOR, and murine double minute 2 (MDM2), are actually important. Recently, other groups have used a combination of biochemistry and bioinformatics to successfully identify novel PI3K/AKT targets in a variety of cell types (3, 4). We investigated whether a similar approach might identify downstream targets of the PI3K pathway in CML cells. We have identified the S6 ribosomal protein as being phosphorylated in a Bcr-Abl kinase-, PI3K-, and mTOR-dependent manner in Bcr-Abl-expressing cell lines and CML cell lines. Another mTOR substrate and important regulator of translation, 4E-BP1, is similarly regulated. The possibility of treating CML cells with a combination of imatinib and an inhibitor of mTOR, rapamycin, was also explored.

## Materials and Methods

**Cell Lines and Cell Culture Conditions.** The murine hematopoietic cell line Ba/F3 was cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/ml murine IL-3. Ba/F3 cells stably transfected with full-length wild-type p210 (Ba/F3-Bcr-Abl-WT) and the T315I mutant (Ba/F3-Bcr-Abl-T315I; Ref. 5; both kindly provided by Charles Sawyers, University of California, Los Angeles, Los Angeles, CA) were grown in RPMI 1640 supplemented with 10% FCS. Ba/F3 cells with amplification of the wild-type *Bcr-Abl* gene (Baf-BCR-ABL-R; Ref. 6) were grown in the presence of 1 μM imatinib, unless otherwise stated. K562 cells were cultured in RPMI supplemented with 10% FCS. In inhibition experiments, three to five million cells were incubated in the following inhibitors for 1 h at 37°C: imatinib (Novartis, Basel, Switzerland), the PI3K inhibitor LY294002 (LY; Calbiochem, San Diego, CA), and rapamycin (Sigma, St. Louis, MO).

**Western Blot Analysis and Immunoprecipitation.** After incubations, cells were collected and washed twice with PBS and incubated on ice for 15 min in 500 μl of lysis buffer. Protein lysates were quantitated by the Bradford assay, 15 μg resolved by SDS-PAGE, and transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The following antibodies were used for immunoblotting: c-abl (clone 8E9; PharMingen, San Diego, CA), phosphotyrosine (clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY), phospho-(Ser/Thr) AKT substrate (AKT-pSub), phospho-S6 ribosomal protein (Ser235/236), S6 ribosomal protein, and 4E-BP1. All antibodies were from Cell Signaling Technology (Beverly, MA), unless otherwise stated. Immunoblotting was performed according to the manufacturers' instructions. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce Chemical Co.) after incubation with a secondary antibody. For immunoprecipitation, K562 cells were incubated for 24 h with or without 10 μM LY. Cells were harvested under nonreducing conditions by removing media and washing cells twice with cold PBS. Cells were then lysed with ice-cold lysis buffer [30 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 20 mM HEPES (pH 7.4), 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride] for 10 min on ice, followed by centrifugation for 10 min at 4°C. Lysate was transferred to a new tube, and 20 μl of immobilized AKT-pSub antibody were used for every 200 μl of lysate

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<sup>3</sup> The abbreviations used are: CML, chronic myelogenous leukemia; PI3K, phosphoinositide 3-kinase; IL-3, interleukin 3; mTOR, mammalian target of rapamycin.

for an overnight incubation at 4°C. After this, each sample was centrifuged for 1 min at 4°C. The pellet was washed five times using lysis buffer, after which 6× SDS sample buffer was added and samples were boiled for 5 min. The samples were loaded onto Bio-Rad (Hercules, CA.) 12% Tris-HCl ReadyGels to perform SDS-PAGE and analyzed by Western blotting.

**Two-Dimensional Gel Electrophoresis.** Lysates of K562 cells that had been treated with or without LY (10 μM) were prepared for two-dimensional electrophoresis. Lysates were prepared with cell lysis buffer containing 30 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 20 mM HEPES (pH 7.4), and 1% Triton X-100. Two-dimensional electrophoresis was performed according to the method of O'Farrell (7) by Kendrick Labs, Inc. (Madison, WI). After slab gel electrophoresis, the gel for blotting was transferred to transfer buffer [12.5 mM Tris (pH 8.8), 86 mM glycine, and 10% methanol] and transblotted onto polyvinylidene difluoride paper overnight at 200 mA and 100 V/two gels. After transfer, polyvinylidene difluoride membranes were stained with Coomassie Blue.

**Patient Samples.** Peripheral blood samples were obtained with appropriate consent and Institutional Review Board approval from CML patients at the University of California at Irvine. Mononuclear cells were obtained by centrifugation through Ficoll-Hypaque, washed twice in PBS, and cryopreserved. Western analysis was performed as described above.

**Tetrazolium-based Proliferation Assays (MTS assay).** Exponentially growing cells were plated in triplicate in 96-well flat-bottomed plates. Cells (20 × 10<sup>3</sup>) were plated in each well in a volume of 100 μl in RPMI with 10% FCS with imatinib (in PBS) at concentrations of 0, 1.0, 5.0, and 10.0 μM with or without rapamycin at 10 ng/ml. Because rapamycin was dissolved in alcohol, controls were also performed with cells incubated in 0.1% ethanol in media. Viability was determined at 48 h by incubating cells in 20 μl of CellTiter 96 AQueous One Solution reagent (Promega, Madison, WI) for 2 h at 37°C in the dark. An EMax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA) was used to read the absorbance at 490 nm. The absorbance of media alone without cells was subtracted from the reading of each well, and the values for the triplicates were averaged to obtain an absorbance for each condition.

## Results

**Identification of Bcr-Abl Kinase- and PI3K-dependent S/T Kinase Substrates in Bcr-Abl Cells.** The recent development of phosphospecific motif antibodies, combined with protein database searches, offers a new approach to identify protein kinase substrates in signaling pathways. We wished to determine whether one such antibody, an AKT phospho-substrate-specific antibody (AKT-pSub), could identify PI3K pathway substrates in Bcr-Abl-expressing cells. The AKT-pSub antibody was raised to a degenerate phosphopeptide of the AKT motif (RxRxxpT). It binds specifically to phosphorylated substrates of S/T kinases that recognize this motif (4). Cell lysates from Bcr-Abl-expressing cells were immunoblotted with the AKT-pSub antibody. To identify bands that corresponded to Bcr-Abl kinase- and PI3K-dependent substrates, lysates from cells treated with the Bcr-Abl kinase inhibitor imatinib and the relatively specific PI3K inhibitor LY294002 (LY) were analyzed in parallel. Multiple bands of identical size were detected in both murine and human Bcr-Abl-expressing cells (Fig. 1A). A prominent band of  $M_r$  28,000 to  $M_r$  32,000 apparent molecular weight was present in both Ba/F3-Bcr-Abl-WT and K562 CML cells, which became markedly attenuated on treatment with either imatinib or LY. Thus, the phosphorylation of this protein occurs in an Abl kinase- and PI3K-dependent manner. The known specificity of the AKT-pSub antibody suggested that the target for phosphorylation was a serine or threonine within the RxRxxS/T consensus motif.

**Identification of the  $M_r$  30,000 Phosphoprotein as Ribosomal Protein S6.** The Scansite program<sup>4</sup> allows protein sequence databases to be searched for motifs within proteins that are likely to be phosphorylated by specific protein kinases. The results of searches may be further

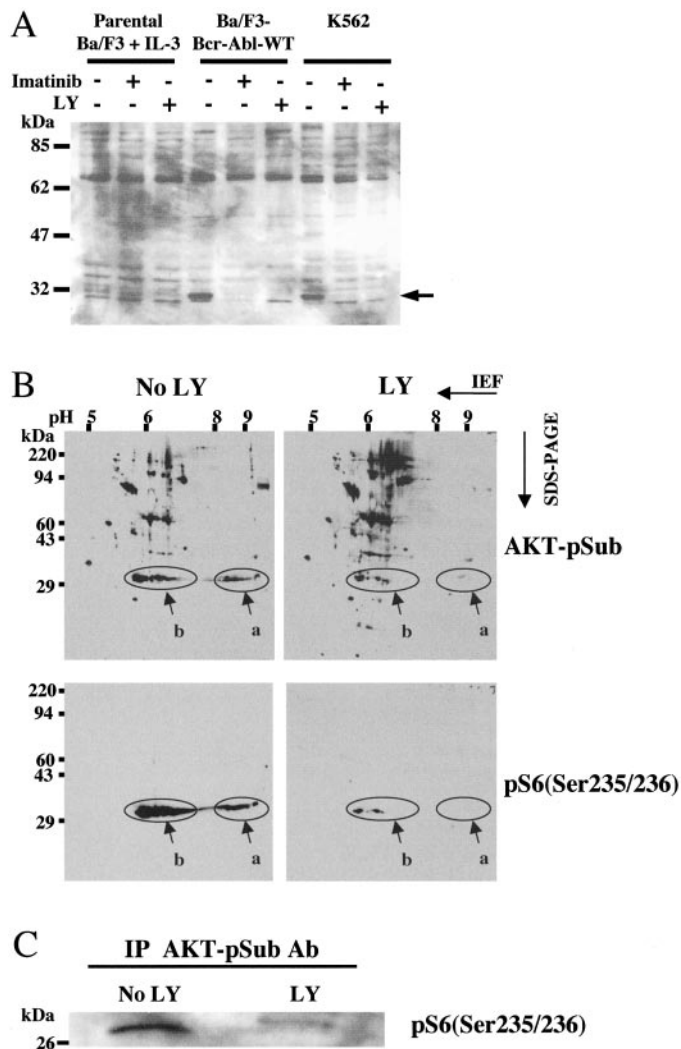


Fig. 1. The phosphospecific motif antibody AKT-pSub identifies ribosomal protein S6 as being phosphorylated in a Bcr-Abl kinase- and PI3K-dependent manner in CML cells. *A*, cell lysates were prepared from parental Ba/F3 grown in the presence of 10 ng/ml IL-3, Ba/F3-Bcr-Abl-WT, and K562 cells incubated with and without 10 μM imatinib or LY for 1 h. Proteins from these lysates were separated by SDS-PAGE and then immunoblotted with the AKT-pSub antibody. *B*, K562 cell lysates, treated without (*left*) or with (*right*) LY, were subjected to two-dimensional gel electrophoresis, followed by Western analysis with the AKT-pSub (*top*) and pS6(Ser235/236; *bottom*) antibodies. Untreated cell lysates (*top left*) demonstrate two prominent groups of phosphoproteins (*a* and *b*) in the range of  $M_r$  28,000 to 32,000. The membranes were stripped and then immunoblotted with the pS6(Ser 235/236) antibody. *C*, cell lysates from K562 cells, treated with and without LY, were immunoprecipitated with the AKT-pSub antibody and then analyzed by Western blotting with the pS6(Ser235/236) antibody.

focused based on the molecular weight and isoelectric point of the protein to be identified. We, thus, determined the isoelectric point of the  $M_r$  28,000 to  $M_r$  32,000 protein by two-dimensional gel electrophoresis on lysates from K562 cells treated with and without LY, followed by Western analysis with the AKT-pSub antibody (Fig. 1B). Two prominent groups of proteins at  $M_r$  30,000 (Fig. 1B, *top left*, circles *a* and *b*) were detected in the lysates from untreated K562 cells. Of these two, the more basic group with a pI between 8 and 11 was no longer detectable in the immunoblot of lysates from LY-treated K562 cells, suggesting that this group represented the LY-sensitive  $M_r$  30,000 protein (Fig. 1B, *top right*, circle *a*).

We searched the GenPept database using the Scansite program with the AKT substrate antibody matrix, limiting the results to proteins with a molecular mass of 28–32 kDa and a pI between 8 and 11. Because the  $M_r$  28,000 to  $M_r$  32,000 protein was found in both murine

<sup>4</sup> Internet address: <http://scansite.mit.edu>.

and human cells, we further narrowed the search to only those results that included both species. This resulted in three candidate substrates, as shown in Table 1. While we were conducting these experiments, another group also used the AKT-pSub antibody and found a similar sized  $M_r$  30,000 protein in ES cells that was phosphorylated in a PI3K-dependent manner; this protein was identified as ribosomal protein S6 (4). The Scansite program predicts that the AKT-pSub antibody would recognize the phosphorylated serine found within the RxRxxS/T consensus sequence at position 236 of S6. The 236 position of S6 ribosomal protein is known to be phosphorylated by p70 S6 kinase in a PI3K- and rapamycin-dependent fashion (8). The detection of S6 using an antibody designed to recognize phosphorylated AKT substrates is likely caused by very similar linear substrate recognition motifs shared between these two families of kinases.

To test whether the  $M_r$  30,000 protein in Fig. 1A was S6, we used the pS6(Ser235/236) antibody, which recognizes only the Ser235/236-phosphorylated form of S6 to probe the two-dimensional electrophoresis. The pS6(Ser235/236) antibody detected the same proteins in circles a and b in untreated K562 cells; in LY-treated cells, no phosphoS6 was observed in circle a, and some residual protein was observed in circle b (Fig. 1B, bottom). These results strongly suggested that the  $M_r$  30,000 protein detected by the AKT-pSub antibody was S6. S6 ribosomal protein is phosphorylated sequentially at Ser235, Ser236, Ser240, and Ser244 (9). As described previously, the pS6(Ser236/235) antibody recognizes two forms of phosphorylated S6: a higher mobility band corresponding to phosphorylation at Ser235/236 only, and a lower mobility band corresponding to phosphorylation at all four sites (10). It is, therefore, likely that the proteins in circles a and b correspond to phosphorylated and hyperphosphorylated S6, respectively, or that the protein species in circle b is a distinct posttranslational modification of S6. An alternative explanation is that the species observed in circle b is entirely distinct from S6. However, because subsequent experiments demonstrate that all AKT-pSub immunoreactive species detected in our Western blots disappear in the presence of rapamycin (see below), this band must necessarily be a downstream substrate of a kinase regulated by mTOR.

To confirm that phosphorylated S6 was indeed the  $M_r$  30,000 protein recognized by the AKT-pSub antibody, we performed immunoprecipitation with this antibody, followed by SDS-PAGE and Western blot analysis for phosphorylated S6, using lysates from untreated and LY-treated K562 cells (Fig. 1C). As expected, the pS6(Ser235/236) antibody detected a  $M_r$  30,000 band from untreated cells and a significantly less prominent band from LY-treated cells.

Phosphorylation of S6 at Ser235/236 is performed by two related kinases, S6K1 and S6K2, both of which are, in turn, activated (phosphorylated) by mTOR. This predicted that treatment of cells with the mTOR inhibitor rapamycin should block S6 phosphorylation. As expected, phosphorylation of the  $M_r$  30,000 protein was sensitive to rapamycin in both murine and human CML cells in immunoblots with either phosphoS6 or the AKT-pSub antibody (Fig. 2A). The levels of total S6 were not affected by imatinib or rapamycin treatment, indicating that the differences in S6 phosphorylation were not because of differences in total S6 (Fig. 2A). Interestingly, in K562 cells, despite complete inhibition of

Bcr-Abl kinase activity by imatinib (as measured by Bcr-Abl autophosphorylation on tyrosines), there was some residual S6 phosphorylation. This indicated that K562 cells have both Abl kinase-dependent and -independent mechanisms for S6 phosphorylation.

To further confirm the Bcr-Abl kinase-dependent nature of S6 phosphorylation, we performed immunoblot analysis of cell lysates from imatinib-resistant Ba/F3 cells that overexpress wild-type Bcr-Abl (Baf-BCR-ABL-R), as well as imatinib-resistant Ba/F3 cells containing a mutant imatinib-resistant form of Bcr-Abl (Ba/F3-Bcr-Abl-T315I). Cells were treated with 0  $\mu$ M, 1  $\mu$ M, or 10  $\mu$ M imatinib, with or without 10 ng/ml rapamycin. As expected, Baf-BCR-ABL-R cells contained phosphorylated S6 at 0  $\mu$ M imatinib, reduced phosphorylation at 1  $\mu$ M, and no phosphorylation at 10  $\mu$ M. In contrast, equal amounts of S6 phosphorylation in Ba/F3-Bcr-Abl-T315I cells was observed at all doses of imatinib, and they were inhibitable by rapamycin (Fig. 2B).

4E-BP1, a negative regulator of cap-dependent mRNA translation, is also phosphorylated by mTOR, but unlike S6 is inactivated by this modification (9). We, therefore, tested whether 4E-BP1 phosphorylation also occurred in CML cells in a Bcr-Abl kinase- and mTOR-dependent manner. We used an antibody to total 4E-BP1 that is able to detect unphosphorylated, phosphorylated, and hyperphosphorylated forms of 4E-BP1 as bands of decreasing mobility. We found that 4E-BP1 was constitutively phosphorylated in parental BaF/3 cells as well as in Ba/F3-Bcr-Abl-WT cells (Fig. 2C). However, a decrease in 4E-BP1 phosphorylation, after imatinib treatment, was only seen in Bcr-Abl-expressing cells, indicating that 4E-BP1 phosphorylation is dependent on Abl kinase activity. In parental Ba/F3 cells, which are IL-3 dependent, 4E-BP1 phosphorylation is likely a result of IL-3-mediated signaling (9). Furthermore, in Baf-BCR-ABL-R cells, increasing concentrations of imatinib resulted in a progressive shift to less phosphorylated 4E-BP1 forms. In contrast, imatinib did not affect Ba/F3-Bcr-Abl-T315I cells at all doses. In all cells, rapamycin resulted in an increase in unphosphorylated 4E-BP1 (Fig. 2C).

To determine whether S6 and 4E-BP1 are similarly regulated in primary CML cells, we performed Western analysis on peripheral blood mononuclear cells from a patient (CML-1) with cytogenetically confirmed CML. In this patient, we find that 4E-BP1 phosphorylation is inhibited by imatinib to the same extent as treatment with rapamycin and that phosphorylated S6 is at least, in part, dependent on Bcr-Abl-kinase activity (Fig. 2D).

These data demonstrate that two regulators of translation, ribosomal protein S6 and 4E-BP1, are constitutively phosphorylated in CML cells and that phosphorylation is dependent on Bcr-Abl and mTOR signaling. These results add mTOR to the list of AKT substrates activated in CML and suggest that targeting this intermediate in Bcr-Abl-expressing leukemias might be therapeutically useful. Because mTOR inhibitors are already in Phase I/II clinical trials for a number of cancers, these findings prompted us to determine whether targeting the mTOR pathway with the inhibitor rapamycin might inhibit growth of CML cells (11).

**The mTOR Inhibitor Rapamycin Enhances Imatinib-mediated Cell Killing.** In initial experiments, we found that rapamycin alone had a minimal or no effect on the viability of either parental Ba/F3 or

Table 1 Scansite results showing potential target proteins recognized by the AKT-pSub antibody

pI	Molecular weight	Predicted best substrates	Position	Sequence	Percentile <sup>a</sup>
10.7	28.8	Murine ribosomal S6	236	AKRRRLSSLRASTSK	0.087
10.7	28.8	Human ribosomal S6	236	AKRRRLSSLRASTSK	0.136
9.0	28.5	Murine cis-Golgi SNARE	230	NLRKRRDSLILGGVI	0.059
9.2	28.7	Human cis-Golgi SNARE	230	NLRKRRDSLILGGVI	0.097
9.9	31.0	Murine Kruppel-like factor 13	268	GGGSRTGSLSDYSRS	0.048
9.5	31.2	Human Kruppel-like factor 13	268	GGGSRTGSLSDYSRS	0.061

<sup>a</sup> The percentile reflects the overall score of the predicted site relative to every other serine and threonine scored in the GenPept database.

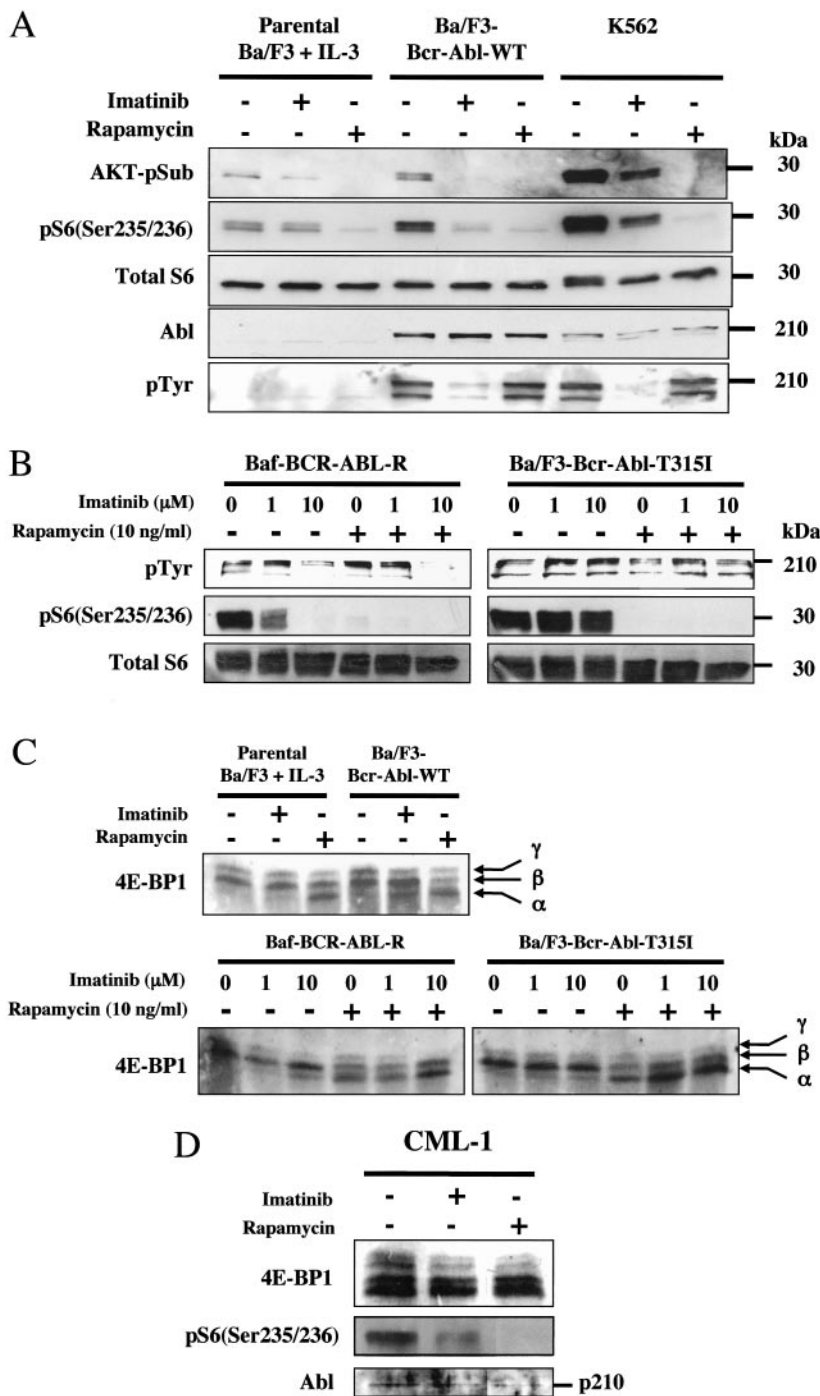


Fig. 2. S6 ribosomal protein and 4E-BP1 are constitutively phosphorylated in a Bcr-Abl kinase-dependent manner, and occurs via mTOR. *A*, cell lysates of untreated Ba/F3 cells grown in IL-3, Ba/F3-Bcr-Abl-WT, and K562 cells, and those treated with 10 μM imatinib or 10 ng/ml rapamycin, were subjected to Western analysis with the AKT-pSub and pS6(Ser235/236) antibodies. In the AKT-pSub and pS6(Ser235/236) panels, the *top* and *bottom* bands correspond to hyperphosphorylated and phosphorylated S6 ribosomal protein, respectively (see text). *B*, cell lysates were made from Baf-BCR-ABL-R and Ba/F3-Bcr-Abl-T315I cells treated with imatinib at 0, 1, and 10 μM for 1 h, with or without 10 ng/ml rapamycin. *C*, *top*, cell lysates were made from parental Ba/F3 cells and Ba/F3-Bcr-Abl-WT cells treated with imatinib and rapamycin and immunoblotted with anti-4E-BP1. This antibody detects unphosphorylated (α), phosphorylated (β), and hyperphosphorylated (γ) forms of 4E-BP1, as bands of decreasing mobility. *Bottom*, Baf-BCR-ABL-R and Ba/F3-Bcr-Abl-T315I cells were treated with increasing doses of imatinib in the presence or absence of rapamycin. *D*, cell lysates of untreated peripheral blood mononuclear cells from a patient with CML, CML-1, or those treated with 10 μM imatinib or 10 ng/ml rapamycin were subjected to Western analysis with anti-4E-BP1, anti-pS6(Ser235/236), and anti-Abl.

Ba/F3-Bcr-Abl-WT cells (Fig. 3A). Because imatinib induces apoptosis in CML cells, we asked whether rapamycin could improve the efficacy of imatinib-mediated cell killing. We found that the combination of rapamycin and imatinib enhanced the killing of Ba/F3-Bcr-Abl-WT cells but had no effect on parental Ba/F3 cells (Fig. 3, B and C). Accordingly, we wondered whether the combination would be able to overcome imatinib resistance in CML cells. Three forms of cellular resistance to imatinib have been described in patients: (a) amplification of the wild-type *Bcr-Abl* gene, resulting in overexpression of the oncoprotein; (b) resistance-conferring mutations in the Abl-kinase domain (12); and (c) LYN kinase overexpression (13). We tested the ability of the combination to overcome two of these forms of resistance by treating imatinib-resistant Ba/F3 cells overexpressing wild-type Bcr-Abl (Baf-BCR-ABL-R) and Ba/F3

cells expressing Bcr-Abl containing the T315I mutation (Ba/F3-Bcr-Abl-T315I) with rapamycin and imatinib. We found that the addition of rapamycin was able to overcome imatinib resistance in Baf-BCR-ABL-R cells at doses of imatinib as low as 1 μM. Baf-BCR-ABL-R cells are completely resistant to imatinib at this concentration and, in fact, are maintained at 1 μM imatinib (14). In contrast, Ba/F3-Bcr-Abl-T315I cells were completely resistant to imatinib and rapamycin (Fig. 3C).

Because previous studies by others have shown that IL-3 protects Bcr-Abl-positive cells from imatinib-induced apoptosis (15, 16), we wondered whether rapamycin would be able to overcome the protective effects of IL-3. We found that in Ba/F3-Bcr-Abl and Baf-BCR-ABL-R cells that rapamycin was not able to overcome IL-3-mediated rescue (Fig. 3D).

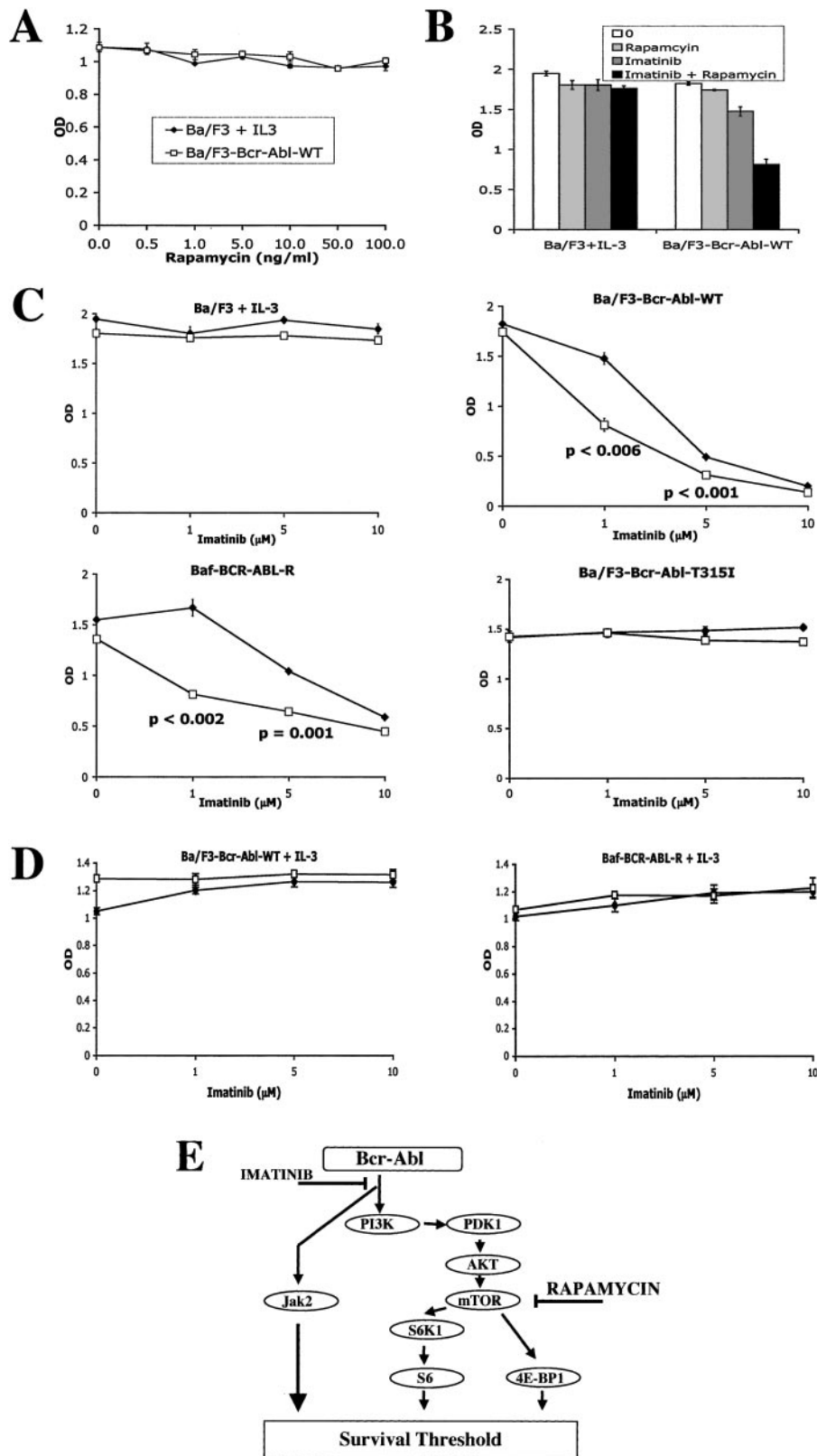


Fig. 3. Rapamycin enhances the ability of imatinib to kill CML cells. *A*, MTS assays were used to assess cell viability at 48 h after treatment of Ba/F3 cells grown in IL-3 or Ba/F3-Bcr-Abl-WT cells in increasing concentrations of rapamycin. Rapamycin has minimal effects on cell viability even at 100 ng/ml, a dose 10-fold higher than that required to inhibit S6 ribosomal protein phosphorylation. *B*, rapamycin (10 ng/ml) in combination with 1 μM imatinib enhances the ability of imatinib to inhibit growth of Ba/F3-Bcr-Abl-WT cells but has no effect on parental Ba/F3 cells. *C*, dose-response curves showing that rapamycin increases the sensitivity of Ba/F3-Bcr-Abl-WT to imatinib and overcomes imatinib resistance in Baf-BCR-ABL-R, but has no effect on Ba/F3-Bcr-Abl-T3151 cells. *Filled and open symbols* represent cells treated without and with rapamycin at 10 ng/ml, respectively. *Bars* indicate the SE. *D*, MTS assays for Ba/F3-Bcr-Abl-WT and Baf-BCR-ABL-R were repeated with the addition of 10 ng/ml IL-3. *E*, proposed signaling pathways mediating mTOR signaling in Bcr-Abl-expressing cells and accounting for the MTS results. *PDK1*, 3-phosphoinositide-dependent protein kinase 1.

**Discussion**

Our findings validate the approach of using kinase substrate-specific antibodies to identify dysregulated signaling pathways in Bcr-Abl-expressing cells. When combined with a Web-based bioinformatics program, this approach has allowed us to identify ribosomal protein S6 as a prominent phosphorylated protein in murine and human CML cells. Furthermore, the specificity of the AKT-pSub antibody for the RxRxxS/T consensus motif allowed the phosphorylation site within S6 to be predicted, and subsequently confirmed. By using a panel of specific inhibitors, we have also been able to identify

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the signaling pathways that control phosphorylation of S6 in CML and demonstrate that it is dependent on Bcr-Abl kinase, PI3K, and mTOR activities. The generation of different phosphospecific motif antibodies recognizing other kinase substrates may allow other signaling pathways in CML to be queried in a similar manner. Because abnormal protein phosphorylation occurs in multiple other cancers and disease states, this approach is likely to be useful in profiling abnormal signaling in many human disorders. As demonstrated here, data gleaned from such screens may help in identifying novel pathways suitable for therapeutic targeting. Clearly, these bioinformatics-based approaches are dependent on proteins already being in databases, along with information regarding their molecular weights, isoelectric points, species specificity, and so forth.

The present study demonstrates that the mTOR pathway is constitutively activated by Bcr-Abl in CML cells and that two of its known substrates, ribosomal protein S6 and 4E-BP1, are constitutively phosphorylated in a Bcr-Abl-dependent manner. mTOR acts as a nutrient and mitogen sensor to positively regulate translation and ribosome biogenesis (17). On activation by mitogens and amino acids, mTOR phosphorylates two key translational regulators: S6K1 and 4E-BP1. S6K1 activation leads to the phosphorylation of the ribosomal protein S6. S6 is important in the translation of mRNAs bearing a 5' terminal oligopolypyrimidine tract (9), such as ribosomal proteins, elongation factors, and growth factors (8). 4E-BP1, in contrast, is a negative regulator of translation. It is phosphorylated in response to growth signals; on phosphorylation, 4E-BP1 dissociates from eIF4E, the translation initiation factor that binds the 5' methyl cap structure on mRNAs, allowing translation to proceed. eIF4E function is particularly important for a subset of genes that are poorly translated in resting cells but are recruited to ribosomes after a proliferative signal (9). The differing translation efficiencies for these mRNAs seem to be a result of a highly structured 5' untranslated region, or the presence of multiple open reading frames, both of which inhibit efficient scanning (9).

Examples of genes with long, highly structured 5' untranslated regions that are translationally regulated by mTOR include *c-myc* and *cyclin D1* (18, 19). Of note, both of these genes have been implicated in the pathogenesis of CML and are essential for Bcr-Abl-mediated transformation (20, 21). Our finding that both ribosomal protein S6 and 4E-BP1 are phosphorylated in a Bcr-Abl kinase-dependent manner in CML cells suggests that deregulated Abl kinase activity leads to activation of critical genes such as *c-myc* and *cyclin D1* by altering their translational control. Post-transcriptional mechanisms mediating Bcr-Abl leukemogenesis have been reviewed recently (22), although the main focus was on the ability of Bcr-Abl to increase the stability of RNA binding proteins involved in regulating translation. Whereas mechanisms and signaling pathways mediating the effect of Bcr-Abl on protein stability remain ill-defined, PI3K signaling has been implicated (23). Thus, Bcr-Abl may signal through PI3K to affect target gene expression post-transcriptionally, both at the level of translational efficiency and protein stability.

How might rapamycin enhance imatinib-mediated killing of CML cells? The combination of imatinib and rapamycin had minimal effect on viability in Ba/F3-Bcr-Abl-T315I cells but was effective in increased killing of Baf-BCR-ABL-R cells (Fig. 3C). This suggests that the combination of imatinib and rapamycin acts to inhibit signals distal to Bcr-Abl and mTOR below a critical threshold required for viability. A model to explain the results is that there are two parallel Bcr-Abl-dependent pathways transmitting survival signals: one that acts via mTOR, and another that is independent of mTOR (Fig. 3E). These two pathways then converge on an integrator of survival signals that transmits such signals only when a threshold level is reached. Thus, inhibition of mTOR alone would have no effect on survival, if

the survival threshold is achieved by mTOR-independent signaling. Conversely, inhibition of Bcr-Abl kinase by imatinib, by affecting both pathways, would lower the signals below the survival threshold, resulting in death. In situations when imatinib is able to partially reduce mTOR-independent signaling, mTOR inhibition could then further reduce the integrated signal below the threshold for survival. In addition, our observation that IL-3 is able to rescue the effects of rapamycin indicates that intermediates in the mTOR-independent pathway might include Jak2 and *c-myc*. This is suggested by the work of Xie *et al.* (16), who have shown that Jak2 is a key mediator of the effects of Bcr-Abl on the IL-3 receptor pathway and is associated with increased *c-myc* expression. The differential effect of the combined treatment on the viability of Ba/F3-Bcr-Abl-WT, Baf-BCR-ABL-R, and Ba/F3-Bcr-Abl-T315I cells is reminiscent of the findings of Hoover *et al.* (24), using the combination of the farnesyl transferase inhibitor SCH66336 and imatinib. These investigators invoked a similar model to explain their findings (24). After the initial report of the T315I kinase domain mutation (which confers a high level of imatinib resistance) in patients with imatinib resistance, several other mutations have been described that confer a moderate degree of imatinib resistance (12). If the threshold model is true, then the combination of rapamycin (or SCH66336) and imatinib may be able to overcome resistance in these patients as well.

In summary, phosphospecific motif antibodies and newly available Web-based bioinformatic programs facilitate the proteomic identification of novel kinase substrates, and can lead to the elucidation of signaling pathways in human malignancies that may be clinically exploitable.

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