Activation of Src Kinases p53/56<sup>lyn</sup> and p59<sup>hck</sup> by p210<sup>bcr/abl</sup> in Myeloid Cells<sup>1</sup>

Susanne Danhauser-Riedl, Markus Warmuth, Brian J. Druker, Bertold Emmerich, and Michael Hallek<sup>2</sup>

Medizinische Klinik, Klinikum Innenstadt, Universität München, Ziemssenstrasse 1, D-80336 München, Germany (S. D.-R., M. W., B. E., M. H.), and Oregon Health Sciences University, Portland, Oregon 97201-3098 [B. J. D.]

ABSTRACT

Chronic myeloid leukemia is characterized by the Philadelphia (Ph<sup>1</sup>) translocation t(9;22) that generates a hybrid gene, bcr/abl, translated to a M<sub>5</sub>210,000 tyrosine kinase (p210<sup>bcr/abl</sup>) with transforming activity for hematopoietic cells. Hematopoietic cell transformation by p210<sup>bcr/abl</sup> seems to involve activation of the Ras signaling pathway by at least two different signaling intermediates, growth factor receptor-bound protein 2 and Src homology and collagen protein, but additional signaling proteins are likely to be required as well. In an effort to identify additional phosphoproteins activated by p210<sup>bcr/abl</sup>, we studied the murine, interleukin 3-dependent, myeloid cell line, 32D, and a bcr/abl-transformed, factor-independent subline, 32Dp210. The analysis of whole-cell lysates of 32D and 32Dp210 cells showed that several proteins with a molecular weight of M<sub>5</sub>50,000—60,000 were phosphorylated on tyrosine residues in 32Dp210 cells. Because Src family kinases have an apparent molecular weight of M<sub>5</sub>50,000—60,000, we asked whether they could become activated by p210<sup>bcr/abl</sup>. Two Src family kinases, p53/56<sup>lyn</sup> and p59<sup>hck</sup>, showed a severalfold higher phosphokinase activity in 32Dp210 cells than in 32D cells. Commounprecipitation experiments with anti-Lyn, anti-Hck, and anti-Abl antibodies demonstrated an intracellular association of p210<sup>bcr/abl</sup> with p53/56<sup>lyn</sup> and p59<sup>hck</sup>. Moreover, the phosphokinase activity of p53/56<sup>lyn</sup> was higher in bcr/abl-positive myeloid cell lines (K562, BV173, and LAMAB4) than in the bcr/abl-negative myeloid cell line JOSK-M. In conclusion, the results show that p210<sup>bcr/abl</sup> induces the activation of at least two Src family kinases, p53/56<sup>lyn</sup> and p59<sup>hck</sup>, in myeloid cells. These findings extend the range of potential targets of p210<sup>bcr/abl</sup> that might mediate its transforming effects.

INTRODUCTION

The Philadelphia translocation t(9;22) (Ph<sup>1</sup>) found in >95% of the patients. It creates a hybrid gene by fusing the 5<sup>'</sup> end of c-abl on the long arm of chromosome 9 to the bcr gene on chromosome 22 (1, 2). The resulting fusion gene, bcr/abl, plays a pivotal role in the pathogenesis of CML. bcr/abl generates a chimeric protein of M<sub>5</sub>210,000, p210<sup>bcr/abl</sup>, which, in contrast to its normal counterpart p145c<sub>abt</sub>, is located in the cytoplasm and has a high, constitutive tyrosine kinase activity (3).

It is thought that the p210<sup>bcr/abl</sup> kinase acts, at least in part, through the (constitutive) phosphorylation and stimulation of cellular signaling proteins, which regulate cell growth, shape, and survival. These proteins include growth factor receptors like c-Kit (4), cytoskeletal proteins like paxillin (5), or downstream signaling proteins like GAP, Shc, Grb2, mitogen-activated protein kinase, CRKL, Crk, phosphatidylase C-y1, phosphotidysinositol 3-kinase, or the 14-3-3 family proteins, which regulate cell growth, shape, and survival. Some Src family kinases have an apparent molecular weight of M<sub>5</sub>50,000-60,000, protein of Mr 210,000, p210<sup>bcr/abl</sup>, which, in contrast to its normal counterpart p145c<sub>abt</sub> is located in the cytoplasm and has a high, constitutive tyrosine kinase activity (3).

Therefore, understanding the pathogenetic principle of this transforming protein requires a detailed investigation of Abl kinase substrates and the regulation of Abl kinase activity (6). Some Src family kinases are activated by various cytokines including IL-3, granulocyte/macrophage-colony-stimulating factor, and IL-6, which are important growth factors for early myeloid progenitors (15–18). Since p210<sup>bcr/abl</sup> is able to replace some of the growth factor requirements of myeloid cells (13, 19), signaling proteins that become activated by hematopoietic growth factors are prime candidates for p210<sup>bcr/abl</sup> substrates. This led us to ask whether p210<sup>bcr/abl</sup> would activate Src family kinases. It has been shown previously that one of these Src family kinases, p53/56<sup>lyn</sup>, becomes activated by IL-3 in myeloid cells (15, 16). Therefore, we decided to focus on the activation of this kinase by IL-3 or p210<sup>bcr/abl</sup> in the murine factor-dependent myeloid cell line, 32D. We could demonstrate that both IL-3 and p210<sup>bcr/abl</sup> activated p53/56<sup>lyn</sup>. We then screened for the activation of other Src family kinases and found that the phosphokinase activity of p59<sup>hck</sup> was also increased by p210<sup>bcr/abl</sup>. The activation of p53/56<sup>lyn</sup> and p59<sup>hck</sup> seemed to involve a (direct or indirect) intracellular association with p210<sup>bcr/abl</sup>, since both Src family kinases could be communoprecipitated with p210<sup>bcr/abl</sup>.

MATERIALS AND METHODS

Reagents and Antibodies. Reagents for cell lysis were all purchased from Sigma Chemical Co. (Deisenhofen, Germany). Ingredients for SDS-PAGE were purchased from Bio-Rad (München, Germany). Recombinant IL-3 was purchased from Genzyme (Rüsselheim, Germany). The mAb against phosphotyrosine residues, 4G10, was prepared and used as described previously (20). The polyclonal Abs against Lyn (44), Hck (N30), and Fyn (3), as well as the anti-Abl mAb 24-11 and the corresponding blocking peptides representing amino acids 44—63 of Lyn and 8—37 of Hck were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal anti-Abl antibody, Ab3, was purchased from Oncogene Sciences (Uniondale, NJ). For immunoblotting, the primary Abs were used at the following dilutions: anti-Lyn 44, 1:250 to 1:1000; anti-Abl 24-11, 1:500; and anti-phosphotyrosine 4G10, 1:2000. Secondary Abs were either purchased from Bio-Rad (coupled with alkaline phosphatase) or Amersham (horseradish peroxidase-coupled goat antimouse mAb; ECL detection system).

Cells and Cell Culture. 32Dc13 (32D) cells were obtained from Dr. James D. Griffin (Dana-Farber Cancer Institute, Boston, MA). K 562, LAMA-84, BV 173, and JOSK-M were cultured in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (Biochrom) and 10–20% of WEHI-3 conditioned medium to provide murine IL-3. 32Dp210 cells as well as the CML cell lines K 562, LAMA-84, BV 173, and JOSK-M were cultured in RPMI 1640 containing 10% FCS. Transfected cells were selected with G418 (1 mg/ml).

Plasmids and Transfection. The full-length bcr/abl cDNA in the expression vector pGPD was transfected by Dr. G. Daley, Boston (21). The bcr/abl plasmid, pGDP210, was introduced into 32Dc13 cells by electroporation as described (11).

Cell Lysis and Stimulation. For cell lysis, appropriate numbers of exponentially growing cells were washed twice in ice-cold PBS (Life Technologies, Inc., Eggenstein, Germany) to remove remaining serum and growth factors. Thereafter, 1 × 10<sup>7</sup> cells were lysed in 100 μl lysis buffer containing 1% Brij96, 20 mm/liter Tris (pH 8.0), 50 mm/liter NaCl, and 10 mm/liter EDTA as well as 1 mm/liter phenylmethylsulfonyl fluoride, 10 μg/ml.
aprotinin, 10 μg/ml leupeptin, and 2 mmol/liter sodium orthovanadate for 25 min at 4°C. Thereafter, unsoluble material was removed by centrifugation at 15,000 × g at 4°C for 15 min. The protein concentration was checked by a protein assay (Bio-Rad). In some experiments, cells were factor deprived prior to lysis by incubation in medium containing only 5% FCS but no growth factors. Before stimulation with rmIL-3, 32D cells were starved for 15 h in FCS and growth factor-free medium containing 1% BSA (Sigma). Thereafter, cells were washed Once in PBS, adjusted to a concentration of 1 × 10⁷ cells per ml, and preincubated at 37°C for 30 min prior to stimulation with 50 units/ml rmIL-3 (Genzyme). Stimulation was terminated by adding ice-cold PBS and subsequently pelleting the cells.

**Gel Electrophoresis and Immunoblotting.** For immunoblots, lysates containing approximately 150 μg of cellular proteins were boiled for 5 min in 1X SDS sample buffer (20% mercaptoethanol, 125 mmol/liter Tris/Cl, 2% SDS, 20% glycerol, and bromphenol blue and resolved by 7.5% SDS-PAGE. Proteins were electrophoretically transferred onto Immobilon P membranes (Millipore, Eschborn, Germany). Unspecific protein binding to these membranes was blocked by incubating for 1 h in TBS [10 mmol/liter Tris base (pH 8.0) and 150 mmol/liter NaCl] containing 5% BSA (for anti-phosphotyrosine blots) or 5% skim milk powder (for all other Abs). Thereafter, blots were incubated with the primary Ab in TBS containing 5% BSA and 0.02% sodium azide. The concentration of primary Abs varied from 1:250 to 1:2500 (see above). Immunoblots with the mAb 4G10 were developed by using alkaline phosphatase-conjugated secondary Abs at a dilution of 1:2000 in TBS. Blots were developed in developing buffer [0.1 mol/liter Tris (pH 9.5), 100 mmol/liter NaCl, and 5 mmol/liter MgCl₂], nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). For blotting with all other Abs, secondary Abs were horseradish peroxidase-conjugated goat Abs used at a dilution of 1:2000 in TBS containing 5% BSA and 0.02% sodium azide; the ECL detection system (Amersham, Braunschweig, Germany) was used according to the guidelines of the manufacturer to visualize the proteins.

**Immunoprecipitation.** IPs were performed with lysates containing approximately 1 mg of cellular proteins, adjusted to a final volume of 600 μl by adding appropriate amounts of IP buffer [0.1% Brij/6, 20 mmol/liter Tris (pH 8.0), 50 mmol/liter NaCl, 10 mmol/liter EDTA, 1 ml/liter phenylmethylsulfonl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mmol/liter sodium orthovanadate]. Lyn, Hck, and Bcr/Abl were precipitated by adding 5 μg of the polyclonal Abs Lyn 44 or Hck N30 or the anti-Abl mAb 24-11, respectively. Following 3 h of incubation at 4°C on a rotating plate, 125 μl of Sepharose 4A beads (Pharmacia Biotech Inc., Freiburg, Germany) diluted 1:1 in IP buffer were added to each sample. For the Ab 24-11, Sepharose G beads were used. After 2 additional h of incubation at 4°C, the precipitates were washed four times with IP buffer, resuspended in 50 μl of IP buffer, and subsequently boiled in 2× sample buffer before loading on SDS gels.

For peptide blocking experiments, Abs and a 10-fold excess of blocking peptide (i.e. 10 μg of peptide per 1 μg of Ab) were preincubated for 2 h at room temperature under permanent agitation. Thereafter, this antibody-peptide mixture was incubated with cell lysates as indicated above.

**Immune Complex Kinase Assay and Reimmunoprecipitation.** For immune complex kinase assays, cell lysis and IP was performed as described above. However, the precipitates were washed three times in IP buffer and once in kinase buffer 1 (K1) containing 50 mmol/liter Tris (pH 7.4) and 10 mmol/liter MnCl₂. For the kinase reaction, the pellet was resuspended in 50 μl kinase buffer containing 10 μCi of [γ-3²P]ATP (Amersham) and incubated at room temperature. The kinase reaction was stopped after 15 min by adding 50 μl SDS sample buffer and boiling the sample for 5 min at 100°C. The reaction was analyzed by separating the proteins by SDS-PAGE and autoradiography on ECL films (Amersham).

In some experiments, enolase was used as an exogenous substrate for Src family kinases (p53/56kN and p59kN). For these experiments, a different kinase buffer (K2) containing 100 mmol/liter HEPES (pH 7.0) and 10 mmol/liter MnCl₂ was used (17). Enolase (Boehringer Mannheim) was prepared by resuspending 20–40 μg of enolase in 20 μl of enolase preparation buffer (50 mmol/liter HEPES (pH 7.0), 0.1 mmol/liter DTT, and 10 mmol/liter MgCl₂) and an equal volume of 25 mmol/liter acidic acid. This reaction mixture was heated for 15 min at 70°C and neutralized by the addition of 1 ml of K2 buffer. Fifty μl of the neutralized enolase preparation were mixed with 10 μCi of [γ-3²P]ATP and used to determine the kinase activity of Lyn and Hck precipitates. The kinase reaction was performed as described above.

For re-immunoprecipitation, the kinase reaction was performed in K1 buffer and terminated after 30 min by washing the precipitates twice in 500 μl lysis buffer. The pellets were then resuspended in 40 μl of disrupting buffer [20 mmol/liter Tris (pH 8.0), 0.5% SDS, and 1 mmol/liter DTT] and heated for 5 min at 100°C. Following centrifugation, 20 μl of the supernatant were diluted in 180 μl lysis buffer, thus reducing the SDS concentration to 0.05%. The remaining, undiluted supernatant was prepared for gel electrophoresis by adding sample buffer as described above. The diluted supernatant (volume, 200 μl) was re-immunoprecipitated with a second Ab for 5 h, with Sepharose A or G beads added for the last 2 h of incubation. The secondary precipitates were washed four times in lysis buffer, prepared for SDS-PAGE, and loaded on SDS gels, together with the primary precipitates as described above.

**RESULTS**

**Stable Transfection of the Myeloid Cell Line 32D with the bcr/abl Gene Induces the Tyrosine Phosphorylation of Multiple Cytosolic Phosphoproteins.** To establish a model system for the study of signaling events induced by p210bcr/abl, the murine, myeloid, IL-3-dependent cell line 32D was transfected with the plasmid pGD210 as described in “Materials and Methods.” The resulting cell line 32Dp210 stably expressed the p210bcr/abl kinase; it could be grown with G418 (1 mg/ml) and without the addition of growth factors as described (11, 13). To investigate the effects of p210bcr/abl on tyrosine phosphorylation of cellular proteins, exponentially growing 32D and 32Dp210 cells were starved for 15 h in media containing 5% FCS without rmIL-3 or conditioned media; thereafter, cells were lysed as described in “Materials and Methods,” and cytosolic proteins were extracted and resolved by 7.5% SDS PAGE. Tyrosine phosphorylation of proteins was assessed by immunoblotting with the 4G10. Fig. 1 demonstrates that 32Dp210 cells displayed dramatically increased levels of tyrosine phosphorylation of more than 20 cellular proteins as compared with 32D cells. At least 20 different, distinct bands were stained by the 4G10 Ab. The apparent molecular weights of these bands were M, 38,000, M, 40,000, M, 42,000, M, 44,000, M, 48,000, M, 50,000, M, 52,000, M, 53,000–57,000, M, 60,000, M, 64,000, M, 72,000, M, 84,000, M, 86,000, M, 90,000, M, 93,000, M, 110,000, M, 117,000, M, 130,000, M, 145,000, M, 180,000, M, 205,000, M, and 210,000 (Fig. 1). Interestingly, phosphorylated protein(s) with an apparent molecular weight of M, 50,000–60,000 were observed, suggesting that Src family kinases known to migrate at this molecular weight might become phosphorylated.

Expression of the p210bcr/abl Kinase in 32D Cells Increases the Kinase Activity of p53/56kN. The Src family kinase Lyn is preferentially expressed in myeloid cells and becomes activated in response to stimulation with IL-3 (15, 16). Since signaling pathways stimulated by IL-3 and p210bcr/abl show a strong overlap suggesting that p210bcr/abl might bypass some of the signaling pathways stimulated by IL-3 (13), we asked whether Lyn was activated by p210bcr/abl. For this purpose, 32D or 32Dp210 cells were lysed and subjected to IP with anti-Lyn 44 and anti-Abl 24-11 Abs to purify Lyn and Bcr/Abl. The immunoprecipitates were incubated with a radiolabeled phosphor (γ-3²P]ATP) to assess the kinase activity of the purified protein complexes. Proteins phosphorylated by this in vitro kinase reaction were resolved by 7.5% SDS-PAGE. Fig. 2A demonstrates that the anti-Lyn Ab 44 allowed to precipitate two proteins of M, 53,000 and M, 56,000, presumably p53/56kN and p56/58kN, the major known isoforms of this kinase. Coimmunoprecipitation with a specific blocking peptide (see “Materials and Methods”) allowed the inhibition of IP of both proteins with this Ab, thus confirming the identity of these proteins. In 32Dp210 cells, autophosphorylation of p53/56kN and p56/58kN was higher than in 32D cells (Fig. 2A). Immune complex kinase assays with the Ab anti-Abl 24-11 resulted in the purification of a M, 210,000 protein, presumably p210bcr/abl, which was detectable in
32Dp210 cells (Fig. 2A) but not in 32D cells (data not shown). Several other proteins coprecipitated with p210bc/abl. Interestingly, one of these coprecipitating proteins had an apparent molecular weight of M, 56,000 and comigrated with p56<sup>tyr</sup> on the gel. In addition, longer exposure of the autoradiographs of anti-Lyn immunocomplex kinase reactions revealed that a M, 210,000 protein coprecipitated with Lyn in 32Dp210 cells (data not shown).

To assess whether equal amounts of Lyn proteins were used in the kinase reactions, aliquots of the immunoprecipitates were removed and analyzed by anti-Lyn immunoblotting with the anti-Lyn Ab 44. Fig. 2B shows that equal amounts of p56<sup>tyr</sup> were precipitated; the analysis of the M, 53,000 isoform in anti-Lyn IPs was hampered by comigration of immunoglobulin heavy chains. Therefore, the content of p53/56<sup>tyr</sup> in total cell lysates of 32D and 32Dp210 cells was analyzed as additional control. Fig. 2B (upper panel), equal amounts of p56<sup>tyr</sup> were precipitated by the anti-Lyn Ab; the analysis of the M, 53,000 isoform was again difficult due to comigration of immunoglobulin heavy chains. Therefore, the content of p53/56<sup>tyr</sup> in total cell lysates of 32D and 32Dp210 cells resulted only in a subtle additional increase of Lyn autophosphorylation (Fig. 3A). To assess whether equal amounts of Lyn proteins were used in the in vitro kinase reactions, aliquots of the immunoprecipitates were removed and analyzed by anti-Lyn immunoblotting with the anti-Lyn Ab 44. As shown in Fig. 3B (upper panel), equal amounts of p56<sup>tyr</sup> were precipitated by the anti-Lyn Ab; the analysis of the M, 53,000 isoform was again difficult due to comigration of immunoglobulin heavy chains. Therefore, the content of p53/56<sup>tyr</sup> in total cell lysates of 32D and 32Dp210 cells was analyzed as additional control. Fig. 3B (lower panel) demonstrates that neither rmIL-3 nor p210bc/abl changed the expression of p53/56<sup>tyr</sup> in 32D or 32Dp210 cells.

Three Human, bcr/abl-positive Cell Lines Show Increased Levels of p53/56<sup>tyr</sup> Kinase Activity. To substantiate the relevance of our findings, in particular with regard to the pathogenesis of human CML, the kinase activity of Lyn purified from Ph<sup>+</sup>-positive and Ph<sup>-</sup>negative myeloid cell lines was determined. Most cell lines were established from bcr/abl-positive myeloid leukemia patients (BV173, LAMA-84, and K562), with the exception of JOSK-M cells. Fig. 4A shows an anti-phosphotyrosine immunoblot of whole-cell lysates of these cell lines. Levels of tyrosine phosphorylation were elevated in the three bcr/abl-positive cell lines as compared with JOSK-M cells. Interestingly, distinct bands of approximately M, 53,000 to M, 56,000 became visible on the blot that were phosphorylated on tyrosine residues. This suggested that proteins with a molecular weight similar to p53/56<sup>tyr</sup> were phosphorylated on tyrosine residues. Therefore, anti-Lyn immune complex kinase assays were performed. As shown in Fig. 4B, the three Ph<sup>+</sup>-positive cell lines displayed elevated levels of Lyn autokinase activity when compared to JOSK-M cells. The two bands detected in Lyn in vitro kinase assays in the four different cell lines (Fig. 4B, left panel) comigrated with two bands detected by the anti-Lyn Ab on immunoblots (Fig. 4B, right panel). Moreover, the levels of p53/56<sup>tyr</sup> precipitated were identical in all four cell lines (Fig. 4B, right panel), demonstrating that the differences in kinase activity did not result from different amounts of purified Lyn protein. Taken together, the results suggest that the activation of p53/56<sup>tyr</sup> might also be relevant in human bcr/abl-positive CML cells.

p210bc/abl Also Activates the Src Family Kinase p59<sup>hck</sup>. After having established that p53/56<sup>tyr</sup> kinase was activated in bcr/abl-positive myeloid cells, we investigated whether additional members of the Src kinase family were activated by p210bc/abl. Therefore, we screened the expression of all Src family kinases in 32D and 32Dp210 cells by in vitro kinase assays and immunoblots and found that, in addition to Lyn, the Src family kinases Hck, Fyn, and Yes were expressed in 32D cells (data not shown). Of these kinases, only Hck was expressed at sufficiently high levels to study its interaction with p210bc/abl in 32Dp210 cells in more detail. A M, 59,000 protein could be precipitated from both cell lines with the anti-Hck Ab, and this

hc/abl KINASE ACTIVATES Src KINASES
bcr/abl KINASE ACTIVATES Sec KINASES

Fig. 2. Activation of p53/56\textsuperscript{yr} by rmIL-3 or p210\textsuperscript{bcr/abl}. A, immune complex kinase assays following immunoprecipitation of 32D or 32Dp210 cell lysates with anti-Lyn Ab 44 and protein A beads (a-lyn), with anti-Lyn Ab, Lyn blocking peptide, and protein A beads (a-lyn + peptide), with protein A beads alone (beads), and with anti-Abl Ab 24-11 and protein G beads (a-abl). For details, see "Materials and Methods." Molecular weight markers are indicated on the right. B, aliquots of the anti-Lyn immunoprecipitates shown in A were used for immunoblotting with the anti-Lyn Ab 44 to assess the amount of p53/56\textsuperscript{yr} precipitated. Similar amounts of p53/56\textsuperscript{yr} were precipitated in each lane. C, aliquots of the anti-Lyn immunoprecipitates shown in A were used to assess the phosphorylation of the Lyn substrate enolase in 32D and 32Dp210 cells. D, aliquots of the anti-Lyn immunoprecipitates shown in A were used to assess the tyrosine phosphorylation of p53/56\textsuperscript{yr} in 32D and 32Dp210 cells.

protein was recognized by the anti-Hck Ab on immunoblots (Fig. 5, right panel). We then performed in vitro kinase assays of anti-Hck and anti-Abl IPs in 32D and 32Dp210 cells. Fig. 5 (left panel) shows that a M\textsubscript{r} 59,000 protein, presumably p59\textsuperscript{hck}, was strongly phosphorylated in 32Dp210 cells but not in 32D cells. Several other proteins of M\textsubscript{r} 79,000, M\textsubscript{r} 84,000, M\textsubscript{r} 117,000, M\textsubscript{r} 190,000, and M\textsubscript{r} 210,000 were also found in the anti-Hck immune complexes, showing that p59\textsuperscript{hck} was present in a complex of several signaling proteins. The addition of the substrate enolase to the kinase reaction showed that p59\textsuperscript{hck} substrate phosphorylation was strongly increased in 32Dp210 cells as compared with 32D cells, similar to our observation with p53/56\textsuperscript{yr} kinase reactions (Fig. 5, left panel). The addition of an excess of a specific Hck peptide was able to block the IP reaction completely, demonstrating the specificity of the Ab used. Anti-Abl kinase reactions were again performed in parallel. The comparison of these kinase reactions revealed that the M\textsubscript{r} 210,000 protein copurified in Hck IPs comigrated with p210\textsuperscript{bcr/abl} on SDS gels, and that the a M\textsubscript{r} 59,000 protein copurified in Abl IPs comigrated with p59\textsuperscript{hck} (Fig. 5). Since these data suggested the coprecipitation of p210\textsuperscript{bcr/abl} with p59\textsuperscript{hck}, this possibility was tested in additional experiments.

p53/56\textsuperscript{yr} and p59\textsuperscript{hck} Coprecipitate with p210\textsuperscript{bcr/abl}. The p210\textsuperscript{bcr/abl} kinase forms complexes with multiple substrates, and binding of these signaling proteins appears to be critical for some transforming effects. Therefore, we asked next whether the activation of p53/56\textsuperscript{yr} or p59\textsuperscript{hck} observed in 32Dp210 cells involved an intracellular association of p53/56\textsuperscript{yr} or p59\textsuperscript{hck} with p210\textsuperscript{bcr/abl}. Whole-cell lysates of 32D and 32Dp210 cells were purified by anti-Hck IP (Fig. 6A). This allowed us to concentrate equal amounts of p59\textsuperscript{hck}, as demonstrated by the appearance of a single M\textsubscript{r} 59,000 band on anti-Hck immunoblots (Fig. 6A, lower panel). The addition of Hck peptide was able to block the anti-Hck IP completely. The analysis of IP-purified p59\textsuperscript{hck} complexes by anti-Abl immunoblotting showed a distinct band of M\textsubscript{r} 210,000, which was detectable only in 32Dp210 cells but not in 32D cells. Blocking the IP reaction with Hck peptide also abrogated the detection of p210\textsuperscript{bcr/abl} in anti-Hck precipitates, demonstrating that p210\textsuperscript{bcr/abl} was present in p59\textsuperscript{hck} protein complexes (Fig. 6A, upper panel).

Since the coprecipitation of p53/56\textsuperscript{yr} and p210\textsuperscript{bcr/abl} could not be demonstrated by simple IP and subsequent immunoblotting (data not shown), we had to choose a more sensitive technique (22). For this purpose, anti-Lyn immunoprecipitates from 32Dp210 cells were subjected to an in vitro kinase assay; the resulting reaction was treated with disruption buffer (see "Materials and Methods") to dissociate protein complexes and then re-precipitated with secondary Abs, either anti-Lyn Ab 44 or anti-Abl 24-11. As shown in Fig. 6B, the anti-Lyn IP precipitated two bands of M\textsubscript{r} 53,000 and M\textsubscript{r} 56,000 but also several

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other proteins, including one of Mr 210,000 protein. When these anti-Lyn immune complex kinase reactions were subjected to a secondary IP with the anti-Abl Ab 24-11, a faint but distinct band of Mr 210,000 was purified from Lyn precipitates (Fig. 6B). This band comigrated with p210 from a primary anti-Abl IP of the same lysate, which strongly suggested that the Mr 210,000 protein in anti-Lyn immune complexes is p210bcr/abl (Fig. 6B). The relatively weak signals from secondary IPs are explained by the loss of more than 90% of precipitated proteins during disruption and re-precipitation. This loss of activity is best demonstrated by experiments where primary and secondary IPs were identical (anti-Lyn or anti-Abl); in secondary IPs, only a small fraction (≤10%) of the originally precipitated Lyn or Abl proteins could be rescued (Fig. 6B).

Experiments were also performed in the opposite direction (first IP anti-Abl and then IP anti-Lyn). Primary anti-Abl IP allowed to co-precipitate proteins of Mr 56,000 that comigrated with p56 from primary anti-Lyn IPs (Fig. 6B). When the primary anti-Abl IP mixture was disrupted and reprecipitated with anti-Lyn Ab 44, a very faint but distinct band became visible at Mr 56,000, which presented p56 (Fig. 6B). p53 was also detected in these secondary IPs when longer exposures were chosen (data not shown). Several other phosphoproteins, including a Mr 72,000 protein, coprecipitated in all anti-Lyn IPs; the identity of these proteins is unknown at present. Taken together, these experiments demonstrate that p53/p56 and p210bcr/abl cophosphorylate in 32Dp210 cells, suggesting that the two kinases exist in an intracellular protein complex.

DISCUSSION

This study demonstrates for the first time that at least two Src family kinases, p53/p56 and 59hck, are activated in bcr/abl-transfected myeloid cells and form an intracellular protein complex with p210bcr/abl. The fact that an increased kinase activity of p53/p56 and 59hck is found in bcr/abl-positive cells obtained from Ph1-positive patients or by bcr/abl transfection suggests that these Src family kinases become activated during the interaction with p210bcr/abl.

It is unclear at present whether Src family kinases contribute to the transforming effects of p210bcr/abl. However, the potential relevance of these findings is underscored by the fact that Src family kinases and p210bcr/abl seem to stimulate identical downstream signaling targets. Bcr/Abl is known to interact with the Ras signaling pathway by binding/activating the growth factor receptor-bound protein 2, Grb2 (10), the Src homology and collagen protein, Shc (9, 12), and the rasGTPase-activating protein, GAP (11), which are all known to modulate the activity of Ras. Their activation ultimately leads to the activation of a pathway commonly referred to as a Ras signaling pathway that involves the coordinate activation of Ras, Raf-1, and MAPK, and which seems to be critical for the regulation of cell growth (23–25). Src family kinases are likely to initiate similar signaling events; GAP is found in complexes with v-src and c-src and seems to be phosphorylated by the activated Src kinase (26–28). Briggs et al. (29) could demonstrate that GAP binds to the SH3 domain of Hck; this interaction was mediated by the highly conserved YXX sequence from the Hck SH3 domain and by a proline-rich N-terminal domain of GAP. She is also phosphorylated in v-src-transformed fibroblasts (30). We could show recently that the stimulation of lymphoid cells with IL-6 induces the complex formation of the Src family kinases Hck or Lyn with Shc/Grb2. Finally, Src family kinases also seem to associate with MAPK (22). Given this apparent overlap of the signal transduction pathways of Src family kinases and p210bcr/abl, it is possible that the activation of Shc, Grb2, GAP, or MAPK by p210bcr/abl may involve the cooperation of Src family kinases that bind to p210bcr/abl or vice versa. In this regard, a similar cooperation has been demonstrated recently for the oncogenic effects of EGFR and c-Src in fibroblasts, where the combined overexpression of EGFR and c-Src increased the DNA synthesis, growth rate, and the tumor formation in nude mice (31). These effects were associated with the formation of a c-Src/EGFR heterocomplex and an enhancement of the phosphorylation of receptor substrates like phospholipase C-γ and Shc (31).

The speculation that p210bcr/abl and Src family kinases might collaborate in stimulating the signaling pathways to Ras is further
Fig. 4. A, analysis of tyrosine phosphorylation of proteins in whole cell lysates from bcr/abl-positive (K562, BV173, and LAMA-84) or bcr/abl-negative (JOSK-M) CML cell lines. B, p53/56m kinase activity in anti-Lyn immunoprecipitates of bcr/abl-positive (K562, BV173, and LAMA-84) or bcr/abl-negative (JOSK-M) myeloid cell lines (left). To control for the amount of Lyn protein used in these kinase assays, aliquots of the anti-Lyn immunoprecipitates were analyzed by anti-Lyn immunoblotting with the anti-Lyn Ab 44 (right). Similar amounts of p53/56m were precipitated in all four lanes.

Fig. 5. Immune complex kinase assays following immunoprecipitation of 32D or 32Dp210 cell lysates with anti-Hck Ab N30 and protein A beads (α-hck), with anti-Hck Ab, blocking peptide, and protein A beads (α-hck + peptide), with protein A beads alone (beads), and with anti-Abl Ab 24-11 and protein G beads (α-abl; left). IP aliquots of the same experiment were used for anti-Hck immunoblotting. Similar amounts of p59hck protein were precipitated by anti-Hck IP (right).
nourished by the observation that Bcr/Abl seems to use at least two independent mechanisms for Ras activation. Y177F point mutations that inactivate the Grb2-binding site in Bcr continue to activate Ras and to transform hematopoietic cells (32, 33). Careful examination of these Y177F mutants revealed that phosphorylation and activation of Shc occurs independently of Grb2 binding (32, 33). Moreover, Grb2 seems to complex with Shc (instead of Bcr) in myeloid cells transfected with Y177F mutants (32, 33). Thus, Shc activation seems to complement Bcr/Abl in myeloid cell transformation. Only simultaneous triple point mutations of the Grb2-binding site in Bcr (Y177L), of the SH2 domain in Abl (R552L), and of an autophosphorylation site in Abl (Y793F) were able to suppress the Shc-Grb2 interaction in hematopoietic cells; these triple mutants seemed also defective in blocking apoptosis, inducing IL-3 independence, activating Ras, and inducing myc mRNA expression (33). However, the precise mechanism by which Bcr/Abl stimulates Shc or other Ras-activating proteins is unknown. Non-receptor tyrosine kinases like Fps/Fes or Src family kinases may mediate some of these effects, since Fps/Fes has been shown to associate with Bcr (34).

At present, the role of Src family kinases for normal hematopoiesis is not fully defined. Single knock-out experiments of different Src kinases did not result in abnormalities of murine myelopoiesis (35). Even when hck- and fgr-deficient animals were interbred to generate double knockout animals, animals appeared completely healthy, and hematopoiesis was not defective (35). Interestingly, hck/src-deficient mice showed hematopoietic deficiencies characterized by anemia, leukopenia, and an accumulation of abnormal immature cells in the spleen (35). This suggests that at least some Src family kinases regulate the differentiation and function of blood cells, probably in a redundant manner. These findings from knock-out experiments are supported by the observation that different Src family kinases are activated by hematopoietic growth factors like IL-3, granulocyte/macrophage-colony-stimulating factor, and IL-6, which promote the growth and differentiation of myeloid progenitors (16–18). Although a role for Src family kinases for myeloid leukemogenesis remains to be established, the potential contribution of Src family kinases to lymphoid leukemogenesis has been demonstrated by Abraham et al. (36), who observed an increased frequency of progression to T-cell leukemias after overexpression of Lck (under the control of a lymphoid-specific promoter and enhancer) in transgenic mice. One might speculate that the constitutive activation of Src family kinases by p210Bcr/Abl may induce similar effects in myeloid cells.

Although the results allow us to hypothesize that Src family kinases might cooperate with p210Bcr/Abl, additional evidence is needed to prove this concept. Future experiments should identify the binding domains that are functionally relevant for mediating the interaction of p210Bcr/Abl with p53/56 or p59hck.

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Activation of Src Kinases p53/56<sup>lyn</sup> and p59<sup>hck</sup> by p210<sup>bcr/abl</sup> in Myeloid Cells

Susanne Danhauser-Riedl, Markus Warmuth, Brian J. Druker, et al.


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