Leukemia (2000) 14, 845–852

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Inactivation of wild-type BCR/ABL tyrosine kinase in hematopoietic cells by mild hyperthermia

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Temperature-sensitive mutants of BCR/ABL tyrosine kinase have been extensively used to study the mechanisms of cell transformation and signal transduction. However, little is known about the effect of temperature on the activity of wild-type BCR/ABL gene product. In this study, we demonstrate that *in vivo* tyrosine kinase activity of p210, p190 BCR/ABL and *v-abl* are temperature-sensitive when expressed in hematopoietic cells and decline when temperature is raised 2°C above normal range. *In vitro* tyrosine kinase activities of purified recombinant Abl and immunoprecipitated p210 BCR/ABL were also sensitive to increased temperature. Tyrosine phosphorylation of cellular proteins was markedly reduced in BCR/ABL transformed cells after 16 h at 39°C, whereas the expression of BCR/ABL was unchanged. Temperature-induced downregulation of BCR/ABL kinase activity was reversible when cells were shifted back to 37°C. The downregulation of Abl tyrosine kinase activity was not influenced by mutation or deletion of SH2 or SH3 domains or mutation of the GRB2 binding site. No increase in functional activity or expression of protein-tyrosine phosphatases, PTP-1B, SH-PTP1 or SH-PTP2 was detected in cells grown at 39°C. Temperature-induced downregulation in tyrosine kinase activity correlated with decline in phosphotyrosine-associated PI 3-kinase whereas there was no change in growth factor independence of transformed hematopoietic cells. In conclusion, Abl tyrosine kinase has intrinsic sensitivity to temperature and BCR/ABL expressed in hematopoietic cells is downregulated by increasing temperature 2°C. These observations provide a unique opportunity to identify cellular factor(s) which regulate BCR/ABL kinase *in vivo* and suggests possible novel treatment of CML by a mild hyperthermia. *Leukemia* (2000) **14,** 845–852.
Keywords: BCR/ABL; temperature sensitivity; tyrosine phosphorylation; tyrosine kinase; chronic myelogenous leukemia

Introduction

The reciprocal translocation between chromosomes 9 and 22 results in the fusion of NH2-terminal portion of the BCR gene upstream of *c-abl* tyrosine kinase gene. The BCR/ABL oncogene most frequently encountered in human leukemias encodes two proteins, p185, frequently referred to as p190, with a smaller BCR domain (amino acids 1–426) and p210, with a larger BCR domain (amino acids 1–902 or 926). p185 is associated with acute lymphocytic leukemia (ALL) and p210 is found in almost all cases of chronic myelogenous leukemia (CML).[1–3] The transforming capacity of BCR/ABL is based in its enhanced tyrosine kinase activity.[4–6] BCR/ABL expression in cytokine-dependent murine hematopoietic cell lines leads to growth factor independence[4,7] and protection of cells from apoptosis induced by irradiation or cytotoxic agents.[6,8–10] Whether CML progenitor cells are growth factor independent in addition to resistant to apoptotic stimuli is currently under discussion.[11–14]

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Received 2 November 1999; accepted 11 January 2000

Temperature-sensitive constructs of *v-abl*[15,16] and BCR/ABL have been used[17–19] in order to test the impact of BCR/ABL expression. Several authors suggested that this approach could alter substrate recognition and specificity and that aberrant temperature could affect signaling pathways other than those which depend on BCR/ABL.[10] Fever physiologically has been recognized as a key feature of infection and inflammation, and is a common feature in autoimmune diseases. *In vitro* hyperthermia has been shown to potentiate immune responses to IL-1, IL-2 and immune response to antigen including enhanced antigen processing.[20–23] There is no previous information on the effect of fever or hyperthermia on chronic myelogenous leukemia cells.

In this study, we have examined the effect of a 2°C increase in temperature on the activity of human wt BCR/ABL protein-tyrosine kinase expressed in different cell types. We found that exposure of BCR/ABL and *v-abl* transformed hematopoietic cells or purified proteins to 39°C downregulates Abl kinase activity and results in a marked reduction in tyrosine phosphorylated substrates in hematopoietic cells. The expression of BCR/ABL is unaffected and tyrosine kinase activity is recovered when cells are shifted back to 37°C. Although PI 3-kinase activity is also inhibited at 39°C, temperature shift is unable to revert growth factor independence of transformed hematopoietic cell lines. These findings provide novel opportunities to understand the mechanisms of regulation of BCR/ABL tyrosine kinase activity.

Materials and methods

Cells

Hematopoietic cell lines BaF-3 (BaF), 32D and FDCP were maintained in RPMI containing 10% FCS and 10–20% conditioned medium from the IL-3 producing cell line, WEHI-3B.[24] The p210-BCR/ABL cDNA was expressed in parental BaF and 32D cells by electroporation and selected for G418 resistance to generate transformed stable cell lines Bp210 and 32D-p210, respectively, as described.[4,25] Bp210-R1053K, Bp210-ΔSH2, Bp190, B-v-abl and B-c-abl-ΔSH3 stable cell lines were generated by electroporation of plasmid DNA encoding various isoform of abl into BaF3 cells. These DNA constructs were kindly provided by R Van Etten (HMS, Boston, MA, USA). Parental and BCR/ABL transformed FDCP cell lines were obtained from Jaqueline Pierce (NCI, Bethesda, MD, USA). Parental and NIH3T3 fibroblast cell lines which express the following constructs: *gag*p210 BCR/ABL, SH3 deleted c-abl (ΔXB), and *v-abl* were grown in DMEM supplemented with 10% calf sera as described.[26,27] Cells were deprived of serum and conditioned media for 14–16 h prior to harvest and whole cell lysates (WCL) were prepared as described.[28]

Antibodies

Anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, NY, USA) and monoclonal and polyclonal anti-abl antibody, AB3 and AB1, respectively were from Calbiochem-Novatech (Cambridge, MA, USA). Anti-SHP1, -SHP2 and -PTP1B antibodies were kindly provided by B Neel (Harvard Institutes of Medicine, HMS, Boston, MA, USA). Goat anti-mouse horseradish peroxidase (HRP)-conjugated and goat anti-rabbit HRP-conjugated second antibodies were from New England Biolab (Beverly, MA, USA) and Promega (Madison, WI, USA), respectively.

Immunoprecipitations and Western blotting

Cells were maintained at 37°C and shifted to 39°C for different time intervals as indicated in the figure legends. Cells were washed in Dulbecco phosphate-buffered saline (D-PBS) prewarmed to a specific temperature and lysed in lysis buffer containing 1% NP-40 in 50 mM HEPES pH 7.5, 150 mM sodium chloride, 50 mM sodium fluoride, 5 mM sodium orthovanadate, 0.5 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 10% glycerol and protease inhibitors (leupeptin and aprotinin at 10 μg/ml, pepstatin at 5 μg/ml and phenylmethylsulfonylfluoride (PMSF) at 0.5 mM). In some cases, an alternative procedure was used: cells were directly lysed in hot SDS sample buffer. For immunoprecipitations, WCL were incubated with the appropriate antibodies for 2 h at 4°C followed by the addition of protein A/Sepharose for 45 min. Immune complexes were isolated by centrifugation, and washed with PBS, 2× with TNE (10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM ethylenediamine-tetraacetic acid (EDTA)) and PBS. Immune precipitates were immediately used for enzyme assays or denatured by boiling in SDS gel loading buffer.

For Western blotting, proteins were separated by SDS-PAGE and transferred on to nitrocellulose membrane. Membranes were blocked in PBS-T (0.2% Tween-20, 7% nonfat dry milk, and 2% bovine serum albumine (BSA)) for 1 h, probed with specific antibodies and incubated with HRP-conjugated second antibodies. Immune reactive bands were detected using chemiluminescence (ECL; Amersham, Arlington Heights, IL, USA). For stripping, membranes were incubated for 10 min either in stripping buffer (150 mM NaCl, 10 mM Tris-HCl, pH 2.3) or in Ponceau-S (Sigma, St Louis, MO, USA) for 20 min at room temperature, washed extensively and reprobed with a different antibody.

PI 3-kinase assay

PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates was assayed using phosphatidylinositol phosphate (PI) and PI 4,5-P_2 as substrates.[29] Reaction products were extracted and subjected to thin layer chromatography. ^{32}P incorporated into PI 3,4,5-P_3 (PIP_3) was quantified by scintillation counting and results expressed as counts/min following background subtraction.

Protein-tyrosine phosphatase activities

The cells were incubated at different temperatures as indicated, harvested, washed once with D-PBS at the appropriate temperature and lysed in phosphatase assay buffer (50 mM HEPES; pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 0.5 mM PMSF). Protein tyrosine kinase substrate, raytide (Oncogene, Cambridge, MA, USA), was phosphorylated in the presence of Mg-γ^{32}P-ATP using purified recombinant Abl kinase (Oncogene) as per the manufacturer's instructions. The products were precipitated by 10% ice-cold TCA containing 0.5% BSA, dried and redissolved in the assay buffer. Protein tyrosine phosphatase activity in the presence or absence of 0.5 mM Na-orthovanadate was measured as described.[17] In brief, reaction mixture containing 100 000 c.p.m. of labeled raytide and WCL or immunoprecipitated PTP-1B used as a positive control was incubated at 30°C for 20 min. Reactions were terminated by addition of 375 μl of cold 4% activated Norit and centrifuged at 4°C for 8 min at 14 000 r.p.m. Two hundred μl of clear supernatant was removed and counted using a beta scintillation counter to determine the amount of ^{32}P released from substrate. Phosphatase activity was calculated as c.p.m. of ^{32}P released/h/mg protein.

In vitro Abl tyrosine kinase activity

The p210 BCR/ABL gene product was immunoprecipitated from Bp210 cells using 1 mg WCL protein/assay and 5 μl of anti-Abl monoclonal antibody at 4°C for 2–3 h. Immune complexes were isolated on Protein A/Sepharose beads (Sigma), washed three times with lysis buffer and once with ice-cold kinase buffer (40 mM HEPES; pH 7.4, 20 mM $MnCl_2$, 20 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.2% Triton X-100). Immunoprecipitated BCR/ABL on beads was mixed with 25 μl of kinase buffer and preincubated for 30 min at different temperatures as indicated. The reaction was started by the addition of 2 μg of raytide and 10 μCi of γ^{32}P-ATP and 25 μM cold ATP for 30 min. Reactions were terminated by transference of the tubes to ice-cold water bath and centrifugation at 12 000 *g* for 5 min. Twenty μl of supernatant was spotted on a phosphocellulose paper (p81, Whatman) and the remainder of the reaction mixture was boiled in SDS gel loading buffer, resolved by SDS-PAGE gel and exposed for autoradiography. The autophosphorylation of BCR/ABL was measured by excising the band from the gel and counting using a beta scintillation counter. The purified recombinant Abl kinase activity was measured by the addition of 2 μg raytide as substrate and processed as above. The filters were washed three times with 0.5% phosphoric acid, air dried and counted using a beta scintillation counter.

Results

Temperature shift to 39°C reduces tyrosine-phosphorylated proteins in BCR/ABL transformed hematopoietic cells

We have previously described mechanisms of p210 BCR/ABL-initiated signal transduction using a temperature-sensitive mutant of BCR/ABL.[30] While performing these experiments, we used BaF cells which are transformed by wt p210 BCR/ABL and found that the major cellular substrates of BCR/ABL (Cbl and Shc) were not tyrosine phosphorylated in these cells

grown at 39°C (Figure 1a, lanes 3 and 4). Subsequent analysis showed that p210 BCR/ABL transformed 32D cells, 32D-p210 (lanes 5 and 6) and FDCP cells, FDCP-p210 (lanes 7 and 8) grown at 39°C also have markedly reduced tyrosine phosphorylated proteins. Similar results were seen in BaF cells transformed by wt p190 BCR/ABL (Figure 1a, lanes 9–12). We were concerned that preparation of cell extracts at 39°C could allow proteins to be dephosphorylated *in vitro*. To avoid the potential effects of lysis procedure, cells were lysed directly in hot SDS sample buffer (Figure 1a, lanes 11 and 12). Similar results were obtained with both methods (Figure 1a, lanes 9–12). These data suggest that wt BCR/ABL tyrosine kinase activity is sensitive to temperature shift in the absence of mutations.

We then analyzed the time course of wt BCR/ABL inactivation at 39°C. Bp210 cells were grown at 37°C and shifted to 39°C for 0.5–16 h and examined for tyrosine phosphorylation of cellular substrates. As shown in Figure 1b, the pattern of tyrosine phosphorylated proteins in cells maintained at 39°C for up to 12 h was similar to that of cells grown at 37°C (lanes 1 and 5). However, after >16 h at 39°C, Bp210 cells contained only a fraction of tyrosine-phosphorylated proteins (lane 6) which persisted when cells were maintained at this temperature for longer periods of time. To determine whether reduced tyrosine phosphorylation of proteins at 39°C could be due to decline in BCR/ABL expression, we performed anti-abl Western blotting of the same membranes. BCR/ABL protein expression remained unaltered (Figure 1b, bottom panel).

To examine whether the downregulation of protein tyrosine phosphorylation at 39°C is reversible, Bp210 cells were grown at 39°C for 16 h and shifted back to 37°C for different time intervals (Figure 1c). Anti-phosphotyrosine Western blots showed that protein-tyrosine phosphorylation recovered gradually over 12 h while expression of BCR/ABL was again unchanged (not shown). Taken together, these results demonstrate that tyrosine phosphorylation of cellular proteins in BCR/ABL transformed hematopoietic cells is sensitive to the relatively small increase in temperature seen in febrile states, whereas the stability and expression of p210 BCR/ABL protein is not affected.

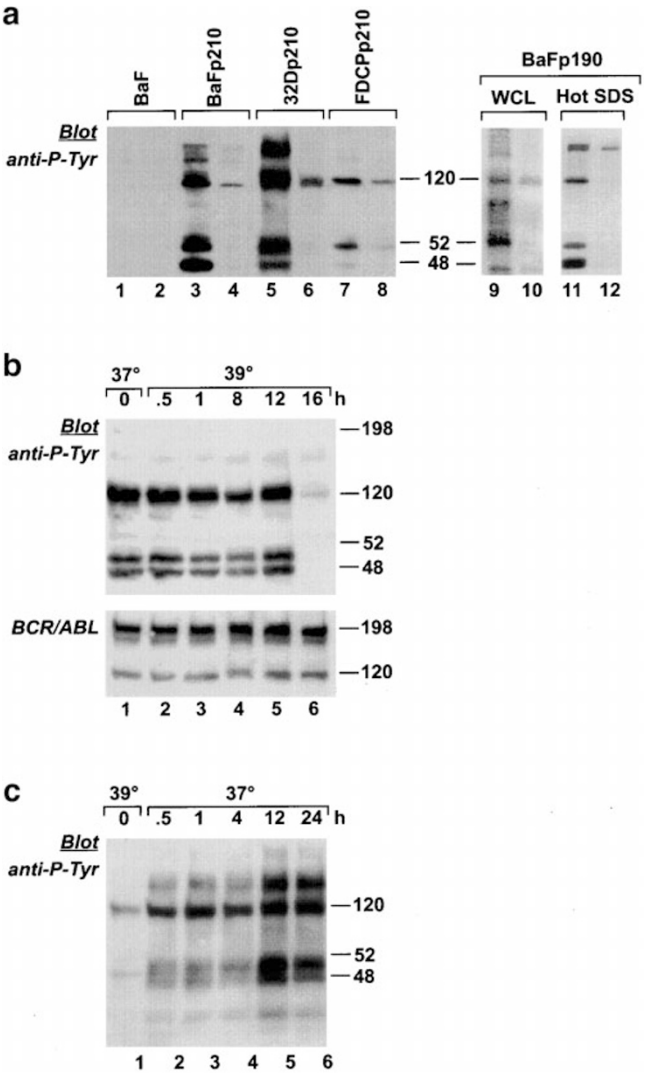

Figure 1 Downregulation of BCR/ABL tyrosine kinase at 39°C in hematopoietic cell lines. (a) Parental BaF (lanes 1, 2), hematopoietic cells expressing wt p210 BCR/ABL, Bp210 (lanes 3, 4), 32Dp210 (lanes 5, 6), FDCPp210 (lanes 7, 8) and Bp190 (lanes 9–12). The cells were grown at 37°C (odd lanes) and shifted to 39°C (even lanes) for 16–18 h. Fifty μg of WCL (lanes 1–10) protein was fractionated on SDS-PAGE gel, transferred on to nitrocellulose membranes and probed with anti-phosphotyrosine antibodies. Position of the molecular weight markers is indicated on the right. Bp190 cells were lysed directly in hot SDS sample buffer (lanes 11, 12). (b) Time course of BCR/ABL tyrosine kinase downregulation at 39°C. Bp210 cells were grown at 37°C (lane 1) and shifted to 39°C for the indicated time intervals (lanes 2–6); cells were harvested and 50 μg WCL protein was fractionated on SDS-PAGE gel, transferred on to nitrocellulose membrane and probed with anti-phosphotyrosine antibodies (upper panel). In the lower panel, the membrane was stripped and reprobed with anti-abl antibodies. Position of the molecular weight markers is indicated on the right. (c) Temperature-induced downregulation of BCR/ABL tyrosine kinase in Bp210 cells is reversible. Bp210 cells were grown at 39°C for 18 h (lane 1) and shifted back to 37°C for the indicated time (lanes 2–6). Cells were harvested and 50 μg WCL protein was fractionated on SDS-PAGE gel, transferred on to nitrocellulose membrane and probed with anti-phosphotyrosine antibodies. The position of the molecular weight markers is indicated on the right.

Structural requirements and specificity of BCR/ABL downregulation upon temperature shift

Leukemogenic potential of BCR/ABL and transformation ability of Abl kinase are influenced by specific structural motifs which determine protein–protein interactions. The reduction in BCR/ABL-induced tyrosine phosphorylation of intracellular substrates could be due to a decrease in the BCR/ABL intrinsic kinase activity and changes in the interaction with potential substrates. To test whether mutation of specific structural domains of BCR/ABL or Abl has an effect on temperature sensitivity of the wt p210 and p190 BCR/ABL, we compared BaF cells which express wt p210 BCR/ABL with cells which express the following mutants (Figure 2a): p210R1053K BCR/ABL, which abolishes the functional interaction with the Abl SH2 domain (Figure 2b, lanes 3 and 4); ΔSH2-p210 BCR/ABL which has a deletion of the SH2 domain (Figure 2b, lanes 5 and 6); *v-abl* (Figure 2b, lanes 7 and 8), ΔSH3-*c-abl*, a transforming variant of *c-abl* which lacks the SH3 domain (Figure 2b, lanes 9 and 10), P190 BCR/ABL and p210Y177F BCR/ABL which fails to bind the adaptor protein, GRB2 (not shown). To avoid clonal variations, G418-resistant mass transfected cells were used for all experiments and cell

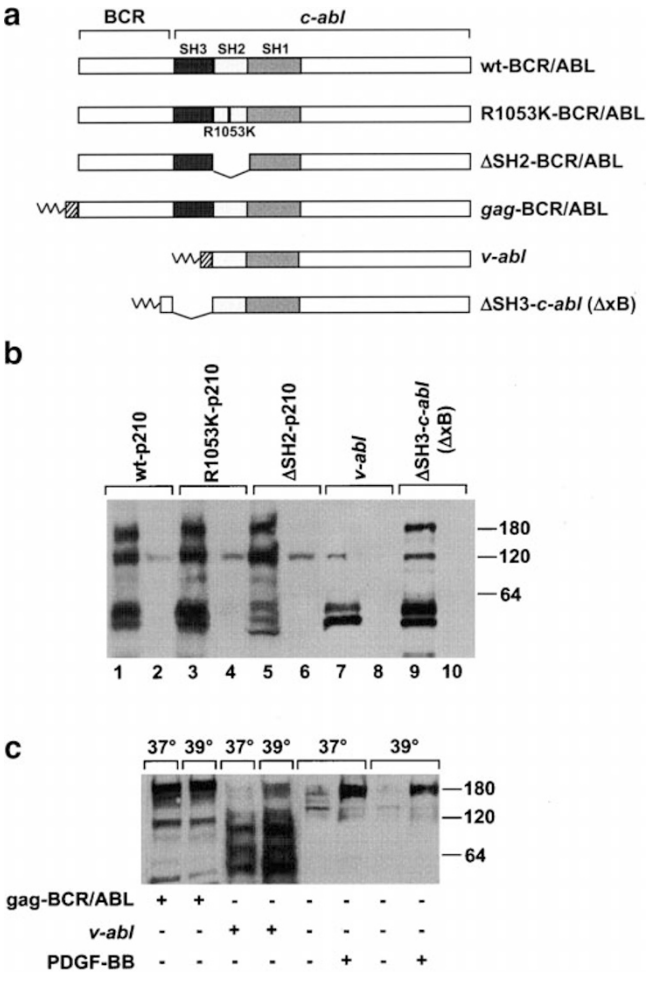

Figure 2 Downregulation of BCR/ABL tyrosine kinase at 39°C is not influenced by BCR/ABL structural motifs and is evident only when expressed in hematopoietic cells. (a) Graphic representation of wt and mutants of BCR/ABL and Abl. The position of various structural motifs is indicated. (b) BCR/ABL structural domains do not affect the kinase activity at 39°C. BaF cells which express different BCR/ABL or *c-abl* mutant proteins were grown as described in the legend to Figure 1 at 37°C (odd lanes) or at 39°C (even lanes) for 16–18 h. Fifty μg of WCL protein was fractionated on SDS-PAGE gel, transferred on to nitrocellulose membranes and probed with anti-phosphotyrosine antibodies. The position of the molecular weight markers is indicated on the right. (c) BCR/ABL, Abl and PDGF-R kinase activities are not downregulated in NIH3T3 fibroblasts by increased temperature. NIH3T3 fibroblasts expressing *gag*-BCR/ABL or *v-abl* were grown as indicated at 37°C or at 39°C for 24 h. Parental NIH3T3 fibroblasts were induced to quiescence for 48, grown for an additional 24 h at 37°C or 39°C and stimulated with 20 ng/ml of PDGF-BB for 10 min. Fifty μg of WCL protein was used for Western blot with anti-phosphotyrosine antibodies. The position of molecular weight markers is indicated on the right.

lines were examined for growth factor independence and expression of BCR/ABL and Abl. To examine the temperature sensitivity, cells were grown at 39°C overnight, WCL normalized to expression of Abl protein and analyzed by Western blotting with anti-phosphotyrosine antibodies. As shown in Figure 2b, a decrease in tyrosine phosphorylated proteins was observed in all cell lines exposed to 39°C which was similar to the results obtained using cells which express the wt p210 BCR/ABL gene product (Figure 2b, lanes 1 and 2). These results suggest that temperature sensitivity of BCR/ABL tyrosine kinase is independent of the BCR, SH2 or SH3 domains and may reside in the tyrosine kinase domain or other domains in Abl not tested.

Previous reports indicate that BCR/ABL signal transduction is uniquely adapted for hematopoietic cells: it is highly transforming for hematopoietic cells whereas it requires plasma membrane targeting for transformation of NIH3T3 fibroblasts.[27] To examine whether temperature-mediated sensitivity of Abl kinase is evident in NIH3T3 fibroblasts, we compared the pattern of tyrosine phosphorylation in cells which express transforming variants of BCR/ABL in NIH3T3 fibroblasts: *gag*BCR/ABL and *v-abl* (Figure 2c). We found no difference between cells grown at 37°C or 39°C. Similar results were obtained using NIH3T3 fibroblasts which express transforming ΔXB-*c-abl* or *v-src* oncogenes (data not shown). Taken together, these results indicate that temperature-induced sensitivity of wt BCR/ABL kinase is only evident in hematopoietic cells and not in NIH3T3 fibroblasts, which further underscores the fundamental differences in signal transduction between these cell types. We also examined the effect of temperature on the activity of an unrelated tyrosine kinase receptor, the platelet-derived growth factor receptor (PDGF-R), in NIH3T3 fibroblasts. As shown in Figure 2c, these cells had no change in activation of PDGF-R upon stimulation with PDGF β at 37°C or 39°C (Figure 2c). These results suggest that temperature-dependent downregulation of BCR/ABL kinase activity is restricted to Abl kinase expressed in hematopoietic cells.

Effect of temperature on Abl and BCR/ABL tyrosine kinase activity in vitro

To determine the optimum temperature for BCR/ABL tyrosine kinase *in vitro*, kinase activity of immunoprecipitated p210 BCR/ABL was tested at different temperatures using tyrosine kinase substrate, raytide. As shown in Figure 3a, p210 BCR/ABL *in vitro* kinase activity was maximal between 24 and 28°C and reduced by 80% and 93% at 37°C and 39°C, respectively. Optimal autophosphorylation of BCR/ABL occurred at 28°C (Figure 3b, lane 4) which was markedly reduced at 39°C to the levels observed at 4°C (Figure 3b, lane 7). We also examined the effect of temperature on the activity of purified recombinant kinase domain of *v-abl*. Abl kinase activity was similar to that of p210 BCR/ABL and was almost undetectable at 39°C (Figure 3c). Time course of inhibition of kinase activity for recombinant Abl revealed that *in vitro* inhibition was rapid and kinase activity was lost within 5 min of preincubation at 39°C (data not shown), which is significantly faster than the effect of temperature on the loss of tyrosine phosphorylated substrates observed *in vivo*. These results indicate that increase in temperature downregulates intrinsic *Abl* tyrosine kinase activity.

Expression and activity of specific protein tyrosine phosphatases in BCR/ABL transformed hematopoietic cells

The differences in the rate of Abl kinase inhibition *in vivo* and *in vitro* suggested that the delay in disappearance of phosphorylated substrates *in vivo* could reflect the expression or activation of protein tyrosine phosphatases. In hematopoietic cells which express ts-p210 BCR/ABL gene product enhanced expression and activation of PTP-1B was linked to BCR/ABL tyrosine kinase activity.[17] Increased expression of SH-PTP1 and SH-PTP2 have been also described in BCR/ABL transfor-

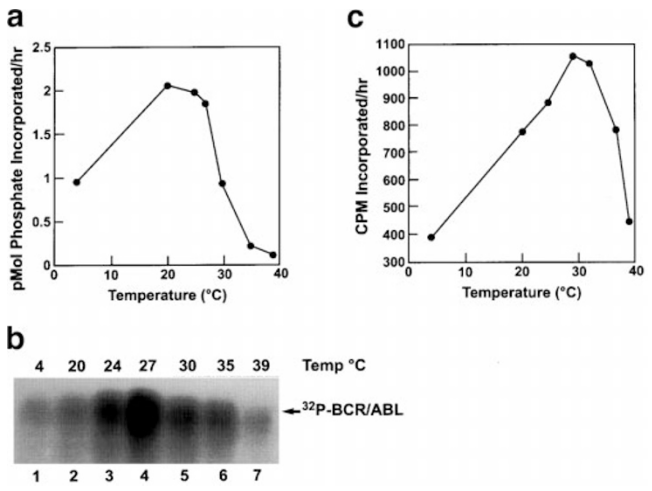

Figure 3 Effect of temperature on p210 BCR/ABL and recombinant purified Abl kinase. (a) p210 BCR/ABL was immunoprecipitated from Bp210 cell lysate and used for *in vitro* kinase activity. BCR/ABL on Protein A/Sepharose beads was preincubated for 30 min at different temperatures ranging from 4 to 39°C. The kinase reactions were carried out at respective temperatures for an additional 30 min, and kinase activity determined by the rate of ^{32}P incorporation into raytide substrate. Data represent one of three independent experiments. (b) Effect of temperature on autophosphorylation of immunoprecipitated BCR/ABL protein. After removing the clear supernatant as described in panel a, remaining samples were boiled in 1 × SDS gel loading buffer, proteins fractionated on SDS-PAGE gels and dried gels were exposed for autoradiography. Data represent one of three independent experiments. (c) Effect of temperature on the kinase activity of purified recombinant Abl. The temperature effect on purified Abl kinase was determined using GST-Crk as substrate. Kinase reactions were carried out at the indicated temperatures as described in panel a. Each reaction contained 2 μg of GST-Crk and 2 units of Abl kinase. Data represent one of three independent experiments.

med hematopoietic cells.[31] To understand whether delay in dephosphorylation of cellular proteins in Bp210 cells at 39°C was due to altered expression or activity of protein tyrosine phosphatases, we performed Western blot analysis and measured *in vitro* phosphatase activity under linear assay conditions. Expression of PTP1B, SH-PTP1 and SH-PTP2 remained constant at 39°C (Figure 4a). The apparent decrease in protein levels of SH-PTP1 was not reproduced in other experiments. Total phosphatase activity in whole cell extracts from cells grown at different temperatures overnight also showed no measurable difference between 37°C and 39°C (Figure 4b). We also measured phosphatase activity in immunodepleted cell extracts using anti-PTP1B, SH-PTP1 or SH-PTP2 antibodies. Although PTP1B, SH-PTP1 and SH-PTP2 were effectively immunoprecipitated with respective antibodies, the tyrosine phosphatase activity in immunodepleted supernatant was not altered (data not shown). Taken together, these results suggests that the loss of tyrosine phosphorylated proteins in cells maintained at 39°C reflects a lack of BCR/ABL tyrosine kinase activity rather than an increase in protein-tyrosine phosphatase activity.

The effect of downregulation of BCR/ABL tyrosine kinase activity on PI 3 kinase activity and cell growth

We and others have shown that activation of PI 3-kinase correlates with tyrosine kinase activity and survival of BCR/ABL transformed hematopoietic cell lines and CML cells[30,32,33]

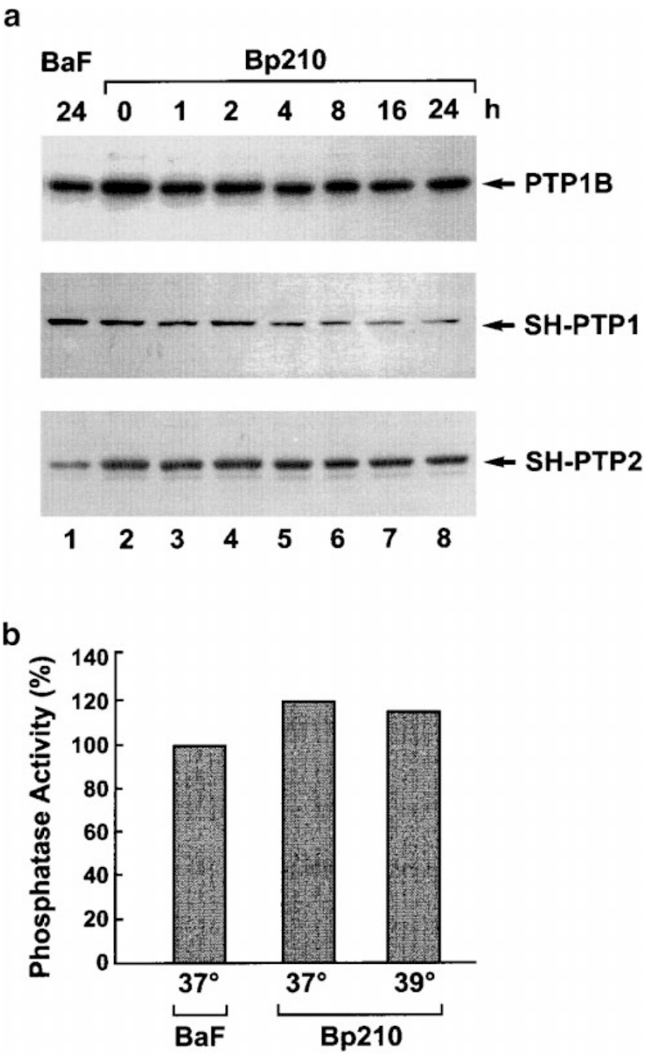

Figure 4 Expression or activity of protein tyrosine phosphatases in hematopoietic cell specific is not altered by temperature shift. (a) Expression of PTP-1B, SH-PTP1 and SH-PTP2 protein tyrosine phosphatases in Bp210 cells at 39°C. BaF (lane 1) or Bp210 cells were grown at 37°C (lane 2) and shifted to 39°C (lanes 3–8) for 24 h. At the indicated time intervals, cells were harvested and 50 μg of WCL protein was fractionated on SDS-PAGE gel, transferred on to nitrocellulose membranes and probed with anti-PTP1B, SH-PTP1 or SH-PTP2 antibodies. (b) Total cellular protein tyrosine phosphatase activity is not altered in Bp210 cells grown at 39°C. Total PTP activity from equal quantities of lysate proteins (1–2 μg) from BaF or Bp210 cells grown at the indicated temperatures was determined using Abl kinase phosphorylated raytide as a phosphate donor.

whereas expression of BCR/ABL in NIH3T3 fibroblasts does not lead to accumulation of PI 3-kinase lipid products.[28] We analyzed the effect of temperature on PI 3-kinase activity in cells shifted to 39°C and during their recovery at 37°C. Total cellular PI 3-kinase activity in Bp210 cells grown at 39°C for 16 or more hours was decreased to the basal levels equal to the levels of quiescent BaF cells (data not shown). Anti-phosphotyrosine immunoprecipitable PI 3-kinase was also decreased by >90% in cells cultured at 39°C (Figure 5a) which correlated with the decrease in tyrosine phosphorylated substrates. Recovery of tyrosine kinase activity by shifting cells back to 37°C resulted in a rapid reactivation of PI 3-kinase in anti-phosphotyrosine immunoprecipitates (Figure 5a). These results are similar to those seen in ts-Bp210 cells grown at

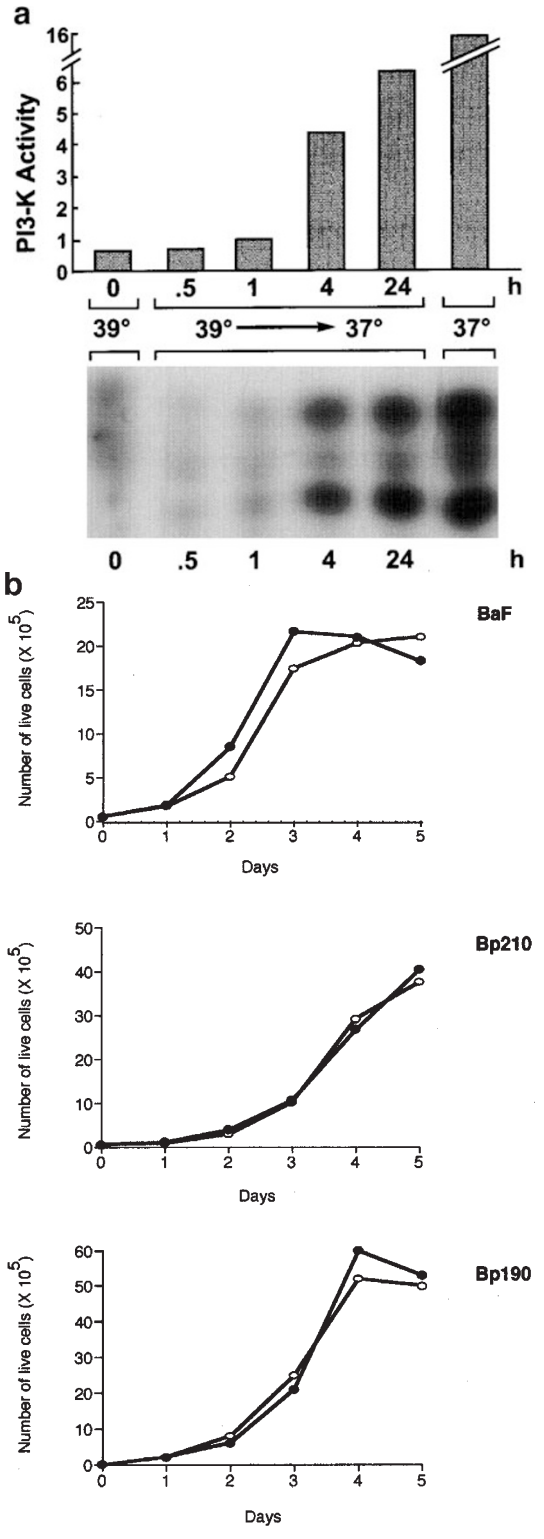

Figure 5 Downregulation of PI 3-kinase activity by temperature shift at 39°C is reversible and does not affect proliferation of hematopoietic cells. (a) Temperature induces a reversible downregulation of PI 3-kinase. Bp210 cells grown at 39°C for 18 h (lane 1) were returned to 37°C for the indicated time (lanes 2–5) or grown at 37°C for 18 h (lane 6). PI 3-kinase activity was measured in anti-phosphotyrosine immunoprecipitates from cells grown at the indicated temperatures using PI and PIP_2 as substrates. Data represent one out of four independent experiments. (b) Downregulation of BCR/ABL activity by temperature does not affect the number of live cells. BaF, Bp210 and Bp190 cells were plated at 0.5×10^5 cells/ml at indicated temperatures (open circles: 37°C, closed circles: 39°C). Parental BaF cells were grown in the presence of 20% conditioned media from WEHI-3B cells. The number of live cells were counted every 24 h by trypan blue exclusion. Data represent one out of six independent experiments.

permissive temperature which allows activation of tsp210 BCR/ABL kinase.[30] Because incubation of primary leukemic cells or BCR/ABL transformed BaF cells with PI 3-kinase inhibitors, such as Wortmannin, partially (30%) blocks proliferation, we examined whether temperature-dependent downregulation of BCR/ABL tyrosine kinase activity and subsequent decrease in PI 3-kinase activity had an effect on cell proliferation. Bp210 cells were grown in the presence or absence of IL-3 at the indicated temperature and assayed for trypan blue exclusion every 24 h. Although tyrosine kinase activity could not be detected in cells grown at 39°C, there was no decrease in proliferation or morphological differences detected in cells grown at 39°C (Figure 5b). The addition of IL-3 to transformed cells did not have an effect on cell growth at either temperature (data not shown). Similar results were obtained using BaF cells transformed by p190 (Figure 5b) and p190Y177F BCR/ABL (data not shown). These data suggest that the decrease in tyrosine kinase activity and shut-down of signaling pathways which require PI 3-kinase and GRB2 are insufficient to induce cell death of BCR/ABL transformed hematopoietic cell lines.

Discussion

Different site-directed mutagenesis strategies have been used to create temperature-sensitive mutants of *v-src*[34] and *v-abl*[16] and, by analogy, two different approaches were used to generate tsp210 BCR/ABL mutants.[18,19] These mutations resulted in reversible suppression of tyrosine kinase activity at 39–40°C without significant change in protein expression. The lack of tyrosine kinase activity has been attributed to improper folding of the mutant proteins at higher temperature. We have found that tyrosine kinase activity of wild-type BCR/ABL is also temperature sensitive *in vitro* and when expressed in hematopoietic cells. Tyrosine phosphorylation of cellular substrates and PI 3-kinase activity in BCR/ABL and *v-abl* transformed hematopoietic cells are abrogated by overnight exposure to 39°C without change in protein expression. This effect is reversible and phosphorylation of intracellular proteins is fully restored at 37°C within 4–12 h. Temperature-induced downregulation of BCR/ABL tyrosine kinase activity *in vivo* is apparent after 16 h, whereas 5 min exposure of purified recombinant Abl kinase to 39°C is sufficient to decrease its activity *in vitro*. These observations suggest that Abl kinase activity is intrinsically sensitive to a 2°C shift above normal body temperature. We can only postulate that the interaction with intracellular proteins and activity of protein-tyrosine phosphatases could determine protein folding which is responsible for maintaining Abl tyrosine kinase activity and the level of tyrosine phosphorylated substrates.

Temperature-induced inhibition of Abl kinase activity *in vivo* was detected only in hematopoietic cells and did not occur in NIH3T3 fibroblasts which express the same gene products. Activation of PDGF-R kinase was also not altered after prolonged incubation of NIH3T3 fibroblasts at 39°C. Several factors may account for specificity of temperature-induced

effects on Abl kinase in hematopoietic cells. In contrast to other non-receptor tyrosine kinases, BCR/ABL gene product is not membrane-bound. p210 BCR/ABL is not transforming when expressed in NIH3T3 fibroblast,[26] fails to induce accumulation of PI 3-kinase lipid products in these cells in spite of the association of PI 3-kinase activity with the BCR/ABL gene product[28] and is only weakly transforming when expressed in rat fibroblast.[35] Mutations which target BCR/ABL to the plasma membrane by myrystoylation of the N-terminus or the addition of the viral *gag* sequence result in activation of PI 3-kinase and transformation of NIH3T3 fibroblasts.[26] Temperature did not induce downregulation of Abl kinase activity when expressed in NIH3T3 fibroblasts, which also support the hypothesis that interaction with cell-specific factors facilitates a continued activation of Abl kinase *in vivo* in spite of intrinsic sensitivity of the kinase to higher temperature.

Previous studies indicate that specific domains of Abl are important in protein–protein interactions; the SH2 domain of Abl or mutation of GRB2 binding site at Y177 are necessary for activation regulation of tyrosine kinase activity, activation of PI 3-kinase and Ras pathways, and is reflected in leukemogenic capacity of BCR/ABL while deletion of the SH3 domain of *c-abl* abolishes interaction with a specific Abl kinase inhibitor and confers transforming capacity to *c-abl*.[36–40] The deletion of SH2 or SH3 domains or mutation at Y177 did not alter temperature-mediated downregulation of Abl kinase in hematopopietic cells. These data suggest that tyrosine kinase activity of BCR/ABL exposed to 2°C above normal body temperature is regulated by interaction with other, still unidentified, domains of the protein which determine proper folding and enzymatic activity.

It has been difficult to generate *ex vivo* models that accurately reflect the biological phenotype of CML. Genetic instability has long been recognized as a main cause of progression of CML into acute leukemia during terminal stages of the disease associated with blast crisis. Previous studies[41] indicate that BCR/ABL tyrosine kinase accelerates mutagenic events in cells through induction of genetic instability. This hypothesis was recently confirmed by analysis of tet-inducible human BCR/ABL gene product expressed in the murine IL-3-dependent hematopoietic cell line, BaF3.[42] To assess the signaling pathways elicited by BCR/ABL in the absence of possible secondary mutations, a glucocorticoid-responsive system[10] and temperature-sensitive conditional mutants of p210 BCR/ABL[18,19] have been used. However, these approaches led to different conclusions: whereas the glucocorticoid-inducible system, which induces expression of fully active BCR/ABL, led to activation of mitogenic signaling and G1-S progression, the primary effect of activation of tsBCR/ABL kinase was inhibition of apoptosis. These discrepancies may be attributed to differences between the effects of *de novo* synthesis of proteins and changes in tyrosine kinase activity. Mutations in BCR/ABL may also impair substrate recognition. As previously reported, activation of PI 3-kinase is required for the leukemogenic capacity of BCR/ABL,[33,43,44] inhibition of apoptotic signaling pathways and resistance to genotoxic damage.[6] Our results show that, in the absence of mutations, human wt BCR/ABL expressed in hematopoietic cells is also sensitive to a 2°C increase in temperature which is in the range of hyperthermia associated with fever. In spite of a significant decrease in tyrosine phosphorylated substrates in BCR/ABL transformed hematopoietic cells maintained at 39°C and lack of detectable PI 3-kinase activity in immune precipitates, there was no change in the number of live cells. These results suggest that BCR/ABL-induced genetic instability may account for secondary mutations which provide alternative mechanisms for cell survival and no longer require active BCR/ABL. Alternatively, remaining low levels of tyrosine kinase activity may be sufficient to maintain IL-3 independence and proliferation whereas higher levels of BCR/ABL kinase activity are necessary for protection against apoptosis. A dose-dependent hierarchy for BCR/ABL-induced biological effects in hematopoietic cells was previously proposed.[13] The increase in temperature may also have an independent effect on activation of other survival pathways which could potentiate biological response to BCR/ABL.

Regulation of the level of BCR/ABL-induced tyrosine phosphorylated intracellular substrates has been attributed to specific protein-tyrosine phosphatases. LaMontagne *et al*[17] reported increased expression of SH2-containing protein tyrosine phosphatase, SH-PTP1, in fibroblasts which express BCR/ABL and in hematopoietic cells which transiently express ts-Bp210. This increase required active BCR/ABL kinase activity. Similarly, their interaction and subsequent tyrosine phosphorylation are important for SH-PTP1 catalytic activity.[17] These data are consistent with the hypothesis that cells may respond to expression of p210 BCR/ABL by increasing the level of a natural antagonist in an attempt to maintain normal levels of tyrosine phosphorylation. In view of these observations, downregulation of BCR/ABL tyrosine kinase activity could affect expression or post-translational modification of cell-specific phosphatases and hence delay the tyrosine dephosphorylation of proteins. However, temperature-induced downregulation of BCR/ABL kinase activity in transformed cells did affect expression or activity of specific tyrosine phosphatases, PTP-1B, SH-PTP1 and SH-PTP2, suggesting that prolonged tyrosine phosphorylation of protein at 39°C is independent of activities of these enzymes. The involvement of other protein-tyrosine phosphatases and molecular mechanisms of temperature-induced changes in BCR/ABL in CML cells require further study. Decreasing BCR/ABL kinase activity has been a goal for the development of novel therapies for CML. Our studies provide a basis for exploring hyperthermia to improve the outcome of treatment and bone marrow transplantation for patients with CML.

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