Changes in the Proteome Associated with the Action of Bcr-Abl Tyrosine Kinase Are Not Related to Transcriptional Regulation*

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Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease, the hallmark of which is the Bcr-Abl protein tyrosine kinase (PTK). Without intervention the disease progresses from a benign chronic phase to a rapidly fatal blast crisis. To identify the molecular mechanisms underlying disease progression we used two-dimensional gel electrophoresis on a model we have previously described using the expression of a conditional mutant of Bcr-Abl PTK in a multipotent stem cell line, FDCP-Mix. Long term exposure of FDCP-Mix cells to Bcr-Abl mimics disease progression in CML. Four major differences were observed as a consequence of long term exposure to the Bcr-Abl PTK compared with cells exposed short term. The proteins were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry-generated peptide mass fingerprint data and liquid chromatography-tandem mass spectrometry-generated sequence information. Leukotriene A4 hydrolase, an enzyme known to be deregulated in CML, was found to be up-regulated. Annexin VI, vacuolar ATP synthase catalytic subunit A, and mortalin were found to be down-regulated. Poly(A) PCR cDNA analysis showed there was no correlation between the protein expression changes and mRNA levels. Western blot analysis also indicated no change in the levels of mortalin or leukotriene A4 hydrolase, indicating that post-translational events may modify protein content of the specific spots. Leukotriene B4 levels (product of leukotriene A4 hydrolase) were, however, reduced in cells exposed long term to Bcr-Abl activity. This study demonstrates the potential of proteomic analysis to define novel effects of oncogenes. _Molecular & Cellular Proteomics_ 1:876–884, 2002.

Mal. The abbreviations used are: CML, chronic myeloid leukemia; PTK, protein tyrosine kinase; FDCP, factor-dependent cells Patterson; mtDNA, mitochondrial DNA; Baf3, a Bcr-Abl-expressing lymphoid cell line; cDNA, complementary DNA; RT, reverse transcriptase; Chaps, 3-[3-cholamido- propyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.

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1 The abbreviations used are: CML, chronic myeloid leukemia; PTK, protein tyrosine kinase; FDCP, factor-dependent cells Patterson; MALDI, matrix-assisted laser desorption ionization; ToF, time of flight; ts, temperature-sensitive; IPG, immobilized pH gradient; LT32, long term 32 °C; LT39, long term 39 °C; ST32, short term 32 °C; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RT, reverse transcriptase; CHAPS, 3-[3-cholamido-propyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.
are suppressed in most of the cells, although they bear morphological characteristics of early and late granulocytes. Thus, Bcr-Abl effects on multipotent cells differ markedly depending on the length of exposure to the tyrosine kinase activity of this oncogene (factor independence is acquired, and the response to differentiation stimuli is altered). The molecular basis of this altered response is unclear. It does, however, reflect the progressive nature of CML, which inevitably progresses to an accelerated phase that is defined by the onset of abnormal stem cell development. Bcr-Abl is already known to initiate the proteasome-mediated turnover of at least three other proteins (7, 8) and post-translationally regulate p53 levels (5). These data represent a compelling case for initiation of a proteomic investigation of Bcr-Abl-mediated effects on multipotent cells. We have therefore studied Bcr-Abl-mediated changes in the proteome of primitive FDCP-Mix cells chronically exposed to Bcr-Abl. This approach has revealed that there are changes in protein expression that can be detected with broad pI range two-dimensional gels. Four proteins altered in expression were characterized, and their mRNA levels were found to be unaltered as a consequence of Bcr-Abl activity, confirming the benefit of proteomic analysis of leukemogenic protein tyrosine kinases on primitive hematopoietic cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—FDCP-Mix cells expressing a temperature-sensitive Bcr-Abl (tsBcr-Abl FDCP-Mix) were prepared as described previously (4). Cells were cultured at the permissive (32 °C) or restrictive (39 °C) temperatures as described previously (5) in Fischer’s medium containing interleukin-3 and 20% (v/v) horse serum. Long term (LT) 39°C Bcr-Abl FDCP-Mix cells were cultured for >12 weeks at the permissive temperature, while the long term 39 °C Bcr-Abl FDCP-Mix cells were held at the restrictive temperature for the same period.

**Two-dimensional Gel Electrophoresis and Image Analysis**—Cells were prepared for two-dimensional PAGE after incubation for various times at the permissive and restrictive temperature for tsBcr-Abl PTK activity. Cells were pelleted by centrifugation at 500 × g for 5 min, and the supernatant was carefully removed. Cells were then washed free of medium in 40 ml of phosphate-buffered saline followed by centrifugation at 500 × g for 5 min. The supernatant was then removed followed by the addition of 350 μl of isoelectric focusing buffer (9 M urea, 2% thiourea, 4% (v/v) CHAPS, 65 mm dithiothreitol, 0.5% (v/v) IPG buffer (Amersham Biosciences)) per 2.5 × 10⁶ cells. The samples were then centrifuged at 12,000 rpm for 15 min at 20 °C. Isoelectric focusing was performed using 18-cm IPG strips (pH 3–10) and the IPGPhor instrument (Amersham Biosciences), focusing for a total of 72 kV-h. The second dimension was a standard SDS-PAGE protocol using the ISODALT system (Amersham Biosciences). Gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid for 30 min and sensitized in 30% (v/v) methanol, 0.2% (w/v) sodium thiosulfate, 6.8% (w/v) sodium acetate for 30 min. The gels were then washed in three changes of water over a period of 15 min, stained in 0.2% (w/v) silver nitrate for 20 min, washed in three changes of water over a period of 3 min, and developed in 2.5% (w/v) sodium carbonate supplemented with 0.015% (v/v) formaldehyde. Development was stopped by removing developer and replacing with 40 mM EDTA. Differential protein expression profiles in silver-stained two-dimensional gel samples from greater than four different experiments were compared using Progenesis software (Non Linear Dynamics, Newcastle, UK).

**Mass Spectrometry**—Spots of interest were excised from the gel followed by destaining, reduction, alklylation, and hydrolysis with modified porcine trypsin (Promega, Southampton, UK) as described previously (9–11). Samples for matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) analysis were prepared by mixing a small aliquot of the digestion supernatant 1:1 with α-cyano-4-hydroxycinnamic acid (10 μg/ml in 1:1 acetonitrile:0.1% (v/v) trifluoro-acetic acid). Peptide mass fingerprinting was performed on the Voyager Elite DE (Applied Biosystems, Warrington, UK) or Benchtop Reflection MALDI (Micromass, Manchester, UK).

For liquid chromatography coupled to electrospray tandem mass spectrometry, low flow rate reverse phase HPLC was used online to a Q-ToF (Micromass). The mobile phase gradient was mixed and delivered by a Merck Hitachi L7100 binary HPLC pump (Hitachi, Wokingham, UK) with the following protocol. Mobile phase A was 2% (v/v) acetonitrile, 0.05% (v/v) formic acid. Mobile phase B was 90% (v/v) acetonitrile, 0.05% (v/v) formic acid. Prior to use columns were equilibrated with 100% A for 20 min. Samples were then loaded and desalted in 100% A for 30 min and eluted with a gradient from 0% B to 80% B over 6 min. The mobile phase composition was then held at 80% B for the remainder of the analysis (45 min). The flow rate was reduced from 130 μl min⁻¹ at the pump to ~200 nl min⁻¹ through the stationary phase with the use of a precolumn split (~99:8.9:0.2). Two empty 1.5-cm × 15-cm HPLC columns connected in series were used as a dead volume reservoir and positioned after the flow splitter but prior to the fused silica capillary column (1 m, 375-μm outer diameter × 50-μm inner diameter; LC Packings NANN75-15-03-C18PM, Presearch Ltd., Hitchin, UK). The outlet of the separation capillary was coupled to a distal coated PicoTip (20-μm inner diameter, 10-μm tip inner diameter; FS360-20-10-o-20, New Objective Inc., Cambridge, MA) via a zero dead volume PRO-ADP ES interface (PRO-ADP, New Objective Inc.). Database searches using trypic peptide masses were performed using Mascot software (Matrix Science, London, UK).

**Transcriptional Analysis**—The expression level of the genes for leukotriene A4 hydrolase, annexin VI, vacular ATP synthase catalytic subunit A, and mortalin were determined using poly(A) PCR cDNA analysis. Poly(A) PCR material was prepared as described previously (12). Poly(A) cDNA samples were used to amplify the genes of interest using the following cycle profile: 22 cycles containing of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min followed by 72 °C for 5 min. Vacular ATP synthase and annexin VI were amplified from a poly(A) cDNA dilution of 1:100. All others were amplified from a dilution of 1:5000. All primers were designed within 250 bp of the poly(A) tail. Primer sequences were: leukotriene A4 hydrolase: atggcaactgaacatgcaatgaggg, ccaatctcatgtctctccca; vacular ATP synthase: tggatggttaggtacttagga, cagggtctaaggaagacgcgg; mortalin: gcagttgtgttagccatatgaaggaagg, ccactttcagatctctccca; vacuolar ATP synthase catalytic subunit A, and mortalin were determined using poly(A) PCR cDNA analysis. Poly(A) PCR material was prepared as described previously (5). The antibodies used were a polyclonal against leukotriene A4 hydrolase (Cayman Chemical), annexin VI (Santa Cruz Biotechnology), and asctes for mortalin/Grp75 (Calbiochem).

**Proteomics Analysis**—For liquid chromatography coupled to electrospray tandem mass spectrometry, low flow rate reverse phase HPLC was used online to a Q-ToF (Micromass). The mobile phase gradient was mixed and delivered by a Merck Hitachi L7100 binary HPLC pump (Hitachi, Wokingham, UK) with the following protocol. Mobile phase A was 2% (v/v) acetonitrile, 0.05% (v/v) formic acid. Mobile phase B was 90% (v/v) acetonitrile, 0.05% (v/v) formic acid. Prior to use columns were equilibrated with 100% A for 20 min. Samples were then loaded and desalted in 100% A for 30 min and eluted with a gradient from 0% B to 80% B over 6 min. The mobile phase composition was then held at 80% B for the remainder of the analysis (45 min). The flow rate was reduced from 130 μl min⁻¹ at the pump to ~200 nl min⁻¹ through the stationary phase with the use of a precolumn split (~99:8.9:0.2). Two empty 1.5-cm × 15-cm HPLC columns connected in series were used as a dead volume reservoir and positioned after the flow splitter but prior to the fused silica capillary column (1 m, 375-μm outer diameter × 50-μm inner diameter; LC Packings NANN75-15-03-C18PM, Presearch Ltd., Hitchin, UK). The outlet of the separation capillary was coupled to a distal coated PicoTip (20-μm inner diameter, 10-μm tip inner diameter; FS360-20-10-o-20, New Objective Inc., Cambridge, MA) via a zero dead volume PRO-ADP ES interface (PRO-ADP, New Objective Inc.). Database searches using trypic peptide masses were performed using Mascot software (Matrix Science, London, UK).

**Measurement of Leukotriene B4 Levels**—Leukotriene B4 levels were determined using an immunoassay according to the manufacturer’s instructions (Amersham Biosciences).

**Western Blotting**—Western blotting was carried out as described previously (5). The antibodies used were a polyclonal against leukotriene A4 hydrolase (Cayman Chemical), annexin VI (Santa Cruz Biotechnology), and asctes for mortalin/Grp75 (Calbiochem).

**Measurement of Leukotriene B4 Levels**—The leukotriene B4 levels in FDCP-Mix cells were assessed using an immunoassay according to the manufacturer’s instructions (Amersham Biosciences).
Chemotaxis and Cell Motility Assay—The migration of primary hematopoietic cells in response to agonists was assessed using a Boyden chamber or Transwell assay system. 24-well Transwell plates were used (Costar, Corning). These consisted of two wells separated by a membrane containing 5-μm pores. Cells (1–2 × 10^6/ml, 100 μl) were placed in the top well, and agonists were added to the top and/or bottom wells (bottom well volume, 600 μl) in Iscove’s medium plus 20% (v/v) batch-tested horse serum. After 4–6 h of incubation at 37 °C in a 5% CO₂-humidified incubator, viable cells in the lower well were counted using trypan blue. In no experiment was cell viability less than 98% in either top or bottom well after incubation. Assays of cell migration in the presence of agonists were linear for more than 10 h.

RESULTS

FDPC-Mix cells expressing Bcr-Abl PTK activity for a prolonged period assume a growth factor-independent phenotype. Furthermore these cells display retarded and abnormal development when the cells are exposed to the cytokines granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor (4, 5). We have shown that FDPC-Mix exposure to Bcr-Abl PTK does not induce detectable cytokine (interleukin-3, granulocyte colony-stimulating factor, or stem cell factor) expression in these cells (5), although p53 expression and Abl inhibitor proteins are post-translationally down-regulated by Bcr-Abl action (5, 8). The objective of this work was to apply proteomic techniques to identify and characterize any gross changes in soluble protein expression as a consequence of prolonged exposure to Bcr-Abl tyrosine kinase activity.

Cell lysates were prepared from tsBcr-Abl FDPC-Mix cells maintained at the permissive (32 °C) and restrictive (39 °C) temperatures for 12 weeks (LT32 and LT39 cells, respectively), tsBcr-Abl FDPC-Mix cells exposed to Bcr-Abl activity for 24 h (ST32), and parental FDPC-Mix at both temperatures. Multiple (n ≥ 4) preparations of cells from each condition were used for two-dimensional polyacrylamide gel electrophoresis. Typical gels are shown in Fig. 1 after silver staining. Protein spot analysis was performed for the various conditions in which FDCP-Mix and tsBcr-Abl FDCP-Mix had been grown. The most noteworthy feature of these analyses is the similarities between the various populations despite their culture at different temperatures and the presence and absence of Bcr-Abl PTK activity. The areas of interest highlighted in Fig. 1 show clearly, however, that there are differences in expression levels: we found four protein spots with substantial changes in expression level that correlated to the effects of...

Fig. 1. Two-dimensional gel analysis of proteins from Bcr-Abl-expressing FDPC-Mix cells. Two-dimensional gels stained with silver are shown of lysates from ST32 tsBcr-Abl FDPC-Mix cells (cells cultured for 24 h at the permissive temperature for the Bcr-Abl PTK) and LT32 tsBcr-Abl FDPC-Mix cells (cells cultured for >12 weeks at the permissive temperature). MALDI-ToF mass spectrometry was used on random spots on the gel to identify specific proteins to calibrate molecular masses. The proteins were: A, Rho GDI-1, 23.5 kDa, gi 12597249; B, tyrosine 3-monooxygenase, 29.3 kDa, gi 5803225; C, eukaryotic translation elongation factor 1, 47.0 kDa, P17182; G, p60, 47.0 kDa, P17182; H, L-plastin, 70.8 kDa, gi 667562; I, lamin B1, 67.0 kDa, gi 675456 (accession numbers beginning with gi are from the NCBI database, and those beginning with P are from the Swiss-Prot database). Proteins that differed in expression levels as a consequence of long term exposure to Bcr-Abl PTK activity in FDPC-Mix cells were identified using Progenesis software and labeled as spots 1, 2, 3, and 4 (see insets for specific regions of interest and the spot labeling).

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long term exposure to the Bcr-Abl PTK. Densitometric analysis of the four protein spots, identified as the major changes on prolonged exposure to Bcr-Abl, revealed changes ranging from a 290% increase in the case of spot 1 to an 80% reduction for spot 4 (Fig. 2). These proteins were consistently altered in all experiments performed (n = 4) and were the largest changes observed. Other minor changes were also seen that were not apparent in all experiments. In some cases other spots showed a mean change in spot volume that did not exceed 50%, and as such, we concentrated on the most consistent and largest effects observed.

The differences between LT32 tsBcr-Abl FDCP-Mix cells and the several control cell populations used confirmed that temperature was not affecting the expression of the proteins nor was the expression of a kinase-inactive Bcr-Abl (seen in lack of expression differences between LT39 tsBcr-Abl FDCP-Mix cells and control FDCP-Mix cells cultured at 39 °C).

Having identified protein spots on gels that are altered as a consequence of Bcr-Abl action over a long period we then determined their identity using mass spectrometry. Initially conventional MS analyses of the products of proteolytic digestion were performed. Comparison of the obtained pattern with computer-simulated digests of all known nucleotide/peptide sequences identifies a protein (13–17). The peptide masses derived from protein spots 1–4 were used in a search against the National Center for Biotechnology Information (NCBI) database using Mascot software, leading to a statistically significant identification for each protein (Table I). The proteins were leukotriene A4 hydrolase (spot 1), mortalin (spot 2), vacuolar ATP synthase catalytic subunit A (spot 3), and annexin VI (spot 4).

Despite the confident identification of all four proteins in question using peptide mass mapping alone, some sequence data were obtained in the case of spot 1 where the number of peptides for which m/z values were obtained was lower than with the other proteins. Partial sequence data were determined using reverse phase LC-MS/MS on a quadrupole/ToF instrument with low energy collisionally induced dissociation of selected doubly protonated peptide ions. Representative product ion spectra of two such peptides are shown in Fig. 3. These spectra were interpreted to generate sequence tags that could then be compared with the sequence of the leukotriene A4 hydrolase. All the product ion spectra obtained were consistent with the sequence proposed confirming the identification of the protein as leukotriene A4 hydrolase.

The question of the mode of regulation of protein expression in these four cases was then considered. Do we see a major shift in mRNA expression for these four proteins? To assay this we used a sensitive, semiquantitative RT-PCR procedure. The method involves an initial global amplification of cDNA derived from all polyadenylated mRNA followed by semiquantitative RT-PCR of individual genes using specific primers. The results and confirmation of the semiquantitative nature of this type of RT-PCR are shown in Fig. 4. There is no significant change in expression of mRNA for any of the above proteins except leukotriene A4 hydrolase. In this case there is a 30% reduction in mRNA levels as a consequence of exposure long term to Bcr-Abl kinase activity. This occurs concomitantly, however, with a 2.6-fold increase in the protein level. Thus the decreased level of mRNA in fact is associated with increased protein levels. Appropriate controls demon-

![Fig. 2. Changes in protein expression levels as a consequence of long term exposure to Bcr-Abl PTK activity.](image)

**Table I**

**Identification of proteins whose expression changes in multipotent hematopoietic FDCP-Mix cells as a consequence of long term exposure to Bcr-Abl PTK activity**

| Spot number (Fig. 1) | Name of protein and NCBI accession number | Number of peptides matched and Mowse score | Percentage of peptide coverage | Effect of long term exposure to Bcr-Abl on protein expression |
|---------------------|------------------------------------------|--------------------------------------------|-------------------------------|----------------------------------------------------------|
| Spot 1              | Leukotriene A4 hydrolase, gi 126354       | Mowse score 126, 8 peptides                | 24.4                          | Increased                                               |
| Spot 2              | Mortalin, gi 4758570                      | Mowse score 212, 13 peptides               | 27.8                          | Decreased                                               |
| Spot 3              | Vacuolar ATP synthase catalytic subunit A, gi 12231040 | Mowse score 199, 13 peptides | 27.6                          | Decreased                                               |
| Spot 4              | Annexin VI, gi 7304891                    | Mowse score 209, 13 peptides               | 29                            | Decreased                                               |

* Proportion of the sequence of the identified protein represented by peptides detected during MALDI MS of the protein digest.
strate this is not a temperature effect. In the case of the other proteins there are no significant mRNA level changes whether the cells have been transfected with Bcr-Abl or not or whether they are cultured at 32 °C or 39 °C.

We next investigated whether the observed protein changes were post-transcriptional or post-translational using Western blot analysis (Fig. 5). Fig. 5 shows that the reported increase in leukotriene A4 hydrolase as a consequence of the prolonged exposure to Bcr-Abl PTK occurs due to post-translational modification, there being no change in the overall level of the protein in the four cell populations. Similarly the changes in mortalin and annexin VI occur due to post-translational modification as the overall protein levels do not change to the degree seen on two-dimensional gel analysis. Thus, over and above p53, Abl inhibitory proteins, and the DNAPKcs (catalytic subunit of DNA-dependent protein kinase) previously reported, we see post-translational regulation of four other proteins by Bcr-Abl. However, as it was with p53, long term exposure to Bcr-Abl activity was required.

The consequence of the altered pattern of leukotriene A4 hydrolase expression in FDCP-Mix cells was examined by assaying the levels of its product, leukotriene B4, in parental FDCP-Mix and tsBcr-Abl FDCP-Mix cells (Table II). The levels of this signaling molecule were slightly decreased in Bcr-Abl-transfected cells compared with control cells. Evidence that this relates to the Bcr-Abl PTK activity was derived from the fact that switching the tsBcr-Abl FDCP-Mix cells to the restrictive temperature increased the levels of leukotriene B4 in
the cells compared to that seen in non-transfected control cells. The decrease seen in leukotriene B4 levels as a consequence of Bcr-Abl activation may alter cellular functions. Using a Transwell migration assay, we demonstrated that exogenous leukotriene B4 is a chemotactic factor for FDCP-Mix cells. In the presence of 10 nM leukotriene B4, there was a 3.1 ± 0.2-fold increase (mean ± S.E. of three observations) in Transwell migration toward the leukotriene B4. Placing the leukotriene in both the top well (with the cells) and the bottom well gave only a 1.08 ± 0.08-fold increase in cell migration (mean ± S.E., n = 3) compared with controls with no leukotriene present, demonstrating that the effect is chemokinetic, not enhanced motility. The magnitude of this effect can best be judged against the effect of the best characterized stem cell chemotactic agent, SDF-1 (stromal derived factor-1) (100 μg/ml); this gave a 9.4 ± 0.2-fold increase in Transwell migration. The efficacy of leukotriene B4 can therefore be seen as a marked and altered production of this agent in hematopoietic microenvironments significant for cellular migration or adhesion.

**DISCUSSION**

The Bcr-Abl oncogene is believed to be the causative agent in chronic myeloid leukemia. This disease is progressive. Initially there is a chronic phase in which granulocytosis is observed; however, the leukemic stem cell clone can differentiate normally. As the disease progresses it enters an accelerated phase where abnormal differentiation is observed, and there is an increased drug resistance. This inevitably leads to a blast crisis where a differentiation-blocked clone predominates. How these changes occur in this disease is unclear. There is some evidence that Bcr-Abl may impose a genetic instability on cells that leads to the inevitable progression observed (18), although evidence from the use of Imatinib (a specific inhibitor of Bcr-Abl kinase activity) suggests that the latter stages of the disease are still, in part, driven by the Bcr-Abl oncogene (19). Using the multipotent FDCP-Mix cell line plus a conditional mutant of the Bcr-Abl PTK we have investigated the progressive effects of the kinase activity on cellular survival, proliferation, and differentiation (3–5).
longed exposure to the Bcr-Abl PTK has an effect on proliferation and also on the ability of the cells to differentiate normally. This is similar to the proliferation and differentiation that is observed in the accelerated phase of CML. The onset of the accelerated phase is insidious, and obtaining appropriate material from patients to study this phase of the disease is problematic; we therefore used the tsBcr-Abl FDCP-Mix cell line to examine the progressive nature of change imposed on multipotent cells by Bcr-Abl. The question we posed was whether there was an overt change in the cellular proteome that correlated with this progression; if there were, then the proteins in question may be valuable prognostic indicators or may tell us something about the mechanism of disease progression.

We have shown that four proteins exhibit marked changes in expression using pI 3–10 two-dimensional gels. The approach we adopted was designed to detect prominent changes in expression of major proteins, hence the use of broad pI range gels. Further analysis of the same samples with narrow pI range gels will identify more subtle changes in the proteome imposed by the oncogene.

The protein that we have found to be increased in expression as a consequence of long term Bcr-Abl action is leukotriene A4 hydrolase. This enzyme, whose product is leukotriene B4, has a role in normal hematopoiesis and in CML. The arachidonic acid metabolite leukotriene B4 is produced in the bone marrow (20, 21) and supports the growth of bone marrow progenitor cells (22, 23). The leukotriene B4 receptor is expressed on a wide variety of hematopoietic cells (24), and its synthesis is stimulated by cytokines (25). In CML patient bone marrow, leukotriene B4 levels are in excess of those found in normal marrow (20). The importance of the leukotriene synthesis pathway in patients with CML has been demonstrated with inhibitors of 5-lipoxygenase, an enzyme that appears further upstream of the leukotriene biosynthetic pathway; such inhibitors reduced the rate of proliferation of CML blast cells and induced their differentiation (26). What is particularly pertinent about our findings is that leukotrienes C and D are often overproduced in CML; this can occur at the expense of leukotriene B4 production given the bifurcating nature of the leukotriene biosynthesis pathway. Thus a slight fall in the level of leukotriene B4 may be indicative of an increased commitment to synthesis of other leukotrienes. This set of events, we would postulate, may arise as a consequence of the post-translational modification of leukotriene A4 hydrolase that gives rise to the increased spot volume on the two-dimensional gel analysis we performed. Furthermore, we have shown that leukotriene B4 is chemotactic for FDCP-Mix cells. Perhaps significantly, polymorphonuclear cells from CML patients taking interferon α, an agent used in the treatment of CML, display decreased leukotriene A4 hydrolase activity (27). These data provide compelling evidence of a role for lipidic mediators in the control of myelopoiesis, control that is loosened in CML. We are currently seeking to identify the significance of these findings using primary normal and CML progenitor cell populations.

### Table II

| Cell line                  | Culture conditions | Leukotriene B4 levels (percentage of control) |
|----------------------------|-------------------|---------------------------------------------|
| FDCP-Mix                   | 32 °C             | (100)                                       |
| FDCP-Mix                   | 39 °C             | 107 ± 5                                     |
| LT32 tsBcr-Abl FDCP-Mix    | 32 °C             | 83 ± 12                                     |
| LT32 tsBcr-Abl FDCP-Mix    | 39 °C             | 108 ± 6                                     |

![Western blot analysis on the effect of Bcr-Abl PTK activity on leukotriene A4 hydrolase, mortalin, and annexin VI. LT32 tsBcr-Abl FDCP-Mix cells and LT39 tsBcr-Abl FDCP-Mix cells were incubated at both 39 °C (Bcr-Abl kinase-inactive) and 32 °C (Bcr-Abl kinase-active). Cell lysates were prepared and resolved by SDS-PAGE using 9% gels prior to Western blot analysis using anti-leukotriene A4 hydrolase, mortalin and annexin VI antibodies. LTA4, leukotriene A4.](image-url)
A second protein, mortalin, has no association with CML of which we are aware, although it is expressed in leukemic cells (28). This protein is decreased in expression as a consequence of long term exposure to Bcr-Abl action. Mortalin has been ascribed a role in transformation (29), and this may be associated with the inactivation of p53 (30), a protein we have shown to be decreased in expression levels as a consequence of exposure to Bcr-Abl kinase activity (5). Whether there is a relationship between decreased p53 levels and a fall in the level of mortalin remains to be determined. Nonetheless this proteomic study has highlighted an area where Bcr-Abl action could contribute to genetic instability. Furthermore mortalin expression is known to alter expression of downstream regulators of p53, such as p21 WAF-1 (30). p21 has an intrinsic role in regulating myeloid cell development (31); thus it may be disrupted by mortalin leading to the abnormalities seen in the accelerated phase of the disease.

Rather more intriguing is the potential role of annexin VI, one of a group of calcium-binding proteins of unknown function but which are implicated in important cellular processes including anticoagulation, ion flux regulation, calcium homeostasis, and endocytosis (32). Annexin VI is expressed in B lineage acute lymphoblastic leukemia cells at higher levels than in normal lymphoid cells, but there is no indication of a role for this protein in leukemogenesis. There is a link to signaling pathways activated by Bcr-Abl in that there is an ability of annexin VI to associate with Ras GAP, Src kinase family members (e.g. Fyn) and negatively charged lipids involved in signaling, such as the phosphatidylinositol phosphates (which mediate Bcr-Abl-stimulated survival signals) (33–36). Whether decreased levels of annexin VI contribute to altered signaling by Bcr-Abl remains to be determined.

The fourth protein to be altered in expression levels by the long term effects of Bcr-Abl is the ATP synthase a subunit (37). The role of this protein in cellular transformation has not been explored. Investigation of the mitochondrial activity of FDCP-Mix cells exposed in the short and long term to Bcr-Abl activity reveals that there is no major difference in mitochondrial activity of these two populations.2

Hematologists and oncologists have a variety of different research issues to address related to proteins, their interactions, and their post-translational modification; all these are approachable rapidly and with great sensitivity using mass spectrometry. Our data show the need for the major transcriptomic and proteomic studies currently underway (for example, see Refs. 38 and 39) to recognize that mRNA level changes do not necessarily correlate with protein level alterations and that post-translational events contribute to the proteome changes (40, 41). Transcriptomic analysis is already a powerful prognostic tool, but the mechanisms underlying malignant disease will require careful examination of the proteome also. Future studies will explore the expression patterns of these proteins in CML samples. Advances in mass spectrometry are taking place (42) that will allow more detailed definition of the proteome, including determination of protein-protein interactions and protein phosphorylation status, using relatively limited material from primary normal and leukemic cell populations.

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