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Cell lines and peripheral blood leukocytes derived from individuals with chronic myelogenous leukemia display virtually identical proteins phosphorylated on tyrosine residues

(protein-tyrosine kinase/anti-phosphotyrosine antibody/*ABL* gene)

RICHARD D. HUHN*, MARSHALL R. POSNER*, SYDONIA I. RAYTER†‡, J. GORDON FOULKES†‡, AND A. RAYMOND FRACKELTON, JR.*§

*Department of Medicine, Roger Williams General Hospital and Brown University, Providence, RI 02908; and †Laboratory of Eukaryotic Molecular Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England

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ABSTRACT **An aberrant p210$^{BCR-ABL}$ protein that possesses constitutive protein-tyrosine kinase activity is presumed to be involved in the development of the neoplastic phenotype in chronic myelogenous leukemia (CML). Using a highly specific antibody against phosphotyrosine, we have isolated the tyrosine-phosphorylated p210$^{BCR-ABL}$ and several other proteins containing phosphotyrosine from a variety of CML cell lines. p210$^{BCR-ABL}$ isolated by the monoclonal anti-phosphotyrosine antibody possessed protein-tyrosine kinase activity *in vitro* comparable to that of the p210$^{BCR-ABL}$ isolated by antibody to a specific peptide sequence in the ABL protein-tyrosine kinase. Other prominent proteins containing phosphorylated tyrosine residues were observed at 185, 150, 120, 105, 63, 56, 36, and 32 kDa, and less prominent proteins were observed at 195, 155, 94, 53, 40, and <29 kDa. Staphylococcal V8 peptide mapping indicated that proteins of similar molecular weights were highly homologous to each other across cell lines, despite the diverse hematopoetic lineages of these cells and the genetic heterogeneity of the patients from whom the CML cell lines were derived. Phosphopeptide mapping also revealed that these proteins were distinct from each other as well as from p210$^{BCR-ABL}$. Because virtually identical phosphotyrosine-containing proteins were found in peripheral blood leukocytes taken directly from CML patients, these proteins are not an artifact of long-term tissue culture but appear to be an integral part of the CML phenotype.**

Hematopoietic cells of over 90% of patients with chronic myelogenous leukemia (CML) have the Philadelphia chromosome (Ph^1), a cytogenetic abnormality resulting from reciprocal translocation of the long arms of chromosome 9 and 22 (1–3). As a consequence of this event, the *ABL* oncogene is translocated from its normal position on chromosome 9 to chromosome 22, resulting in the fusion of the 5′ sequence of the *BCR* gene on chromosome 22 to 3′-*ABL* sequences (4–7). A specific 8-kilobase mRNA transcript of the *BCR-ABL* fusion gene can be isolated from Ph^1-positive CML cell lines and patient samples (8–11). This transcript is translated into a chimeric BCR-ABL phosphoprotein of ≈210-kDa (p210$^{BCR-ABL}$) that exhibits constitutive protein-tyrosine kinase activity (EC 2.7.1.112) (12, 13).

Two independent lines of research have provided much circumstantial evidence implicating protein-tyrosine kinases in both neoplastic transformation and in the control of normal cell growth (reviewed in refs. 14 and 15). First, the transforming proteins of many retroviruses [e.g., ABL (Abelson murine leukemia virus), SRC (Rous sarcoma virus), FMS (McDonough feline sarcoma virus), and ERBB (avian erythroblastosis virus)] are constitutively active protein-tyrosine kinases that phosphorylate themselves and/or various cellular proteins (16–20). Studies with temperature-sensitive and deletion mutants of several of these viruses have demonstrated that their ability to transform cells is dependent on their expression of an active protein-tyrosine kinase (21–24).

Second, tyrosine phosphorylation has been implicated in the control of cell growth because cellular receptors for several polypeptide growth factors are protein-tyrosine kinases that are activated by the binding of their respective ligands to phosphorylate themselves as well as other cellular proteins (25–29).

Attempts to identify and purify physiologically relevant phosphotyrosine-containing proteins have been hampered by the low abundance of those proteins and the problems involved in distinguishing them from proteins containing only phosphoserine and phosphothreonine (14, 15, 23, 30, 31). To overcome these difficulties anti-phosphotyrosine antibodies have been developed that have enabled the identification and purification of specific phosphotyrosine-containing proteins from a variety of normal and neoplastic cells, including the transforming proteins ABL and SRC, as well as the activated receptors for epidermal growth factor, platelet-derived growth factor, and insulin (29, 32–37).

As a first step to determine the role of the ABL protein-tyrosine kinase in CML, we have used a highly specific monoclonal antibody to phosphotyrosine to affinity-purify tyrosine-phosphorylated proteins from Ph^1-positive CML cell lines and from peripheral blood leukocytes of CML patients. In addition to the tyrosine-phosphorylated p210$^{BCR-ABL}$, we have isolated several other phosphotyrosine-containing proteins that are candidates for cellular substrates of the aberrant ABL protein-tyrosine kinase. Remarkably, both the peripheral leukocytes from patients and each of the cultured CML cell lines harbor virtually identical phosphotyrosine-containing proteins.

MATERIALS AND METHODS

Cells. K562, BV173 (obtained from J. Ritz), and RWLeu4 are Ph^1-positive cell lines derived from individual patients in the acute phase of CML (38–40). These cells and HL-60 (acute promyelocytic) (41), HEL (human erythroleukemic), ANN-1 (Abelson murine leukemia virus-transformed fibroblasts) (42), 298-18 (Abelson murine leukemia virus-transformed murine pre-B lymphocytes) (43), and BALB/c 3T3 (a nontransformed murine fibroblast cell line) were grown in

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Abbreviations: CML, chronic myelogenous leukemia; Ph^1, the Philadelphia chromosome.

‡Present address: Oncogene Science Inc., Manhasset, NY 11030.
§To whom reprint requests should be addressed.

supplemented RPMI 1640 as described (33). K562, HL-60, HEL, and BALB/c 3T3 cells were obtained from the American Type Culture Collection. Peripheral blood leukocytes from two patients (A.G. and C.C.) in the acute phase of CML were prepared by Ficoll-Hypaque density zone centrifugation and then incubated briefly in supplemented RPMI 1640 at 2×10^6 cells per ml in 5% CO_2/95% air at 37°C prior to labeling and extraction.

Metabolic Labeling and Immunoprecipitations. Exponentially growing cells were incubated with $^{32}P_i$ (1 mCi per 10^6 cells per ml) for 3 hr at 37°C and then extracted at 0°C with 1% Triton X-100 in a buffer containing proteinase, kinase, and phosphatase inhibitors (29, 33). Tyrosine-phosphorylated proteins were isolated from the extracts by micro-batch affinity chromatography, as described (29, 33), except that a different more specific anti-phosphotyrosine antibody (antibody 1G2) was employed (A.R.F., unpublished data). This monoclonal antibody has high affinity and specificity for phosphotyrosine; phosphoserine, phosphothreonine, phosphohistidine, or mono- or triphosphonucleotides are not recognized (unpublished data). Phosphotyrosine-containing proteins were eluted specifically from the immunosorbent with the hapten phenyl phosphate (29, 33), resolved by $NaDodSO_4$/PAGE (7.5% gels) under reducing conditions (44), and visualized by autoradiography.

For immunoprecipitation of ABL proteins, cell extracts or hapten eluates (diluted to 1 ml with the extraction buffer) were precleared of nonspecifically adsorbing proteins by two 30-min incubations with 5 μl of nonimmunized rabbit serum and with 50 μl of protein A-Sepharose CL4B beads. The supernatant after centrifugation was retained, and 5 μl of specific anti-ABL, anti-Moloney GAG, or control nonimmune rabbit serum was added. The anti-ABL antiserum was raised by immunization of rabbits with a peptide homologous to the v-abl sequence, Tyr-Ile-Ser-Asp-Glu-Val-Glu-Lys-Glu-Leu-Gly-Lys (corresponding to peptide 4 in ref. 45). After a 2-hr incubation, immune complexes were precipitated by 50 μl of protein A-Sepharose CL4B. The beads were washed four times in protein-free extraction buffer, and the immune complexes were solubilized by boiling in $NaDodSO_4$/gel sample buffer.

Phosphoamino Acid Analysis. Phosphoproteins extracted from individual bands were analyzed by partial acid hydrolysis and thin layer electrophoresis (30, 33). Labeled phosphoamino acids were detected by autoradiography and quantitated by video densitometry (46).

Staphylococcal V8 Protease Peptide Mapping. Proteins were extracted from individual gel bands and precipitated with trichloroacetic acid as for phosphoamino acid analysis. The precipitated proteins were digested with staphylococcal V8 protease (type XVIII, Sigma) (47), and the resulting peptide fragments were resolved by $NaDodSO_4$/PAGE (15% acrylamide) and visualized by autoradiography.

***In Vitro* Kinase Reactions.** The ABL protein was immunoprecipitated by either anti-phosphotyrosine or anti-ABL antibodies as described above (using extracted or purified proteins derived from 10^6 cells) and washed four times in kinase buffer (0.015% Brij 35/50 mM Hepes/0.1 mM EDTA, pH 7.5). The beads were resuspended directly in 20 μl of kinase buffer and acid-denatured rabbit muscle enolase (48) (5 μl at 1 μg/μl) was added to serve as exogenous substrate. The reaction was initiated by the addition of 5 μl of 50 mM $MgCl_2$ and 5 μl of 1 mM ATP containing 2 μCi of [γ-^{32}P]ATP per μl (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and continued for 30 min at 30°C.

RESULTS

Affinity-Purification of Proteins Phosphorylated on Tyrosine Residues from CML Cell Lines. To identify candidate substrates of the aberrant ABL protein-tyrosine kinase, phosphotyrosine-containing proteins were isolated from three [$^{32}P_i$]-labeled CML cell lines by affinity chromatography on monoclonal anti-phosphotyrosine antibody-Sepharose. The nine major proteins isolated were 210, 185, 150, 120, 105, 63, 56, 36, and 32 kDa (Fig. 1*A*, lanes 1). Few of the phosphotyrosine-containing proteins isolated from CML cell lines were prominent in either the Ph[1]-negative promyelocytic cell

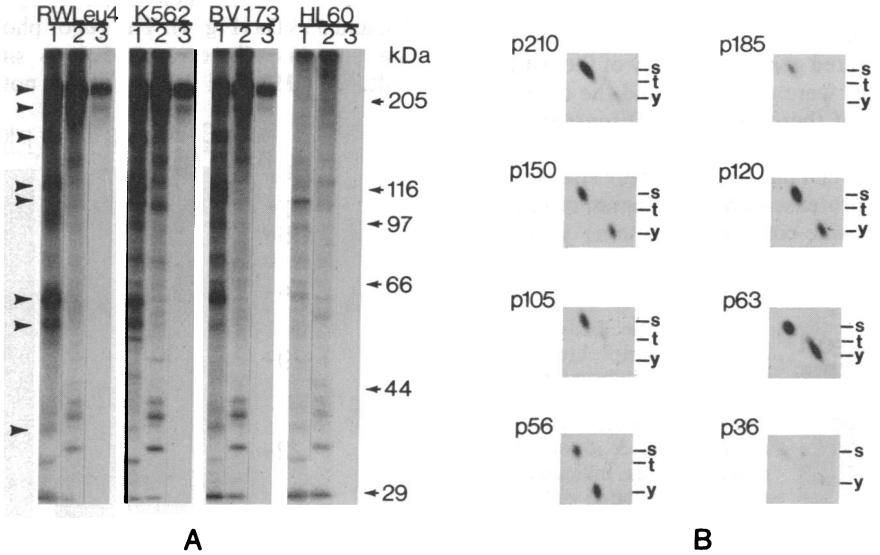

FIG. 1. Phosphotyrosine-containing proteins isolated from CML cell lines. (*A*) $NaDodSO_4$/PAGE of proteins isolated by anti-phosphotyrosine antibody and by anti-ABL antibody. Cells from the CML cell lines RWLeu4 (10^6 cells), K562 (2×10^6 cells), and BV173 (2.4×10^6 cells) and the acute promyelocytic leukemia cell line HL-60 (2×10^6 cells) were incubated for 3 hr with $^{32}P_i$ and extracted with Triton X-100 in a buffer containing kinase, phosphatase, and proteinase inhibitors. Specific proteins were immunopurified as indicated below, resolved by $NaDodSO_4$/PAGE (7.5% acrylamide), and visualized by autoradiography. Lanes: 1, proteins purified with the anti-phosphotyrosine immunosorbent; 2, proteins purified with anti-ABL antibody; 3, proteins purified first with the anti-phosphotyrosine immunosorbent and then with anti-ABL antibody. (*B*) Phosphoamino acid analysis of phosphotyrosine-containing proteins from RWLeu4 cells. Proteins were eluted from excised gel bands (arrowheads in *A*, lanes 1) and subjected to partial acid hydrolysis and two-dimensional thin layer electrophoresis. Phosphoamino acids were visualized by autoradiography (y, phosphotyrosine; t, phosphothreonine; s, phosphoserine).

line HL-60 (Fig. 1*A*) or other Ph^1-negative cell lines, including quiescent mouse fibroblasts and cells from Burkitt lymphoma, from T-cell leukemia, from acute myelogenous leukemia, and from human erythroleukemia (HEL) (data not shown).

The most intense labeling was seen in a 210-kDa protein that we suspected was the aberrant ABL protein ($p210^{BCR-ABL}$) characteristic of Ph^1-positive cells (12, 13). A phosphorylated protein of identical electrophoretic mobility was immunoprecipitated by the affinity-purified anti-ABL antibody (Fig. 1*A*, lanes 2). This protein was absent in the Ph^1-negative acute promyelocytic leukemia cell line HL-60 (Fig. 1*A*). Anti-ABL antibody also immunoprecipitated the labeled 210-kDa protein from anti-phosphotyrosine-purified proteins of CML cell lines, indicating its identity as $p210^{BCR-ABL}$ (Fig. 1*A*, lanes 3). Confirming this conclusion, staphylococcal V8 protease-peptide mapping (47) of the ^{32}P-labeled 210-kDa proteins isolated by anti-ABL and by anti-phosphotyrosine antibody were indistinguishable (data not shown).

Specificity of the purification procedure was demonstrated by the ability of the phosphotyrosine analog phenyl phosphate to inhibit isolation of all of the ^{32}P-labeled bands shown in Fig. 1, lanes 1 (data not shown), and by phosphoamino acid analysis of the gel bands. The nine most prominent ^{32}P-labeled proteins, as well as six less-prominent proteins of 195, 155, 94, 53, 40, and <29 kDa from RWLeu4 cells contained 20%–60% phosphotyrosine relative to the total acid-stable phosphoamino acids (Fig. 1*B* and data not shown).

The specificity of the anti-ABL antibody was ascertained by determining its ability to react with various ABL protein species in extracts of ^{32}P-labeled cells. This antibody immunoprecipitated the $p120^{v-abl}$ and $p160^{v-abl}$ from Abelson murine leukemia virus-transformed ANN-1 cells (Fig. 2*A*) and 298-18 cells (data not shown), respectively, as did an antibody directed against v-gag determinants. The v-gag sequences are expressed on the chimeric v-abl proteins but not on the $p210^{BCR-ABL}$. Thus only the anti-ABL antibody was able to immunoprecipitate $p210^{BCR-ABL}$ from RWLeu4 cells (Fig. 2*B* and data not shown). Immunoprecipitation of $p210^{BCR-ABL}$ from RWLeu4 cells by anti-ABL antibody was blocked by 1 mM peptide 4 (Fig. 2*B*), again confirming the specificity of this antibody.

Despite both the expected genetic diversity of the patients from whom the cell lines were developed and the divergent hematopoietic lineages of these cells (see *Discussion*), the electrophoretic patterns of the phosphotyrosine-containing proteins from the various cell lines are strikingly similar (Fig. 1). Staphylococcal V8 protease-peptide mapping of each of the major phosphotyrosine-containing proteins was performed to examine the extent of their relatedness. Both ^{32}P-labeled and ^{35}S-labeled peptide maps of the 210-, 185-, 120-, 105-, 56-, and 32-kDa proteins from RWLeu4 cells were indistinguishable from those of proteins of corresponding molecular sizes from BV173 and K562 (data not shown). The maps of the 63-kDa protein of RWLeu4 and BV173 cells were also identical but dissimilar to the map of the 63-kDa protein of K562. Proteins of dissimilar molecular sizes displayed dissimilar maps (Fig. 3). Therefore, none of these proteins appear to be proteolytic products of either $p210^{BCR-ABL}$ or each other, nor do they appear to bear any precursor/product relationship.

***In Vitro* Kinase Activity of the Anti-ABL- and Anti-Phosphotyrosine-Isolated Proteins.** $p210^{BCR-ABL}$ immunoprecipitated by at least certain antibodies is an active protein-tyrosine kinase *in vitro*, capable of phosphorylating both itself and exogenous substrates such as enolase (49, 50). To establish clearly the identity of the 210-kDa protein isolated by the anti-ABL and anti-phosphotyrosine procedures, we assessed their activities as protein-tyrosine kinases. *In vitro* kinase reactions were performed directly on the anti-ABL- or anti-phosphotyrosine-Sepharose complexes, in which $p210^{BCR-ABL}$ and the exogenous substrate enolase were both phosphorylated. Phosphoamino acid analysis revealed that enolase was phosphorylated predominantly on tyrosine.

Phosphotyrosine-Containing Proteins Isolated Directly from Peripheral Blood Leukocytes of Patients with Acute Phase CML. The CML cell lines examined in this study have been passaged repeatedly in culture. We were concerned, therefore, about the relevance of their phosphotyrosine-containing proteins to the natural pathogenesis of neoplastic transformation. To extend our observations, therefore, CML cells were obtained directly from patients. Peripheral blood mononuclear leukocytes (blast cells and lymphocytes) from CML patients were separated by Ficoll-Hypaque density zone centrifugation, incubated with $^{32}P_i$, and extracted as described for the cultured cells. Many phosphotyrosine-containing proteins were affinity purified from these leukocytes, including $p210^{BCR-ABL}$ (ascertained by immunoprecipitation with anti-ABL) and heavily labeled proteins of 150, 105, 63, 56, and 53 kDa (Fig. 4). The major phosphotyrosine-containing proteins all corresponded to similar proteins in the cultured CML cell lines although not all were represented

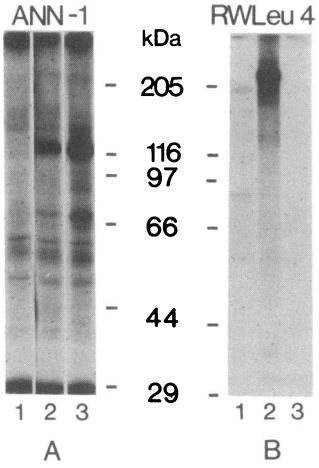

FIG. 2. Specificity of anti-ABL immunoprecipitations. Cells were incubated for 3 hr with $^{32}P_i$ and then extracted with detergent. The extracts were incubated with 5 μl of specific antibodies (as indicated) and then coprecipitated with protein A-Sepharose. (*A*) Cell line ANN-1 (Abelson murine leukemia virus-transformed fibroblasts). Lanes: 1, nonimmune rabbit serum; 2, anti-Moloney GAG serum; 3, anti-ABL antibody. (*B*) Cell line RWLeu4 (acute phase CML). Lanes: 1, nonimmune rabbit serum; 2, anti-ABL antibody; 3, anti-ABL antibody incubated with 1 mM ABL peptide 4.

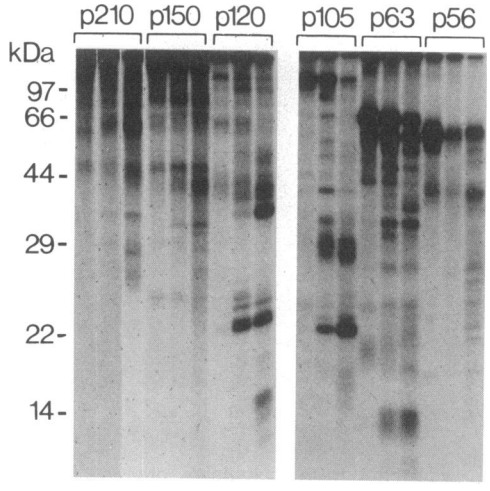

FIG. 3. Peptide mapping of major phosphotyrosine proteins isolated from $^{32}P_i$-labeled RWLeu4 CML cells. RWLeu4 proteins were extracted from gel bands and subjected to partial proteolysis with 1, 7, and 30 μg of V8 protease per ml (lanes from left to right, respectively, for each protein). The resulting proteolytic fragments were resolved on 15% $NaDodSO_4$ gels and visualized by autoradiography.

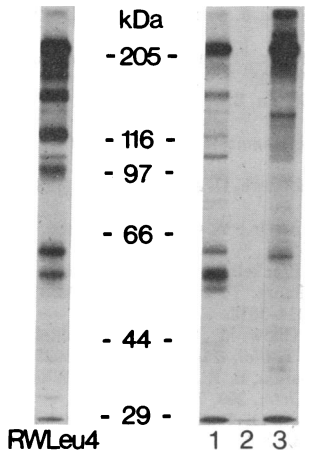

FIG. 4. Phosphotyrosine-containing proteins isolated directly from peripheral blood leukocytes of a CML patient. Peripheral blood mononuclear cells (2×10^6 cells) from a patient (A.G.) in the acute phase of CML were labeled with $^{32}P_i$ and extracted with detergent. Lanes: 1, proteins isolated from the detergent extract by anti-phosphotyrosine antibody; 2, 40 mM phenyl phosphate competitively inhibited the purification of the phosphotyrosine-containing proteins; 3, 210-kDa protein immunoprecipitated from the detergent extract by anti-ABL. Proteins phosphorylated on tyrosine residues from RWLeu4 cells are shown for comparison.

equally. Virtually identical results were obtained with acute phase cells from a separate individual (patient C.C.). Staphylococcal V8 protease peptide maps of the 105-, 63-, and 56-kDa proteins from leukocytes of patients were identical to those of RWLeu4 cells (data not shown).

DISCUSSION

In this study, numerous phosphotyrosine-containing proteins were affinity purified from both CML cell lines and from peripheral blood leukocytes of patients in the acute phase of CML. Most prominent was a 210-kDa protein that displayed protein-tyrosine kinase activity *in vitro*, was immunoprecipitated from the hapten eluate by anti-ABL antibodies, and was indistinguishable from $p210^{BCR\text{-}ABL}$ by peptide mapping. On this basis, we identify this protein as $p210^{BCR\text{-}ABL}$.

In addition to $p210^{BCR\text{-}ABL}$, 15 other proteins phosphorylated on tyrosine residues were isolated from CML cell lines and peripheral blood leukocytes of CML patients. Most of the phosphotyrosine-containing proteins from peripheral blood leukocytes correspond to proteins in the cultured CML cell lines, both in terms of molecular size (Fig. 4) and V8 peptide maps, demonstrating that the tyrosine-phosphorylated proteins in these cell lines are not artifacts induced by long passage in tissue culture. Furthermore, electrophoretic patterns of these phosphotyrosine-containing proteins are remarkably similar across the cell lines, despite the expected genetic heterogeneity of the patients from whom they were derived and the diverse hematopoetic lineages of these cells.

Of the three CML cell lines studied in this paper, BV173 is the most primitive, probably representing a pluripotent stem cell (39). BV173 cells do not react with monoclonal antibodies specific for myeloid or monocytic cells, nor do they differentiate to macrophages or granulocytes in response to phorbol esters or retinoic acid. Although they lack markers for mature B or T cells (e.g., surface immunoglobulin, OKT3 antigen, or sheep erythrocyte rosette-forming antigen), they display Ia(DR) antigen characteristic of B cells and progenitor myeloid cells, as well as the common acute lymphoblastic leukemia antigens and other antigens characteristic of lymphopoietic progenitor cells.

In contrast, K562 cells are well along the path toward erythroid differentiation (38, 39). These cells have glycophorin A, spectrin, and the "i" surface antigen characteristic of erythrocytes (51–54) and are readily induced by hemin and other chemical agents to differentiate into erythroid cells that synthesize hemoglobin (51, 55).

RWLeu4 cells, on the other hand, are quite different. This CML cell line clearly lies on the myelomonocytic pathway: it lacks Fc receptors, has a basophilic cytoplasm with prominent cytoplasmic granules, has irregularly shaped nuclei, bears β_2-microglobulin on its surface, lacks immunoglobulin, and histocytochemically displays both peroxidase and esterase activity. Furthermore, these cells are induced to differentiate along the myeloid pathway into granulocytes by polar solvents and along the monocytic pathway into macrophages by phorbol esters (40).

The similarity of phosphotyrosine-containing proteins displayed by all of the CML cells is further surprising in that variable expression of tyrosine-phosphorylated substrates has been reported in other systems in which protein-tyrosine kinases have been implicated in cell transformation and regulation of cell growth (31, 56–59). Particularly relevant is a report that pre-T cells and pre-B cells that have been transformed by v-*abl* share only three out of eight phosphotyrosine-containing proteins (58). Furthermore, v-*abl*-transformed pre-B cells and fibroblasts contain many diverse, unshared proteins phosphorylated on tyrosine residues, even though they do display the ABL-transforming protein and share in common phosphotyrosine-containing proteins of 63 and 56 kDa (59).

Clearly, phosphotyrosine-containing proteins that are essential for the maintenance of the CML phenotype should be present in all CML cells. By this criterion, virtually all of the 15 cellular substrates remain candidates except one of the bands at 63 kDa (this band is actually a doublet), which appears to be different in K562. Of particular interest is the other band at 63 kDa and the band at 56 kDa, which may be related to similar-size proteins found in cells transformed by the Abelson murine leukemia virus (59).

However, one might also expect that a candidate substrate(s) would not be phosphorylated in quiescent, nonneoplastic cells. Thus we would elminiate the 105-kDa protein, which was found in all cells examined, including nonmitotic fibroblasts (unpublished results).

Because some of the tyrosine-phosphorylated proteins in CML cells might be phosphorylated by tyrosine kinases that are normally expressed in cells susceptible to the Ph^1 translocation, it was important to examine cells at a similar state of differentiation that lacked the Ph^1. Although normal cells of this description are not available, two Ph^1-negative human leukemias provided close alternatives. The promyelocytic leukemia cell line HL-60 lacks the aberrant Abelson tyrosine kinase but is antigenically and developmentally nearly identical to the CML cell line RWLeu4. HL-60 and RWLeu4 cells differentiate to granulocytes and to macrophages in response to polar solvents and to phorbol esters, respectively (40, 60). HEL cells lack the aberrant Abelson kinase yet are developmentally nearly identical to the CML cell line K562 (61). HL-60 and HEL cells contained anti-phosphotyrosine-isolated proteins of 94, 63, 36, and 32 kDa. But while the 36- and 32-kDa proteins in these cells were phosphorylated with nearly as great an intensity as the corresponding proteins in the CML cells, the other proteins were considerably less pronounced. These may be related to the 56-kDa protein described in LSTRA (a mouse T-cell lymphoma cell line) or the 38- and 55- to 68-kDa proteins described in cells of hemopoietic origin (62–67) and may represent targets for another protein-tyrosine kinase(s). Of course, some of these targets may also reflect physiologically irrelevant phos-

phorylations by kinases with relaxed substrate specificity (14, 15, 31).

Naldini *et al.* (68) and Ogawa *et al.* (69) used anti-phosphotyrosine antibodies to detect tyrosine-phosphorylated proteins in the CML line K562. Each group detected different proteins phosphorylated on tyrosines, and both groups failed to detect several of the phosphotyrosine-containing proteins that we observed in this study. The reasons for these discrepancies are unclear, although we have found that reproducible isolation of large numbers of phosphotyrosine-containing proteins requires critical attention to the inhibition of protein phosphatases and proteases.

It will be important to establish whether the CML cell phosphotyrosine-containing proteins are specific ABL substrates and whether their phosphorylation is correlated with the transition from the chronic to the acute phase of this disease.

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