MOLECULAR AND CELLULAR BIOLOGY, Nov. 1985, p. 3116–3123
0270-7306/85/113116-08$02.00/0

Vol. 5, No. 11

Detection of c-*abl* Tyrosine Kinase Activity In Vitro Permits Direct Comparison of Normal and Altered *abl* Gene Products

JAMES B. KONOPKA AND OWEN N. WITTE*

Department of Microbiology and Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024

Received 21 May 1985/Accepted 12 August 1985

The v-*abl* transforming protein $P160^{v\text{-}abl}$ and the $P210^{c\text{-}abl}$ gene product of the translocated c-*abl* gene in Philadelphia chromosome-positive chronic myelogenous leukemia cells have tyrosine-specific protein kinase activity. Under similar assay conditions the normal c-*abl* gene products, murine $P150^{c\text{-}abl}$ and human $P145^{c\text{-}abl}$, lacked detectable kinase activity. Reaction conditions were modified to identify conditions which would permit the detection of c-*abl* tyrosine kinase activity. It was found that the Formalin-fixed *Staphylococcus aureus* formerly used for immunoprecipitation inhibits in vitro *abl* kinase activity. In addition, the sodium dodecyl sulfate and deoxycholate detergents formerly used in the cell lysis buffer were found to decrease recovered *abl* kinase activity. The discovery of assay conditions for c-*abl* kinase activity now makes it possible to compare $P150^{c\text{-}abl}$ and $P145^{c\text{-}abl}$ kinase activity with the altered *abl* proteins $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$. Although all of the *abl* proteins have in vitro tyrosine kinase activity, they differ in the way they utilize themselves as substrates in vitro. Comparison of in vitro and in vivo tyrosine phosphorylation sites of the *abl* proteins suggests that they function differently in vivo. The development of c-*abl* kinase assay conditions should be useful in elucidating c-*abl* function.

The c-*abl* oncogene was initially identified as the normal cellular homolog of the v-*abl* transforming gene of Abelson murine leukemia virus (13, 15, 41, 46). The human c-*abl* gene has been shown to be altered by translocation from chromosome 9 to 22 in Philadelphia chromosome (Ph^1)-positive chronic myelogenous leukemia cells (2, 14, 16). Viral transduction and chromosomal translocation of c-*abl* are similar in that they result in the synthesis of *abl* proteins with structurally altered amino termini (27, 28, 47). The v-*abl* gene product, $P160^{v\text{-}abl}$, and the gene product of the translocated c-*abl* gene, $P210^{c\text{-}abl}$, have similar in vitro tyrosine kinase activities (10, 25, 27, 28). When assayed under similar conditions, the murine c-*abl* protein, $P150^{c\text{-}abl}$, and human c-*abl* protein, $P145^{c\text{-}abl}$, lacked detectable tyrosine kinase activity (10, 25, 28). Altered *abl* kinase activity is strongly implicated in the pathogenesis of Abelson murine leukemia virus-induced leukemia and chronic myelogenous leukemia.

Previous studies have suggested that amino-terminal alteration of the v-*abl* and t-*abl* proteins is important for altering c-*abl* tyrosine kinase activity (28). In addition to lacking detectable in vitro tyrosine kinase activity, the c-*abl* protein is not detectably phosphorylated on tyrosine in vivo, but the v-*abl* and t-*abl* proteins are phosphorylated on tyrosine in vivo (28, 36, 37). Since it is the c-*abl*-derived sequences that encode the kinase domain, it was surprising that kinase activity was not detected for the $P150^{c\text{-}abl}$ or $P145^{c\text{-}abl}$. This raised the possibility that the c-*abl* protein is regulated and that amino-terminal alteration of the v-*abl* and t-*abl* proteins disrupts normal regulation. Another possibility is that c-*abl* tyrosine kinase activity differs from that of $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$ and may not autophosphorylate efficiently in vitro. The ability of amino-terminal sequences to influence kinase activity was also demonstrated by analysis of a carbohydrate-modified form of the v-*abl* protein synthesized with an amino-terminal leader sequence (43). This v-*abl* protein lacks detectable kinase activity in vitro and is not phosphorylated in vivo (43).

To examine the possibility that the kinase activity of the c-*abl* protein in some way differs from that of $P160^{v\text{-}abl}$ or $P210^{c\text{-}abl}$, reaction conditions were varied to determine conditions which would permit the detection of c-*abl* kinase activity. When reaction conditions were optimized for the detection of $P160^{v\text{-}abl}$ autophosphorylation, it was found that c-*abl* kinase activity could also be detected. Under the new reaction conditions, kinase activity continued for a longer period of time, resulting in increased detection of autophosphorylation and substrate phosphorylation. Comparison of the in vitro autophosphorylation sites indicates that the c-*abl* proteins differ from altered *abl* proteins $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$ in the way they utilize themselves as substrates in vitro.

MATERIALS AND METHODS

Cells and antisera. Murine NIH 3T3 fibroblasts were transformed with the P160 strain of Abelson murine leukemia virus. SCRF60A is a murine thymoma cell line (30). Human cell lines included Ph^1-negative HL-60 (6) and Ph^1-positive K562 (31) and EM-2 (22).

abl-specific serum was prepared as previously described (26). Sera harvested from rabbits immunized with regions of v-*abl* expressed as *trpE-abl* fusion proteins in *Escherichia coli* are designated αpEX-2, αpEX-4, or αpEX-5. Serum harvested from rabbits immunized with a chemically synthesized peptide (peptide 5) corresponding to the predicted v-*abl* protein sequence was designated αpep5. The kinase-inhibiting sera are directed against v-*abl* residues 374 to 384 (αpep5) or residues 466 to 593 (αpEX-2). For use in kinase inhibition studies, immunoglobulin G (IgG) was purified

* Corresponding author.

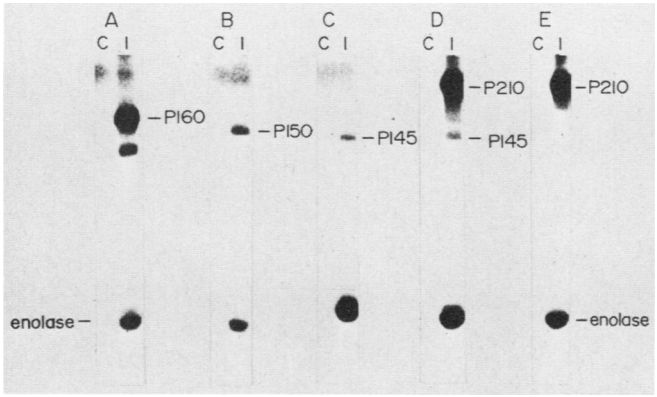

FIG. 1. In vitro phosphorylation of *abl* proteins. P160 A-MuLV-transformed NIH 3T3 fibroblasts (A), murine SCRF60A (B), human HL-60 (C), and Ph^1-positive human K562 (D) and EM-2 (E) cells were extracted and immunoprecipitated with control normal serum (lanes C) or α*abl* sera (lanes 1). Murine cell extracts were precipitated with α*abl* pEX-4 (A and B), and human cell extracts were precipitated with α*abl* pEX-5 (C, D, and E). Immune complexes were collected on protein A-Sepharose, washed twice with extraction buffer lacking SDS, washed once with 50 mM Tris (pH 7.0), and then suspended in 20 μl of 20 mM PIPES (pH 7.0) with 20 $MnCl_2$. Acid-denatured enolase (5 μg) was added, and then reactions were initiated by the addition of 10 to 20 μCi of [γ-^{32}P]ATP. Samples were then electrophoresed on an 8% SDS-polyacrylamide gel, and then the gel was treated with 1 M KOH at 55°C for 2 h to preferentially hydrolyze phosphoserine but not phosphotyrosine (8). Phosphorylated proteins were detected by autoradiography with an intensifying screen at 24 h.

from αpEX-2 sera, and the αpep5 sera were affinity purified as described (10). α*abl* pEX-4 sera were used to analyze murine *abl* proteins, and α*abl* pEX-5 sera were used to analyze human *abl* proteins.

Cell extraction and immunoprecipitation. Exponentially growing hematopoietic cells (1×10^7 to 3×10^7) or P160 Abelson murine leukemia virus-transformed NIH 3T3 fibroblasts (2×10^6 to 4×10^6) growing in suspension were washed twice in phosphate-buffered saline, and then the pellet was suspended in about 50 μl of phosphate-buffered saline at 4°C. All subsequent steps must be done at 4°C or on ice. The cells were then extracted into 3 to 4 ml of ice-cold kinase lysis buffer (1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], 10 mM Na_2HPO_4-NaH_2PO_4 [pH 7.0], 150 mM NaCl) plus 5 mM EDTA–5 mM phenylmethylsulfonyl fluoride (Sigma)–100 μM Na_3VO_4 (Fisher). Kinase lysis buffer differs from the phosphate lysis buffer used in previous studies (28) in that the SDS concentration was reduced from 0.1 to 0.05% and the deoxycholate was omitted. These detergents were reduced or omitted because they decreased recovered amounts of *abl* kinase activity. The SDS could not be completely omitted without significantly increasing the nonspecific background. The presence of SDS also helped prevent the degradation of *abl* proteins during cell lysis. Cell extracts were clarified by centrifugation at 100,000 × *g* for 2 h. Before immunoprecipitation, extracts of P160-transformed cells were diluted 50- to 100-fold into extraction buffer to approximately match the levels of $P160^{v\text{-}abl}$ and $P150^{c\text{-}abl}$ kinase activity that would be recovered. A 1-ml sample of clarified extract was incubated with 5 μl of control preimmune sera or α*abl* sera for 4 to 12 h at 4°C. To harvest immune complexes, a 15-μl packed volume of preswollen protein A-Sepharose beads (Bio-Rad) was added to each sample and incubated for 1 h at 4°C, with gentle shaking to keep the beads in suspension. The stocks of protein A-Sepharose beads were stored in kinase extraction buffer plus 0.1% sodium azide at 4°C and were washed one time in fresh buffer before use. Formalin-fixed *Staphylococcus aureus* (23) cannot be used to collect immune complexes, because it inhibits *abl* kinase activity.

In vitro phosphorylation reactions. *abl* proteins immunoprecipitated as described above were prepared for assay by washing the precipitates two times with extraction buffer lacking SDS. The precipitates were then washed once with 50 mM Tris (pH 7.0) and suspended in 20 μl of 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0)–20 mM $MnCl_2$. Acid-denatured rabbit muscle enolase (5 μg) (Boehringer Mannheim) was added as a substrate for the *abl* kinase as previously described (7). Reactions were initiated with the addition of 5 μl of [γ-^{32}P]ATP (200 Ci/mmol; final concentration of about 2 μM for 10 μCi per sample) (ICN) and were incubated at 30°C. Reactions were terminated by the addition of 25 μl of 2× SDS-gel sample buffer and heated at 95°C for 5 min. Kinase inhibition assays were performed in a similar manner, except that before the kinase reaction, samples were incubated with 30 μg of purified IgG from the αpEX-2 sera or αpep-5 sera in 100 μl of phosphate-buffered saline. As a control, IgG purified from preimmune serum or αpep5 incubated with an excess of peptide 5 (30 μg) to block immunoreactivity was also used. Samples were then washed with 50 mM Tris (pH 7.0) and analyzed as described above.

Gel electrophoresis, peptide mapping, and phosphoamino acid analysis. Samples were eluted off the protein-A Sepharose by boiling in SDS gel sample buffer and were then analyzed by electrophoresis on an 8% SDS-polyacrylamide gel and by autoradiography (29). The α*abl* pEX-5 serum used to immunoprecipitate human cell extracts reacted with a non-*abl*-related serine kinase activity. The gel shown in Fig. 1 was alkali treated with 1 M KOH for 2 h at 55°C to preferentially visualize phosphotyrosine (8). Proteins were eluted from polyacrylamide gels by incubating crushed gel slices in 50 mM NH_4HCO_3–0.1% SDS–5% 2-mercaptoethanol for 16 h at 37°C. Eluted proteins were concentrated by trichloroacetic acid precipitation with 50 μg of bovine serum albumin as the carrier. Samples were prepared for two-dimensional peptide analysis by performic acid oxidation and digestion with tosyl-L-phenylalanine chloromethyl ketone–trypsin as previously described (28). Tryptic phosphopeptides were analyzed by electrophoresis for 1 h in 1% $(NH_4)_2CO_3$ (pH 8.9) on cellulose thin-layer plates. Ascending chromatography was performed in the second dimension in *n*-butanol–pyridine–acetic acid–H_2O (15:10:3:12). The plates were dried and autoradiographed with an intensifying screen.

RESULTS

Murine and human c-*abl* proteins have kinase activity in vitro. Previous studies have not detected tyrosine kinase activity for the c-*abl* protein. One major problem in the analysis of the c-*abl* protein is that it is synthesized at a 50- to 100-fold lower level than the v-*abl* protein (28, 36). A low level of activity could have easily escaped detection. To improve the sensitivity of the in vitro kinase assay, the cell extraction, immunoprecipitation, and kinase reaction conditions were modified to optimize detection of v-*abl* autophosphorylation (see Materials and Methods for details). Utilizing new conditions, the *abl* proteins were reex-

amined, and phosphorylation of $P150^{c\text{-}abl}$ and $P145^{c\text{-}abl}$ could be detected (Fig. 1B and C). $P150^{c\text{-}abl}$ and $P145^{c\text{-}abl}$ had levels of activity similar to the P160 v-*abl* protein (panel A). $P145^{c\text{-}abl}$ was also phosphorylated in addition to the $P210^{c\text{-}abl}$ in the Ph^1-positive K562 cell line (panel D). The phosphorylation of $P145^{c\text{-}abl}$ could not be detected in the Ph^1-positive EM-2 cell line (panel E). The EM-2 cell line lacks a normal chromosome 9, thereby lacking a normal c-*abl* gene which correlated with the lack of P145 detection after metabolic labeling (27).

Two major factors contributed to the detection of in vitro phosphorylation of c-*abl*. The detergents SDS and deoxycholate formerly used in the cell extraction buffer were found to decrease recovered kinase activity. Leaving out deoxycholate, reducing the amount of SDS from 0.1 to 0.05%, and washing precipitates with buffered 1% Triton X-100 increased recovered *abl* kinase activity without significantly increasing the background. The major factor was the discovery that the Formalin-fixed *S. aureus* used for immunoprecipitation inhibited *abl* kinase activity. When protein A-Sepharose beads were used, increased v-*abl* kinase activity was detected, and c-*abl* phosphorylation could also be detected (Fig. 1). *S. aureus* appears to inhibit kinase activity in part by adsorbing the [γ-^{32}P]ATP (data not shown). Preincubation of a reaction mixture with *S. aureus* prevented subsequent phosphorylation of *abl* proteins. The inhibition was not due to a soluble factor, as preincubation of the reaction buffer only did not significantly affect subsequent *abl* autophosphorylation (data not shown). These results may explain why the addition of various nucleotides in addition to the [γ-^{32}P]ATP was found to enhance v-*abl* autophosphorylation (44).

Tyrosine kinase activity is intrinsic to the c-*abl* proteins. The specificity of the c-*abl* kinase activity was examined, as the $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$ proteins are both known to autophosphorylate on tyrosine in vitro (28). The phosphoamino acid content of in vitro-phosphorylated *abl* proteins was determined after partial acid hydrolysis and thin-layer electrophoresis (Fig. 2). $P150^{c\text{-}abl}$ and $P145^{c\text{-}abl}$ (Fig. 2A and C) were phosphorylated on tyrosine similarly to $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$ (lanes B and D). A low level of serine phosphorylation was detected in all samples, but it is probably not *abl* related (9). Phosphoserine is not apparent in the $P160^{v\text{-}abl}$ sample (Fig. 2, lane B) at this level of exposure.

The tyrosine kinase activity associated with the c-*abl* proteins could be intrinsic to the c-*abl* protein or could be due to a coprecipitating kinase. The phosphorylation of exogenously added enolase on tyrosine was only detected in α*abl* serum precipitates (Fig. 1, lanes 1) and not in normal serum precipitates (lanes C). This indicates that the kinase activity was not nonspecifically precipitating with the protein A-Sepharose. However, it is possible that the c-*abl* protein is coprecipitating with another tyrosine kinase under the mild detergent extraction conditions employed. To examine this possibility, site-directed α*abl* antibodies which block $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$ kinase activity were tested for their ability to block $P150^{c\text{-}abl}$ kinase activity (Fig. 3). The kinase-blocking antibodies are directed against sequences in the kinase catalytic domain of $P160^{v\text{-}abl}$ (26). These antibodies cross-react with $P150^{c\text{-}abl}$ but not with unrelated tyrosine kinases (28; unpublished data). $P150^{c\text{-}abl}$ was first immunoprecipitated with noninhibiting sera (α*abl* pEX-4) to tether it to the protein A-Sepharose (Fig. 3, lanes 1 to 5). $P150^{c\text{-}abl}$ was then incubated with IgG purified from control sera (lanes 2 and 4) or kinase-inhibiting sera (lanes 3 and 5) before the phosphorylation reaction. The results demonstrated that $P150^{c\text{-}abl}$ autophosphorylation and enolase phosphorylation were blocked by preincubation with IgG from α*abl* pEX-2 (Fig. 3, lane 3) or α*abl* pep5 (lane 5). $P150^{c\text{-}abl}$ kinase activity was not affected by preincubation with IgG from normal serum (lane 2) or IgG from αpep5 sera when competed with an excess of peptide 5 to block immunoreactivity (lane 4). These results indicate that tyrosine kinase activity is intrinsic to the c-*abl* proteins.

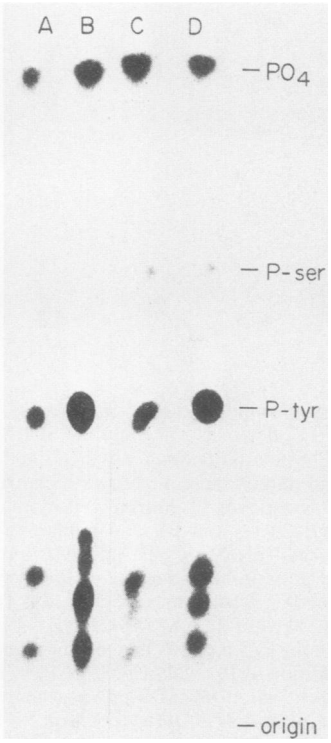

FIG. 2. Phosphoamino acid analysis of in vitro-phosphorylated *abl* proteins. $P150^{c\text{-}abl}$ (A), $P160^{v\text{-}abl}$ (B), $P145^{c\text{-}abl}$ (C), and $P210^{c\text{-}abl}$ (D) were autophosphorylated in vitro with [γ-^{32}P]ATP as described in the legend to Fig. 1. Phosphorylated *abl* proteins were electrophoresed on an 8% SDS-polyacrylamide gel, eluted from the gel, and then hydrolyzed for 2 h at 100°C in 6 N HCl. Hydrolysates were concentrated by lyophilization and suspended in a mixture of nonradioactive phosphoamino acid standards, and then approximately 2,000 cpm of each sample was electrophoresed on a thin-layer cellulose plate. Samples were electrophoresed toward the anode at the top in pyridine-acetic acid-H_2O (5:50:945). Phosphoamino acid composition was detected by autoradiography for 24 h with an intensifying screen. Nonradioactive standards were detected by ninhydrin staining (data not shown).

Improved reaction conditions permit *abl* kinase to function for longer times in vitro. Previous studies have reported that the in vitro phosphorylation of $P160^{v\text{-}abl}$ and $P210^{c\text{-}abl}$ is over in 1 to 2 min (28, 44). This short time course of activity raised the possibility that tyrosine phosphorylation may represent an intermediate and not the true physiological activity (44). Reexamination of *abl* kinase activity with new reaction conditions demonstrated that $P160^{v\text{-}abl}$ (Fig. 4A) and $P150^{c\text{-}abl}$ (Fig. 4B) continued to autophosphorylate and phosphorylate enolase for >60 min. Purified, bacterially expressed v-*abl* protein was also found to be active for long periods and catalyzed phosphotransferase activity at 170 μmol of phosphate min^{-1} μmol^{-1} (11a). These results indicate that tyrosine kinase activity is an important function of the *abl* proteins.

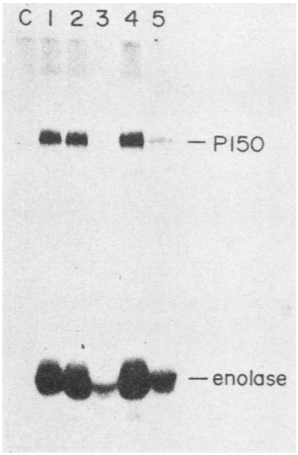

FIG. 3. Antibodies binding to the *abl* kinase domain block c-*abl* kinase activity. Murine SCRF60A cells were extracted and immunoprecipitated with control normal serum (lane C) or with α*abl* pEX-4 (lanes 1 to 5). Immune complexes were collected on protein A-Sepharose, washed, and then suspended in 100 μl of phosphate-buffered saline. IgG purified from normal serum (lane 2), α*abl* pEX-2 (lane 3), α*abl* pep5 previously incubated with an excess of peptide 5 to block immunoreactivity (lane 4), or α*abl* pep5 (lane 5) was added, and samples were incubated at 4°C for 1 h. Samples were then washed with 50 mM Tris (pH 7.0) and in vitro phosphorylated with [γ-^{32}P]ATP as described in the legend to Fig. 1. Phosphorylated proteins were electrophoresed on an 8% SDS-polyacrylamide gel and detected by autoradiography with an intensifying screen for 24 h. α*abl* pEX-2 and α*abl* pep5 are site-directed antisera that cross-react with the *abl* kinase domain.

During the time course of autophosphorylation, P150$^{c\text{-}abl}$ becomes slightly retarded in electrophoretic mobility, but this was not detected for P160$^{v\text{-}abl}$ (Fig. 4). It is not known whether P210$^{c\text{-}abl}$ mobility is altered during autophosphorylation, because it migrates in a high-molecular-weight, nonlinear region of the gel. Altered electrophoretic mobility due to phosphorylation has also been detected for the v-*fps* protein (1). Possibly, phosphorylation disrupts SDS binding.

Additional aspects of the *abl* kinase activity (Table 1) were also examined. ATP is used as a phosphate donor, but GTP can be used to a lesser extent. All of the *abl* proteins preferred Mn^{2+} as a cofactor, Mg^{2+} can be used to a lesser extent, and Ca^{2+} alone resulted in no activity. This differs from results reported for bacterially expressed v-*abl* protein which preferred Mg^{2+} to Mn^{2+} (11a). It has been proposed that Mn^{2+} may inhibit phosphatases, resulting in higher recovered kinase activity (4, 11a). A mixture of Mn^{2+} and Mg^{2+} did not result in increased P160$^{v\text{-}abl}$ or P160$^{c\text{-}abl}$ kinase activity. Otherwise, *abl* proteins isolated from mammalian cells were similar to the bacterially expressed v-*abl* protein. Analysis of the thermal stability of P160$^{v\text{-}abl}$ and P150$^{c\text{-}abl}$ demonstrated that 70 to 80% activity could be recovered after 5 min at 35°C, and 10% of activity was detected after 5 min at 40°C (Table 1). This suggests that amino-terminal alteration of P160$^{v\text{-}abl}$ does not grossly alter the stability of its catalytic domain.

***abl* proteins phosphorylate on different sites in vitro.** The ability of the *abl* proteins to serve as their own substrates in vitro was compared by examining their sites of phosphorylation. Two-dimensional tryptic peptide map analysis demonstrated that P160$^{v\text{-}abl}$ and P210$^{c\text{-}abl}$ are phosphorylated on two to three major sites and several minor sites, in agreement with previous studies (Fig. 5A and C) (10). The major phosphorylation sites are not the same for P160$^{v\text{-}abl}$ and P210$^{c\text{-}abl}$. In contrast, P150$^{c\text{-}abl}$ and P145$^{c\text{-}abl}$ are each phosphorylated at one major site in vitro (Fig. 5B and D). P145$^{c\text{-}abl}$ isolated from Ph^1-positive K562 cells (data not shown) was phosphorylated at the same major site as P145$^{c\text{-}abl}$ isolated from Ph^1-negative HL-60 cells (panel D). The major phosphotryptic peptide detected in murine and human c-*abl* proteins comigrated in mixing experiments, indicating that they are phosphorylated at the same site in vitro. This suggests that amino-terminal structural alteration of the c-*abl* protein alters the way it utilizes itself as a substrate in vitro and may affect interaction with substrates in vivo.

Comparison of the in vitro and in vivo sites of tyrosine phosphorylation of the *abl* proteins further supports the idea that they function differently in vivo. P160 is phosphorylated at v-*abl* tyrosine residue 514 in vivo as is the homologous tyrosine of v-*src* and other tyrosine kinases (Fig. 5E) (25, 26). P160$^{v\text{-}abl}$ is phosphorylated at several sites in vitro but only a low level of phosphorylation, if any, at tyrosine 514 can be detected. The c-*abl* protein is not detectably phosphorylated on tyrosine in vivo (28, 36) but is phosphorylated on tyrosine 514 in vitro. The identity of the P160$^{v\text{-}abl}$ in vivo and P150$^{c\text{-}abl}$ in vitro tyrosine phosphorylation site was determined by demonstrating that they comigrate in two-dimensional separation (Fig. 5F). In addition, when eluted from the peptide map, these peptides comigrated during electrophoresis at pH 3.5 and were both sensitive to digestion with α-chymotrypsin but not *S. aureus* V-8 protease (data not shown). The in vivo and in vitro tyrosine phosphorylation of P160$^{v\text{-}abl}$ and P150$^{c\text{-}abl}$ is summarized in Fig. 6. These results indicate that P150$^{c\text{-}abl}$ is capable of being phosphorylated at the tyrosine 514 homologous residue, although P150$^{c\text{-}abl}$ is not detectably phosphorylated at this site in vivo. P210$^{c\text{-}abl}$ is not detectably phosphorylated at the tyrosine 514 homologous residue in vivo but is

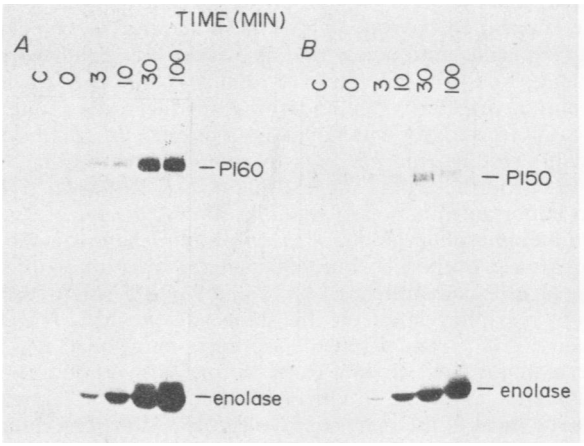

FIG. 4. Time course of in vitro *abl* kinase activity. P160$^{v\text{-}abl}$ (A) or P150$^{c\text{-}abl}$ (B) were immunoprecipitated with control normal serum (lanes C) or α*abl* pEX-4 (all other lanes) and prepared for kinase assay as described in the legend to Fig. 1. Reactions were commenced by the addition of [γ-^{32}P]ATP and incubated for the indicated amount of time. Samples in lanes C were incubated for 30 min. Reactions were terminated by the addition of an equal volume of 2× SDS gel sample buffer and heated at 95°C for 5 min. Samples were electrophoresed on an 8% SDS-polyacrylamide gel, and the phosphorylated proteins were detected by autoradiography for 24 h with an intensifying screen.

TABLE 1. Comparison of normal and altered *abl* protein tyrosine kinase activity

Protein	Presence of p-*tyr*		Phosphorylate enolase	Kinase inhibition with α-kinase domain sera	ATP > GTP[a]	Reaction with cation cofactor[b]					% Thermal stability[d] (recovered activity) after 5 min at the following temp (°C):		
	In vivo	In vitro				Mn	Mg	Ca	Mn + Mg	Mn + Ca	4	35	40
P160	Yes	Yes	Yes	Yes	Yes	++++	+	−	++++	++++	100	70–80	10
P150	No	Yes	Yes	Yes	Yes	++++	+	−	++++	++++	100	70–80	10
P210	Yes	Yes	Yes	Yes	Yes	++++	+	−	++++	++++	100	70–80	10
P145	No	Yes	Yes	Yes	Yes	++++	+	−	ND[c]	ND	ND	ND	ND

[a] Ability to use ATP better than GTP as a substrate.

[b] Various cations or combinations of cations were examined for their ability to act as cofactors for the *abl* tyrosine kinase activity. Kinase reactions were performed as described in the legend to Fig. 1, except that different cations at 20 mM were substituted for Mn^{2+}. Three- to fivefold greater activity was detected with Mn than Mg, and Ca^{+} resulted in no detectable activity.

[c] ND, Not determined.

[d] *abl* proteins were prepared for kinase assay as described in the legend to Fig. 1 and then incubated at 0, 35, or 40°C for 5 min. Samples were then cooled on ice, and kinase reactions were initiated by the addition of [γ-^{32}P]ATP and incubated for 10 min at 30°C.

phosphorylated at another site that has not been localized yet (28; unpublished data).

DISCUSSION

Detection of c-*abl* kinase activity. Detection of c-*abl* kinase activity was facilitated by the observation that Formalin-fixed *S. aureus* used for immunoprecipitating *abl* proteins inhibited in vitro kinase activity. *abl* kinase activity was not inhibited when protein A-Sepharose was used instead of *S. aureus*. The new reaction conditions described in this paper should also be useful for studying other kinases, particularly those forms of the v-*mos* protein which have homology to the kinase family but no detectable kinase activity (33), in contrast to *gag-mos* strains with serine kinase activity (24).

Previously, the short time course of in vitro kinase activity of P160$^{v\text{-}abl}$ and the lack of detectable kinase activity for P150$^{c\text{-}abl}$ suggested that tyrosine kinase activity may represent an intermediate of the true c-*abl* enzymatic activity (44). However, with modified reaction conditions, all of the *abl* proteins had the ability to autophosphorylate and phosphorylate enolase on tyrosine. This kinase activity was blocked by *abl*-specific antibodies binding within the region corresponding to the v-*abl* catalytic domain. Although the *abl* proteins have different amino termini, the thermal stability of the catalytic activity was not grossly altered. In contrast to previous studies, the *abl* kinases remained active for >60 min in vitro. These data suggest that tyrosine kinase activity is an important function of the *abl* proteins in vivo.

***abl* autophosphorylation.** The *abl* proteins have similar in vitro kinase properties but differ in the way they utilize themselves as substrates. P150$^{c\text{-}abl}$ and P145$^{c\text{-}abl}$ are predominantly phosphorylated on the same site in vitro, but the altered P210$^{c\text{-}abl}$ and P160$^{v\text{-}abl}$ are each phosphorylated at several major sites distinct from the major c-*abl* phosphorylation site. The c-*abl* in vitro phosphorylation site appears to be identical to the major in vivo tyrosine phosphorylation site of P160$^{v\text{-}abl}$ tyrosine 514. The in vitro phosphorylation sites of P160$^{v\text{-}abl}$ have been mapped to the amino-terminal *gag* sequences (45).

The different manner in which the *abl* proteins utilize themselves as substrates in vitro suggests that they may interact differently with substrates in vivo. This idea is supported by the fact that P150$^{c\text{-}abl}$ and P145$^{c\text{-}abl}$ are not detectably phosphorylated on tyrosine in vivo, but P160$^{v\text{-}abl}$ and P210$^{c\text{-}abl}$ are. Novel phosphotyrosine-containing proteins have been detected in v-*abl*-transformed cells and in Ph^{1}-positive cells (8; E. Freed and T. Hunter, personal communication). However, detection of these putative substrates may be due to the higher levels of P160$^{v\text{-}abl}$ and P210$^{c\text{-}abl}$ synthesis than to P150$^{c\text{-}abl}$ and P145$^{c\text{-}abl}$ synthesis. Analysis of the EM-2 cell line suggests that P210$^{c\text{-}abl}$ can replace the function of P145$^{c\text{-}abl}$. EM-2 cells synthesize P210$^{c\text{-}abl}$, but P145$^{c\text{-}abl}$ is not detected (Fig. 1), correlating with the presence of a translocated *abl* gene but not a normal c-*abl* gene (22, 27). Alternatively, P210$^{c\text{-}abl}$ and P145$^{c\text{-}abl}$ could have different functions in vivo if P145$^{c\text{-}abl}$ is not essential for growth. Qualitative differences in the *abl* kinase activity may be important for inducing altered growth control. Qualitative differences between v-*src* and c-*src* proteins have been shown to be important in the activation of *src* oncogenic potential (19, 20, 34).

It is surprising that c-*abl* protein is not detectably phosphorylated on tyrosine in vivo since it can be in vitro. Even bacterially expressed v-*abl* is phosphorylated on tyrosine in vivo (42). The *src* and *fps* proteins are similar to *abl* proteins in that the v-*src* and v-*fps* proteins are phosphorylated on tyrosine in vivo, but c-*src* and c-*fps* are not (18, 21, 32, 35, 38). Another pattern is represented by the *erb*B protein and the epidermal growth factor (EGF) receptor. The *erb*B oncogene is thought to be derived from the EGF receptor (or a very similar gene) (11, 40, 48). The v-*erb*B protein is not detectably phosphorylated on tyrosine in vivo but in vivo phosphorylation of the EGF receptor is stimulated upon binding of EGF (12, 17).

Deletion or mutation of the phosphate-accepting tyrosine in v-*src* does not block kinase activity but decreases tumorigenicity of the v-*src* protein (9, 39). Although not essential, phosphorylation of the v-*src* protein on a tyrosine not within the catalytic site accompanies increased activity (3, 5). This phosphorylation may represent a regulatory modification. Possibly, amino-terminal structural alteration of the *abl* protein influences which tyrosine is available for phosphorylation in vivo. This may explain why P160$^{v\text{-}abl}$ is phosphorylated at tyrosine 514 and at tyrosine 385 in vivo (26, 37), the P210 tyrosine phosphorylation site has not been localized but it is apparently different from v-*abl* tyrosine 514 (28), and the c-*abl* protein is not detectably phosphorylated on tyrosine in vivo (28, 36). Alternatively, in vivo phosphorylation may be due to differential interaction with tyrosine-specific phosphatases. This possibility was examined by incubating cells with vanadate, an inhibitor of tyrosine phosphatases that results in increased tyrosine phosphorylation of the v-*src* protein (3, 5). Preliminary results indicate that treatment of cells with 10 μM or 100 μM vanadate did

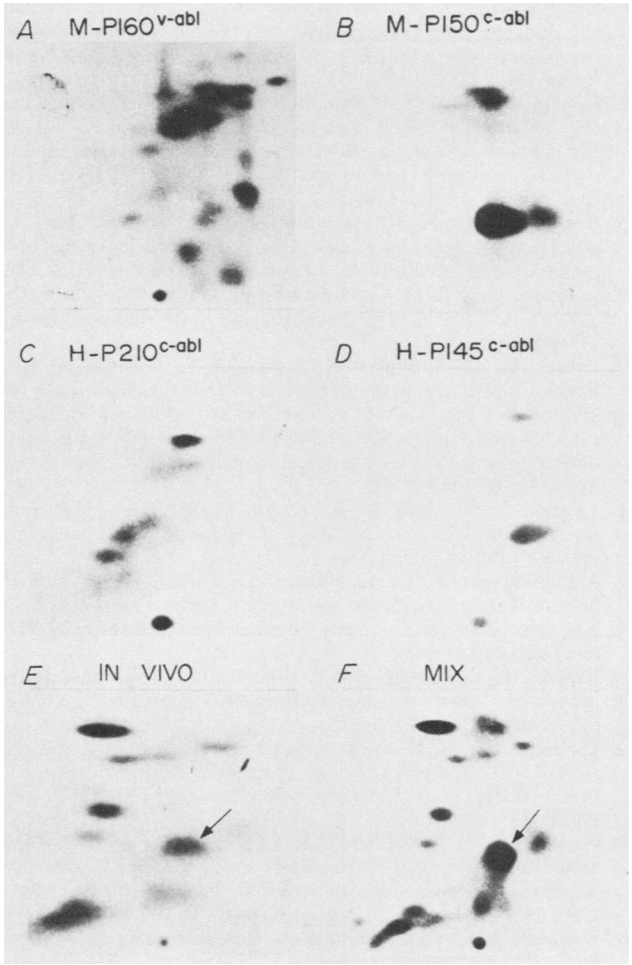

FIG. 5. Two-dimensional phosphopeptide analysis of in vitro-autophosphorylated *abl* proteins. $P160^{v\text{-}abl}$ (A), $P150^{c\text{-}abl}$ (B), $P210^{c\text{-}abl}$ (C), and $P145^{c\text{-}abl}$ (D) were autophosphorylated in vitro with [γ-^{32}P]ATP as described in the legend to Fig. 1. For comparison, analysis of $P160^{v\text{-}abl}$ (E) labeled in vivo with $^{32}P_i$ (28) and a mix of in vivo-labeled $P160^{v\text{-}abl}$ and in vitro-phosphorylated $P150^{c\text{-}abl}$ is also shown (F). An arrow points to the major phosphotyrosine-containing peptide of in vivo-labeled $P160^{v\text{-}abl}$ in panels E and F. Phosphorylated *abl* proteins were eluted from polyacrylamide gels and concentrated by trichloroacetic acid precipitation. Three thousand to five thousand counts per minute of each sample was performic acid oxidized, completely digested with trypsin, and then electrophoresed on thin-layer cellulose plates for 1 h at 400 V in 1% ammonium carbonate (pH 8.9). The origin is at the center of the baseline, and the cathode is on the right. The plates were then chromatographed in the second dimension in *n*-butanol–pyridine–acetic acid–H_2O (15:10:3:12). Plates were autoradiographed for 1 to 2 days.

not result in the detection of phosphotyrosine on the c-*abl* protein (unpublished data).

The significance of the phosphorylation of *abl* proteins on tyrosine in vivo is unclear, but the development of c-*abl* kinase assay conditions makes it possible to further examine the relationship between in vivo phosphorylation and in vitro kinase activity. Possibly, in vivo tyrosine phosphorylation of c-*abl* will correspond to in vivo c-*abl* kinase activity. The EGF receptor becomes phosphorylated on tyrosine in vivo after the stimulation of its kinase activity by EGF (17). In this regard, it will be interesting to examine c-*abl* proteins

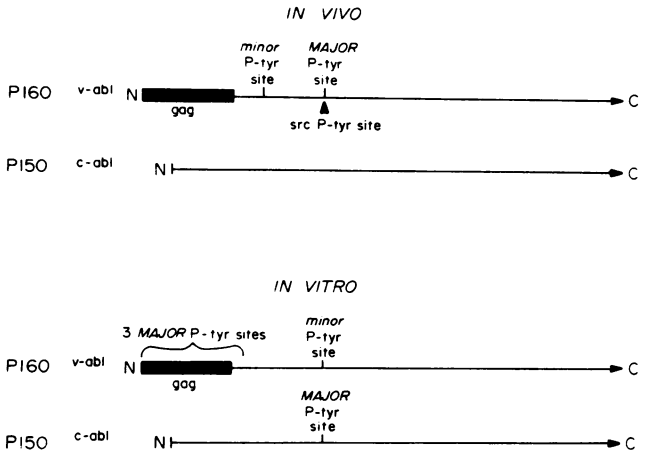

FIG. 6. Comparison of in vivo and in vitro *abl* tyrosine phosphorylation sites. The in vivo tyrosine phosphorylation sites of $P160^{v\text{-}abl}$ correspond to v-*abl* residues 385 and 514 (26). The major in vivo $P160^{v\text{-}abl}$ tyrosine phosphorylation site is homologous to the major tyrosine phosphorylation site of the v-*src* protein and other tyrosine kinases (35). $P150^{c\text{-}abl}$ is not detectably phosphorylated on tyrosine in vivo (28, 36). In vitro, $P160^{v\text{-}abl}$ is phosphorylated on three major sites which have been localized to the amino-terminal *gag* sequences (45). Only a low level of phosphorylation on tyrosine 514 is detected in vitro. In contrast, $P150^{c\text{-}abl}$ is phosphorylated on one major site which is homologous to the major in vivo tyrosine phosphorylation of $P160^{v\text{-}abl}$ (Fig. 5).

isolated from different cell types, different phases of the cell cycle, and during treatment of cells with different growth factors. Analysis of c-*abl* activity during growth should also be useful in elucidating *abl* function.

ACKNOWLEDGMENTS

This work was supported by research grants from the National Institutes of Health and a Faculty Scholar Award from the American Cancer Society to O.N.W. J.B.K. was supported as a predoctoral fellow on U.S. Public Health Service grant GM 07185.

We thank Debra Bomar for excellent secretarial assistance.

LITERATURE CITED

1. **Adkins, B., and T. Hunter.** 1982. Two structurally and functionally different forms of the transforming protein of PRC II avian sarcoma virus. Mol. Cell. Biol. **2**:890–896.
2. **Bartram, C. R., A. de Klein, A. Hagemeijer, T. van Agthoven, A. G. van Kessel, D. Bootsma, G. Grosveld, M. A. Ferguson-Smith, T. Davies, M. Stone, N. Heisterkamp, J. R. Stephenson, and J. Groffen.** 1983. Translocation of c-*abl* oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukemia. Nature (London) **306**:277–280.
3. **Brown, D. J., and J. A. Gordon.** 1984. The stimulation of $pp60^{v\text{-}src}$ kinase activity by Vanadate in intact cells accompanies a new phosphorylation state of the enzyme. J. Biol. Chem. **259**:9580–9586.
4. **Casnellie, J. E., M. L. Harrison, L. J. Pike, K. E. Hellstrom, and E. G. Krebbs.** 1982. Phosphorylation of synthetic peptides by a tyrosine protein kinase from the particulate fraction of a lymphoma cell line. Proc. Natl. Acad. Sci. USA **79**:282–286.
5. **Collett, M. S., S. K. Belzer, and A. F. Purchio.** 1984. Structurally and functionally modified forms of $pp60^{v\text{-}src}$ in Rous sarcoma virus-transformed cell lysates. Mol. Cell. Biol. **4**:1213–1220.
6. **Collins, S. J., R. C. Gallo, and R. E. Gallagher.** 1977. Continuous growth and differentiation of human myeloid leukemia cells in suspension culture. Nature (London) **270**:347–349.
7. **Cooper, J. A., F. S. Esch, S. S. Taylor, and T. Hunter.** 1984.

Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases in vivo and in vitro. J. Biol. Chem. **259**:7835–7841.

8. **Cooper, J. A., and T. Hunter.** 1981. Four different classes of retroviruses induce phosphorylation of tyrosines present in similar cellular proteins. Mol. Cell. Biol. **1**:394–407.
9. **Cross, F. R., and H. Hanafusa.** 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation are dispensable for transformation. Cell **34**:597–607.
10. **Davis, R. L., J. B. Konopka, and O. N. Witte.** 1985. Activation of the c-*abl* oncogene by viral transduction or chromosomal translocation generates altered c-*abl* proteins with similar in vitro kinase properties. Mol. Cell. Biol. **5**:204–213.
11. **Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield.** 1984. Close similarity of epidermal growth factor receptor and v-*erb*B oncogene protein sequences. Nature (London) **307**:521–527.

11a. **Foulkes, J. G., M. Chow, C. Gorka, A. R. Frackelton, Jr., and D. Baltimore.** 1985. Purification and characterization of a protein-tyrosine kinase encoded by the Abelson murine leukemia virus. J. Biol. Chem. **260**:8070–8077.

12. **Gilmore, T., J. E. De Clue, and G. S. Martin.** 1985. Protein phosphorylation at tyrosine is induced by the v-*erb* B gene product in vivo and in vitro. Cell **40**:609–618.
13. **Goff, S. P., E. Gilboa, O. N. Witte, and D. Baltimore.** 1980. Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell **22**:777–785.
14. **Groffen, J., J. R. Stephenson, N. Heisterkamp, A. de Klein, C. R. Bartram, and G. Grosveld.** 1984. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell **36**:93–99.
15. **Heisterkamp, N., J. Groffen, and J. R. Stephenson.** 1983. The human v-*abl* cellular homologue. J. Mol. Appl. Gen. **2**:57–68.
16. **Heisterkamp, N., J. R. Stephenson, J. Groffen, P. F. Hansen, A. de Klein, C. R. Bartram, and G. Grosveld.** 1983. Localization of the c-*abl* oncogene adjacent to a translocation point in chronic myelocytotic leukemia. Nature (London) **306**:239–242.
17. **Hunter, T., and J. A. Cooper.** 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell **24**:741–752.
18. **Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa.** 1985. Low level of cellular protein phosphorylation by nontransforming overproduced $p60^{c\text{-}src}$. Mol. Cell. Biol. **5**:1058–1066.
19. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
20. **Jakobovits, E. B., J. E. Majors, and H. E. Varmus.** 1984. Hormonal regulation of the Rous sarcoma virus *src* gene via a heterologous promoter defines a threshold dose for cellular transformation. Cell **38**:757–765.
21. **Karess, R. E., and H. Hanafusa.** 1981. Viral and cellular *src* genes contribute to the structure of recovered avian sarcoma virus transforming protein. Cell **24**:155–164.
22. **Keating, A., P. J. Martin, I. D. Bernstein, T. Papayannopoulou, W. Raskind, and J. W. Singer.** 1983. EM-2 and EM-3: two new Ph^1+ myeloid cell lines, P. 513–520. *In* D. Golde and P. Marks (ed.), Proceedings of the UCLA Symposia on Molecular and Cellular Biology. Alan R. Liss, Inc., New York.
23. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent. J. Immunol. **115**:1617–1624.
24. **Kloetzer, W., and R. Arlinghaus.** 1983. $P85^{gag\text{-}mos}$ encoded by ts110 Moloney murine sarcoma virus has an associated protein kinase activity. Proc. Natl. Acad. Sci. USA **80**:412–416.
25. **Kloetzer, W., R. Kurzrock, L. Smith, M. Talpaz, M. Spiller, J. Gutterman, and R. Arlinghaus.** 1985. The human cellular abl gene product in the chronic myelogenous leukemia cell line has an associated tyrosine protein kinase activity. Virology **140**:230–238.
26. **Konopka, J. B., R. L. Davis, S. M. Watanabe, A. S. Ponticelli, L. Schiff-Maker, N. Rosenberg, and O. N. Witte.** 1984. Only site-directed antibodies reactive with the highly conserved *src*-homologous region of the v-*abl* protein neutralize kinase activity. J. Virol. **51**:223–232.
27. **Konopka, J. B., S. M. Watanabe, J. W. Singer, S. J. Collins, and O. N. Witte.** 1985. Cell lines and clinical isolates derived from Ph^1-positive chronic myelogenous leukemia patients express c-*abl* proteins with a common structural alteration. Proc. Natl. Acad. Sci. USA **82**:1810–1814.
28. **Konopka, J. B., S. M. Watanabe, and O. N. Witte.** 1984. An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell **37**:1035–1042.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
30. **Lerner, R. A., F. Jensen, S. J. Kennel, F. J. Dixon, F. Des-Roches, and U. Francke.** 1972. Karyotypic, virologic, and immunological analyses of two continuous lymphocyte cell lines established from New Zealand black mice: possible relationship of chromosomal mosaicism to autoimmunity. Proc. Natl. Acad. Sci. USA **69**:2965–2969.
31. **Lozzio, C. B., and B. B. Lozzio.** 1975. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood **45**:321–334.
32. **Mathey-Prevot, B., H. Hanafusa, and S. Kawai.** 1982. A cellular protein is immunologically crossreactive with and functionally homologous to the Fujinami sarcoma virus transforming protein. Cell **28**:897–906.
33. **Papkoff, J., E. A. Nigg, and T. Hunter.** 1983. The transforming protein of Moloney murine sarcoma virus is a soluble cytoplasmic protein. Cell **33**:161–172.
34. **Parker, R. C., H. E. Varmus, and J. M. Bishop.** 1984. Expression of v-src and chicken c-src in rat cells demonstrates qualitative differences between $pp60^{u\text{-}src}$ and $pp60^{c\text{-}src}$. Cell **37**:131–139.
35. **Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton.** 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA **79**:973–977.
36. **Ponticelli, A. S., C. A. Whitlock, N. Rosenberg, and O. N. Witte.** 1982. In vivo tyrosine phosphorylation of the Abelson virus transforming protein are absent in its normal cell homolog. Cell **29**:953–960.
37. **Sefton, B. M., T. Hunter, and W. C. Raschke.** 1981. Evidence that the Abelson virus functions as a protein kinase that phosphorylates on tyrosine in vivo. Proc. Natl. Acad. Sci. USA **78**:1552–1556.
38. **Smart, J. E., H. Oppermann, A. P. Czernilofsky, A. F. Purchio, R. L. Erikson, and J. M. Bishop.** 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus ($pp60^{v\text{-}src}$) and its normal cellular homologue ($pp60^{c\text{-}src}$). Proc. Natl. Acad. Sci. USA **78**:6013–6017.
39. **Snyder, M. A., J. M. Bishop, W. W. Colby, and A. D. Levinson.** 1983. Phosphorylation of tyrosine 416 is not required for the transforming properties and kinase activity of $pp60^{v\text{-}src}$. Cell **32**:891–901.
40. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. Tam, W. Lee, Y. Yarden, T. A. Liberman, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg.** 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (London) **309**:418–425.
41. **Wang, J. Y. J., F. Ledley, S. Goff, R. Lee, Y. Groner, and D. Baltimore.** 1984. The mouse c-abl locus: molecular cloning and characterization. Cell **36**:349–356.
42. **Wang, J. Y. J., C. Queen, and D. Baltimore.** 1982. Expression of an Abelson murine leukemia virus encoded protein in *Escherichia coli* causes extensive phosphorylation of tyrosine residues. J. Biol. Chem. **257**:13181–13184.
43. **Watanabe, S. M., N. E. Rosenberg, and O. N. Witte.** 1984. A membrane-associated, carbohydrate-modified form of the v-*abl* protein that cannot be phosphorylated in vivo or in vitro. J.

Virol. **51**:620–627.
44. **Witte, O. M., A. Dasgupta, and D. Baltimore.** 1980. Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature (London) **283**:826–831.
45. **Witte, O. N., A. Ponticelli, A. Gifford, D. Baltimore, N. Rosenberg, and J. Elder.** 1981. Phosphorylation of the Abelson murine leukemia virus transforming protein. J. Virol. **39**:870–878.
46. **Witte, O. N., N. Rosenberg, and D. Baltimore.** 1979. A normal cell protein cross reactive to the major Abelson murine leukemia virus gene product. Nature (London) **281**:396–398.
47. **Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore.** 1978. Identification of an A-MuLV encoded protein present in transformed fibroblasts and lymphoid cells. Proc. Natl. Acad. Sci. USA **75**:2488–2492.
48. **Yamamoto, T., T. Nishida, N. Miyajima, S. Kawai, T. Ooi, and K. Toyashima.** 1983. The *erb*B gene of Avian erythroblastosis virus is a member of the src gene family. Cell **35**:71–78.