Only Site-Directed Antibodies Reactive with the Highly Conserved *src*-Homologous Region of the *v-abl* Protein Neutralize Kinase Activity

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Received 14 December 1983/Accepted 24 February 1984

Antisera specific for six regions of the *v-abl* protein were used to serologically characterize the Abelson murine leukemia virus tyrosine kinase. Chemically synthesized peptides corresponding to the predicted *v-abl* protein sequence and larger regions of the *v-abl* protein expressed as fusion proteins in bacteria were used as immunogens. The specificity of each antiserum was confirmed by immunoprecipitation analysis with defined deletion mutants of Abelson murine leukemia virus. Several of these *v-abl*-specific antisera display much higher titers and avidities than serum harvested from mice bearing Abelson murine leukemia virus-induced tumors, previously the only source of anti-*abl*-specific serum. Two antisera were found to block the *in vitro* autophosphorylation of the *v-abl* protein as well as its ability to phosphorylate a peptide substrate. Examination of the sites against which the kinase-blocking antisera were prepared revealed that both are in close proximity to the *in vivo* sites of tyrosine phosphorylation, which fall within the region of high homology with *v-src* and other tyrosine kinases. Antisera directed against other regions of *v-abl* did not inhibit kinase activity.

Tyrosine-specific protein kinases have been closely linked to the growth-promoting activities of many viral (3, 16, 52) and cellular genes (8, 17, 47). The transforming protein of Abelson murine leukemia virus (A-MuLV) is a tyrosine kinase (36, 50, 52) which mediates the ability of this virus to transform fibroblast (35) and lymphoid (33) cells *in vitro* and rapidly induce B cell lymphomas *in vivo* (1). Mutant strains of A-MuLV which lack tyrosine kinase activity are transformation defective (32, 53), and mutants with reduced kinase activity have been found to be less efficient at lymphoid transformation (34).

A-MuLV is the product of a recombination event between Moloney murine leukemia virus and a normal cellular gene called *c-abl* (12, 55), resulting in the expression of a *gag-abl* fusion protein (31, 56). Deletion mutagenesis of the A-MuLV gene has localized the transforming activity and the tyrosine kinase activity to a 45-kilodalton (kd) region of the A-MuLV protein on the carboxyl side of the *gag-abl* junction (29). In addition, a transformation-defective mutant of A-MuLV which lacks kinase activity has a 700-base-pair deletion within this region (14, 53). Examination of the predicted amino acid sequence for this region has shown that it contains homology with the Rous sarcoma virus-transforming protein pp60^{src} and other tyrosine kinases (30, 15). The remaining N-terminal and C-terminal sequences are not essential for transformation. When present, they correlate with an increased transforming potency (29, 51, 58) and a lethal (13, 51, 58) function of A-MuLV on certain cell types.

The *v-abl* protein has been characterized with anti-Moloney virus *gag* gene sera (31, 56) and antitumor sera from mice bearing A-MuLV-induced lymphomas (54). The antitumor sera recognize the *abl*-specific region of the *v-abl* protein and also cross-react with the *c-abl* gene product,

NCP150 (55). Antitumor sera are difficult to prepare, and the titer varies significantly between different batches. Therefore, to further characterize the structure and function of the *v-abl* tyrosine kinase, we have made a series of antisera directed against defined regions of the *v-abl* protein. Antisera of predetermined specificity have been used to characterize the functional domains of several proteins (23). Investigators working with pp60^{src} have identified regions that may be functionally important for its kinase activity with antisera of predetermined specificity, which can block kinase activity *in vitro* (10, 37, 46). In applying this approach to A-MuLV, we have observed that only two of the antisera neutralized kinase activity. Both of these antisera are directed against the region of the *v-abl* protein that is homologous with other tyrosine kinases (15, 30).

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 fibroblasts transformed with the P160 (14), P120 (14), or P87 (51) strain of A-MuLV were grown in Dulbecco modified Eagle medium with 10% calf serum.

Metabolic cell labeling. Exponentially growing cells (5×10^6) were washed two times in 150 mM NaCl and then suspended in 2 ml of the appropriate Dulbecco modified Eagle minimal media lacking either phosphate or methionine and containing 5% dialyzed fetal calf serum. Cell labelings were then started by the addition of 50 μ Ci of [³⁵S]methionine (Amersham Corp., Arlington Heights, Ill.) or 250 μ Ci of [³²P]ATP (ICN Nutritional Biochemicals, Cleveland, Ohio) per ml. The cells were incubated for 2 h at 37°C.

Immunoprecipitation. Cells (5×10^6) were washed once in phosphate-buffered saline and then lysed in 5 ml of cold phosphate lysis buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.01 M NaH₂PO₄-Na₂HPO₄ [pH 7.5], 0.1 M NaCl, 5 mM EDTA). The detergent extracts were incubated with 250 μ l of a 10% (wt/vol) suspension of Formalin-fixed *Staphylococcus aureus* Cowan

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strain (18) and then clarified by ultracentrifugation at 35,000 rpm for 2 h. One-milliliter samples of clarified extract were incubated overnight with 5 μ l of the appropriate antisera at 4°C, and then samples were collected after the addition of 50 μ l of *S. aureus* and incubation on ice for 1 h. Where indicated, *v-abl* immunoprecipitation with the antipeptide sera was blocked by the addition of 20 μ g of the appropriate peptide before the addition of the antisera to the extract. The

immunoprecipitates were washed twice with phosphate lysis buffer, and then the bound proteins were eluted off with SDS gel sample buffer for subsequent analysis by SDS-polyacrylamide gel electrophoresis (20). Gels were prepared for fluorography by incubation in 1 M sodium salicylate before drying (4).

Kinase reactions. For use in kinase assays, *v-abl* protein was immunoprecipitated from cell extracts as described

FIG. 1. Construction of *v-abl* bacterial expression plasmids. (A) A 2.2-kb *Bgl*II fragment was subcloned from pABI60 into the inducible expression plasmid pKRS101. This new construct, pEX-1, was subsequently modified by deleting an internal *Sst*I fragment to generate the *v-abl* expression vector pEX-2. (B) A 0.9-kb *Pst*I fragment was subcloned from pABI60 into pUC12 to generate pSMW100. An internal *Sst*I fragment was then deleted from pSMW100 to generate pSMW101. The *v-abl* sequences were removed from SMW101 by *Eco*RI and *Hind*III digestion and subcloned into the inducible expression vector pJH14 to generate the *v-abl* expression vector pEX-4. (C) A 1.1-kb *Sal*I-*Hind*III fragment was subcloned from pABI60 into the inducible expression vector pJH12 to generate the *v-abl* expression vector pEX-5. See the text for a more detailed description of the constructs.

above. After collecting the samples on *S. aureus*, they were washed two times with phosphate lysis buffer, washed once with 50 mM Tris (pH 7.5), and then suspended in 45 μ l of 20 mM PIPES (1,4-piperazinediethanesulfonate) (pH 7.0) with 20 mM MnCl_2 . Autophosphorylation reactions were started by the addition of 5 μ l of [γ - ^{32}P]ATP (1 μM ; 1,000 Ci/mmol) (ICN) and then incubated at 30°C for 5 min. Reactions were terminated by the addition of 1 ml of cold phosphate lysis buffer plus 5 mM EDTA. The immune complexes were recovered by centrifugation, the bound proteins were eluted in SDS gel sample buffer, and then the autophosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Phosphorylation of the peptide substrate angiotensin (Sigma Chemical Co., St. Louis, Mo.) (57) was performed with immunoprecipitated *v-abl* suspended in 20 mM PIPES, 20 mM MnCl_2 , and 2 mM angiotensin. Reactions were started by the addition of 1 μ l of [γ - ^{32}P]ATP (1 μM ; 5,000 Ci/mmol) to 9 μ l of reaction mix and incubated at 30°C for 5 min. The reactions were terminated with the addition of an equal volume of 50 μM pyrophosphate and 50 μM EDTA. The immune complex was pelleted by centrifugation, and then the peptide-containing supernatant was quantitated. The supernatant was diluted with an equal volume of 60% acetic acid, spotted onto a 2- by 2-cm phosphocellulose paper square, washed for 15 min in 30% acetic acid, washed two times for 15 min each in 15% acetic acid, washed for 5 min in acetone, and then dried. Under these conditions, the phosphorylated peptide remains quantitatively bound, and the excess [γ - ^{32}P]ATP from the reaction is washed away (11). The amount of ^{32}P bound to the phosphocellulose squares was quantitated by scintillation counting in a toluene-based cocktail. All peptide phosphorylation reactions were performed in duplicate with and without peptide added, using preimmune and immune sera to determine the amount of background. In all peptide phosphorylation assays, background levels were less than 0.1% of input radioactivity.

Peptide synthesis materials. *t*-Butyloxycarbonyl (Boc)-protected amino acids were purchased from Vega Biochemicals, except for lysine, which was purchased from Bachem Fine Chemicals, Inc. Boc-[^{14}C]glycine and Boc-[^{14}C]alanine were prepared by diluting 0.25 mCi of free [^{14}C]glycine or [^{14}C]alanine (Amersham) into the appropriate non-radioactive amino acid, followed by reaction with di-*t*-butyl-dicarbonate [(Boc $_2$) $_2$ O] (26). *N,N'*-Dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, and 99% distilled trifluoroacetic acid were obtained from Aldrich Chemical Co.; hydrogen bromide gas was purchased in a Lecture bottle from Matheson. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem-Behring. Complete and incomplete Freund adjuvant were purchased from GIBCO.

Synthesis and purification. Peptides were synthesized by the solid-phase method of Merrifield, using *N,N'*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole activation (44). A ^{14}C -labeled glycine or alanine residue was incorporated into each peptide as a radiolabeled tracer for later processing. Peptides 1, 2, 3, and 4 have an artificial addition of tyrosine at their amino termini to allow for possible iodination in radioimmunoassay, as well as for possible alternative coupling to carrier protein through the tyrosine hydroxyl group. Peptide 2 has an artificial addition of lysine penultimate to the amino-terminal tyrosine. Peptide 5 has a substitution of phenylalanine for tryptophan in the Abelson sequence, since the indole ring of tryptophan is highly sensitive to the conditions used for cleaving the completed peptide from the support matrix. Thus, the closest related

amino acid, phenylalanine, replaced the tryptophan to make peptide 5 amenable to our established synthesis procedure. Peptides were cleaved from their support resins by a 90-min incubation in 99% trifluoroacetic acid in the presence of bubbling hydrogen bromide gas. To prevent the formation of bromotyrosine, the hydrogen bromide gas was scrubbed in the presence of a 5 $\times$ molar excess to peptide of anisole as a scavenger. After cleavage, the peptides were repeatedly washed with methanol and dried by evaporation. Several other extractions were performed to remove the anisole and other organic contaminants. One cycle of lyophilization was performed to yield a crude crystalline product. The peptides were purified by subjecting them to gel filtration chromatography, using either Bio-Rad P2 or P4 in 50 mM formic acid, pH 2.5. Column fractions were analyzed by optical density, at 280 nm for molecular weight standards and by ^{14}C scintillation counting for peptides. Fractions overlapping with the single ^{14}C activity peak observed for each peptide were pooled and lyophilized to a white, fluffy crystalline product.

Coupling to carrier. Crystalline peptide was dissolved in 0.1 M NaH_2PO_4 - Na_2HPO_4 (pH 7.2)-0.15 M NaCl at room temperature. KLH in 0.15 M NaCl was added such that the peptide was at a 75 $\times$ molar excess per 100,000-molecular-weight of KLH. The reaction was performed by slow addition of 20 mM glutaraldehyde to a final concentration of 6.7 mM. After a 30-min incubation with stirring, the conjugate was dialyzed extensively against phosphate-buffered saline at 4°C. Peptide-KLH conjugate was stored at 4°C. Efficiency of coupling was determined by measuring ^{14}C activity in the reaction mixture before the addition of glutaraldehyde and after dialysis. On average, the coupling was 50% efficient.

Bleedings and immunizations. Peptide-KLH conjugates were emulsified in an equal volume of complete Freund adjuvant. For each peptide-KLH conjugate, two adult, female New Zealand White rabbits were prebled for normal sera on day 0 and injected subcutaneously at four to six sites

FIG. 2. Regions of the *v-abl* protein corresponding to the chemically synthesized peptides and the bacterially expressed fusion proteins used as immunogens. Regions corresponding to chemically synthesized peptides are indicated below the P160 protein, and regions expressed as fusion proteins in bacteria are indicated above the P160 protein. The predicted structures of the *v-abl* proteins expressed by A-MuLV strains P120 and P87 are included for comparison. The amino acid sequences of the 5 chemically synthesized peptides are as follows: Tyr¹LysGluArgGlyProProAspGlySerLeu (peptide 1), Tyr¹Lys¹GlyAlaSerGluAspAspSerArgGluLeu (peptide 2), TyrGluGluAlaAlaGluGluGlyPheLysAspThrGluSerSerProGlySerSerProProSerLeu (peptide 3), Tyr¹SerAspGluValGluLysGluLeuGlyLys (peptide 4), and TyrGlyGluValTyrGluGlyValPhe¹LysLys (peptide 5). ¹, Addition of a residue not found in the A-MuLV protein sequence; ², substitution of phenylalanine for tryptophan.

in their posterior, axial lymph node regions, with 1 mg of conjugate (ca. 300 μ g of peptide). The rabbits were boosted at the same dosage of conjugate emulsified in incomplete Freund adjuvant every 2 to 3 weeks. Intraperitoneal injections were interspersed with the regular subcutaneous injections in the schedule.

Construction of plasmids expressing the *trpE-abl* fusion protein. The Abelson sequences expressed in all of the fusion proteins were subcloned from pAB160 (22). The 2.2-kilobase (kb) *Bgl*II fragment subcloned to construct pEX-1 was purified by low melting point agarose gels (Bethesda Research Laboratories, Inc., Rockville, Md.) and then extracted with hexadecyltrimethylammonium bromide (21). The fragment was ligated to pKRS101 (42) treated with *Bgl*II (P-L Biochemicals, Inc., Milwaukee, Wis.) and calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Ligations were transformed into *CaCl*₂-shocked (24) *Escherichia coli* HB101. Ampicillin-resistant colonies were screened by minilysates for the presence of the 2.2-kb *Bgl*II fragment and for the correct orientation of that fragment. Minilysates from the appropriate colonies were then used to transform *CaCl*₂-shocked *E. coli* C600. These cells were induced (see below) for expression of pEX-1. pEX-2 was derived from pEX-1 by digesting cesium chloride-ethidium bromide-purified pEX-1 plasmid with *Sst*I (P-L Biochemicals). Ligations were then carried out under dilute conditions (10 μ g/ml) and transformed into *CaCl*₂-shocked *E. coli* C600. Colonies were screened by minilysate for deletion of a 0.7-kb *Sst*I fragment.

The *abl* sequence expressed in pEX-4 was subcloned by digesting pAB160 with *Pst*I and *Hind*III (P-L Biochemicals). Fragments were ligated to pUC12 (48) which had been treated with *Pst*I and calf intestinal phosphatase. Ligation reactions were transformed into *CaCl*₂-shocked *E. coli* JM83 and grown in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -galactoside (X-gal) (Sigma). White colonies were screened by minilysates for plasmids containing a 0.9-kb *Pst*I fragment in the correct orientation. An appropriate colony was picked, and supercoiled DNA was purified by cesium chloride-ethidium bromide centrifugation. This DNA was then digested with *Sst*I, which cuts in the polylinker region of pUC12 and in the *abl* sequences. The restricted DNA was religated under dilute conditions (1 μ g/ml) and transformed into *CaCl*₂-shocked *E. coli* HB101. Colonies were screened for loss of the *Sst*I fragment. An appropriate colony was selected, and plasmid DNA was purified by cesium chloride-ethidium bromide centrifugation. Plasmid DNA was restricted by *Hind*III (International Biotechnologies, Inc.) and *Eco*RI. The *Eco*RI-*Hind*III fragment was purified through an agarose gel, hexadecyltrimethylammonium bromide extracted, and ligated to pJH14 (generously contributed by T. J. Koerner), which had been digested by *Eco*RI and *Hind*III. Ligations were transformed into *CaCl*₂-shocked *E. coli* HB101. Colonies were screened by minilysates for the presence of the insert. Minilysate DNA from appropriate colonies was then used to transform *E. coli* C600. Ampicillin-resistant colonies were rescreened for presence of pEX-4 and then induced (see below). The construction of pEX-5 involved digestion of pAB160 with *Hind*III and *Sal*I (P-L Biochemicals). The 1.1-kb *Hind*III-*Sal*I *abl* fragment was purified on low melting point agarose and hexadecyltrimethylammonium bromide extracted. pJH12 (a generous gift of T. J. Koerner) was *Hind*III and *Sal*I digested and then ligated to the gel-purified Abelson insert. Ligation reactions were transformed into *CaCl*₂-shocked *E. coli* HB101. The resulting colonies were

screened for the insert by minilysates. Minilysates from selected colonies were used to transform *CaCl*₂-shocked *E. coli* C600. Ampicillin-resistant colonies were screened for production of pEX-4 by minilysates and then induced. Construction of the *trpE-abl* fusion proteins is diagrammed in Fig. 1. Regions of the *v-abl* protein expressed as fusion proteins are diagrammed in Fig. 2.

Induction of *trpE-abl* fusion plasmids. *E. coli* C600 cells carrying the *trpE-abl* fusion plasmids were induced as previously described (19, 42). Cultures were inoculated from frozen stocks (-70°C) and grown overnight at 37°C in M9 (25) plus 1% Casamino Acids and 100 μ g of ampicillin and 20 μ g of tryptophan per ml. Overnight cultures were diluted 1:100 into fresh M9 with 1% Casamino Acids plus 100 μ g of ampicillin per ml and grown to an optical density at 600 nm of 0.2. Expression of the *trpE-abl* fusion proteins was then induced by adding 100 μ l of a 20- μ g/ml solution of 3- β indoleacrylic acid (Sigma) in ethanol to 100 ml of culture. Growth was continued to late log phase (8 h), and then the cells were harvested by centrifugation and stored at -20°C . Induction of the expected fusion protein was monitored by lysing induced and uninduced cells directly in $2\times$ concentrated SDS gel sample buffer and then analyzing the expressed proteins by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie brilliant blue (Fig. 3). As a control, induced and uninduced cells carrying the parental *trpE* expression vector were also analyzed.

Fusion proteins were partially purified from induced cell pellets as described previously (19, 42). Frozen cell pellets from 100 ml of culture were suspended in 10 ml of TEN (50 mM Tris-hydrochloride [pH 7.5], 0.05 mM EDTA, 0.3 M NaCl) and incubated for 15 min on ice with lysozyme (1 mg/ml) and then 220 μ l of a 10% Nonidet P-40 solution. After a 10-min incubation, 15 ml of 1.5 M NaCl, 12 mM *MgCl*₂, and 100 μ l of 2 mg of DNase I (Sigma) per ml was added. The DNase I treatment continued for 1 h on ice with shaking. The fusion proteins were recovered as insoluble protein after centrifugation for 15 min at $8,000\times g$. The insoluble protein pellets were washed twice with 4 ml of TEN. For gel electrophoresis, the insoluble protein pellets were solubilized by boiling in $2\times$ SDS gel sample buffer for 20 min.

FIG. 3. Bacterial expression of *trpE-abl* fusion proteins. Proteins expressed by *E. coli* carrying the *trpE-abl* fusion plasmids pEX-1 (A), pEX-2 (B), pEX-4 (C), or pEX-5 (D) were analyzed by electrophoresis on an 8% SDS-polyacrylamide gel. Total cell lysates prepared from 0.1 ml of late-log-phase cultures grown in the absence (lane 1) or presence (lane 2) of the *trp* operon inducing agent 3- β -indoleacrylic acid (20 μ g/ml) were analyzed. The insoluble proteins extracted from the equivalent of 0.1 ml of an induced cell culture were analyzed in lane 3. The gel was stained with Coomassie brilliant blue to visualize the proteins.

pEX-4 could not be purified by this procedure, as it was degraded. Instead, conditions for the expression of pEX-4 were optimized after a time course of induction, and a total cell lysate of induced pEX-4 cells was prepared by boiling in $2\times$ SDS gel sample buffer for 20 min.

Immunizations with *trpE-abl* fusion proteins. Fusion proteins were further purified by preparative SDS gel electrophoresis on 1.5- or 3.0-mm-thick 8% polyacrylamide gels as described previously (19, 42). The side lanes of the gel were stained with Coomassie brilliant blue to identify the position of the fusion protein in the gel. The area of the gel containing the fusion protein was excised and homogenized in complete Freund adjuvant (GIBCO) by serial passage through 18-, 20-, and 22-gauge syringe needles. New Zealand white female rabbits (Mission Laboratories) weighing 2 kg were immunized with 500 μ g of protein each. The rabbits were then boosted with 250 μ g of fusion protein every two weeks, and serum was collected 7 days after each boost.

RESULTS

Synthesis of peptides corresponding to the A-MuLV protein sequence. Five peptides corresponding to different regions of the predicted amino acid sequence of the A-MuLV protein (30; M. Paskind and D. Baltimore, personal communication) were synthesized with a modification of the Merrifield method for solid-phase peptide synthesis (44, 45, 49). Details of the synthesis, coupling to carrier molecules, and immunizations are described above. These peptides were chosen for synthesis by scanning the desired region of the *v-abl* protein sequence for a series of hydrophilic amino acids. The sequences of the peptides and their correspondence with the *v-abl* protein are shown in Fig. 2. Peptides 1, 2, and 3 correspond to the "P160 unique" region, which is the region present in the P160 strain of A-MuLV but deleted in the P120 strain. Deletion mutagenesis studies have demonstrated that A-MuLV strains which retain the P160 unique region have an increased lethal function on certain fibroblast cell lines (51). Peptides 4 and 5 were synthesized to correspond with sequences in the region between the *gag-abl* junction and the start of the P160 unique region. This region of the *v-abl* protein encompasses the sequences essential for kinase and transforming activities (29) and is phosphorylated on tyrosine at two distinct sites in vivo (28, 51). We were able to predict which tyrosines are phosphorylated by comparison of data characterizing the phosphotyrosine-containing tryptic peptides with the *v-abl* protein sequence (30). Two-dimensional phosphopeptide analysis of A-MuLV deletion mutants has localized the phosphotyrosine sites to the 45-kd transformation-essential region (28, 51). Patschinsky et al. found that both of the *v-abl* phosphotyrosine-containing tryptic peptides are not digestible with *S. aureus* V8 protease, indicating that they do not contain glutamic acid residues (27). Furthermore, Patchinsky et al. found that during automated Edmann degradation, the phosphotyrosine was the first residue of one tryptic peptide and the seventh residue of the other (27). Analysis of this information indicates that *v-abl* tyrosine residues 385 and 514 are phosphorylated in vivo. The phosphotyrosine at residue 514 is homologous with the in vivo phosphotyrosine site in pp60^{src} and other tyrosine kinases (15, 30). The tyrosine phosphorylated at residue 385 is unique to *v-abl* and is not found in pp60^{src}. The anti-peptide 5 serum is directed against a sequence adjacent to the site of tyrosine phosphorylation at residue 385.

Bacterial expression of A-MuLV. Antisera were also raised against bacterially expressed regions of the *v-abl* protein as

indicated in Fig. 2. These *v-abl* regions, between 10 to 20 kd in size, were expressed in *E. coli* as *trpE-abl* fusion proteins with three related, inducible expression plasmids (42; T. J. Koerner, personal communication). These plasmid vectors contain the *E. coli trp* operator, promoter, *trpL* (leader), and attenuator sequences, as well as all or part of the *trpE* structural gene. The cloning steps involved in joining restriction fragments from the cloned A-MuLV genome (22) in frame behind the amino two-thirds of the *trpE* structural gene are described and diagrammed in detail above and in Fig. 2. This approach was used in addition to the antipeptide sera because it generated higher titered *v-abl* antisera as monitored by immunoprecipitation. In addition, the fusion protein antigens generated strong positive responses in a shorter amount of time (4 weeks) compared with peptide immunogens (8 to 20 weeks).

The *v-abl* sequences expressed in pEX-2 (Fig. 2) overlap the common site of in vivo tyrosine phosphorylation between *v-src* and *v-abl* (30). The pEX-2 sequences also lie within the 45-kd kinase region identified by deletion mutagenesis as essential for transformation (29). The *v-abl* sequences expressed in pEX-4 and pEX-5 are derived from the P160 unique region and the *v-abl* carboxyl terminus, respectively. After transformation of the *trpE-abl* fusion plasmids into *E. coli*, the cells were tested for their ability to express the predicted fusion proteins upon induction of the *trp* operon. The appearance of a specific polypeptide in the expected 55-kd range was detected by SDS-polyacrylamide gel analysis of cell extracts after induction (see above) of pEX-2, pEX-4, and pEX-5 (see lane 2 in Fig. 3B, C, and D). It is interesting to note that during the construction of pEX-2, a 2.2-kb *Bgl*II fragment containing the coding sequence for the carboxyl 80 kd of A-MuLV strain P160 was inserted in frame into the *trpE* expression vector pKRS101, resulting in the construct pEX-1. The *trpE-abl* fusion protein expressed by pEX-1 was predicted to be 120 kd but was not stably expressed upon induction (Fig. 3A, lane 2). This is in agreement with the results of others who have found that high-molecular-weight *trpE* fusion proteins are not stably expressed (42). However, when pEX-1 was modified with an internal deletion to create pEX-2, a truncated fusion protein of ca. 55 kd was now stably produced (Fig. 3B, lane 2).

***abl*-Specific antisera differentially immunoprecipitate deletion mutants of A-MuLV.** The specificity of the antisera collected from rabbits immunized with one of the synthetic peptides coupled to KLH or with one of the *trpE-abl* fusion proteins was examined by immunoprecipitation analysis of ³²PO₄-labeled, A-MuLV P160-infected cell lysates as described above. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography. The results show that P160 is precipitated by a mixture of α p15 and α p12 *gag* monoclonal antibodies (5) (Fig. 41A, lane 2). This band was not detected with any of the preimmune sera (lane 1) but was detected with antisera (lane 2) against peptide 3 (B), peptide 5 (C), pEX-2 (D), pEX-4 (E), pEX-5 (F), and peptide 4 (G). Addition of the appropriate competing peptide to the immunoprecipitation blocked the ability of the antipeptide sera to precipitate P160 (Fig. 41B-D, lane 3). To confirm that each antiserum specifically recognized the expected region of the A-MuLV protein, the antisera were tested for their ability to immunoprecipitate A-MuLV deletion strains P120 and P87. The predicted structures of the proteins expressed by these strains and the P160 strain are shown in Fig. 2. The P120 strain has an internal deletion, thereby lacking the P160 unique region, whereas P87 is a truncation mutant that expresses a protein that

FIG. 4. Immunoprecipitation analysis of the specificity of anti-peptide and antifusion protein antisera for the *v-abl* proteins P160, P120, and P87. (I) P160-transformed NIH 3T3 fibroblasts were metabolically labeled with $^{32}\text{PO}_4$ and extracted. Portions were immunoprecipitated with a mixture of αp15 and αp12 *gag* monoclonal antibodies (A) or sera against peptide 3 (B), peptide 5 (C), pEX-2 (D), pEX-4 (E), pEX-5 (F), or peptide 4 (G). For each antisera, lane 1 is preimmune serum, lane 2 is immune serum, and where indicated, lane 3 is immune serum plus homologous competing peptide. The immunoprecipitates were analyzed by 8% SDS-polyacrylamide gel electrophoresis, followed by autoradiography. (A) through (F) were exposed for 4 h, and (G) was exposed for 15 h with an intensifying screen. (II) P120-transformed NIH 3T3 fibroblasts were metabolically labeled with $^{32}\text{PO}_4$ and extracted. Portions were immunoprecipitated with a mixture of αp15 and αp12 *gag* monoclonal antibodies (A) and with sera against peptide 3 (B), pEX-2 (C), and pEX-5 (D). For each antisera, lane 1 is preimmune serum, lane 2 is immune serum, and where indicated, lane 3 is immune serum plus appropriate competing peptide. The immunoprecipitates were analyzed by electrophoresis on an 8% SDS-polyacrylamide gel, followed by autoradiography. Exposure time was 4 h with an intensifying screen. (III) P87-transformed NIH 3T3 fibroblasts were metabolically labeled with [^{35}S]methionine and extracted. Portions were immunoprecipitated with a mixture of αp15 and αp12 monoclonal antibodies (A), and sera against peptide 3 (B), pEX-2 (C), and pEX-5 (D). For each antisera, lane 1 is preimmune serum, lane 2 is immune serum, and where indicated, lane 3 is immune serum plus appropriate competing peptide. The immunoprecipitates were analyzed by electrophoresis on an 8% SDS-polyacrylamide gel, followed by fluorography. Exposure time was 3 days.

terminates near the 5' border of the P160 unique region. Antisera directed against the 45-kd transformation-essential region, anti-pEX-2 sera (Fig. 4IIC, lane 2), and antisera against the carboxyl terminus, anti-pEX-5 sera (Fig. 4IID, lane 2), immunoprecipitated ^{32}P -labeled P120. However, as predicted, antisera directed against the P160 unique region, anti-peptide 3 sera (Fig. 4IIB, lane 2), did not precipitate P120. In a similar fashion, the antisera were tested for their ability to immunoprecipitate ^{35}S -labeled P87 strain of A-MuLV. P87 lacks the P160 unique region and the carboxyl terminus of the P160 strain. As expected, only antisera

directed against the 45-kd transformation-essential region, anti-pEX-2 sera (Fig. 4IIC, lane 2), precipitated P87. Antisera directed against the P160 unique region, anti-peptide 3 sera (Fig. 4IIB, lane 2), and antisera against the carboxyl terminus, anti-pEX-5 sera (Fig. 4IID, lane 2), did not precipitate P87. The proteins expressed by A-MuLV strains derived from the P160 strain are differentially modified post-translationally by carbohydrate addition and phosphorylation (58; S. M. Watanabe, manuscript in preparation). Figure 3C demonstrates that the anti-pEX-2 sera precipitated the lower-apparent-molecular-weight, phosphorylated form of P87 as well as the higher-apparent-molecular-weight, non-phosphorylated, carbohydrate-modified form of P87 from the ^{35}S -labeled cell extracts. All of the *v-abl* antisera will precipitate both the carbohydrate- and the non-carbohydrate-modified form of the *v-abl* protein when analyzed with the appropriate A-MuLV strain (data not shown). When analyzed in similar experiments, none of the antisera precipitated any Moloney murine leukemia virus gene products (data not shown).

Serum from rabbits immunized with peptides 1 and 2 did not detectably immunoprecipitate P160 after 4 to 5 months of immunization, although anti-peptide 2 sera had detectable anti-peptide 2 titers (N. Rosenberg, unpublished data). Anti-peptide 4 sera only weakly cross-reacted with P160, but antisera against peptides 3 and 5 readily detected P160 by immunoprecipitation (Fig. 4I). Antisera directed against fusion proteins pEX-2, pEX-4, and pEX-5 were highly cross-reactive with P160 after 4 weeks of immunization, whereas it took up to 3 months for rabbits immunized with synthetic peptides to produce antisera capable of immunoprecipitating P160.

Antibodies binding to the region of *v-abl* that is highly conserved among tyrosine kinases block *v-abl* autophosphorylation. Having confirmed that the new antisera had the expected specificity, we tested their ability to detect P160 with an *in vitro* autophosphorylation assay (52). To compare the amount of immunoprecipitated, P160-transforming protein with its autophosphorylation activity, P160-infected cells were metabolically labeled with [^{35}S]methionine, lysed, and then immunoprecipitated with each antisera. Half of each immune complex was electrophoresed on an SDS-polyacrylamide gel and fluorographed to determine the relative amounts of P160 precipitated (Fig. 5A). The second half of each immunoprecipitate was washed in Tris buffer, suspended in kinase buffer, and then incubated in the presence of [γ - ^{32}P]ATP to allow the P160 molecules to autophosphorylate (52). The autophosphorylated P160 molecules were then electrophoresed on SDS-polyacrylamide gels and analyzed by autoradiography without fluorography (Fig. 5B).

Comparison of the amount of P160 autophosphorylation with the relative amount of ^{35}S -P160 recovered indicates that antisera directed against the N-terminal *gag* sequences (Fig. 5A and B, lane 1), the P160 unique region (α peptide 3, lane 2; $\alpha\text{pEX-4}$, lane 5), and the carboxyl terminus ($\alpha\text{pEX-5}$, lane 6) allow P160 to autophosphorylate efficiently. However, P160 precipitated with antisera directed against the 45-kd transformation-essential region (α peptide 5, lane 3; and $\alpha\text{pEX-2}$, lane 4) resulted in at least 20-fold lower autophosphorylation than was detected with antisera against other regions of *v-abl*. Similar experiments with non-radiolabeled ATP in the autophosphorylation reaction mixture have shown that the P160 molecules remain quantitatively bound to the immune complex during the autophosphorylation reaction (data not shown). Mixing immunoprecipitates or coimmunoprecipitating with kinase-blocking sera and a

FIG. 5. Comparison of P160 in vitro autophosphorylation activity detected after immunoprecipitation with antisera directed against different regions of the *v-abl* protein. P160-transformed NIH 3T3 fibroblasts were metabolically labeled with [35 S]methionine and extracted, and then portions were immunoprecipitated. Half of each immunoprecipitate was analyzed for the recovery of metabolically labeled P160 protein by electrophoresis on an 8% SDS-polyacrylamide gel, followed by fluorography (A). The second half of each immunoprecipitate was allowed to autophosphorylate with [γ - 32 P]ATP as described in the text. Autophosphorylated proteins were analyzed by electrophoresis on an 8% SDS-polyacrylamide gel, followed by autoradiography without fluorography (B). For this analysis, P160 extracts were immunoprecipitated with a mixture of α p15 and α p12 *gag* monoclonal antibodies (lane 1) and with antisera against peptide 3 (lane 2), peptide 5 (lane 3), pEX-2 (lane 4), pEX-4 (lane 5), pEX-5 (lane 6), and peptide 4 (lane 7). Exposure time for (A) was 6 days. Exposure time for (B), lanes 1 through 6, was 3 h with an intensifying screen; lane 7 was exposed for 15 h.

nonblocking serum results in detectable levels of autophosphorylation. This indicates that the kinase-blocking sera do not contain high levels of nonspecific inhibitory agents (data not shown). Furthermore, a small amount of kinase-active *v-abl* protein was eluted from a 10- μ l anti-peptide 5 precipitate after incubation for 2 h at 4°C in 100 μ l of 1.0 M NaCl with 30 μ g of peptide 5 (data not shown). Therefore, it appears that precipitation with anti-peptide 5 antibodies or anti-pEX-2 antibodies inhibits *v-abl* autophosphorylation. None of the antisera were phosphorylated on their heavy chain during the autophosphorylation reaction, as is commonly seen for the related tyrosine kinase pp60^{src} (9, 16).

Effects of antibody binding on phosphorylation of a peptide substrate. The *v-abl* protein precipitated with the autophosphorylation-blocking antisera was assayed for its ability to phosphorylate a peptide substrate. The phosphorylation of a peptide substrate was examined to determine whether the autophosphorylation-blocking antisera would also block the *v-abl* phosphorylation of a small substrate molecule. Angiotensin, a tyrosine-containing peptide that serves as a phosphate acceptor for the Rous sarcoma virus pp60^{src} tyrosine kinase (57) was used as a substrate for the A-MuLV kinase. Angiotensin was also selected as a substrate because it contains tyrosine but not the other phosphate-accepting residues, serine and threonine. Therefore, it was unnecessary to perform phosphoamino acid analysis before interpreting the results of each assay. This makes it possible to rapidly quantitate the results with a phosphocellulose paper binding assay which rapidly purifies phosphorylated angiotensin away from unincorporated [γ - 32 P]ATP in the reaction mixture (11).

Table 1 shows the quantitation of angiotensin phosphorylation by P160 precipitated with various sera. P160 precipitated with anti-pEX-2 and anti-peptide 5 sera phosphorylated angiotensin ca. 10- and 20-fold less than control antisera, respectively. Therefore, precipitation with the autophosphorylation-blocking antisera also blocked the ability of P160 to phosphorylate a small peptide substrate. This suggests that the kinase-blocking antisera may act by neutralizing catalytic activity.

DISCUSSION

Production of antisera. Utilizing two different approaches for generating the antisera used in this study has enabled us

to observe several advantages and disadvantages intrinsic to each approach. In our experience, only three of the five peptide immunogens resulted in antisera that would immunoprecipitate *v-abl* after 3 months of immunizations. Also, sera from two rabbits immunized with the same peptide immunogen differed in *v-abl* cross-reactivity by 2- to 10-fold (data not shown). However, both rabbits of each pair immunized with the fusion proteins produced antisera that were as avid and as high a titer as preparations of goat or rabbit antisera raised against Moloney murine leukemia virus proteins previously used in detecting the *v-abl* protein by immunoprecipitation (56). In addition, these *abl*-specific antisera had a higher titer than antitumor sera collected from mice bearing A-MuLV-induced lymphomas (54). For some purposes, the peptide antisera have an advantage in that they

TABLE 1. Tyrosine kinase activity of P160 immunoprecipitated with various *v-abl* antisera

Sera	Autophosphorylation ^a		Peptide phosphorylation ^b	
	cpm ($\times 10^3$)	Relative activity (%)	cpm ($\times 10^3$)	Relative activity (%)
α p15 + α p12	16.0	100	810	100
α peptide 3	13.4	84	NT	NT
α peptide 5	≤ 0.5	≤ 5	25	≤ 5
α pEX-2	0.7	≤ 5	120	15
α pEX-4	14.8	93	NT	NT
α pEX-5	11.0	69	860	106

^a P160 immunoprecipitated from cell extracts with each antiserum was autophosphorylated with [γ - 32 P]ATP and electrophoresed on an 8% SDS-polyacrylamide gel. The amount of 32 P incorporated was quantitated by scintillation counting of P160-containing gel slices. All values listed were normalized for the amount of P160 protein precipitated with each antiserum. The relative amount of P160 precipitated by each antiserum was determined by quantitating the amount of P160 precipitated from an extract of P160-transformed cells that were metabolically labeled with 32 PO₄. The tabulated results represent the average of two independent experiments. The extent of autophosphorylation was linear with the amount of P160 protein precipitated.

^b P160 was immunoprecipitated from cell extracts and subsequently assayed for phosphorylation of a peptide substrate, angiotensin (57), with [γ - 32 P]ATP, as described in the text. All values listed were normalized for the amount of P160 protein precipitated with each antiserum. The relative amount of P160 precipitated by each antiserum was determined by quantitating the amount of P160 precipitated from an extract of P160-transformed fibroblasts that were metabolically labeled with 32 PO₄. The tabulated results represent the average of two independent experiments. The extent of angiotensin phosphorylation was linear with the amount of P160 protein precipitated. NT, Not tested.

FIG. 6. Summary of the analysis of the *v-abl* tyrosine kinase activity with various anti-*v-abl* sera. The regions of the P160 molecule recognized by antisera which either block or do not block *v-abl* kinase activity are indicated. The close proximity of the in vivo *v-abl* phosphotyrosine sites to regions recognized by kinase-blocking antisera is also shown. Both of the regions recognized by the kinase-blocking antisera occur within sequences known to be highly homologous with the *v-src* protein (30). For comparison, the regions of *v-src* recognized by anti-*v-src* kinase-blocking sera (10, 43, 46) are shown.

are specific for 10 to 20 amino acid sequences of *v-abl*. The larger 101 to 142 amino acid sequences of *v-abl* expressed as fusion proteins consistently generated higher titer antisera.

When tested by immunoprecipitation analysis with A-MuLV deletion mutants, all of the antisera had the expected specificity for *v-abl*. Each antipeptide serum also immunoprecipitated proteins from uninfected cells. The precipitation of these proteins could be blocked by the addition of competing peptide to the immunoprecipitate. However, none of these proteins was precipitated by other *v-abl* peptide or fusion protein sera, and probably none are *v-abl* related. The peptide antisera can be made more specific by affinity purification with the peptide cross-linked to a column matrix. Affinity purification of the fusion protein sera can be accomplished in a similar manner, but an additional step is required to adsorb the anti-*trpE* antibodies.

Aside from their use in the present study, the new *v-abl* antisera will also have other applications, such as cell staining by immunofluorescence and immunoaffinity chromatography. In addition, the *v-abl* antisera are being tested for their ability to cross-react with the *c-abl* gene product NCP150. The new antisera will make it possible to study the expression of *c-abl* in more detail than previous antisera allowed.

Neutralization of the *v-abl* kinase. The kinase activity of P160 precipitated by the various sera was examined, and it was observed that two of the antisera blocked autophosphorylation and the phosphorylation of a peptide substrate. The sequences against which the kinase-blocking antisera are directed are diagrammed in Fig. 6. Both of the kinase-blocking antisera are directed against the 45-kd region identified by deletion mutagenesis as essential for kinase and transforming activities (29). Previously, it has been shown that a transformation-defective A-MuLV mutant lacking kinase activity has a 700-base-pair deletion within this region. Antibodies binding to this region can block the *v-abl* kinase activity in vitro, but antibodies binding to other regions of the *v-abl* protein had no significant effect on kinase activity.

Inspection of the homology between *v-abl* and other kinases suggests that the kinase-blocking sera bind to sequences that are functionally important for kinase activity. The kinase-blocking pEX-2 serum is directed against the most highly conserved region between *v-abl* and other tyrosine

kinases. It was not unexpected that the anti-pEX-2 serum blocks kinase activity, because sera against the homologous region in pp60^{src} block its kinase activity (10, 43, 46). Also, the anti-pEX-2 serum is directed against a sequence that is centered around the predicted in vivo site of tyrosine phosphorylation at *v-abl* residue 514, which is homologous with the in vivo phosphotyrosine site of pp60^{src} (15, 30). The other kinase-blocking serum (anti-peptide 5) was directed against a site that is more amino terminal than previously reported kinase-blocking sera for pp60^{src} and other tyrosine kinases (10, 43, 46). The peptide 5 antiserum is directed against a sequence on the N-terminal side of the other predicted in vivo site of *v-abl* tyrosine phosphorylation at residue 385 (27, 30). The homologous position in *v-src* is not phosphorylated in vivo. However, based on reported homology between the catalytic subunit of the cyclic AMP-dependent protein kinase and several tyrosine kinases (2, 38), the peptide 5 antiserum binds to a region that is 8 residues away from a conserved lysine residue at position 391. This lysine residue has been identified as the ATP binding site for the cyclic AMP-dependent kinase (39) and for pp60^{src} (B. M. Sefton and M. Kamps, personal communication) with reactive ATP analogs. Therefore, we have observed that antibodies binding within the 45-kd kinase domain identified by deletion mutagenesis to two distinct sites which are conserved in other kinases inactivate the *v-abl* kinase. This strongly suggests that the sequences bound by the kinase-blocking sera are functionally important for *v-abl* kinase activity.

The role of *v-abl* phosphorylation on tyrosine in vivo. The fact that both of the kinase-blocking antisera bind to *v-abl* near sites of in vivo tyrosine phosphorylation suggests that phosphorylation on these sites may be important for kinase function. A-MuLV mutants which lack kinase activity are not phosphorylated on tyrosine in vivo (28). However, analysis of in vitro-generated mutants of pp60^{src} have shown that the major in vivo site of tyrosine phosphorylation is dispensable for kinase and transforming activities (6, 41). Perhaps the in vivo phosphorylation on tyrosine of viral tyrosine kinases is not a biologically important modification but rather a consequence of sequences situated close to the active site. In view of this, it will be interesting to examine *c-abl* (28) which, unlike *c-src* (40), is not detectably phosphorylated on tyrosine in vivo. *c-src* has tyrosine kinase activity (40), but it is presently not known whether *c-abl* is a tyrosine kinase. The antisera generated in this study should make it feasible to test this possibility.

ACKNOWLEDGMENTS

We gratefully acknowledge the contribution of plasmids from T. J. Koerner and K. R. Spindler. Unpublished *v-abl* sequence was generously provided by M. Paskind, R. Lee, and D. Baltimore.

This work was supported by Public Health Service grant 2T32-CA-9030 from the National Cancer Institute to S.M.W. and by a Faculty Scholar Award and a Research Career Development Award from the American Cancer Society to O.N.W. and N.E.R., respectively. J.B.K. is a predoctoral trainee supported by the National Institute of Health.

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