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Monoclonal Antibodies Specific for v-*abl*- and c-*abl*-Encoded Molecules

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Monoclonal antibodies specific for regions of the transforming protein of Abelson murine leukemia virus were prepared. Antibodies directed against the kinase domain inhibited the autophosphorylation of v-*abl* proteins, and all of the antibodies reacted with the products of the murine and human c-*abl* loci.

Many studies of the mechanism of transformation by retroviruses which carry oncogenes have focused on a comparison of the structure and function of the products of viral oncogenes and their normal cellular homologs. Studies characterizing the transforming protein of Abelson murine leukemia virus (A-MuLV) have used antiserum reactive with Moloney murine leukemia virus-derived determinants (14, 26) as well as tumor regressor antiserum (24) and rabbit antisera raised against v-*abl*-specific synthetic antigens (8). With these reagents, the v-*abl* gene product has been identified as a 160,000-molecular-weight protein containing Moloney murine leukemia virus- and v-*abl*-derived determinants, whereas the product of the mouse c-*abl* locus has been identified as a related 150,000-molecular-weight molecule (25). An altered form of the c-*abl* product, P210, is found in most human chronic myelogenous leukemia cells (9). All of these molecules are tyrosine-specific protein kinases (2, 10, 20, 21). To more fully understand the structure-function relationship of these *abl*-encoded proteins, we have begun to generate a panel of monoclonal antibodies directed against specific regions of the v-*abl*-encoded molecule. In this report, we describe the isolation and characterization of four monoclonal antibodies specific for v-*abl* sequences in the kinase and carboxy-terminal regions of the molecule.

BALB/c mice were immunized with gel-purified *trpE-abl* fusion proteins (8) representing the regions of the v-*abl* protein derived from the v-*src*-homologous, kinase-active domain (pEX-2) and the carboxy-terminal domain (pEX-5), which has been suggested to be exposed on the surfaces of transformed cells (13, 24). Splenocytes from mice responding to these antigens were fused to the myeloma cell line P.3U1 (6, 7), and supernatant fluids from 100 to 500 wells containg cells from each of four fusions were analyzed for the ability to immunoprecipitate P160 v-*abl*. Using this screening approach, we identified two clones from a single fusion by using splenocytes from an animal immunized with pEX-2 (19-110 and 19-84) and two clones from a single fusion by using splenocytes from an animal immunized with pEX-5 (24-21 and 24-430) (data not shown). These isolates were subcloned two or three times until stable clones were identified. Three of these antibodies were immunoglobulin G1 (κ) (19-84, 19-110, and 24-21), and one, 24-430, was immunoglobulin M (κ) (data not shown).

To determine whether the v-*abl*-specific monoclonal antibodies had the expected specificities for regions of the v-*abl*-encoded transforming protein, we analyzed their ability to bind to Abelson protein molecules synthesized by cells infected with A-MuLV strains which differed in their v-*abl* sequences (Fig. 1). Cells (5×10^6) infected with P120 (lanes 1), P90 (lanes 2), and P92 (lanes 3) were lysed in ice-cold Tris lysis buffer (0.01 M Tris [pH 7.5], 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). The extracts were clarified for 15 min at 4°C at $13,000 \times g$, adjusted to contain a 1× concentration of SDS gel sample buffer (26), and run on a 7% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose by the Western blotting technique (19), and the panels of nitrocellulose were reacted with an anti-p12 monoclonal antibody, H548 (1) (panel A); the anti-pEX-2 monoclonal antibodies 19-110 (panel B) and 19-84 (panel C); the anti-pEX-5 monoclonal antibodies 24-21 (panel D) and 24-430 (panel E); and the control, immunoglobulin G1 (κ) monoclonal antibody 71A7 against concanavalin A (17) (panel F). After being washed, the nitrocellulose was reacted with horseradish peroxidase-conjugated, affinity-purified rabbit antimouse immunoglobulin (Cappel Laboratories), and antibodies which had bound to Abelson proteins were visualized by reacting the nitrocellulose with the peroxidase substrate 3,3-diaminobenzidine.

As expected (Fig. 1), H548 (panel A) bound to the P120 (lane 1), P90 (lane 2), and P92 (lane 3) Abelson proteins, all of which contain the p12 determinant recognized by the antibody. Both 19-84 (panel C) and 19-110 (panel B) anti-pEX-2 antibodies directed against determinants within the kinase region reacted with P120 (lanes 1) and P90 (lanes 2), which retain this region, but failed to bind to P92 (lanes 3). The P92 protein is encoded by a transformation-defective virus containing a 700-base-pair deletion in v-*abl* sequences in the kinase region (4, 22), including those expressed by pEX-2 (8). Anti-pEX-5 clones 24-21 (panel D) and 24-430 (panel E) secreted antibodies which bound to the P120 and P92 proteins (lanes 1 and 3, respectively), which contain the complete carboxy-terminal region, but failed to bind to the truncated P90 protein (lanes 2), which is encoded by a mutant virus which lacks the sequences expressed in pEX-5 (15, 23). The results of the Western blotting experiment were confirmed by immunoprecipitation and SDS-polyacrylamide gel analysis (data not shown) and indicated that the monoclonal antibodies raised against synthetic antigens had the

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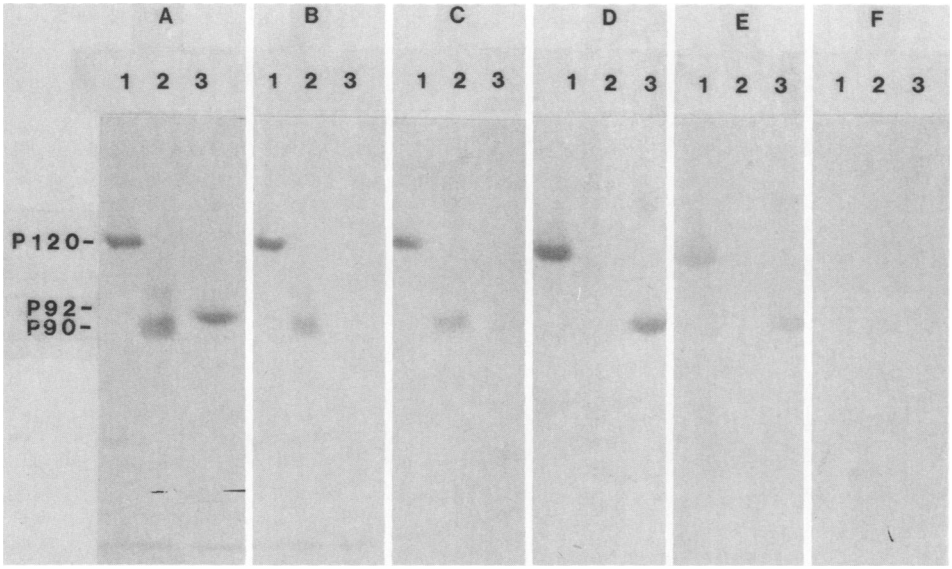

FIG. 1. Western blot analysis of the specificities of anti-fusion protein monoclonal antibodies. Lysates of 5×10^6 P120 (lanes 1)-, P90 (lanes 2)-, and P92 (lanes 3)-infected cells were run on 7% SDS-polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose (27), additional protein-binding sites were blocked, and the nitrocellulose was reacted with 1 ml of culture fluid from the anti-p12 clone H548 (panel A) and 3 ml of culture fluid from the anti-pEX-2 clones 19-110 (panel B) and 19-84 (panel C), the anti-pEX-5 clones 24-21 (panel D) and 24-430 (panel E), and the anti-concanavalin A clone 71A7 (panel F). Monoclonal antibodies bound to immobilized proteins on the nitrocellulose were detected by incubating the nitrocellulose with horseradish peroxidase-conjugated antimouse immunoglobulin and then the peroxidase substrate 3,3-diaminobenzidine. The brown reaction products on the nitrocellulose were photographed with a Polaroid MP-4 camera and 55 P/N film.

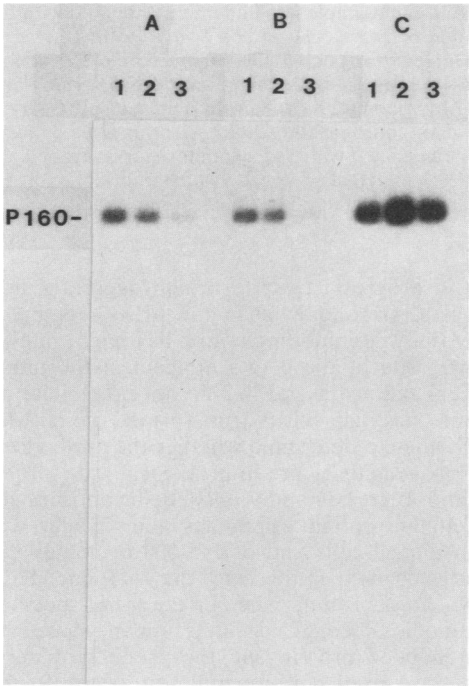

FIG. 2. Inhibition of kinase activity by monoclonal antibodies against pEX-2. Extracts were prepared from 10^6 A-MuLV P160-transformed lymphoid nonproducer cells (15) and immunoprecipitated with 50 μl of culture fluid from the anti-p12 clone H548 (1). Immune complexes were collected on *S. aureus* (IgGsorb), washed in lysis buffer, and resuspended in 50 μl (lanes 1), 100 μl (lanes 2), or 500 μl (lanes 3) of culture fluid from the anti-pEX-2 clones 19-84 (panel A) and 19-110 (panel B) or the isotype-matched anti-p15 clone 13E10 (panel C). After incubation for 1 h at 4°C, the immune complexes were centrifuged to remove inhibiting antibody, washed, and reacted with 2 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; New England Nuclear Corp.) in the standard in vitro protein kinase assay (21). The samples were analyzed on a 10% SDS-polyacrylamide gel. The gel was fixed, dried, and exposed to Kodak XAR-5 film for 5 days at −70°C by using a Cronex Lightning-Plus intensifying screen (E. I. Du Pont de Nemours & Co., Inc.).

expected specificities for the regions of the v-*abl* protein which were specified by the antigens.

Antisera of predetermined specificities have been used to characterize the functional domains of several proteins (11), including the transforming protein of Rous sarcoma virus, pp60 *src* (3, 16, 18). Antibodies reactive with some determinants within the kinase domain of pp60 *src* inhibit kinase activity. Because v-*src*-homologous sequences are present in the pEX-2 fusion protein, we wondered whether monoclonal antibodies directed against the v-*abl* kinase domain would inhibit protein kinase function. We analyzed the ability of culture fluid from the anti-pEX-2 clones 19-84 and 19-110 to inhibit the autophosphorylation of the P160 protein in a standard in vitro protein kinase assay (21). Increasing amounts of culture fluid from the pEX-2-specific clones 19-84 and 19-110 and the anti-p15 clone 13E10 (L. Schiff-Maker and N. Rosenberg, manuscript in preparation) were added to immune complexes of the P160 protein precipitated by the anti-p12 monoclonal antibody H548. The immune complexes were resuspended in inhibiting culture fluid and incubated at 4°C afor 1 h. The complexes were then centrifuged to remove the culture fluid, washed, and reacted with [γ-^{32}P]ATP in the standard in vitro protein kinase assay (8, 21), and labeled proteins were analyzed on a 10% SDS-polyacrylamide gel (Fig. 2). Increasing amounts (lanes 1 to 3) of culture fluid from clones 13E10 (panel C), 19-84 (panel A),

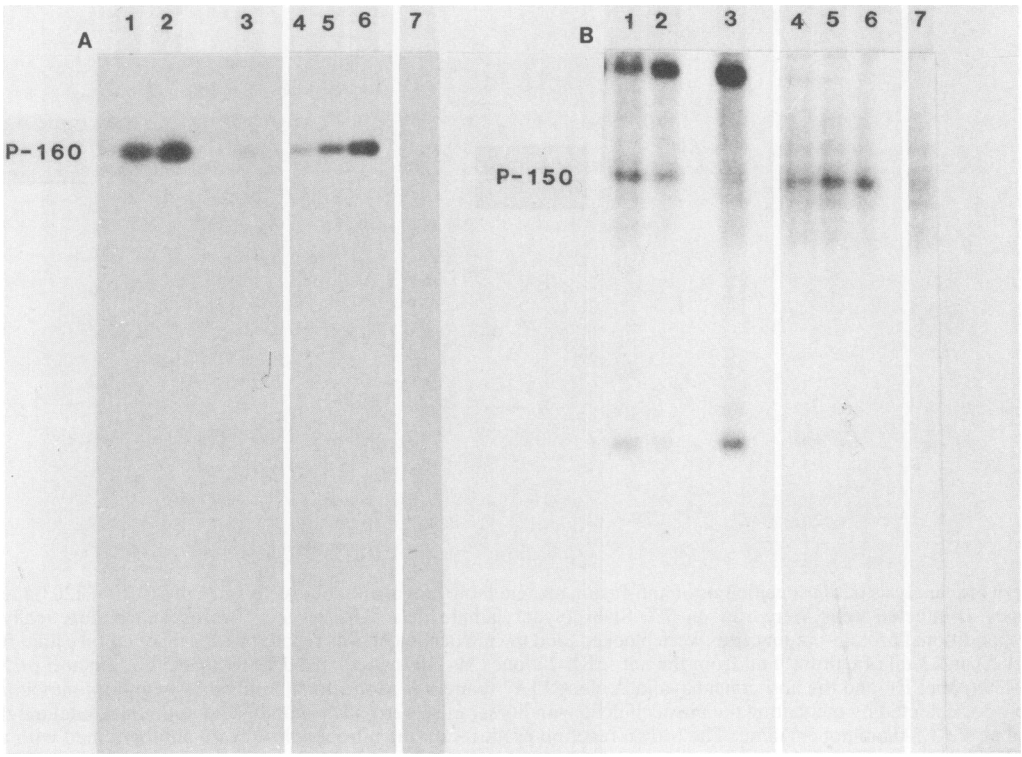

FIG. 3. Reactivity of the v-*abl*-specific monoclonal antibodies with P150 c-abl. A-MuLV P160-transformed lymphoid nonproducer cells (4×10^7) (panel A) and RL 11 cells (8×10^7) (panel B) were labeled for 4 h with $^{32}P_i$ (1 mCi/ml). Lysates were preincubated with *S. aureus* (IgGsorb) before being clarified. Clarified extracts were divided into seven samples, and each sample was immunoprecipitated with 15 μl each of rabbit anti-pEX-4 serum and rabbit anti-pEX-5 serum for 1 h at 4°C. Immune complexes were collected on *S. aureus* for 1 h, washed, and eluted in 50 μl of sample buffer lacking glycerol and bromphenol blue. The eluted samples were pooled, diluted in 3.5 ml of extraction buffer, divided into seven samples, and reprecipitated either with 15 μl of rabbit anti-pEX-4 serum (lanes 1), rabbit anti-pEX-5 serum (lanes 2), or preimmune rabbit serum (lanes 3) or with 300 μl of culture fluid from the anti-pEX-2 hybridoma 19-110 (lanes 4), the anti-pEX-5 hybridomas 24-430 (lanes 5) and 24-21 (lanes 6), or the control hybridoma 71A7, which is specific for concanavalin A (lanes 7). Immune complexes were collected on *S. aureus*. *S. aureus* for samples involving monoclonal antibodies was preincubated with 5 μg of affinity-purified rabbit antimouse immunoglobulin per sample. Labeled proteins were analyzed on a 10% SDS-polyacrylamide gel. The gel was fixed, dried, and exposed to Kodak XAR-5 film for 45 min (panel A) or 3 days (panel B) at −70°C by using a Cronex Lightning-Plus intensifying screen.

and 19-110 (panel B) were assayed for their inhibitory activity. Whereas culture fluid from the anti-p15 clone 13E10 failed to inhibit the autophosphorylation activity of the P160 protein (panel C), both of the anti-pEX-2 clones (panels A and B) secreted antibodies capable of inhibiting the kinase activity in the autophosphorylation assay, as detected by the decreasing intensity of the P160 band as the amount of inhibiting culture fluid increased (compare lanes 1 and 3 in panels A and B). Because all of the antibodies tested for inhibitory activity were of the same isotype and failed to bind *Staphylococcus aureus*, this assay is a direct measure of their ability to interact with Abelson protein molecules. These results suggest that the sequences bound by the kinase-blocking antibodies are functionally important for v-*abl* kinase activity.

The monoclonal antibodies could inhibit kinase activity either by binding near the site of in vivo tyrosine phosphorylation (5, 13) or by interacting with the catalytic site of the enzyme. Rabbit antisera against pEX-2 are capable of inhibiting the phosphorylation of exogenous substrates, such as the peptide angiotensin and the enzyme enolase, indicating that some antibodies in these polyclonal sera probably bind in or near the active site of the kinase domain (2). Experiments to determine whether the pEX-2-specific monoclonal antibodies inhibit the ability of v-*abl* kinase to function in *trans* are in progress. Despite a high degree of homology between pp60 *src* and P160 in the pEX-2 region and the possibility that the inhibiting antibodies might interact with the catalytic site of these two molecules, the monoclonal antibodies raised against pEX-2 do not precipitate pp60 *src* from Rous sarcoma virus-transformed cells (data not shown). Studies to determine whether the antibodies inhibit pp60 *src* kinase activity are in progress.

Both tumor regressor and v-*abl*-specific antisera identify a normal cellular protein which has a molecular weight of 150,000 in mouse cells and of 145,000 in human cells and which shares tryptic peptides with the v-*abl*-encoded protein (9, 12, 25). To determine whether the v-*abl*-specific monoclonal antibodies recognized a determinant shared between the v-*abl*-encoded protein and the product of the murine c-*abl* gene, we used a reprecipitation assay (9) in which extracts of the P150 c-*abl*-positive thymoma cell line RL 11 were first immunoprecipitated with a mixture of rabbit anti-pEX-4 and anti-pEX-5 sera, both of which are able to precipitate *abl*-encoded proteins. After the immunoprecipitated molecules were recovered, the extracts were reprecipitated with either the rabbit anti-pEX-4 and anti-pEX-5 sera or the monoclonal antibodies. The labeled molecules were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 3). As expected, rabbit anti-pEX-4

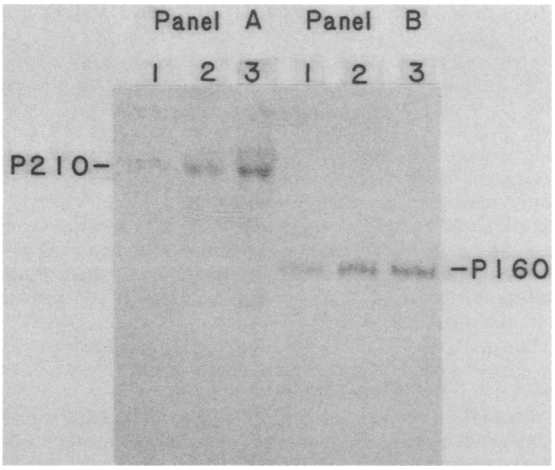

FIG. 4. Western blot analysis of the reactivity of the v-*abl*-specific monoclonal antibodies with P210 c-*abl*. K562 cells (5×10^7) (panel A) were lysed in phosphate lysis buffer (26) and immunoprecipitated with 25 μl of rabbit anti-pEX-5 serum (8). Immune complexes were isolated on *S. aureus* and suspended in sample buffer such that increasing cell equivalents could be run in each lane of an 8% SDS-polyacrylamide gel. In panel A, lanes 1, 2, and 3 represent the immunoprecipitated extracts of 12×10^6, 24×10^6, and 48×10^6 K562 cells, respectively. A-MuLV P160-transformed NIH 3T3 cells (5×10^6) (panel B) were similarly lysed and immunoprecipitated. In panel B, lanes 1, 2, and 3 represent the immunoprecipitated extracts of 0.8×10^6, 1.2×10^6, and 4×10^6 P160-transformed cells, respectively. The samples were run on an 8% SDS-polyacrylamide gel before electrophoretic transfer of the proteins to nitrocellulose. The additional protein-binding sites on the membrane were blocked with 10% powdered milk, and the membrane was incubated overnight with 0.5 ml of culture fluid from a mixture of 19-84 and 19-110. A 1:1,000 dilution of horseradish peroxidase-conjugated goat antimouse immunoglobulin and horseradish peroxidase Color Development Reagent (Bio-Rad Laboratories), which contains the peroxidase substrate 4-chloro-1-naphthol, were used to visualize the monoclonal antibody-binding reaction.

and anti-pEX-5 sera were able to reprecipitate the 150,000-molecular-weight product of the murine c-*abl* locus (panel B, lanes 1 and 2, respectively), whereas preimmune rabbit serum (lane 3) and culture fluid from the anti-concanavalin A hybridoma 71A7 (lane 7) failed to reprecipitate any labeled protein. Culture fluids from the anti-pEX-2 hybridoma 19-110 (panel B, lane 4), specific for a determinant within the kinase domain, and the anti-pEX-5 hybridomas 24-430 (lane 5) and 24-21 (lane 6), specific for a determinant within the carboxy-terminal domain, were able to reprecipitate a 150,000-molecular-weight protein which comigrated with that reprecipitated by the rabbit anti-pEX sera (lanes 4 to 6). Control reprecipitation of P160 v-*abl* is shown in panel A. As expected, this molecule was reprecipitated by anti-pEX-4, anti-pEX-5, and all of the v-*abl*-specific monoclonal antibodies (panel A, lanes 1, 2, and 4 to 6) but not by control antibodies (lanes 3 and 7). Both higher- and lower-molecular-weight bands were nonspecifically reprecipitated by all of the antibodies from samples of extracts of the RL 11 cell line. These results indicate that the v-*abl*-specific monoclonal antibodies are capable of recognizing the product of the murine c-*abl* locus. The fact that these antibodies are capable of reprecipitating Abelson protein that has been denatured in an SDS-containing buffer as well as of recognizing the protein after Western transfer (see above) supports the conclusion that they do not recognize conformationally specific determinants. In other experiments (data not shown), these antibodies were also able to identify P145, c-*abl*, the product of the human c-*abl* locus.

In a study surveying c-*abl* gene products in murine and human cell lines, an altered, higher-molecular-weight c-*abl* gene product, P210 c-*abl*, was identified in K562 human leukemia cells by using rabbit antisera raised against synthetic antigens (9). Because monoclonal antibodies might be able to identify structural differences among viral, normal cellular, and altered cellular *abl* gene products, we analyzed the ability of the v-*abl*-specific monoclonal antibodies to bind to P210 c-*abl* in a Western blotting assay (Fig. 4). A-MuLV P160-transformed NIH 3T3 cells and K562 cells were extracted and immunoprecipitated with rabbit antiserum directed against the pEX-5 protein. Increasing cell equivalents of protein (lanes 1 to 3) recovered from immune complexes were run on an 8% SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose. The nitrocellulose was probed with a mixture of 19-84 and 19-110, both of which recognize the kinase domain of v-*abl*. Bound antibody was detected by using a second antibody, goat antimouse immunoglobulin conjugated to horseradish peroxidase. Both the v-*abl*-encoded P160 protein (panel B) and the P210 c-*abl* protein (panel A) were detected by the v-*abl*-specific monoclonal antibodies. The v-*abl*-encoded P160 protein was not detected in extracts of K562 cells (panel A), and the P210 c-*abl* protein was not detected in extracts of A-MuLV P160-transformed cells (panel B). Other experiments demonstrated that clones 19-84, 19-110, and 24-21 each secreted antibodies capable of binding to P210 (data not shown). Thus, monoclonal antibodies raised against both pEX-2 and pEX-5 were capable of reacting with P150 c-*abl*, the 150,000-molecular-weight product of the murine c-*abl* locus, as well as with P145 c-*abl* and P210 c-*abl*, the products of the normal and altered human c-*abl* genes, respectively. We have yet to determine whether the *abl* protein from lower eucaryotes can be recognized by any of these monoclonal antibodies.

The monoclonal antibodies described here will be useful in providing additional information about the relationship between the v-*abl*- and c-*abl*-encoded proteins in terms of their structure, activities, and regulation. In addition, studies on the subcellular localization of the v-*abl*- and c-*abl*-encoded proteins, made difficult by the viral cross-reactivity in tumor regressor serum and the nonspecific mouse reactivity in the site-directed rabbit sera, should be facilitated by the availability of *abl*-specific monoclonal antibodies that recognize both the cellular and viral forms of the *abl*-encoded protein. Finally, large-scale purification of these proteins is possible by using the monoclonal antibodies linked to resins for immunoaffinity chromatography.

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