Abelson Murine Leukemia Virus Transformation-Defective Mutants with Impaired P120-Associated Protein Kinase Activity

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Several transformation-defective (td) mutants of Abelson murine leukemia virus (AbLV) are described. Cells nonproductively infected with such mutants exhibited a high degree of growth contact inhibition, failed to form colonies in soft agar, lacked rescuable transforming virus, and were as susceptible as uninfected control cells to transformation by wild-type (wt) AbLV pseudotype virus. In addition, each of several td AbLV nonproductively infected cell clones analyzed was found to be nontumorigenic in vivo. Biochemical analysis of td mutant AbLV-infected clones revealed levels of expression of the major AbLV translational product, P120, and a highly related 80,000-*M*_r AbLV-encoded protein, P80, at concentrations analogous to those in wt AbLV-transformed cells. Although the AbLV-specific 120,000-*M*_r polypeptides expressed in td mutant AbLV-infected clones were indistinguishable from those in wt AbLV-transformed lines with respect to molecular weight and [³⁵S]methionine tryptic peptide composition, they each differed from wt AbLV P120 in their patterns of post-translational phosphorylation. A previously described AbLV-associated protein kinase activity is shown to recognize as substrate a major tyrosine-specific acceptor site(s) contained within a single well-resolved tryptic peptide common to both AbLV P120 and P80. In vitro [γ -³²P]ATP-mediated labeling of this phosphorylation site was reduced to below detectable levels in td mutant nonproductively infected cell clones. These findings establish that the AbLV-encoded polypeptide P120 and its associated protein kinase activity are involved in AbLV tumorigenesis.

Abelson murine leukemia virus (AbLV) was isolated by passage of Moloney murine leukemia virus (Moloney-MuLV) in prednisolone-treated BALB/c mice (1). Although replication-defective, AbLV can be propagated by coinfection with appropriate type C helper virus (10, 17, 18). In the presence of such helper virus, AbLV transforms lymphoid cells (15) and embryo fibroblasts (17) in cell culture and induces a rapid B-cell lymphoma in vivo (1, 15). AbLV encodes, as its major translational product, a 120,000-dalton polypeptide, P120, containing Moloney-MuLV *gag* gene structural components (p15, p12, and a minor portion of p30) and a less well defined nonstructural component (12, 14, 28). AbLV P120 lacks immunological determinants cross-reactive with Moloney-MuLV p10, reverse transcriptase, or envelope glycoprotein (12, 28). By tryptic peptide mapping analysis, AbLV P120 has been shown to contain 13 major well-resolved [³⁵S]methionine-labeled peptides (25). Of these, one is p12 specific, and the others are localized within the nonstructural component of AbLV P120 and are apparently encoded by ac-

quired cellular sequences within the AbLV genome.

The nonstructural component of AbLV P120 has been shown to be highly acidic (12) and to contain six to eight well-resolved phosphopeptides which are labeled in vivo after metabolic labeling with [³²P]orthophosphate (26). In addition, AbLV P120 exhibits an intrinsic or highly associated protein kinase (26, 27). This enzymatic activity is present in immunoprecipitates of AbLV P120, efficiently incorporated into AbLV pseudotype virions, and causes phosphorylation of AbLV P120 under in vitro labeling conditions. In this latter respect, this enzymatic activity differs from a protein kinase associated with avian sarcoma virus pp60^{src} which, in immune complex form, recognizes the heavy chain of immunoglobulin as its major substrate (3, 8).

We previously reported the isolation of a number of transformation-defective (td) AbLV mutants cloned by endpoint dilution in mink cells (16). In the present study, we have extended our studies to the isolation of td AbLV mutants cloned in Fisher rat embryo cells and have ana-

lyzed both groups of mutants with respect to polyprotein expression and associated protein kinase activity. In addition, the substrate specificity of the AbLV-associated protein kinase activity has been further analyzed. Our results argue that the protein kinase activity associated with AbLV recognizes a single tyrosine acceptor site common to both AbLV and a highly related 80,000-*M*, probable posttranslational cleavage product and that this activity is involved in AbLV-mediated transformation.

MATERIALS AND METHODS

Cells and virus. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) in petri dishes (60 by 15 mm; Falcon Plastics, Los Angeles, Calif.). Cell lines have been previously described and included NIH/3T3 mouse cells, CCL64 mink cells, and a highly contact-inhibited Fisher rat embryo line, designated FRE (12). ANN-1, a subclone of NIH/3T3 nonproductively transformed by AbLV (17), as well as an AbLV-transformed subclone of the CCL64 mink cell line designated 64 Ab-2 (12), have also been described. Several CCL64 mink clones infected with td variants of AbLV, including 3-1-1, 9-104-7, and 9-124-2 (16), were also used. Type C viruses included Moloney-MuLV and a 4070-A wild mouse amphotropic virus pseudotype of AbLV, designated AbLV(4070-A) (12).

Competition immunoassays. Type C virus structural proteins, including Moloney-MuLV p12 and p30, were purified and labeled with ^{125}I at high specific activity (5 to 20 $\mu\text{Ci}/\mu\text{g}$) according to previously reported procedures (21). Competition immunoassays were performed by testing unlabeled cell extracts at serial twofold dilutions for ability to compete with ^{125}I -labeled viral proteins for binding limiting amounts of goat antiserum directed against detergent-disrupted Moloney-MuLV. Reaction mixtures contained 0.01 M Tris-hydrochloride (pH 7.8), 1.0 mM EDTA, 0.4% Triton X-100, 1% bovine serum albumin, and 0.05 M NaCl in a total volume of 0.2 ml. Antiserum and unlabeled competing antigen were incubated at 37°C for 1 h followed by addition of 10,000 cpm of ^{125}I -labeled antigen. After further incubation for 3 h at 37°C and 18 h at 4°C, antigen-antibody complexes were immunoprecipitated as previously described (21).

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Exponentially growing cells were radioactively labeled by incubation in medium containing [^{35}S]methionine (1,075 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or [^{32}P]orthophosphate (carrier-free, New England Nuclear Corp., Boston, Mass.) as previously described (26). After labeling, cell monolayers (1.0×10^7 cells) were washed twice with serum-free medium and disrupted at 4°C in 5 ml of PBSTDS (10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) lysis buffer. Extracts (1 ml) were incubated at 4°C for 18 h after addition of normal goat serum (5 μl), protein A-Sepharose C1-4B (0.2 ml of a 10% [vol/

vol] suspension) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). After clarification by centrifugation for 1 h at $100,000 \times g$, virus-specific proteins were immunoprecipitated by incubation for 18 h at 4°C in the presence of 5 μl of either goat anti-Moloney-MuLV, goat anti-Rauscher-MuLV p12, or normal goat serum (14). Finally, 50 μl of a 10% (vol/vol) protein A-Sepharose C1-4B suspension was added and extracts were subjected to centrifugation at $2,000 \times g$ for 10 min, washed three times in PBSTDS, and analyzed by SDS-PAGE on 5 to 20% polyacrylamide gradient slab gels according to the method of Laemmli (7).

Protein kinase assay. Cells were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM Tris-hydrochloride (pH 7.2)-100 mM NaCl-1 mM EDTA-5 mM MgCl_2 buffer containing 0.1% Triton X-100 and clarified by centrifugation at $2,000 \times g$ for 10 min. Virus-specific proteins were immunoprecipitated as described above with the exception that SDS was omitted from the PBSTDS precipitation buffer and protein kinase activity was assayed by suspension of immunoprecipitates in 20 mM Tris-hydrochloride (pH 7.2)-5 mM MgCl_2 buffer containing 1 μCi of [γ - ^{32}P]ATP (2,000 Ci/mmol; New England Nuclear Corp.) and incubation for 10 min at 30°C. Reactions were terminated by addition of 3 ml of ice-cold PBSTDS. After repeated washing in PBSTDS, immunoprecipitates were suspended in 20 μl of 0.65 M Tris-hydrochloride (pH 6.7), 1% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromophenol blue, heated for 2 min at 90°C, and analyzed by SDS-PAGE as described above.

Two-dimensional tryptic peptide analysis. Virus-specific proteins were purified by immunoprecipitation and SDS-PAGE as previously described (25). Proteins were digested by incubation of selected gel slices in tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemicals Corp., Freehold, N.J.) (50 $\mu\text{g}/\text{ml}$ in 0.05 M ammonium bicarbonate, pH 8.0) for 6 h at 37°C. Supernatant fluids were removed and replaced by a fresh TPCK-trypsin solution for additional digestion at room temperature for 16 h. Supernatant fractions were pooled, filtered, lyophilized, washed by resuspension in water, and re-lyophilized. Labeled digests were incubated for 2 h at 4°C in 0.1 ml of chilled performic acid (30% H_2O_2 and 90% formic acid [1:9] preincubated for 2 h at room temperature). Samples were subsequently diluted with 2 ml of water, lyophilized, suspended in electrophoresis buffer (acetic acid-formic acid-water, 15:5:80) (pH 1.9), and spotted on cellulose-coated thin-layer chromatography (TLC) glass plates (10 by 10 cm; EM Laboratories, Inc., Elmsford, N.Y.). Electrophoresis was performed for 30 min at 500 V, and chromatography was conducted in the second dimension in buffer containing butanol-pyridine-acetic acid-water (32:25:5:20). Radiolabeled tryptic peptides were visualized by autoradiography, using Kodak X-Omat R film. Detection of [^{35}S]methionine-labeled peptides was enhanced by spraying TLC plates with ether containing 7% diphenylloxazole (2).

Phosphoamino acid identification. Proteins purified by immunoprecipitation and SDS-PAGE and eluted from selected gel slices as described above, or individual tryptic phosphopeptides scraped from TLC

plates (3,000 to 10,000 cpm), were heated to 110°C for 3 h in 50 μ l of 6 N hydrochloric acid containing 0.1 μ mol each of appropriate phosphoamino acid standards. Samples were lyophilized, suspended in 1 ml of deionized water, lyophilized a second time, and dissolved in buffer containing acetic acid-formic acid-water (15:5:80), pH 1.9. Three authentic phosphoamino acid standards, *o*-phospho-D-serine, *o*-phospho-L-arginine, and *o*-phospho-DL-threonine, were purchased from Sigma Chemical Co., St Louis, Mo. *o*-Phospho-L-tyrosine was synthesized by heating a mixture containing 5 g of L-tyrosine, 35 g of phosphoric acid (85%), and 15 g of P₂O₅ at 100°C for 3 days; 5 g of additional P₂O₅ was added on the 2nd and 3rd days. The reaction was terminated by dilution into a 200-ml ice water mixture, and the *o*-phospho-L-tyrosine was purified by chromatography over Dowex 50 $\times$ 8, hydrogen form, and crystallization from ethanol (9, 11). Two-dimensional analysis of phosphoamino acid samples using TLC plates (10 by 10 cm) was performed by electrophoresis for 1 h in pyridine-acetic acid-water (1:10:100) buffer and chromatography in the second dimension in 0.5 M ammonium hydroxide-isobutyric acid (3:5). Unlabeled phosphoamino acid standards were detected by ninhydrin staining, and ³²P-labeled amino acids were visualized by autoradiography on X-Omat R film with the use of calcium tungstate intensifying screens (Eastman Kodak, Rochester, N.Y.).

RESULTS

Isolation and biological characterization of td AbLV mutants. In an effort to isolate mutants of AbLV defective in expression of the transformed phenotype, FRE rat cells were in-

fectured with AbLV(4070-A) pseudotype virus at a multiplicity of around 1. Cells were trypsinized 18 h later and immediately cloned in microtiter plates. Upon scoring after 14 days of incubation at 37°C, 380 of 810 clones examined were morphologically transformed. Of the nontransformed clones, 65 exhibited readily detectable levels of Moloney-MuLV p12 reactivity in absence of either Moloney-MuLV p30 or infectious virus production. Nine of the 65 Moloney-MuLV p12-positive, nontransformed clones as well as 10 nonproductively transformed clones were twice recloned in microtiter plates and subjected to further analysis. Representative nontransformed clones lacked ability to form colonies in soft agar but expressed levels of Moloney-MuLV p12 antigenic reactivity comparable to that exhibited by wt AbLV transformants (Table 1).

To further characterize the above p12-positive nontransformed clones and to establish whether the defect in expression of transformation was virus specific or cellular in nature, each was analyzed for susceptibility to transformation by wt AbLV and for rescuable transforming virus. All nine of the Moloney-MuLV p12-positive, nontransformed clones were as susceptible as control FRE rat cells to retransformation by AbLV(4070-A) pseudotype virus, arguing that the defect in transformation is specific to the AbLV genome rather than a cellular gene whose expression is required for transformation. This conclusion is supported by the inability to rescue

TABLE 1. *Biological characterization of AbLV mutants defective in expression of transformation*

Cell clone	Growth in soft agar (%) ^a	Transformation by AbLV(4070-A) (log ₁₀ foci/10 ⁵ cells) ^b	Rescue of wt AbLV (log ₁₀ FFU/ml) ^c	Moloney-MuLV p12 expression (ng/mg of cell protein) ^d
Control, FRE C13A	<0.01	4.2	0.0	0.8
wt AbLV infected				
4-4	16		4.6	510
2-9-T2	19		5.3	460
td AbLV infected				
34-F2	<0.01	4.1	0.0	405
2-262-F4	<0.01	3.8	0.0	340
411-F2	<0.01	4.6	0.0	605
98-F6	<0.01	4.2	0.0	480
2-112-F2	<0.01	3.5	0.0	410

^a Plating efficiency in soft agar was determined as previously described (16).

^b Susceptibility of individual cell clones to transformation was determined by infection with wt AbLV(4070-A) pseudotype virus at serial 10-fold dilutions. Results are expressed as number of foci per 10⁵ cells extrapolated back to the highest virus concentration tested.

^c Control (CCL64) and AbLV-nonproducing cell clones were infected with 4070-A virus at a multiplicity of infection of around 1. Fourteen days later, culture fluids were harvested and assayed at serial 10-fold dilutions for focus-forming activity on control FRE rat cells, and plates were scored for transformed foci at 10 days after infection. FFU, Focus-forming units.

^d Cell extracts were prepared and tested at serial twofold dilutions in homologous competition immunoassay for Moloney-MuLV p12 as described in Materials and Methods.

wt AbLV from such clones after superinfection with 4070-A helper virus. In contrast, all 10 wt AbLV-transformed clones were found to contain readily detectable levels of rescuable AbLV. Representative data for two wt and five td AbLV clones are summarized in Table 1. Of interest, the nonproductively transformed clones exhibited only minimal frequencies of reversion to the nontransformed phenotype (data not shown) and thus differed markedly from previously described AbLV-transformed CCL64 mink clones, which were found to revert at relatively high frequency.

Expression in wt AbLV-transformed clones of AbLV P120 and a highly related 80,000-*M_r* AbLV-encoded protein. In an initial effort to establish the involvement of the AbLV-encoded polyprotein P120 in transformation, each of 10 wt AbLV-transformed FRE clones were tested for AbLV P120 expression. Three wt AbLV-transformed CCL64 mink clones, five previously described morphological revertants of one such clone, 64 Ab2, and a single AbLV-transformed mouse cell line, ANN-1, were similarly analyzed. Cultures were pulse-labeled with [³⁵S]methionine, immunoprecipitated by using either anti-Moloney-MuLV or normal goat

serum, and analyzed by SDS-PAGE. AbLV P120 was expressed at readily detectable levels in each of the wt AbLV-transformed CCL64 and FRE clones examined (Fig. 1). In contrast, loss of phenotypic transformation was associated with loss of polyprotein expression in each of the revertant clones (data not shown). The fact that transformation was never observed in the absence of AbLV P120 expression argues its involvement in transformation.

In addition to AbLV P120, low levels of a serologically related 80,000-*M_r* polyprotein were also expressed in each of the AbLV-transformed clones. In each case, P80 was efficiently immunoprecipitated by antisera directed against detergent-disrupted Moloney-MuLV but not by normal goat serum (Fig. 1). AbLV P120, purified from either mouse (Fig. 2A) or rat (Fig. 2B) cells by immunoprecipitation and SDS-PAGE, contained a total of 13 well-resolved methionine-containing tryptic peptides. Nine of these peptides, including peptide "13" previously shown (25) to be specific to the p12 component of AbLV P120 (Fig. 2D), were also present in P80 (Fig. 2C). Moreover, none of the AbLV P120- and P80-specific methionine-containing peptides, with the exception of peptide "13," corresponded

FIG. 1. Immunoprecipitation and SDS-PAGE analysis of AbLV and Moloney-MuLV translational products. NIH/3T3 cells productively infected with Moloney-MuLV (A, B) and AbLV nonproductively transformed mouse ANN-1 (C, D), rat C1 4-4 (E, F), and mink 64 Ab2 cells (G, H) were pulse-labeled in [³⁵S]methionine (250 μ Ci/ml) containing medium for 30 min, immunoprecipitated with goat antiserum directed against detergent-disrupted Moloney-MuLV (A, C, E, G) or normal goat serum (B, D, F, H), and analyzed by SDS-PAGE. Molecular weight standards include ¹⁴C-labeled phosphorylase B (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome (12,000).

FIG. 2. Tryptic peptide analysis of [^{35}S]methionine-labeled AbLV and Moloney-MuLV translational products. Proteins were purified by immunoprecipitation and SDS-PAGE and digested with TPCCK-trypsin as described in the text. Tryptic digests (6,000 to 10,000 cpm) were resuspended in electrophoresis buffer and spotted on cellulose TLC glass plates (O). Separation was by electrophoresis in the first dimension (left to right) and by ascending chromatography in the second dimension. Proteins analyzed included AbLV P120 isolated from ANN-1 (A) or C1 4-4 (B) cells; AbLV P80 isolated from C1 4-4 cells (C); Pr65^{gag} (D) and Pr180^{gag/pol} (E) isolated from NIH/3T3 cells productively infected with Moloney-MuLV; a 1:1 mixture of Moloney-MuLV Pr180^{gag/pol} and ANN-1 derived AbLV P120 (F). Tryptic peptides contained within AbLV P120 are designated 1 through 13 whereas those specific to Moloney-MuLV p12 and p30 are labeled "a" and "b, c," respectively.

in position to any of the major methionine-containing peptides contained within Moloney-MuLV Pr180^{gag/pol} (Fig. 2E, F).

We have previously shown that, under *in vivo* labeling conditions, AbLV P120 exhibits two p12-specific phosphorylation sites as well as sev-

eral major sites of phosphorylation within its nonstructural component (26). As an extension of these studies, we undertook to determine which, if any, of these sites of phosphorylation are also represented within P80. AbLV nonproductively transformed mouse and rat cells were pulse-labeled in [32 P]orthophosphate-containing medium and analyzed by immunoprecipitation and SDS-PAGE. Both P120 and P80 were efficiently labeled by this means (Fig. 3). Tryptic peptide analysis revealed a total of seven major phosphopeptides in AbLV P120, five of which, including the two specific to the p12 component, were also present in P80 (Fig. 4). The fact that the phosphopeptide maps for these polyproteins were indistinguishable, irrespective of whether they were isolated from transformed mouse or rat cells, confirms their virus specificity.

AbLV P120 and P80 expression in td AbLV nonproductively infected clones. The presence of Moloney-MuLV p12 immunological reactivity in extracts of cells nonproductively infected by representative td AbLV mutants raised the possibility that such reactivity might be associated with either AbLV P120 or P80. To test this possibility, several representative td mutant clones were analyzed by immunoprecipitation and SDS-PAGE. Proteins of around 120,000 and 80,000 *M_r* were expressed in each of five td mutant AbLV-infected clones at levels comparable to those in four wt AbLV-transformed clones examined in the same experiment (Fig. 3). Analysis of 10 td AbLV mink cell clones also revealed high levels of AbLV P120 and P80 expression (data not shown). Although these findings exclude the possibility that polyproteins expressed in any of the td mutant clones examined to date possess major deletions, as compared with wt AbLV-encoded P120 and P80, minor deletions might not be resolved by the procedures used in the present study.

In a further attempt to demonstrate differences between AbLV-specific polyproteins expressed in wt and td mutant clones, the 120,000 *M_r* polyproteins expressed in each of six td mutant clones were analyzed with respect to number and distribution of phosphopeptides. As indicated from a comparison of the results summarized in Fig. 4 and 5, phosphopeptide tryptic maps of each of six of the td AbLV 120,000 *M_r* polyproteins examined were distinct from the phosphopeptide tryptic map characteristic of wt AbLV P120. In particular, one relatively intensely labeled wt AbLV P120 phosphopeptide (designated "f") was consistently absent from polyproteins expressed in each of the mutant clones. In contrast to the striking differences between the phosphopeptide tryptic maps of the 120,000-*M_r* polyproteins expressed in wt and td

FIG. 3. Immunoprecipitation and SDS-PAGE analysis of [32 P]orthophosphate-labeled polyproteins encoded by wt and td clones of AbLV. Cells were pulse-labeled for 2 h in medium containing [32 P]orthophosphate (100 μ Ci/ml), immunoprecipitated by anti-Rauscher-MuLV p12 (A-D, F-L) or control goat serum (E, M), and analyzed by SDS-PAGE. Cell lines tested included four wt AbLV-transformed FRE clones, 4-4 (A, E), 2-9-T2 (B), 2-5-T2 (C), and 6-T1 (D); control FRE rat cells (F); and five td AbLV nonproductively infected FRE clones, 2-262-F4 (H, M), 411-F2 (I), 98-F6 (J), 2-112-F2 (K), and 34-F2 (L). Molecular weight standards include 14 C-labeled phosphorylase B (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome (12,000).

clones, no significant differences were apparent when tryptic peptides were prepared after labeling with [35 S]methionine. The results of such an analysis of wt AbLV P120 and one representative td AbLV mutant clone are shown in Fig. 6. Thus the major differences between polypro-

FIG. 4. Tryptic analysis of [32 P]orthophosphate-labeled 120,000 (A, C)- and 80,000 (B, D)-*M*, polypeptides expressed in wt AbLV nonproductively transformed mink 64 Ab2 (A, B) and rat Cl 4-4 (C, D) cells. Polypeptides were purified by immunoprecipitation and SDS-PAGE as described in the legend to Fig. 2. Tryptic digests (6,000 to 10,000 cpm) were prepared, suspended in electrophoresis buffer, spotted on cellulose-coated TLC glass plates, and analyzed as described in the legend to Fig. 1. Individual peptides are designated "a" through "g."

teins expressed in wt and td AbLV nonproductively infected clones appear to primarily involve altered patterns of post-translational phosphorylation rather than differences in primary structure.

td AbLV mutant nonproductively-infected clones lack AbLV-associated protein kinase activity. The demonstration of AbLV P120 expression in FRE Fisher rat and CCL64 mink cells nonproductively infected with td variants of AbLV suggested that if AbLV P120 is involved in transformation, the polypeptide as it exists in the mutant clones must be functionally defective. One possibility would be a reduction or loss of AbLV P120-associated protein kinase activity. To explore this possibility, polypeptides expressed in representative wt and td AbLV nonproductively infected cultures were partially purified by immunoprecipitation and analyzed for protein kinase activity as described in Materials and Methods. Labeled substrates were identified by SDS-PAGE. Both AbLV P120 and P80 immunoprecipitated from wt AbLV-trans-

formed clones were efficiently labeled by this means (Fig. 7). In contrast, only minimal labeling of AbLV P120 and no detectable labeling of P80 were observed upon similar analysis of the five td AbLV mutant clones.

AbLV-associated protein kinase preferentially recognizes a tyrosine acceptor site contained within a tryptic peptide common to AbLV P120 and P80. In an effort to determine whether the same acceptor sites are recognized by the AbLV P120-associated protein kinase as are phosphorylated under *in vivo* labeling conditions, tryptic peptide maps of *in vitro* [γ - 32 P]ATP-labeled AbLV P120 and P80 were examined. The results indicate preferential recognition by the AbLV P120-associated protein kinase of a single major well-resolved phosphopeptide common to P120 and P80 (Fig. 8). Although low-level labeling of several additional peptides was frequently observed, this was somewhat variable. Tryptic peptide analysis of a weakly *in vitro* [γ - 32 P]ATP-labeled 120,000-*M*, polypeptide in immunoprecipitates of a repre-

FIG. 5. Tryptic peptide analysis of [32 P]orthophosphate-labeled 120,000-M, polypeptides expressed in *td* mutant AbLV nonproductively infected FRE rat clones 262-F4 (A), 411-F2 (B), and 98-F6 (C), and FRE rat and 3-1-1 (D), 9-104-7 (E), and 9-124-2 (F) CCL 64 mink clones. Polypeptides were purified and subjected to tryptic peptide analysis as described in Fig. 3. Individual 32 P-labeled tryptic peptides are designated "a" through "g" on the basis of their map positions relative to wt AbLV P120 phosphopeptides shown in Fig. 4.

sentative AbLV *td* mutant, 2-109-F5, revealed no detectable labeling of the major wt AbLV *in vitro* 32 P-labeled peptide (data not shown). In contrast, background levels of weakly labeled peptides were comparable to those observed in wild-type AbLV P120 immunoprecipitates. Thus, phosphorylation of peptides other than the major peptide shared by P120 and P80 may be nonspecific due to contaminating cellular protein kinases.

To further characterize the major well-resolved AbLV P120 phosphopeptide recognized

by the polypeptide-associated protein kinase, phosphoamino acid analysis was performed. The results of two-dimensional analysis of unlabeled amino acid standards, including phosphoarginine, phosphoserine, and phosphothreonine obtained commercially, and of phosphotyrosine synthesized and purified as described in Materials and Methods, are shown in Fig. 9. Under the separation conditions used, the four standards were well resolved when mixed in equimolar ratios and could be individually identified. In a mixing experiment between a hydrolysate of in

FIG. 6. Comparison of 120,000-M_r polypeptides expressed in representative wt AbLV, Cl 4-4 (A) and td mutant AbLV, 411-F2 (B) nonproductively infected FRE rat cells. After purification by immunoprecipitation and SDS-PAGE, proteins were analyzed by two-dimensional tryptic peptide mapping as described in Fig. 2, with the exception that analysis was performed on 20 by 20-cm TLC plates and the time of electrophoresis and chromatography was increased from 0.5 to 1.0 h and 1.0 to 3.0 h, respectively. Peptides in parentheses (1, 2, 9, and 12) correspond to those present in P120 but not P80; the single peptide in brackets [13] is specific to the Moloney-MuLV p12-derived component of AbLV P120.

FIG. 7. Analysis of protein kinase activities associated with polypeptides expressed in rat and mink cells nonproductively infected with either wt or td AbLV. Cells were disrupted, immunoprecipitated by either anti-Rauscher-MuLV p12 (A, C-H, J, K) or normal goat serum (B, I), assayed for [γ -³²P]ATP phosphotransferase activity, and analyzed by SDS-PAGE as previously described (26). Cell lines tested included wt AbLV-transformed FRE rat cells, Cl 4-4 (A, B); five td mutant AbLV FRE nonproductively infected clones, 2-262-F4 (C), 411-F2 (D), 98-F6 (E), 2-112-F2 (F), and 34-F2 (G); wt AbLV-transformed CCL64 mink cells, 64 Ab2 (H, I); and two td mutant AbLV CCL64 nonproductively infected clones, 9-124-2 (J) and 9-104-7 (K). Molecular weight standards are described as in the legend to Fig. 1.

vitro ³²P-labeled AbLV P120 and unlabeled phosphoamino acid standards, essentially all of the radioactivity was seen to comigrate with

phosphotyrosine.

Comparison of tumorigenic potential of wt and td AbLV nonproductively infected

rat cells. The availability of Fisher rat cells nonproductively infected by AbLV mutants defective in their associated protein kinase activities provided a syngeneic model system to test the involvement of the AbLV P120-associated protein kinase in AbLV tumor induction. Each of the two AbLV-transformed FRE clones examined was found to be highly tumorigenic (Table 2). In vivo inoculation of as few as 10^5 transformed cells resulted in tumor development in 100% of the animals within 2 to 3 weeks. In contrast, even when assayed at cell doses as high as 10^7 cells per animal, none of the td AbLV mutant clones induced tumors during the 3-month observation period. Thus, the defect in polyprotein-associated protein kinase activity

exhibited by these mutants is correlated not only with increased contact inhibition and loss of ability to grow in soft agar, but also with a marked reduction of in vivo tumor-inducing potential.

DISCUSSION

In the present study, we describe the isolation and characterization of several td mutants of

TABLE 2. Tumorigenicity of wt and td mutant AbLV nonproductively infected fisher rat cells

Cell clone	Fraction of animals developing tumors after inoculation of the following cell number ^a		
	10^5	10^6	10^7
wt			
4-4	20/20	10/10	10/10
2-9-T2	39/39	10/10	10/10
Mutant			
34-F2	NT	0/10	0/10
2-262-F4	NT	0/10	0/10
411-F2	NT	0/10	0/10
98-F6	NT	0/10	0/10
2-112-F2	NT	0/10	0/10

^a Six- to eight-week-old Fisher rats were inoculated subcutaneously with the indicated numbers of cells and observed at weekly intervals. Animals scored as positive developed massive tumors within 2 to 3 weeks and died by week 6, whereas the remaining animals failed to exhibit any obvious evidence of tumor development over an observation period of 3 months. NT, Not tested.

FIG. 8. Identification by tryptic peptide analysis of sites of phosphorylation within AbLV P120 (A) and P80 (B), labeled in vitro by AbLV-associated protein kinase. Proteins were labeled in the presence of [γ - 32 P]ATP as described in Fig. 7, purified by SDS-PAGE, immunoprecipitated, and subjected to tryptic peptide analysis as described in Fig. 2.

FIG. 9. Identification of AbLV P120 amino acid acceptor residues phosphorylated in vitro by AbLV-associated protein kinase activity. The major [γ - 32 P]ATP in vitro labeled AbLV P120 tryptic peptide was purified as described in Fig. 8, hydrolyzed, and subjected to two-dimensional analysis according to procedures outlined in the text. Phosphoamino acid standards including phosphoarginine [arg(P)], phosphotyrosine [tyr(P)], phosphothreonine [thr(P)], and phosphoserine [ser(P)] were identified by ninhydrin staining. For identification of AbLV P120 phosphoamino acid residues labeled in vitro, equimolar concentrations of all four standards were mixed with a 32 P-labeled hydrolysate of AbLV P120 and analyzed by either ninhydrin staining (A) or autoradiography (B). The dotted-line circles in panel B represent the positions of phosphoamino acid standards shown in panel A.

AbLV and provide evidence arguing for the involvement of AbLV P120 and/or P80 in AbLV-associated transformation. Mink or rat cells nonproductively infected with these newly isolated mutants express AbLV P120 and P80 levels comparable to those characteristic of wt AbLV transformants. Such clones exhibit a degree of contact inhibition similar to that of uninfected control cells, lack ability to grow in soft agar, and, in the case of nonproductively infected Fisher rat cells, fail to produce tumors after *in vivo* inoculation in syngeneic hosts. Cells nonproductively infected with such mutants are as susceptible to retransformation by wt AbLV as are control cells but lack rescuable AbLV competent for malignant transformation. The rate of reversion to wt transformation is negligible, arguing for a defect in the viral genome involving more than a simple point mutation.

The isolation of td mutants of AbLV provides a means of beginning to define the potential roles of AbLV P120 and P80 in AbLV-mediated transformation. Each of 10 wt AbLV-transformed clones is shown to express comparable levels of AbLV P120 and P80. Such expression is invariably lost subsequent to reversion of representative AbLV-transformed mink clones to the nontransformed phenotype. The demonstration of 120,000-dalton polypeptides in each of the mutant clones and the high degree of relatedness of such proteins to AbLV P120 as indicated by immunoprecipitation and [³⁵S]methionine tryptic peptide analysis establishes that AbLV genomic RNA is both present and expressed in these cell lines. These data do not exclude the possibility of minor in-phase deletions present within the individual P120s not involving methionine-containing tryptic peptides. Of six mutant clones examined with respect to [³²P]orthophosphate tryptic peptide composition, each was strikingly different from wt AbLV P120. As one possibility, similar conformational changes in polypeptides in the td mutant-infected clones may have resulted in altered patterns of post-translational phosphorylation. Although it will be necessary to examine additional mutant clones to resolve whether a particular site of phosphorylation is critical for the transforming function apparently associated with AbLV 120, it is of interest that one particular phosphopeptide (designated "f") was consistently missing from polypeptides expressed in the td mutant clones.

Of interest, despite the demonstration of AbLV P120 and P80 expression in each of the mutant clones examined, protein kinase activity associated with AbLV in td AbLV transformants is greatly diminished. AbLV-associated protein kinase is also shown to be lost in morphological

revertants of wt AbLV-transformed mink cells, although this reflects complete loss of polyprotein expression. As described previously, in immunocomplex form, AbLV P120 and P80 represent the major substrates for the AbLV-associated protein kinase (26, 27). Thus, an impairment to *in vitro* [γ -³²P]ATP-mediated phosphorylation of AbLV P120 and P80 in AbLV td mutant clones could reflect a defect in the capacity of the virus-coded polypeptide to act as substrate. Alternatively, if the observed kinase activity is in fact intrinsic to AbLV P120, the defect could be in the enzymatic activity itself.

The demonstration of an 80,000-*M_r* protein in AbLV-transformed cells raises the question of the relatedness of this protein to AbLV P120. Although each of the methionine-containing tryptic peptides present in P80 is also represented in P120, no tryptic peptides unique to P80 were observed. Thus, P80 appears to lack amino acid sequences other than those shared with P120 and probably represents a subcomponent of P120. Whether P80 is generated by post-translational cleavage of AbLV P120, premature termination of translation, or some more complex mechanism, possibly involving a secondary "spliced" mRNA species, is, however, as yet unresolved. The functional significance of AbLV P80 also remains to be established. In this respect it is of interest that cells transformed by either the Gardner or Snyder-Theilen strain of feline sarcoma virus also express, in addition to their major 115,000-*M_r* polypeptides, highly related proteins of around 70,000 to 80,000 *M_r*. (24).

The protein kinase activity associated with AbLV is shown to exhibit apparent specificity for a tyrosine residue represented in both AbLV P120 and P80. After completion of the present study, Witte et al. (27) also reported that the AbLV-associated protein kinase recognized tyrosine as its major acceptor *in vitro*. Although [γ -³²P]ATP labeling mediated by the AbLV-associated protein kinase is shown to primarily involve a single well-resolved tryptic peptide, the possibility that more than one tyrosine acceptors are present within this peptide cannot be excluded. It should, however, be emphasized that the pattern of *in vitro* phosphorylation of the tyrosine acceptor site(s) may not necessarily reflect the *in vivo* situation. Also, although the method of amino acid analysis used argues against serine and threonine as phosphate acceptors, phosphorylation by the AbLV P120-associated protein kinase of rare, acid-labile amino acid acceptors such as histidine and lysine cannot be excluded. It also remains to be resolved whether the major [γ -³²P]ATP-labeled peptide is contained within the structural (p15, p12, and a minor protein of p30) or nonstructural com-

ponents of P120 and P80. In its unusual property of recognizing tyrosine as an acceptor, the AbLV P120-associated protein kinase resembles enzymatic activities associated with polyoma middle T antigen (5), avian sarcoma virus pp60^{src} (4, 6), and a 115,000-M_r polypeptide encoded by the Gardner strain of feline sarcoma virus (13).

The isolation of AbLV mutants defective in transformation and the demonstration that the 120,000- and 80,000-M_r polypeptides expressed in cells nonproductively infected by such mutants lack detectable protein kinase activity argues that P120 and P80 are involved in AbLV transformation. Although the present findings demonstrate that AbLV-associated protein kinase has specificity for tyrosine, the phosphorylation site involved in AbLV transformation is not identified. As one possibility, the tyrosine acceptor site for this enzyme, shared by P120 and P80, may be critical to the transforming process. Alternatively, the existence of an acceptor site shared by AbLV P120 and P80 could be fortuitous and the tyrosine acceptor site involved in transformation may be on an as yet unidentified cellular substrate. The availability of AbLV mutants exhibiting simultaneous defects in *in vitro* protein kinase labeling of AbLV-encoded polypeptides and in transformation should be of value in resolving these questions.

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