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Localization of the Abelson Murine Leukemia Virus Protein in a Detergent-Insoluble Subcellular Matrix: Architecture of the Protein

MICHAEL A. BOSS, GIDEON DREYFUSS,† AND DAVID BALTIMORE*

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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We examined the interaction of Abelson murine leukemia virus protein P120 with other cellular components after extraction with the nonionic detergent Triton X-100. Most of the Abelson murine leukemia virus P120-associated kinase activity was found in the detergent-insoluble matrix in both lymphoid and fibroblast cell lines. The P120 labeled during a short exposure of cells to [^{35}S]-methionine was mainly in the detergent-insoluble matrix (lymphoid cells) or equally distributed in the detergent-insoluble matrix and the soluble fraction (fibroblasts). Steady-state-labeled P120 was distributed equally in the two fractions (lymphoid cells) or mostly in the soluble portion (fibroblasts). Thus, there was an apparent movement of P120 from the detergent-insoluble matrix to the detergent-soluble fraction and a concomitant loss of enzymatic activity. When the detergent-insoluble matrix was incubated with [^{32}P]ATP in situ, phosphorylation of tyrosine residues of P120 was observed. We found an 80,000-molecular-weight fragment of P120 (designated F80) after extraction of fibroblast cells with detergent. F80 was not found in extracted lymphoid cells, but mixing labeled lymphoid cells and unlabeled fibroblasts before extraction produced the fragment. F80 contained the *gag* determinants of P120 but did not react with Abelson-specific serum. These data allowed us to assign various features of the protein to regions of the P120 molecule and to localize the Abelson-specific antigenic determinants to the C-terminal region of the molecule.

Abelson murine leukemia virus (A-MuLV) is a replication-defective, rapidly transforming retrovirus. It can induce leukemia in vivo (1) and transform bone marrow cells and some established mouse cell lines in vitro (16, 18, 23), and a number of A-MuLV strains have been identified (7a, 17). The sole A-MuLV-encoded product is a protein consisting of the amino-terminal 30,000 daltons of the Moloney murine leukemia virus (M-MuLV) *gag* gene precursor attached to the carboxy-terminal region of a polypeptide encoded by a mouse gene (28; S. P. Goff, R. Lee, and D. Baltimore, unpublished data). This protein is responsible for transformation of cells by A-MuLV (28; S. P. Goff and D. Baltimore, unpublished data). A-MuLV proteins have molecular weights ranging from 160,000 to 90,000; the prototype A-MuLV strain encodes a 120,000-molecular-weight protein designated P120. P120 is a phosphorylated surface membrane protein which becomes labeled with ^{32}P on tyrosine residues when it is incubated with [^{32}P]ATP after immunoprecipitation (25). This behavior is similar to the protein kinase activities associated with proteins encoded by certain other transforming viruses, notably pp60^{src} of Rous sarcoma virus (6, 8) and the proteins of Fujinami sarcoma virus (7), Y73 (9), PRC II (13), and feline sarcoma virus (24).

Detergent extraction of intact cells yields insoluble residues which retain the major morphological features of the cells from which they were derived (2). This extraction procedure operationally defines the following two fractions: a soluble fraction (designated SOL) and a detergent-insoluble subcellular matrix (designated DIM), commonly referred to as the cytoskeleton (2, 4, 22). In this work we studied the interaction of P120 with other cellular components after extraction with the nonionic detergent Triton X-100. Although numerous cell types have been extracted with Triton X-100 (2, 3, 12, 22), the insoluble matrix has been best characterized from human erythrocytes, where it has been demonstrated that only the cytoskeleton and linked components remain after extraction (19, 20, 29).

† Present address: Department of Biochemistry and Molecular and Cellular Biology, Northwestern University, Evanston, IL 60201.

Recently, it has been shown that $pp60^{src}$ is associated with the DIM fraction of Rous sarcoma virus-infected chicken embryo fibroblasts after Triton X-100 extraction (3). In this paper we show that most of the A-MuLV P120-associated kinase activity is found in the DIM fraction in both lymphoid and fibroblast cell lines. However, studies of the distribution of [^{35}S]methionine and in vivo $^{32}PO_4$-labeled protein revealed a more complex pattern. When the DIM fraction was incubated with [^{32}P]ATP in situ, phosphorylation of the tyrosine residues of P120 occurred. This fractionation procedure has helped to localize the functional regions of the P120 molecule and proteins derived from it.

MATERIALS AND METHODS

Cell lines. The A-MuLV-transformed fibroblast cell lines which we used were as follows: ANN-1, an A-MuLV(P120)-transformed NIH/3T3 nonproducer cell line (18); A2, an A-MuLV(P120)-transformed NIH/3T3 nonproducer cell line that makes both a P120 protein and a P90 protein; P160N54, an A-MuLV(P160)-transformed NIH/3T3 nonproducer cell line; P90B-3, an A-MuLV(P90)-transformed NIH/3T3 cell line; and P92td, an NIH/3T3 cell line nonproductively infected with the transformation-defective A-MuLV strain A-MuLV(P92td) (17, 26). The A-MuLV-transformed lymphoid cell lines used were as follows: 2M3, a P120 nonproducer cell line; an M-MuLV-superinfected derivative of 2M3, 2M3/M (28); 18-81, a P120 producer cell line; and 18-48, a P100 producer cell line (21). We also used 70Z, a BALB/c-derived, chemically transformed, pre-B-lymphoid cell line (14). Lymphoid cells were grown in RPMI 1640 medium containing 10% calf serum and 5×10^{-5} M β-mercaptoethanol. Other lines were grown in Dulbecco modified Eagle medium containing 10% calf serum.

Immune sera. Goat anti-M-MuLV serum and anti-Moloney *gag*-specific sera were provided by the Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md. Anti-AbT serum containing antibodies that immunoprecipitated A-MuLV proteins by virtue of their non-*gag* determinants has been described previously (27). Rabbit anti-vimentin was a gift from Richard Hynes, Massachusetts Institute of Technology.

Cell labeling. Radioactive chemicals were purchased from New England Nuclear Corp., Boston, Mass. Cells were washed with phosphate-buffered saline and incubated in labeling medium.

When labeled with [^{35}S]methionine, cells were incubated in Dulbecco modified Eagle medium lacking unlabeled methionine but supplemented with 2% dialyzed fetal calf serum and 125 μCi of [^{35}S]methionine per ml for 1 to 2 h. For overnight labeling under nonlimiting conditions, [^{35}S]methionine was added to whole Dulbecco modified Eagle medium. When labeled with $^{32}PO_4$, cells were incubated in Dulbecco modified Eagle medium lacking unlabeled PO_4 but supplemented with 2% fetal calf serum and 375 to 1,000 μCi of $^{32}PO_4$ per ml for 2 to 6 h.

Triton X-100 extraction. Usually, Triton X-100 extraction was carried out on monolayer cells in 3.5-cm dishes, although occasionally extraction was performed on cells in suspension. Adherent cells were allowed to attach overnight. Nonadherent cells were allowed to settle for 1 to 2 h onto dishes coated with poly-L-lysine (type V; Sigma Chemical Co., St. Louis, Mo.) to induce attachment. Dishes were coated by incubating them with 1 ml of 0.1% poly-L-lysine for 45 min at room temperature, followed by one distilled water rinse and the addition of the cell suspension in Dulbecco modified Eagle medium containing 2% calf serum. For extractions, dishes were washed twice with phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$ at 4°C. The cells were then incubated at 4°C for 2 min with 400 μl of extraction buffer (10 mM PIPES [1,4-piperazinediethanesulfonic acid], pH 6.8, 100 mM KCl, 300 mM sucrose, 2.5 mM $MgCl_2$) containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 1% aprotinin (Sigma), and 0.5% Triton X-100. The SOL fraction was removed, and 400 μl of extraction buffer was added to the DIM fraction. The two fractions were solubilized either in gel sample buffer for direct application to a polyacrylamide gel or in lysis buffer (10 mM $NaHPO_4$-Na_2HPO_4, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride) for clarification by centrifugation at 150,000 $\times$ *g* for 2 h and subsequent immunoprecipitation. Fractions were immunoprecipitated with 5 to 10 μl of normal or immune serum. The immune complexes were isolated by binding to *Staphylococcus aureus*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by using the discontinuous stacking system of Laemmli (10). To prepare nuclei, 10 mM Tris (pH 7.4), 10 mM NaCl, 25 mM $MgCl_2$, and 0.5% Triton X-100 were added to the DIM fractions. The DIM fractions were scraped off the dishes and homogenized with 0.5% sodium deoxycholate and 1% Tween 40. The nuclei were isolated by low-speed centrifugation.

In vitro and in situ kinase assays. We measured the kinase activities of samples after immunoprecipitation by incubating the isolated immune complexes at 30°C for 9 min in 20 mM Tris (pH 8.0)-10 mM $MnCl_2$ containing 1 μCi of [^{32}P]ATP.

In situ kinase assays of DIM fractions were performed by incubating the preparations with 200 μl of extraction buffer containing 50 μCi of [^{32}P]ATP (specific activity, 7,000 Ci/mmol) for 2 min on ice. The DIM fractions were then treated as described above.

High-voltage electrophoresis of phosphorylated amino acids. After immunoprecipitation, phosphorylated proteins were precipitated with trichloroacetic acid and washed in absolute alcohol. About 5,000 cpm of phosphate-labeled protein was hydrolyzed for 2 h in 6 N HCl at 100°C. Hydrolyzed samples were suspended in 5 μl of a solution containing unlabeled phosphoserine, phosphothreonine, and phosphotyrosine (concentration, 1 μg/ml each). Samples were analyzed by high-voltage electrophoresis in pyridine acetic acid (pH 3.5) on Whatman 3MM paper at 2,400 V for 1 h (25).

RESULTS

To determine whether the A-MuLV-encoded protein and its associated kinase activity were associated with the DIM fraction or the SOL

fraction, we have studied the following two A-MuLV-transformed cell lines: 2M3, a lymphoid, nonproducer cell line (28), and ANN-1, a fibroblast nonproducer cell line (18). The cells were extracted with a buffer containing Triton X-100, which was developed previously to optimize preservation of cytoskeletal elements and retention of polyribosomes (4). Because A-MuLV-transformed fibroblasts and lymphoid cells do not adhere tightly to plastic, the cells were plated onto dishes coated with poly-L-lysine, which allowed the cells to form monolayers.

Cell fractionation. To examine the efficacy of the extraction, ANN-1 and 2M3 cells were labeled overnight with [^{35}S]methionine and treated with extraction buffer to produce the DIM and SOL fractions. About 60% of the alkali-resistant (0.33 N NaOH, 37°C, 15 min), trichloroacetic acid-precipitable, [^{35}S]methionine-labeled protein was recovered in the SOL fraction. The radioactivity remaining with the DIM fraction was distributed 25% in the cytoskeletal matrix and 15% in the nuclear fraction. Electrophoretic fractionation revealed a complex spectrum of proteins in both the DIM fraction and the SOL fraction (Fig. 1, lanes 1 and 2); some bands were unique to each fraction (Fig. 1, arrows), and the fractions had numerous bands in common.

To determine more exactly the purity of the fractions, labeled cells were extracted, and vimentin distribution was assessed by immunoprecipitation. Vimentin is an intermediate filament protein (11) that should be mainly in the DIM fraction. In fibroblasts the vimentin was recovered almost entirely in the DIM fraction (Fig. 1, lanes 9 and 10), and in lymphoid cells a significant amount of vimentin was recovered in the SOL fraction (Fig. 1, lanes 5 and 6). The specificity of immunoprecipitation was shown by the absence or low level of vimentin in fractions treated with normal rabbit serum (Fig. 1, lanes 3, 4, 7, and 8). We are not aware that vimentin has been identified previously in lymphoid cells (11), and thus it is not clear whether the extraction method which we used released some cytoskeletal elements from lymphoid cells or whether there was a normal pool of soluble vimentin in such cells. Light microscopy observations of the extracted monolayers showed that the overall morphology of the cells was maintained (2, 4; data not shown). We concluded from these experiments that the fractionation of transformed fibroblasts held to dishes with poly-L-lysine was effective in distinguishing the DIM and SOL fractions; the definition of the lymphoid cell fractions was less certain, with a possibility of loss of some cytoskeletal elements from the DIM fraction.

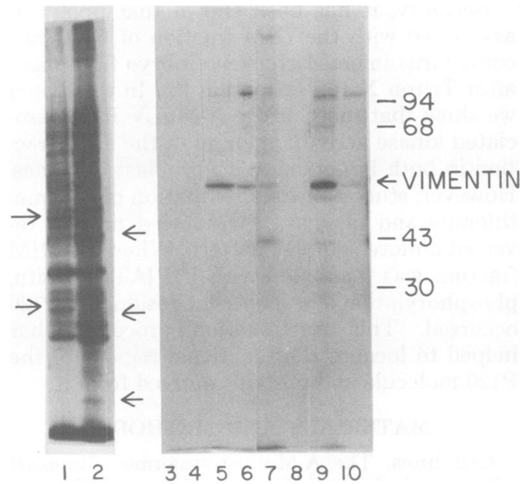

FIG. 1. *Specificity of the fractionation procedure. ANN-1, 2M3, and P90B-3 cells were labeled with [^{35}S]methionine overnight in complete medium; this was followed by fractionation into DIM and SOL fractions after attachment to dishes coated with poly-L-lysine. Samples containing equal cell number equivalents were analyzed with or without specific immunoprecipitation by electrophoretic separation and autoradiography. Samples from ANN-1 cells were analyzed without immunoprecipitation from the DIM fraction (lane 1) and the SOL fraction (lane 2). Some bands unique to each fraction are indicated by arrows. Lanes 3 and 7, DIM fractions from lines 2M3 and P90B-3, respectively, immunoprecipitated with normal rabbit serum; lanes 4 and 8, SOL fractions from lines 2M3 and P90B-3, respectively, immunoprecipitated with normal rabbit serum; lanes 5 and 9, DIM fractions from lines 2M3 and P90B-3, respectively, immunoprecipitated with rabbit anti-vimentin serum; lanes 6 and 10, SOL fractions from lines 2M3 and P90B-3, respectively, immunoprecipitated with rabbit anti-vimentin serum. The positions of unlabeled marker proteins of known molecular weights (× 10^3) are indicated on the right.*

P120-associated kinase activity in the DIM fraction. To examine the subcellular localization of the A-MuLV P120-associated protein kinase activity, both SOL fractions and DIM fractions were assayed for kinase activity after immunoprecipitation. With ANN-1 cells, the DIM fraction contained two prominent protein bands that became phosphorylated when an anti-M-MuLV serum precipitate was incubated with [γ-^{32}P]ATP (Fig. 2, lane 2). One band had a molecular weight of 120,000 and thus was the P120 which becomes labeled by $^{32}PO_4$ on a tyrosine residue under these conditions (25). The other band had a molecular weight of 75,000 to 80,000; this band was designated F80 (fragment of about 80,000 molecular weight) and is discussed further below. The only other significant

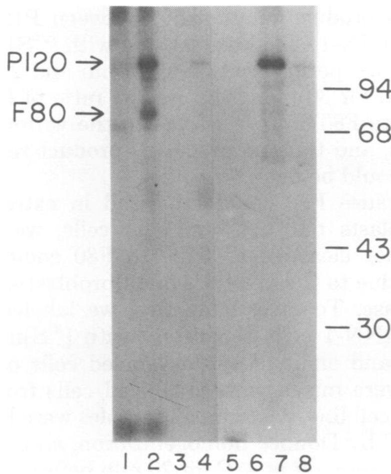

FIG. 2. *In vitro protein kinase assay after fractionation. ANN-1 and 2M3 cells were fractionated into DIM and SOL fractions after attachment to dishes coated with poly-L-lysine. Each fraction was solubilized in lysis buffer and clarified by ultracentrifugation. After immunoprecipitation and isolation of the immune complexes with S. aureus, samples were incubated in 20 mM Tris (pH 8.0)–10 mM $MnCl_2$ containing 1 μCi of [γ-^{32}P]ATP for 9 min at 30°C. The products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1 and 5, DIM fractions from ANN-1 and 2M3 cells, respectively, immunoprecipitated with normal goat serum; lanes 3 and 6, SOL fractions from ANN-1 and 2M3 cells, respectively, immunoprecipitated with normal goat serum; lanes 2 and 7, DIM fractions from ANN-1 and 2M3 cells, respectively, immunoprecipitated with goat anti-M-MuLV serum; lanes 4 and 8, SOL fractions from ANN-1 and 2M3 cells, respectively, immunoprecipitated with goat anti-M-MuLV serum. The positions of molecular weight markers (× 10^3) are indicated on the right.*

band (a band having a low molecular weight) was also evident when normal serum was used (Fig. 2, lane 1). The SOL fraction showed a weak band of labeled P120 after incubation with [γ-^{32}P]ATP, along with a small amount of F80 (Fig. 2, lane 4). Both the SOL fraction and the DIM fraction had a number of other weaker specific bands that have yet to be characterized. Precipitation of 2M3 cell extracts followed by labeling with [γ-^{32}P]ATP produced a dark P120 band which was recovered exclusively in the DIM fraction (Fig. 2, lanes 5 through 8).

The labeling of P120 and F80 in extracts of A-MuLV-transformed cells was specific to such cells. For instance, when 70Z cells were extracted, no phosphorylatable P120 or F80 was detected (data not shown); 70Z cells are very similar in their differentiated state to 2M3 cells, but they are not transformed by A-MuLV (14). Also, fibroblasts transformed by other agents or untransformed fibroblasts produced no labeled P120 and F80 bands. The lack of kinase activity in the SOL fraction was not due to inhibitors; when partially purified P120 was added to each fraction, it was as effectively phosphorylated after immunoprecipitation from the SOL fraction as after immunoprecipitation from the DIM fraction (data not shown). As an additional control, both ANN-1 and 2M3 cells were extracted in suspension rather than bound to dishes with poly-L-lysine and the same localization to the DIM fraction was observed.

The distribution of P120 in ANN-1 cells, as determined by [^{35}S]methionine labeling, did not follow the distribution of enzymatic activity. After a 1-h pulse of [^{35}S]methionine or $^{32}PO_4$, the distribution of P120 was about equal in the SOL and DIM fractions (data not shown). After overnight steady-state labeling with [^{35}S]methionine, most of the P120 was found in the SOL fraction, whereas a majority of the F80 protein was found in the DIM fraction (Fig. 3, lanes 1 through 4). In 2M3 cells, after 2 h of labeling, the [^{35}S]methionine-labeled P120 profile was very similar to the enzymatic distribution; most

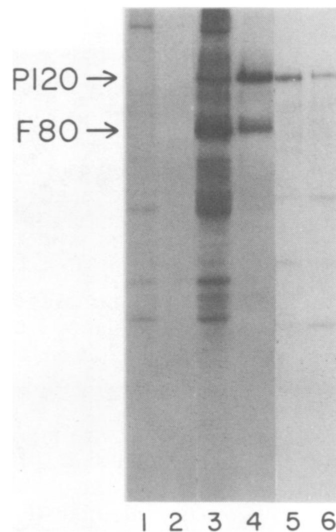

FIG. 3. *Distribution of [^{35}S]methionine-labeled P120. ANN-1 and 2M3 cells were labeled with [^{35}S]-methionine overnight in complete medium; this was followed by fractionation into DIM and SOL fractions, and preparations were immunoprecipitated as described in the legend to Fig. 1. Lane 1, DIM fraction from ANN-1 cells immunoprecipitated with normal goat serum; lane 2, SOL fraction from ANN-1 cells immunoprecipitated with normal goat serum; lanes 3 and 5, DIM fractions from ANN-1 and 2M3 cells, respectively, immunoprecipitated with goat anti-M-MuLV serum; lanes 4 and 6, SOL fractions from ANN-1 and 2M3 cells, respectively, immunoprecipitated with goat anti-M-MuLV serum.*

of the P120 was found in the DIM fraction (data not shown). After overnight labeling of 2M3 cells with [^{35}S]methionine, a significant proportion of the P120 was found in the SOL fraction (Fig. 3, lanes 5 and 6).

Origin of F80. The labeled F80 in ANN-1 extracts might have been either a protease digestion product of P120 or a kinase substrate. To distinguish between these two possibilities, we performed partial protease digestion on the two proteins (5), and the electrophoretic similarity of these digests suggested that P120 and F80 were related (data not shown). Immunoprecipitation with sera specific for individual *gag* proteins showed that when P120 was immunoprecipitated, so was F80 (Fig. 4, lanes 1 through 5). This was not a consequence of a P120-F80 complex because serum specific for non-*gag* determinants on P120 (anti-AbT serum [27]) immunoprecipitated P120 but not F80 (Fig. 4, lane 7), whereas normal mouse serum precipitated neither protein (Fig. 4, lane 6). The immunoprecipitation data implied that F80 contains the *gag* determinants of P120 but lacks the determinants recognized by at least anti-AbT serum.

To investigate whether there might be a precursor-product relationship between P120 and F80, ANN-1 cells were labeled with [^{35}S]methionine for periods between 10 min and 2 h and chased for 30 min to 18 h. The ratio of labeled P120 to F80 did not vary with the various protocols, and thus no precursor-product relationship could be detected.

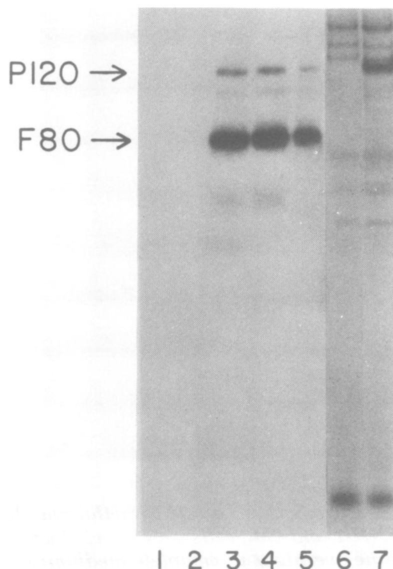

FIG. 4. *Antigenic relationship between P120 and F80. ANN-1 cells were pulsed for 1 h with [^{35}S]methionine, and the unfractionated cells were extracted and immunoprecipitated as described in the legend to Fig. 1 with the following sera: normal goat serum (lane 1), anti-p10 serum (lane 2), anti-p30 serum (lane 3), anti-p12 serum (lane 4), anti-reverse transcriptase/p15 serum (lane 5), normal mouse serum (lane 6), and anti-AbT serum (lane 7).*

Because F80 could be found in extracts of fibroblasts but not lymphoid cells, we asked whether cleavage of P120 to F80 could have been due to the presence of a fibroblast-specific protease. To investigate this, we labeled 2M3 and ANN-1 cells separately with [^{35}S]methionine, and one-half of the labeled cells of each line were mixed with unlabeled cells from the other cell line. All four cell samples were broken at 4°C by Dounce homogenization, and the extracts were left at 4°C for 20 min before solubilization and immunoprecipitation. 2M3 cells extracted alone showed the presence of only P120 (Fig. 5, lane 2), whereas 2M3 cells extracted in the presence of unlabeled ANN-1 cells showed the presence of an F80 band (Fig. 5, lane 3); the mixture had a pattern very similar to the pattern of labeled ANN-1 cells (Fig. 5, lane 6). Thus, fibroblasts but not lymphoid cells appeared to contain a protease which cleaved P120 at a specific site to generate F80.

In situ kinase assay. Because kinase activity was demonstrable readily in immunoprecipitates from the solubilized DIM fraction, we investigated the kinase activity of the DIM fraction itself. Cells were extracted, and the DIM fraction was incubated in situ with [γ-^{32}P]ATP. The in situ kinase assay was performed originally by incubation in 20 mM Tris (pH 8.0) containing 10 mM $MnCl_2$ because these conditions were optimal for phosphorylation of P120 in the in vitro kinase assay (25). In the in situ system, however, we found that equivalent results were obtained if the DIM fraction was incubated directly in extraction buffer. We continued to use these latter conditions for optimal preservation of the DIM fraction structure. After the reaction, the DIM fraction was solubilized and immunoprecipitated with anti-M-MuLV serum. ANN-1 cells yielded a prominent F80 band, a weak P120 band, and an additional band having a molecular weight of about 100,000 (Fig. 6, lane 1). Another cell line that made a P120 protein (A2) also produced labeled P120 and F80 (Fig. 6, lane 4).

We examined cells transformed by A-MuLV strains that made different sizes of Abelson proteins in the same way. A cell line transformed by A-MuLV(P90) gave rise to a weakly labeled P90 band plus a strong band of F80 (Fig. 6, lane 2). A-MuLV(P160) gave rise to a labeled P160 band and three other bands, all of which were

larger than F80 (Fig. 6, lane 3). The 90,000-molecular-weight band was the most intense; it may be homologous to the F80 produced by the P120 strains.

In situ labeling of lymphoid cell DIM fractions from 2M3 and 2M3/M cells, both transformed by A-MuLV(P120), gave rise almost exclusively to radioactive P120 (Fig. 6, lanes 5 and 6). This again emphasized that the protease which cut F80 from P120 was not present in lymphoid cell extracts.

To determine what proportion of the $^{32}PO_4$ incorporated by the DIM fraction was found in A-MuLV proteins, we examined the fraction without immunoprecipitation (Fig. 6, lanes 7 through 15). Uninfected NIH/3T3 cells contained a number of labeled proteins, but none was very prominent (Fig. 6, lane 11). Infected fibroblast cells produced a number of common bands, which sometimes also appeared in uninfected cells and were of unknown significance. More importantly, the F80 fragment and the F90 fragment from A-MuLV(P160) were clearly evident, showing that these compounds were major products of the in situ kinase assay. In contrast, the in vivo-labeled A-MuLV phosphoprotein could not be distinguished without immunoprecipitation (unpublished data).

Extracts of lymphoid cells that were not immunoprecipitated produced much heavier bands of labeled A-MuLV proteins than fibroblasts, and they were also largely undegraded (Fig. 6, lanes 12 through 14). Cell line 2M3 and an equivalent cell line, 18-81, produced P120 as their most prominent in situ-labeled band (Fig. 6, lanes 12 and 13). Cell line 18-48 produced a prominent P100 band (lane 14); its A-MuLV had been shown previously to synthesize P100 (7a). As a control, chemically transformed 70Z cells were labeled in situ, and they were shown to lack any prominent labeled band (lane 15).

To identify the amino acid phosphorylated in the in situ reactions, the A-MuLV proteins labeled in situ were collected by immunoprecipitation with an anti-M-MuLV serum and subjected to partial acid hydrolysis. The combination of P120 and F80 contained exclusively phosphorylated tyrosine residues when it was labeled in the presence of Mn^{2+} (Fig. 7, lane 2) and at most a hint of phosphoserine when it was labeled in the presence of Mg^{2+} (Fig. 7, lane 1). In contrast, the total DIM fraction showed extensive labeling of serine residues (Fig. 7, lane 3).

These experiments showed that the in situ activity of the A-MuLV-associated tyrosine kinase activity is the most prominent activity in DIM fractions from transformed lymphoid cells and is also an easily detected activity in transformed fibroblast cells. This is in marked contrast to results obtained with cells labeled in culture with $^{32}PO_4$, where the A-MuLV proteins are not major bands and most of the PO_4 in the A-MuLV protein is on serine residues (25).

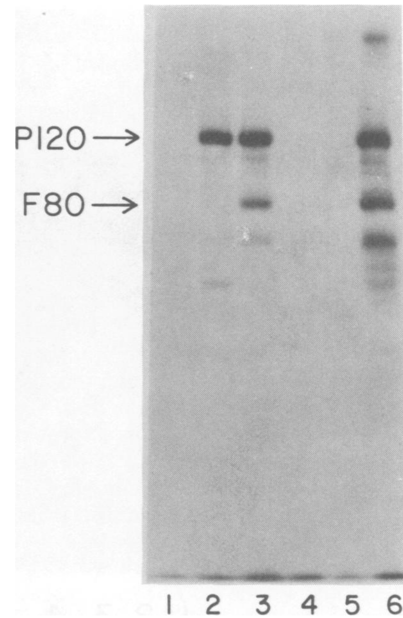

FIG. 5. *Effect of mixing 2M3 and ANN-1 cell extracts. ANN-1 and 2M3 cells were labeled separately with [^{35}S]methionine for 1.5 h. After labeling, the cells were collected by centrifugation and suspended in cold phosphate-buffered saline. Labeled and unlabeled cells were mixed as indicated below and were allowed to swell for 10 min at 4°C in 10 mM Tris (pH 7.4)-1 mM $MgCl_2$ before being homogenized. The cell extracts were left for 20 min at 4°C before solubilization in lysis buffer and immunoprecipitation. Lanes 1, 4, and 5, labeled 2M3 cell extract, labeled 2M3 cell extract plus unlabeled ANN-1 cell extract, and labeled ANN-1 cell extract, respectively, all immunoprecipitated with normal goat serum; lanes 2, 3, and 6, labeled 2M3 cell extract, labeled 2M3 cell extract plus unlabeled ANN-1 cell extract, and labeled ANN-1 cell extract, respectively, all immunoprecipitated with goat anti-M-MuLV serum.*

DISCUSSION

It has been shown previously that $pp60^{src}$ is associated with a DIM fraction in Rous sarcoma virus-transformed chicken embryo fibroblasts (3). In this paper we show that the A-MuLV P120-associated kinase activity is also located mainly in the DIM fraction of both transformed fibroblast and lymphoid cells. Unlike $^{32}PO_4$-labeled $pp60^{src}$, however, the P120 distribution, as measured by $^{32}PO_4$- or ^{35}S-labeled protein, was not the same as the distribution of enzyme ac-

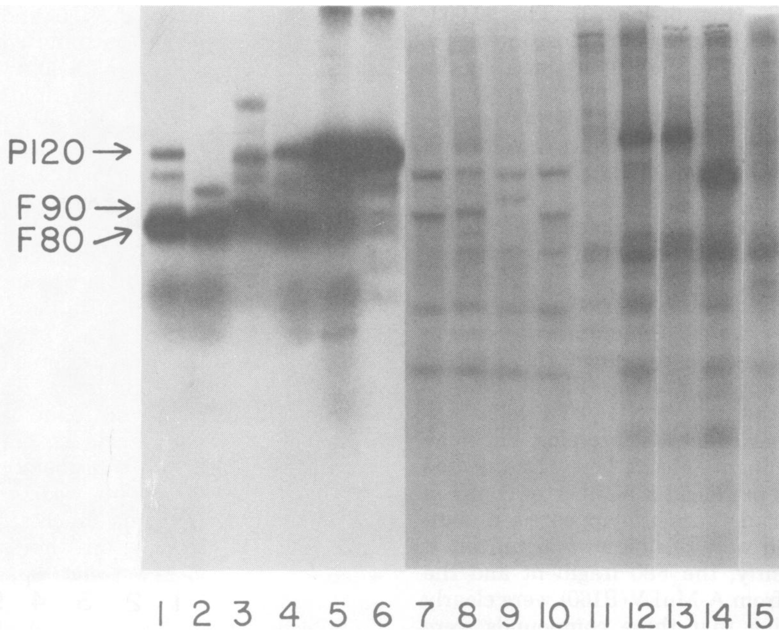

FIG. 6. *In situ protein kinase assay of different cell lines. Cells were allowed to attach to dishes coated with poly-L-lysine and were fractionated with extraction buffer into DIM and SOL fractions. The SOL fraction was removed, and the DIM fraction was incubated with 200 μl of extraction buffer containing 50 μCi of [γ-^{32}P]ATP for 2 min at 4°C. The DIM fraction was then solubilized and subjected to sodium lauryl sulfate polyacrylamide gel electrophoresis. Lanes 1 through 6 contained samples which were immunoprecipitated with goat anti-M-MuLV serum. Lane 1, ANN-1 cells; lane 2, P90B-3 cells; lane 3, P160N54 cells; lane 4, A2 cells; lane 5, 2M3 cells; lane 6, 2M3/M cells. Lanes 7 through 15 contained samples which were not immunoprecipitated. Lane 7, ANN-1 cells; lane 8, A2 cells; lane 9, P160N54 cells; lane 10, P90B-3 cells; lane 11, NIH/3T3 cells; lane 12, 2M3 cells; lane 13, 18-81 cells; lane 14, 18-48 cells; lane 15, 70Z cells.*

tivity. In 2M3 cells, a short labeling period did produce a distribution equivalent to the enzyme activity distribution, but after a longer labeling period equal amounts of P120 were found in the DIM and SOL fractions. Similarly, briefly labeled ANN-1 cells showed equal amounts of P120 in both fractions, but after longer labeling most of the P120 was found in the SOL fraction. Thus, in both lymphoid and fibroblast cells, we found an apparent movement of P120 from the DIM fraction to the SOL fraction. It is possible that newly synthesized P120 is located in the DIM fraction and is enzymatically active and that it then moves to the SOL fraction, losing enzymatic activity. It is also possible that the kinase activity in immunoprecipitates of P120 is due to an associated protein which is not bound to the form of P120 found in the SOL fraction. The P120-associated kinase activity purifies with P120 through a number of chromatographic purification steps (A. Dasgupta and D. Baltimore, unpublished data), but it has not been shown rigorously that P120 is itself a kinase.

An 80,000-molecular-weight protein (F80) antigenically related to P120 was identified previously (15). F80 showed a different distribution profile than P120; it was always recovered predominantly with the DIM fraction. We found that F80 is a cleavage product of P120 and contains the *gag* portion of the molecule. F80 also contains many, if not most, of the sites phosphorylated by the P120-associated kinase, but it is not known whether F80 is itself a kinase. Witte et al. (26a) have shown that most of the phosphorylatable sites on P120 are located in the amino-terminal 43,000 daltons of the protein; thus, the presence of phosphorylatable sites in F80 would be expected. Although fibroblast cell extracts were capable of cleaving P120 in a lymphoid cell extract, we do not know whether any cleavage of P120 to F80 occurs in vivo in fibroblasts. Our failure to chase P120 to F80 suggests that some cleavage may occur during experimental manipulations, but both in vivo and experimentally induced cleavages may occur.

Most of the F80 is recovered in the DIM fraction whether the protein is labeled in vitro or in vivo. It is difficult to know whether this means that P120 is most susceptible to cleavage

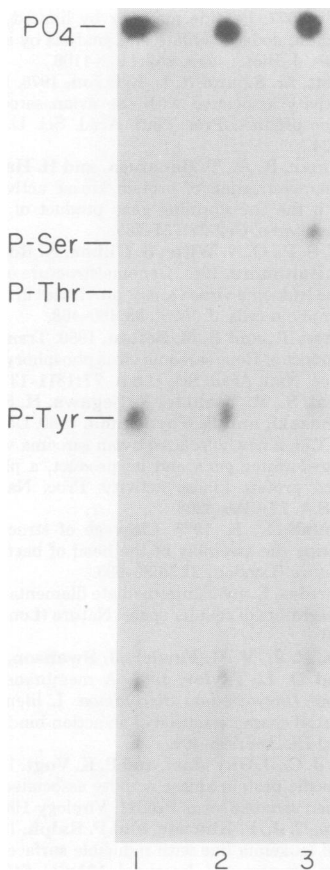

FIG. 7. *Characterization of the phosphoamino acids after in situ protein kinase assay. ANN-1 cells were fractionated with extraction buffer into DIM and SOL fractions. The SOL fraction was removed, and the DIM fraction was incubated with 50 μCi of [γ-^{32}P]ATP in situ at 4°C for 2 min. The DIM fraction was solubilized in lysis buffer, clarified by ultracentrifugation, and immunoprecipitated with goat anti-M-MuLV serum. Samples containing about 5,000 cpm were hydrolyzed in 6 N HCl at 100°C for 2 h, applied to Whatman 3MM paper, and subjected to electrophoresis in pyridine acetic acid buffer (pH 3.5) at 2,400 V for 1 h. Each of the hydrolyzed samples was mixed with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine markers, and the electrophoresis paper was stained with ninhydrin to locate the markers. Lane 1, DIM fraction reacted in the presence of Mg^{2+} and immunoprecipitated with goat anti-M-MuLV serum; lane 2, DIM fraction reacted in the presence of Mn^{2+} and immunoprecipitated with goat anti-M-MuLV serum; lane 3, DIM fraction reacted in the presence of Mn^{2+} and analyzed without immunoprecipitation.*

when it is bound or that F80 is bound selectively. Whatever the interpretation, this result implies that the F80 portion of P120 probably contains the binding site for the DIM fraction. F80 retains the *gag* determinants of P120, and it could be the *gag* determinants that bind it to the DIM fraction. Perhaps P120 binds less well because of competing interactions with lipids, detergents, or other proteins.

These experiments, as well as others (26a), have provided evidence for the localization of the A-MuLV protein in transformed cells and the position of antigenic determinants on the protein. Because the F80 fragment contains all of the *gag* determinants but does not react with anti-AbT serum, the 40,000-molecular-weight fragment lost during cleavage must be part of the Abelson virus-specific region of the protein. Anti-AbT serum but not anti-*gag* serum reacts with determinants on P120 found on the surface of the cell. Thus, we concluded previously that some of the P120 is a transmembrane protein (28). In similar experiments, we have shown that the P90 protein exhibits much less reactivity with anti-AbT serum on the cell surface, whereas P160- and P120-transformed fibroblasts have similar intensities of surface fluorescence (unpublished data). Furthermore, absorption of anti-P120 serum with extracts containing the P90 protein left antibodies that were reactive with P120, implying that the serum reacted with the carboxy-terminal portion of P120, which is absent in P90 (unpublished data). Like F80, P90 lacks the carboxy-terminal Abelson virus-specific sequence found in P120. These results are consistent with the hypothesis that a majority of the anti-AbT-reactive determinants are on the cell surface and that these constitute the carboxy-terminal portion of the A-MuLV protein. This region is lost either by protease fragmentation to generate F80 or by the mutation that truncates P120 to generate P90. Figure 8 shows a model for the organization of the P120 and P90 A-MuLV proteins. Because A-MuLV(P120) transforms both lymphoid and fibroblastic cells but A-MuLV(P90) has very little

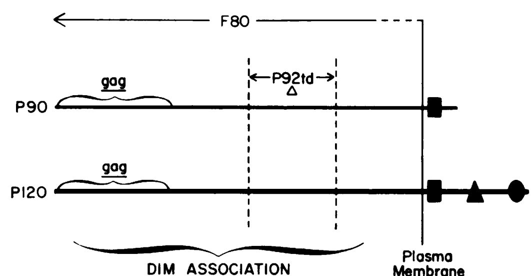

FIG. 8. *Model for the organization of A-MuLV proteins. Symbols: ■-▲-●, antigenic determinants recognized by anti-AbT serum; ▲-●, those antigenic determinants present on P120 but not on P90; Δ, region deleted in the transformation-defective P92td strain.*

lymphoid cell-transforming activity compared with its fibroblast-transforming activity, one implication of this model for the organization of the proteins is that determinants on the outer face of the cell may play a role in the lymphoid cell-transforming ability of the A-MuLV(P120) strain.

The largest A-MuLV-specific protein is P160, which is made by A-MuLV(P160) strains of the virus. This protein did not give rise to F80 in extracts of transformed fibroblasts but rather gave rise to a major cleavage product of about 90,000 daltons. Thus, it appears that P160 is cleaved at a position in the protein which was deleted in the process of generating P120.

It is interesting that the fractionation procedure used here distinguished quite well between two apparently different forms of P120. The form retained by the DIM fraction has associated catalytic activity in vitro and in situ, whereas most of the P120 found in the SOL fraction has little associated catalytic activity. Without a better understanding of the factors that control the kinase activity associated with P120 and without a more complete knowledge of the interactions that lead proteins to bind to elements of the DIM fraction, it is difficult to explain the significance of these observations.

Our results indicate some potentially fundamental similarities in the behaviors of A-MuLV P120 and Rous sarcoma virus pp60^{src}, suggesting a common pathway(s) of transformation for the two viruses, which may involve interactions with both the plasma membrane of the cell and the subcellular DIM fraction.

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