ABSTRACT

The Abelson murine leukemia virus (A-MuLV)-transformed lymphoid cell line L-1-2 is exceptional since it is rejected following transplantation into syngeneic C57L mice. Furthermore, the serum from recovered mice (Abelson tumor serum) has been used as a source of antibodies to abl antigens present in the M, 120,000 A-MuLV-encoded gag-abl fusion protein (p120). In addition to p120, this antiserum precipitates from the serum from recovered mice (Abelson tumor serum) has provided the only reliable source of antibody reactive with abl antigens (18, 26).

Recent evidence has indicated that p120 is a tyrosine-phosphorylating protein kinase (23). In addition, analysis of several A-MuLV strain variants encoding gag-abl fusion proteins with apparent molecular weights between 90,000 and 160,000 has demonstrated a direct correlation between the protein kinase activity of the molecules and their ability to transform lymphoid or fibroblast cultures (16, 24). Furthermore, abl antigens have been detected on the outer surfaces of cells infected with A-MuLV (26). However, p120 occurs predominantly in intracellular site(s) (17), and it is not known whether the cell surface component is p120 or a processed derivative.

When cells transformed by A-MuLV are injected into syngeneic mice, massive tumors form rapidly and cause death within 1 to 2 months (26). Only one Abelson-transformed lymphoid cell line, the L-1-2 of C57L origin, is reproducibly rejected by its syngeneic host (26). Furthermore, the serum from recovered mice (Abelson tumor serum) has provided the only reliable source of antibody reactive with abl antigens (18, 26).

For these reasons, we initiated a study of the plasma membranes of L-1-2 cells. The goals were to analyze the metabolism of gag-abl fusion protein(s) and to identify plasma membrane targets of the immune response in the recovered C57L mice.

MATERIALS AND METHODS

Cells and Virus. The C57L-derived A-MuLV-transformed lymphoid cell line L-1-2 was obtained from N. Rosenberg (Cancer Research Center, Tufts University School of Medicine, Boston, Mass.) and grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum and 2 × 10⁻⁶ M β-mercaptoethanol. The SWR/4, 230-23-8, and 230-37 A-MuLV-transformed lymphoid cell lines (27) were kindly provided by S. Goff (Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Mass.). The ANN-1 cell line, an A-MuLV-transformed nonproducer cell line of NIH-3T3 origin, was obtained from J. Stephenson (Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Md.) and grown in McCoy’s modified Medium 5a (Grand Island Biological Co.) containing 10% calf serum and antibiotics. F-MuLV was obtained from the Medium of logarithmically growing Eveline cells and purified as described by Evans et al. (6).

Immune Sera. Abelson tumor serum was obtained from C57L mice (The Jackson Laboratory, Bar Harbor, Maine) that successfully rejected an inoculum of approximately 10⁵ Abelson tumor cells. Abelson tumor serum is a rapidly fatal nonthymic lymphosarcoma in mice (1) and can transform certain bone marrow and fibroblast cultures in vitro (15). The tumorigenic potential of A-MuLV has been generally attributed to the expression of the virus’ own gene product, p120 (13, 27), a recombinant-type molecule containing serological determinants related to the M-MuLV amino-terminal gag gene products, p15 and p12 (13, 27), and also containing determinants related to a protein encoded by the abl gene found in the normal mouse genome (9, 22, 25).

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ected tumor challenge induced by s.c. injections of 5 x 10^5 viable L1-2 cells. Immunization and bleeding schedules used in obtaining this serum were by the procedure of Witte et al. (26). Monospecific antisera to Rauscher murine leukemia virus p30, p12, and gp70, were obtained from the Office of Program Resources and Logistics, National Cancer Institute, NIH, Bethesda, Md., and have been characterized previously (6).

Labeling and Extraction of Cells, Immunoprecipitation Procedures, and SDS-PAGE. Conditions for labeling cells with L-[35S]methionine and for conducting efficient cell extraction have been described previously (19). Procedures for the immunoprecipitation of proteins from labeled cells extracts with antisera (3, 19), electrophoresis of immunoprecipitated proteins in polyacrylamide gels in the presence of SDS (3, 11, 19), fluorographic detection of radioactive protein components on the dried gels (2, 12, 19), and the quantitative estimation of radioactive components by densitometric scanning of appropriately exposed autoradiographic films (20) have been described previously. The [35S]protein standards (New England Nuclear, Boston, Mass.) were used in all gels and had molecular weights of 92,500, 69,000, 46,000, 30,000, and 12,300.

Endogenous Labeling of Cell Surface Proteins. The selective binding and purification of cell surface antigens using specific antisera was performed essentially as described by Krangel et al. (10) with modifications specified by Fitting and Kabat (7). Essentially, A-MuLV-transformed cells (5 x 10^6/ml) were pulse labeled by incubation with L-[35S]methionine (100 μCi/ml of medium) for 2 hr at 37° and subsequently incubated with specific antibody for 45 min at 4°. These cells were then washed twice with cold phosphate-buffered saline (2.7 mM KCl:1.5 mM KH2PO4:0.14 M NaCl:0.015 M Na2HPO4:7H2O; pH 7.2; Grand Island Biological Co.) and extracted in the presence of a 10-fold excess of nonradioactive cellular lysate for 30 min at 4°. In these extraction conditions, antibodies which may dissociate from their immune complexes are competitively inhibited from rebinding to other labeled, immunologically reactive molecules (10). The lysates were subsequently clarified by centrifugation at 15,000 x g for 20 min. The antigen:antibody complexes were then collected with Staphylococcus aureus protein A (Pansorbin; Sigma Chemical Co., St. Louis, Mo.), and the immunoprecipitates were subjected to SDS-PAGE as described above.

Rosette Assay. Sheep erythrocytes were coupled with S. aureus protein A by the chromium chloride method of Goding (6). These protein A-conjugated erythrocytes were then allowed to bind to surface antigen in the presence of specific antibodies as described previously (19).

Protein Kinase Assay. A-MuLV-specific proteins, immunoprecipitated from nonradioactive L1-2 lysates with antisera prepared against the p12E molecule, were phosphorylated in vitro with [γ-32P]ATP using the method of Witte et al. (23). Briefly, immunoprecipitates were suspended in 20 mM Tris-HCl (pH 7.2):5 mM MgCl2 buffer containing 1 μCi of [γ-32P]ATP (New England Nuclear) and incubated for 10 min at 30°. Reactions were terminated by addition of ice-cold 20 mM Tris-HCl (pH 7.2) and washed repeatedly by centrifugation. The immunoprecipitates were then resuspended in 15 μl Laemmli electrophoresis buffer (11), boiled for 5 to 10 min, and then analyzed by SDS-PAGE as described above.

Tryptic Peptide Analysis. Peptide maps were performed by a modification of a procedure described by Elder et al. (5). Proteins either metabolically labeled with L-[35S]methionine or labeled in vitro with [γ-32P]ATP were purified by immunoprecipitation with antisera to p12E and by SDS-PAGE. These proteins were subsequently digested by incubation of selected gel sections in tosylsulfonylphenylalanichloromethyl ketone:trypsin (50 μg/ml) for 24 hr at 37°. Samples were then oxidized with performic acid for 4 hr at 0°, subsequently diluted in 2 ml of water and concentrated to dryness. The peptide digest was then washed twice with distilled water, subsequently resuspended in electrophoresis solution (28% formic acid), and spotted on cellulose thin-layer chromatography glass plates (CEL 300, 20 x 20 cm, 0.25 mm thick; Brinkmann Instruments, Inc., Westbury, N. Y.). Electrophoresis was conducted for 5 hr at 150 V in the first dimension, and ascending chromatography was performed in the second dimension in solution containing either isomy alcohol:pyridine:glacial acetic acid:H2O (70:70:40:10:60) for L-[35S]methionine-labeled peptides or 1-butanol:pyridine:glacial acetic acid:H2O (32:25:5:20) for 32P-labeled peptides. Detection of L-[35S]methionine-labeled peptides was enhanced by spraying plates with 7% diphenyl oxalate in ether. Radiolabeled peptides were visualized by autoradiography using Kodak XAR-5 film and intensifed in the case of 32P-labeled peptides with the use of calcium tungstate intensifying screens (Cronex Xtra Life Screens; Dupont Instruments, Wilmington, Del.).

Phosphoamino Acid Analysis. 32P-labeled proteins, phosphorylated in vitro, purified by immunoprecipitation with antisera to p12E and by SDS-PAGE, and collected by in situ trypsinization as described above, were heated to 110° for 1 hr in 200 μl of 6 n hydrochloric acid. Samples were washed repeatedly by concentration under vacuum and then resuspended in electrophoresis solution containing acetic acid:formic acid:H2O (15:5:80), pH 1.9. Phosphoamino acid standards, O-phospho-d-serine and O-phospho-dL-threonine, were purchased from Sigma Chemical Co. O-phospho-t-tyrosine was kindly provided by Tony Hunter (Salk Institute, San Diego, Calif.). Hydrolyzed samples containing 1.65 μg of each phosphoamino acid standard were applied to cellulose thin-layer plates and analyzed by 2-dimensional high-voltage electrophoresis. Electrophoresis was conducted with pH 1.9 buffer for 2.5 hr at 1000 V in the first dimension and with pyridine:glacial acetic acid:H2O (10:100:1890), pH 3.5, for 1 hr at 1000 V in the second dimension. Unlabeled standards were detected by ninhydrin staining, and 32P-labeled amino acids were visualized by autoradiography using calcium tungstate intensifying screens.

RESULTS

Virus-related Proteins in L1-2 Cells. Extracts of L-[35S]methionine-labeled L1-2 cells contain at least 2 proteins which can be precipitated either with antisera recognizing the M-MuLV gag gene products, p15 and p12, or with serum collected from C57L mice that have rejected A-MuLV-induced tumors (Abelson tumor serum). These proteins include the well-characterized A-MuLV p120 molecule (27) and a second minor component with an apparent molecular weight of 95,000 (p95) (Fig. 1, Lanes 1 and 5). Upon extended electrophoresis, p95 often appears as a doublet on SDS-PAGE. Preadsorption of the antisera to gag proteins with concentrated F-MuLV quantitatively eliminates the ability of this serum to precipitate p120 and p95 from L-[35S]methionine-labeled L1-2 extracts (Fig. 1, Lane 2; Table 1). Similar preadsorption of the Abelson tumor serum with this concentrated virus preparation does not appreciably affect the precipitation of either of these 2 molecules (Fig. 1, Lane 6; Table 1). These observations imply that this tumor serum precipitates p120 with antibodies reactive with abl antigens rather than with gag antigens. Therefore, based on this immunological criterion, both p120 and p95 are fusion proteins containing gag and abl determinants.

In addition to the A-MuLV p120 and p95 molecules, the L1-2 cell line contains other viral proteins encoded by the M-MuLV gag and env genes. Sera-recognizing determinants encoded by these 2 genes precipitate the previously characterized M-MuLV proteins (4, 27), Pr6559a9, Pr759a9, gp80env, gp70env, and p15E from L-[35S]methionine-labeled L1-2 extracts (Fig. 1, Lanes 1 and 3). The precipitation of the env-related molecules p15E and its precursor, gp80env, by the Abelson tumor serum (Fig. 1, Lane 5) implies that this serum contains a p15E antibody subpopulation. The precipitation of these 2 env pro-
for the A-MuLV p120 molecule (23). In addition, phosphorylated p120 and p95 have nearly identical phosphotryptic peptide maps (Fig. 5), each containing one major and several minor phosphopeptides.

Detection of abl Antigens on the Surface of Cells Transformed by A-MuLV. Evidence that the surface of Abelson-transformed cells contains abl antigens was provided by an antibody-dependent rosetting assay. In this rosetting assay, sheep erythrocytes coated with protein A are incubated with cell monolayers in the presence of specific antisera (see "Materials and Methods"). Binding of erythrocytes onto the surface of the Abelson nonproducer cell line ANN-1 occurred in the presence of tumor serum but not in the presence of either preimmune mouse serum or antiserum to p12 (Fig. 6). The ANN-1 cell line was chosen for this study because it contains only A-MuLV- and not M-MuLV-encoded proteins. The use of this cell line obviated the necessity of absorbing the p15E antibody subpopulation of the tumor serum prior to its application in the rosetting assay. The results in Fig. 6 imply that abl antigens occur on the surfaces of the A-MuLV-transformed cell line, ANN-1.

Further evidence to support the presence of abl antigens on the surface of A-MuLV-transformed cells was provided by serum absorption experiments. As shown in Fig. 7, Abelson tumor serum preadsorbed with 10^6 ANN-1 cells (Lane 3) cannot precipitate p120 from L-[35S]methionine-labeled L1-2 cellular lysates. Alternatively, tumor serum preadsorbed with 10^6 or even 10^7 NIH-3T3 cells (Fig. 7, Lanes 6 and 7) precipitates p120 efficiently.

Use of Abelson Tumor Serum to Detect a M, 95,000 Protein on the Surface of L1-2 Cells. We attempted to identify specific gag-abl surface molecules on the L1-2 cell line by a method that involves direct absorption of antibodies onto L-[35S]methionine-labeled intact cells, followed by cell washing, lysis with detergents in the presence of an excess of nonradioactive cellular extract, and rapid isolation of antigen:antibody complexes (see "Materials and Methods"). The M-MuLV gp70-env and p15E molecules were detected in these surface prepara-

Table 1
Relative amounts of viral proteins precipitated by various antisera from L-[35S]methionine-labeled L1-2 cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Anti-p12 serum</th>
<th>Anti-p12 serum preadsorbed with F-MuLV</th>
<th>Anti-gp70 serum</th>
<th>Anti-gp70 serum preadsorbed with F-MuLV</th>
<th>Tumor serum</th>
<th>Tumor serum preadsorbed with F-MuLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>p120</td>
<td>11.9</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>11.3</td>
<td>10.8</td>
</tr>
<tr>
<td>p95</td>
<td>1.5</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>gp80-env</td>
<td>0</td>
<td>0</td>
<td>80.0</td>
<td>14.3</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Pr65-92 envelope</td>
<td>5.1</td>
<td>0.1</td>
<td>19.1</td>
<td>1.0</td>
<td>17.4</td>
<td>12.2</td>
</tr>
<tr>
<td>p15E</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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Surface Membranes of A-MuLV-transformed Cells

Fig. 1. Identification of viral proteins in the L1-2 cell line. L1-2 cells were pulse labeled with L-[35S]methionine (2 hr, 50 µCi/ml). The labeled cells were subsequently extracted, and viral proteins were immunoprecipitated from the lysates with anti-p12 (Lane 1), anti-gp70 (Lane 3), or an Abelson tumor serum obtained from C57L mice (Lane 5). Immunoprecipitations were also conducted with serum that had been preadsorbed with 100 µl of concentrated F-MuLV (preadsorption of anti-p12, anti-gp70, and tumor serum conducted in Lanes 2, 4, and 6, respectively).

p120 and p95 Are Structurally Related Molecules and Contain Apparently Identical Tyrosine-containing Phosphorylation Sites. To determine the structural relatedness of the p95 molecule to A-MuLV p120 and M-MuLV Pr65-92 envelope, trypic peptide analyses of L-[35S]methionine-labeled proteins were conducted. A-MuLV p120 contained 16 major and several minor methionine peptides (Fig. 2). Of the 16 major peptides found in p120, all but 2 appear to be present in the p95 molecule. These 2 missing peptides (Fig. 2, arrows) do not appear in the tryptic peptide map of the M-MuLV Pr65-92 envelope protein. We infer that the portion of p120 which is absent from p95 occurs in the abl region, consistent with our immunological evidence that p95 contains the M-MuLV p15 (data not shown) and p120 envelope regions found in p120.

Both of these gag-abl fusion proteins could be labeled by incubation of nonradioactive immunoprecipitates with [γ-32P]-ATP in an in vitro protein kinase assay (Fig. 3). Two-dimensional electrophoretic analyses of acid hydrolysates of proteins phosphorylated in the L1-2 immunoprecipitate show that the amino acid acceptor for phosphorylation on p95 is at tyrosine residues (Fig. 4), consistent with results described previously.
tions when antisera to gp70
was used (Fig. 8, Lane 3). The p15E protein binds weakly to gp70
and is partially coprecipitated with the surface gp70
: antibody complexes. When the Abelson tumor serum was used to isolate antigenically reactive molecules from the L1-2 cell surface, a diffuse band with an apparent molecular weight of 95,000 was observed in SDS-PAGE (Fig. 8, Lane 4). This molecule was detected on the L1-2 surface only with tumor serum and not with preimmune mouse serum or antisera recognizing p120
 or gp70
 (Fig. 8). The A-MuLV p120 molecule was not detected by this method (Fig. 8, Lane 4).

Is the M, 95,000 Cell Surface Protein the gag-abl p95 Molecule? The above results do not establish that the cell surface M, 95,000 protein is the A-MuLV p95 molecule. The tumor serum could possibly contain antibodies against 2 distinct components. The p95 gag-abl protein would presumably be the major component in whole-cell lysates, but it might be absent or present in only small amounts on the cell surface. We analyzed this question by absorbing the tumor serum with cells containing abl antigens on their surface membranes. As shown in a previous experiment (Fig. 7), as few as 10
 ANN-1 cells are able to quantitatively absorb the abl antibodies present in 5 µl of tumor serum. We used 10 times this number of cells to conduct tumor serum preadsorptions prior to application of the serum in the extracellular antibody adsorption procedure. As can be seen (Fig. 9, Lanes 3 and 4), preadsorption of tumor serum with either ANN-1, an A-MuLV-transformed NIH-3T3 cell line, or uninfected NIH-3T3 cells does not block the precipitation of the M, 95,000 surface molecule from L1-2 plasma membranes. Therefore, we conclude that the M, 95,000 surface protein found on the L1-2 cell line is not the A-MuLV p95 gag-abl protein. The M, 95,000 surface protein is detected only under selective conditions (i.e., use of the extracellular antibody adsorption technique to isolate plasma membrane proteins) and is presumably produced to a much lesser extent than is the A-MuLV p95 molecule. Furthermore, the M, 95,000 surface molecule appears to be absent from the surfaces of all other A-MuLV-transformed lymphoid cell lines tested (Fig. 9, Lanes 5 and 7).* These tested cell lines include the SWR/J cell line derived from the SWR/J mouse and the 230-23-8 and 230-37 cell lines of C57L origin. The A-MuLV-transformed nonproducer fibroblast cell line, ANN-1, and its NIH-3T3-uninfected cell line counterpart also appear to lack the M, 95,000 surface molecule, consistent with the inability described previously of these cell lines to adsorb antibodies which react with this determinant.

DISCUSSION

Regression of L1-2 Tumor Transplants in Syngeneic C57L mice. A major advance in understanding the molecular biology of A-MuLV and for defining the transforming protein of the virus was the development of a mouse tumor serum reactive to the host-encoded abl determinant found in the virus’ major gene product, p120 (26, 27). This serum can only be produced in the C57L mouse by the repeated injection of only one A-MuLV-transformed syngeneic lymphoid cell line, the L1-2 (18, 26). Rotter et al. (18) have established that the uniqueness of tumor regression in this one system lies in the properties of the cell line and not in the genetics of C57L animals. Other A-MuLV-transformed lymphoid cell lines of C57L origin have been found to be highly lethal to their syngeneic hosts, consistent with studies of Abelson tumorigenesis in other mouse strains (26). This evidence suggested that L1-2 cells differ from other A-MuLV-infected cells in their immunological interactions with the host.

abl Antigens Occur in Plasma Membranes. The extracellular antibody adsorption procedure described in this study has been used previously to investigate the membrane processing of human histocompatibility antigens (10) and the surface molecules encoded by the gag and env genes of F-MuLV (7). Although the direct identification of p120 or an antigenically related derivative on the plasma membranes of A-MuLV-transformed cells was not achieved in our study, observations obtained by other techniques are consistent with the model proposed by Witte et al. (26) for the orientation of Abelson-encoded gag-abl molecules in cellular membranes. Thus, our results are consistent with the idea that abl antigen(s) may be a transmembrane protein with its amino-terminal gag-related region embedded in the plasma membrane and its host-encoded abl determinant protruding to the exterior of the cell. Our studies have shown that intact ANN-1 cells can absorb antibodies recognizing the abl antigen from the tumor serum (Fig. 7). Furthermore, binding of protein A-coupled sheep erythrocytes to the surface of the A-MuLV-transformed nonproducer fibroblast, ANN-1, was achieved only when the tumor serum was used and not with serum recognizing p120
 antibodies (Fig. 6).

The apparent paradox of why the extracellular antibody adsorption technique is able to detect M-MuLV gp70
 on the surface of the L1-2 cell and not A-MuLV gag-abl fusion proteins is presumably related to the different antisera used. Isolation of membrane proteins using this procedure requires highly avid antibodies (7). Antisera with a low avidity can dissociate from their antigens during the manipulations (i.e., cell lysis and ultracentrifugation) required for the isolation of the immune complexes. Alternatively, the abl determinant on the cell surface may be partially blocked by steric hindrance and therefore less accessible to certain classes of extracellular antibody.

Significance of Minor gag-abl Components. There are several possible explanations for the appearance of the minor p95
 component in L1-2 cellular lysates. Similar minor components with heterogeneous molecular weights between 80,000 and 110,000 have been noted by previous investigators (27) in various clonally derived A-MuLV-transformed cell lines. First, p95 might be a proteolytic fragment of p120, artifically produced during the extraction of L-[35S]methionine-labeled L1-2 cells. This explanation seems unlikely because the concentration of this molecule was unaffected by the use of different lysis procedures or protease inhibitors. A second possibility which has been suggested previously for other cell lines (27) is that the different gag-abl components may be encoded by distinct A-MuLV proviruses which might coexist in the same cells. A third possibility is that processing or structural modification of gag-abl fusion proteins might occur intracellularly and that different components may occur in distinct subcellular sites. This possibility is intriguing because MuLV-encoded gag gene products are heterogeneous processed in both the cytoplasm and in subcellular membranous organelles (4, 21). In this context, it is interesting to note that

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* C. Machida and D. Kabat, unpublished observations.
**abi** antigens also occur in both cytoplasmic and plasma membrane locales (17, 26).

**M, 95,000 Protein on the Surface Membranes of L1-2 Cells.** The M, 95,000 protein that occurs on the surface of L1-2 cells seems highly significant because it reacts strongly with antibodies present in the serum of mice which have rejected L1-2 tumor challenge. Furthermore, this molecule is not detected by **abi** antibodies that recognize A-MuLV p120 (Fig. 9) and appears to be absent from the surfaces of other A-MuLV-transformed lymphoid and fibroblast cell lines, including those of C57L origin. Unlike L1-2 cells, the latter transformed cell lines are highly tumorigenic when transplanted onto C57L mice (18, 26). These results suggest that the M, 95,000 cell surface protein might be an important target for the immune response of host C57L mice to the L1-2 tumor cell challenge. Presumably, the M, 95,000 cell surface protein is distinct from the surface antigen described by Risser et al. (14). The latter antigen is common to different A-MuLV-transformed lymphoid tumors (14), whereas the M, 95,000 protein occurs only, or in substantially higher concentrations, on L1-2 cells.

Although our results are consistent with the possibility that the M, 95,000 protein plays an important role as a target for immune rejection of L1-2 tumor cells in C57L mice, many aspects of its structure and function remain unknown. For example, immune response to this component and to L1-2 cells might involve cellular as well as humoral mechanisms. Furthermore, the observed immune response to this molecule suggests that the host mice are not tolerized to all of its antigens. Therefore, we suggest that it might be encoded by a derepressed host gene or by a passenger virus which has somehow infected the L1-2 tumor cells. If these ideas are correct, passenger virus infection of other tumor cell lines might enhance their rejectability and thereby facilitate preparation of tumor-specific antisera.

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REFERENCES


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Fig. 2. Tryptic peptide analyses of L-[\textsuperscript{35}S]methionine-labeled p120, p95, and M-MuLV Pr65\textsuperscript{pp60} L-[\textsuperscript{35}S]Methionine-labeled proteins, purified by immunoprecipitation with anti-p120\textsuperscript{pp60} serum and by SDS-PAGE, were digested by incubation of selected gel sections in tolylsulfonylphenylalanyllchloromethyl ketone:trypsin for 24 hr at 37\textdegree. Following performic acid oxidation, these samples were diluted in 2 ml of H\textsubscript{2}O, concentrated under vacuum, resuspended in electrophoresis buffer (28\% formic acid), and spotted on cellulose thin-layer chromatography plates. Electrophoresis was conducted at 150 V for 5 hr in the horizontal dimension, and chromatography was performed in buffer containing isoamyl alcohol:pyridine:ethanol:glacial acetic acid:H\textsubscript{2}O (70:70:40:10:60) in the vertical dimension. Detection of L-[\textsuperscript{35}S]methionine-labeled peptides was enhanced by spraying thin-layer chromatography plates with 7\% diphenyloxazole in ether. Proteins analyzed included A-MuLV p120 (A), A-MuLV p95 (B), and M-MuLV Pr65\textsuperscript{pp60} (C). Arrows, representative peptides found in p120 that are absent in p95 and Pr65\textsuperscript{pp60}. Origin, lower right.

Fig. 3. Analysis of proteins phosphorylated by \([\gamma-\textsuperscript{32}P]\)ATP in the L1-2 immunoprecipitate. Virus-specific proteins from nonradioactive L1-2 lysates were immunoprecipitated with anti-p12 serum. In vitro phosphorylation was conducted by suspending immunoprecipitates in 20 mm Tris-HCl (pH 7.2):5 mm MgCl\textsubscript{2} buffer containing 1 \mu Ci of \([\gamma-\textsuperscript{32}P]\)ATP and incubating them for 10 min at 30\textdegree. Reactions were terminated by addition of ice-cold 20 mm Tris-HCl (pH 7.2) and after repeated washings were analyzed by SDS-PAGE as described in ‘Materials and Methods.’

Fig. 4. Phosphoamino acid analysis of A-MuLV p120 and p95 phosphorylated in vitro with \([\gamma-\textsuperscript{32}P]\)ATP. A-MuLV p120 and p95, labeled in vitro with \([\gamma-\textsuperscript{32}P]\)ATP, were purified by immunoprecipitation with anti-p12\textsuperscript{pp60} serum and by SDS-PAGE, hydrolyzed with 6 N HCl, and subjected to 2-dimensional electrophoretic analyses following procedures outlined in ‘Materials and Methods’ (pH 1.9 buffer in horizontal dimension; pH 3.5 buffer in vertical dimension). A and B, analyses for p120 and p95, respectively; C, unlabeled phosphoamino acid standards [phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr)] detected by ninhydrin staining. Origin, lower right.
Fig. 5. Tryptic peptide analyses of A-MuLV p120 (A) and p95 (B) phosphorylated in vitro with [γ-32P]ATP. Proteins were labeled in vitro with [γ-32P]ATP, purified by immunoprecipitation with anti-p120 serum and by SDS-PAGE, and subjected to tryptic proteolysis as described in “Materials and Methods.” Electrophoresis was conducted in the horizontal dimension, and chromatography was conducted in the vertical dimension. Arrows, major phosphotryptic peptide found in both phosphorylated p120 and p95. This phosphopeptide comprises >75% of the total radioactivity found in the digest. The other minor phosphopeptides can only be detected after prolonged exposures of the film; x, origin.

Fig. 6. Erythrocyte rosette assay for detection of A-MuLV-encoded cell surface antigens. The binding of sheep erythrocytes coated with protein A to the A-MuLV-transformed nonproducer fibroblast, ANN-1, occurred only in the presence of tumor serum containing antibodies reactive to the unique ab1 portion of the Abelson-encoded protein (A). No binding of erythrocytes was observed with either nonimmune mouse (B) or anti-p12 sera (data not shown). Bar, 20-μm length. Erythrocytes in A are seen as highly refractile small white circles.

Fig. 7. Absorption of tumor serum with ANN-1 or NIH-3T3 cells. Tumor serum was preadsorbed with either 10^5, 10^6, or 10^7 ANN-1 cells (Lanes 2, 3, and 4, respectively) or NIH-3T3 cells (Lanes 5, 6, and 7, respectively) prior to the application of the serum in the immunoprecipitation of p120 from L-[35S]methionine-labeled L1-2 lysates. Tumor serum which did not undergo preadsorption with intact cells (Lane 1) was also used to precipitate p120 from the radioactive lysates. Antigen:antibody complexes were then collected with S. aureus protein A and subjected to SDS-PAGE.

Fig. 8. Analysis of MuLV-encoded proteins on the surface of L1-2 cells. L1-2 cells were pulse labeled by incubation with L-[35S]methionine (2 hr, 100 μCi/ml). The labeled cells were subsequently extracted, and viral proteins were immunoprecipitated from the lysates with either 5 μl of anti-gp70 (Lane 1) or Abelson tumor serum (Lane 2). Alternatively, the L1-2 cells, similarly labeled, were incubated directly with either 5 μl of anti-gp70 (Lane 3) or Abelson tumor serum (Lane 4) for 45 min at 4°C. After washing to remove unbound antibodies, lysates were prepared as described in “Materials and Methods.” Surface antigen:antibody complexes were then collected with S. aureus protein A and subjected to SDS-PAGE. M, 95,000 surface molecule appears as a diffuse band on SDS-PAGE (Lane 4).

Fig. 9. Analysis of M, 95,000 surface molecule on A-MuLV-transformed cells. The surface membranes of L1-2 cells were analyzed by the extracellular antibody adsorption technique (see “Materials and Methods”) using normal mouse serum (Lane 1), Abelson tumor serum (Lane 2), or tumor serum preadsorbed with either 10^7 NIH-3T3 or ANN-1 cells (Lanes 3 and 4, respectively). The same extracellular antibody adsorption technique using tumor serum as the probe was used with 2 other A-MuLV-transformed lymphoid cell lines, 230-23-8 (Lane 5) and SWR/4 (Lane 7). Surface preparations from the L1-2 cell line, illustrating the M, 95,000 molecule, are shown in Lane 6 for comparison. M, 95,000 surface molecule appears as a diffuse band on SDS-PAGE.
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