IDENTIFICATION OF A 36,000-MOLECULAR WEIGHT, GAG-RELATED PHOSPHOPROTEIN IN LYMPHOMA CELLS TRANSFORMED BY RADIATION LEUKEMIA VIRUS

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Many RNA tumor viruses preferentially induce neoplasms of hematologic or lymphoid tissue in susceptible animals. According to their oncogenic potential, these retroviruses are divisible into two groups, the acute leukemia viruses and the slow or chronic leukemia viruses. The acute viruses, such as Abelson murine leukemia virus (AbLV), cause rapid onset of neoplastic disease (2-4 wk) following infection in vivo and can transform appropriate target cells in tissue culture (1, 2). Inoculation with slow leukemia viruses, which include the avian and murine leukemia viruses (ALV and MuLV), results in neoplasia only after a prolonged latent period of 3-12 months. They have not been found to cause morphological transformation of tissue culture cells (3, 4).

The mechanism(s) of oncogenesis by these viruses is not yet understood. The transforming activity of the acute viruses has been attributed to a particular genomic region termed the onc gene. Onc sequences do not encode viral structural proteins, but rather are thought to have been acquired by recombination from cellular DNA at the expense of replicative genes. As a result, these viruses are uniformly replication defective (1, 5, 6). The translation product of such an onc gene in transformed cells appears as a "fusion" protein, consisting of the putative transforming protein colinear with viral peptides. In the example of AbLV, a 120,000-dalton protein (p120) containing portions of Mo-MuLV p15, p12, and p30 gag polypeptides covalently linked to a highly phosphorylated cell-derived fragment is thought responsible for transformation (7). The slow leukemia viruses, in contrast, contain no readily definable transforming genes. Neel et al. (8) and Payne et al. (9) have acquired evidence suggesting that the majority of bursal lymphomas induced in chickens by the slow ALV result from activation of a cellular oncogene by vicinal integration of viral promoter elements. How-

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Abbreviations used in this paper: AbLV, Abelson murine leukemia virus; ALV, avian leukemia virus; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MuLV, murine leukemia virus; p120, 120,000-dalton protein; PBS, phosphate-buffered saline; RadLV, radiation leukemia virus; R-MuLV, Rauscher virus; SAC, Staphylococcus aureus A, Cowan strain; SFFV, spleen focus-forming virus of Friend complex; TCA, trichloroacetic acid; TLC, thin layer chromatography; TNE, 50 mM Tris-HCl (pH 7.5)/100 mM NaCl/1 mM EDTA.
ever, oncogenesis by promoter insertion has not been documented for MuLV lymphomagenesis.

Recently Manteuil-Brutlag et al. (10) described an analysis of radiation leukemia virus (RadLV) RNA. Their results indicate that leukemogenic preparations of RadLV contain a presumed defective 5.6-kb RNA along with full-length 8.0-kb molecules. Closely related, but nonleukemogenic BL/Ka (B) virus has only the 8.0-kb species. When translated in vitro, the 5.6-kb RNA produced polypeptides of 100 kdaltons, 36 kdaltons, and 30 kdaltons, which expressed gag antigenic determinants. By analogy to the acute defective leukemia viruses, these investigators propose an oncogenic function for the products of the 5.6-kb RNA. Thus the leukemogenic agent may be a replication-defective agent associated with helper viruses and not the complete virus, as suggested by Decleve et al. (11) and Lieberman et al. (12).

The present study reports analysis of a gag-associated 36,000 molecular weight protein (p36) expressed by thymic lymphoma cell lines induced by RadLV. Evidence differentiating p36 from gag intermediates and supporting its association with cells transformed by RadLV is presented. The possible origins and involvement of this protein in RadLV leukemogenesis are discussed.

Materials and Methods

Virus and Antisera. RadLV was prepared as 20% cell-free extracts of thymomas induced by intrathymic injection of similarly in vivo passaged RadLV. It was originally derived from radiation-induced murine lymphomas by Lieberman and Kaplan (13). Goat anti-viral antisera were kindly provided by Dr. J. Cole, National Cancer Institute, Bethesda, MD. These included anti-Tween-ether-disrupted AKR MuLV (4S-97), anti-Tween-ether-disrupted BALB: virus-2 (5S-91), anti-Rauscher virus (R-MuLV) p30 (6S-492), anti-R-MuLV p12 (5S-37), anti-AKR-MuLV p10 (5S-465), anti-R-MuLV p15 (7S-30), and R-MuLV gp70 (5S-167); also provided was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin for indirect immunofluorescence assays. Monoclonal anti-p15(E) was a gift of Dr. Bruce Chesebro (14).

Cell Lines

The AQR-V, B10.G-V, B10.S-V, and B6-V1 lines were established into tissue culture from RadLV-induced thymomas from mice of C57BL lineages, with the line name reflecting mouse strain of origin. Likewise, AQR-X1 and B6-X1 were established from radiation-induced thymomas. The RL-12 and L591-6 cell lines were originally adapted to tissue culture elsewhere from radiation lymphomas of C57BL/Ka and C57L mice, respectively (15, 16). The III6A clone of Sc-1 cells, obtained from Dr. T. Pincus (Vanderbilt University), was originally derived from a feral mouse embryo by Hartley and Rowe (17), and lacks Fv-I type restriction of ecotropic MuLV. The mink lung cell line CCL64 was a generous gift of Dr. A. Mayer (New York University Medical center). Cells were routinely maintained in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum (FCS) supplemented with antibiotics (complete DME), except RL-12, which grew in RPMI-1640 with 10% FCS and antibiotics.

Infection Procedures

Assay. The methods used for infecting cells with MuLV were minor modifications of those described (18, 19). For monolayer cells, 2 × 10^5 were seeded into 25-cm² T-flasks (Corning Glass Works) in 5 ml of complete DME; the following day, the medium was removed and the cells treated with 25 µg/ml of diethylaminoethyl (DEAE)-dextran (Sigma Chemical Co., St. Louis, MO) in DME for 1 h at 37°C, after which the DEAE-dextran solution was removed and 0.5 ml of AQR-V culture supernatant sterilized by filtering
through a nitrocellulose membrane (0.45 μm, Millipore Corp., Bedford, MA) and containing 4 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI), was added. After incubation at 37°C for 2 h, the flasks were fed with 5 ml of complete DME, and passaged at confluency, approximately every 6–7 d.

For suspension cells, 1 × 10⁶ cells in log phase were mixed in 24-well Linbro cluster plates with an equal volume (0.2 ml) of filtered (0.45 μm, Millipore) culture supernatant containing 4 μg/ml polybrene. After a 2 h adsorption in humidified 5% CO₂ incubator at 37°C, 1 ml of medium was added. Medium was changed three times weekly.

The number of virus-positive cells was determined at various intervals after infection using an indirect immunofluorescence procedure on fixed cells (18, 20).

**Radiolabeling and Cell Extraction**

Suspension cells were radiolabeled metabolically with [³H]leucine as follows. Cells were washed 3X in cold sterile PBS. For 1–5 h steady-state or pulse-chase labeling periods, cells were resuspended at 25 × 10⁶ cells per ml with DME minus leucine (or RPMI-1640 minus leucine for RL-12 cells) (Gibco Laboratories, Grand Island, NY) supplemented with 2% FCS, 1% penicillin/streptomycin, and 25 μCi/ml of L-[³,4,5-³H(N)]-leucine (148-152 Ci/mmol; New England Nuclear [NEN], Boston, MA). Cells were incubated in humidified 5% CO₂ at 37°C with frequent mixing. For overnight labeling, cells were resuspended to 1 × 10⁶/ml in DME plus one-tenth the normal concentration of leucine, 2% FCS, penicillin/streptomycin, and 25 μCi/ml [³H]leucine. When [³⁵S]methionine (1,300 Ci/mmol; Amersham Corp., Arlington Heights, IL) was used, DME minus methionine (Gibco) was substituted in the procedure. Monolayer cells were labeled just as they reached confluency by washing 3X with sterile PBS, then adding 0.8 ml of the above labeling medium. For incorporation of radioactive phosphate, cells were washed 3X in phosphate-free DME (Gibco), then resuspended to 25 × 10⁶/ml in phosphate-free DME with 2% FCS, 1% penicillin/streptomycin, and 300 μCi/ml [³²P]-orthophosphoric acid (3,000 Ci/mmol; NEN) for 4 h.

Following labeling, cells were washed 3X with ice-cold PBS and disrupted in lysing buffer consisting of 0.5% NP-40/20 mM Tris-HCl (pH 7.5)/100 mM NaCl/2 mM EDTA/2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) on ice for 45 min with frequent vortexing. Lysates were clarified by centrifugation at 38,000 g and stored at −70°C until use. To determine incorporated trichloroacetic acid (TCA)–precipitable radioactivity, 1-μl samples were added to 0.5 ml PBS containing 5% FCS and precipitated with 0.5 ml of 20% TCA for 30 min on ice. Precipitates were collected by centrifugation for 10 min at 11,000 g in a microfuge (Beckman Instruments, Spinco Div., Palo Alto, CA), redissolved in 100 μl PBS, and added to 2 ml of Betaphase scintillation solution (WestChem Products, San Diego, CA). Radioactivity was assayed using a Beckman LS-250 liquid scintillation counter.

**Virus Purification**

Supernatant from 20 h labeled cells was clarified by centrifugation at 2,000 g at 4°C and spun through discontinuous 25–45% (wt/vol) gradients of sucrose in 50 mM Tris-HCl (pH 7.5)/100 mM NaCl/2 mM EDTA (TNE) at 26,000 rpm for 2 h in an SW-27 rotor (Beckman). Material sedimenting at the interphase was diluted in TNE and layered onto 15 ml preformed 15–60% continuous sucrose gradients and spun for 17 h at 26,000 rpm in an SW-27 rotor. Fractions of 0.8 ml were collected and refractive indices measured; fractions containing sucrose densities of 1.14 to 1.16 g/cm³ were pooled and pelletted at 39,000 rpm in a type 40 rotor. Viral pellets were either resuspended in electrophoresis sample buffer (2% NaDodSO₄/80 mM Tris-HCl (pH 6.8)/10% glycerol/2 mM EDTA/5% β-mercaptoethanol/0.001% bromophenol blue) or in lysing buffer for immunoprecipitation analysis.

**Immunoprecipitation**

Aliquots of cellular lysates containing 10–20 × 10⁶ TCA-precipitable cpm were pre-cleared by incubation for 30 min at 4°C with 20 μl of normal rabbit serum, followed by
addition of 200 µl of 10% formalin-fixed Staphylococcus aureus A (Cowan strain) (SAC; The Enzyme Center, Inc., Boston, MA) suspended in lysing buffer containing 1% bovine serum albumin (BSA) for 60 min at 4°C. The SAC was removed by centrifugation at 1,000 g in a microfuge for 5 min, and the precleared supernatant was then incubated overnight at 4°C with 5–20 µl of the appropriate antiserum or ascites fluid. The next morning, 200 µl of SAC suspension was added for 60 min at 4°C and pelleted by centrifugation at 2,000 g for 10 min. SAC pellets were washed 5x with 1% deoxycholate/1% Triton X-100/0.1% SDS in TNE buffer, then resuspended in 80 µl of electrophoresis sample buffer.

**Gel Electrophoresis**

NaDodSO4/polyacrylamide gel electrophoresis was routinely run using the discontinuous Tris-glycine system of Laemmli (21). Samples were treated to 100°C for 5 min, SAC removed by centrifugation, and eluted proteins electrophoresed on 25-cm gradient gels of 7.5–18% or 10–18% acrylamide (30:0.8, acrylamide to bisacrylamide; Bio-Rad Laboratories), topped with 2.5-cm stacking gels of 4.5% acrylamide, for 20 h at 130V. Molecular weight marker proteins (Bio-Rad, Richmond, CA) were coelectrophoresed included phosphorylase b (molecular weight 92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Molecular weights were confirmed using 14C-labeled standard proteins (NEN). Gels were fixed and stained in 0.1% Coomassie Brilliant Blue R in 50% TCA for 30 min and destained in several changes of 7% glacial acetic acid. The gels were processed for fluorography by washing in cold running water for 15 min, soaking in 3-gel volumes of Autofluor (National Diagnostics Inc., Somerville, NJ) containing 1% glycerol for 60 min at 20°C, and drying on Whatman 3MM paper under vacuum at 80°C. Dried gels were exposed to Kodak XAR-5 film at −70°C and developed using an automated Kodak X-OMAT processor.

**Tryptic Peptide Analysis**

Radiolabeled proteins to be fingerprinted using the general method of Elder (22), were resolved by NaDodSO4/polyacrylamide gel electrophoresis of immunoprecipitates. Bands were cut from fixed and dried gels using a razor blade with the fluorogram as a template guide. Gel slices were placed in 12 × 75 mm tubes and washed overnight in three changes of 2 ml dH2O at 4°C to remove water-soluble fluoros and glycerol, then in 2 ml 0.05 M NH4HCO3 buffer (pH 8.0) for 4–6 h at 4°C. Supernatant was then completely aspirated and 0.5 ml of 0.05 M NH4HCO3 (pH 8.0) containing 50 µg/ml of tosylsulfonylphenylalanyi chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp., Freehold, NJ) was added. Tubes were covered and incubated overnight at 37°C in a shaking water bath, after which supernatants were removed and lyophilized. Samples were dissolved in 10 µl of Buffer I (acetic acid–formic acid–water, 15:5:80) and carefully spotted onto 10 × 10 cm cellulose-coated thin layer chromatograph (TLC) plates (EM Laboratories, Elmsford, NY). The samples were then electrophoresed using Buffer I at 500 mV for ~20 min on a water-cooled Multiphor 2117 apparatus (LKB Instruments, Inc., Rockville, MD). To monitor consistent electrophoresis, a mixture of 2% Orange G (wt/vol) and 1% acid-fuchsin (wt/vol) in Buffer I was spotted at the opposite end from the sample and migrated as an internal control. The plates were dried and peptides chromatographed in the second dimension using butanol–pyridine–acetic acid–water, 32.5:25:5:20. The plates were again dried, then dipped in 20% 2,5-diphenyloxazole (PPO) in acetone, redried, and exposed to Kodak XAR-5 film at −70°C and developed as above.

**Phosphoamino Acid Analysis**

To determine sites of protein phosphorylation, amino acid analysis was performed essentially according to the method of Beemon and Hunter (23), with modifications. Immunoprecipitated proteins labeled in vivo with 32P-orthophosphate were separated by SDS-polyacrylamide gel electrophoresis and radioactive bands of interest cut from dried, unfixed, unstained gels. Slices were rehydrated with 0.5 M NH4CO3 (pH 8.0)/0.1% SDS
Identification of a Novel gag-Related Protein p36 in RadLV-induced Lymphoma Cell Lines. In order to characterize the virally encoded polypeptides expressed by a RadLV-induced thymic lymphoma, the AQR-V cell line was metabolically labeled with [3H]leucine, detergent lysed, and immunoprecipitated using antisera prepared against MuLV proteins. The immunoprecipitates were analyzed by NaDodSO4/polyacrylamide gel electrophoresis (Fig. 1A). Normal goat serum (lane 1) precipitated no detectable radiolabeled proteins from these extracts; antisera prepared against disrupted ecotropic AKR virus (anti-AKR-MuLV) or xenotropic BALB:ivirus-2 (anti-BV2), which react with virtually all of the viral structural proteins, precipitated several distinct bands (lanes 2 and 6) corresponding to major env and gag gene products. Readily visualized are the env-coded Pr80env and Pr15(E), which are both precipitable by anti-p15(E) (lane 7) and anti-Rauscher gp70 (not shown). By use of specific antisera, various gag-related molecules were identified. Anti-p30 (lane 3), anti-12 (lane 4), and anti-p15 (lane 5) all precipitated their respective mature structural products along with the polyprotein precursor Pr65gag and a 94,000-molecular weight band that likely corresponds to the glycosylated cell surface polyprotein, gp94gag.

In addition to these commonly recognized viral proteins and precursor molecules that have been defined for a variety of MuLV-infected cells (25-27), a major polypeptide of 36,000 molecular weight (p36) was present in anti-gag (Fig. 1B).
Identification of p36 in a RadLV-induced lymphoma cell line. (A) Immunoprecipitates of 4-h $[^{35}S]$leucine-labeled AQR-V cell extracts were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and visualized by fluorography. Antisera used include normal goat serum (lane 1), goat anti-AKR MuLV (lane 2), goat anti-p30 (lane 3), goat anti-p12 (lane 4), goat anti-p15 (lane 5), goat anti-BV2 (lane 6), and monoclonal anti-p15(E) ascites (lane 7). (B) Virus purified from 20-h $[^{35}S]$leucine-labeled AQR-V culture supernatant was immunoprecipitated with goat anti-AKR/MuLV (lane 2) or electrophoresed intact (lane 1). Labeled supernatant cleared of virus was also immunoprecipitated with goat anti-AKR-MuLV (lane 3). The position of molecular weight standards is indicated, as are the identified viral proteins.

1A, lanes 3–5) immunoprecipitates, but absent from anti-env analyses (Fig. 1A, lane 7) for anti p15E; similar results were obtained when several anti-gp70 reagents were used, data not shown) of AQR-V cellular extracts. Anti-p30 (lane 3), anti-p12 (lane 4), anti-p15 (lane 5), and anti-p10 (not shown) precipitate p36 in different amounts, which may reflect antibody titer or avidity differences. As observed by others (28), the heteroantisera reactive with p10, p12, and p15 may be contaminated with low levels of antibody to p30; therefore, the precise antigenic determinants present on p36 are ambiguous. However, the observation that anti-p12 (lane 4) precipitates larger amounts of p36 than of p30 suggests that its reactivity with p36 is not due solely to these contaminating anti-p30 antibodies. It may indicate that the anti-gag activity seen results from p12 sequences present in p36.
p36 Is Not Incorporated into Virions. It has been reported that RadLV preparations may contain mixtures of viruses differing in host range and tissue tropism (29). To determine whether p36 was the product of a selected component among these viruses that could be passaged from AQR-V supernatant, culture fluid from the cell line was inoculated onto mouse Sc-1, mink CCL64, RL-12, and L691-6 cells. The latter two lines are non-producer radiation-induced lymphomas that are susceptible to MuLV infection, and reported to allow replication of thymotropic virus (19). The infected cultures were followed for the onset of viral expression by an indirect immunofluorescence assay using anti-MuLV antibody; after 5–8 passages all cultures became 80–100% viral antigen positive. The cultures were then metabolically labeled, extracted, and analyzed as before (Fig. 2) to test for production of p36. It was found that neither the infected Sc-1 cells, replicating ecotropic virus(es), nor the infected CCL64 cells replicating xenotropic virus synthesized detectable p36 (Fig. 2A, lanes 1 and 2, respectively). By contrast, it was quite easy to immunoprecipitate viral structural molecules from these infected cell lines (data not shown).

Figure 2. p36 absence from cells infected with passaged AQR-V viruses. All cells were labeled for 4 h with [3H]leucine (A and B) or [35S]methionine (C), extracted, and equal amounts of TCA-precipitable radioactivity used for immunoprecipitation analysis. AQR-V is included for comparison. (A) Goat anti-BV2 immunoprecipitate of infected CCL64 cells (lane 1), infected Sc-1 cells (lane 2), and AQR-V (lane 3). (B) Goat anti-AKR MuLV (lanes 1–3) and goat anti-p12 (lanes 4–6) were used to precipitate AQR-V (lanes 1 and 4), uninfected RL-12 cells (lanes 2 and 5), and RL-12 cells infected with AQR-V supernatant virus (lanes 3 and 6). (C) Analysis of [35S]methionine-labeled cells; normal goat serum (lane 7), goat anti-AKR MuLV (lanes 1–3) and goat anti-p12 (lanes 4–6) were used to precipitate AQR-V (lanes 1 and 6), uninfected L691-6 cells (lanes 2 and 5), and L691-6 infected with AQR-V supernatant virus (lanes 3 and 4).
The RL-12 and L691-6 cells also were found to replicate virus passaged from AQR-V supernatant. Fig. 2, B and C show analyses of their cellular lysates for presence of p36. Extracts of uninfected RL-12 cells labeled with [3H]leucine and immunoprecipitated with anti-viral antibodies (Fig. 2B, lanes 2 and 5) show no detectable synthesis of viral proteins. Following infection with AQR-V supernatant, the cells synthesize Pr65gp and p30, which are precipitated with either anti-AKR MuLV (lane 3) or anti-p12 (lane 6). However, no p36 band is visible in these precipitates. Equal amounts of TCA-precipitable radioactivity of [3H]-leucine-labeled AQR-V cellular extract were also immunoprecipitated and co-electrophoresed for comparison (lanes 1 and 4). The levels of env and p30 proteins expressed by infected RL-12 cells is proportionally lower than in AQR-V cells over this labeling period, although the radioactivity in the Pr65gp bands is comparable.

A similar analysis of [35S]methionine-labeled L691-6 cell extracts is presented in Fig. 2C. The precipitation using normal goat serum (lane 7) demonstrates the increased background of nonspecific bands detected using 35S-labeled lysates. Uninfected L691-6 cells precipitated with anti-AKR MuLV or anti-p12 show no evidence of gag protein synthesis. But L691-6 infected with AQR-V supernatant, synthesize amounts of Pr65gp and p30 (lanes 3 and 4) comparable to AQR-V cells (lanes 1 and 6). Nevertheless, p36 is not detectable in extracts of infected L691-6 precipitated with anti-AKR MuLV or anti-p12 (lanes 3 and 4).

These findings were reproduced using infectious virus derived from other RadLV-induced lymphoma cells that produced p36 (see Table I). Recipient cells infected with virus from the B6-V1 and B10.S-V lines and from in vivo primary RadLV lymphomas were found to produce easily precipitable env and gag proteins, but no p36 was detectable in anti-virus immunoprecipitates (data not shown).

Since p36 was not detected after passage of AQR-V viruses in other cells, we checked AQR-V supernatant (which contains abundant amounts of infectious

| Cell line   | Envelope-related (gp70) | Env-related (p15E) | Gag-related (p30) | Gag-related (p36) |
|-------------|-------------------------|--------------------|-------------------|-------------------|
| RadLV-induced: |                         |                    |                   |                   |
| AQR-V       | +                       | +                  | +                 | +                 |
| B10.G-V     | +                       | +                  | +                 | +                 |
| B10.S-V     | +                       | +                  | +                 | +                 |
| B6-V1       | +                       | +                  | +                 | +                 |
| Radiation-induced: |                   |                    |                   |                   |
| RL-12       | ±                       | ±                  |                     |                    |
| L691-6      | ±                       | ±                  |                     |                    |
| AQR-X1      | +                       | +                  | +                 | +                 |
| B6-X1       | +                       | +                  | +                 | +                 |

* Determined by gel electrophoresis of specific immunoprecipitates of [3H]leucine-labeled cell extracts.

1 ± indicates presence of very low quantities of protein as visualized on fluorograms as faint bands following long exposures.
viruses) directly for p36 activity. To test if p36 were incorporated into virions or secreted into culture medium, virus banding at 1.14-1.16 g/cm² was purified from 24-h radiolabeled AQR-V supernatant. The virus was pelleted, extracted, and immunoprecipitated as above. No p36 was detectable in the virion preparations precipitated with anti-AKR-MuLV (Fig. 1B, lane 2), although several of the mature viral polypeptides are readily apparent. Aliquots of culture supernatant from which virus had been pelleted were also immunoprecipitated with anti-AKR-MuLV (lane 3). The supernatant contained p30 and gp70, perhaps from lysed virions, but no p36 was found. These findings strongly suggest that p36 is either encoded by a defective viral genome (i.e., no replication capacity) or by cellular sequences which contain fused viral information (i.e., gag coded).

p36 Independence from gag Gene Products Synthesis Suggests it Might Be the Product of Fused Viral gag and Cellular Sequences. The RadLV-induced B10.G-V lymphoma line appears unique in that it produces relatively large amounts of p36 in the absence of detectable p30 or Pr65<sup>gag</sup> (Fig. 3A, lanes 1 and 2). This lack of p30 synthesis allowed us to establish the independence of p36 biosynthesis from that of other gag-related proteins. B10.G-V cells were analyzed in a pulse-chase labeling experiment and immunoprecipitated with anti-AKR-MuLV (Fig. 3B). Significant amounts of p36 have been synthesized after the 20-min pulse period, while no Pr65<sup>gag</sup> or p30 bands are apparent. The quantity of p36 peaks at ~30 min of chase, after which there is a gradual decline. The kinetics of p36 synthesis in B10.G-V cells are not substantially different from the p36 kinetics in a cell line such as AQR-V, which produces large quantities of viruses. Thus, in AQR-V cells most p36 is present after the initial 20-min pulse, along with a prominent Pr65<sup>gag</sup> band (Fig. 3C). As radioactivity is chased from Pr65<sup>gag</sup>, it does not appear to end up in p36, which also gradually decreases over the chase period. However, radioactivity in p30 increases over the first hour of chase, consistent with its derivation from cleavage of Pr65<sup>gag</sup>.

To confirm directly that p36 is not an intermediate between Pr65<sup>gag</sup> and p30, the radiolabeled proteins were purified by immunoprecipitation and NaDodSO₄/polyacrylamide gel electrophoresis, and subjected to two-dimensional tryptic peptide mapping (Fig. 4). Several p36 peptides differ from the p30 and Pr65<sup>gag</sup> peptides, emphasizing the fact that novel, cellular sequences are probably part of the p36 molecules.

p36 Is Phosphorylated In Vivo, but Does Not Express Detectable Protein Kinase Activity. The fact that p36 appears to be derived from fused gag (viral) and cellular genetic information and does not appear to be encoded by a replication competent virus, makes this protein homologous in many respects with several viral oncogene proteins described in other systems. Many of these proteins, exemplified by AbLV p120, are phosphorylated and/or have protein kinase activity (24, 26, 30). Therefore, we wished to examine p36 with respect to these two parameters. The B10.G-V cell line was metabolically labeled with [³²P]-orthophosphate, and cellular extracts subjected to immunoprecipitation and NaDodSO₄/polyacrylamide gel electrophoresis. Fig. 3A shows the results of immune precipitations using normal serum (lane 3) or anti-AKR MuLV (lane 4). The p36 protein is the most prominently labeled phosphoprotein specifically precipitated from the B10.G-V extract with anti-viral antisera. Similar results
were obtained with anti-gag sera. Analysis of $^{32}$P-labeled AQR-V cells also revealed a phosphorylated p36 band which co-migrated with $^3$H-labeled p36 on gel electrophoresis (data not shown).

The in vivo $^{32}$P-labeled p36 was subjected to acid hydrolysis and two-dimensional analysis for identification of phosphorylated amino acids (23). By this method, p36 was found to contain abundant phosphoserine, but no phosphotyrosine or phosphothreonine were detected (data not shown).

We also tested anti-gag protein immune precipitates of AQR-V and B10.G-V extracts for in vitro phosphorylating activity using $[^{32}P]ATP$ by the protocol of Witte et al. (24). Neither p36 nor other proteins precipitated by anti-viral antisera became detectably phosphorylated in these assays (data not shown).

*p36 Is Associated with RadLV-induced Transformation.* Several other thymic
lymphoma cell lines were assayed for production of p36, including other than AQR-V RadLV-induced lines and lines adapted from thymomas caused in vivo by x-irradiation. These data are shown in Figs. 2 and 5(A) and summarized in Table I. Although the amounts of p36 synthesized in pulse-labeling experiments varied among the RadLV-induced cell lines, all expressed detectable levels of p36. None of the radiation-induced lymphoma cell line extracts contained detectable p36. Notably, two of these lines, AQR-X1 and B6-X1, release infectious virus into the culture medium and express readily precipitable gag proteins (Fig. 5A, lane 5), yet do not produce p36. P36 was also detected in extracts of primary RadLV-induced tumors but was not detected in extracts of normal thymocytes of C57BL mice (Fig. 5B, lanes 1–3). In addition, primary spontaneous thymomas of AKR/J mice, which do express the MuLV structural proteins and their precursors, do not express p36 (Fig. 5B, lane 4). These findings are consistent with a role for p36 in RadLV leukemogenesis.
FIGURE 5. p36 expression in (A) lymphoma cell lines and (B) in vivo-derived thymic cells of varied etiology. (A) Goat anti-BV2 immunoprecipitates of [3H]leucine-labeled cell extracts: RadLV induced cell lines B6-V1 (1) and B10.S-V (2); x-irradiation induced cell lines RL-12 (3), B10.G-X (4), and AQR-X (5). (B) Goat-anti-BV2 immunoprecipitates of [3H]leucine-labeled normal B10.Q mouse thymocytes (1), and thymomas from RadLV-inoculated B10.AQR (2) and B10.Q (3) mice, and a thymoma spontaneously arising in an AKR/J mouse (4). Comparable amounts of TCA-precipitable radioactivity were used for each analysis.

Discussion

Our analysis of the polypeptides precipitated by anti-viral antisera from RadLV-induced lymphoma cell lines has resulted in the identification of a novel 36,000-molecular weight gag-associated protein, p36. This polypeptide appears to be specific to in vivo transformation by RadLV (and persists in in vitro grown RadLV-transformed cell lines), as it is present in several thymoma cell lines induced in vivo by RadLV, but not in normal thymocytes, Sc-1 mouse fibroblasts, nor any cell line adapted from thymic tumors induced in vivo by X-irradiation.

Several lines of evidence suggest that p36 is not merely a gag processing intermediate, such as Pr40gag. The kinetics of appearance and loss of p36 during pulse-chase experiments show that p36 is synthesized rapidly, without the considerable lag seen for p30. The production of p36 in the B10.G-V lymphoma lines, which does not release infectious virus into the culture medium and lacks detectable synthesis of Pr65gag and p30 over the entire pulse-chase period, supports the independence of p36 expression from normal gag processing. Furthermore, the finding that p36 is precipitated by anti-p12 and anti-p15 and is phosphorylated in vivo are unusual for a gag intermediate of its size. Of viral molecules in this molecular weight range, Pr40gag contains only p30 linked to p10 and does not incorporate radiolabeled phosphate (26). Finally, the comparison of two-dimensional maps of [3H]leucine containing tryptic peptides indicates
that Pr65\textsuperscript{p6} and p30 share several peptides as expected but p36 has unique peptides.

The failure of p36 to appear in AQR-V supernatants containing virus-infected cells with a known capacity to replicate a wide range of murine leukemia viruses strongly suggests that it is either encoded for by a defective virus or it is a cellular protein(s) expressed only in cells transformed by RadLV. The fact that it is precipitated by viral anti-gag antibodies, suggests it might be encoded in a gag-cell gene fusion and be the product of a defective component of RadLV that participates in lymphomagenesis. This situation would be analogous to the acute murine leukemia viruses AbLV (7, 31) and spleen focus-forming virus (SFFV) (32, 33). The expression of p36 in the absence of helper virus gag protein synthesis in the B10.G-V line could then be attributed to loss of the nonessential helper function while acquiring the capability of p36 synthesis that would be required for maintenance of the transformed leukemic phenotype.

Consistent with this hypothesis are the additional features of p36 that resemble those of transformation-specific proteins of certain acute defective leukemia viruses, such as AbLV p120 (7): p36 is precipitable with anti-gag but not anti-env sera, and is found phosphorylated in vivo. P36 is not expressed by fibroblasts or lymphoid cells infected in vitro with viruses passaged from AQR-V supernatant. Similarly, as for AbLV, p120 is not expressed by L691-6 cells infected in vitro with AbLV and Mo-MuLV (7). Like AbLV p120 (7) and SFFV gp55 (33, 34), p36 is not found incorporated in significant amounts into mature virions. Unlike AbLV p120, p36 was not found to contain phosytosine and did not show any detectable ATP-specific protein kinase activity. However, these findings were not unexpected, as the phosphorylation of tyrosine has not been found associated with tumorigenesis by MuLV; additionally several other defined onc gene products, including those of the murine sarcoma viruses (35, 36), do not possess any ATP-specific protein kinase function.

It is possible that p36 may correspond to the 36,000-molecular weight in vitro translation product of the defective 5.6-kb RNA of leukemogenic RadLV/VL\textsubscript{s} described by Manteuil-Brutlag et al. (10), which has never been demonstrated in vivo in lymphoma cells. The presence of the 5.6-kb RNA of RadLV was correlated in their studies with leukemogenic potential. When isolated 5.6-kb RNA, which resembles other acute retroviruses in containing a centrally substituted region, was used to program a reticulocyte lysate, proteins of 100,000, 36,000, and 30,000 molecular weight were translated (10). These could be precipitated by antibody against p12 and p15. In our analysis of RadLV-induced lymphoma lines, we have failed to identify a 100,000 molecular weight polypeptide with these characteristics. The products identified by Manteuil-Brutlag et al. (10) were not precipitated with anti-p30 antisera, while p36 is readily detected in anti-p30 immune precipitates of cellular extracts. This may indicate lack of identity of these proteins, or reflect heterogeneity of anti-viral heteroantisera and contaminating reactivities. The relatedness of these proteins remains to be clarified by further investigation.

As an alternative to expression of p36 from defective RadLV RNA molecules, p36 might be induced from the host cell genome by transformation with RadLV. In this case, p36 would resemble the 53,000-molecular weight protein induced
in a number of cell types of different modes of transformation (37), or the c-myc gene product, whose expression is thought to be increased in ALV-induced lymphomas of chickens by nearby integration of the ALV provirus promoter region (38). That RadLV may act in such a fashion is supported by the suggestion that RadLV integrates into a preferred site in the host genome (39). The consistent size of p36 among independently derived RadLV lymphomas, however, would suggest a mechanism of induction differing from ALV promoter insertion, which can occur at variable distances from the c-myc gene. Current work in our laboratory should help distinguish between these possibilities.

Summary

Radiation leukemia virus (RadLV) causes thymic lymphoma in 90% of susceptible mice after a latent period of several months. The virally encoded polypeptides produced by RadLV-induced lymphoma cells were analyzed by immunoprecipitation and NaDodSO4/polyacrylamide gel electrophoresis. Along with the expected precursor and mature forms of gag and env gene products, a polypeptide of 36,000 molecular weight (p36) was precipitated by anti-gag antiserum. It was not precipitable by normal sera or anti-env antibodies. Like the gag-associated fusion proteins of some acute leukemia viruses, p36 was found to be phosphorylated in vivo, although it lacked detectable ATP-specific protein kinase activity in vitro. By kinetics during pulse-chase labeling experiments and by comparison of two-dimensional tryptic peptide maps, this protein is not an intermediate in gag precursor processing. One lymphoma cell line is described that resembles a nonproducer RadLV-transformant, synthesizing relatively large amounts of p36 in the absence of Pr66\textsuperscript{Kr} or p30 production. Several RadLV-induced lymphoma cell lines also produce p36, while it was not detectable in the radiation-induced lines tested. In addition, p36 was not produced by mouse or mink fibroblasts or cultured thymocyte cell lines infected with virus passaged from the RadLV-induced lymphomas. We conclude that p36 may represent a previously unrecognized transformation-related protein induced directly or indirectly by infection with RadLV.

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