Monoclonal Antibodies Specific to Transforming Polyproteins Encoded by Independent Isolates of Feline Sarcoma Virus

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Hybridomas secreting monoclonal antibodies directed against polyprotein gene products of the Gardner, Snyder-Theilen, and McDonough strain of feline sarcoma virus have been isolated. Antibody produced by one hybridoma recognizes immunological determinants localized within a feline leukemia virus *gag* gene structural component (p15) common to polyproteins encoded by each feline sarcoma virus isolate while antibody produced by a second is specific for p30 determinants unique to P170^{*gag-fms*}. Additional hybridomas secrete antibody directed against *v-fes* specific determinants common to the Gardner and Snyder-Theilen feline sarcoma virus-encoded polyproteins and to *v-fms* determinants unique to P170^{*gag-fms*} polyprotein. GA P110^{*gag-fes*} and ST P85^{*gag-fes*} immunoprecipitated by antibody directed against p15 exhibit readily detectable levels of protein kinase activity but lack such activity when precipitated by antibody specific for their acquired sequence (*v-fes*) components. P170^{*gag-fms*} immunoprecipitated by monoclonal antibody to either p15 or p30 lacks detectable levels of autophosphorylation but represents a substrate for the GA P110^{*gag-fes*} and ST P85^{*gag-fes*} enzymatic activities. These findings argue that the *v-fes*-associated protein kinase represents an intrinsic property of the *v-fes* gene product and recognizes tyrosine acceptor sites within polyprotein gene products of all three strains of feline sarcoma virus.

Retroviruses with acute transforming function represent genetic recombinants between cellular genes and nontransforming viral sequences (5, 16). These viruses provide a potential means for identifying and molecularly cloning cellular genes involved in malignant transformation. This approach has led to the identification of two different cellular genetic loci represented within known acute transforming viruses of feline origin (10, 25). These include the *v-fes* gene common to Gardner and Snyder-Theilen strains of feline sarcoma virus (FeSV) (3, 9, 11, 14, 26) and the *v-fms* gene which is unique to McDonough FeSV (3, 7, 27). The translational products of these acquired cellular sequences are expressed covalently linked to amino terminal feline leukemia virus (FeLV) structural components in the form of high-molecular-weight (M_r) polyproteins (22, 24). These include 85,000- and 110,000- M_r polyproteins encoded by two independent isolates of Snyder-Theilen FeSV (22, 24, 26), and 110,000- and 170,000- M_r polyprotein gene products of Gardner (3, 26) and McDonough (3, 27) FeSV, respectively. The Snyder-Theilen and Gardner FeSV-encoded polyproteins contain FeLV p15 and p12 amino terminal structural components (3, 22, 24, 26), whereas the McDon-

ough FeSV translational product, designated P170^{*gag-fms*} according to convention (5), contains both of these proteins in addition to an FeLV p30 structural component (3, 27).

Functional analysis of the Gardner and Snyder-Theilen FeSV gene products has led to the identification of a protein kinase activity with specificity for tyrosine residues (1, 20, 28). The involvement of this activity in transformation has been demonstrated through the analysis of a series of transformation-defective viral mutants (8, 18), and single preferred tyrosine phosphorylation acceptor sites have been described (4; F. H. Reynolds, Jr., S. Oroszlan, J. Blomberg, and J. R. Stephenson, *Virology*, in press). Analogous enzymatic activities have been described in association with several other well-defined transforming proteins (16), including those encoded by an avian retrovirus transformation specific gene (*v-fps*) genetically related to the mammalian *v-fes* gene (23). In contrast, the *v-fms* gene product, represented within the McDonough FeSV-encoded polyprotein P170^{*gag-fms*}, lacks detectable enzymatic activity when assayed under similar conditions (18). Although there has been a report of low levels of both serine and tyrosine phosphorylation of P170^{*gag-*}

fms in immunoprecipitates, the possibility that this is due to contaminating cellular enzymes has not been excluded (2).

Because of the striking conservation of the *fes-fps* genetic locus throughout vertebrate evolution and its representation within several independent feline and avian transforming retrovirus isolates (23), it represents a likely candidate for involvement in spontaneously arising human cancers. In an effort to better define the *fes* and *fms* gene products and, in particular, to determine the functional significance of proteins encoded by their cellular homologues, specific immunological reagents are required. For this purpose, we have undertaken the development of hybridomas producing antibody with specificity for both the viral and acquired sequence-encoded components of polyprotein gene products of the Gardner, Snyder-Theilen, and McDonough strains of FeSV.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.). Cell lines used have been described previously and include a highly contact-inhibited Fisher rat embryo line, FRE 3A (21), as well as subclones of FRE 3A cells nonproductively transformed by the Gardner, Snyder-Theilen, and McDonough strains of FeSV, designated GA-FeSV FRE C14, ST-FeSV FRE C10, and SM-FeSV FRE C13, respectively (18, 21). In addition, CCL64 mink cells and nonproductively transformed subclones designated GA-FeSV 64 C12, ST-FeSV 64 C156, and SM-FeSV 64 C115 (18, 21) were used. A clonal isolate of feline leukemia virus (FeLV) subgroup A, propagated on CCL64 mink cells has been previously described (15). The Lou rat myeloma line, Y3-Ag 1.2.3 (13) was a gift of C. Milstein, Cambridge, England.

Isolation of hybridoma clones. Weanling Fisher rats were immunized subcutaneously with 1.0×10^7 disrupted cells resuspended in 0.2 ml of Freund complete adjuvant and 2 weeks later were challenged with 1.0×10^6 live cells. Animals in which large tumors developed at the site of inoculation, and subsequently regressed, were reimmunized with 1.0×10^7 live cells every 2 weeks thereafter for a period of 3 to 4 months. From 3 to 5 days after the final immunization, spleens were removed, and splenic lymphocytes (2.0×10^8) were fused with an equal number of Y3-Ag 1.2.3 rat myeloma cells by previously described procedures (13). Fused cells were resuspended in Dulbecco modified Eagle medium supplemented with 15% fetal calf serum, 1 mM sodium pyruvate, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine and were plated in microtiter plates (Costar, Cambridge, Mass.) at a concentration of 1.0×10^6 cells per well. After 10 to 12 days of incubation at 37°C, culture fluids of wells containing actively growing hybrid colonies were harvested, pooled in groups of 3 to 10, and screened by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cultures scored as positive were subcloned initially at a concentration of five cells per well and subsequently as single cell clones. To

obtain large pools of high-titer antibody, hybridoma cells (3.0×10^7) were cultured for 2 hours in serum-free Dulbecco modified Eagle medium and injected into the peritoneal cavity of pristane-pretreated Lou rats. Ascites fluid was harvested within 7 to 10 days, clarified by centrifugation at $2,500 \times g$ for 10 min, and stored at -20°C .

Metabolic labeling, immunoprecipitation, and SDS-PAGE. Tissue culture cells were radioactively labeled by incubation for 2 h in medium containing either $^{32}\text{P}_i$ (100 $\mu\text{Ci}/\text{ml}$, carrier-free) or [^{35}S]methionine (100 $\mu\text{Ci}/\text{ml}$, 800 Ci/mmol, New England Nuclear Corp., Boston, Mass.). Labeled cell monolayers were washed twice in serum-free Dulbecco modified Eagle medium and disrupted at 4°C by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM sodium phosphate (pH 7.2)–0.9% NaCl–1% Triton X-100–0.5 sodium deoxycholate–0.1% SDS (PBSTDS). Immunoprecipitation analysis was performed by addition of either 0.1 ml of hybridoma culture fluid or 0.2 to 1.0 μl of ascites fluid, 0.1 ml of goat anti-rat immunoglobulin G (IgG), and 0.1 ml of a 10% suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) to 2.0 ml of labeled extract and by incubation for 18 h at 4°C . Immunoprecipitates were collected by centrifugation at $2,000 \times g$ for 10 min, washed repeatedly in PBSTDS, suspended in 20 μl of 0.65 M Tris-hydrochloride (pH 6.7) 1% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromophenol blue, heated for 2 min at 90°C , and analyzed by SDS-PAGE (21).

In vitro phosphotransferase reactions. Tissue culture cells (1.0×10^7) were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM Tris-hydrochloride (pH 7.2) 200 mM NaCl, 1.0% Triton X-100, and 5 mM MnCl_2 (TBSTM), and lysates were clarified by centrifugation at $100,000 \times g$ for 1 h. After the addition of 10 μl of specific antiserum, lysates were incubated for 18 h at 4°C , 100 μl of a 10% suspension of protein A-Sepharose was added, and incubation continued for 2 h at 4°C . Immunocomplexes were collected by centrifugation for 10 min at $2,000 \times g$, washed in TBSTM buffer, resuspended in the same buffer containing 2.5 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP (2,000 Ci/mmol, New England Nuclear Corp., Boston, Mass.), and incubated for 10 min at 30°C . Reactions were terminated by the addition of 3 ml ice-cold PSBTDs, and immunoprecipitates were washed and analyzed by SDS-PAGE as described above.

Phosphoamino acid analysis. Proteins were digested by incubation of SDS-PAGE slices in tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Biochemicals, Freehold, N.J.; 50 $\mu\text{g}/\text{ml}$ in 0.05 M ammonium bicarbonate [pH 8.0]) for 6 h at 37°C . Hydrolysis was performed by a 1-h incubation at 100°C in 0.2 ml of 6 N HCl under nitrogen. After hydrolysis, HCl was removed by lyophilization, and phosphoamino acids were dissolved in 10 μl of acetic acid, formic acid, and water (15:5:80) and spotted on thin-layer chromatography plates. Two-dimensional electrophoretic and chromatographic analysis was performed as described previously (26).

Immunoprecipitation of ^{125}I -labeled FeLV structural proteins. FeLV structural proteins were purified and ^{125}I -labeled to high specific activity by previously described methods (15). Hybridoma ascites fluids were tested at serial twofold dilutions for immunoprecipitation of ^{125}I -labeled FeLV proteins. Reaction mix-

TABLE 1. Isolation of hybridomas with specificity for FeSV-encoded polypeptides

Immunogen	No. of rats immunized	No. of hybrid clones ^a		Hybridoma designation
		Screened	Positive	
SM-FeSV FRE Cl3	12	1,860	3	F40, F161, F170
GA-FeSV FRE Cl4	6	1,350	1	F123
ST-FeSV FRE Cl10	19	2,280	3	F72, F113, F115

^a Culture fluids of hybrid clones were scored for antibody to the appropriate FeSV-encoded polypeptides by immunoprecipitation and SDS-PAGE as described in Materials and Methods. Clones scored as positive included only those which continued to produce high-titer antibody through several successive cycles of subcloning.

tures containing 0.01 M Tris-hydrochloride (pH 7.8), 1.0 mM EDTA, 0.4% Triton X-100, 1% bovine serum albumin, 0.05 M NaCl, 10,000 cpm of ¹²⁵I-labeled FeLV protein, and 2.0 µl of normal rat serum in a total volume of 0.2 ml were incubated for 3 h at 37°C and a further 18 h at 4°C. After the addition of 0.025 ml of goat serum directed against rat IgG, incubation at 37°C for 1 and 3 h at 4°C, immunoprecipitates were collected by centrifugation at 2,500 × g for 15 min. Radioactivity was either measured directly in a Searle model 1285 gamma counter, or ¹²⁵I-labeled proteins were immunoprecipitated and subjected to SDS-PAGE analysis as described above.

Immunodiffusion. Immunoglobulin subclasses were determined by the Ouchterlony technique by using immunodiffusion agarose gels (Hyland Diagnostics, Deerfield, Ill.). Immunoglobulins were prepared from conditioned media obtained from twice-cloned, growing hybrid cell lines. The supernatant fluids were dialyzed in phosphate-buffered saline for 24 h and lyophilized, and the resulting preparations were reconstituted in distilled water as a 10-fold concentrate. Typing antisera to the rat IgG subclasses and rat IgM were obtained from Miles Laboratories, Inc., Elkhart, Ind. Precipitin lines were read after incubation for 24 h at 37°C.

RESULTS

Hybridomas with specificity for FeSV-encoded polypeptides. As an initial step in the generation of hybridoma clones secreting antibody against FeSV-encoded polypeptides, we isolated a series of Fisher rat embryo cells nonproductively transformed by the Gardner, Snyder-Theilen, and McDonough strains of FeSV. These clones were highly tumorigenic generally producing progressively growing fibrosarcomas in newborn and weanling Fisher rats which eventually killed their host. However, when rats were preimmunized with disrupted cells, before tumor challenge, resulting tumors frequently regressed. After repeated immunization of such

animals with large numbers of transformed cells, high-titered antibody was found against the appropriate FeSV polypeptide gene product.

Spleen cells from 37 hyperimmunized rats were fused with the Y3-Ag 1.2.3 rat myeloma line, and cellular fluids from approximately 100 to 150 hybrid clones from each fusion were assayed for antibody against the appropriate viral polypeptide. Of the 12 to 15 clones initially scored as positive by immunoprecipitation SDS-PAGE analysis, 8 were highly stable through repeated cycles of subcloning. These included single hybridomas from spleen cells of rats immunized with either McDonough or Gardner FeSV-transformed cells and three hybridomas from spleen cells of animals immunized with cells transformed by Snyder-Theilen FeSV (Table 1). An additional hybridoma, F118, secreted

FIG. 1. Immunoprecipitation SDS-PAGE analysis of monoclonal antibodies in culture fluids of hybrid clones prepared from spleen cells of rats immunized with syngeneic FeSV-transformed cells. Cell lines including SM-FeSV FRE Cl3 (IA, IIA to C), ST-FeSV FRE Cl10 (IB, IC, and ID), and GA-FeSV FRE Cl4 (IE) were metabolically labeled by 30-min incubation in [³⁵S]methionine-containing medium (100 µCi/ml), were immunoprecipitated with 0.1 ml of undiluted culture fluid from individual hybrids including F40 (IA, IIA), F72 (IB), F113 (IC), F115 (ID), F123 (IE), F170 (IIB), and F161 (IIC) and were analyzed by SDS-PAGE as described in Materials and Methods. Molecular weight standards are shown in Fig. 2 and include ¹⁴C-labeled myosin (200,000), phosphorylase B (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome c (12,000).

TABLE 2. Analysis of monoclonal antibodies for immunoglobulin subclass and immunoprecipitation of FeLV structural proteins

Antibody	Immuno- globulin class ^a	Immunoprecipitation of ¹²⁵ I-labeled proteins (titer log ₁₀) ^b				
		p15	p12	p30	p10	gp70
Hybridoma						
F40	IgG1	<1.0	<1.0	6.2	<1.0	<1.0
F123	IgG2a	<1.0	<1.0	<1.0	<1.0	<1.0
F72	IgG1	5.3	<1.0	<1.0	<1.0	<1.0
F113	IgG2b	<1.0	<1.0	<1.0	<1.0	<1.0
F115	IgM	<1.0	<1.0	<1.0	<1.0	<1.0
F118	IgG2c	<1.0	<1.0	<1.0	<1.0	<1.0
F161	NT	<1.0	<1.0	<1.0	<1.0	<1.0
Goat anti- FeLV		3.8	3.2	5.3	2.1	3.9

^a Hybridoma culture fluids were analyzed with respect to immunoglobulin class and subclass by immunodiffusion as described in Materials and Methods.

^b Immunoprecipitation of ¹²⁵I-labeled FeLV structural proteins was performed as described in Materials and Methods. Antibody titers are expressed as the reciprocal of the highest dilution of ascites fluid at which 10% precipitation was observed and represent mean values from three separate determinations.

antibody specific for an 85,000-*M_r* cellular phosphoprotein while F161 immunoprecipitated both P170^{gag-fms} and its major 120,000-*M_r* cleavage product. F170 antibody reacted predominantly with the P170^{gag-fms} cleavage product but has not as yet been characterized further. Culture fluids from each of the hybridomas, with the exception of F118, immunoprecipitated [³⁵S]methionine-labeled proteins of the expected molecular weight from both mink and rat transformed cells, but not from nontransformed control cell lines (Fig. 1). The identity of proteins immunoprecipitated by cultured fluids of these hybridomas, as viral encoded polyprotein gene product was confirmed by tryptic peptide analysis (data not shown).

Characterization of immunoglobulin class produced by clonal hybrid cell lines. As an initial step in establishing the monoclonality of the hybrid cell lines and because of the functional significance of the different classes of immunoglobulin, we determined the immunoglobulin subclass produced by each. Each hybrid clone produced a single subclass of immunoglobulin: F72 and F40 produced IgG1, F113 produced IgG2b, and F123 produced IgG2a (Table 2). Antibody produced by an additional hybrid clone, F118, described further below, was of the IgG2c subclass. Although F115 immunoglobulin was significantly reactive with the broad IgG typing antiserum it did not react with any of the

more specific IgG subclass typing sera. Upon further analysis, F115 immunoglobulin proved to be highly reactive with IgM typing antiserum, whereas immunoglobulins produced by each of the other hybrids were nonreactive.

High-titer monoclonal antibody in ascites fluids. Inoculation of cloned hybrid cells into the peritoneal cavity of Lou rats resulted in growing hybridomas that secreted high concentrations of monoclonal antibodies into the ascites fluids. Although antibodies obtained by this means exhibited the same specificity as those in the originally tested hybrid cell culture fluids, the titers were 100- to 1,000-fold higher. After a 1,000-fold dilution, ascites fluids from hybridomas F72 (Fig. 2, lane C), F113 (Fig. 2, lane E), F115 (Fig. 2, lane F), and F123 (Fig. 2, lane G) immunoprecipitated ST P85^{gag-fes} to extents comparable to those obtained with undiluted hyperimmune goat serum (Fig. 2, lane A). In contrast, ascites fluid from hybridoma F40 failed to precipitate ST P85^{gag-fes} (Fig. 2, lane B) but reacted strongly with [³⁵S]methionine-labeled P170^{gag-fms} (data not shown). Ascites fluid from F118 also immunoprecipitated a phosphoprotein of around 85,000 *M_r* from Snyder-Theilen FeSV-transformed rat (Fig. 2, lane D) and mink (data not shown) cells. On further analysis, F118 antibody proved to be directed against a cellular protein unrelated to the FeSV-encoded polyproteins and was only included in subsequent studies for control purposes.

FIG. 2. Analysis of ascites fluids from six individual hybrid clones for immunoprecipitation of ³²P-labeled ST P85^{gag-fes}. The ST-FeSV FRE C110 cells were incubated overnight in ³²P_i-containing medium and were subjected to immunoprecipitation analysis with 0.01 ml of goat anti-FeLV (A) or 10 μ l of a 1:1,000 dilution of ascites from hybrid clones F40 (B), F72 (C), F118 (D), F113 (E), F123 (F), and F115 (G). Molecular weight standards are as described in the legend to Fig. 1.

FIG. 3. Immunoprecipitation SDS-PAGE analysis of ascites fluids from hybrid clones with specificity for FeLV *gag* gene structural proteins. Cell lines including SM-FeSV FRE Cl3 (A and B); GA-FeSV FRE Cl4 (C and D); ST-FeSV FRE Cl10 (E and F); and control FRE 3A (G and H) were metabolically labeled by 30 min incubation in [35 S]methionine-containing medium (100 μ Ci/ml), immunoprecipitated with 10 μ l of 1:1,000 of ascites fluid from either the F40 (A, C, E, and G) or F72 (B, D, F, and H) hybrid clones, and analyzed by SDS-PAGE as described in Materials and Methods. Molecular weight standards are as described in the legend to Fig. 1.

Characterization of hybridomas specific to FeLV structural components of FeSV polyproteins. To define the specificities of the above-described hybridomas, we tested the possibility that one or more might produce antibody specifically directed against FeLV structural proteins. Ascites fluids from each of the hybrid clones were analyzed for immunoprecipitation of [125 I]-labeled FeLV *gag* and *env* gene-encoded structural proteins. Antibody, produced by hybridoma F40, immunoprecipitated FeLV p30, while another hybridoma clone, F72, produced antibody directed against p15 (Table 2). The identities of these antigens were confirmed by SDS-PAGE analysis of mixtures of [125 I]-labeled antigens after immunoprecipitation. Of the three remaining hybridoma ascites fluids analyzed, none contained antibody which precipitated any of the [125 I]-labeled FeLV structural proteins to a significant extent. These findings identify FeLV structural components recognized by antibodies produced by two of the hybridoma clones, F40 and F72, as p30 and p15, respectively, and raised the possibility that the antibody produced by each of four remaining clones may be directed against FeSV-acquired sequence-encoded components.

To further define monoclonal antibodies produced by hybridoma clones F72 and F40, both

were tested for immunoprecipitation of the polyprotein gene products of all three strains of FeSV. Ascites fluids from both clones precipitated P170^{*gag-fms*}, whereas only F72 antibody reacted with GA P110^{*gag-fes*} and ST P85^{*gag-fes*} (Fig. 3). This observation is consistent with the fact that, of these polyproteins, only FeSV P170^{*gag-fms*} contains an extensive p30 component (27) and localizes the immunological determinants recognized by F40 within the domain of p30 unique to P170^{*gag-fms*}.

Hybridomas producing antibody specific for *v-fes* and *v-fms* translational products. To further test whether antibodies produced by hybridomas F113, F115, and F123 may be specific for Gardner and Snyder-Theilen FeSV-acquired sequence (*v-fes*) gene products, ascites fluids corresponding to each were tested for immunoprecipitation of GA P110^{*gag-fes*}, P170^{*gag-fms*}, and FeLV Pr65^{*gag*}. Hybridoma F72 ascites fluid precipitated all three [35 S]methionine-labeled viral polyproteins (Fig. 4). Immunoprecipitation of FeLV Pr65^{*gag*} in addition to the two FeSV-encoded polyproteins is consistent with the demonstration of specific recognition of FeLV p15 structural components by F72 antibody. In contrast, although F113 and F115 specific monoclonal antibodies each precipitated GA P110^{*gag-fes*}, neither recognized either P170^{*gag-fms*} or FeLV Pr65^{*gag*} (Fig. 4). Analogous results were obtained upon analysis of F123 hybridoma culture and ascites fluids (data not shown). Moreover, monoclonal antibodies produced by

FIG. 4. Analysis of monoclonal antibodies with specificity for acquired encoded components common to the Gardner and Snyder-Theilen FeSV polyproteins. Cell lines including SM-FeSV FRE Cl3 (a), GA-FeSV FRE Cl4 (b), and FRE 3A Fisher rat cells chronically infected with FeLV subgroup A (c) were metabolically labeled by 30-min incubation in [35 S]methionine-containing medium (100 μ Ci/ml) and immunoprecipitated with 10 μ l of a 1:1,000 dilution of ascites fluid from hybrid clones F72, F113, and F115 as indicated.

all three hybridomas efficiently immunoprecipitated ST P85^{gag-fes}. These findings argue that the immunological determinants recognized by F113, F115, and F123 map within acquired sequence-encoded components common to the Gardner and Snyder-Theilen FeSV polyproteins. Hybridoma F161 antibody was apparently *v-fms* specific in that it immunoprecipitated both P170^{gag-fms} and a 120,000-*M_r* cleavage product lacking known FeLV structural components (Fig. 1) but was nonreactive with either GA P110^{gag-fes} or ST P85^{gag-fes} (data not shown).

Tyrosine phosphorylation of P170^{gag-fms} by ST P85^{gag-fes} and GA P110^{gag-fes} protein kinase activities. By use of the monoclonal antibodies specifically directed against the Gardner, Snyder-Theilen and McDonough, FeSV-encoded polyprotein structural components, it was possible to reexamine the question of protein kinase activity associated with the McDonough FeSV polyprotein gene product, P170^{gag-fms}. Under conditions in which both GA P110^{gag-fes} (Fig. 5, lane A) and ST P85^{gag-fes} (Fig. 5, lanes D and H) were efficiently phosphorylated after precipitation from either transformed mink or rat cells by

FIG. 5. Analysis of the Gardner, Snyder-Theilen, and McDonough FeSV-encoded polyproteins for protein kinase activity after immunoprecipitation by monoclonal antibodies directed against their FeLV *gag* gene structural components. Viral polyproteins were immunoprecipitated from extracts of FeSV-transformed cell clones including GA-FeSV FRE C14 (A), SM-FeSV FRE C13 (B), ST-FeSV FRE C110 (D and F), ST-FeSV 64 C156 (H), and 1:1 mixtures of SM-FeSV FRE C13 with GA-FeSV FRE C14 (C), ST-FeSV FRE C110 (E and G), and ST-FeSV 64 C156 (I) with 10 μ l of a 1:1,000 dilution of ascites fluid from hybridomas F72 (A, B, C, D, E, H, and I), and F118 (F and G). Immunoprecipitates were assayed for phosphotransferase activity by using [γ -³²P]ATP as substrate and analyzed by SDS-PAGE. Molecular weight standards are as described in the legend to Fig. 1.

FIG. 6. Phosphoamino acid analysis of P170^{gag-fms} labeled in the presence of [γ -³²P]ATP by coimmunoprecipitation with either GA P110^{gag-fes} (A) or ST P85^{gag-fes} (B), and purification by SDS-PAGE. The positions of unlabeled phosphoamino acid standards including phosphothreonine (p-thr), phosphotyrosine (p-tyr), and phosphoserine (p-ser) are indicated by dotted circles.

F72 antibody, no detectable labeling of P170^{gag-fms} (Fig. 5, lane B) was observed. The above-described monoclonal antibody recognizing a nonviral 85,000-*M_r* cellular protein, F118, was included as a control (Fig. 5, lanes F and G). Analogous results were obtained upon immunoprecipitation of P170^{gag-fms} from either of these transformed clones with hybridoma F40 antibody (data not shown). In contrast, when either GA P110^{gag-fes} (Fig. 5, lane C) or ST P85^{gag-fes} (Fig. 5, lanes E and I) were coimmunoprecipitated with the McDonough FeSV gene product, a low level of P170^{gag-fes} phosphorylation was observed. By two-dimensional phosphoamino acid analysis, the only P170^{gag-fms} residue phosphorylated to significant extent was identified as tyrosine (Fig. 6). Thus, although P170^{gag-fes} itself, lacks detectable protein kinase activity, it contains one or more acceptor sites recognized by protein kinases associated with the Snyder-Theilen and Gardner FeSV-encoded transforming proteins.

Inhibition of protein kinase activity by antibody specific to *v-fes* specific polyprotein components.

FIG. 7. Inhibition of the Gardner and Snyder-Theilen FeSV-associated protein kinase activities after immunoprecipitation of polyproteins by monoclonal antibodies against their nonstructural components. Viral polyproteins were immunoprecipitated from extracts of ST-FeSV FRE Cl10 (A, B, C, and D) and GA-FeSV FRE Cl4 (E, F, and G) transformed cells by 10 μ l of a 1:1,000 dilution of ascites fluids from hybridomas F72 (A and E), F115 (B and F), F123 (C), and F113 (D and G), assayed for phosphotransferase activity by using [γ - 32 P]ATP as substrate and analyzed by SDS-PAGE as described in the legend to Fig. 5.

It was of interest to examine the influence of antibody specifically directed against FeSV polyprotein v-*fes* encoded components on the polyprotein-associated protein kinase activities. As shown in Fig. 7A, ST P85^{gag-fes} immunoprecipitated by F72 antibody exhibited readily detectable levels of *in vitro* autophosphorylation. In contrast, when ST P85^{gag-fes} was similarly tested after precipitation with ascites fluids from each of the three hybridomas directed against ST P85^{gag-fes} nonstructural components, no detectable protein kinase was observed (Fig. 7, lanes B, C, and D). Analogous results were obtained upon analysis of GA FeSV^{gag-fes} (Fig. 7, lanes E, F, and G), with the exception that after precipitation with one of the acquired sequence-specific monoclonal reagents, F113, a low but detectable level of *in vitro* phosphorylation was observed (Fig. 7, lane G).

Further evidence for inhibition of the Gardner and Snyder-Theilen FeSV-encoded protein kinases by monoclonal antibodies specifically directed against the polyprotein nonstructural components was obtained by immunoprecipitation of either GA P110^{gag-fes} or ST P85^{gag-fes}, using 1:1 mixtures of F72 monoclonal antibody in combination with antibodies produced by the F113, F115, and F123 hybridomas. In each case, protein kinase activity, as measured by auto-

phosphorylation, was markedly lower than that observed with F72 antibody alone.

DISCUSSION

A major difficulty encountered in the determination of the role of retrovirus-encoded proteins in transformation has been the lack of appropriate immunological reagents. In particular, high-titer antisera with specificity for the acquired sequence-encoded transforming proteins of the various FeSV isolates have been unavailable. In this study, we describe the isolation of a series of hybridomas derived from spleen cells of Fisher rats immunized with syngeneic Gardner, Snyder-Theilen, and McDonough FeSV-transformed cells. These include two hybridomas producing IgG1 antibody against FeLV *gag* gene structural components, one which is directed against FeLV p15 and one which is specific for FeLV p30. The FeLV p15 determinants recognized by the first clone are shared by polyprotein gene products of all three strains of FeSV, whereas the p30 determinants recognized by the second clone are unique to P170^{gag-fms}. Three of the remaining hybridomas, one initially isolated from spleen cells of Gardner FeSV-immunized rats and two from spleen cells of Snyder-Theilen FeSV-immunized rats, lack detectable reactivity with FeLV structural proteins (Fig. 8). Of these latter monoclonal antibody reagents, one is an IgG2a, one is an IgG2b, and the remaining one is an IgM. An additional hybrid, F161, not as yet tested with respect to immunoglobulin subclass, was v-*fms* specific.

The demonstration of tyrosine-specific protein kinase activity in the Gardner and Snyder-Theilen FeSV-encoded polyproteins after immunoprecipitation by F72 hybridoma antibody supports the possibility that the observed reactivities represent intrinsic properties of the polyproteins themselves, rather than associated cellular reactivities. In contrast, P170^{gag-fms}, when assayed under similar conditions, lacked detectable protein kinase activity, but was phosphorylated in tyrosine when coimmunoprecipitated with either the Gardner or Snyder-Theilen FeSV-encoded polyproteins. These findings are consistent with our previous results indicating that P170^{gag-fms} lacks intrinsic protein kinase enzymatic activity (18) and may account for a reported low level of P170^{gag-fms} phosphorylation in immunoprecipitates obtained with conventional hyperimmune antisera (2). Whether the phosphorylation of tyrosine acceptor site(s) within P170^{gag-fms} by the Snyder-Theilen and Gardner FeSV polyprotein-associated protein kinase is of physiological significance remains to be determined.

Several lines of evidence indicate that monoclonal antibodies produced by hybridomas F113,

FIG. 8. Localization, within FeSV-encoded polyproteins, of antigenic determinants recognized by monoclonal antibodies with specificity for their structural components and acquired sequence-encoded nonstructural components.

F115, and F123 are specific for the acquired sequence (*v-fes*) components of the Gardner and Snyder-Theilen FeLV-encoded polyproteins. None of these three reagents exhibit detectable cross-reactivity with FeLV Pr65^{gag}, or with any of the FeLV *gag* and *env* gene-encoded structural proteins. In contrast to polyproteins immunoprecipitated by F72 antibody, the F113-, F115-, and F123-precipitated polyproteins lack detectable enzymatic activity, and in appropriate mixing experiments, direct inhibition of the protein kinase by these latter monoclonal reagents can be demonstrated. These findings are consistent with previous reports indicating hyperimmune sera directed against Rous sarcoma virus pp60^{src} to block autophosphorylation (6, 17), whereas in each case where antisera used for precipitation are directed against the structural components of retrovirus-encoded polyproteins, readily detectable levels of polyprotein phosphorylation are observed (16, 25). Immunoprecipitation of ST P85^{gag-fes} by antibody secreted by F123, the one hybridoma originally isolated from spleen cells of a Gardner FeSV-transformed cell-immunized rat, argues that it is specific for immunological determinants mapping within the region of GA P110^{gag-fes} shared with ST 85^{gag-fes}. Finally, the lack of detectable immunoprecipitation of P170^{gag-fms} by any of these three *v-fes*-specific reagents is consistent with previous studies indicating the translational products of the *v-fes* and *v-fms* genes to be structurally and immunologically unrelated (3, 7, 12, 26, 27).

The immunological reagents developed in this study should be of value for further studies of the functional and structural organization of the various FeSV polyprotein gene products. In particular, those monoclonal antibodies with specificity for the *fes* gene product may allow a determination of the role of this gene in naturally occurring tumors.

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