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Phosphorylation of the Nonstructural Proteins Encoded by Three Avian Acute Leukemia Viruses and by Avian Fujinami Sarcoma Virus

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The *gag* gene-related, nonstructural proteins of three avian acute leukemia viruses (namely, myelocytomatosis viruses MC29 and CMII and avian erythroblastosis virus) and of avian Fujinami sarcoma virus (FSV) isolated by immunoprecipitation from cellular lysates with anti-*gag* serum were shown to be phosphoproteins in vivo. The specific ^{32}P radioactivity of the nonstructural proteins of MC29, CMII, and FSV was significantly higher than that of helper viral, intracellular *gag* proteins. Two of these proteins, i.e., the 140,000-dalton FSV and the 110,000-dalton MC29 proteins, were also phosphorylated in vitro by a kinase activity associated with immunocomplexes. This kinase activity is either separated from these proteins or inactivated by incubation of cellular lysates with normal serum followed by adsorption to staphylococcal protein A or sedimentation at 100,000 × *g* or both. It remains to be resolved whether the 110,000-dalton MC29 and 140,000-dalton FSV proteins, in addition to being substrates for phosphorylation, also have intrinsic kinase activity.

Highly oncogenic sarcoma and acute leukemia viruses of the retrovirus family code for nonstructural proteins which are known or thought to be necessary for oncogenic function (2–5, 8, 9, 14, 15, 17). Some of these viral proteins, i.e., the 60-kilodalton (kd) *src* protein of Rous sarcoma virus (RSV) (7, 10) and the *gag* gene-related, nonstructural proteins of Abelson murine leukemia virus and feline sarcoma virus were shown either to have phosphokinase activity or to be closely associated with a phosphokinase (15, 16). The kinase activity of the 60-kd *src* gene product of RSV is primarily directed to other proteins, particularly to antibody used to precipitate this protein from cellular lysates, although autophosphorylation has also been observed (7, 7a, 10, 12). By contrast, the *gag*-related, nonstructural proteins of Abelson murine leukemia virus and feline sarcoma virus are reported to be autophosphorylating in vitro and transfer little or no phosphate to antibody used for immune precipitation (15, 16). In addition, the nonstructural proteins of RSV, Abelson murine leukemia virus, and feline sarcoma virus are reported to be phosphoproteins in vivo (6, 10, 11, 15–17). However, since the kinases associated with some of these viral proteins are very active in vitro (they work at 0°C in cellular lysates [13, 16]), an unequivocal distinction between in vivo phosphorylation and in vitro phosphorylation in extracts of ^{32}P-labeled cells is not always available, except in the case of the 60-kd *src* protein. The *src* protein of RSV was extracted from lysates of ^{32}P-labeled cells as phosphoprotein under conditions that inhibit known kinase activities in vitro, i.e., in the presence of EDTA and unlabeled ATP (11).

Here we ask whether the *gag* gene-related, nonstructural proteins of the defective avian acute leukemia viruses MC29 (myelocytomatosis virus) and CMII of the MC29 subgroup (2–4), avian erythroblastosis virus (AEV) ES4 of the AEV subgroup (1, 2, 8), and the defective avian Fujinami sarcoma virus (FSV) (3a, 9) are phosphoproteins in the cell and are associated with kinase activity. Lysates of cells transformed by one of these viruses, MC29, were examined previously for the presence of phosphoproteins immunoprecipitable by sera directed against *gag*-related proteins and were found to be negative (10). Here we report that the *gag* gene-related proteins of each of the four defective, transforming avian tumor viruses studied (MC29, CMII, AEV, and FSV) are phosphoproteins in vivo. The anti-*gag* immunocomplex of the FSV protein and, to a lesser degree, that of the MC29 protein have kinase activity in vitro which phosphorylates the FSV and MC29 proteins. Since nonviral kinase activities were found in such complexes, it is possible that these proteins function as substrates rather than as enzymes.

(A preliminary account of this work was given at the ICN-UCLA Symposium on Animal Virus Genetics at Keystone, 1980 [3a].)

To determine whether the nonstructural, *gag* gene-related proteins of MC29, CMII, AEV, and FSV are phosphorylated in vivo, these proteins were isolated by immunoprecipitation with sera against the *gag* proteins of nondefective avian tumor viruses from lysates of ^{32}P-labeled cells. Lysis of cells and immunoprecipitation was performed by a modification of the methods of Bister et al. (3, 4) and Oppermann et al. (11) in the presence of EDTA and unlabeled ATP to prevent extracellular phosphorylation of *gag* gene-related viral proteins during extraction. The immunocomplexes were adsorbed to staphylococcal protein A linked to Sepharose beads (Sigma Chemical Co., St. Louis, Mo.) and recovered by centrifugation. After solubilization of the adsorbed immunocomplexes in electrophoresis sample buffer, the proteins were analyzed by electrophoresis in polyacrylamide gels and detected by autoradiography of dried gels. In parallel experiments, the same proteins recovered from lysates of [^{35}S]methionine-labeled cells were analyzed. The intracellular ^{35}S-labeled 110-kd MC29, 90-kd CMII, 75-kd AEV, and 140-kd FSV *gag*-related proteins can also be detected as ^{32}P-proteins with the same electrophoretic and obviously similar or identical serological properties (Fig. 1). Therefore, it is concluded that the *gag*-related, nonstructural proteins of each of these viruses are phosphoproteins in vivo.

The Pr76 *gag* gene products of the nondefective helper viruses CMIIAV and FAV which are associated with the defective CMII and FSV (4, 9) were also phosphorylated (Fig. 1). However, the specific ^{32}P radioactivity of the *gag*-related, nonstructural 90-kd CMII and 140-kd FSV proteins was much higher than that of the respective helper virus Pr76 proteins, based on the respective $^{32}P/^{35}S$ ratios. The same appears to be true for the 110-kd MC29 protein (unpublished data), although this cannot be deduced directly from the data shown here since nonproducer cells transformed by MC29 in the absence of helper virus encoding Pr76 protein were analyzed (Fig. 1). In contrast, the specific ^{32}P radioactivity of the 75-kd AEV protein was very low and only detectable after prolonged autoradiography, which exposed other nonviral ^{32}P-proteins.

To examine whether the immunocomplexes of the *gag*-related proteins of the three acute leukemia viruses and of FSV have phosphokinase activity, the immunocomplexes prepared from nonradioactive cells and adsorbed to staphylococcal A protein were washed and subsequently

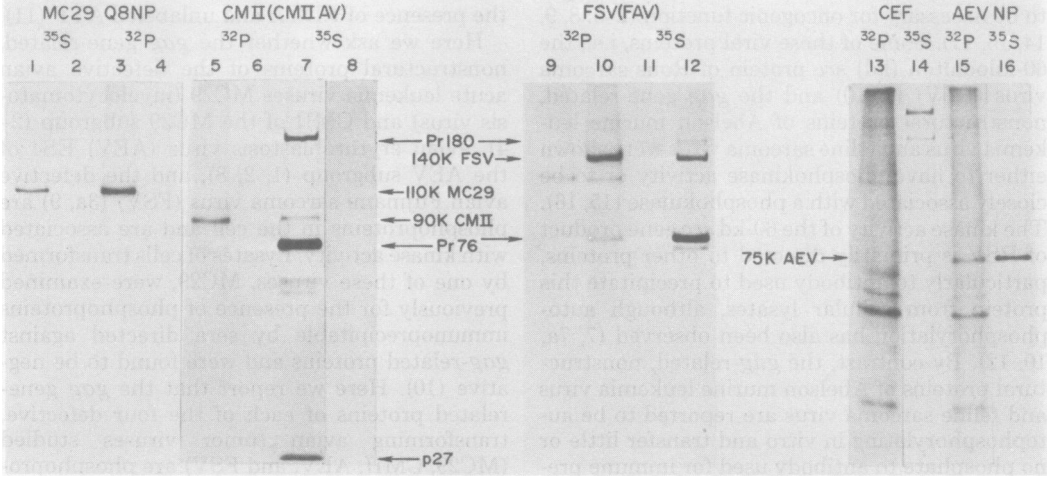

FIG. 1. *^{32}P- and ^{35}S-labeled proteins immunoprecipitated from extracts of cells transformed by acute leukemia viruses MC29, CMII, or AEV or by FSV. The control is an extract from uninfected chicken embryo fibroblasts (CEF). Q8NP is a nonproducer line of MC29-transformed quail cells (3), and AEV NP is a nonproducer line of AEV-transformed CEF, clone C23 (kind gift of G. S. Martin, Berkeley). Labeling was for 2 h with $H_3{}^{32}PO_4$ (500 μCi/ml) in phosphate-free medium or [^{35}S]methionine (100 μCi/ml) in methionine-free medium. Cell extracts were prepared in lysis buffer (3, 4) containing EDTA (1 mM) and unlabeled ATP (2 mM) to prevent phosphorylation of proteins with labeled ATP during preparation. Immunoprecipitation was carried out with normal rabbit serum (lanes 2, 4, 6, 8, 9, and 11), anti-whole RSV serum (lanes 1, 3, 5, 7, 10, and 12), or anti-p27/p19 serum (lanes 13 to 16). Staphylococcal protein A coupled with Sepharose (Sigma Chemical Co.) or fixed to bacterial cell walls (IgGsorb; Enzyme Center Inc., Boston, Mass.) was used as immunocomplex adsorbent. Gel electrophoresis was in 7.5% sodium dodecyl sulfate-polyacrylamide gels and as described previously (3, 4).*

incubated with [γ-^{32}P]ATP to allow self-phosphorylation or phosphorylation of immunoglobulin proteins to occur. Incubation was for 20 min at 20°C in 50 μl of kinase reaction buffer as described in the legend to Fig. 2. Phosphorylation of protein was detected by autoradiography after electrophoresis as described above (Fig. 1), using ^{35}S-labeled viral proteins extracted from infected cells as markers. The 140-kd FSV and the 110-kd MC29 proteins were phosphorylated in vitro, whereas the 90-kd CMII and the 75-kd AEV proteins, prepared from approximately the same number of infected cells as were used to obtain the FSV and MC29 proteins, remained essentially unphosphorylated under these conditions (Fig. 2).

Moreover, a protein of about 120 kd, nonspecifically precipitated by preimmune as well as immune serum from lysates of all virus-infected and uninfected cells (not shown) tested, was found to be phosphorylated under these conditions (Fig. 2). This indicated that a nonviral, cellular kinase activity is present in such immunoprecipitates that could have phosphorylated the *gag*-related viral proteins. Hence, the 140-kd FSV and 110-kd MC29 proteins could have functioned either as kinases or as substrates in the above in vitro reactions. A distinction between these alternatives appeared feasible when the lysates of FSV- and MC29-infected cells were analyzed for kinase activity after sequential steps of clarification by differential centrifugation and preincubation with normal rabbit serum and protein A adsorbent. Immunoprecipitates from these lysates, formed with anti-*gag* serum, were then incubated in an in vitro kinase reaction. Figure 3A shows that kinase activity strongly decreased with clarification of the lysates, although control experiments precipitating ^{35}S-proteins indicated that the *gag*-related viral proteins were not lost during clarification of ^{35}S-labeled lysates by identical procedures (Fig. 3B). This could indicate that the kinase activity of the virus-infected cells is serologically distinct from the *gag*-related proteins of these viruses and that the *gag*-related proteins of MC29 and FSV functioned as substrates rather than as kinases in our assay conditions or that a putative intrinsic kinase activity of these proteins was inactivated by these procedures. In addition, incorporation of ^{32}P-phosphate in the heavy chain of immunoglobulin was not observed in any of the immunocomplexes of the *gag*-related proteins of these defective viruses. It was, however, readily observed when the immunoprecipitate of the *src* gene product of Schmidt-Ruppin RSV-transformed cells was tested after centrifugation of the lysate at 100,000 × *g* (Fig. 3A) or after preincubation of the lysate of Schmidt-Ruppin RSV-infected cells with anti-*gag* serum, followed by precipitation with anti-*src* serum (not shown).

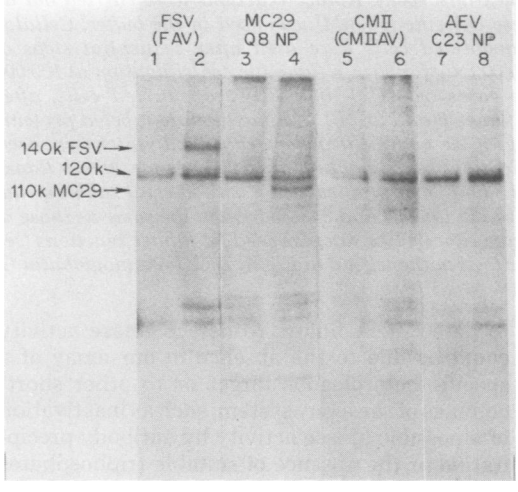

FIG. 2. *Protein kinase activity in immunoprecipitates from lysates of cells transformed by acute leukemia viruses MC29, CMII, or AEV or by FSV. Immunoprecipitates were formed at 0°C with normal rabbit serum (lanes 1, 3, 5, and 7) or anti-whole RSV serum (lanes 2, 4, 6, and 8), adsorbed by protein A coupled with Sepharose (Sigma Chemical Co.), and subsequently washed with solutions of (i) 0.5 M NaCl, 0.02 M Tris-hydrochloride (pH 7.4), 1 mM EDTA, 0.2% Nonidet P-40; (ii) RIPA buffer (5); (iii) 0.15 M NaCl, 0.05 M Tris-hydrochloride (pH 7.4), 0.1% Nonidet P-40; and (iv) 0.02 M Tris-hydrochloride (pH 8.0). The pellets were then incubated for 20 min at 20°C with 50 μl of a kinase reaction buffer containing 0.02 M Tris-hydrochloride (pH 8.0), 10 mM magnesium chloride, and 10 μCi of [γ-^{32}P]ATP (4,200 Ci/mmol; ICN, Irvine, Calif.). The reaction was stopped by the addition of lysis buffer containing 1 mM EDTA. The immunoprecipitates were washed twice in the same buffer and then analyzed by gel electrophoresis as described in the legend to Fig. 1.*

Here we have demonstrated that the *gag*-related, nonstructural proteins of MC29 and CMII, two closely related avian acute leukemia viruses (2, 4), avian acute leukemia virus AEV, and avian FSV are all phosphorylated in vivo. In addition, we provide suggestive evidence that each of these nonstructural, viral proteins, with the possible exception of the AEV protein, contains specific phosphorylation sites which are not shared with the structural *gag* protein of nondefective avian tumor viruses. Our data cannot determine whether these specific sites reside in the *gag*-related, *gag*-unrelated, or both domains of these proteins. However, the 140-kd FSV protein was recently shown to contain six

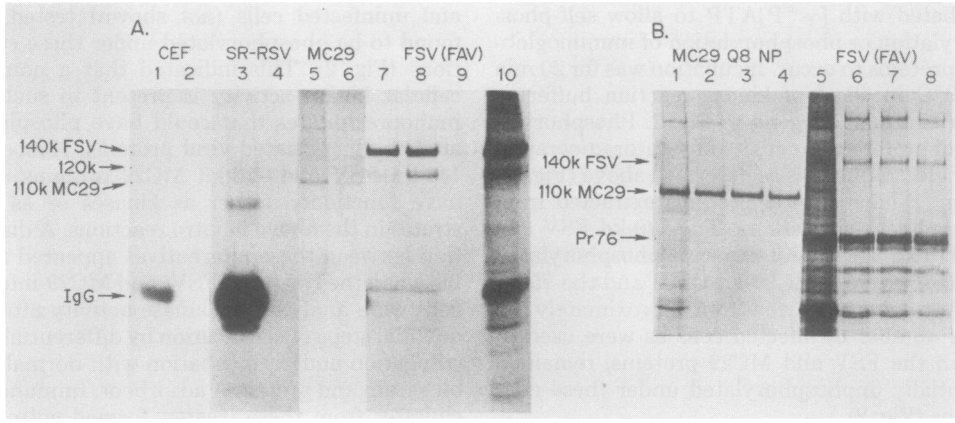

FIG. 3. *(A) Protein kinase activity in immunoprecipitates from lysates of normal chicken embryo fibroblasts (CEF), Schmidt-Ruppin RSV (SR-RSV) subgroup D-transformed CEF, or cells transformed by MC29 or FSV. Immunoprecipitates were formed with anti-whole RSV serum (lanes 2, 4, 5, 6, 7, 9, and 10), rabbit tumor antiserum no. 13 (kind gift of H. Oppermann and J. M. Bishop, San Francisco) (lanes 1 and 3), or anti-p27/p19 serum (lane 8). IgGsorb was used as immunocomplex adsorbent. Kinase reactions were carried out as described in the legend to Fig. 2, except that manganese chloride (10 mM) was used in the buffer. Cellular lysates of MC29, FSV, and SR-RSV subgroup D-transformed cells were used after sequential steps of clarification carried out at 0 to 4°C: centrifugation at 10,000 × g for 30 min (lane 10), centrifugation at 100,000 × g for 30 min (lanes 3, 4, 5, 7, and 8), and in the cases of MC29- and FSV-transformed cells, after preincubation with normal rabbit serum and IgGsorb (lanes 6 and 9). (B) [^{35}S]methionine-labeled proteins precipitated with anti-whole RSV serum from lysates of MC29- or FSV-transformed cells. Lysates were used for immunoprecipitation after sequential steps of clarification: centrifugation at 10,000 × g for 30 min (lanes 1 and 5), centrifugation at 100,000 × g for 30 min (lanes 3 and 7), or preincubation with normal rabbit serum and IgGsorb (lanes 2 and 6). Immunoprecipitates analyzed in lanes 4 and 8 were treated the same as those in lanes 3 and 7 and, in addition, were washed and incubated exactly like samples used for kinase reactions (see legend to Fig. 2). Electrophoresis was performed as described in the legend to Fig. 1. IgG, Immunoglobulin G.*

phosphopeptides, two of which are known *gag*-phosphopeptides, and four of which are not; hence, these four peptides may derive from non-*gag* sequences (A. Pawson and G. S. Martin, personal communication). Analogy with *gag*-related, nonstructural proteins of mammalian defective transforming retroviruses described above, which have been shown to contain specific phosphopeptides (15), supports the view that specific phosphorylation sites are also present in the nonstructural proteins of avian viruses studied here.

The lack of intrinsic in vitro kinase activity demonstrated here for the *gag*-related, nonstructural proteins of CMII and AEV and the possibility that the FSV and MC29 proteins function as substrates only do not conform with the pattern established for the *gag*-related proteins of the mammalian Abelson leukemia and several feline sarcoma viruses (15, 16) which are thought to have intrinsic kinase activity. This could either indicate that some or all of the viral proteins studied here are indeed different from their mammalian counterparts and devoid of kinase activity or that our assay was insufficient to detect it. A failure to detect kinase activity could be due to the absence in our assay of a specific heterologous target or to other shortcomings of the assay system, such as inactivation of a possible kinase activity by antibody precipitation or the absence of suitable triphosphates or ions. The latter two possibilities are considered unlikely since, under our conditions, a non-viral kinase activity has been detected, and kinase activity of the 60-kd *src* protein was readily observed (Fig. 3A).

If the nonstructural, *gag*-related viral proteins described are indeed devoid of kinase activity but necessary for oncogenic function, it would appear that phosphorylation is not a general mechanism by which viral proteins transform cells.

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