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Synthetic peptide fragment of *src* gene product inhibits the *src* protein kinase and crossreacts immunologically with avian *onc* kinases and cellular phosphoproteins

(pp60^{src}/peptide synthesis/avian sarcoma viruses)

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ABSTRACT **All the known avian sarcoma viruses have associated protein kinase activities that phosphorylate tyrosine residues of their target proteins. A decapeptide fragment of pp60^{src} of Rous sarcoma virus (RSV), residues 415–424, and an analog of that sequence have been chemically synthesized by solid-phase methods. The two decapeptides were not phosphorylated by pp60^{src} of RSV, P90 of Y73 avian sarcoma virus, or P140 of Fujinami sarcoma virus. However, both peptides were able to inhibit competitively the kinase activities associated with the transforming proteins. Antiserum was raised against one of the peptides and IgG was purified from the serum by affinity chromatography. The antibody was able to precipitate pp60^{src} of RSV as well as P90 of Y73 virus from cells infected with these viruses. The antibody also precipitated a number of high molecular weight phosphoproteins from normal chicken and rat fibroblasts and from several lines of virus-transformed cells.**

The recent molecular cloning and sequence analysis of the *src* gene of Rous sarcoma virus (RSV) (ref. 1; T. Takeya and H. Hanafusa, personal communication; D. Schwartz, R. Tizard, W. Gilbert, J. Taylor, and R. Guntaka, personal communication) have made it feasible to study the characteristics of different structural and functional domains of the *src* gene product, pp60^{src}. pp60^{src} has been shown to be a phosphoprotein with two possible sites of phosphorylation (2, 3). One of these is a serine residue that is phosphorylated by a cyclic AMP (cAMP)-dependent protein kinase; the other is a tyrosine residue that is phosphorylated by a cAMP-independent kinase. The cAMP-independent protein kinase activity has been shown to be associated with pp60^{src} itself and appears to be required for the acquisition and maintenance of the transformed phenotype in Rous sarcoma virus (RSV)-infected cells. A number of other oncogenic viruses also have been shown to have associated protein kinases that phosphorylate tyrosine residues and that are transformation-specific (4–12).

The sequence of a tryptic fragment of pp60^{src} that contains the tyrosine phosphate acceptor site has been reported recently (13). These analyses have identified tyrosine-419 as the residue phosphorylated by the pp60^{src} protein kinase. The peptide sequence data are consistent with DNA sequence data. It is of considerable interest that the same phosphotyrosine-containing peptide was also found in the transforming proteins of two other avian sarcoma viruses, the Y73 sarcoma virus and the Esh sarcoma virus (13), although RSV and Y73 have been shown to have no measurable homology by nucleic acid hybridization (14, 15). The limited and yet exact homology in the three different transforming genes suggests that the sequence may be a general tyrosine phosphate acceptor sequence for cellular and viral proteins and may be present in a number of cellular proteins, among them the cellular oncogenes.

There has been increasing interest in the use of chemically synthesized peptides as immunogens for studying antigenic determinants of viral polyproteins (16, 17). This method offers the advantage of allowing the production of monospecific antibody which may be used for probing known proteins or for identifying previously undiscovered ones. We have been prompted by the information available on the protein sequence of pp60^{src} to use a similar approach for studying the properties of the protein and its associated protein kinase activity. In this report we describe the use of synthetic peptides as inhibitors of tyrosine-specific kinases and also as immunogens to obtain antibody for studying the presence of homologous sequences in cellular proteins.

MATERIALS AND METHODS

Cells and Viruses. Chicken embryo fibroblasts, rat fibroblasts, and RR1022 cells [a line of rat fibroblasts transformed by the Schmidt–Ruppin strain (subgroup D) of RSV(SRD)] were maintained in culture as described (18, 19). The Schmidt–Ruppin strain (subgroup B) of RSV was used to transform chicken embryo fibroblasts. A SRD-transformed goat cell line was initiated by infecting primary goat fibroblasts with that virus. The line was maintained in culture by procedures similar to those used for RR1022 cells. Fujinami sarcoma virus (FSV) and Y73 avian sarcoma virus were generously provided by H. Hanafusa and K. Toyoshima.

Antigen Preparation and Purification of IgG. Decapeptides were synthesized on polystyrene resin by the solid-phase method of Merrifield (20). The crude product obtained after hydrofluoric acid cleavage and deprotection was found to consist predominantly (80%) of the desired product by a number of analytical techniques including thin-layer chromatography, high-pressure liquid chromatography, ion exchange chromatography, and amino acid analysis. The peptides were purified to homogeneity by chromatography on a DEAE-Sephadex column. Amino acid analysis of acid hydrolysates of the purified products showed that they had the correct composition.

The purified decapeptide, containing residues 415–424 of pp60^{src}, was coupled to keyhole limpet hemocyanin by using glutaraldehyde as described (21). The reaction was monitored by the addition of a tracer amount of ^{125}I-labeled peptide, and the average coupling yield was 12%. The coupled peptide was separated from unreacted peptide and glutaraldehyde by gel filtration on a Sephadex G-50 column. The conjugated immu-

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Abbreviations: cAMP, cyclic AMP; RSV, Rous sarcoma virus; SRD, Schmidt–Ruppin strain (subgroup D) of Rous sarcoma virus; TBR, tumor-bearing rabbit; FSV, Fujinami sarcoma virus.

419

I NH_2-Glu Asp Asn Glu Tyr Thr Ala Arg Gln Gly-COOH

II NH_2——————Val——————COOH

FIG. 1. Amino acid sequence of synthetic decapeptides. Sequence and numbering are from Czernilofsky *et al.* (1).

nogen (200 μg) was emulsified in complete Freund's adjuvant and injected into New Zealand White rabbits intramuscularly and subcutaneously at several sites. Booster doses were administered subcutaneously on a weekly basis for 3 weeks and intraperitoneally on the fourth week before the bleeding. Sera were tested for the presence of IgG that would immunoprecipitate the ^{125}I-labeled peptide. Significant titers were observed within 2 weeks after initial immunization. The IgG fraction was obtained from the sera by ammonium sulfate precipitation and chromatography on DEAE-cellulose. IgG was purified by affinity chromatography on a decapeptide-Sepharose column. The peptide-specific IgG was eluted with 0.2 M glycine·HCl buffer at pH 2.7

Metabolic Labeling and Immunoprecipitation. Cells were incubated for 1–2 hr in medium lacking phosphate or methionine. Medium was changed to phosphate- or methionine-free medium supplemented with carrier-free [^{32}P]orthophosphate (Amersham) at 0.5 mCi/ml (1 Ci = 3.7 × 10^{10} becquerels) or with L-[^{35}S]methionine (1400 Ci/mmol, Amersham) at 125 μCi/ml, and incubation was continued for 5 hr. Cells were washed and lysed in detergent-containing buffer as described (22). Aliquots of cell lysate were incubated with 2–18 μl of tumor-bearing rabbit (TBR) serum or affinity-purified antipeptide IgG for 2 hr. Immunoprecipitates were adsorbed on protein A-Sepharose and washed with buffers as described (22). They were then analyzed by electrophoresis on 7.5% NaDodSO$_4$/polyacrylamide gel and subsequent autoradiography.

***In Vitro* Kinase Assays.** Purified pp60^{src}, prepared according to Erikson *et al.* (23), was used to phosphorylate casein. Reactions were carried out for 20 min in a total volume of 50 μl containing 0.1 μM [γ-^{32}P]ATP (3000 Ci/mmol), 20 mM Tris·HCl (pH 7.4), and 5 mM $MgCl_2$ at 30°C. Electrophoresis sample buffer was added to terminate reactions and samples were boiled for 2 min. Reaction products were electrophoresed on 10% NaDodSO$_4$/polyacrylamide gels. After electrophoresis, gels were fixed and dried. Radioactive bands were located by autoradiography, excised, and quantitated by scintillation counting. For assays with P140 of FSV and P90 of Y73 virus, the protein kinases were immunoprecipitated from virus-transformed cells with anti-gag serum that contained anti-p19 activity as described (7). In these assays, $MgCl_2$ in the kinase buffer was replaced with 10 mM $MnCl_2$. The hexapeptide inhibitor of cAMP-dependent protein kinases, Arg-Gly-Tyr-Ala-Leu-Gly, was obtained from Peninsula Laboratories (San Carlos, CA). Phosphorylation of peptides was carried out in a similar fashion except that the reaction products were analyzed by high-voltage paper electrophoresis on Whatman 3MM paper in a pH 3.5 buffer (pyridine/acetic acid/water, 1:10:90, vol/vol).

Phosphopeptide and Phosphoamino Acid Analyses. Cells were labeled with [^{32}P]orthophosphate (3 mCi/ml), and immunoprecipitated proteins were obtained as described above.

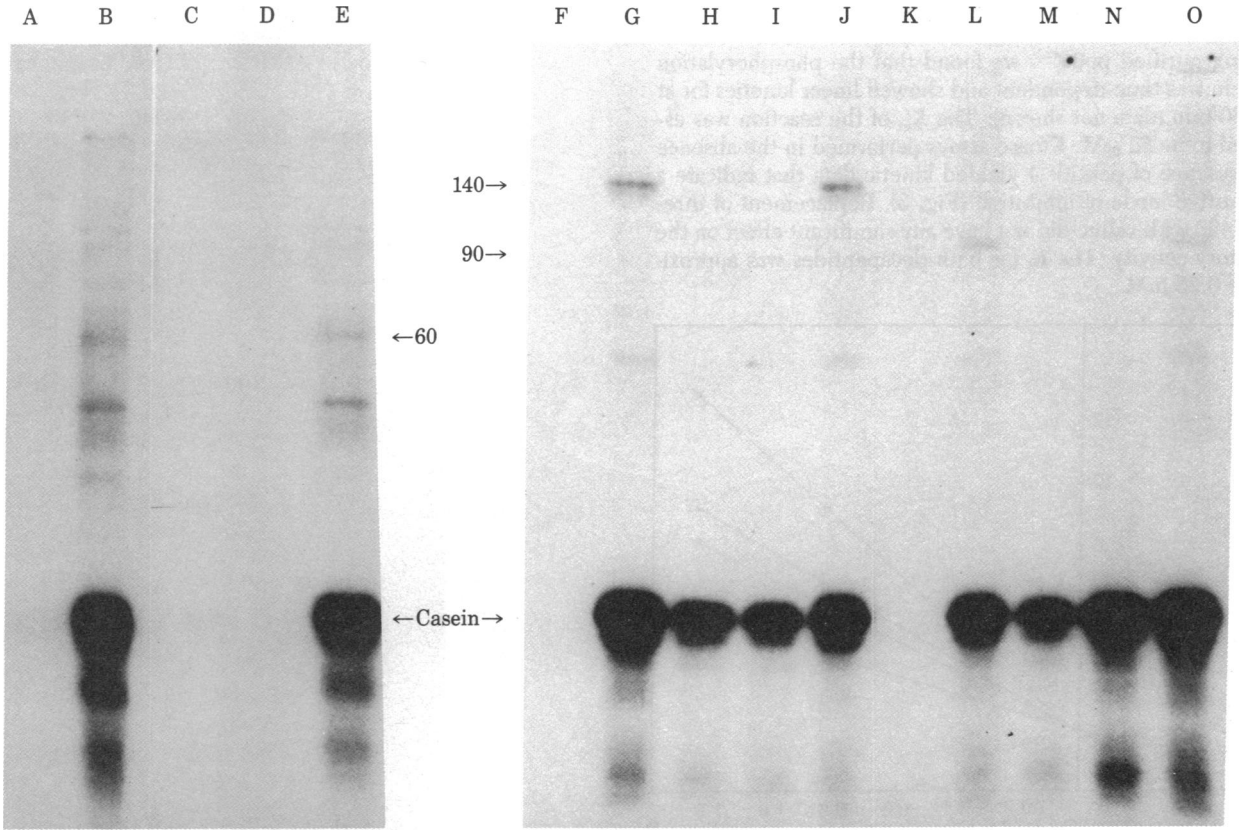

FIG. 2. Synthetic peptides inhibit the protein kinase activities associated with viral transforming proteins. Casein (0.8 mg/ml) was treated with pp60^{src} (lanes A–E), P140 of FSV (lanes F–J), or P90 of Y73 virus (lanes K–O). Peptides I (lanes C, H, and M), II (lanes D, I, and N), and Arg-Gly-Tyr-Ala-Leu-Gly (an inhibitor of cAMP-dependent protein kinases) (lanes E, J, and O) were present at 5 mM. No peptide was added in lanes B, G, and L. Lanes A, F, and K are controls in which either no enzyme was added (lane A) or normal rabbit serum was used in place of TBR or anti-gag serum in the immunoprecipitation of viral protein (lanes F and K). Molecular weights are shown × 10^{-3}.

After electrophoresis, the gels were dried directly and bands of interest were located by autoradiography. Phosphoproteins were oxidized with performic acid and digested with TPCK-trypsin as described (24). Tryptic peptides were separated as described (3). Samples were electrophoresed on cellulose plates in 1% ammonium carbonate (pH 8.9) at 1000 V for 27 min, followed by chromatography for 4 hr in *n*-butanol/acetic acid/pyridine/water, 74:15:60:60 (vol/vol). For phosphoamino acid analyses, samples were hydrolyzed in 6 M HCl for 2 hr and the hydrolysates were separated by two-dimensional electrophoresis as described (3).

RESULTS

Effect of Synthetic Peptides on Protein Kinase Activities Associated with Transformation-Specific Proteins. The amino acid sequences of the two peptides that were synthesized are shown in Fig. 1. Peptide I is residues 415–424 of pp60^{src} and includes the tyrosine phosphorylation site. Peptide II is an analog in which threonine-420 was replaced with valine in order to eliminate the possibility of the threonine residue serving as a phosphate acceptor. Peptide I was used in immunization procedures as well as in affinity purification of antisera. Using several different sources of kinase activity, we found that, over a wide concentration range of peptide I or II, neither peptide became phosphorylated (data not shown). The protein kinases used were pp60^{src} of RSV, immunoprecipitate of FSV-encoded P140, and immunoprecipitate of Y73 virus-encoded P90. However, both of the decapeptides inhibited the phosphorylation of casein (Fig. 2). Moreover, we observed that the phosphorylation of pp60^{src} was completely abolished by the peptides. The inhibitory effects appeared to be specific because they were not observed with a tyrosine-containing hexapeptide that is a well-known inhibitor of cAMP-dependent protein kinases.

Using purified pp60^{src}, we found that the phosphorylation of casein was time-dependent and showed linear kinetics for at least 30 min (data not shown). The K_m of the reaction was estimated to be 62 μM. Kinase assays performed in the absence and presence of peptide I yielded kinetic data that indicate a competitive mode of inhibition (Fig. 3). Replacement of threonine-420 with valine did not have any significant effect on the inhibitory activity. The K_i for both decapeptides was approximately 0.25 mM.

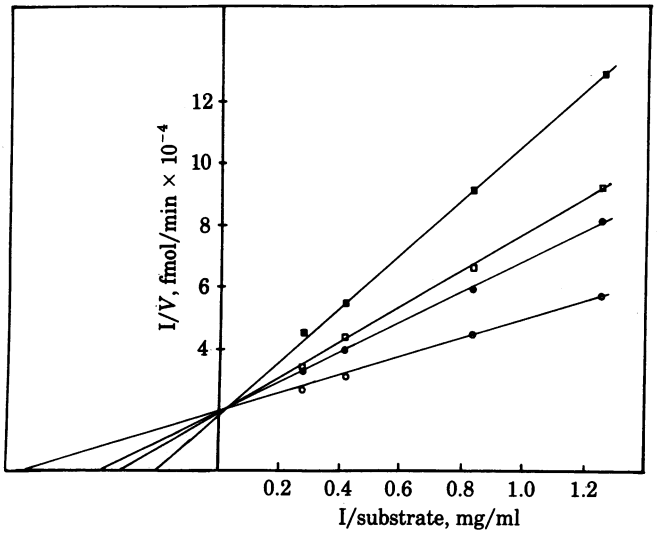

FIG. 3. Competitive inhibition of protein kinase associated with pp60^{src}. Casein phosphorylation was assayed in a 20-min reaction in the absence (○) or presence of peptide I (●, 0.8 mM; □, 1.0 mM; ■, 1.3 mM).

We also examined the kinase activities associated with two other avian sarcoma viruses, FSV and Y73 sarcoma virus. As shown in Fig. 2 (lanes F–O), peptide I inhibited the phosphorylation of casein by approximately 40% and that of P140 and P90 completely. The same results were obtained with peptide II as inhibitor, except that the phosphorylation of casein by P90 did not seem to be affected by presence of the peptide. The apparent differences among the three *onc* kinases in the extent of inhibition by the peptides of phosphorylation of casein and the viral proteins may reflect differences in the enzyme specificities.

Immunoprecipitation of Viral Proteins with Antipeptide IgG. Affinity-purified IgG from antipeptide serum was used to immunoprecipitate pp60^{src} in order to test whether or not residues 415–424 of the protein constitute an important antigenic determinant. The antipeptide IgG precipitated a number of phosphoproteins from RSV-transformed cells (Fig. 4). One of these proteins comigrated with pp60^{src} that had been immunoprecipitated with TBR serum and was shown to contain phosphotyrosine. Peptide mapping of *Staphylococcus aureus* V8 protease-digested 60,000-dalton protein indicated identity with pp60^{src} (data not shown). We therefore concluded that antipeptide IgG did recognize pp60^{src}. However, the amount of pp60^{src} that could be precipitated by antipeptide IgG varied

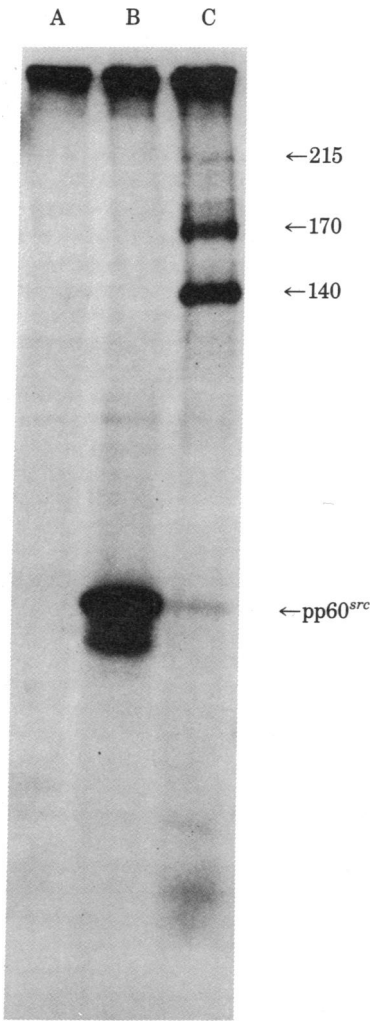

FIG. 4. NaDodSO$_4$/polyacrylamide gel analysis of immunoprecipitates from ^{32}P-labeled extracts of SRD-transformed goat cells. Cell lysates were incubated with normal rabbit serum (lane A), TBR serum (lane B), or affinity-purified IgG against peptide I (lane C).

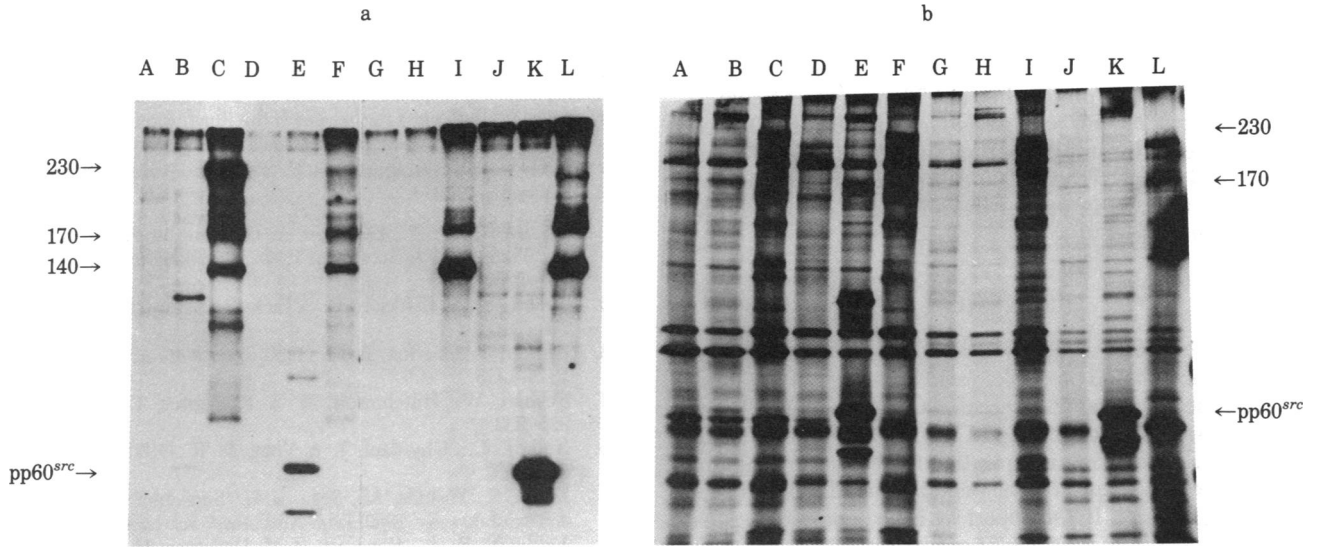

FIG. 5. NaDodSO$_4$/polyacrylamide gel analysis of ^{32}P-labeled (*a*) and ^{35}S-labeled (*b*) immunoprecipitates from normal and RSV-transformed cells. Lanes A–C, normal chicken embryo fibroblasts; D–F, infected chicken embryo fibroblasts; G–I, normal rat fibroblasts; J–L, RR1022 cells. Antisera used were: lanes A, D, G, and J, normal rabbit serum; lanes B, E, H, and K, TBR serum; lanes C, F, I, and L, affinity-purified antipeptide IgG. Molecular weights are shown × 10^{-3}.

with the type of transformed cells. Of all the transformed cell lines tested, the SRD-transformed goat cells consistently showed the highest level of immunoprecipitable pp60^{src} with antipeptide IgG. However, immunoprecipitation with TBR serum showed no detectable differences in pp60^{src} levels between SRD-transformed goat cells and RR1022 cells.

Other major proteins that were precipitated with antipeptide IgG all were of high molecular weight. The apparent molecular weights of these proteins in both uninfected and infected chicken embryo fibroblasts were 140,000, 170,000, and 230,000 (Fig. 5*a* and *b*). There were also three or four minor bands between 69,000 and 195,000 daltons. Transformation by avian sarcoma virus seemed to have caused a decrease in phosphorylation of these proteins (Fig. 5*a*) although there appeared to be no difference in the amounts of these proteins (Fig. 5*b*). The two major phosphoproteins immunoprecipitated from rat fibroblasts were the same 140,000- and 170,000-dalton proteins precipitated from chicken embryo fibroblasts. However, we did not observe the 230,000-dalton protein in normal and transformed rat cells. Instead, we noted a 215,000-dalton protein that was phosphorylated in SRD-transformed rat cells (Fig. 5*a*, lanes I and L). Phosphoamino acid analyses of these phosphoproteins showed that they all contained phosphoserine, small amounts of phosphothreonine, and no phosphotyrosine. This finding was

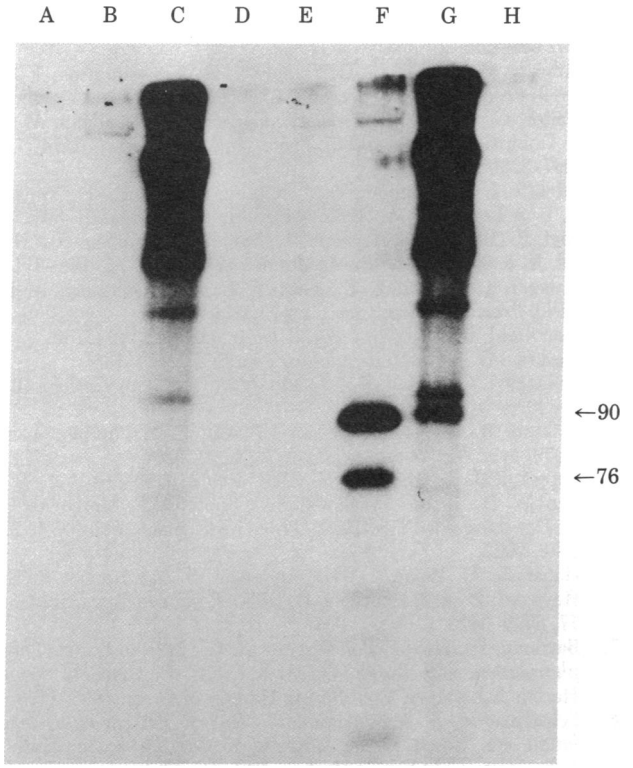

FIG. 6. NaDodSO$_4$/polyacrylamide gel analysis of P90 from chicken embryo fibroblasts transformed with Y73 avian sarcoma virus. ^{32}P-Labeled extracts of normal (lanes A–D) and of Y73 virus-infected cells (lanes E–H) were incubated with normal rabbit serum (lanes A and E), anti-gag serum (lanes B and F), and antipeptide I IgG (lanes C and G). In lanes D and H, immunoprecipitation was with antipeptide IgG in the presence of 200 μg of peptide I. Molecular weights are shown × 10^{-3}.

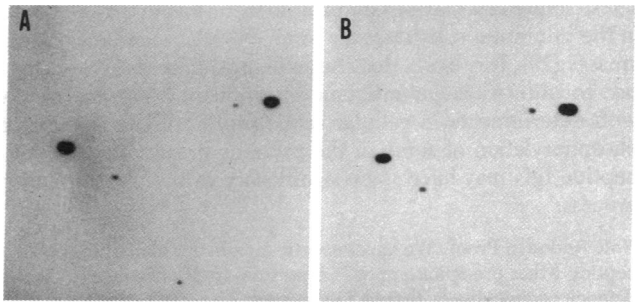

FIG. 7. Tryptic peptide analysis of P90 immunoprecipitated from Y73 virus-infected chicken embryo fibroblasts. ^{32}P-Labeled cell lysate was prepared and immunoprecipitated with anti-gag serum (*A*) or with antipeptide IgG (*B*). Proteins were isolated by gel electrophoresis, oxidized with performic acid, and digested with TPCK-trypsin. Peptides were separated by electrophoresis and ascending thin-layer chromatography.

surprising because the immunogen used to produce the antibody lacked serine.

Antipeptide IgG also was examined for its ability to precipitate the transforming protein P90 of Y73 avian sarcoma virus. Fig. 6 shows that P90 was precipitated from lysates of Y73 virus-transformed cells by anti-gag serum (lane F) as well as by antipeptide IgG (lane G). The 90,000-dalton phosphoproteins precipitated by the two antibodies were found to have identical tryptic peptides (Fig. 7).

DISCUSSION

To date three different classes of avian sarcoma viruses have been recognized based on nucleic acid homology of the transformation-specific genes within each class of virus (14, 15). Two of these classes, of which RSV and Y73 virus are representative, bear a remarkable relationship in that their transforming proteins share a homologous tryptic peptide around their tyrosine phosphorylation site. In this report we have demonstrated that a synthetic decapeptide which encompasses this common sequence is able to inhibit competitively the phosphorylation of the transforming proteins of these two viruses by their associated protein kinases. We also observed such inhibition with P140 of FSV, which shares no detectable homology with transforming proteins of the other two subclasses (25, 26). These results suggest that the different *onc* protein kinases may have similar substrate specificities. The reason for the inability of the decapeptide to serve as a substrate for the *onc* kinases is unclear.

Antiserum against the synthetic peptide enabled us to immunoprecipitate pp60^{src} and P90 from RSV- and Y73 virus-transformed cells, respectively. These findings confirm the published nucleic acid and protein sequence data for the transforming sequences. The interaction between the transforming proteins and antipeptide IgG was relatively weak and may be due to the small size of the immunogen. We have observed that the immunoprecipitation of pp60^{src} with TBR serum can be completely abolished by treatment of the antigen with denaturing agents. This result implies that the major antigenic determinants of pp60^{src} are most likely represented in the conformational profile of the polyprotein. The decapeptide used as an immunogen was probably not sufficiently large to represent one of the native determinants, and the amount of pp60^{src} precipitated was therefore limited by the recognition of the primary sequence. An alternative explanation for the weak antigenic interaction is that the decapeptide sequence may not be readily accessible to antipeptide IgG in the native protein.

Several reports have indicated that the substrates for a number of protein kinases contain clusters of acidic residues (27, 28). These sequences may be comparable to the basic residues found in the minimum substrates for some cAMP-dependent protein kinases (29). It is likely that the acidic residues of the decapeptide constitute a major antigenic determinant which crossreacts with determinants in cellular acidic proteins. The changes in phosphorylation of some of the proteins precipitated by antipeptide IgG may have some significance in the transformation process.

Note Added in Proof. We have recently obtained other antisera against peptide I that precipitate pp60^{src} from RSV-transformed cells with efficiency comparable to that of TBR serum.

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