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Avian Retrovirus pp32 DNA Endonuclease Is Phosphorylated on Ser in the Carboxyl-Terminal Region

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The avian retrovirus pp32 DNA endonuclease and the β polypeptide of the reverse transcriptase contain the same three phosphoserine (p-Ser) tryptic peptides. At least 95% of the P_i label is nearly equally distributed between two major p-Ser tryptic peptides derived from either β or pp32. These polymerase gene-derived proteins were metabolically labeled with various radioactive amino acids or P_i, and the purified protein was subjected to cyanogen bromide or hydroxylamine cleavage. The results indicated that the two major p-Ser tryptic peptides map to the COOH-termini of both proteins. The two major p-Ser tryptic peptides isolated from P_i-labeled pp32 were subjected to proteolysis by three separate specific proteases. Analysis of the data suggested that these p-Ser are located on pp32 at amino acid positions 262 and 282 from the amino terminus of pp32 (286 amino acids in length). At present, we cannot exclude the possibility that one or both p-Ser peptides map between amino acid positions 124 to 150. The role of this site-specific phosphorylation of pp32 and β is also discussed.

Retroviruses require a function(s) located at the 3′ terminus of the polymerase gene (*pol*) for integration (6, 32, 41). In avian retroviruses, the pp32 DNA endonuclease is located in this region of *pol* (15, 38). The pp32 protein is derived from the COOH-terminus of the *pol* β polypeptide by proteolytic cleavage in vivo (5, 8, 35). Hizi and Joklik (19) first demonstrated that the β-polymerase subunit and not the α-polymerase subunit was phosphorylated in vivo. This observation was confirmed by us, and it was further demonstrated that the pp32 protein exists as a mixture of phosphorylated and nonphosphorylated polypeptides in vivo (39). Two-dimensional tryptic peptide analysis of both ^{32}P-labeled *pol* proteins (β and pp32) of Rous sarcoma virus (RSV), Prague C (PrC) strain, revealed that each protein contained three labeled peptides. Site-specific phosphorylation of *pol* proteins in vivo may (i) affect the DNA endonuclease activities associated with pp32 (14, 15) or αβ (7, 11, 18, 36); (ii) affect proteolytic processing of the apparent immediate *pol* precursor to the β polypeptide (1) to yield αβ DNA polymerase, the dimeric pp32 protein, or the recently identified COOH-terminal *pol* (4,100-dalton) polypeptide (1, 13); (iii) affect protein-protein interactions or DNA-binding properties of pp32; or (iv) affect integration of viral DNA by pp32. In vitro phosphorylation-dephosphorylation of αβ DNA polymerase does affect DNA polymerase activity but not RNase H activity (26). Using RSV Prague A (PrA) strain, we have tentatively localized the two major tryptic peptides containing phosphorylated Ser to the COOH-terminus of β or pp32.

MATERIALS AND METHODS

In vivo labeling of pp32 and β. Chicken embryo fibroblast cells (C/E, leukosis-free, chf^-, gs^-) were obtained from SPAFAS, Inc. Stocks of PrA virus were obtained from an infectious PrA DNA clone (pJD-100) generously supplied by T. J. Parsons. Nearly confluent monolayer cultures of PrA-infected chicken embryo fibroblast cells (typically 18 150-cm^2 flasks per experiment) were labeled with 270 μCi of $^{32}P_i$ (New England Nuclear Corp.) per ml in phosphate-free Eagle minimal essential medium (GIBCO Laboratories) containing 5% dialyzed fetal bovine serum, 1% dimethyl sulfoxide, 0.22% sodium bicarbonate, 2% medium 199 (GIBCO), and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.2) (39). Supernatants were harvested at approximately 48-h intervals and were replaced with the above medium minus $^{32}P_i$. Virus from the supernatants was purified by low- and high-speed clarification steps and by sedimentation through discontinuous sucrose gradients. The purified virus was suspended in NTE buffer (100 mM NaCl, 20 mM Tris hydrochloride [pH 7.5], 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA) and trichloroacetic acid-precipitable counts per minute were determined.

PrA-infected cells were labeled with [^{3}H]valine (130 μCi/ml, 58.1 Ci/mmol; New England Nuclear) in minimal essential medium (GIBCO) containing 5% of the normal concentration of valine and 4% dialyzed calf serum for 18 h at 41°C (33). L-[^{35}S]cystine (6.1 Ci/mmol; New England Nuclear) was used at 21 μCi/ml in cystine-free medium which contained 25% of the normal amount of methionine (35). Cultures were incubated for two successive 15-h intervals in radioactive cystine containing medium, followed by a 15-h incubation in complete medium. [^{35}S]methionine-labeled PrA was obtained as described previously (39). Virus from the above labeling procedures was purified as described for P_i labeling.

Polyacrylamide gel electrophoresis. For analytical analysis of cyanogen bromide- and hydroxylamine-cleaved pp32 or β, two different denaturing gel electrophoresis systems were used, for maximal separation of phosphate-containing polypeptides and for estimation of molecular masses. A 6 M urea-sodium dodecyl sulfate (SDS)-polyacrylamide gel system containing phosphate buffer (42) and a modified Laemmli discontinuous buffer system containing a high concentration of Tris (0.75 M) (9) in the resolving gel were used. With the urea-containing system, there is a direct dependence of mobility on the logarithm of the molecule weight of polypeptide chains between molecular weights of 1,700 and approximately 17,000. A plot of log molecular

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weight versus distance migrated for polypeptide markers (4,000 to 60,000) on the high-concentration Tris-polyacrylamide gel is linear, except for an inflection at approximately 14,000 (9).

Preparative immunoprecipitation of radiolabeled pp32 and β. Immunoprecipitation of detergent-lysed virions was previously described (39), except that protein A-Sepharose CL-4B (Pharmacia) was the adsorbent and rabbit antipeptide sera directed against the NH_2 terminus of pp32 (13) was utilized. Sodium PP_i (50 mM) was added to immunoprecipitation reactions to inhibit virion-associated phosphatases. Immunoprecipitated ^{32}P-, ^{3}H-, or ^{35}S-labeled *pol* proteins were purified on preparative SDS-10% polyacrylamide gels (25). The labeled proteins were autoradiographed on wet or dried gels by using Kodak X-Omat film and an X-ray intensifying screen. The desired bands were excised from the gels.

Phosphoamino acid analysis. Phosphoamino acid analysis of phosphorylated PrA pp32 and β was performed by high-voltage paper electrophoresis (4, 28). Immunoprecipitated and SDS-polyacrylamide gel-purified pp32 or β were hydrolysed by 6 N HCl at 110°C for 2 h and processed for electrophoresis. Samples were spotted on Whatman no. 1 filter paper with unlabeled marker phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr). After electrophoresis, the paper was dried and the unlabeled amino acids were detected by ninhydrin staining. The ^{32}P-labeled phosphoamino acids were detected by autoradiography.

Cyanogen bromide cleavage. The CNBr mapping technique has been described elsewhere (34). Gel slices containing ^{32}P- or ^{35}S-labeled pp32 or β were washed briefly with water and incubated with CNBr for 1 h at 23°C in 0.1 N HCl plus 0.4% 2-mercaptoethanol (BSH). The concentration of CNBr was varied from 10 to 150 mg/ml in some experiments, resulting in the same cleavage pattern. The treated gel slices were washed, eluted with 6 M urea–1% SDS–0.01 M sodium phosphate (pH 7.2)–1% BSH overnight at 25°C and eluted further at 37°C for 4 h. CNBr-cleaved myoglobin (20 μg) low-molecular-weight markers (Pharmacia) were included in the above elution step. The supernatant from each slice was removed, and the gel slice was further washed with water to quantitatively recover the labeled protein fragments. The appropriate combined supernatant and wash samples were lyophilized. Water and BSH were added to bring the urea concentration to 6 M and 1% BSH, respectively. Samples were denatured at 92°C for 5 min and analyzed on 5% stacking-12 or 15% resolving polyacrylamide gels containing 0.1 M sodium phosphate (pH 7.2), 0.1% SDS, and 6 M urea (42). For analysis on the high-concentration Tris-polyacrylamide gels (9), the washed CNBr-treated gel slices were dounced in 0.05 M ammonium bicarbonate–0.1% SDS–0.3 M BSH and heated at 92°C for 5 min and then at 37°C for 1 h (T. Hunter, personal communication). After centrifugation, the supernatant was removed and the gel slices were incubated with additional bicarbonate buffer. The appropriate combined samples were lyophilized and analyzed. Carrier proteins (15 μg) were always added to the above extraction procedure.

Hydroxylamine cleavage. Hydroxylamine cleavage of proteins immobilized in the matrix of a polyacrylamide gel is described elsewhere (37). After treatment with hydroxylamine and various wash steps, the gel slices containing ^{3}H-, ^{35}S-, or ^{32}P-labeled pp32 or β were submerged in 6 M urea–62 mM Tris (pH 7.5)–3% SDS–5% BSH. The samples were incubated at 37°C for 2 h and then at 95°C for 5 min. Molecular weight protein markers (20 μg) were added before the urea elution step. The samples were analyzed on the high-concentration Tris-polyacrylamide gel or the urea SDS-polyacrylamide phosphate gel systems.

Tryptic peptide analysis. Immunoprecipitated and SDS-polyacrylamide gel-purified ^{32}P-labeled pp32 or β were eluted with the ammonium bicarbonate-SDS buffer system as described above. The eluted proteins were trichloroacetic acid precipitated and were treated with 50 μl of performic acid (100 μl of 30% H_2O_2 and 900 μl of 98% formic acid) for 2 h on ice (Hunter, personal communication). After the addition of water and lyophilization, the samples were dissolved in 0.05 ml of 0.05 M ammonium bicarbonate (pH 7.8) and digested with trypsin (10 μg) (Millipore Corp.; tolylsulfonyl phenylalanyl chloromethyl ketone treated) at 37°C for 5 h (twice) and lyophilized repeatedly. The samples were dissolved in 5 μl of buffer (15% [vol/vol] acetic acid, 5% [vol/vol] 88% formic acid) (pH 1.9). The samples were applied to thin-layered cellulose plates and electrophoresed by using the above-mentioned acetic acid-formic acid buffer (39). The plates were dried and chromatographed in isobutyric acid-pyridine-glacial acetic acid-*n*-butanol-water (65:5:3:2:29, by volume) (16). The plates were dried and autoradiographed with an intensifying screen at −70°C. Other proteases were obtained as follows: chymotrypsin (Millipore), *Staphylococcus aureus* V-8 (Boehringer Mannheim Biochemicals), and prolyl endopeptidase (ICN Immunobiologicals).

RESULTS

Predicted amino acid sequence of pp32. The amino acid sequence of the avian retrovirus *pol* gene is highly conserved among various avian retroviruses as deduced by tryptic peptide mapping of *pol* proteins, restriction enzyme mapping of cloned viral DNAs, amino acid sequence analysis of *pol* products, and direct DNA sequencing of various cloned viruses (5, 8, 10, 13, 17, 35, 40; unpublished data). But the existence of single-amino-acid substitutions along the entire *pol* gene cannot be ruled out. A schematic representation of the pp32 DNA endonuclease domain as deduced from the PrC sequence (40) is shown (Fig. 1). Locations of several specific amino acids essential to this report, potential chemical cleavage sites, and predicted tryptic peptides of pp32 are illustrated.

Ratio of phosphorylated to unphosphorylated pp32 in different avian retroviruses. The ratio of phosphorylated to unphosphorylated pp32 varies among the different avian retroviruses. We have previously demonstrated (39) that only the top polypeptide comprising the pp32 doublet as revealed by SDS-polyacrylamide gel analysis is phosphorylated (Fig. 2). With the purified avian myeloblastosis virus pp32 protein, the ratio of phosphorylated protein to unphosphorylated protein is approximately 1 to 3, respectively (Fig. 2A), while with ^{35}S-labeled PrA pp32 immunoprecipitated by anti-pp32 rabbit polyclonal sera, the ratio is approximately 3 to 1 (Fig. 2B). The ratio of phosphorylated to unphosphorylated pp32 when immunoprecipitated from PrC virus is approximately equal (39). This variance of phosphorylation of pp32 could be due to protein phosphatases and cellular kinases located in virions. To minimize the potential phosphatase problem in these present studies, 50 mM sodium PP_i was always included in the immunoprecipitation reactions.

Phosphoamino acid analysis of in vivo-labeled β and pp32. To determine which amino acids on the β and pp32 proteins were phosphorylated, PrA virions were labeled in vivo,

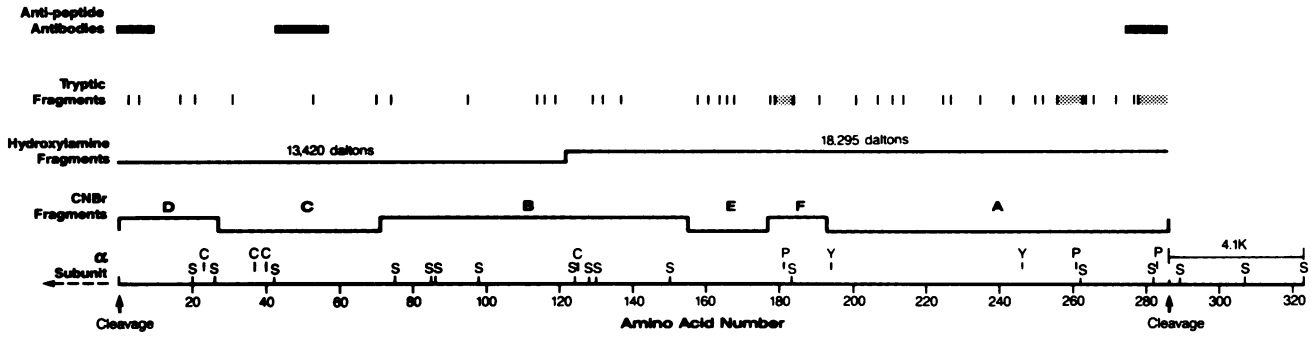

FIG. 1. Schematic for potential phosphorylation sites on pp32. The bottom line identifies the number and location of predicted amino acids in pp32; the locations of Ser (S), Cys (C), Pro (P), and Tyr (Y); the identified in vivo proteolytic cleavage sites; and the 4,100-dalton protein found at the very COOH-terminal domain of avian retrovirus *pol*. The predicted cleavage sites for CNBr and hydroxylamine on pp32 are illustrated as potential fragments. The predicted tryptic fragments are marked by vertical lines, and three possible p-Ser tryptic fragments located in the COOH-terminal region are marked with stippling. The top three horizontal bars are peptides which have generated rabbit immunoprecipitating antibodies.

using $^{32}P_i$. The labeled virions were lysed and immunoprecipitated by antisera directed against pp32. The labeled β and pp32 proteins were purified by SDS-polyacrylamide gel electrophoresis (39). The excised proteins were hydrolyzed to free amino acids (28). The amino acids p-Tyr, p-Thr, and p-Ser were resolved by high-voltage paper electrophoresis, and the presence of ^{32}P was detected by autoradiography. In three separate experiments, only p-Ser was detected with either β or pp32 (Fig. 3). Phosphoamino acid analysis of purified phosphorus-labeled tryptic peptides 1 and 2 (see below) resulted only in the identification of p-Ser (data not shown).

Tryptic peptide analysis of ^{32}P-labeled β and pp32. It was previously demonstrated that β or pp32 purified from ^{32}P-labeled PrC contained three phosphorylated tryptic peptides

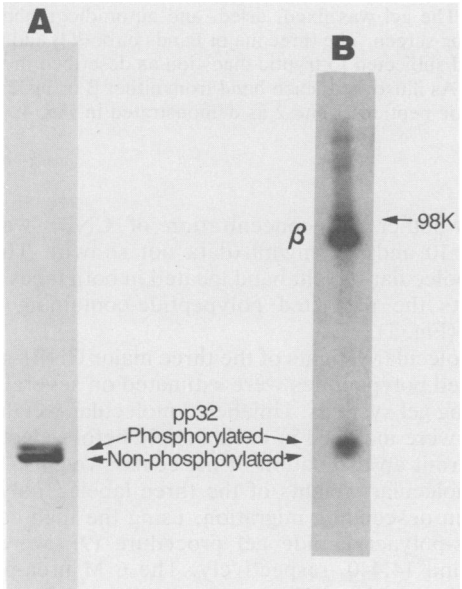

FIG. 2. Ratio of phosphorylated and nonphosphorylated pp32 in different avian retroviruses. Discontinuous SDS-10% polyacrylamide gel electrophoresis was used to define the ratio of phosphorylated and nonphosphorylated forms of pp32. (A) Glycerol gradient-purified avian myeloblastosis virus pp32 (2.8 μg). (B) ^{35}S-labeled pp32 immunoprecipitated by anti-pp32 rabbit serum from detergent-lyzed PrA labeled with methionine.

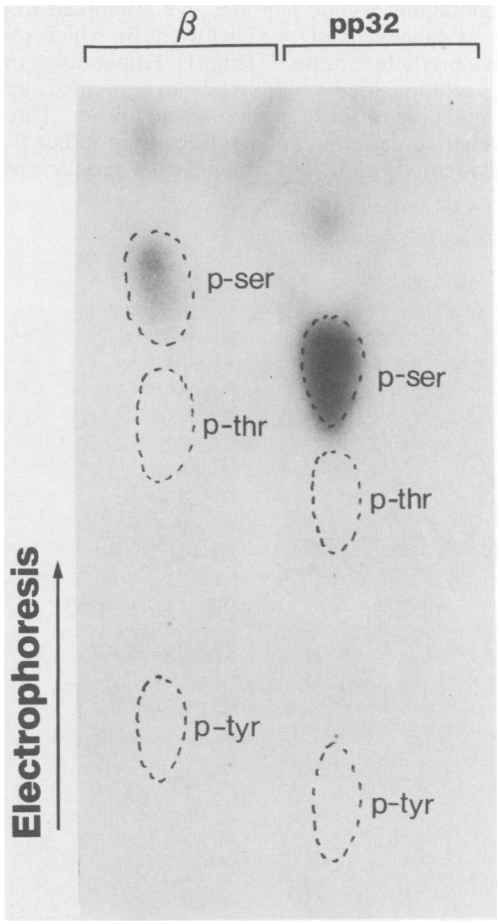

FIG. 3. Phosphoamino acid analysis of PrA ^{32}P-labeled β and pp32. Immunoprecipitated SDS-polyacrylamide gel-purified β and pp32 were isolated as described in Materials and Methods. The bands of protein were hydrolyzed to free amino acids and p-Tyr, p-Thr, and p-Ser were resolved by paper electrophoresis. The marker phosphorylated amino acids were visualized by ninhydrin staining and are circled. The total counts per minute applied for β and pp32 were 1,400 and 2,500, respectively. The different migration patterns of the β and pp32 samples were due to sample preparation.

(39). Both β and pp32 isolated from in vivo-labeled PrA also contained three ^{32}P-labeled tryptic peptides (Fig. 4). The same three tryptic peptides (no. 1, 2, and 3 in Fig. 4) were reproducibly observed in numerous experiments. These three tryptic peptides from PrA β or pp32 also comigrated in a mixing experiment (Fig. 4D), suggesting that the same three p-Ser-containing tryptic peptides of these two proteins are identical. The relative ratio of the labeled peptides 1, 2, and 3, as determined by Cerenkov counting, was 28, 21, and 1, respectively. The same three phosphorus-containing peptides were also observed whether the β or pp32 proteins were not treated with performic acid before trypsin digestion or whether the electrophoresis buffer was changed to ammonium carbonate (pH 8.9) (data not shown). Although we cannot exclude the existence of other minor phosphorus-containing peptides, it appears that PrA β and pp32 (Fig. 4) contain only three p-Ser tryptic peptides.

CNBr cleavage of ^{32}P-labeled β and pp32. There are 14 Ser predicted for pp32 (40), and their positions are illustrated in Fig. 1. The majority of these Ser are located in the NH_2-terminal half of pp32. To map the location of the three p-Ser-containing tryptic peptides, we attempted to partially cleave ^{32}P-labeled β or pp32 with CNBr which cleaves at Met (five predicted for pp32) (Fig. 1). Hopefully, a precursor product relationship would have been generated, permitting partial mapping of these in vivo-labeled p-Ser. Three major ^{32}P-labeled comigrating polypeptides from either β or pp32 were evident (Fig. 5). No other partial products were observed whether the concentration of CNBr was varied between 10 and 150 mg/ml (data not shown). The fainter higher-molecular-weight band located in both lanes probably represents the predicted polypeptide-containing fragment CBEFA (Fig. 1).

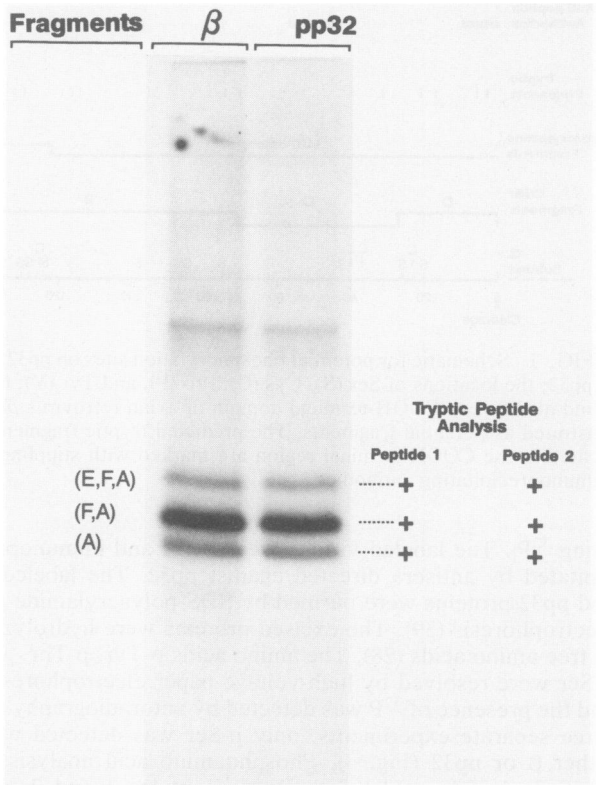

FIG. 5. CNBr cleavage of PrA ^{32}P-labeled β and pp32. Immunoprecipitated ^{32}P-labeled pp32 and β purified from discontinuous SDS-polyacrylamide gels were subjected to CNBr (100 mg/ml) cleavage as described in Materials and Methods. The treated and eluted samples were electrophoresed on a high-concentration Tris-15% polyacrylamide gel. Fragments EFA, FA, and A probably correspond to the CNBr fragments illustrated in Fig. 1. Tryptic peptide analysis was also performed on these CNBr-cleaved *pol* proteins. The gel was fixed, dried, and autoradiographed with an intensifying screen. The three major bands of both β and pp32 were eluted and subjected to tryptic digestion as described in the legend to Fig. 4. As illustrated, each band from either β or pp32 contained both tryptic peptides 1 and 2 as demonstrated in Fig. 4.

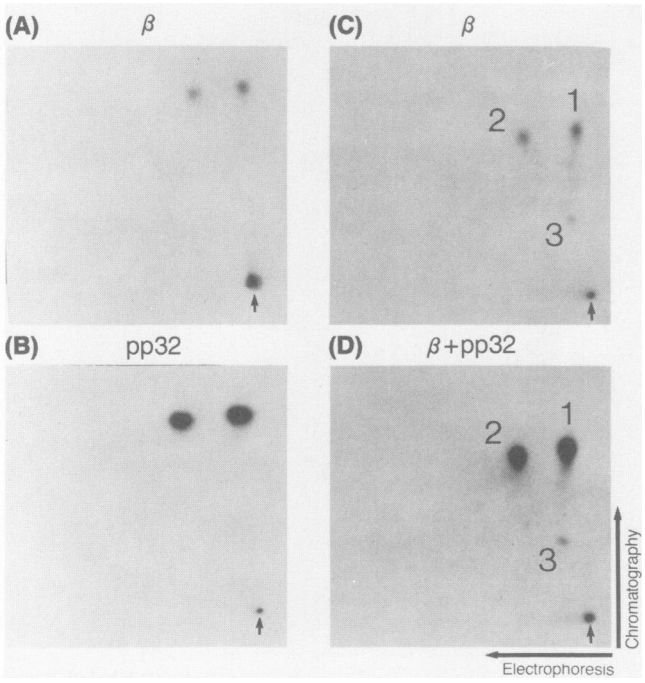

FIG. 4. Tryptic peptide analysis of phosphorus-labeled β and pp32. (A) Purified β was subjected to exhaustive trypsin digestion as described in Materials and Methods. After digestion, the sample (165 cpm) was lyophilized and processed on thin-layered cellulose plates. (B) Purified pp32 (450 cpm). (C) Different digested sample of β (190 cpm). (D) Purified pp32 (480 cpm) plus β (190 cpm). Panels A and B and panels C and D were processed on different days. The plates were exposed to autoradiography by using an intensifying screen for 2 weeks. The arrows indicate sites at which the sample was applied.

The molecular weights of the three major CNBr-generated ^{32}P-labeled polypeptides were estimated on several different denaturing gel systems. Unlabeled molecular weight protein markers were included in each sample before electrophoresis to permit an estimation of molecular weights. The estimated molecular weights of the three labeled polypeptides (Fig. 5) in descending migration, using the high-concentration Tris-polyacrylamide gel procedure (9), were 16,900, 15,500, and 14,440, respectively. The 6 M urea-polyacrylamide gel procedure (42) gave molecular weights of 18,400, 14,050, and 11,700 for the same three labeled polypeptides. Clearly, no accurate assessment of molecular weights or positive identification of these CNBr fragments was possible due to the presence of the negatively charged phosphate group(s) on these polypeptides.

The three CNBr ^{32}P-labeled polypeptides resolved on the

high-concentration Tris-polyacrylamide gel system (Fig. 5) were analyzed to determine if a specific tryptic peptide (Fig. 4) originated from a specific CNBr fragment. All three labeled polypeptides derived from either β or pp32 were eluted and subjected to tryptic peptide analysis (data not shown). As illustrated in Fig. 5, all six polypeptides contained both tryptic peptides 1 and 2 as shown in Fig. 4. The lightly labeled tryptic peptide (no. 3, Fig. 4) was not evident in these analyses. Therefore, there data demonstrate that all three CNBr fragments share the same sites of phosphorylation.

^{32}P-CNBr *pol* cleavage products lack [^{35}S]Cys. There are only four Cys predicted for pp32 (4), three of which are localized to the NH_2-terminal one-third of pp32 (Fig. 1). We wanted to determine if the ^{32}P-labeled CNBr cleavage fragments (Fig. 5) also contained Cys residues. PrA-infected chicken embryo fibroblast cells were labeled with [^{35}S]-cystine in cystine-free medium with 25% the normal concentration of methionine (35). This labeling procedure minimizes conversion of [^{35}S]Cys to Met. CNBr cleavage of [^{35}S]Cys-labeled pp32 or β as well as their ^{32}P-labeled counterparts is shown in Fig. 6. No [^{35}S]Cys-labeled fragments derived from pp32 comigrated with either of the two lower-molecular-weight ^{32}P-labeled fragments derived from β or pp32 in this 6 M urea-polyacrylamide gel system. The same results were obtained with the high-concentration Tris-polyacrylamide gel system (data not shown). There is no apparent comigration of a [^{35}S]Cys fragment with the top ^{32}P-labeled fragment (Fig. 6) or with the high-concentration Tris-polyacrylamide gel system (data not shown). For an internal [^{35}S]Cys-labeling control, the major labeled [^{35}S]Cys fragment derived from pp32 by CNBr cleavage is approximately 7,800 daltons, consistent with the localization of three Cys in the CNBr-derived fragment labeled DC (see Fig. 1). The partial CNBr digest pattern derived from [^{35}S]Cys-labeled β is too complex to interpret because it contains 12 predicted Cys.

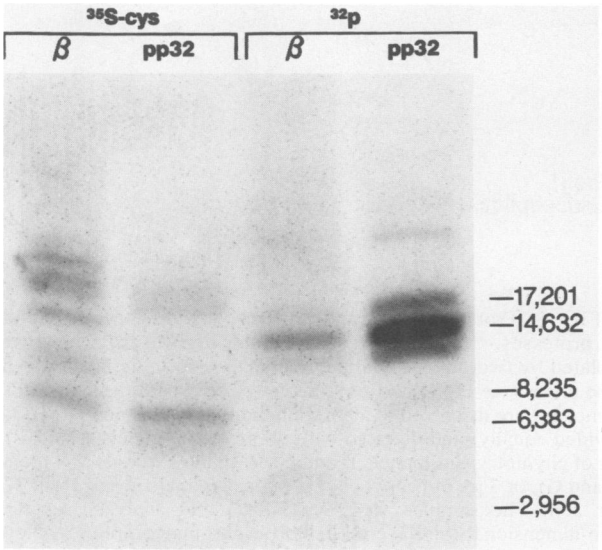

FIG. 6. CNBr cleavage of PrA [^{35}S]Cys or ^{32}P-labeled pp32 and β. After immunoprecipitation, both [^{35}S]Cys- or ^{32}P-labeled pp32 and β were isolated from discontinuous SDS-polyacrylamide gels and were treated with CNBr (100 mg/ml). The samples were eluted, myoglobin markers (Pharmacia) were added, and the samples were electrophoresed on a 6 M urea-SDS-polyacrylamide gel. The gel was stained with Coomassie blue, dried, and autoradiographed with an intensifying screen for 2 weeks. The molecular weights of the labeled fragments were estimated from myoglobin markers included in each sample. The numbers on the right side of the figure define the locations of each marker.

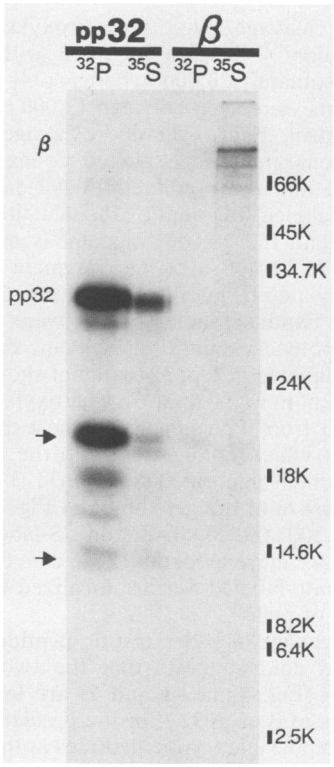

FIG. 7. Hydroxylamine cleavage of ^{35}S- or ^{32}P-labeled pp32 and β. The appropriate labeled proteins were immunoprecipitated and purified by SDS-polyacrylamide gels. The eluted proteins were subjected to hydroxylamine cleavage as described in Materials and Methods. The treated proteins were analyzed on an SDS–high-concentration Tris–17% polyacrylamide gel. The gel was stained with Coomassie blue, dried, and autoradiographed with an intensifying screen for 2 weeks. The locations of various molecular weight protein markers included in the samples are illustrated on the right. The arrows on the left indicate the appropriate cleavage bands for ^{32}P- and ^{35}S-labeled fragments derived from pp32.

In summary, these data indicate that the two major p-Ser-containing tryptic peptides are not present in predicted CNBr fragments D, C, or B (Fig. 1) and that at least these two major tryptic peptides map toward the COOH terminus of pp32.

Hydroxylamine cleavage of ^{32}P- and [^{35}S]Met-labeled β and pp32. Hydroxylamine specifically cleaves at rarely found Asn-Gly bonds, although other Asn-X bonds are slightly attacked (2, 37). There is only one Asn-Gly bond predicted in PrC for β or pp32 which is located 122 amino acids in from the NH_2 terminus of pp32 (17) (Fig. 1). The infectious PrA DNA clone used in this study also contains the above Asn-Gly bond, as predicted from DNA sequence analysis (T. Quinn, unpublished data). Therefore, cleavage of PrA pp32 by hydroxylamine should also permit the mapping of the labeled p-Ser to either the NH_2-terminal fragment (13,420 daltons) or to the COOH-terminal fragment (18,295 daltons) (see Fig. 1).

Purified [^{35}S]Met- or ^{32}P-labeled pp32 was subjected to

hydroxylamine cleavage (Fig. 7). Hydroxylamine cleavage generated a major ^{32}P-labeled fragment with a molecular weight of approximately 20,000, whereas only several minor labeled fragments were seen between 13,000 and 15,000. As an internal control, hydroxylamine cleavage of [^{35}S]Met-labeled pp32 generated the expected comigrating 20,000-dalton fragment and the predicted 13,400 dalton fragment (Fig. 7). A ^{35}S-labeled fragment (~18,500 daltons) migrating slightly faster than the 20,000 fragment is most likely the unphosphorylated 18,400 cleavage fragment derived from the unphosphorylated form of PrA pp32 (Fig. 2B, see similar ratio of protein bands). Similar results were obtained with the 6 M urea-polyacrylamide gel system, employing ^{32}P-labeled and ^{3}H-labeled PrA pp32 (data not shown). Although not clearly evident in Fig. 7 (lane 3), hydroxylamine cleavage of ^{32}P-labeled β from PrA generated the expected 20,000-dalton fragment which comigrated with the corresponding ^{32}P-labeled fragment generated from pp32 (Fig. 7, lane 1). Upon long exposure of the gel shown in Fig. 7 (lane 4), the approximate 18,500- and 20,000-dalton ^{35}S-labeled fragments derived from β are more evident. These data further suggest that the phosphate-labeled Ser are localized to the COOH-terminal region of pp32.

Proteolytic cleavage of p-Ser tryptic peptides. The previously presented data suggests that the two major p-Ser tryptic peptides (Fig. 4, no. 1 and 2) are localized in the COOH terminus of β or pp32. For the predicted amino acid sequence of two possible tryptic peptides at the very COOH terminus of pp32, including several specific proteolytic cleavage sites found within them, see Fig. 9. Digestion of the isolated p-Ser tryptic peptides derived from pp32 with specific proteases is shown in Fig. 8. Chymotrypsin preferentially cleaves at the W, F, or Y amino acids, prolyl endopeptidase cleaves at P residues (44), and *S. aureus* V-8 cleaves at E residues (20). Both isolated p-Ser tryptic peptides (Fig. 4 and 9) were partially cleaved by both chymotrypsin and prolyl endopeptidase, whereas only tryptic peptide 2 was digested with *S. aureus* V-8 protease (Fig. 8). These data are consistent with the predicted amino acid composition of each tryptic peptide. The p-Ser residues appear to be located on pp32 at amino acid positions 262 and 282, respectively (Fig. 1) (40).

DISCUSSION

The avian retrovirus pp32 protein and the corresponding protein moiety on the β polypeptide contain the same three p-Ser tryptic peptides. The two major peptides appear to map to the COOH terminus of these proteins. The location of these two p-Ser tryptic peptides on pp32 and β was established by (i) using various radiolabeled compounds to differentially label pp32 or β in vivo followed by specific protein chemical cleavages, (ii) tryptic peptide analysis, and (iii) specific proteolytic cleavage of isolated p-Ser tryptic peptides.

Immunoprecipitation of the three major ^{32}P-labeled CNBr fragments (Fig. 5) generated from pp32 by either two separate NH_2-terminal antipeptide antibodies or one COOH-terminal antibody (Fig. 1) (13) resulted in the immunoprecipitation of all three fragments by the COOH-terminal antisera only (data not shown). In comparison with polyclonal antisera directed against pp32, the ability of the COOH-terminal antisera to immunoprecipitate the three CNBr fragments was greatly diminished. The decreased sensitivity of this antipeptide serum is probably due to the chemical denaturation of a tertiary structure essential for recognition of this particular antipeptide serum. All three antipeptide sera effectively immunoprecipitated intact pp32. These data further support the conclusion that the p-Ser-containing CNBr fragments are derived from the COOH-terminal region of pp32.

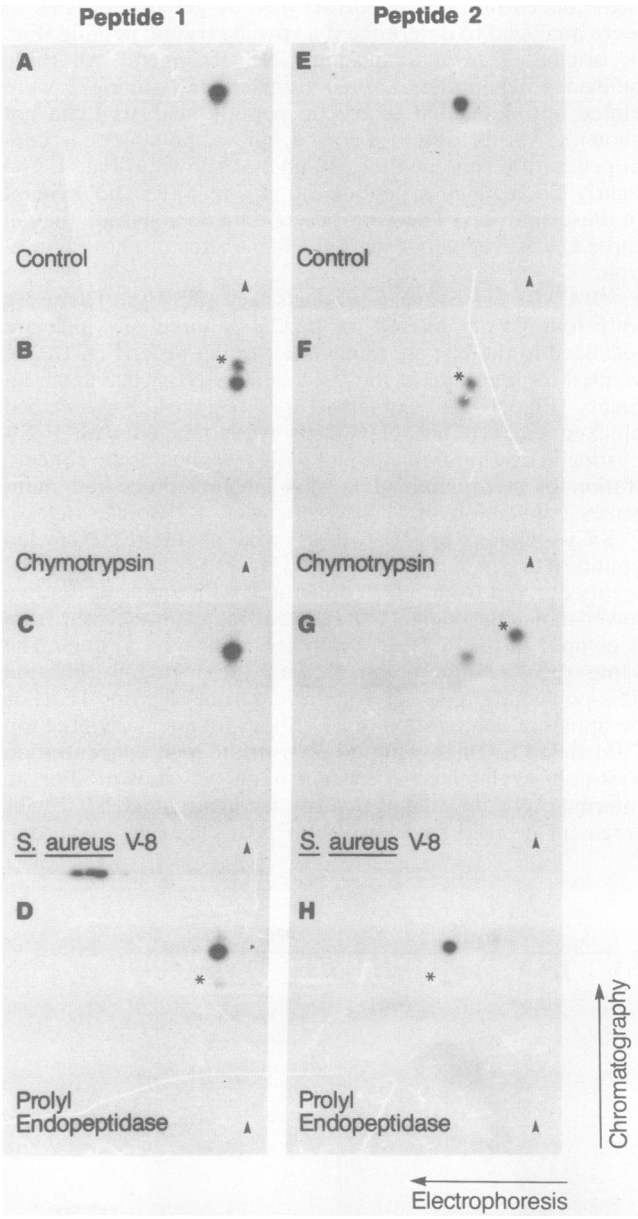

FIG. 8. Proteolytic cleavage of p-Ser-containing tryptic peptides by proteases. ^{32}P-labeled tryptic peptides derived from pp32 were isolated by two-dimensional peptide analysis on thin-layered cellulose plates (see Fig. 4). After recovery of both peptides 1 and 2, the samples were dissolved in 50 mM ammonium bicarbonate (pH 7.8), divided equally, and digested with no protease (panels A and E), 3 μg of chymotrypsin (panels B and F), 2 μg of *S. aureus* V-8 (panels C and G), or 3 μg of propyl endopeptidase (panels D and H) at 37°C for 2 h. The samples were lyophilized and analyzed again by two-dimensional thin-layered cellulose chromatography. *, Newly generated p-Ser peptide. These analyses were done in duplicate with identical results. See Fig. 9 for predicted sequence of the proposed tryptic peptides and specific protease cleavage sites.

The p-Ser tryptic peptides (no. 1 and 2) derived from PrA pp32 or β appear similar to p-Ser tryptic peptides (no. 1 and

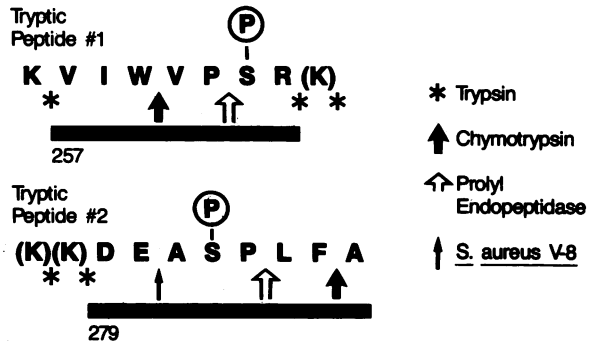

FIG. 9. Sequence of amino acids contained in the two proposed p-Ser tryptic peptides. The amino acid sequence and the numbering of residues are from Schwartz et al. (40). The darkened line indicates the length of each tryptic peptide, and the symbols indicate protease cleavage sites. The number below the darkened line identifies the first amino acid position of the tryptic peptide in pp32. The total number of amino acids in pp32 is 286.

2) previously reported for the PrC *pol* proteins (39) (data not shown). The PrA *pol* peptides appear to chromatograph similarly to the PrC peptides, using the slightly different two-dimensional thin-layered cellulose procedure previously described (39). Further analysis is needed to definitely establish this result.

The exact structure of the PrA p-Ser-containing tryptic peptides is unknown. As illustrated in Fig. 9, both peptides contain two adjacent trypsin digestion sites. Trypsin does not act as an efficient exopeptidase (21). We cannot exclude the possibility that each tryptic peptide contains one or two basic residues even under our exhaustive trypsin digestion conditions. However, redigestion of each isolated tryptic peptide by trypsin did not result in the generation of new peptides (data not shown).

Results of this study suggest but do not prove that p-Ser peptides 1 and 2 map to the COOH terminus of pp32. It should be noted that the DNA sequence of PrA is identical to that of PrC (40) throughout this COOH-terminal region of PrA pp32 (nucleotide numbers 4975 to 5097) (unpublished data). The Schmidt-Ruppin B strain of RSV also contains these predicted Ser (23). The possibility exists that these p-Ser map between amino acid positions 124 to 150 (40). Peptide 2 contains Glu (Fig. 9). No Glu residues exist in the three predicted p-Ser tryptic peptides between amino acid positions 120 and 150, suggesting that peptide 2 maps to the COOH terminus of pp32. However, one predicted p-Ser tryptic peptide in this region contains both Pro and Trp residues. Our protease digestion results do not exclude peptide 1 (Fig. 9) being located in the middle of pp32, except that all three phospholabeled CNBr fragments do not contain Cys residues and conversly, no significant CNBr ^{32}P-labeled fragments were evident which comigrated with any of the lower-molecular-weight CNBr [^{35}S]Cys bands (Fig. 1 and 6; data not shown). Finally, single-amino-acid changes between PrA and PrC in this middle region may prevent an exact prediction of tryptic peptides or the presence of another Ser residue.

The location of the minor, labeled tryptic peptide (Fig. 4, no. 3) on either pp32 or β is unknown. Tryptic digestion of the three major p-Ser-containing CNBr cleavage fragments revealed the presence of the two major tryptic peptides only. The absence of the minor tryptic peptide was probably due to the lack of sensitivity. Tryptic peptide 3 could possibly be located at amino acid position 180 of pp32 or elsewhere (Fig. 1). Tryptic peptides 1 and 2 do not appear to map to amino acid position 180 because CNBr fragments EFA, FA, and A (Fig. 1 and 5) contain equivalent ratios of tryptic peptides 1 and 2. Second, the predicted p-Ser tryptic peptide at position 180 does not contain *S. aureus* V-8 or chymotrypsin cleavage sites.

A phosphorus-containing tryptic peptide (no. 3) derived from PrC β or pp32 was phosphorylated to a greater extent on pp32 than on β (39). The significance of this differential labeling of PrC peptide 3 on pp32 is unknown. This PrC peptide could have been the result of incomplete tryptic digestion conditions. Its relationship to the minor p-Ser peptide 3 of PrA is unknown. In contrast, both PrC and PrA peptides 1 and 2 contain nearly equivalent amounts of phosphate label (Fig. 4) (39). These results suggest that p-Ser peptides 1 and 2 of both viruses may play a significant role in defining the phosphorylated properties of pp32 and β.

The amino acid sequence of the 3′-terminal end of the *pol* gene is highly conserved among various classes of retroviruses, although the NH_2-terminal one-half is most conserved (3, 30). The 3′ terminus of *pol* encodes a function(s) essential for integration of viral DNA (6, 32, 41; T. Quinn and D. P. Grandgenett, unpublished data). The Moloney murine leukemia virus encodes a *pol* polypeptide of 46,000 daltons (20, 24, 43) which is not phosphorylated (43). It is unknown whether other retrovirus COOH-terminal *pol* polypeptides are phosphorylated, including human immunodeficiency virus (27) or spleen necrosis virus (29). If it is shown that other retrovirus COOH-terminal *pol* proteins are not phosphorylated, it would suggest a fundamental difference exists between RSV pp32 and other retrovirus *pol* products. At present, it is unclear at the biochemical level whether other retroviruses have a DNA endonuclease activity associated with their respective COOH-terminal *pol* polypeptides (20, 22, 27, 29, 31, 43).

What is the biological function(s) associated with this site-specific phosphorylation of Ser residues on both β and pp32? Site-directed mutagenesis studies directed at modifying these p-Ser residues to other amino acids will be necessary to define what biological function(s), if any, phosphorylation of *pol* has in the life cycle of this virus. These studies are currently in progress.

Is the Mg^{2+}-dependent DNA endonuclease of pp32 which generates apparently biologically pertinent nicks at the circle junction site of tandem avian retrovirus long terminal repeats (14) regulated by this phosphorylation? The αβ DNA polymerase does not have an associated Mg^{2+}-dependent DNA endonuclease activity under normal conditions of incubation (12). Partial chymotrypsin cleavage of the αβ DNA polymerase does result in the release of the pp32 moiety which possesses Mg^{2+}-dependent DNA endonuclease activity (12). Since both pp32 and αβ are apparently phosphorylated on the same Ser residues, it appears that the covalent association of the pp32 moiety with the α subunit in this αβ complex is sufficient to prevent expression of the Mg^{2+}-dependent activity. The contribution of both the phosphorylated and unphosphorylated forms of pp32 in controlling its associated DNA endonuclease activity is presently unknown.

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