Partial Phosphorylation In Vivo of the Avian Retrovirus pp32 DNA Endonuclease

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Avian retrovirus pp32, a DNA endonuclease which is structurally related to the avian retrovirus DNA polymerase β polypeptide, has been demonstrated to be partially phosphorylated in vivo. Unlabeled or [^{35}S]methionine-labeled pp32 from avian sarcoma virus or avian myeloblastosis virus migrated as an electrophoretic doublet on discontinuous sodium dodecyl sulfate-polyacrylamide slab gels. However, pp32 immunoprecipitated from avian sarcoma virus labeled in vivo with [^{32}P]orthophosphoric acid migrated as a single band, which co-electrophoresed with the slower-moving band of the doublet represented by unlabeled or ^{35}S -labeled pp32. The presence of a slower-migrating phosphorylated band in pp32 suggests that the observed electrophoretic heterogeneity of purified pp32 is due to partial phosphorylation. Tryptic peptide analysis of ^{32}P -labeled avian sarcoma virus β and pp32 demonstrated that all the three labeled peptides in the β polypeptide were also present in pp32. However, pp32 had one tryptic peptide which was preferentially labeled in comparison to the comigrating peptide found in β digests, suggesting that phosphorylation may play a role in the processing of pp32 from β or in the regulation of its associated DNA endonuclease activity.

Three enzymatic activities are associated with avian retrovirus $\alpha\beta$ DNA polymerase (for review, see reference 4). Two well-characterized activities, RNA-directed or DNA-directed DNA polymerase and RNase H, are associated with the free α polypeptide ($M_r = 62,000$) as well as with $\alpha\beta$ DNA polymerase itself. All of the amino acid sequences in the α polypeptide are also found in the β polypeptide ($M_r = 92,000$). The third activity, a DNA endonuclease which preferentially utilizes supercoiled DNA as substrate, appears to be associated with the portion of β containing amino acid sequences which are not found in α (6, 16). These non- α sequences of the β polypeptide are similar, if not identical, to those found in the avian retrovirus DNA endonuclease pp32 ($M_r = 32,000$) (6, 7, 16).

These polymerase-associated activities appear to reside in separate domains on the β polypeptide. Indirect experiments with various enzymatic inhibitors indicate that DNA polymerase and RNase H are located on different active sites (4). A polypeptide fragment ($M_r = 24,000$) having only RNase H activity can be isolated by partial chymotryptic digestion of the α moiety (11). Recently, non- α fragments of the β polypeptide ($M_r = 32,000$ to $34,000$) containing DNA endonuclease activity were derived from β by in vitro proteolytic cleavage (6). Thus, these enzymatic activities may be located in separate do-

maines linked by protease-sensitive hinge regions.

One evolutionary mechanism by which a protein may acquire structural diversity and, therefore, diversity of function is through a posttranslational modification such as selective phosphorylation (15). Two of the nucleic acid-binding proteins of avian retroviruses, pp19 and pp12, have been shown to be partially phosphorylated in vivo, with the phosphorylated form of each protein migrating more slowly than the non-phosphorylated form when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3, 12). Furthermore, it was previously reported that the β polypeptide of avian sarcoma virus (ASV) $\alpha\beta$ DNA polymerase, but not the α polypeptide, is phosphorylated in vivo (8). Thus, it was important to establish whether pp32, which is structurally related to the non- α portion of β , is itself phosphorylated in vivo and, if so, whether the phosphorylated regions of β and pp32 are similar. We report that pp32 from ASV or avian myeloblastosis virus (AMV) is partially phosphorylated in vivo and that the ^{32}P -labeled tryptic peptides of β and pp32 are similar.

To determine whether pp32 is phosphorylated in vivo and to obtain pure polypeptide samples for tryptic peptide analysis, it was necessary to label avian retrovirus phosphoproteins in vivo with [^{32}P]orthophosphoric acid and then to immunoprecipitate selectively the small quantities of labeled polymerase gene products from virions which contained more than 95% of their

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protein-bound radiophosphorus in nonpolymerase gene products (3, 12). As illustrated in Fig. 1, [^{35}S]methionine-labeled β , α , and pp32 (electrophoresing as a visible doublet) were selectively immunoprecipitated from AMV virions by rabbit antiserum directed against purified AMV $\alpha\beta$ DNA polymerase. Figure 1B also shows that antiserum directed against pp32 selectively immunoprecipitated the β polypeptide as well as pp32 from ^{35}S -labeled AMV. Since pp32 shares no detectable proteolytic peptides with the α polypeptide (6, 16), α was not specifically precipitated by this antiserum. A minute amount of the α polypeptide is present in our immunoprecipitates, presumably due to nonspecific immunoprecipitation or "trapping." Similar results were obtained when ^{35}S -labeled ASV was subjected to immunoprecipitation by these antisera (data not shown). These data demonstrated that the immunoprecipitates obtained with these two antisera were sufficiently specific for use in our *in vivo* phosphorylation studies and tryptic peptide analyses.

To determine whether the pp32 protein incorporates radioactive phosphorus, it was necessary to electrophorese both ^{35}S - and ^{32}P -labeled pp32 in the same gels. To obtain sufficient phosphorylated protein for analysis, we had to use approximately ninefold more ^{32}P -labeled viral protein as starting material than ^{35}S -labeled protein. Figure 2 depicts a typical autoradiogram showing the immunoprecipitation of ASV proteins labeled *in vivo* with [^{35}S]methionine (lane A) or [^{32}P]orthophosphoric acid (lane B) by antiserum directed against pp32. As previously reported (8), the β polypeptide was found to be phosphorylated. In addition, a single band labeled with radiophosphorus co-electrophoresed with the slower-migrating band of the ^{35}S -labeled pp32 doublet. The same results were obtained when antiserum directed against AMV $\alpha\beta$ DNA polymerase was used and when the corresponding polypeptides were immunoprecipitated from ^{32}P -labeled AMV by antisera directed against pp32 or $\alpha\beta$ DNA polymerase (data not shown). No incorporation of radiophosphorus into α was detected (see also reference 8). The relatively small amounts of ^{32}P -labeled pp19 and pp12 present in our samples (in comparison with the quantities in unfractionated virions) were due to trapping of these proteins in our immunoprecipitates.

These findings indicate that, like the other avian retrovirus nucleic acid-binding proteins, pp32 is partially phosphorylated *in vivo*. Polyuridylic acid-Sepharose-purified pp32 and pp32 immunoprecipitated from virions labeled *in vivo* with [^{35}S]methionine exhibited electrophoretic

heterogeneity, each migrating as a doublet in discontinuous sodium dodecyl sulfate-polyacrylamide slab gels (16; Fig. 1). In contrast, pp32 immunoprecipitated from virions labeled *in vivo* with [^{32}P]orthophosphoric acid migrated as a

FIG. 1. Specificity of antiserum directed against AMV pp32. Rabbit antiserum directed against AMV $\alpha\beta$ DNA polymerase (A) or AMV pp32 (B) was used to immunoprecipitate [^{35}S]methionine-labeled AMV as described in the legend to Fig. 2. Approximately 10^6 trichloroacetic acid-precipitable cpm of [^{35}S]methionine-labeled AMV was immunoprecipitated by each antiserum, and then a portion of each sample was analyzed as shown in Fig. 2, except that the polyacrylamide gel was 32 cm in length. Left lane: top band, β ; middle band, α ; bottom doublet, pp32. Right lane: top band, β ; bottom doublet, pp32.

FIG. 2. Immunoprecipitation of [35 S]methionine- and [32 P]orthophosphoric acid-labeled ASV. Secondary cultures of C/E chicken embryo fibroblast (CEF) cells (SPAFAS, Inc., Norwich, Conn.) were infected with ASV, strain Prague C (initial virus stock provided by P. K. Vogt). Nearly confluent monolayer cultures of ASV-infected CEF cells were labeled for 24 h at 37°C with 200 μ Ci of [32 P]orthophosphoric acid per ml (carrier-free in 0.02 N HCl; New England Nuclear Corp.) in phosphate-free Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% dialyzed fetal bovine serum (KC Biological, Inc.), 1% dimethyl sulfoxide, 2% medium 199 (GIBCO Laboratories), and 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer; the supernatant media were harvested at 24-h intervals for several days and were replaced with phosphate-free Eagle minimum essential medium containing dialyzed fetal bovine serum, medium 199, and HEPES buffer, but no label. Other subconfluent monolayers of virus-infected CEF cells were labeled for 24 h at 37°C with 50 μ Ci of L-[35 S]methionine (New England Nuclear Corp.) in methionine-free Eagle minimum essential medium (GIBCO) containing 5% dialyzed fetal bovine serum, 0.25% nondialyzed

single band which co-electrophoresed with the slower-moving band of the 35 S-labeled pp32 doublet. Thus, the avian retrovirus pp32 protein exists in two forms—a slower-migrating phosphorylated form and a faster-migrating non-phosphorylated form; examination of the relative intensities of the two bands of the doublet produced by slab gel electrophoresis of polyuridylic acid-Sepharose-purified, unlabeled AMV pp32 (16) reveals that the faster-migrating non-phosphorylated species is the predominant form of the protein under the purification conditions employed.

To determine whether the β polypeptide and pp32 contained similar phosphorylated regions, β and pp32 were immunoprecipitated from 32 P-labeled ASV by antiserum directed against pp32 and were purified by gel electrophoresis (Fig. 2).

fetal bovine serum, 1% dimethyl sulfoxide, and 5% medium 199 (equivalent to a 50-fold excess of unlabeled methionine). The 32 P- or 35 S-labeled virions were purified from the harvested supernatants, and the virion proteins were immunoprecipitated by the Staphylococcus aureus protein A-antibody adsorbent method of Kessler (9) with rabbit antiserum directed against heparin-Sepharose-purified AMV $\alpha\beta$ DNA polymerase (5) or AMV pp32 purified through preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16). Certain samples were also treated for 30 min at 37°C with 25 μ g of bovine pancreatic DNase per ml, 20 μ g of bovine pancreatic RNase A per ml, and 20 μ g of bovine pancreatic RNase T₁ per ml immediately before immunoprecipitation. DNase and RNase digestion did not alter the electrophoretic characteristics of pp32 immunoprecipitated from 35 S- or 32 P-labeled virions (data not shown), suggesting that the incorporation of phosphorus into the pp32 protein was nuclease resistant and therefore specific for phosphoamino acid residues. The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the discontinuous Tris-glycine buffer system (10). Resolving gels (1.5 mm thick) contained a linear gradient of 8 to 22% acrylamide (30 acrylamide:0.8 N,N'-methylene-bis-acrylamide by weight); stacking gels contained 4.65% acrylamide. After electrophoresis at a constant current of 25 mA for approximately 5 h, the gel (16 cm) was processed for fluorography by the method of Bonner and Laskey (1), dried under vacuum, and exposed to Kodak RP Royal X-Omat film for 6 to 14 days at -70°C in the presence of Du Pont Cronex Lightning-Plus X-ray intensifying screens. Samples containing either 10⁶ trichloroacetic acid-precipitable cpm of [35 S]methionine-labeled ASV (A, 9 μ g of protein) or 10⁷ trichloroacetic acid-precipitable cpm of [32 P]orthophosphoric acid-labeled ASV (B, 80 μ g of protein) were immunoprecipitated by rabbit antiserum directed against pp32; 15 μ l of antiserum was used with the 35 S-labeled ASV, whereas 40 μ l of antiserum was used with 32 P-labeled ASV.

The appropriate labeled polypeptide bands were excised from the gels and subjected to tryptic peptide analysis as illustrated in Fig. 3. Only two prominently labeled peptides (Fig. 3A, no. 1 and 2) and a very faintly labeled peptide (no. 3) were present in the digest of the ^{32}P -labeled β polypeptide. In contrast, the corresponding peptides (no. 1, 2, and 3) in pp32 digests were labeled with nearly equal intensity (Fig. 3B, C, and D). The same relative proportions of the three labeled peptides in β and pp32 were found in three separate experiments. It should be noted that there are contaminating spots (not numbered) in Fig. 3B which do not represent peptides and are not present in other pp32 digests (Fig. 3C).

The region of the avian retrovirus polymerase gene encoding those sequences of the β polypeptide which correspond to pp32 appears to be at or near the 3'-end of the gene (14, 17; R. Eisen-

man, personal communication). We have recently determined the amino acid sequences at the amino termini of β and α and have arrived at the same conclusion (T. Copeland, D. P. Grandgenett, and S. Oroszlan, submitted for publication). The biological function of the partial in vivo phosphorylation of the avian retrovirus pp32 protein and of the specific phosphorylation of the non- α portion of the β polypeptide is not understood at present. Figure 3 demonstrates the presence of one peptide (spot no. 3) in tryptic digests of pp32 which was preferentially labeled with radiophosphorus in comparison with the comigrating peptide in β digests. This finding suggests that site-specific phosphorylation may be involved in the processing of the β polypeptide to yield pp32. Alternatively, the phosphorylation of β may affect the DNA polymerase, RNase H, or DNA endonu-

FIG. 3. Tryptic peptide analysis of ^{32}P -labeled β and pp32. ^{32}P -labeled ASV was immunoprecipitated by anti-pp32, and the sample was subjected to electrophoresis as described in the legend to Fig. 2. Without the aid of fluorography, the β and pp32 bands were visualized and excised from the gel, washed in 10% methanol, lyophilized, and then exhaustively digested with trypsin as previously described (6). After digestion, the samples were eluted and processed on thin-layer cellulose plates as described elsewhere (6) and exposed to autoradiography for 3 weeks under the same conditions as described in Fig. 2. (A) β polypeptide. (B) pp32. (C) Repeated pp32. (D) Schematic of B for labeling of peptides with numbers.

clease activity associated with the $\alpha\beta$ DNA polymerase molecule. Similarly, phosphorylation may be a critical determinant of the DNA endonuclease activity associated with pp32.

Finally, the fact that the avian retrovirus pp32 protein is only partially phosphorylated *in vivo* suggests that a biologically significant equilibrium exists between its phosphorylated and non-phosphorylated forms. Perhaps the presumptive precursor of pp32, the β polypeptide, is also only partially phosphorylated; it should be noted that the polyacrylamide gel system used in this study would not be able to separate phosphorylated and nonphosphorylated β polypeptides. Several reports (13, 18) have indicated that the degree of phosphorylation of the avian retrovirus DNA polymerase enzyme or of some phosphate acceptor protein closely associated with it is proportional to the DNA polymerase activity exhibited by the enzyme *in vitro*. Our findings suggest that this effect may be mediated through the pp32 protein or the phosphorylated pp32-specific (non- α) moiety of the β polypeptide. Further studies of the biosynthesis, processing, and enzymatic functions of all of the avian retrovirus polymerase gene products are in progress, and the possible existence and enzymatic consequences of protein-protein interactions involving $\alpha\beta$ DNA polymerase and pp32 are also currently under investigation.

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