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RNA TUMOR VIRUS PHOSPHOPROTEINS: PHOSPHORYLATION OF PRECURSOR AND PROCESSED POLYPEPTIDES

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Summary: Monospecific antisera made against the 30,000 molecular weight major internal polypeptide (p30) and the 12,000 molecular weight phosphorylated polypeptide (pp12) of a wild mouse type C oncovirus were used to immunoprecipitate precursor polypeptides from extracts of isotopically labeled cells infected with the oncovirus. Analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis led to the detection of several precursor polypeptide (Pr) species containing the determinants of both p30 and pp12. These species, namely Pr>100, Pr100, Pr77, Pr62, and Pr50, were all found to be phosphorylated in pulse experiments. The polypeptide pp12 was, however, the major phosphorylated species immunoprecipitated by anti-pp12 sera in pulse and chase experiments. These data and a relatively high degree of phosphorylation in the processed pp12 suggested that phosphorylation of oncovirus protein is initiated at the polyprotein level and the phosphoprotein moiety is further phosphorylated subsequent to processing.

Mature viral proteins of type C RNA tumor viruses are formed by the cleavage of intracellular precursor polyproteins (1-3). In Rauscher murine leukemia virus, several virus-specific precursor polypeptides have been identified (4-6). Among these precursors, the approximate size of the polypeptides containing determinants of the viral group-specific antigens (gag) ranges from 55,000 to 200,000 daltons (Pr55 to Pr200) (7). The gag gene codes for four low molecular weight (10,000 to 30,000 daltons) structural proteins termed p30, p15, p12, and p10. In type C viruses of the lower mammalian species p12 is phosphorylated (pp12), whereas the analogous phosphoprotein in primate viruses is pp15 (8-10) and in avian viruses it is pp19 (11). Since one of the gag proteins is modified by phosphorylation, it is of considerable interest that protein phosphorylation may have a significant role in the processing of the precursors to the mature viral gag proteins. In this paper we present results that show that the gag precursors of a wild mouse strain of murine leukemia virus are phosphorylated and that the processed pp12 product undergoes further intracellular phosphorylation.

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MATERIALS AND METHODS

Cells and viruses. Feral mouse embryo cell line SC-1 (13) chronically infected with wild mouse (WM) 292 virus (14) was maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Cells were grown in 60 mm^2 Petri dishes to near confluency, washed three times with warm Earle's balanced salt solution (EBSS) and labeled with [^{3}H]-amino acid mixture (10 μCi/ml) and [^{32}P]-phosphate (250 μCi/ml) in EBSS. Cells were incubated for 10 min in pulse experiments and 10 min followed by 2 hr chase in complete growth medium (MEM containing 10% FBS) in pulse-chase experiments. After incubation the medium was removed, the cells were washed twice with ice cold EBSS, and then immediately collected by scraping. An NP-40-deoxycholate lysate of the cells was prepared by a modification of the procedure of Vogt and Eisenman (1) with the exception that protease inhibitor, phenylmethylsulfonylfluoride, was added at a concentration of 300 μg/ml (15). The cell lysate was then centrifuged at 15,000 g for 15 min to obtain the supernatant (cell extract) for the immunoprecipitation experiments.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Specific antisera against gel filtration purified proteins (16), p30 and pp12, of WM-292 virus were made in New Zealand white rabbits as described (17). Immunoprecipitation reactions were carried out by mixing cell extracts from pulse or pulse-chase experiments, with the optimal amount of specific anti-p30 or anti-pp12 serum. The mixture was incubated at 37° for 15 min and then 16 to 18 hr at 4°. Immunoprecipitates formed were collected by centrifugation at 2000 g for 20 min at 4°, washed three times with ice-cold STE (100 mM NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH 7.4), and analyzed by SDS-polyacrylamide gel electrophoresis (16, 18). Standard proteins, bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome C were included in identical gels as molecular weight markers. Gels containing marker proteins were fixed and stained with Coomassie blue and those containing radioactive samples were fractionated by slicing and the radioactivity in the slices determined by liquid scintillation counting (19).

RESULTS

Specificity of WM-292 virus anti-p30 and anti-pp12 sera. To determine the specificity of the antisera, purified WM-292 virus was disrupted with NP-40, and radioiodinated by the chloramine-T method (20), and immunoprecipitated with the anti-p30 and anti-pp12 sera. Analyses of the immunoprecipitates in SDS-polyacrylamide gels show that the anti-p30 and anti-pp12 sera are monospecific and possess mutually exclusive immunological determinants (Fig. 1). These antisera did not react with extracts of uninfected SC-1 cells.

Precursor phosphorylation. Extracts from WM-292 virus-infected SC-1 cells, pulse labeled for 10 minutes with [^{3}H]-amino acid mixture and [^{32}P]-phosphate, were immunoprecipitated with anti-pp12 sera and the immunoprecipitates were studied by SDS-polyacrylamide gel electrophoresis. The results (Fig. 2A) show that several polypeptides containing both [^{3}H] and [^{32}P]-labels

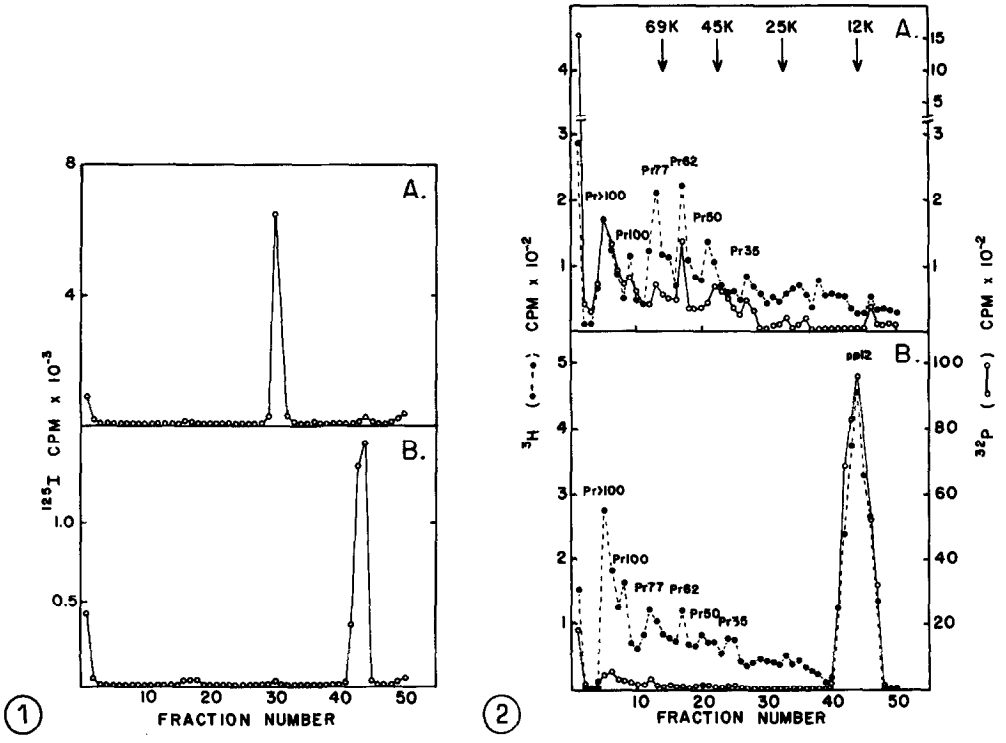

Fig. 1. Specificity of anti-p30 and anti-ppl2 sera. Immunoprecipitates obtained from radioiodinated disrupted WM-292 virus with anti-p30 sera (A), and with anti-ppl2 sera (B), were electrophoresed in 7.5% SDS-polyacrylamide gels.

Fig. 2. SDS-polyacrylamide gel electrophoresis of immunoprecipitates obtained with antisera against WM-292 virus ppl2. A. Immunoprecipitate formed with extract of virus infected cells pulse-labeled for 10 min. with [^{3}H]-amino acid mixture and [^{32}P]-phosphate. B. Immunoprecipitate obtained from extract of cells pulse-labeled for 10 min and then chased for 2 hr in unlabeled growth medium.

were resolved. Since these polypeptide species contained ppl2 determinants, they were considered to be precursors of this protein. Based on their approximate molecular weights, the species were designated as Pr>100, Pr100, Pr77, Pr62, Pr50, and Pr35. The mature viral polypeptide, ppl2, however, was not labeled in such a short pulse. It should be noted that all the precursor polypeptides were phosphorylated and that Pr>100 and Pr62 species contained relatively higher amounts of phosphate (Fig. 2A). In pulse-chase experiments, anti-ppl2 serum precipitated the mature ppl2 protein in addition to the pre-

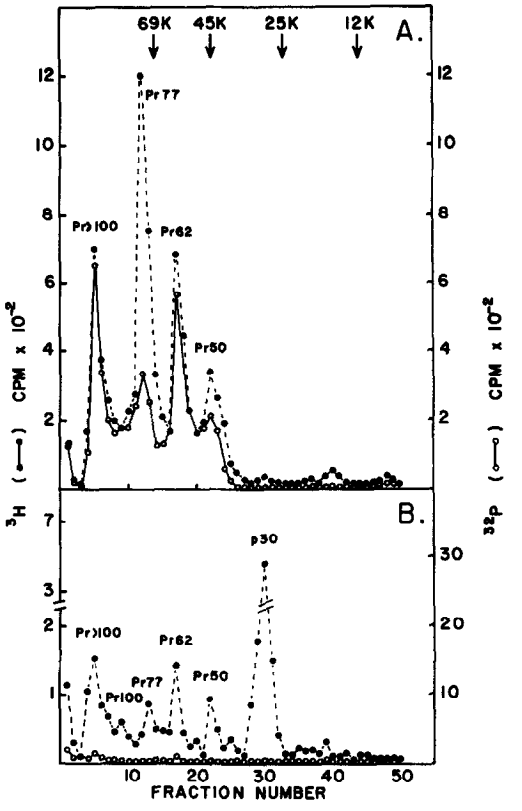

Fig. 3. SDS-polyacrylamide gel electrophoresis of immunoprecipitates obtained with antisera against WM-292 virus p30. A. Immunoprecipitate formed with extract of virus infected cells pulse-labeled for 10 min with [^{3}H]-amino acid mixture and [^{32}P]-phosphate. B. Immunoprecipitate formed with extract of cells pulse labeled for 10 min followed by 2 hr chase in unlabeled growth medium.

cursors described above (Fig. 2B). Although all major precursor species remained phosphorylated, the extent of phosphorylation of the pp12 species was the highest (Fig. 2B). The ratio of [^{32}P] to [^{3}H]-counts was about 20 for pp12 whereas that for a major phosphorylated precursor, such as Pr62, was less than one. Assuming that Pr62 was phosphorylated only at the pp12 moiety, and normalizing the [^{3}H]-counts for the molecular size difference between Pr62 and pp12, the phosphate content of the processed pp12 was still at least four-fold higher. These data indicated that the processed pp12 is further phosphorylated subsequent to cleavage from the precursor polypeptide.

Figure 3 shows our results with anti-p30 sera. This sera recognized all

the precursor species detected by the anti-pp12 sera except Pr35. The small amount of Pr100 was not resolved in the pulse experiments, although it was visible in the pulse and chase experiments using anti-p30 sera (Fig. 3A and 3B). Four major phosphorylated precursors, Pr>100, Pr77, Pr62, and Pr50, were well resolved (Fig. 3A). In addition, anti-p30 also precipitated nonphosphorylated mature p30 formed in the chase experiment (Fig. 3B). A comparison of the $^{32}P/^{3}H$ ratio of the various precursors indicates that the phosphorylation was highest in the case of Pr>100 and lowest with the Pr77. Such variation in the degree of phosphorylation was also noted in experiments described in Figure 2.

DISCUSSION

The results in this report show that the major intracellular precursor polypeptides, Pr>100, Pr77, Pr62, and Pr50, share antigenic determinants with the gag gene proteins, p30 and pp12, of the wild mouse leukemia virus. It is very likely that these precursors are equivalent to the corresponding Pr200, Pr80, Pr65, and Pr55 gag precursor polyproteins detected in Rauscher murine leukemia virus infected cells (7). Another polypeptide (Pr35), containing the determinants of pp12 but not p30, that we have detected in the wild mouse virus-infected cells is apparently different from Pr45 (7), which contains both p30 and pp12 moieties. This difference may be related to their representation of different portions of the murine gag-gene coded precursor polypeptide whose order has been determined as NH_2-p15-p12-p30-p10-COOH (21). Since Pr45 does not contain p15 determinants (7), it probably represents the p12-p30- part, while Pr35 may be the p15-p12-fraction. We find that all intracellular precursors, including Pr35, are phosphorylated. The degree of phosphorylation, however, seems to vary, Pr>100 and Pr62 being relatively highly phosphorylated. Whether this observation is related to sequential phosphorylation and dephosphorylation of the precursors at the pp12 moiety or due to phosphorylation at various sites remains unclarified in the present study. However, the stable virion or intracellular polyprotein, P60, has been

reported to be phosphorylated primarily on the pp12 moiety (12). Differential levels of phosphorylation of the mouse gag precursor polypeptides have also been detected by other authors; Pr65 is phosphorylated whereas Pr80 is not detectably phosphorylated (unpublished results cited in reference number 7). In addition to the variation in the level of precursor phosphorylation, our results suggest that the mature viral pp12 is further phosphorylated in the cell after cleavage from the precursor polypeptide. It would be important to examine whether all the intracellular mature pp12 molecules are equally phosphorylated or if they also exist in non-random multiple phosphorylated species as in the mature extracellular virion (9, 10, 22, 23). This aspect and the possible role of phosphorylation/dephosphorylation in the cleavage processes of the gag precursor polypeptides are being currently investigated.

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REFERENCES

1. Vogt, V. M. and R. Eisenman. (1973) Proc. Natl. Acad. Sci. USA 70, 1734-1738.
2. Naso, R. B., L. J. Arcement and R. B. Arlinghaus. (1975) Cell 4, 31-36.
3. Okasinski, G. F. and L. J. Velicer. (1976) J. Virol. 20, 96-106.
4. Arcement, L. J., W. L. Karshin, R. B. Naso, G. A. Jamjoom and R. B. Arlinghaus. (1976) Virology 69, 763-774.
5. Van Zaane, D., A. L. J. Gielkens, M. J. A. Dekker-Michielsen and H. P. J. Bloemers. (1975) Virology 67, 544-552.
6. Jamjoom, G. A., R. B. Naso and R. B. Arlinghaus. (1977) Virology 78, 11-34.
7. Arcement, L. J., W. L. Karshin, R. B. Naso and R. B. Arlinghaus. (1977) Virology 81, 284-297.
8. Pal, B. K. and P. Roy-Burman. (1975) J. Virol. 15, 540-549.
9. Pal, B. K., R. M. McAllister, M. B. Gardner and P. Roy-Burman. (1975) J. Virol. 16, 123-131.
10. Hayman, E. G., B. K. Pal and P. Roy-Burman. (1977) J. Gen. Virol. 36, 459-469.
11. Lai, M. M. C. (1976) Virology 74, 287-301.
12. Oskarsson, M. K., C. W. Long, W. G. Robey, M. A. Scherer and G. F. Vande Woude. (1977) J. Virol. 23, 196-204.
13. Hartley, J. W. and W. P. Rowe. (1975) Virology 65, 128-134.
14. Gardner, M. B., B. E. Henderson, J. D. Estes, R. W. Rongey, J. Casagrande, M. Pike and R. J. Huebner. (1976) Cancer Res. 36, 574-581.
15. Fahrney, D. E. and A. M. Gold. (1963) J. Amer. Chem. Soc. 85, 997-1000.
16. Pal, B. K., M. Wright, J. E. Officer, M. B. Gardner and P. Roy-Burman. (1973) Virology 56, 189-197.

17. Strand, M. and J. T. August. (1973) J. Biol. Chem. 248, 5627-5633.
18. Summers, D. F., J. V. Maizel and J. E. Darnell. (1965) Proc. Natl. Acad. Sci. USA 54, 505-513.
19. Roy-Burman, P., B. K. Pal, M. B. Gardner and R. M. McAllister. (1974) Biochem. Biophys. Res. Commun. 56, 543-551.
20. Greenwood, F. C., W. M. Hunter and J. S. Glover. (1963) Biochem. J. 89, 114-123.
21. Barbacid, M., J. R. Stephenson and S. A. Aaronson. (1976) Nature 262, 554-559.
22. Hayman, E. G., B. K. Pal, M. M. C. Lai and P. Roy-Burman. (1977) Biochem. Biophys. Res. Commun. 78, 1156-1161.
23. Pal, B. K. and P. Roy-Burman. (1977) Virology 83, 423-427.