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Two Structurally and Functionally Different Forms of the Transforming Protein of PRC II Avian Sarcoma Virus

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The primary translation product of the PRC II avian sarcoma virus genome is a protein of 105,000 daltons (P105), and we have previously shown that approximately 50% of the P105 molecules are converted to molecules of 110,000 daltons (P110) by posttranslational modification. Fractionation of PRC II-infected cells showed that P105 was contained primarily in a nonionic detergent-soluble compartment, whereas P110 partitioned almost exclusively with a nonionic detergent-insoluble or crude cytoskeletal fraction. The tyrosine-specific protein kinase activity previously observed in immunoprecipitates which presumably contained both P110 and P105 was found predominantly in the P110-containing immunoprecipitates made from the cytoskeletal fraction and was essentially absent from the P105-containing immunoprecipitates prepared from the soluble fraction. Individual analysis of ^{32}P-labeled P110 and P105 prepared by this fractionation technique revealed that P110 contained more phosphotyrosine per mole of protein than did P105. Examination of the tryptic peptide maps of ^{32}P-labeled P110 and P105 suggested that the additional phosphotyrosine in P110 resulted from phosphorylation at discrete sites within the protein. From these experiments, we conclude that PRC II-infected cells contain two discrete forms, P105 and P110, of the transforming protein and that each of these proteins exhibits distinct structural and functional characteristics.

A number of different RNA tumor viruses have been identified which code for transforming proteins with an associated tyrosine-specific protein kinase activity (2, 4, 12, 16, 21, 22, 27, 30, 37, 40, 43). Viruses of this type induce transformation of fibroblasts in vitro and include the avian sarcoma viruses (ASVs), Abelson murine leukemia virus (AbMuLV), and two strains of feline sarcoma virus. A role for this enzymatic activity in the initiation or maintenance of the transformed phenotype has been suggested by the observation that cells transformed by these viruses contain elevated levels of phosphotyrosine in protein (2, 4, 36, 37). Analysis of the phosphotyrosine-containing proteins present in transformed cells has revealed a number of possible cellular substrates for the associated protein kinase activities of these viruses (13, 15, 32, 35; for a review of Rous sarcoma virus [RSV] see reference 20). It is possible that the putative transforming proteins of these viruses are also substrates for their own enzymatic activity in an "autophosphorylation" reaction. This idea is suggested by the observation that these viral proteins can act as phosphate acceptors for the tyrosine-specific protein kinase activity in immune complexes containing these proteins (2, 4, 16, 22, 30, 43). Although it is not certain whether autophosphorylation occurs in vivo, it is clear that the transforming proteins are substrates for some tyrosine-specific protein kinase(s) since all of these proteins contain phosphotyrosine in vivo (2, 16, 21, 22, 30, 31, 37).

The factors which are involved in the regulation of the associated protein kinase activity in vivo are not known, although there is some evidence with the ASVs that phosphorylation at tyrosine residues may be important in this regard. Mutants of ASV have been isolated which are temperature sensitive for the maintenance of the transformed phenotype (17, 19, 32; for review of RSV temperature-sensitive mutants, see reference 4). This type of temperature-sensitive mutant has been described for RSV and for two ASVs, Fujinami sarcoma virus (FSV), and PRC II virus, which are closely related to one another but unrelated to RSV (3, 4, 18, 25, 29, 31, 38). When cells transformed with a temperature-sensitive mutant of RSV, FSV, or PRC II are shifted to the restrictive temperature, the amount of phosphotyrosine in total cellular protein decreases nearly to the level seen in uninfected cells, and there is a concomitant loss in

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the protein kinase activity associated with the putative transforming proteins of these viruses as assayed in vitro in the immunoprecipitate (12, 17, 21, 27, 32, 36; P. K. Vogt, personal communication). At the permissive temperature, the viral proteins of these temperature-sensitive mutants contain both phosphoserine and phosphotyrosine, but at the restrictive temperature, they contain mainly phosphoserine. These results are compatible with a model in which phosphorylation of the putative transforming protein at tyrosine residues is essential for associated protein kinase activity.

It is also possible that the location of the protein inside the cell contributes to the specificity of enzymatic activity. Immunocytochemical studies on RSV-transformed cells have shown $pp60^{src}$-specific antibody staining at the plasma membrane (42), and immunofluorescent analysis has shown $pp60^{src}$ in areas of cell-to-cell contact (10, 39) and in those structures involved in attachment of cells to the substratum, called adhesion plaques (33, 39). Cell fractionation techniques have been used to localize $pp60^{src}$ and its associated kinase activity to the plasma membrane (14, 23, 24) and to the cytoskeleton (11). These studies suggest a compartmentalization of $pp60^{src}$ to the plasma membrane or membrane-associated structures.

PRC II is a replication-defective ASV whose RNA genome and putative transforming protein are unrelated to the RSV genome and to $pp60^{src}$, respectively (4, 7, 29, 31, 38). The PRC II transforming protein, P105, has been shown to contain NH_2-terminal sequences derived from the helper virus *gag* gene and, in addition, a portion which is coded for by the transformation-specific sequences, termed the *fps* region (4, 28). We have previously observed (1) that approximately 50% of the P105 molecules are modified posttranslationally to yield a 110,000-dalton protein (P110) and that these proteins are present in roughly equimolar amounts at steady state. It was of interest to determine whether these two proteins might be found in different subcellular locations. By using a modification of the fractionation techniques described for RSV-transformed cells (11), we have separated PRC II-transformed cells into a cytoskeletal and a soluble compartment. This analysis revealed that P105 is found almost exclusively in the soluble fraction, whereas P110 is greatly enriched in the cytoskeletal fraction. We have used this method preparatively to study the individual structural and functional characteristics of P110 and P105.

MATERIALS AND METHODS

Cells and viruses. The PRC II virus used in these experiments was a cloned isolate prepared from stocks harvested from PRC II-infected chicken cells which were originally provided by P. K. Vogt (University of Southern California). Rous-associated virus type 2 was also from P. K. Vogt. Infection with PRC II cloned virus was done on gs^-chf^- chicken embryo fibroblasts (CEF) prepared from eggs supplied by SPAFAS, Inc., as described previously (5).

Radiolabeling and immunoprecipitation. Procedures for labeling cells with [^{35}S]methionine (Amersham) or $^{32}P_i$ (ICN) and immunoprecipitation have been described (34). Tumor-bearing rabbit (TBR) serum was obtained by injection of the Schmidt-Ruppin strain of RSV subgroup D into newborn rabbits (9). Rabbit antiserum to AMV p19 was provided by D. P. Bolognesi (Duke University Medical Center).

Cell fractionation. The procedure used was similar to those described by Brown et al. (8), Lenk et al. (26), and Burr et al. (11) to remove cytoplasmic proteins but to preserve most of the cytoskeletal components except microtubules. A monolayer of PRC II-transformed CEF was placed at 4°C and washed once with ice-cold Tris-buffered saline. The cells were then treated with a buffer solution containing 0.1 M KCl, 0.5% Nonidet P-40, 0.003 M $MgCl_2$, 0.01 M PIPES, pH 6.8, 0.3 M sucrose, 0.001 M ATP, and 1% Trasylol (Mobay Chemical Corp.) at 4°C for 10 min with intermittent gentle rocking. The supernatant or "soluble" fraction was removed, and the dish was washed with the same buffer. The soluble fraction was adjusted to 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 1% sodium deoxycholate, 1% Trasylol, and 0.002 M EDTA (RIPA with EDTA) and kept at 4°C for 20 min. The material remaining on the dish, the cytoskeletal fraction, was solubilized in RIPA with EDTA, scraped with a rubber policeman, and kept at 4°C for 20 min. Both fractions were then treated as described above for immunoprecipitation of total cell lysates.

Protein kinase assay and phosphoamino acid analysis. The procedures for assay of protein kinase activity present in immune complexes (1) and for analysis of phosphoamino acids in protein have been described before (21).

Tryptic peptide mapping. ^{32}P-labeled proteins were obtained by labeling PRC II-transformed CEF for 16 to 20 h with 1.0 mCi of $^{32}P_i$ per ml, followed by immunoprecipitation with appropriate antisera and preparative sodium dodecyl sulfate gel electrophoresis. Preparation of the samples and digestion with trypsin were performed as previously described (5). The digestion products were separated on 100-μm cellulose thin-layer plates by electrophoresis at pH 8.9 in 1% ammonium carbonate for 20 min at 1 kV in the horizontal dimension, followed by ascending chromatography in *n*-butanol–pyridine–acetic acid–water (75:50:15:60) in the vertical dimension.

RESULTS

P110 and associated kinase activity are found in the cytoskeleton. The generation and maintenance of two electrophoretically distinct forms, P110 and P105, of the PRC II transforming protein suggested the possibility that these two proteins might be found in different subcellular

compartments. To test this idea, we utilized a fractionation technique similar to that reported by Burr et al. (11) for RSV-transformed cells. This method separates cells into a nonionic detergent-soluble fraction and a nonionic detergent-insoluble or crude cytoskeletal fraction. A soluble fraction and a cytoskeletal fraction were prepared as described above from [^{35}S]methionine-labeled PRC II-transformed CEF. Each fraction was then immunoprecipitated with anti-p19 serum, which precipitates P110 and P105 by recognition of the p19 determinants at the NH_2 termini of the molecules (4, 28).

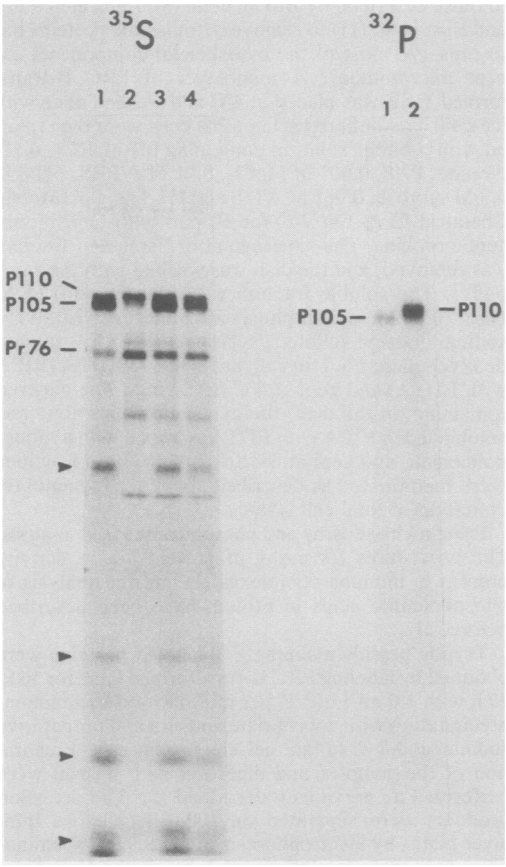

FIG. 1. Fractionation of PRC II-transformed cells. PRC II-transformed CEF were labeled for approximately 18 h with [^{35}S]methionine or $^{32}P_i$. The cells were separated into cytoskeletal and soluble fractions as described in the text, and each fraction was immunoprecipitated with anti-p19 serum. [^{35}S]methionine-labeled PRC II-transformed CEF lanes are as follows: soluble fraction (1); cytoskeletal fraction (2); and a mixture of equal volumes of the soluble and cytoskeletal fractions made before (3) and after (4) immunoprecipitation. ^{32}P-labeled PRC II-transformed CEF lanes are as follows: soluble fraction (1); cytoskeletal fraction (2). The bands indicated by the arrowheads are discussed in the text.

Figure 1 shows that P105 fractionated almost exclusively with the soluble preparation (lane 1), whereas the cytoskeletal fraction (lane 2) was highly enriched for P110. The precursor to the internal structural proteins, Pr76^{gag}, encoded by the helper avian leukosis virus also appeared to be enriched in the cytoskeletal fraction. Interestingly, anti-p19 serum immunoprecipitated a number of smaller polypeptides, many of which are likely to be intermediate cleavage products of Pr76^{gag}, and these partitioned with the soluble fraction (lane 1, arrowheads). The small amount of P110 present in the soluble fraction may have resulted from the removal of whole cells from the dish during the pipetting procedure. We have observed that the state of the cells, in particular their degree of adherence to the dish, is a critical factor for optimal fractionation of P110 and P105. The same pattern of fractionation was also observed with ^{32}P-labeled PRC II-transformed CEF (Fig. 1).

A tyrosine-specific protein kinase activity has previously been observed in immunoprecipitates containing both P110 and P105 (4, 30). This activity results in the phosphorylation of P110/P105 (autophosphorylation) when anti-p19 serum is used and of both P110/P105 and the heavy chain of immunoglobulin when TBR serum is used. The ability to separate P110 and P105 by a simple fractionation method allowed us to address the question of whether one or both of these proteins displays the associated kinase activity. For this purpose, cytoskeletal and soluble fractions were prepared in parallel from unlabeled and from [^{35}S]methionine-labeled PRC II-transformed CEF. Each fraction was immunoprecipitated with anti-p19 serum or with TBR serum. Analysis of the [^{35}S]methionine-labeled samples showed that P110 and P105 were present in approximately equimolar amounts in the cytoskeletal and soluble fractions, respectively (data not shown). Kinase assays were then performed on the unlabeled samples in this immunoprecipitate.

Figure 2 shows that although the soluble fraction contained a small amount of kinase activity, the majority of the activity was found in the cytoskeletal fraction. The kinase activity partitioned in a similar fashion with anti-p19 serum and with TBR serum. In the latter case, the autophosphorylating activity and the activity phosphorylating a heterologous substrate, the heavy chain of immunoglobulin G, were both found primarily in the cytoskeletal fraction. When an equal amount of the supernatant fraction was mixed with the cytoskeletal fraction, the protein kinase activity was undiminished (lanes 3A and B). The activity specific for the cytoskeletal fraction was also unaffected when the soluble and cytoskeletal preparations were

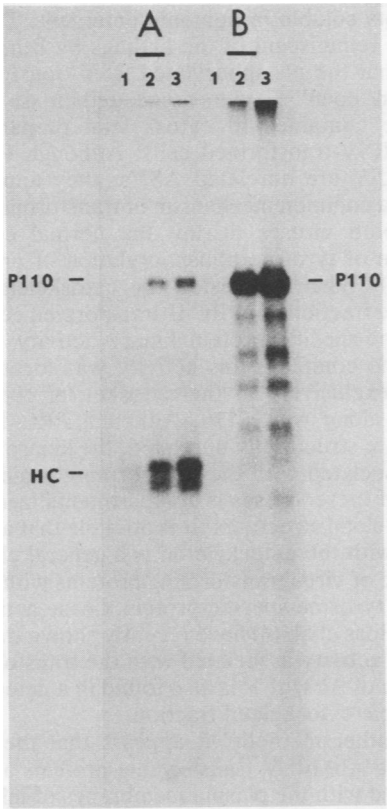

FIG. 2. Kinase activity fractionates with P110. PRC II-transformed CEF were separated into cytoskeletal and soluble fractions, and one-half of each fraction was immunoprecipitated with either TBR serum (A) or anti-p19 serum (B). Kinase assays in the immune complex were performed as described in the text. Lanes for both (A) and (B) show kinase activity in (1) the soluble fraction, (2) the cytoskeletal fraction, and (3) a mixture of an equal volume of the soluble and the cytoskeletal fractions made after immunoprecipitation. HC denotes the position of the heavy chain of immunoglobulin visualized by staining.

mixed before immunoprecipitation (data not shown). Therefore, it seemed that the lack of kinase activity in the P105-containing fraction was not due to the presence of a diffusible substance(s) inhibitory to this reaction.

Differences in the degree and sites of tyrosine phosphorylation between P110 and P105. From the experiments described above, it appeared that the protein kinase activity associated with unfractionated PRC II proteins could be attributed almost exclusively to P110. We were interested in whether other factors aside from subcellular locale might be correlated with this functional difference between P110 and P105. Reports of a relationship between tyrosine phosphorylation and kinase activity with temperature-sensitive mutants of RSV, FSV, and PRC II (12, 17, 21, 27, 32, 36; P. K. Vogt, personal communication) suggested the possibility that P110 is more highly phosphorylated at tyrosine residues than is P105. Initial experiments with ^{32}P-labeled PRC II-transformed CEF demonstrated that, at steady state, P110 consistently contained 5- to 10-fold more ^{32}P radioactivity when normalized to ^{35}S radioactivity than did P105. For example, in the experiment shown in Fig. 1, a lysate of [^{35}S]methionine-labeled PRC II-transformed cells fractionated and immunoprecipitated in parallel with the ^{32}P-labeled preparation yielded approximately equal radioactivities in P110 in the cytoskeletal fraction and P105 in the soluble fraction (data not shown). We wished to determine whether the additional ^{32}P label in P110 was due to phosphorylation at tyrosine or serine residues or both. For this purpose, ^{32}P-labeled preparations of P110 and P105 were isolated from cytoskeletal and soluble fractions, respectively, and the phosphoamino acid content of the proteins was determined after acid hydrolysis (Fig. 3). Elution of the phosphoamino acids from each preparation and quantitation by liquid scintillation counting showed that the phosphotyrosine-to-phosphoserine ratio was 4:1 for P110 and 1:1 for P105. The precise ratios were found to vary somewhat from experiment to experiment, which may be due to variability in the extent of cross-contamination between P110 and P105. However, P110 consistently contained more phosphotyrosine than did P105, and the ratio of phosphotyrosine to phosphoserine was characteristically higher for P110 than for P105.

An interesting question raised by these experiments was whether the additional phosphotyrosine in P110 resulted from the phosphorylation

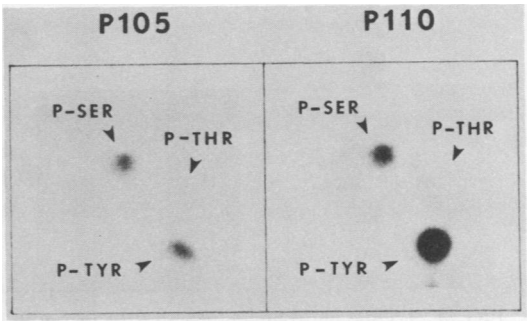

FIG. 3. Phosphoamino acid analysis of P105 and P110. PRC II-transformed CEF were labeled for 20 h with $^{32}P_i$, separated into soluble and cytoskeletal fractions, and immunoprecipitated with anti-p19 serum. The P105 and P110 bands were excised from the gel and treated as described in the text for analysis of phosphoamino acids.

of tyrosine residues at distinct sites. We tested this idea by performing tryptic peptide analysis on ^{32}P-labeled P110 and P105 prepared from a cytoskeletal fraction and a soluble fraction, respectively (Fig. 4). Although the tryptic peptide maps of P110 and P105 were very similar qualitatively, three major peptides contained in P110 were underrepresented in the P105 preparation (these peptides are numbered 2, 5, and 8). Elution of these peptides from the P110 preparation and subsequent phosphoamino acid analysis revealed that all three peptides contained predominantly phosphotyrosine (Fig. 4C). From these experiments, we conclude that P110 contains more phosphotyrosine per mole of protein than does P105 and that this additional phosphorylation occurs at discrete sites within the protein.

DISCUSSION

The primary translation product of the PRC II ASV genome is a protein of 105,000 daltons (P105) (1, 3, 4, 28). Within 15 min of synthesis, some proportion of the P105 molecules are modified to yield molecules of 110,000 daltons (P110), and at steady state, P110 and P105 are present in approximately equimolar amounts (1). In this paper, we report that P110 and P105 are localized in different subcellular compartments in PRC II-transformed CEF. P110 was found primarily in a crude cytoskeletal fraction which presumably contained primarily cytoskeletal structural proteins and residual nuclear matrix (8, 11, 26). P105, on the other hand, fractionated with the cytoplasm and plasma membrane components soluble in nonionic detergent. This result is reminiscent of the findings by Burr et al. (11) that the phosphorylated RSV transforming protein, pp60^{src}, and its associated kinase activity are contained in cytoskeletal preparations from RSV-transformed cells. Although PRC II and RSV are unrelated ASVs, they appear to share a common mechanism of transformation in that both viruses disrupt the normal cellular pattern of tyrosine phosphorylation of proteins (4, 36). When we tested the cytoskeletal and soluble fractions of PRC II-transformed cells for tyrosine-specific protein kinase activity in the immune complex, this activity was located almost exclusively in the cytoskeletal compartment, along with P110. Although PRC II and RSV are structurally unrelated, the kinase activity associated with the transforming proteins of both of these viruses is compartmentalized with cytoskeletal structures. It is possible that association with the cytoskeleton is a general characteristic of virus transforming proteins with associated tyrosine-specific protein kinase activities since Boss et al. (6) have recently shown that the kinase activity associated with the transforming protein of AbMuLV is also found in a detergent-insoluble cytoskeletal fraction.

By other methods, it appears that the RSV and the AbMuLV transforming proteins are associated with the plasma membranes of infected cells (14, 23, 24, 42, 44). A possible explanation for these seemingly contradictory results is that there may be two domains in the transforming protein, one which associates with the plasma

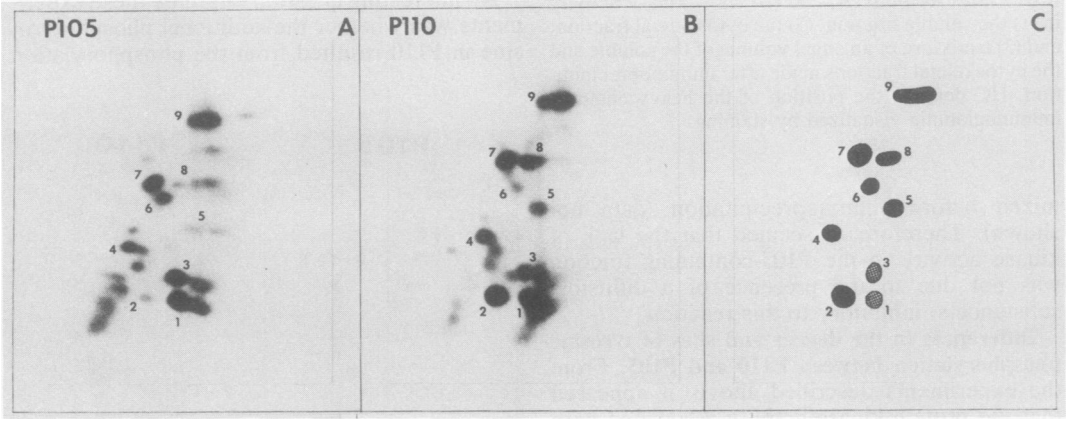

FIG. 4. Tryptic phosphopeptides in P105 and in P110. ^{32}P-labeled P105 and P110 were prepared as indicated in the legend to Fig. 3. Digestion with trypsin and separation of the phosphopeptides in two dimensions were carried out as described in the text. The origin is approximately in the middle of the plates, and the cathode is at the right. The radioactivity was visualized through the use of an intensifying screen, and exposure times were 5 days (A) and 36 h (B). Each of the numbered phosphopeptides in the P110 preparation was eluted and analyzed for phosphoamino acid content as described by Patschinsky and Sefton (31). The phosphoamino acids in each phosphopeptide are depicted schematically in (C). Symbols: ●, predominantly phosphotyrosine; ◕, phosphotyrosine and phosphoserine (approximately 1:1).

membrane and one which attaches to the cytoskeleton. It is not certain, however, that this model need be invoked since cytoskeletal fractions prepared by extraction with nonionic detergents contain several enzymatic activities which usually fractionate with the plasma membrane (S. Courtneidge, personal communication). It is possible that, instead of attachment to cytoskeletal structures, these proteins may actually be associated with plasma membrane components not extracted in the soluble fraction. Similarly, although PRC II P110 fractionates with the cytoskeleton, this does not preclude the possibility that P110 may be a plasma membrane protein. Indeed, it seems likely that one or both of the PRC II proteins is associated with the plasma membrane since the closely related FSV transforming protein appears predominantly in plasma membrane fractions (D. Moss, personal communication). Clearly, additional methods of localization must be employed before assigning definitive subcellular locations to P110 and P105.

Fractionation of PRC II-transformed cells into a crude cytoskeletal and a soluble compartment yielded preparations of P110 and P105 which were minimally cross-contaminated. Individual examination of ^{32}P-labeled P110 and P105 showed that P110 is more highly phosphorylated than P105 at tyrosine residues and that these residues map to distinct sites within the protein. It is tempting to speculate that the differential modification of P110 and P105 could explain the relative enzymatic activities of these two proteins. There is a strong positive correlation between the phosphorylation of the tyrosine in pp60^{src} and the presence of pp60^{src} kinase activity (27). By analogy with pp60^{src}, it is possible that the associated enzymatic activity of P110 is regulated by phosphorylation at one or more specific tyrosine residues. However, the issue is complicated by the observation that there are two parameters which correlate with kinase activity, that of cytoskeletal location and that of additional phosphorylation at tyrosine residues. It will be necessary to analyze the enzymatic activity of purified P110 free of any cytoskeletal components which might be contaminating the immunoprecipitates to clarify the roles of cytoskeletal location and tyrosine phosphorylation independently.

We had previously observed (1) that the tryptic peptide maps of [^{35}S]methionine-labeled P110 and P105 were identical, although there was a difference of approximately 5,000 in the apparent molecular weights of these proteins. It is possible that the additional phosphorylation of P110 might explain the difference in the electrophoretic mobilities of P110 and P105, perhaps by altering local sodium dodecyl sulfate binding to the protein and, therefore, decreasing the mobility more than expected from the simple addition in mass of the phosphate groups. We are currently doing experiments to map the additional phosphotyrosine groups to the *fps* or the *gag* portion of the molecule by cleaving with the viral protease p15.

The kinase activity in immunoprecipitates of cytoskeletal preparations from PRC II-transformed cells seems to be specific for the presence of P110 since similar preparations from uninfected cells do not display this activity (data not shown). In addition, attempts to confer kinase activity on P105 by mixing soluble fractions from PRC II-infected cells with cytoskeletal preparations from uninfected cells have been unsuccessful. The observation that P110-containing fractions have kinase activity but that P105-containing fractions do not allows a tentative assignment of this enzymatic function to P110. It is possible that P105 can act as a kinase under certain circumstances but that we did not observe this activity because substances inhibitory to this reaction might be complexed with P105. Alternatively, it is feasible that P105 is performing a function in the noncytoskeletal portions of the cell which we have not yet envisioned and therefore cannot assay. Nonetheless, these observations suggest a function for the generation and maintenance of P110 in the induction of the transformed phenotype by PRC II virus.

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