Proteolytic digestion of the transforming protein of Rous sarcoma virus (pp60src) with trypsin, chymotrypsin, or thermolysin generated a 29,000-dalton fragment representing the carboxyl half of this molecule. This proteolytic fragment was able to phosphorylate pp60src-specific immunoglobulin as well as exogenous substrates such as angiotensin, casein, and tubulin. When quantitated on a molar basis, the protease-resistant fragment of pp60src had a greater specific activity than the intact enzyme. Digestion of pp90yes, the transforming protein of Y73 sarcoma virus with these proteases yielded a peptide of similar molecular weight which was capable of autophosphorylation as well as the phosphorylation of exogenous substrates. The proteolytic fragments of both pp60src and pp90yes displayed the same strict specificity for phosphorylation of tyrosine as the intact enzymes. These results indicate that the 29,000-dalton carboxyl end of pp60src and pp90yes can function independently as phosphotransferases and indicate that the catalytic domains of these molecules have a conformation which confers protection against limited conditions of proteolysis.

Oncogenic transformation by RSV1 is mediated by the protein product of a single virus-encoded gene, designated src. This phosphoprotein of M, = 60,000 has been shown to possess tyrosine-specific phosphotransferase activity (1-5). Since the expression of the transformed phenotype in cells infected with RSV is associated with 7- to 10-fold elevated levels of cellular phosphotyrosine, it is postulated that tyrosine-specific protein phosphorylation is involved in oncogenic transformation by RSV (6). Tyrosine-specific phosphotransferase activity has also been shown to be associated with the transforming proteins encoded by other oncogenic retroviruses indicating that this enzymatic activity is a common though not universal feature of retrovirus-transforming proteins (for review see Ref. 7).

The complete nucleotide sequences of several of the retrovirus-transforming genes have been determined. The deduced amino acid sequences of these gene products show a high degree of homology among the products of the viral genes denoted src (8, 9), fps (10), fes (11), yes (12), and abl (13). These results support the hypothesis that the viral genes were acquired from the cellular genome by recombination.

In this report, we further probe this domain of pp60src and the related gene product pp90yes encoded by the Y73 sarcoma virus. It is shown that a 29,000-dalton fragment from the carboxyl domain of pp60src and pp90yes is highly resistant to digestion by a variety of proteases and can function as a tyrosine-specific protein kinase. These results suggest that sequences in the NH2-terminal half of pp60src and pp90yes are not necessary for phosphotransferase activity and that structural and functional properties of the catalytic domain may be shared among different tyrosine kinase-transforming proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

H2PO4 carrier-free (285 Ci/mg), and (γ-32P)ATP, crude, carrier-free, were purchased from ICN. [3H]Lysine (75 Ci/mM) was purchased from Amersham Corp. TPCK-trypsin and chymotrypsin were purchased from Worthington, thermolysin from Boehringer Mannheim, [Val3]angiotensin II, chymotrypsinogen, bovine serum albumin (fraction V), ovalbumin, and cytochrome c from Sigma, aprotinin from FBA Pharmaceutical, Staphylococcus V8 protease from Miles, Whatman 3MM and P81 filter paper from Fisher. Bio-Gel P-60 was purchased from Bio-Rad and XAR x-ray film and Lightning Plus intensifying screens from DuPont.

**Cells and Virus**

Chicken embryo fibroblasts were prepared from virus-free gs-minus embryos (Spafas) and transformed by the Y73 sarcoma virus (obtained from H. Hanafusa, Rockefeller University, New York) as described previously (1). Cells were used in these experiments when they were greater than 90% transformed based on morphology. SRD-
3T3 cells were obtained by transformation of mouse Balb 3T3 cells with the SR-D strain of RSV using polyethylene glycol as described by Kawai (20).

**Serum**

TBR serum was prepared from rabbits bearing tumors induced by the SR-D strain of RSV from L. Behrenschilder as described previously (1).

Monoclonal antibody to pp60<sup>Src</sup> was provided by D. Boettiger. Whole medium from the hybridoma cells were used for immunoprecipitation. Monoclonal antibodies to pp60<sup>Src</sup> were prepared from hybridoma cells with spleen cells from a mouse immunized with pp60<sup>Src</sup> produced in Escherichia coli (21). Purified IgG was used for the immunoprecipitation experiments. Details of the preparation of these reagents are described elsewhere (22). Antiserum to mouse immunoglobulin was obtained from Miles.

**Immunoprecipitation and Sample Analysis**

Cells were washed three times in 0.15 M NaCl, 601 M Tris hydrochloride (pH 7.2) and lysed at 4 °C in modified RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% NaDodSO4, 0.81 M ethylene glycol bis(p-aminomethylene)trimethylene tetracetic acid), and the lysate was clarified at 40,000 x g for 30 min. The lysate was incubated with antibody for 60 min, and the immune complexes were absorbed to formalin-fixed Staphylococcus aureus bacteria (23) for 15 min. In experiments using monoclonal antibodies to pp60<sup>Src</sup>, anti-mouse IgG was included in the reaction to enhance binding of the monoclonal antibody to the immunoadsorbent. The bacteria were washed three times with RIPA and ether incubated with sample buffer for direct analysis or treated further as described in the figure legends. The samples were analyzed on 7.5 or 10% NaDodSO4-polyacrylamide gels as described previously (1). The fixed and stained blue-stained gel from this experiment indicated that the immune complex whereas only 30-50% of the PTR was released by chymotrypsin. Examination of the Coomassie polypeptides with electrophoretic mobilities similar to the lower mobility of sample buffer or aprotinin as indicated.

**Gel Filtration**

Three hundred microliters of trypsin-treated pp60<sup>Src</sup> (above) were loaded onto a 100-cm column containing Bio-Gel P 60, fine grade, equilibrated with 10% glycerol. 0.5 ml fractions were collected at a flow rate of 2.5 ml/h. The column was calibrated using 300 µg of bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c. The elution of these proteins was monitored by absorbance at 280 nm. Ass. Protease phosphotransferase assays were performed using 50 µl of alternating column fraction, 4 µl of 50 mM magnesium or 2 µl of 10% of RIPA serum, and 10 µl of 50 mM Tris, pH 7.5, 5 mM MgCl<sub>2</sub>, and 0.01% bovine serum albumin. 1% ethylene glycol ad-

**RESULTS**

**Proteolytic Cleavage of pp60<sup>Src</sup>**—Fig. 1 displays the products generated by enzymatic cleavage of the proteins immunoprecipitated from <sup>32</sup>P-labeled RSV-transformed mouse cell lysates with serum from a rabbit bearing a tumor induced by Rous sarcoma virus (TBR serum). The major protein immunoprecipitated by TBR serum is pp60<sup>Src</sup>, the protein of the RSV src gene. Other minor phosphoproteins precipitated by this serum included Pr76, the viral gag-gene translation product, and pp52, a proteolytic product of pp60<sup>Src</sup> generated during cell lysis. Incubation of this immune complex with trypsin resulted in the digestion of all of these protein species (Fig. 1A, lanes 2-7). A single polypeptide of Mr = 29,000 was resistant to further cleavage even at concentrations of 2 mg/ml. This polypeptide fragment appeared to be derived from pp60<sup>Src</sup> since the level of <sup>32</sup>P incorporation into this species was greater than that of the minor phosphoproteins precipitated by TBR serum. In addition, this PTR fragment was not generated after digestion of proteins precipitated nonspecifically with normal rabbit serum; and preincubation of TBR serum with disrupted virus to block the precipitation of viral structural proteins (in this case Pr76) did not prevent the production of the 29-kDa PTR fragment (data not shown).

**Enzymatic digestion with either chymotrypsin (Fig. 1A, lanes 8-13) or thermolysin (Fig. 1B, lanes 3-6) generated polypeptides with electrophoretic mobilities similar to the trypsin-resistant PTR fragment. Chymotrypsin digestion produced two protein species which migrated as a doublet on NaDodSO4-polyacrylamide gel electrophoresis (Fig. 1A, lanes 8-13). Digestion with 20 µg/ml of thermolysin generated a single protein species of Mr = 29,000 (Fig. 1B, lane 3). Incubation with higher concentrations of thermolysin resulted in the generation of another species which migrated slightly faster than the trypsin-generated PTR fragment (lanes 4-6). These results suggest that a 29,000 fragment of immunoprecipitated pp60<sup>Src</sup> is protected from digestion by these three proteolytic enzymes.

**In Vitro Phosphorylation**

In Fig. 1A, the products of proteolytic digestion which were released from the immunoadsorbent (lanes 2, 4, 6, 8, 10, 12) were separated from the proteins which remained bound to the bacterial adsorbent (lanes 1, 3, 5, 7, 9, 11, 13). Digestion with trypsin released the majority of the PTR fragment from the immune complex whereas only 30-50% of the PTR was released by chymotrypsin. Examination of the Coomassie blue-stained gel from this experiment indicated that the

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For a complete understanding, please refer to the original document for detailed methodology and results.
amount of the PTR fragment found in the supernatant after protease treatment was proportional to the amount of IgG dissociated from the immunoadsorbent. (No reduction in the mobility of the IgG heavy chain was detectable after proteolytic digestion.) We have found that approximately half of the PTR fragment sediments with IgG on a glycerol gradient, indicating that some of the PTR molecules are still bound to IgG after release from the immunoadsorbent (data not shown).

Protein Kinase Activity—In order to determine whether the protease-resistant fragment described in Fig. 1 is active as a protein kinase, we examined the ability of the trypsin-treated pp60

 fragments to phosphorylate TBR-IgG. It has been shown previously that pp60

 can phosphorylate the heavy chain of IgG from TBR serum when bound in an immune complex (2, 4). Fig. 2B displays the phosphorylation of TBR-IgG after treatment of immunoprecipitated pp60

 with trypsin. In order to approximate the per cent of pp60

 converted to the PTR fragment, the cells were labeled for 16 h with [3H]lysine. After immunoprecipitation by normal rabbit serum or TBR and incubation in the presence or absence of trypsin, the samples were treated with the protease inhibitor aprotinin and then divided into two fractions. One-half of the sample was analyzed directly (Fig. 2A), and the other half was incubated with [32P]ATP and Mg

 (Fig. 2B). The relative intensity of the pp60

 band was compared to that of the PTR fragment band by densiometric tracing of the autoradiogram in Fig. 2A. The densiometric quantitation revealed that approximately 20% of pp60

 is resistant to proteolysis in this assay. Fig. 2B demonstrates that the incorporation of 32P into IgG in the trypsin-treated immunoprecipitates (lane 4) was approximately double that found in the untreated sample (lane 3). This result suggests that the trypsin treatment of pp60

 increases its phosphotransferase activity using TBR-IgG as substrate.

To determine whether the increase in pp60

 activity correlated with the generation of the PTR fragment, we examined IgG phosphorylation after incubation with various concentrations of trypsin (Fig. 3). The fate of pp60

 was followed by polyacrylamide gel electrophoresis of [3H]leucine-labeled pp60

. Treatment with 2.5 μg/ml of trypsin resulted in a loss of 75% of intact pp60

 and no 60-kDa protein was detectable after treatment with 5 or 10 μg/ml of trypsin. An intermediate 47-kDa fragment of pp60

 was detectable in immunoprecipitates incubated with 2.5–5 μg of trypsin. The 29-kDa PTR fragment was detectable in samples treated with 5 μg/ml of trypsin and the levels of this peptide increased with higher doses of trypsin. The increased incorporation of 32P into TBR-IgG closely correlated with the appearance of the PTR fragment. Under conditions which allowed the highest level of 32P incorporation, there was no detectable 60-kDa or 47-kDa form of pp60

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Since TBR-IgG is tightly associated with pp60

, this phosphorylation reaction represents an unusual enzyme-substrate interaction. In order to examine the phosphorylation of exogenous substrates after trypsin treatment of pp60

, we assayed the phosphorylation of the [Val]angiotensin II. Angiotensin has been shown to serve as a substrate for pp60

 in in vitro reactions (27, 28). In these assays, pp60

 was immunoprecipitated by a monoclonal antibody to pp60

. This

Fig. 1. Proteolytic digestion of pp60

. A, lane 1, no proteolytic enzyme; lane 2, 20 μg/ml of trypsin, released; lane 3, 20 μg/ml of trypsin, bound; lane 4, 200 μg/ml of trypsin, released; lane 5, 200 μg/ml of trypsin, bound; lane 6, 2 mg/ml of trypsin, released; lane 7, 2 mg/ml of trypsin, bound; lane 8, 20 μg/ml of chymotrypsin, released; lane 9, 20 μg/ml of chymotrypsin, bound; lane 10, 200 μg/ml of chymotrypsin, released; lane 11, 200 μg/ml of chymotrypsin, bound; lane 12, 2 mg/ml of chymotrypsin, released; lane 13, 2 mg/ml of chymotrypsin, bound. B, lane 1, no proteolytic enzyme; lane 2, 20 μg/ml of trypsin; lane 3, 2 μg/ml of thermolysin; lane 4, 20 μg/ml of thermolysin; lane 5, 200 μg/ml of thermolysin; lane 6, 2 mg/ml of thermolysin. SRD-3T3 cells (5 × 10⁶ cells) were labeled with [32P]ATP and then divided into two fractions. One-half of the sample was incubated with sample buffer, boiled for 60 s, and electrophoresed on a 7.5% gel, autoradiography 24 h.
monoclonal antibody does not interfere with the phosphorylation of angiotensin, and the PTR fragment generated after trypsin digestion of immune complex-bound pp60^c-src appears to be identical to that found after treatment of TBR-precipitated pp60^c-src. Table I shows the incorporation of ^32P into angiotensin after incubation of immunoprecipitated pp60^c-src in the presence or absence of trypsin. The trypsin-treated immunoprecipitate phosphorylated angiotensin to a 5-fold higher level than the untreated immunoprecipitated sample. Phosphoamino acid analysis of both samples revealed that phosphotyrosine was the only phosphorylated amino acid. All of the negative control samples had background levels of ^32P binding to the filters.

In both the TBR-IgG and angiotensin phosphotransferase reactions, the activity of pp60 was undiminished or stimulated 2- to 5-fold by proteolytic digestion under conditions in which intact pp60 was not detectable. This result suggests that the

### Table I

| Trypsin | Angiotensin | Filter |
|---------|-------------|--------|
|         | IP:Control  | 898    |
|         | +           | 1,115  |
|         | -           | 757    |
|         | +           | 1,648  |
|         |             |        |
| IP:anti-pp60^c-src:A |             |        |
| -       | +           | 1,999  |
| +       | -           | 15,400 |
| +       | +           | 3,499  |
| +       |             | 44,087 |
| IP:anti-pp60^c-src:B |             |        |
| -       | +           | 2,043  |
| +       | -           | 31,226 |
| +       | +           | 3,400  |
| +       |             | 153,103|

![Fig. 2. Phosphorylation of IgG after trypsin treatment of immune complex-bound pp60^c-src.](http://www.jbc.org/)

![Fig. 3. Phosphorylation of TBR-IgG after treatment with various concentrations of trypsin.](http://www.jbc.org/)
protease-resistant fragment(s) of pp60 has a higher specific activity than the intact molecule.

**Gel Filtration of Trypsin-treated pp60**—In each of the above phosphotransferase assays the reactions were carried out on the total digestion products of the immunoprecipitates. In order to distinguish which trypsin-generated fragment was responsible for the activity detected in these assays, the trypsin-treated fraction was fractionated on a Bio-gel P-60 column (Fig. 4). The sample was prepared for fractionation by trypsin treatment of pp60° bound to an immunoaffinity column containing covalently bound monoclonal antibodies directed against pp60°. This procedure generated proteolytic fragments which were free of immunoglobulin molecules. The only phosphate-containing fragment which is released by trypsin comigrates was the 29-kDa PTR fragment. This fragment would be produced if the V8 protease resistant fragment detectable on NaDdSO₄-polyacrylamide gels (Figs. 1 and 2), this suggests that this PTR fragment is responsible for the enzymatic activity detected after proteolysis. Using the PTR fragment fractionated on this column we have shown that this fragment of pp60° can also phosphorylate tubulin and casein and retains the strict specificity for phosphorylation of tyrosine using these proteins as substrates (data not shown).

**Trypsin Resistance of pp90**—The transforming protein of Yamaguchi 73 (Y73) sarcoma virus also displays tyrosine-specific protein kinase activity. Although the viral gene encoding this protein was derived from a cellular gene distinct from the cellular homologue of the v-src gene, the amino acid sequence of pp90°° shares considerable amino acid homology with pp60°° (12). In order to determine whether pp90°° contains a protease-resistant domain similar to the PTR fragment of pp60°°, cell lysates of 3°P-labeled Y73 transformed cells were immunoprecipitated with monoclonal antibody to the gag gene-encoded protein pp19. pp90°° is precipitated by anti-pp19 since the Y73-transforming protein is a chimeric protein encoded by a fused gene containing a portion of the viral gag gene-linked to yes gene-specific sequences, pp90°° was the only detectable protein precipitated with this antibody (Fig. 5). Incubation of pp90°° with trypsin resulted in the generation of a polypeptide which comigrated with the PTR fragment from pp60°° (lanes 2 and 4). This suggests that the conformation of this domain of pp90°° is very similar to that of pp60°° in that both domains are protected from proteolytic digestion. Since pp90°° was immunoprecipitated with antibody to p19, it is clear that antibody to the transformation-specific regions of pp90°° is not necessary for the protease protection demonstrated in these assays. In order to determine whether the PTR fragment from pp90°° was able to phosphorylate exogenous substrates, angiotensin I phosphorylation was assayed. Table II shows that the trypsin-treated sample phosphorylated angiotensin to 2-fold greater levels than the untreated samples. The percentage of pp90°° resistant to proteolysis was approximately 10–20% (data not shown). Thus, trypsin treatment of pp90°° increases the phosphotransferase of this molecule similarly to that found for pp60°°. These results suggest that the conformation of pp60°° responsible for protease resistance is conserved in pp90°° and that this region of each molecule can function independent of the remainder of the molecule as a protein kinase.

**Peptide Mapping of the PTR Fragments**—Fig. 5B shows the partial proteolytic digestion pattern of intact pp60°° and pp90°° and their respective PTR fragments with Staphylococcus V8 protease (V8). It has been shown previously that reelectrophoresis of pp60°° with low concentrations of V8 protease produces a single cut in pp60°° which generates a 34-kDa phosphoserine-containing fragment derived from the amino end of pp60°° and a 26-kDa phosphotyrosine-containing fragment from the carboxyl end of pp60°° (30, 31). Incubation of the pp60°°-derived PTR with V8 protease generated a fragment which comigrated with the COOH-derived 26-kDa V8 fragment of pp60°° (Fig. 5, lane 1). This result suggests that the PTR fragment is derived from the COOH domain of pp60°° (9). Incubation of pp90 with V8 protease produces a fragment which comigrates with the 26-kDa fragment of pp60°° (lane 4). This fragment would be produced if the V8 protease cleaved pp90°° at the Glu residue corresponding to...
FIG. 5. Digestion of $^{32}$P-labeled pp60$^{c-src}$ and pp90$^{y-ras}$ with trypsin. SRD-3T3 cells (A, lanes 1 and 2) or Y73-transformed cells (A, lanes 3 and 4) were labeled with $^{32}$P as described in Fig. 1, lysed with RIPA, and the clarified lysate incubated with monoclonal antibody to pp60$^{c-src}$ (273) (SRD-3T3 cells) or monoclonal antibody to p19 (Y73-chicken cells). The washed immunoprecipitates were incubated with 0.05 M ammonium carbonate in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 20 $\mu$g/ml of trypsin as described under "Experimental Procedures." The reaction was terminated by the addition of sample buffer and the samples electrophoresed on a 10% gel and dried. After autoradiography the proteins of interest were excised and re-electrophoresed on a 12.5% gel in the presence of 25 ng of V8 protease (B): lane 1, pp60$^{c-src}$ from A, lane 1; lane 2, PTR from A, lane 2; lane 3, PTR from A, lane 3; lane 4, pp90$^{y-ras}$ from A, lane 4.

FIG. 6. Digestion of in vitro phosphorylated pp60$^{c-src}$ and pp90$^{y-ras}$ with trypsin and peptide analyses of the protease-resistant product. SRD-3T3 cells (1.5 x 10$^5$ cells) (lanes 1 and 2) or Y73-transformed chicken cells (lanes 3 and 4) were lysed in RIPA, and the clarified lysate incubated with monoclonal antibody to pp60$^{c-src}$ (lanes 1 and 2) or to p19$^{y-ras}$ (lanes 3 and 4). After washing the immunoabsorbent-bound immune complexes, they were incubated with $^{32}$P]ATP as described under "Experimental Procedures." The complexes were then washed and incubated with 0.05 M ammonium carbonate alone (lanes 1 and 3) or with 20 $\mu$g/ml of trypsin as described under "Experimental Procedures." Sample buffer was added to stop the proteolysis, and the samples were electrophoresed on a 10% acrylamide gel. The gel was dried after electrophoresis and radioactive bands detected by autoradiography. The bands were excised and subjected to re-electrophoresis on a 12.5% gel alone; lane 5 (pp60 from lane 1); lane 6 (PTR from lane 2); lane 9 (pp90 from lane 3), lane 10 (PTR from lane 4); or in the presence of 50 ng of V8 protease; lane 7 (pp60 from lane 1); lane 8 (PTR from lane 2); lane 11 (pp90 from lane 3); lane 12 (PTR from lane 4).

### Table II

Phosphorylation of angiotensin after trypsin treatment of pp90$^{y-ras}$

A lysate from Y73-transformed chicken cells was incubated with 500 $\mu$l of media from a nonproducer hybridoma cell line (IP:Control) or from hybridoma cells secreting monoclonal antibody to pp19$^{y-ras}$ (IP:anti-pp19$^{y-ras}$). Angiotensin phosphorylation was assayed as in Table I. IP:anti-pp19$^{y-ras}$ B represents a different experiment in which only anti-pp19$^{y-ras}$ media was used for the immunoprecipitation.

| Trypsin          | Angiotensin | Filter |
|------------------|-------------|--------|
| IP:Control       |             | cpm    |
| -                | -           | 1,136  |
| +                |             | 1,047  |
| IP:anti-pp19$^{y-ras}$-A |   | + 1,413 |
| -                | +           | 1,249  |
| +                |             | 1,163  |
| IP:anti-pp19$^{y-ras}$-B |   | + 16,033 |
| -                | +           | 1,718  |
| +                |             | 25,482 |
| +                |             | 2,006  |
| +                |             | 12,670 |
| +                |             | 1,854  |
| +                |             | 24,502 |
pp60· and pp90· were immunoprecipitated from 5 x 106 SRD-3T3 cells (lanes 1 and 2) or Y73-transformed chicken cells (lanes 3 and 4) as described in Fig. 6. The washed immunoprecipitates were incubated with 0.05 M ammonium carbonate alone or (y-32P)ATP and 10 mM Tris, pH 7.2, 5 mM MgCl2, and pp90ye·. The transforming protein of Y73 sarcoma virus was shown to contain a similar protease-resistant phosphotransferase-active domain, suggesting that this structural feature is conserved in other tyrosine kinases. In addition, Weinmester and co-workers have reported that limited proteolysis of the Fu-gam sarcoma virus pp140· gene protein releases COOH-terminal 29-kDa and 45-kDa peptides which are phosphorylated upon addition of ATP and Mg (38). Within this family of related viral tyrosine kinase-transforming proteins there is extensive homology in the amino acid sequences corresponding to the PTR domains of pp60· and pp90· while sequences outside of this domain show little or no homology with large differences in size. This suggests that the PTR domain of these enzymes might have evolved as separate genetic entities which have become linked with different genetic elements. It is possible that the non-PTR domain of these enzymes could regulate the functional activity of the phosphotransferase domain, possibly affecting the substrate specificity and/or the specific activity of the enzyme. The genetic linkage of the catalytic domain of tyrosine kinases with different regulatory elements would allow for functional diversification of tyrosine-specific protein kinases. For instance, sequences encoding the tyrosine kinase domain of cellular growth hormone receptors might have become genetically linked to sequences which confer hormone responsiveness to the kinase domain. This would be analogous to the family of cellular dehydrogenases which share dinucleotide binding domains and differ in the domains which confer substrate specificity (for review see Ref. 39). In many other enzyme systems, limited proteolytic digestion has been used to separate different domains of activity. In the case of rabbit muscle phosphorylase, subtilisin cleaves the molecule into two fragments, a 30-kDa NH2-terminal peptide which carries the regulatory binding site for allosteric effectors and a 70-kDa COOH-terminal fragment containing the catalytic site (36, 37).

**DISCUSSION**

The experiments in this report extend previous analyses of the phosphotransferase activity of the carboxyl domain of pp60· and another viral tyrosine-specific protein kinase, pp90·. It is shown that the COOH-terminal 29,000 daltons of pp60· can be released as an active phosphotransferase by limited proteolysis with trypsin. This fragment can phosphorylate IgG from serum of animals bearing tumors induced by RSV as well as exogenous substrates such as angiotensin, tubulin, and casein. Proteolysis of pp60· does not alter the strict specificity of this enzyme for phosphorylation of tyrosine. In each immunoprecipitate-bound phosphotransferase assay, proteolytic digestion of pp60· resulted in higher levels of substrate phosphorylation despite an apparent 5-fold difference in the molar amounts of the PTR fragment relative to intact pp60·. These results suggest that the PTR fragment has a higher specific activity than intact pp60· and thus raises the possibility that structural constraints on this domain imposed by the NH2-terminal half of pp60· could regulate the phosphotransferase activity of the carboxyl domain. Indeed, Purchio and co-workers have evidence which suggests that autophosphorylation within the NH2-terminal domain of pp60· might increase the specific activity of pp60· 5- to 8-fold (34, 35).

The configuration within the carboxyl half of pp60· confers protection against limited proteolytic digestion. Analysis of the amino acid sequences in this domain does not reveal any obvious structural features which would account for this behavior. It is clear that the protease resistance of this domain is not dependent on antibody binding since pp90 was immuno precipitated by antibody to the gag portion of this chimeric protein. In addition, the PTR fragment is generated after precipitation of pp60· with monoclonal antibodies which recognize different epitopes on pp60· (data not shown). It will be of interest to determine whether loss of protease resistance correlates with the loss of the functional integrity of this domain of pp60·, i.e. do mutations which inactivate the phosphotransferase activity of pp60· alter the protease-resistant configuration of this substructural domain?

The transforming protein of Y73 sarcoma virus was shown to contain a similar protease-resistant phosphotransferase-active domain, suggesting that this structural feature is conserved in other tyrosine kinases. In addition, Weinmester and co-workers have reported that limited proteolysis of the Fuganami sarcoma virus pp140· protein releases COOH-terminal 29-kDa and 45-kDa peptides which are phosphorylated upon addition of ATP and Mg (38). Within this family of related viral tyrosine kinase-transforming proteins there is extensive homology in the amino acid sequences corresponding to the PTR domains of pp60· and pp90· while sequences outside of this domain show little or no homology with large differences in size. This suggests that the PTR domain of these enzymes might have evolved as separate genetic entities which have become linked with different genetic elements. It is possible that the non-PTR domain of these enzymes could regulate the functional activity of the phosphotransferase domain, possibly affecting the substrate specificity and/or the specific activity of the enzyme. The genetic linkage of the catalytic domain of tyrosine kinases with different regulatory elements would allow for functional diversification of tyrosine-specific protein kinases. For instance, sequences encoding the tyrosine kinase domain of cellular growth hormone receptors might have become genetically linked to sequences which confer hormone responsiveness to the kinase domain. This would be analogous to the family of cellular dehydrogenases which share dinucleotide binding domains and differ in the domains which confer substrate specificity (for review see Ref. 39). In many other enzyme systems, limited proteolytic digestion has been used to separate different domains of activity. In the case of rabbit muscle phosphorylase, subtilisin cleaves the molecule into two fragments, a 30-kDa NH2-terminal peptide which carries the regulatory binding site for allosteric effectors and a 70-kDa COOH-terminal fragment containing the catalytic site (36, 37).
The isolation of this domain of kinase activity provides the means to investigate various aspects of the regulation of the enzymatic activity of the tyrosine kinase-transforming proteins.

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