Synthetic Peptide Substrates for a Tyrosine Protein Kinase*

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Immunoprecipitates containing the transforming protein of the avian sarcoma virus, Y73, together with its associated tyrosine-specific protein kinase, have an activity which will phosphorylate the synthetic peptide Lys-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg at the tyrosine residue. This peptide corresponds to 10 out of 11 amino acids surrounding the phosphorylated tyrosine in both pp60" or p90, the transforming proteins of Rous sarcoma virus and Y73 virus, respectively. The apparent 

The CAMP-dependent protein kinase and the casein protein kinases appear to recognize their substrates at least partly on the basis of the primary amino acid sequence surrounding the target residue. The serines phosphorylated by the cAMP-dependent protein kinase usually have one or two basic amino acids placed close by on the NH2-terminal side (see Ref. 10 for review). In contrast, the serines and threonines phosphorylated by the casein protein kinases often have neighboring acidic residues which are on the NH2-terminal side for casein kinase I and on the COOH-terminal side for casein kinase II (see Ref. 11 for review). The identification of a new type of protein kinase-phosphorylating tyrosine has prompted investigation of whether there are primary sequence requirements in the recognition of target tyrosine residues. By partial sequence analysis, we and others have found that there are striking homologies between several of the tyrosine phosphorylation sites in the viral transforming proteins (12-15). They are characterized by the presence of a lysine or arginine 7 residues to the NH2-terminal side of the phosphorylated tyrosine and one or more acidic residues, most commonly glutamic acid, in the intervening distance. Sites of tyrosine phosphorylation in cellular proteins are less evidently homologous to those in the viral transforming proteins, but most of them appear to have a glutamic acid close to the tyrosine on the NH2-terminal side (15). While it is almost certain that there will be secondary and tertiary structure requirements, these primary sequence features may be determinants in the recognition of specific tyrosines by tyrosine protein kinases.

The primary sequence requirements for phosphorylation by the cAMP-dependent protein kinase were assessed directly by using as substrates for the purified enzyme synthetic peptides corresponding in sequence to known phosphorylation sites (16-19). We have begun to use a similar approach for the tyrosine protein kinases. The only site of tyrosine phosphorylation for which the complete sequence is known is that of pp60" (13, 15). Accordingly, we have synthesized the peptide Lys-Leu-Ile-Glu-Asp-Asn-Tyr-Thr-Ala-Arg which, with the substitution of a lysine for an arginine in the NH2-terminal position, corresponds to this tyrosine phosphorylation site (20). We have found that the tyrosine protein kinase associated with the transforming protein of the avian sarcoma virus, Y73 (21, 22), is able to phosphorylate the tyrosine in this peptide. We have also tested a number of related peptides as substrates to determine the influence of the amino acid sequence embedding the tyrosine in this phosphorylation reaction.

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1 The abbreviations used are: RSV, Rous sarcoma virus; Boc, t-butoxycarbonyl; PIPES, 1,4-piperazinediethanesulfonic acid.
**Experimental Procedures**

**Synthesis and Purification of Peptides**—The peptide Lys-Leu-Ile-Glu-Asp-Glu-Tyr-Thr-Ala (src I) was synthesized using a Beckman Synthesizer Model 990 as described (23). Boc-alanine, Boc-aspartic acid (benzyl), Boc-asparagine (xanthyl), Boc-glutamic acid (benzyl), Boc-glutamine (2-chlorobenzoxycarbonyl), Boc-arginine (benzyl), and Boc-lysine (2,6-dichlorobenzyl) were used together with Boc-arginine (tosyl) resin ester. Cleavage from the resin and deprotection were performed with HF. Analysis of the peptide material by thin layer electrophoresis and chromatography showed two major ninhydrin-staining components. To purify these peptides, 200 mg of crude material dissolved in 50 ml of H2O and adjusted to pH 8.9 was applied to a Sephadex QAE-A25 column (35 × 1.5 cm) in the HCO3- form equilibrated with 0.1 M NH4HCO3, pH 8.9. The peptides were eluted with a linear gradient from 0.1 M NH4HCO3, pH 8.9, to 0.5 M NH4HCO3, pH 8.9, using 500 ml of each buffer. Two main peaks of material absorbing at 280 nm were detected eluting at 0.34 M (I) and 0.25 M (II) salt. A minor peak was observed at 0.23 M (III). These three peaks were pooled separately and lyophilized several times from H2O until a constant weight was attained. Amino acid analysis of these peaks gave the following amino acid compositions normalized to alanine: Ala, 1.05; Arg, 1.08; Asp, 1.39; Glu, 1.99; Ile, 1.06; Leu, 1.01; Lys, 1.13; Thr, 1.12; Tyr, 0.83. IIA: Ala, 1.00; Arg, 1.05; Asp, 1.19; Glu, 1.10, Leu, 1.07; Lys, 1.20, Thr, 1.11; Tyr, 0.83. IIIA: Ala, 1.00; Arg, 0.96; Asp, 2.05; Glu, 1.19; Leu, 0.91; Thr, 1.00; Tyr, 0.90. The hydrolyses were not performed in the presence of O2 and the tyrosine values were not corrected for the resultant losses. The sequence of the peptide, src I, therefore had the correct composition to be the peptide required. Its sequence was confirmed by automated analysis in a Beckman 890C sequencer as described (14) followed by high performance thin layer chromatography analysis of the phenylthiohydantoin (29). The peptide, src II, lacked a glutamic acid residue. Its sequence was shown to be Lys-Leu-Ile-Glu-Asp-Glu-Tyr-Thr-Ala by automated Edman analysis. The position of the glutamic acid in src II was confirmed by digestion of the peptide with Staphylococcus aureus V8 protease. The digest contained two peptides, one of which was found to be Tyr-Thr-Ala. The peptide, src III, lacked both isoleucine and glutamic acid. Digestion of src III with S. aureus protease also yielded the peptide Tyr-Thr-Ala. Therefore src III has the sequence Lys-Leu-Ile-Glu-Glu-Tyr-Thr-Ala.

Several other peptides were derived from src I as follows. The peptide Lys-Leu-Ile-Glu-Asp-Glu-Tyr-Thr-Ala (src IV) was prepared by digestion of 1 mg of src I in 0.5 ml of 0.05 M NH4HCO3 with 150 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin ( Worthington, TRTPCK) for 16 h at 23 °C. Following two cycles of lyophilization the digest was separated by electrophoresis at pH 4.7 at 1 kV for 27 min on a 100-μm cellulose thin layer plate (25). Peptides were detected by staining marker strips. The released lysine was well resolved from the other main hydrolysis products. The residue peptide product was eluted. Upon acid hydrolysis and analysis of the hydrolysate by two-dimensional electrophoresis and chromatography this peptide was found to contain Ala, Arg, Asp, Glu, Ile, Leu, Thr, and Tyr. The peptide Lys-Leu-Ile-Glu-Asp-Glu-Tyr-Thr-Ala (src V) was prepared by digestion of 500 μg of src I in 0.05 M NH4HCO3 with 50 μg of n-chymotrypsin ( Worthington, CD1) for 12 h at 23 °C. After lyophilization, the digest was separated by electrophoresis at pH 5.3 in a buffer containing 5% 1-butanol, 2.5% acetic acid, 5% pyridine for 20 min at 1 kV on a 100-μm cellulose thin layer plate. The released arginine was well separated from the other major peptide product which was eluted. Upon acid hydrolysis this peptide was found to contain Ala, Glu, Ile, Leu, Lys, Thr, and Tyr. The peptide Lys-Leu-Ile-Glu-Asp-Glu-Tyr-Thr-Ala (src VI) was prepared by digestion of 500 μg of src I in 0.05 M NH4HCO3, pH 5.3 with 50 μg of n-chymotrypsin ( Worthington, CD1) for 12 h at 23 °C. After lyophilization, the digest was separated by electrophoresis at pH 5.3 in a buffer containing 5% 1-butanol, 2.5% acetic acid, 5% pyridine for 20 min at 1 kV on a 100-μm cellulose thin layer plate. The released arginine was well separated from the other major peptide product which was eluted. Upon acid hydrolysis this peptide was found to contain Ala, Arg, Asp, Glu, Ile, Leu, Thr, and Tyr. The peptide Tyr-Thr-Ala-Arg (src VII) was prepared by digesting 500 μg of src I in 200 μl of 0.05 M NH4HCO3, with 50 μg of S. aureus V8 protease ( Miles Laboratories, Inc., Elkhart, IN) for 12 h at 23 °C. After lyophilization, the digest was separated by electrophoresis at pH 8.9 at 1 kV for 20 min on a 100-μm cellulose thin layer plate. The released arginine was well separated from the other major peptide product which was eluted. Upon acid hydrolysis this peptide was found to contain Ala, Arg, Thr, and Tyr. The peptides were all dissolved at 10 mg/ml in H2O and the pH adjusted to 7. Their concentrations were checked by measurement of absorbance at 278 nm (εw = 1300).

**Cell culture and Immunoprecipitation**—Chick cells transformed with Y73 virus were grown as described previously (14). The Y73 virus-transforming protein, P90, was isolated from lysates of Y73 virus-transformed chick cells by immunoprecipitation with a rabbit antiserum raised against disrupted virions of the Prague strain of RSV (the kind gift of Jim Neil, the University of Southern California) (14). The titre of this serum had been determined, and the immunoprecipitates were prepared in antigen excess with about 5 × 10⁵ cells/μl of serum. The immune complexes were collected by absorption to S. aureus (Pansorbin, Calbiochem) using 500 μg/ml of serum and washed according to our usual procedure at 4 °C (27). After a final wash in Tris-buffered saline, the S. aureus pellet was resuspended in a buffer containing 20 mM PIPES, pH 7.0, 16 mM MgCl₂, using 10 μl for each microcrystall of precipitating serum and stored in liquid N₂.

**Peptide Phosphorylation**—Each reaction contained 5 μl of immunoprecipitate in the form of the bacterial transformation described above (equivalent to 2.5 × 10⁵ cells), 2 μl of peptide in H₂O, and 1 μl of [γ-32P]ATP (5 mCi/ml; 40 mCi/μmol, Amersham/Searle). The mixtures were set up on ice and the reaction started by addition of the ATP followed by incubation at 30 °C for 10 min. The reaction was stopped by transfer to an ice bath followed rapidly by centrifugation for 2 min in a microcentrifuge. The supernatants were aspirated and 1 μl of each sample was spotted onto a nitrocellulose sheet and exposed to Kodak X-AR-5 film with a fluorescent screen at −70 °C for 12 h. In each series a control incubation without added peptide was included. Areas of the autoradiogram corresponding to the phosphorylated peptides and a similar region from the control were aspirated from the plate. The radioactivity was eluted with pH 4.7 buffer and counted with an aqueous scintillator. Phosphorylated peptides were eluted in a similar fashion for phosphoamino acid analysis and automated sequencing which were performed as described (5, 15).

**Results**

To test whether the synthetic peptide Lys-Leu-Ile-Glu-Asp-Glu-Tyr-Thr-Ala (src I) is a substrate for tyrosine protein kinase, it was incubated with a phosphorylated protein kinase known to phosphorylate this sequence in a native protein. In the absence of such a purified enzyme, we chose to use preparations of P90, the transforming protein of the avian sarcoma virus Y73. When isolated by immunoprecipitation from Y73 virus-transformed chick cells, P90 has an associated tyrosine protein kinase activity (22) which will phosphorylate a site in P90 itself with a sequence identical to that in pp60⁵© (12, 14, 15). There is reason to believe, therefore, that the synthetic peptide might be a proper substrate for the P90-associated protein kinase activity.

P90 is a chimeric protein containing vitally specified sequences at its NH₂-terminus joined to sequences encoded by the cellular information in the Y73 virus genome (22). The immunoprecipitates were made with antisera directed against the NH₂-terminal viral portion of P90. Since the cellular COOH-terminal domain is believed to specify the tyrosine protein kinase activity associated with P90, we thought that the active site of P90 would be available to phosphorylate exogenous substrates in this type of preparation. Such immunoprecipitates contain the enzyme activity bound to antibody molecules which in turn are complexed with protein A molecules on the surface of the S. aureus cells. It is possible that the immobilized state of the enzyme in this type of preparation may affect the absolute kinetic parameters of the reaction (see "Discussion"). For this reason, the terms apparent Kₘ and apparent Vₘₐₓ have been used throughout. Nevertheless...
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**FIG. 1.** Electrophoretic separation of phosphorylated peptides. Phosphorylation reactions were carried out as described under “Experimental Procedures” with the following approximate peptide concentrations for both src I and src II: 1, 2 mM; 2, 0.7 mM; 3, 0.25 mM. One-µl samples from each reaction were electrophoresed at pH 3.5. A control (C) sample was also run. The direction of electrophoresis is indicated and the origin is denoted by horizontal arrows. N indicates the position of a neutral marker dye. Some radioactive material is apparently trapped in this region in each lane. P indicates the position of the phosphorylated peptides. 0.05 pmol of phosphate were incorporated/mol of src I peptide in 10 min at 30 °C.

**FIG. 2.** Phosphoamino acid composition of src I phosphorylated in vitro. Two µl of a phosphorylation reaction containing 1 mM src I was electrophoresed at pH 3.5 as described under “Experimental Procedures.” The phosphorylated peptide was located by autoradiography and eluted from the plate. The eluate was lyophilized and hydrolyzed in 6 N HCl at 110 °C for 1 h. Half the hydrolysate (2,000 cpm) was resolved in 2 dimensions on a cellulose thin layer plate by electrophoresis at pH 1.9 for 20 min at 1.5 kV (horizontal direction) followed by electrophoresis at pH 3.5 for 16 min at 1 kV (vertical direction). The plate was exposed to film with a fluorescent screen for 10 h. The positions of the origin (O), the phosphoserine (P.SER) and phosphothreonine (P.THR) markers (dotted circles) and phosphotyrosine (P.TYR) are indicated.

Nevertheless, it should be valid to compare the kinetic constants determined for different synthetic peptides. Another drawback with the use of an immunoprecipitate as the source of protein kinase is that the amount of enzyme is not defined. One advantage of the system is that the enzyme can readily be removed from the reaction mixture.

To assay for the phosphorylation of the peptide src I, we used incubation conditions which are suitable for the phosphorylation of P90 itself, namely 10 mM MnCl₂, 20 mM PIPES, pH 7.0. When added to P90-containing immunoprecipitates under these conditions in the presence of 5 µM [γ-³²P]ATP, the peptide was phosphorylated. Electrophoresis at pH 3.5 proved to be the best system for separating the phosphorylated src I from the unincorporated ATP (see Fig. 1), although for some of the more anionic peptides tested subsequently, electrophoresis at pH 4.0 was employed. Initial experiments showed that incorporation into phosphorylated peptides was linear at the concentrations used here for 10 min at 30 °C under standard conditions. This incubation time was used for all experiments.

In addition to the tyrosine residue, src I also contains a threonine which could potentially be phosphorylated. Two-dimensional separation of an acid hydrolysate of ³²P-labeled phosphorylated src I, however, showed that phosphotyrosine was the only phosphoamino acid present (Fig. 2). This was confirmed by automated sequence analysis of phosphorylated src I which resulted in the release of the phenylthiodyantoin of phosphotyrosine at cycle 8 as expected (Fig. 3).

By varying the concentration of peptide in the phosphorylation reaction, the apparent K₉ for src I was found to be approximately 5 mM (Fig. 4, Table I). As pointed out above, this value should not be construed as a true K₉. Moreover, because of the limited solubility of the peptide a value this high is difficult to determine with precision. Using the same assay, we have measured approximate apparent K₉ values for two other synthetic peptides related to src I which were by-

**FIG. 3.** Sequence analysis of src I phosphorylated in vitro. Five µl of a phosphorylation reaction containing 1 mM src I was electrophoresed at pH 3.5 as described under “Experimental Procedures.” The phosphorylated peptide was located by autoradiography and eluted from the plate. The eluate (21,000 cpm) was subjected to automated sequence analysis. The radioactivity released at each cycle was counted directly using Cerenkov radiation.

**FIG. 4.** Initial rates of phosphorylation of src I and src II. A double reciprocal plot of v⁻¹ against [peptide]¹⁻ is given. Because the precise amount of enzyme in each assay was unknown the initial rates were determined simply as the radioactivity incorporated into peptide at each concentration.
products in the original peptide preparation and four further peptides which were derived from src I by proteolytic treatment. The phosphorylation of src II is illustrated in Fig. 1. It can be seen that the phosphorylated peptide migrates slightly faster toward the cathode than the phosphorylated form of src I, reflecting both the extra glutamic acid in src I and the decreased mass of src II. The phosphorylated form of each of the peptides tested here had a characteristic and distinguishable mobility upon electrophoresis at pH 3.5 and pH 4.0 (data not shown). By this criterion, none of the peptides was significantly contaminated with another phosphorylatable peptide. The sequences of all the peptides tested are listed together in Table I. The two peptides, src II and src III, which lack the glutamic acid at position 4, have much higher apparent \( K_a \) values than src I. The two peptides, src VI and src VII, in which the tyrosine is terminal were poor substrates. Src V, the peptide lacking the COOH-terminal arginine, has an apparent \( K_a \) slightly lower than src I. We encountered some difficulty testing src IV, the peptide without the NH\(_2\)-terminal lysine. The presence of this peptide in the assay causes a major fraction of the radioactivity to adhere to the bacteria. Although this could be eluted by adjusting the pH of the reaction to 4, comparison with the other peptides may be vitiated.

Since an unknown amount of enzyme was present in the reaction, absolute \( V_{\text{max}} \) values cannot be determined. The relative apparent \( V_{\text{max}} \) for src I, however, is lower than for src II (Table I). Relative apparent \( V_{\text{max}} \) values are also listed for src III to V in Table I. The rates of phosphorylation of src VI and src VII were too low to estimate an apparent \( V_{\text{max}} \). Instead some indication of the efficiency of phosphorylation of these peptides relative to src I is given in Table I as defined by the incorporation of \( ^{32} \text{P} \) at a peptide concentration of 2 mM under our standard assay conditions. We have not determined the \( K_a \) for inhibition of phosphorylation of protein substrates by any of the peptides nor have we measured the \( K_a \) for ATP.

We have tested a variety of other tyrosine-containing peptides in our system. For example, the peptide Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu, corresponding to the sequence around tyrosine 315 in the polyoma virus middle T antigen (28), which has been reported to be the site of tyrosine phosphorylation of the middle T antigen in vitro (29), was phosphorylated with an apparent \( K_a \) of 3 mM. Luteinizing hormone release factor (Pyr-His-Tyr-Tyr-Gly-Leu-Arg-Pro-Gly-NH\(_2\)) was phosphorylated on tyrosine, but its \( K_a \) was unmeasurably high. The peptide Arg-Gly-Tyr-Ala-Leu-Gly was also phosphorylated but extremely poorly. Free tyrosine was not phosphorylated.

We have also assessed the ability of the tyrosine protein kinases associated with a number of other viral transforming proteins to phosphorylate src I. Immunoprecipitates of pp60\(^{src}\) of RSV made with a serum directed against the COOH-terminal hexapeptide of pp60\(^{src}\), in which the active site of pp60\(^{src}\) is likely to be exposed were able to phosphorylate src I. Surprisingly, this was also true of immunoprecipitates of pp60\(^{src}\) made with a rabbit antitumor serum in which pp60\(^{src}\) molecules are able to phosphorylate the immunoglobulin heavy chain but not exogenous protein substrates (30). Immunoprecipitates containing pp60\(^{src}\), the cellular homologue of pp60\(^{src}\), made with antitumor serum were likewise able to phosphorylate src I. The tyrosine protein kinases associated with the transforming proteins of PRCI virus (31) and Abelson virus (32) and the middle T antigen of polyoma virus (33) phosphorylated src I as well, although the efficiency of this reaction varied widely. The epidermal growth factor-stimulated tyrosine protein kinase associated with the plasma membrane of A431 human tumor cells (3) was also active in the phosphorylation of src I.

**DISCUSSION**

Through the use of synthetic peptides we have begun to assess to what extent particular features of the primary sequence of the tyrosine phosphorylation site in pp60\(^{src}\) influence the phosphorylation of this tyrosine by tyrosine-specific protein kinases. Our preliminary results indicate that primary sequence does indeed play some role in the recognition of this tyrosine. A synthetic peptide (src I) corresponding to 10 out of 11 residues around the phosphorylated tyrosine in pp60\(^{src}\) and P90 serves as a substrate for the tyrosine protein kinase activity associated with the transforming protein of Y73 virus.

The apparent \( K_a \) for the peptide in the phosphorylation reaction is about 5 mM. This value is about 2 orders of magnitude higher than that obtained for the best synthetic peptide substrates of the cAMP-dependent protein kinases (18, 19). This difference might reflect the unusual nature of an assay in which the enzyme is immobilized. However, the phosphorylation of a synthetic peptide very similar to src I in a soluble enzyme system has been found to exhibit a similarly high apparent \( K_a \) value (34). Furthermore, in the parallel situation offered by serine protein kinases which are associated with membranes and thereby immobilized, phosphorylation of exogenous substrates displays normal kinetics (35). Since many of the tyrosine protein kinases are themselves membrane-affiliated (36, 37), it might even be misleading to attempt to determine kinetic parameters in solution. The worry that the measured kinetic values might be the result of a situation in which the "autophosphorylation" of P90 is competing with peptide phosphorylation appears to be unfounded. The phosphorylation of P90 is complete within a few seconds of incubation at 30 °C and the extent of this reaction

| Peptide | Sequence | Apparent \( K_a \) (mM) | Relative \( V_{\text{max}} \) | Relative rate at 2 mM |
|---------|----------|-------------------|-------------------|-------------------|
| Src I   | Lys-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg | 5 | 1.0 | 1.0 |
| Src II  | Lys-Leu-Ile-Asp-Asn-Glu-Tyr-Thr-Ala-Arg | 37 | 2.3 | 0.5 |
| Src III | Lys-Leu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg | 40 | 2.5 | 0.4 |
| Src IV  | Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg | 7 | 0.6 | 0.5 |
| Src V   | Lys-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala | 2 | 0.9 | 1.6 |
| Src VI  | Lys-Leu-Ile-Glu-Asp-Asn-Glu-Tyr | nd | nd | 0.1 |
| Src VII | Tyr-Thr-Ala-Arg | nd | nd | 0.01 |

**Table I**

*Phosphorylation of synthetic peptides*

Kinetic constants were determined as described in the legend to Fig. 4 and under "Experimental Procedures." The values were calculated from at least six initial rate measurements in each case. The apparent \( V_{\text{max}} \) values are expressed relative to the value for src I which has been given the value of 1. Likewise, the relative rates of reaction at a peptide concentration of 2 mM are expressed relative to the rate for src I. nd indicates that the values were not determined for the reasons given in the text.
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is less than 1% of that of peptide phosphorylation.

If the apparent $K_v$ value determined here were real, the
high value for phosphorylation of src I might indicate that the
primary structure of the phosphorylation site does not provide
such an important recognition determinant as tertiary struc-
ture. Before concluding this, however, it should be noted that
for technical reasons src I was synthesized with a lysine at its
NH$_2$ terminus rather than the arginine found in the sequence
of pp60$^{c-src}$. The possibility that lysine does not substitute
effectively for this arginine should be borne in mind, since in the
case of the peptide substrates for the cAMP-dependent
protein kinase replacement of arginine by lysine causes a 10-
to 100-fold increase in the apparent $K_v$ (18, 19). The studies
of Casnellie et al. (34), however, showed that a peptide with
an arginine in this position still had a $K_v$ value as high as the
one determined here for src I. The nature of the protein kinase
phosphorylating the peptide is another consideration. The
decay curve for thermal inactivation of the peptide-phospho-
rylating activity in P90-containing immunoprecipitates shows
only a single component. This means that a single enzyme is
responsible for phosphorylation of the peptide. Presumably,
this activity is the same as that which phosphorylates P90
itself at a sequence identical in 10 out of 11 positions with that
of src I. Therefore, it seems likely that the peptide is an
appropriate substrate for this enzyme. It would be desirable,
however, to repeat these experiments with a purified soluble
tyrosine protein kinase.

Regardless of the potential problems with interpreting the
values of the kinetic constants determined in this assay,
comparison of the kinetic parameters for the phosphorylation
of different peptides would seem to be justified. As a result of
this comparison, there do appear to be elements of the primary
sequence surrounding the target tyrosine which are recog-
nized. Clearly, the tyrosine is inefficiently phosphorylated if
it is either the NH$_2$ or COOH terminus of the peptide. Re-
moval of the COOH-terminal arginine from src I led to a
decrease in apparent $K_v$. Whether this reflects an inhibiting
influence of a basic residue so close to the tyrosine on the
COOH-terminal side is not clear. The effect is not simply due
to the loss of a positive charge from the peptide, since the
removal of the NH$_2$-terminal lysine did not lead to such a
change. Other synthetic peptides with longer COOH-terminal
extensions in which the arginine is replaced by neutral amino
acids should be tested.

The removal of the NH$_2$-terminal lysine did not alter the
properties of the peptide as a substrate substantially. In some
ways, this is surprising since a lysine or arginine 7 residues to the
NH$_2$-terminal side of the phosphorylated tyrosine is a wides-
pread feature of the sites of tyrosine phosphorylation in
viral transforming proteins (15). Several possibilities could
account for this. The basic amino acid found at this site may not
play a role in substrate recognition in short peptides, for
instance because it is the NH$_2$-terminal residue. Alternatively,
if a basic residue were important, lysine may not be able to
substitute for arginine at this site. Finally, a requirement for
a positively charged residue at this position might be fulfilled
by the $\alpha$-amino group of the NH$_2$-terminal leucine in src IV.

Perhaps the most striking result obtained with the ana-
gologies of src I is the 10-fold increase in apparent $K_v$ observed
for those peptides lacking the glutamic acid residue up-
stream of the tyrosine. This probably does not simply reflect
a decrease in the anionic character of the peptide since src I
does not have a higher apparent $K_v$ than src IV. Moreover,
the effect is not due to the decreased distance between the
lysine and the tyrosine, since in the case of src II removal of

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