TPK-IIB, a spleen tyrosine protein kinase devoid of autophosphorylation activity (Brunati, A. M., and Pinna, L. A. (1988) Eur. J. Biochem. 172, 451-457), has been purified to near homogeneity and assayed for its ability to phosphorylate the synthetic peptides EDNEYTA and EPQYQPA reproducing the two conserved phosphorylation sites of pp60-src (Tyr-416 and Tyr-527). While EPQYQPA was phosphorylated with low efficiency ($K_m = 16.7 \text{ mM}$, $K_{cat} = 14.4$), EDNEYTA is an excellent substrate displaying a $K_m$ value of 58 $\mu\text{M}$ and a $K_{cat}$ value of 31.2. The single substitution, in the latter peptide, of the glutamic acid adjacent to the tyrosine by alanine to give EDNAYTA caused a 6-fold increase in the $K_m$. The positive influence on the phosphorylation of the acidic residues at -3 and -4 relative to the tyrosine is indicated by comparison of the kinetic constants for peptides EDAYAA ({$K_{cat} = 4.6$, $K_m = 0.325$ mM}) and QNAAYAA ({$K_{cat} = 2.4$, $K_m = 1.7$ mM}). Furthermore, when residues in the peptide NEYTA were replaced by alanine, the phosphorylation of the peptides NAYTA and AAYAA, was almost negligible (in terms of $K_{cat}/K_m$ ratio). However AEYTA, NEYAA and AEYAA were still substrate phosphorylated, albeit less efficiently than NEYTA. The probability that these peptides will adopt a $\beta$-turn is EDNAYTA $>$ EDNEYTA, NYTA $>$ NEYTA, and no predicted $\beta$-turn for AEYTA, NEYAA, and AEYAA. Therefore these results support the concept that an amino-terminal acidic residue(s) is strictly required by TPK-II, irrespective of peptide conformation, although a $\beta$-turn may enhance the phosphorylation of those peptides that satisfy this requirement.

Two other spleen tyrosine kinases, TPK-I/lyn and TPK-III, both related to the src family, also have a far greater preference for the peptide EDNEYTA over EPQYQPA. However, they can be distinguished from TPK-II by their lower affinity for the peptides EDNEYTA and NEYTA and by their different specificity towards the substituted derivatives of NEYTA. TPK-I/lyn, accepts most of the substitutions that are detrimental to TPK-II, the triply substituted peptide AA-YAA being actually preferred over the parent peptide NEYTA. The substitution of glutamic acid by alanine is also tolerated by TPK-III, although, in contrast to TPK-II, the phosphorylation efficiency is drastically decreased by the substitution of the aspartic acid at position -2. Taken together these data would indicate that the highly conserved phosphoacceptor site, homologous to pp60-src Tyr-416, is optimally configured for the specificity requirements of TPK-II, suggesting that TPK-II or tyrosine protein kinases with similar specificity might be involved in the phosphorylation of the members of the src family.

All the tyrosine protein kinases encoded by cellular genes of the src family contain two major phosphoacceptor sites which are homologous to Tyr-416 and Tyr-527 of pp60-src (reviewed in (1)). The former represents the main autophosphorylation site in vitro, and its phosphorylation correlates with increased kinase activity (2). The latter, which is close to the carboxyl terminus and is absent in the oncogenic forms (v-src), appears to be responsible for the down-regulation of the kinase itself (1, 3). Recently a brain TPKI uncapable of autophosphorylation has been reported to specifically phosphorylate in vitro the carboxy-terminal phosphoacceptor site of pp60-src (4). Such an unusual lack of autophosphorylation activity is also shared by TPK-II (5), a spleen enzyme which can be resolved by chromatographic procedures from three other forms of tyrosine kinase two of which (TPK-I and TPK-IIA) are closely related to each other. While TPK-I and II are immunologically indistinguishable from the product of the lyn oncogene (6), which is a member of the src family (7), TPK-II does not cross-react with any of the nonspecific antibodies against TPKs of the src family tested so far. Such behavior and its inability to undergo autophosphorylation argue against any close relationship of TPK-II to the src family, whose members invariably include a highly conserved autophosphorylation site (see Ref. 1). These findings prompted us to undertake a study aimed at assessing whether TPK-II, like the brain "c-src-kinase" (4), might be involved in the phosphorylation and regulation of the TPKs belonging to the src family. Here we present data concerning the phosphorylation of peptides reproducing the two phosphoacceptor sites of the src proteins, and supporting the concept that Tyr-416 but not Tyr-527 is an excellent target for TPK-II.

MATERIALS AND METHODS

The peptides EDNEYTA, ENEYTA, EDNAYTA, EDAAYAA, QNAAYAA, NEYTA, NEYAA, AYTA, NAYTA, and EPQYQPA

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1 The abbreviations used are: TPK, tyrosine protein kinase; HPLC, high pressure (performance) liquid chromatography; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Fmoc, N-(a-fluorenyl)methoxycarbonyl.
were synthesized by solid-phase technique from Fmoc amino acids using a manual synthesizer (Model Bomil 4175, LKB). The side-chain functional groups of glutamic acid, aspartic acid, threonine, and tyrosine were blocked using acid-labile t-butyl groups. Syntheses were performed in continuous flow on a 0.1-mmol scale using the Fmoc amino acid active esters derived from pentafluorophenol or 3,4-dihydroxybenzoic acid-1,2,5-benzo triazole. The syntheses with pentafluorophenol esters was carried out in the presence of N-hydroxybenzotriazole as a catalyst. Synthetic peptides were cleaved from the resin and side chain protecting groups removed using 95% aqueous trifluoroacetic acid for 1 or 2 h. Reagents were evaporated and checked by analytical HPLC on a reversed-phase Waters Delta-Pak C18 300 A column (7.8 x 300 mm) using a Perkin-Elmer 410 LC B10 HPLC apparatus. Elution was performed with linear gradient from 0.1% aqueous trifluoroacetic acid to 30% acetonitrile containing 0.08% trifluoroacetic acid in 35 min at 3 ml/min with the eluent monitored at 215 nm. The purity of the peptides (95% or more) was checked by analytical HPLC on a reversed-phase Waters Delta-Pak C18, 300 A column (3.9 mm x 30 cm) column and by amino acid analysis.

IIB has been shown not to cross-react with various monospecific antibodies raised against tyrosine kinases of the src family, namely those expressed by lck, hck, lyn, and fyn (6).

TPK-IIB also fails to cross-react with anti-c-src antibodies (m AS 327), with anti-cst-1 antibodies which recognize pp60c-src, pp60c-src and pp59fyn, and with anti-SEEP antibodies raised against the 330-345 segment of pp60c-src, which is highly conserved in all the tyrosine kinases of the src family (not shown).

After the heparin sepharose chromatography to separate TPK-IIB from TPK-IIA (5), TPK-IIB underwent three additional purification steps as shown in Table I. On Mono Q HPLC the final preparation exhibits an individual sharp protein peak coinciding with tyrosine kinase activity (Fig. 1). This peak gives rise to a single prominent protein band of the expected Mr (52,000) upon SDS-PAGE (Fig. 1, inset). The identification of this band as the tyrosine kinase itself is also consistent with the high specific activity of the final preparation of TPK-IIB which is comparable with the activity exhibited by other highly purified preparation of tyrosine kinases (13, 14 and references cited therein). Such a high activity would hardly be compatible with the single prominent band merely representing a contaminating component.

As pointed out previously, TPK-IIB does not undergo any detectable autophosphorylation (5). Its lack of autophosphorylation sites has been now corroborated by its failure to cross-react with antiphosphotyrosine monoclonal antibodies (Fig. 2): no phosphotyrosine signal could be detected with TPK-IIB, with or without preincubation in the autophosphorylation medium. On similar blots, lysates from Jurkat cells expressing high levels of pp60-src, a tyrosine kinase of the src family (1), resulted in strong positive signal, of the expected molecular weight. The same signal is evident with spleen TPK-1/lyn, another src-related tyrosine kinase capable of autophosphorylation (5, 6). The specificity of the reaction was established by preincubation of the antibody with phosphotyrosine which totally eliminated the signal.

This finding clearly confirms that TPK-IIB is devoid of phosphotyrosyl sites, a quite unusual feature, distinguishing it from all the known tyrosine kinases of the src-family. TPK-IIB nevertheless phosphorylates synthetic peptides reproducing the two main tyrosyl phosphoacceptor sites that are conserved in all the cellular members of the src family. As shown in Table II the peptide EDNEYTA, reproducing the sequence around pp60c-src Tyr-416, is an especially good substrate, particularly by virtue of its Kcat value (58 μM), which is one of the lowest ever reported for peptides of comparable size used as substrates for tyrosine kinases (e.g. see Ref. 15). The phosphorylation efficiency of EDNEYTA by TPK-IIB is more than 30 times higher than that of angiotensin II, a widely employed substrate of tyrosine protein kinases, while the peptide EPQYQPA, reproducing the down-regulation site corresponding to pp56fyn Tyr-637, is a much poorer substrate: its Kcat value is more than two orders of magnitude higher and its Kcat is about two-fold lower than that of EDNEYTA (Table II).

In order to obtain a better insight into the particular susceptibility of the peptide EDNEYTA to phosphorylation by TPK-IIB, a number of derivatives have been synthesized and analyzed for their kinetic parameters (Table III). It is evident that, except for the conservative replacement of Glu for Asp at position -3 relative to tyrosine, all the other substitutions tested are unfavorable, giving rise to peptides that invariably display higher Kcat and sometimes also lower Kcat values. The replacement of the glutamic acid adjacent to tyrosine is especially unfavorable. It is more detrimental however in the pentapeptide NAYTA than in the heptapep-
Phosphorylation of src Peptides

The isolation of the particulate fraction from 3.5 kg of spleen from freshly slaughtered beef and its extraction with 1% Nonidet P-40 were performed essentially as previously described (5). The crude extract was resolved into four fractions (TPK-I, TPK-IIA, TPK-IIB, and TPK-III) by combining DEAE-Sepharose and heparin-Sepharose chromatographies, as in a previous study (5). TPK-IIB was further purified by phosphocellulose chromatography (5) and by Sephacryl S-200 gel filtration and Mono Q HPLC as described under "Materials and Methods." The activity in the crude extract and after the first purification step is underestimated due to the presence of tyrosine protein phosphatase activity not completely inhibited by the vanadate present in the kinase assay. This may also account for the higher than 100% apparent recovery of activity after the DEAE-Sepharose and heparin-Sepharose steps if the activities of the other tyrosine kinase fractions, TPK-I/TPK-III and TPK-IIA, resolved by DEAE-Sepharose, and heparin-Sepharose, respectively (5), have been taken into account. These overall yields are shown in parentheses.

| Purification step | Protein | Total activity | Specific activity | Purification | Yield |
|-------------------|---------|----------------|------------------|--------------|-------|
| Crude extract     | 57,450  | 387            | 0.0067           | 1            | 100   |
| DEAE-Sepharose    | 2,275   | 252            | 0.11             | 16.7         | 65 (150) |
| Heparin-Sepharose | 58      | 232            | 0.02             | 590          | 59 (89) |
| Phosphocellulose  | 3.5     | 133            | 35.42            | 5,220        | 34    |
| Sephacryl S-200   | 1.2     | 124            | 103.33           | 16,410       | 32    |
| Mono Q            | 0.2     | 105            | 525.00           | 78,350       | 27    |

Fig. 1. Mono Q HPLC and SDS-PAGE of TPK-IIB. An aliquot of TPK-IIB after the first Mono Q purification step (see Table I) was subjected to a second Mono Q HPLC by applying a discontinuous gradient (dotted line). Proteins were automatically recorded at 280 nm (solid line). 0.4-ml fractions were collected and 10-µl aliquots were assayed for tyrosine kinase activity (dashed line). The inset shows the SDS-PAGE Coomassie Blue patterns of TPK-IIB after the second (lane 1) and first (lane 2) Mono Q and after the Sephacryl S200 purification step (lane 3). The position of molecular mass markers (in kilodaltons) are as indicated.

tide EDNAYTA, suggesting that the additional acidic residues at position -3 and -4, present only in the heptapeptide, may exert a positive influence, reinforcing the effect of glutamic acid at position -1. Consistent with this hypothesis EDNEYTA, EDNAYTA and EDAAYAA are much better substrates than NEYTA, NAYTA, and AAYAA, respectively, and EDAAYAA is phosphorylated more efficiently than QNAAYAA. The striking superiority of EDNEYTA over its triply substituted EDAAYAA derivative, however, highlights the crucial relevance of the amino acids nearer to the tyrosyl residue. The comparative analysis of the individual mono- and disubstituted derivatives of NEYTA, namely, AEYTA, NAYTA, and NEYAA clearly indicates the most harmful substitution to be that of the glutamic acid adjacent to the amino-terminal side of tyrosine, NAYTA exhibiting a 10-fold higher $K_{m}$ and a more than 3-fold lower $K_{m}$ than the parent peptide NEYTA. It should be noted that the propensity to adopt a $\beta$-turn conformation is similar for NAYTA and NEYTA and for EDNAYTA and EDNEYTA, despite their sharply different susceptibility to phosphorylation. Apparently therefore an acidic side chain adjacent to the amino-terminal side of tyrosine acts as a powerful specificity determinant independent of its ability to confer a $\beta$-turn conformation.

Fig. 2. Western blots of TPK-IIB (lane 1), TPK-I/lyn (lane 3) and p56$^{++}$ (lane 4) developed with monoclonal antiphosphotyrosine antibodies. Lane 2 refers to TPK-IIB previously incubated with unlabelled ATP in the autophosphorylation medium. TPK-I/lyn was isolated from spleen (6) and further purified by Mono Q HPLC. p56$^{++}$ was obtained by immunoprecipitation of Jurkat cells lysates (22). TPK-IIB (1 µg), TPK-I/lyn (0.1 µg), and p56$^{++}$ (0.1 µg) were resolved on 10% SDS-PAGE followed by electrophoretic transfer onto nitrocellulose filters. The blot was decorated with monoclonal antibodies anti-TyrP (2 µg/ml) followed by 125I-rabbit anti-mouse. Lanes 5 and 6 were treated with monoclonal antibody previously kept overnight at 4 °C in the presence of 10 mM phosphotyrosine. The autoradiography of the transblotted filters is shown.

On the other hand the unfavorable effect of substituting the neutral residues at positions -2 and/or +1 with alanine could be interpreted in terms of conformational alterations, since the singly and doubly substituted derivatives AEYTA, NEYAA and AAYAA have lost the predicted $\beta$-turn conformation of the parent pentapeptide NEYTA (see Table II). The same effect could account for the decreased efficiency of EDAAYAA compared with EDNAYTA.

The excellent phosphorylation efficiency of EDNEYTA by TPK-IIB, by virtue of its low $K_{m}$, is especially remarkable if
TABLE II
Kinetic constants of src-derived peptides for purified TPK-IIB

The kinetic constants were determined by double-reciprocal plots constructed from initial rate measurements fitted to the Michaelis-Menten equation. The ATP concentration was kept constant at 20 μM. Average values calculated from three or more experiments are shown. The standard error for all reported values was ±15%. The Km values were calculated from the Vmax values assuming 95% purity and a molecular mass of 52 kDa for TPK-IIB. Km values are defined and were calculated according to Chou and Fasman (20) for all the quartets of amino acids including tyrosine. Only the highest value for each peptide is reported and the corresponding predicted β-turn is shown if the pβ value is higher than the cut-off value of pβ = 0.75.10^(-4) (20).

| Peptides | Km(mM) | Apparent Km(mM) | Km/n | pβ | β-turn |
|----------|--------|----------------|------|----|--------|
| EDNEYYTA| 31.2   | 0.698          | 537.9| 1.17| DNEY   |
| EDNEYYTA| 26.3   | 0.037          | 710.8| 0.96| NEY    |
| EDNYAYTA| 24.0   | 0.322          | 74.3 | 1.10| NAVT   |
| EDAVAYTA| 4.6    | 0.325          | 14.1 | 0.48| No β-turn |
| QNAYAYTA| 24.0   | 1.700          | 1.41 | 0.53| No β-turn |
| NEYAYTA | 57.2   | 0.500          | 74.4 | 0.86| NEYT   |
| NAVAYTA | 4.6    | 0.325          | 14.1 | 0.48| No β-turn |
| NEAYTA  | 51.6   | 0.800          | 39.3 | 0.65| No β-turn |
| AEFAYTA | 26.9   | 1.500          | 17.9 | 0.32| No β-turn |
| AEAYTA  | 33.6   | 3.000          | 11.2 | 0.23| No β-turn |
| AAYAA   | 4.6    | 1.000          | 0.46 | 0.30| No β-turn |
| EPQYPQA | 14.4   | 16.700         | 0.8  | 1.11| PQYQ   |
| Angiotensin II | 39.8 | 2.000 | 15.4 | 0.54| No β-turn |

Table III
Kinetic constants of src-derived peptides for partially purified tyrosine kinases TPK-I/lyn and TPK-III

Vmax (expressed as nmol.min⁻¹mg⁻¹) and apparent Km values were determined as detailed in Table II for TPK-IIB. In the case of TPK-I/lyn and TPK-III, however, Km values could not be calculated since the enzyme preparations were only partially purified.

| Peptides | Vmax(mM) | Km(mM) | Km/Vmax | Km (mM) | Km/Vmax |
|----------|----------|--------|---------|---------|---------|
| EDNEYYTA| 18.6     | 3.8    | 4.7     | 9.4     | 0.43    |
| NEYAYTA | 39.3     | 6.2    | 6.3     | 11.1    | 0.55    |
| NAVAYTA | 52.5     | 3.0    | 17.5    | 10.7    | 1.3     |
| NEAYTA  | 56.7     | 7.0    | 8.1     | 17.8    | 1.7     |
| AEFAYTA | 27.9     | 12.0   | 2.3     | 7.2     | 5.3     |
| AEAYTA  | 58.2     | 3.5    | 16.6    | 4.1     | 5.3     |
| AAYAA   | 87.6     | 5.0    | 17.5    | 3.5     | 4.5     |
| EPQYPQA | 19.6     | 8.3    | 2.3     | 3.9     | 0.43    |
| Angiotensin II | 34.1 | 1.8 | 19.0 | 6.2 | 1.2 |

specification distinct from those of either TPK-IIB and TPK-I/lyn. It is reminiscent of TPK-IIB in that the parentage of penapetide is preferred over the substituted derivatives (Table III). The replacement of the asparagine at position = 2 however is much more detrimental than that of the glutamic acid adjacent to the tyrosyl residue (Table III).

Any more direct and exhaustive comparison of the catalytic efficiencies of the three spleen tyrosine kinases considered here was hampered by the fact that TPK-I/lyn and TPK-III are still only partly purified, so that their Kcat values could not be calculated nor compared with those of TPK-IIB.

The possibility that the lower Kcat values of TPK-IIB might merely reflect its higher degree of purification was ruled out by the fact that identical values were obtained when the kinetic experiments were performed with TPK-IIB after heparin sepharose, the degree of purification of which is comparable to that of TPK-I/lyn and TPK-III (not shown).

In order to assess the actual ability of TPK-IIB to phosphorylate tyrosine kinases of the src family, TPK-I/lyn, whose autophosphorylation site is exactly reproduced by the epta- peptide EDNEYTA, was incubated with TPK-IIB in the absence and presence of heparin, which is a powerful inhibitor of TPK-IIB (5), while it stimulates TPK-I/lyn activity (5, 6). As shown in Fig. 3A TPK-IIB promotes an increased radiolabeling of TPK-I/lyn which is suppressed by heparin. The 32P-peptide maps obtained from TPK-I/lyn either autophosphorylated or phosphorylated in the presence of TPK-IIB are identical (Fig. 3B) suggesting that the same phosphoacceptor site(s) are involved. This would indicate that the sequence EDNEYTA, is preferentially affected by TPK-IIB even if it is included into the parent proteins.

DISCUSSION

A somewhat paradoxical outcome of this work is that peptides reproducing the highly conserved autophosphorylation site shared by all TPKs of the src family (Tyr-416 of pp60 src) are more efficiently phosphorylated by a spleen TPK presumably unrelated to the src family (TPK-IIB) than they are by members of the src family such as pp60 src (16), p56 src(14).

Fig. 3. Phosphorylation of TPK-I/lyn by TPK-IIB. A. 32P incorporation into TPK-I/lyn (6.5 μg) either in the absence or presence of TPK-IIB (25 units) and of heparin (20 μg/ml) was performed and evaluated as described under “Materials and Methods.” The autoradiography of SDS-PAGE is shown. The control with TPK-IIB alone plus heparin displayed no detectable radioactive bands (not shown). B. The radiolabeled bands of TPK-I/lyn either autophosphorylated (lane 1) or phosphorylated in the presence of TPK-IIB (lane 2) were electroeluted, digested with V8 protease from Staphylococcus aureus (200 μg/ml) for 15 min at 37 °C in the presence of 0.5% SDS and subjected to 20% SDS-PAGE. The autoradiograph of the gel is presented. Marker proteins were: glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1), and α-lactalbumin (14.2 kDa).

2 A. M. Brunati, R. Kypta, A. Donella-Deana, and L. A. Pinna, unpublished data.
Phosphorylation of src Peptides

(18), and by two src-related spleen TPKs, namely TPK-I/lyn and TPK-III. These peptides are excellent substrates for TPK-IIB, displaying $K_m$ values in the micromolar range. The fact that substitution of any of the amino acids surrounding tyrosine decreases their ability to serve as substrates for TPK-IIB suggests that all the features of the main phosphoacceptor site of the src family contribute to the specificity for this kinase. The membership of this tyrosine kinase to the src family, though not completely ruled out, is rendered extremely unlikely by its lack of autophosphorylation activity, a property shared by all the src-related TPKs known so far and by its markedly different peptide substrate specificity. Furthermore, the modifications that decrease the phosphorylation of synthetic peptides by TPK-IIB either do not affect or even improve the phosphorylation of the same peptides by TPK-I/lyn. Such a behavior, albeit somewhat paradoxical, was not totally unexpected considering that amino-terminal acidic residues (which are invariably found at the src autophosphorylation sites) are not required for the phosphorylation of synthetic peptides by TPKs related to pp60$^{c-src}$ (16, 19), and may even exert an unfavorable effect (8).

In this connection it would be interesting to establish the identity of TPK-III, which is also a member of the src family according to its reactivity with anti-c-src-1 and anti-SEEP antibodies raised against conserved segments of src protein kinases. Its substrate specificity however is significantly distinct from that of TPK-I/lyn. The finding that TPK-III is not recognized by a variety of monospecific antibodies raised against the products of c-src, lek, hck, lyn, lyn (6), and yes$^2$ oncogenes, increases the probability that it might be identical or very closely related to the last member of the src family, namely fgr. Interestingly the fgr tyrosine kinase differentiates for having the least conserved autophosphorylation site among the members of the src family (see Ref. 1). This might correlate with the distinct site specificity of TPK-III.

It is possible that the great susceptibility of EDNEYTA and NEYTA to phosphorylation by TPK-IIB might partially derive from their adopting a $\beta$-turn conformation, which is predicted by the method of Chou and Fasman (20). However, the probability of adopting a $\beta$-turn conformation is either the same or even higher for EDNAYTA and NAYTA, which are much worse substrates than EDNEYTA and NEYTA, respectively. It is reasonable therefore to conclude that the favorable effect of glutamic acid at position $-1$ is accounted for predominantly by the acidic nature of its side chain, rather than by any effect of conformation. The intrinsic importance of the acidic nature of the residue adjacent to the amino-terminal side of tyrosine is also corroborated by the very poor phosphorylation efficiency of the peptides AEYTA, NEYAA, and AEYAA which have lost the predicted $\beta$-turn conformation of NEYTA.

Taken together these observations would suggest that TPK-IIB or other TPK(s) with similar site specificity are involved in the phosphorylation of the src products in vivo. It should be recalled in this connection that although pp60$^{c-src}$ Tyr-416 (and the homologous tyrosines of the other src-TPKs) undergo in vitro autophosphorylation, no incontrovertible evidence is available that their in vivo phosphorylation invariably occurs by the same mechanism. Rather, the possibility is still open that heterologous phosphorylation of Tyr-416 by a distinct kinase could contribute to the activation of pp60$^{c-src}$ (see Ref. 1). The inter-, rather than intramolecular mechanism of in vitro autophosphorylation (21) would be consistent with this hypothesis, assuming that a kinase with higher affinity for the phosphorylation site (like TPK-IIB) is present. On the other hand, it is also possible that the auto-phosphorylation efficiency of the src-related tyrosine kinase is increased either by conformational features inherent in the tertiary structure of the kinase itself or by so far unidentified endogenous effectors. In such a case Tyr-416 phosphorylation/activation by a heterologous kinase may still become relevant whenever the cellular src-TPKs have an intrinsically low activity, because of down-regulation by concomitant Tyr-527 phosphorylation. In any event the hypothesis that the autophosphorylation site(s) of src tyrosine kinases might be targeted by TPK-IIB has been corroborated, at least in vitro, by showing that TPK-IIB promotes an increased phosphorylation of TPK-I/lyn, giving rise to the same $\gamma$-peptide map obtained with autophosphorylated TPK-I/lyn.

In this respect TPK-IIB seems to be different from the brain TPK reported to specifically phosphorylate pp60$^{c-src}$ at its carboxyl-terminal site (Tyr-527) (4) despite sharing the same inability to perform autophosphorylation, a most unusual property among tyrosine kinases. TPK-IIB actually displays a very low phosphorylation efficiency toward the synthetic peptide EPQYQPA reproducing the sequence around Tyr-527. It should be noted however that such a peptide is an extremely poor substrate for TPK-I/lyn too (Table II), consistent with the concept that the carboxyl-terminal tyrosine is not an autophosphorylation site but, rather, a target for another TPK(s) involved in down-regulation of src-TPKs.

Although TPK-IIB poorly phosphorylates the peptide EPQYQPA, it is still conceivable, that its efficiency might increase once this sequence is included in a more extended protein domain. Considering, however, the opposite regulatory functions of Tyr-416 and Tyr-527 it seems unlikely that they might be targets for the same kinase. All the data obtained with synthetic peptides support the concept that TPK-IIB displays a remarkable affinity for the site including Tyr-416 but not for the one including Tyr-527. A similar preference for EDNEYTA over EPQYQPA is shared by the other spleen tyrosine protein kinases characterized so far. In this respect the synthetic peptide EPQYQPA could prove a suitable substrate for monitoring and detecting the tyrosine protein kinase(s) which are able to down-regulate the TPKs of the src family by phosphorylating their carboxyl-terminal site.

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Phosphorylation of src Peptides

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