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Spleen protein tyrosine kinases TPK-IIB and CSK display different immunoreactivity and opposite specificities toward c-*src*-derived peptides

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Polyclonal antibodies have been raised against two synthetic peptides reproducing the 48–64 and 353–369 sequences of CSK, a protein tyrosine kinase implicated in the down-regulation of *src*-related protein kinases. Both antibodies specifically recognize recombinant CSK and a CSK-related 49 kDa protein tyrosine kinase present in spleen but they do not cross-react with purified TPK-IIB, a spleen protein tyrosine kinase sharing with CSK catalytic activity toward *src* kinases and incapability to autophosphorylate. CSK and TPK-IIB once resolved from each other by heparin-Sepharose affinity chromatography, display opposite specificities toward synthetic peptides reproducing the sequences around the main phosphoacceptor residues of pp60$^{c\text{-}src}$, namely Tyr-416 and Tyr-527. These data support the view that TPK-IIB and CSK may exert opposite effects on the activity of *src*-related protein tyrosine kinases.

Protein tyrosine kinase; pp60$^{c\text{-}src}$ regulation; Phosphotyrosine; Peptide; Spleen

1. INTRODUCTION

A common feature of most protein tyrosine kinases is their capability to autophosphorylate [1,2]. Such an autophosphorylation primarily occurs at a site which is homologous to the main autophosphorylation site of pp60$^{c\text{-}src}$, i.e. Tyr-416, and correlates with an activation of the kinase [1,3]. A notable exception is provided by a protein tyrosine kinase initially isolated from brain [4,5] and subsequently found in a variety of tissues including spleen and lymphoid cells [5,6] which is devoid of any evident autophosphorylation activity and has been termed CSK (c-*src* kinase) after its capability to phosphorylate c-*src* at its C-terminal, down-regulating site, i.e. Tyr-527 [5]. CSK can also phosphorylate and inactivate the *src* family kinases p56*lyn*, p59*fyn* [6] and p56*lck* [7]. The deduced amino acid sequence of CSK revealed a certain extent of homology with other known tyrosine protein kinases, but it lacks any potential phosphoacceptor site in the region homologous to that harbouring Tyr-416 in pp60$^{c\text{-}src}$ [5].

The unusual property of lacking autophosphorylation activity is shared by a tyrosine kinase isolated from spleen and conventionally termed TPK-IIB [8]. TPK-IIB proved immunologically unrelated to the *src* family, while capable to phosphorylate in vitro the *src*-related *lyn* product [9]. To check the possible relation between TPK-IIB and CSK we have now raised antibodies against two CSK synthetic peptides. Here we show that these antisera do not cross-react with purified TPK-IIB, while they readily recognize a spleen 49 kDa protein tyrosine kinase the activity ratio of which toward synthetic peptides reproducing the two main phosphoacceptor sites of pp60$^{c\text{-}src}$ (Tyr-416 and Tyr-527) is opposite to that exhibited by TPK-IIB.

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2. MATERIALS AND METHODS

Angiotensin II was purchased from Sigma. The peptide EDNEYTA was synthesized by a manual synthesizer (Model Biolinx 4175, LKB) using the continuous flow variant of the 'Fmoc-polyamide' method on the Kieselguhr-supported polydimethylacrylamide resin functionalized with 4-hydroxymethylphenoxy-acetic acid (NovaSynR KA-Novabiochem AG) [9]. Other peptides were synthesized by an automated synthesizer from Applied Biosystems (Model 431-A), using Fmoc/ NMP (*N*-methylpyrrolidone) chemistry on hydroxymethylphenoxymethyl-resin (HMP-Applied Biosystems).

2.1. *Antibodies*

The peptides reproducing the sequences 48–64 and 353–369 of CSK were coupled to keyhole limpet hemocyanin with a coupling kit (Pierce Chemical Co.). Four rabbits were injected and the sera were monitored by Western blots of spleen soluble and particulate fractions. All sera detected a 49 kDa band that could be abolished by preincubation of the sera with the corresponding peptides (300 μM).

2.2. *Western blots*

Enzyme fractions were subjected to 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose filters in a Hoefer apparatus at 400 mV for 2 h. The filters were blocked for 1 h at room temperature with 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl. The rabbit antisera were used at a dilution of 1:200. Immunoreactive proteins were incubated

with donkey anti-rabbit Ig, biotinylated and detected by incubation with streptavidin alkaline phosphatase conjugate.

Purification of TPK-IIB (and CSK) from spleen soluble and particulate fractions was performed essentially as described in [8,9] except for the disruption of cells, which was obtained in isotonic medium containing 0.25 M sucrose saccharose, 1 mM EDTA, 2 mM $MgCl_2$, 100 μM PMSF, 100 mM Tris-HCl, pH 7.5.

Tyrosine protein kinase activity was routinely tested on poly(Glu^4,Tyr^1) as in [8].

Phosphorylation of peptide substrates was assayed as previously described [9].

3. RESULTS

Antisera were raised against the synthetic peptides YKAKNKVGREGIIPANY and TAPEALREKKFSTKSDV reproducing the 48–64 and 353–369 sequences of CSK (see section 2). Both antisera recognize recombinant glutathione-*S*-transferase-CSK expressed in *E. coli* and p49 from Jurkat cells on Western blots (unpublished data).

As shown in Fig. 1 both AB (48–64) and AB (353–369) interact on a Western blot with a 49 kDa component present in partially purified preparations of TPK-IIB obtained by DEAE-Sepharose chromatography of both cytosol and extracts of the particulate fraction

Table I

Different peptide substrate specificity of CSK-like kinase and TPK-IIB

Peptide substrate	Phosphorylation rate by	
	CSK-like	TPK-IIB
DRVYIHPF angiotensin II	100	100
AFLEDYFTSTEPQYQPGENL (c-*src* [514–533])	894	587
AFLEDFFTSTEPQYQPGENL (c-*src* [514–533]Phe^{519})	704	77
AFLEAFFTSTEPQYQPGENL (c-*src* [514–533]Ala^{518}Phe^{519})	258	33
EDNEYTA (c-*src* [412–418])	225	2275
KDDEYNPA (*fgr* [408–415])	5	192
RLMTGDTYTAHAGAK (*abl* [386–400])	6	15
DENYYKA (*syk* [515–521])	203	218
NIDGDGEVNYEE (CaM loop IV)	220	950
EKEYHAE (EGFR[842–848])	12	49

The phosphorylation rate is expressed relative to that of angiotensin II (=100%). The concentration of peptides was 250 μM. CSK-like kinase and TPK-IIB were resolved from each other by heparin–Sepharose chromatography and collected as indicated in Fig. 2. The same amounts of enzymes in terms of kinase activity toward 2 mM angiotensin (3 pmol · min^{-1}) were used. Peptide nomenclature (between brackets) is reminiscent of which sequence of the parent protein they reproduce. Tyrosines are in bold type. Underlining denotes those residues that have been substituted relative to the parent peptide.

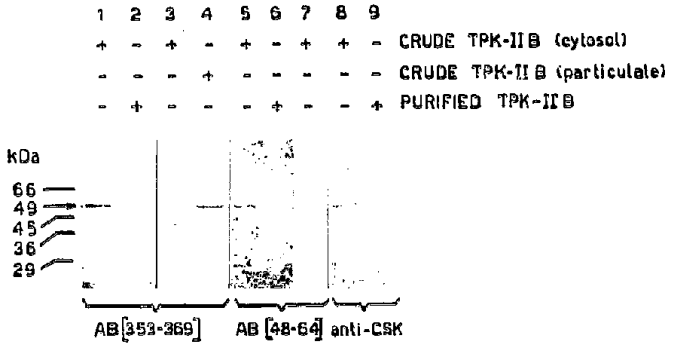

Fig. 1. Western blots of crude spleen protein tyrosine kinase fractions and purified TPK-IIB with various anti-CSK antisera. TPK-IIB from either rat spleen cytosol (lanes 1, 3, 5, 7 and 8) or particulate fraction (lane 4) was partially purified by DEAE–Sepharose, subjected to SDS-PAGE, and immunoreacted with the indicated antisera either as such (lanes 1, 4, 5 and 8) or after preincubation of the antisera with the corresponding peptides (lanes 3 and 7) (for details see section 2). Lanes 2, 6 and 9 refer to purified TPK-IIB after Mono-Q. The amount of purified TPK-IIB was more than twice that of the partially purified enzyme, as judged from kinase activity.

from rat spleen (lanes 1, 4 and 5). The signal is specific since it is readily abolished if the antisera are preincubated with excess of the antigen peptides (lanes 3 and 7). The same 49 kDa band is also recognized by anti-CSK antisera (raised against bacterially expressed protein) provided by Dr. M. Okada [5] (lane 8).

On the other hand, highly purified TPK-IIB preparations from either cytosol or the particulate fraction are not recognized by any of the 3 antibodies used (Fig. 1, lanes 2, 6 and 9), thus strongly suggesting that TPK-IIB is not itself responsible for the immunoreactivity displayed by partially purified preparations. The clear-cut demonstration that indeed CSK and TPK-IIB are distinct entities was provided by subjecting the DEAE-cellulose fraction exhibiting both tyrosine kinase activity and CSK immunoreactivity to heparin-Sepharose chromatography and by probing the individual fractions for their reactivity with AB (48–64). As already known [8] TPK-IIB eluted from heparin-Sepharose with 0.45 M NaCl (see Fig. 2). It is preceded, however, by a smaller peak of activity eluted with 0.27 M NaCl. While the activity of the small peak coelutes with immunoreactivity against antisera AB [48–64], the main peak of TPK-IIB activity is entirely devoid of such a property (inset of Fig. 2).

The two peaks were separately purified by phosphocellulose column chromatography and Mono-Q FPLC where the immunoreactive CSK-like protein kinase is eluted with 0.22 M NaCl, whereas non-immunoreactive TPK-IIB is less retarded, and eluted with 0.18 M NaCl (data not shown).

The peptide substrate specificities of these two kinases are sharply different as outlined in Tables I and II. In particular CSK and TPK-IIB display an opposite preference toward peptides reproducing the two main phosphoacceptor sites of pp60$^{c\text{-}src}$, namely Tyr-416 and

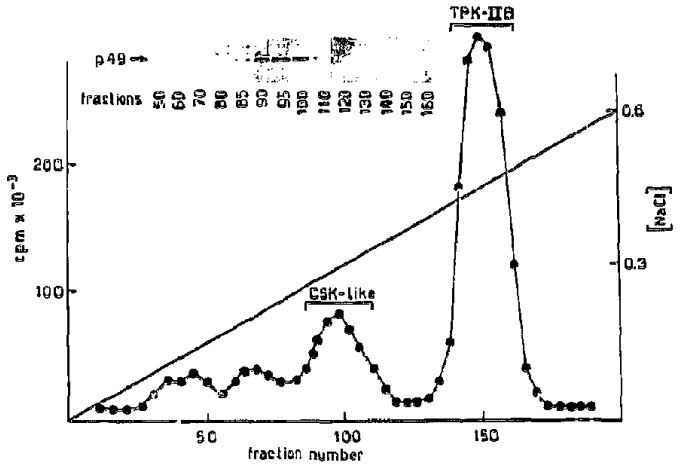

Fig. 2. Resolution of TPK-IIB and CSK-like activities by heparin–Sepharose. Aliquots of 20 and 40 μl were assayed for protein tyrosine kinase activity and immunoreactivity with antisera AB(48–64), respectively. Western blots of individual fractions, as indicated, are shown in the inset. Other experimental conditions are detailed in section 2.

Tyr-527. While, in fact, the best substrates for the CSK-like enzyme are two peptides (c-*src*[514–533] and c-*src*[514–533]Phe^{519}), reproducing the C-terminal site responsible for down-regulation, TPK-IIB by far prefers a heptapeptide substrate (c-*src*[412–418]) that reproduces the autophosphorylation site of pp60$^{c\text{-}src}$ (Tyr-416). The phosphorylation of the C-terminal peptide *src*[514–533] by TPK-IIB is moreover dramatically reduced when Tyr-6 is replaced by Phe, while this substitution only slightly affects the phosphorylation by the CSK-like kinase. This clearly indicates that the phosphorylation of the C-terminal *src* peptide by TPK-IIB occurs at a residue (Tyr-6 in the peptide, equivalent to Tyr-519 in pp60$^{c\text{-}src}$) which is not phosphorylated in vivo, whilst the tyrosyl residue responsible for down-regulation (Tyr-527, equivalent to Tyr-14 in the peptide) is the one preferentially affected by the CSK-like kinase. While the peptide reproducing the C-terminal segment of c-*src* is phosphorylated by CSK more readily than all the other tyrosyl peptides tested so far, reproducing the phospho-acceptor sites of a variety of proteins (see Table I), its phosphorylation is seriously hampered upon replacement of Ala for Asp-5, equivalent to Asp-518 of c-*src*, which is supposed to be essential for the phosphorylation of Tyr-527 in vivo [10]. As shown in Table II such a negative effect of substitution is accounted for by a drop in V_{max}, whereas the K_m value is almost unchanged.

4. DISCUSSION

This report provides the clear-cut demonstration that the protein tyrosine kinases termed CSK [5] and TPK-IIB [8], sharing the unique property of lacking autophosphorylation activity, are present in rat spleen cytosol as two definitely distinct enzymes, based on the following criteria: (1) immunoreactivity with various anti-CSK antisera that do not recognize TPK-IIB; (2) different chromatographic behaviour on heparin–Sepharose and mono-Q; and (3) opposite specificity toward *src*-derived peptides. In particular, while CSK exhibits its highest activity toward an eicosapeptide reproducing the carboxyl terminal segment of pp60$^{c\text{-}src}$, affecting its tyrosyl residue that corresponds to Tyr-527, responsible for down-regulation of pp60$^{c\text{-}src}$, TPK-IIB by far prefers the heptapeptide EDNEYTA reproducing the main autophosphorylation site of pp60$^{c\text{-}src}$ and its relatively modest phosphorylation of the C-terminal peptide is mostly accounted for by the tyrosyl residue corresponding to Tyr-519, the phosphorylation of which in pp60$^{c\text{-}src}$ has never been reported.

The remarkable specificity of spleen CSK for Tyr-527 of pp60$^{c\text{-}src}$ is also outlined by the observation that the two peptides reproducing this site, either as such or with Phe substituted for Tyr-519 are by far preferred over all the other peptide substrates tested so far.

The additional finding that the substitution of Ala for the aspartyl residue equivalent to Asp-518, essential for down regulation of c-*src*, slows down the phosphorylation of the C-terminal peptide by CSK corroborates the concept that the substrate specificity of this enzyme closely reflects its putative role in phosphorylating and downregulating the *src* related protein tyrosine kinases.

On the other hand the relatively high K_m values of CSK for its best peptide substrates (Table II) would indicate that the structural information contained in the eicosapeptide *src*[514–533] are not sufficient to ensure

Table II

Kinetic constants of *src*-derived peptides for CSK

Peptide substrate	V_{max} (nmol · min^{-1} · mg^{-1})	K_m (mM)	Efficiency (V_{max}/K_m)
AFLEDYFTSTEPQYQPGENL (*src*[514–533])	4.00	0.95	4.21
AFLEDFFTSTEPQYQPGENL (*src*[514–533]Phe^{519})	2.66	0.97	2.74
AFLEAFFTSTEPQYQPGENL (*src*[514–533]Ala^{518}Phe^{519})	0.92	1.10	0.83
EDNEYTA (*src*[412–418])	1.35	2.80	0.48

CSK was resolved from TPK-IIB by heparin–Sepharose chromatography, as in Fig. 2, and partially purified by phosphocellulose column chromatography. Peptides are represented as in Table I. Kinetic constants were determined by regression analysis of double reciprocal plots constructed from initial rate measurements.

a high affinity binding to CSK, suggesting that higher order structural features or perhaps the integrity of the whole protein substrate are required in order to optimize the phosphorylation efficiency by this enzyme. The behaviour of TPK-IIB is different also in this respect since it displays with the heptapeptide EDNEYTA, which is its best substrate, a quite low K_m value, in the μmolar range [9]. Such a high affinity for the peptide reproducing a site of c-*src* the phosphorylation of which correlates with a stimulation of activity suggests that TPK-IIB may exert a positive control on c-*src*. The possibility therefore that TPK-IIB could play a role antagonistic to that of CSK by stimulating instead of inhibiting the kinases of the *src* family should be taken into account in further studies.

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