Site specificity of p72^{syk} protein tyrosine kinase: efficient phosphorylation of motifs recognized by Src homology 2 domains of the Src family

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Received 4 April 1995

Abstract Protein tyrosine kinase p72^{syk} purified from rat spleen has been assayed for its ability to phosphorylate a number of peptide substrates derived from naturally occurring phospho-acceptor sites. The phosphorylation efficiency is extremely variable, depending on the peptide sequence, with K_m values in the 3–1500 μM range. The by far best peptide substrates, with K_m values of 3 and 4 μM are those reproducing the phospho-acceptor sites of Vav and HS1 proteins, respectively. These sites include multiple acidic residues flanking tyrosine on both sides and they also display the consensus sequences (YEDL and YEEV) preferred by the SH2 domains of the Src family. Alteration of this consensus in the HS1 peptide, by replacing either the glutamic acid or valine, also reduces the phosphorylation efficiency by p72^{syk}. Also the replacement of acidic residues at position −1 and, to a lesser extent at positions −3 and −4 (but not at positions +3 and +5) are detrimental. These observations may suggest a role of p72^{syk} in the recruitment of ligands/substrates for the Src family enzymes. We also show that the HS1 peptide can be used for the specific monitoring of p72^{syk} since neither the two Src-related c-Fgr and Lyn kinases (needing a hydrophobic instead of acidic residue at position −1) nor CSK appreciably phosphorylate it.

Key words: p72^{syk}; c-Fgr; Lyn; c-Src kinase (CSK); Tyrosine kinase specificity; Kinase peptide substrate

1. Introduction

Non receptor-type protein tyrosine kinases make up an important class of enzymes that are deeply implicated in signal transduction and in the regulation of cell growth and differentiation [1,2]. They play an especially crucial role in hematopoietic cells, where they are associated with both the B- and T-cell antigen–receptor complexes (BCR and TCR, respectively) and they participate in the mechanism of activation of these cells [3,4]. Among this class of enzymes those belonging to the family of Src related tyrosine kinases, including now-a-days not less than 10 members [5], probably are the best known. They share a common structure consisting of a N-terminal poorly conserved myristoylated domain followed by a SH3, a SH2 and a catalytic (SH1) domains and ending with a short regulatory C-terminal tail including a tyrosyl residue whose phosphorylation by C-terminal domain Src kinase (CSK) inhibits kinase activity [6–8]. Also Src unrelated protein tyrosine kinases however are implicated in signal transduction generated from both BCR and TCR. The recently characterized p72^{syk} kinase [9,10] appears to play an especially crucial role in B-cells, although it is also expressed in many other tissues [11–17]. Unlike the Src kinases, p72^{syk} bears two SH2 and no SH3 domains and it lacks both the C-terminal down-regulation site and the N-terminal myristoylation site. Consequently p72^{syk} is predominant in the cytosol, whereas the Src kinases are membrane bound. This may also imply a different mechanism of targeting, which in the case of p72^{syk} could be less dependent on compartimentation and more on the recognition of specificity determinants. In order to check this possibility, we have analysed the kinetic constants of p72^{syk} with a number of peptide substrates derived from naturally occurring phospho-acceptor sites potentially susceptible to p72^{syk} mediated phosphorylation. The results, presented in this report, unambiguously show that p72^{syk} unlike the Src-related kinases Lyn and c-Fgr and CSK is endowed with a very marked site specificity mostly determined by acidic residues both upstream and downstream from tyrosine where they also generate the motif preferentially recognized by the SH2 domains of Src kinases.

2. Materials and methods

The peptide EDNEYTA and its derivatives were synthesized as previously described [18]. The phospho-peptides and their unphosphorylated analogues were obtained as in [19]. The other peptides were prepared by an automated synthesizer from Applied Biosystems (Model 431-A) using Fmoc/N-methyl-pirrolidone chemistry on 4-hydroxymethyl-phenoxymethyl- copolystirene-1% divinylbenzene-resin.

Anti-Syk (13–28) antibody was kindly provided by Dr. K. Sada (Matsuoka, Japan). This antibody was raised in rabbit employing a peptide corresponding to a N-terminal sequence (13–28) of p72^{syk} and was affinity purified using the peptide-BSA coupled column.

p72^{syk} was purified from rat spleen by a four-steps procedure including phospho-cellulose, ammonium sulfate precipitation, heparin-sepharose and Mono-Q/FPLC as in [10]. The last step (MonoQ/FPLC in the presence of 0.2 mM EDTA [10]) completely resolved genuine 72 kDa Mr, p72^{syk}, eluted with 0.8 M NaCl and immunoreacting with anti-Syk (13–28) antibodies from contaminant protein tyrosine kinase TPK-IIB [18], eluted with 0.1 M NaCl, which is not recognized by the anti-Syk (13–28) antibodies.

Lyn, c-Fgr and CSK were purified as previously described [20–22]. Peptide phosphorylation assay was performed in a final volume of 50 μl containing 50 mM Tris-HCl, pH 7.5, 5 mM $MnCl_2$, 100 μM sodium vanadate, 20 μM [γ-^{32}P]ATP (specific radioactivity 2,000 cpm/pmol), 20 ng of p72^{syk} and peptide sustrate. Reactions were terminated after 10 min incubation by addition of 1 ml of 1 N HCl and labeled phosphopeptides were quantitated as previously described [23]. Briefly: samples were heated for 15 min at 100°C in order to convert [γ-^{32}P]ATP into $^{32}P_i$, which was removed by conversion into phosphomolybdic complex and extractions with isobutanol-toluene. The radioactivity, present in the aqueous phase, due to phosphopeptides, was then measured in a scintillation counter.

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SSDI 0014-5793(95)00555-2

K_m and V_{max} values were determined by double-reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis–Menten equation.

With all peptides the only radiolabeled phosphoaminoacid was Tyr-P; no radioactive Thr-P or Ser-P could be detected.

3. Results and discussion

The peptides listed in Table 1 reproduce a number of naturally occurring tyrosyl phospho-acceptor sites present in proteins that are implicated in signal transduction. Their phosphorylation efficiency by p72^{syk} has been estimated by determining the kinetic constants, that are also shown in Table 1. Two outcomes are noteworthy: firstly the phosphorylation efficiencies are dramatically variable especially due to the K_m values that diversify in a range of three orders of magnitude; secondly two peptides exhibiting strikingly low K_m values share the presence of multiple acidic residues on both sides of tyrosine. The sequence of these peptides, derived from HS1 and Vav, respectively, is also suited for binding Src SH2 domains (once the tyrosine is phosphorylated) by virtue of the motif TyrP-acidic-acidic-Val/Leu [24]. In contrast the poorest substrates of p72^{syk} are characterized by the absence of acidic residues adjacent to either the N- or C-terminal side of tyrosine and by the presence of basic residues nearby as exemplified by the p72^{syk} and the cdc2 peptides. This observation is noteworthy in two respects: on one side it argues against efficient autophosphorylation of p72^{syk} , in agreement with the finding that the putative 'autophosphorylation' site of p72^{syk}-related ZAP kinase is rather phosphorylated by heterologous kinases [25]; on the other side it discloses a sharp difference between p72^{syk} and Src-related kinases which very efficiently phosphorylate the cdc2 derived peptide [26]. The stringent requirement of acidic residues by p72^{syk} is corroborated by the finding that the tyrosyl residue preferentially phosphorylated by it in the Src C-terminal peptide is not the physiological one (i.e. Tyr-14, equivalent to Tyr-527 of c-Src, responsible for the down-regulation), which lacks acidic residues on both sides, but the one upstream (Tyr-6), just C-terminal to two acidic residues, as outlined by comparing the phosphorylation rates of peptides 8 and 9 in Table 1.

In order to check whether and to what extent acidic residues flanking tyrosine act as positive determinants for p72^{syk} mediated phosphorylation a number of derivatives of the HS1 (393–402) peptide in which acidic residues were individually replaced by neutral ones have been prepared and assayed for their phosphorylation efficiency. As shown in Table 2A the replacement of the two glutamic acids at positions +1 and +2 relative to tyrosine by their amides, glutamine and asparagine, respectively (or, even more, by alanines), is deleterious, causing a dramatic rise of K_m. The individual substitution of aspartic acid at position −1 is equally detrimental inducing a 24-fold increase of K_m. In contrast Glu −3 appears to be less important, its substitution just doubling the K_m value and the Glu at +5 position is irrelevant, since its substitution with Ala actually slightly increases the phosphorylation efficiency.

On the other hand it should be noted that the replacement of Val +3 with Glu, is not beneficial as one could expect but, rather, detrimental. The motif recognized by p72^{syk} (YEEV) is therefore superimposable to that recognized by the SH2 domains of Src kinases after the tyrosyl residue is phosphorylated. It is tempting to speculate that p72^{syk} and possibly other related protein tyrosine kinases with the same site specificity, might be committed with the recruitment of ligands for kinases of the Src family, by generating the phosphotyrosyl residues that specifically bind to Src SH2 domains.

The positive role of acidic residues upstream from tyrosine was confirmed using derivatives of the Src (412–418) peptide EDNEYTA, in which there are no acidic residues on the C-terminal side and which consequently is a substrate not as good as the HS1 (388–402) peptide (see Table 1). As shown in Table 2B the individual replacements of each of the 3 acidic residues on the peptide EDNEYTA are detrimental, to quite different extents, however. The most detrimental replacement, as in the case of the HS1 peptide is the one affecting position −1. Also position −3 is quite important in the Src peptide, which was not the case of the HS1 peptide, and even the Ala for Glu(−4) substitution substantially decreases the phosphorylation efficiency. This may suggest that whenever the acidic determinants adjacent to the C-terminal side of tyrosine are lacking, the importance of additional N-terminal acidic residues (besides the crucial one at position −1) increases. In contrast the replacement of Asn at position −2 with Ala is almost ineffective. Interestingly the replacement of this Asn with Glu only slightly improves the phosphorylation efficiency (compare peptide 13 with 8), suggesting that acidic residues at position −2 may be not so important for p72^{syk} recognition as those at positions −3, −1, +1 and +2.

Table 1
Kinetic constants of p72^{syk} for synthetic peptides reproducing naturally occurring phospho-acceptor sites

Sequence	Nomenclature	k_{cat} (min^{-1})	K_m (μM)	$10^{-6} \cdot k_{cat} \cdot K_m^{-1}$ ($M^{-1} \cdot min^{-1}$)
1 EQEDEPEGD**Y**EEVLE	HS1 (388–402)	70.4	4	17.60
2 PEGD**Y**EEVLE	HS1 (393–402)	48.4	12	4.03
3 VEVNEEAEGDEI**Y**EDLM	Vav (165–179)Val[1]	55.4	3	18.50
4 DENY**Y**KA	Syk (515–521)	41.0	750	0.05
5 KVEKIGEGT**Y**GVVYK	cdc2 (6–20)	17.3	1500	0.01
6 EDENL**Y**EGLNLDDCSM**Y**EDI	ARAM Ig-α (177–196)	87.8	25	3.50
7 EDNE**Y**TA	Src (412–418)	72.5	67	1.08
8 AFLED**Y**FTSTEPQ**Y**QPGENL	Src (514–533)	90.6	500	0.18
9 AFLEDFFTSTEPQ**Y**QPGENL	Src (514–533)Phe[6]	9.7	550	0.02

Nomenclature is reminiscent of the amino acid sequence of the parent protein. Substituted positions are numbered as they occur in the parent peptide and are underlined in the sequence. Experimental conditions are detailed in section 2. Values are means of at least three determinations with a standard error less than 16%. HS1, hematopoietic-lineage cell-specific protein; Vav, product of the proto-oncogene *vav*; cdc2, p34^{cdc2} kinase; ARAM, Antigen Recognition Activation Motif.

Table 2
Effects of substitutions in peptide HS1 [388–402] (A) and Src [412–418] (B) on phosphorylation efficiency by p72^{syk}

		k_{cat} (min^{-1})	K_m (μM)	$10^{-6} \cdot k_{cat} \cdot K_m^{-1}$ (M^{-1} · min^{-1})
A				
1	PEGD**Y**EEVLE	48.4	12.0	4.03
2	PEGD**Y**QNVLE	52.1	116.0	0.45
3	PEGD**Y**AAVLE	42.3	250.0	0.17
4	PAGD**Y**EEVLE	41.7	33.3	1.25
5	PEGA**Y**EEVLE	50.0	290.0	0.17
6	PEGD**Y**EEVLA	37.6	8.3	4.50
7	PEGD**Y**EEELA	38.7	36.0	1.07
B				
8	EDNE**Y**TA	72.5	67	1.08
9	ADNE**Y**TA	60.5	220	0.27
10	EANE**Y**TA	61.3	360	0.17
11	EDAE**Y**TA	47.8	70	0.68
12	EDNA**Y**TA	83.7	1100	0.07
13	EDEE**Y**TA	69.5	40	1.74

Substituted residues are underlined in the sequence. Experimental conditions are detailed in section 2. Values are means of at least three determinations with a standard error less than 14%.

The excellent phosphorylation of the Vav peptide, however , where positions +1, +2 and −2 (but not −1) are occupied by acidic residues supports the view that under certain circumstances an acidic residue at position −2 can surrogate the one at position −1. In any case the requirement of acidic residues on both sides of tyrosine in order to optimize the phosphorylation by p72^{syk} is outlined by comparing the HS1 and Vav derived peptides with the ARAM and Src peptides which lack either the N-terminal or the C-terminal acidic determinants, respectively (see Table 1).

The outstanding phosphorylation efficiency of the HS1 and Vav peptides makes them potential candidates for the sensitive and specific monitoring of p72^{syk}. As shown in Table 3, where the phosphorylation of a number of peptides by p72^{syk}, two Src related kinases (c-Fgr and Lyn) and CSK are compared, the HS1 peptide is much better suited than the Vav one for the specific assay of p72^{syk}, since it is very poorly phosphorylated by both the Src kinases and CSK, whereas the Vav peptide is phosphorylated by either c-Fgr or Lyn almost as efficiently as the cdc2 peptide, which is considered a first-choice substrate for Src family kinases [26]. It is likely that this difference is at least partially due to the nature of the residue occupying the −1 position, acidic in the case of the HS1 peptide, hydrophobic in the Vav peptide. While we have shown here that an acidic residue in that position is a strong positive determinant with p72^{syk}, a hydrophobic residue appears to be preferred by the Src kinases. This view is also confirmed by comparing two C-terminal Src peptide derivatives whose phosphorylation is varied in opposite directions by replacing the glutamic acid adjacent to the N-terminal side of tyrosine with valine: p72^{syk} dependent phosphorylation is decreased, while c-Fgr and Lyn dependent phosphorylation is increased. This conclusion is in agreement with recent data obtained using a degenerated peptide library, showing that Ile, Val and Leu, in this order, are the residues preferred at position −1 by v-Src and Lck [27]. The markedly acidophilic nature of p72^{syk} and in particular its preference for an acidic residue at position −1 is reminiscent of a lower Mr tyrosine kinase previously isolated from spleen and conventionally termed TPK-IIB [18]. In order to check further the analogies between p72^{syk} and TPK-IIB advantage has been taken of the unique property of TPK-IIB to recognize phosphorylated residues at position −1 as specificity determinants as good as, or even better than, glutamic acid [19]. The same series of phosphorylated peptides used in that study, as well as their dephosphorylated derivatives were tested as substrates for p72^{syk} and the results, shown in Table 4 clearly show that p72^{syk} also perceives phospho-threonine, phospho-tyrosine and phospho-serine, in this order, as powerful specificity determinants. These data, besides corroborating the concept that TPK-IIB, despite its much lower M_r, is cognate to p72^{syk}, also

Table 3
Kinetic constants of four different PTKs for a selection of synthetic peptides

		p72^{syk}			**Fgr**		
		k_{cat} (min^{-1})	K_m (μM)	$10^{-6} \cdot k_{cat} \cdot K_m^{-1}$ (M^{-1} · min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$10^{-6} \cdot k_{cat} \cdot K_m^{-1}$ (M^{-1} · min^{-1})
1	EQEDEPEGD**Y**EEVLE	70.4	4	17.60	n.m.	n.m.	n.m.
3	VEVNEEAEGDEI**Y**EDLM	55.4	3	18.50	53.0	100	0.530
5	KVEKIGEGT**Y**GVVYK	17.3	1500	0.01	26.5	80	0.331
6	EDENL**Y**EGLNLDDCSM**Y**EDI	87.8	25	3.50	25.0	33	0.757
9′	AFLEDSFTGTEPL**Y**QPGENL	51.7	760	0.07	17.2	1130	0.015
9″	AFLEDSFTGTEPE**Y**QPGENL	60.4	200	0.30	3.0	640	0.005
		Lyn			**CSK**		
		k_{cat} (min^{-1})	K_m (μM)	$10^{-6} \cdot k_{cat} \cdot K_m^{-1}$ (M^{-1} · min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$10^{-6} \cdot k_{cat} \cdot K_m^{-1}$ (M^{-1} · min^{-1})
1	EQEDEPEGD**Y**EEVLE	10.2	5000	0.002	9.0	625	0.014
3	VEVNEEAEGDEI**Y**EDLM	23.0	152	0.152	13.5	308	0.044
5	KVEKIGEGT**Y**GVVYK	34.7	1250	0.028	11.8	4160	0.003
6	EDENL**Y**EGLNLDDCSM**Y**EDI	22.6	98	0.230	12.2	211	0.058
9′	AFLEDSFTGTEPL**Y**QPGENL	17.4	2000	0.009	n.d.	n.d.	n.d.
9″	AFLEDSFTGTEPE**Y**QPGENL	8.3	2630	0.003	n.d.	n.d.	n.d.

Peptides are numbered as in Table 1 except for peptides 9′ and 9″ which are derivatives of peptide 9 of Table 1 (Src[514–533]Phe^6) where the substitutions denoted by underlining were made. Values are means of at least three determinations with a standard error less than 18%. n.m., not measurable due to too low phosphorylation rate; n.d., not determined.

Table 4
Phosphorylated residues as specificity determinants for p72^{syk}

Peptide	Phosphorylation rate (pmol/min)
NEYTA	6.6
NTYTA	1.0
NTpYTA	15.2
NYYTA	2.1
NYpYTA	10.7
NSYTA	0.6
NSpYTA	5.4
GEGTYGV	0.7
GEGTpYGV	11.7

Peptide concentration was 0.4 mM. The peptides NEYTA and GEGTYGV reproduce the main autophosphorylation site of pp60$^{c\text{-}src}$ (Y416) and the down-regulatory site of p34^{cdc2} (Y15), respectively. Sp, Tp, Yp denote Ser-P, Thr-P and Tyr-P, respectively. Values are means of at least three determinations with a standard error less than 13%.

disclose the possibility that p72^{syk} phospho-acceptor sites could be generated or potentiated by the previous phosphorylation of residues nearby by either Ser/Thr or Tyr specific protein kinases.

4. Conclusions

The main outcome of this paper is that p72^{syk} is a very acidophilic protein tyrosine kinase that preferentially phosphorylates a consensus sequence (acidic)$_n$-Tyr-acidic-acidic-hydrophobic which is including the motif recognized by the Src family SH2 domains (TyrP-acidic-acidic-hydrophobic) and which is not susceptible to efficient phosphorylation by Src related kinases by virtue of an acidic residue at position −1, where a hydrophobic residue is conversely preferred by Src kinases. The overlapping between p72^{syk} and Src SH2 consensus sequences disclose the possibility that p72^{syk} might be implicated in the recruitment of ligands for the Src kinases, possibly giving rise to a hierarchical mechanism where p72^{syk} mediated phosphorylation is a pre-requisite for subsequent phosphorylation by Src kinases. On the other hand the finding that phosphorylated residues can effectively replace carboxylic amino acids as specificity determinants for p72^{syk} outlines the possible occurrence of an inverted cascade, where new sites for p72^{syk} are either created or potentiated by the previous phosphorylation of the target by either Ser/Thr or Tyr specific kinases. Such an intricated network of reciprocal influences would be well consistent with the crucial yet still elusive and in some respects contradictory involvement of p72^{syk} in the complex machinery of BCR signaling [11,13]. Since both kinds of hierarchical implications of p72^{syk} have been also described of a spleen PTK conventionally termed TPK-IIB, distinguishable from p72^{syk} for both its lower Mr and different chromatographic behaviour (see section 2 and [18]), the relatedness of TPK-IIB to p72^{syk} should be carefully inspected.

Acknowledgements: This work was supported by AIRC, Italian Ministry of Health, (Project AIDS), Italian MURST and CNR (Target Project on ACRO) The skilful technical assistance of Mr G. Tasinato is gratefully acknowledged.

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