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Specific monitoring of Syk protein kinase activity by peptide substrates including constrained analogs of tyrosine

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Abstract The ability of Syk protein tyrosine kinase (PTK) to phosphorylate peptides, where tyrosine had been replaced by conformationally constrained analogs, has been exploited to develop highly selective substrates suitable for the specific monitoring of Syk activity. In particular we have synthesized a peptidomimetic, RRRAAEDDE(L-Htc)EEV (syktide), with the 3(*S*)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid residue (L-Htc) replaced for tyrosine, which is phosphorylated by Syk with remarkable efficiency (K_{cat} = 73 min^{-1}, K_m = 11 μM), while it is not affected to any appreciable extent by a number of PTKs tested so far. These properties make syktide the first choice substrate for the specific monitoring of Syk.

Key words: Protein kinase; Syk tyrosine kinase; Peptide; Tyrosine analog; Peptide phosphorylation

1. Introduction

Protein phosphorylation is the most general and frequent mechanism controlling nearly all aspects of cell life. About one third of mammalian proteins contain covalently bound phosphate and the phosphorylating enzymes, protein kinases, encoded by the human genome, are more than 500 [1]. It is not surprising therefore that protein kinases play a key role in nearly all signal transduction pathways and that altered functions of individual protein kinases underlie numerous pathological conditions with special reference to neoplastic growth [2]. Nowadays, a large proportion of potential targets for pharmacological treatment are indeed protein kinases. Given these premises, the development of reagents suitable for the specific monitoring and inhibition of individual protein kinases is a crucial goal in the field of signal transduction and related pathologies.

Ser/Thr specific protein kinases often display a stringent site specificity dictated by a number of local determinants [3] which can be exploited to generate highly discriminatory peptide substrates, readily phosphorylated by just one or a few related enzymes, while being almost unaffected by most of the others [4]. In contrast, the specificity of tyrosine kinases is not strictly dependent on the sequence of the phosphoacceptor site [3]. This feature, which may well reflect the tendency of protein tyrosine kinases (PTKs) to recognize their targets through adhesion modules located outside the catalytic site, has hampered the design of highly selective peptide substrates based on sequence distinctiveness, like those successfully used for the specific monitoring of many Ser/Thr protein kinases. Such a situation is exemplified by the widespread use of two 'universal' substrates, random polymers of glutamic acid and tyrosine (notably polyE_4Y) and angiotensin II (DRVYIHPF), respectively, to assay nearly every kind of PTK [4]. Especially telling is, in this connection, the observation that these two quite unrelated substrates are readily phosphorylated by PTKs belonging either to the Src family, whose optimal recognition sequence includes a hydrophobic residue at position $n-1$ [5] (present in angiotensin but not in polyE_4Y) or to the Syk family, which instead displays a marked preference for acidic residues [6,7], an obvious feature of polyE_4Y but not of angiotensin.

An alternative strategy for rendering the selectivity of peptide substrates of PTKs more stringent than that afforded by sequence specificity could take advantage of the observation that the replacement of tyrosine by artificial phosphorylatable analogs with conformational constraints is variably tolerated by the different classes of tyrosine kinases [8–12]. Here we show the usefulness of this approach for the development of peptide substrates which can be readily phosphorylated by Syk, while being practically unaffected by Src family enzymes as well as by other tyrosine protein kinases including those present in crude spleen extracts.

2. Materials and methods

2.1. Materials

[γ^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Protease inhibitor cocktail and PP2 inhibitor were obtained from Boehringer and Calbiochem, respectively. Modified angiotensin II (DRVYIHPFR) was kindly provided by Dr. Oriano Marin (University of Padua, Italy). Anti-Syk, anti-Lyn, anti-c-Fgr and anti-Csk were from Santa Cruz Biotechnology.

2.2. Peptides

Peptides were synthesized by solid phase using Fmoc chemistry in 0.25 mM scale on an ABI 431A peptide synthesizer according to the protocol provided by ABI [13] using a Wang resin [14]. Fmoc-[Hba-Gly]-OH was synthesized starting from phthaloyl-Tyr(Bzl)-Gly-OH [15] adapting the method described by Flynn and de Laszlo for the

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Abbreviations: PTK, protein tyrosine kinase; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate

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corresponding Phe analog. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole activation employed a three-fold molar excess (1 mM) of Fmoc-amino acids in *N*-methyl pyrrolidone–dimethylformamide (1:1) solution for each coupling cycle. Deprotection was performed with 20% piperidine. In the coupling to the secondary amino groups of L- or D-Htc, the activation of the α-carboxylic moiety of glutamic acid derivative was performed using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate instead of HBTU [16]. Cleavage from the resin and deprotection were performed by treatment with trifluoroacetic acid–anisole–triisopropylsilane–H_2O (82:8.5:8.0:1.5 v/v). Peptides were purified by preparative reversed-phase HPLC and characterized by analytical HPLC, amino acid analysis of the acidic hydrolysates, and matrix assisted laser desorption ionization–mass spectrometry.

2.3. PTKs

Lyn, c-Fgr, Syk and Csk were purified from rat spleen to near homogeneity as previously described [17–20]. Recombinant ZAP70 was purchased from Upstate (Lake Placid, NY, USA). They were routinely assayed on polyGlu$_4$Tyr [17]. One unit was defined as the amount of enzyme transferring 1 pmol phosphate per minute to 0.1 mg/ml polyGlu$_4$Tyr under standard conditions.

2.4. Resolution of PTKs by heparin-Sepharose chromatography

Crude extract from rat spleen particulate fraction was subjected to heparin-Sepharose column chromatography [17]. Tyrosine kinase activity was monitored by assaying 10 μl of the fractions on either polyGlu$_4$Tyr [17] or syktide (see Section 2.5). 30 μl of fractions was subjected to SDS–PAGE. Proteins were transferred to nitrocellulose membranes, incubated with the indicated antibody followed by the appropriate biotinylated second antibody and developed using an enhanced chemiluminescent detection system.

2.5. Peptide phosphorylation

Peptides were phosphorylated in 40 μl of a medium containing 50 mM Tris–HCl, pH 7.5, 5 mM $MnCl_2$ (5 mM $MgCl_2$ in the case of c-Fgr), 30 μM [γ^{32}P]ATP (specific activity 1000 cpm/pmol) and 10 U enzyme. The reactions were terminated after 10 min by addition of 1 ml HCl and processed as described elsewhere [6]. The phosphorylation of EDDE(L-Htc)EEV was also assessed by reversed-phase HPLC. The purified phosphoderivative was analyzed by ESI–MS, giving the expected MW of 1157.32 ($M+K^+$). In the case of angiotensin-R and syktide, which contain three positively charged residues, the phosphocellulose paper procedure to quantify ^{32}P incorporation into peptides was applied [21]. K_m and K_{cat} values were calculated from double-reciprocal plots constructed from initial rate measurements fitted linearly to the Lineweaver–Burk representation of the Michaelis–Menten equation.

2.6. Tyrosine kinase autophosphorylation

Autophosphorylation was performed as in Section 2.5, omitting the peptide substrate. Samples were then subjected to SDS–PAGE and ^{32}P incorporated was evaluated by a Packard imager.

3. Results

The structures of the constrained tyrosine analogs, 3(*S*)- or 3(*R*)-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid (either the L or D isomer) and 8-hydroxy-4(*S*)-amino-1,3,4,5-tetrahydro-3-oxo-2*H*-benzazepine-2-acetic acid, henceforth termed Htc and Hba-Gly, respectively, are shown in Fig. 1. These analogs were replaced for tyrosine in peptides already known to undergo phosphorylation by non-receptor PTKs Syk, Lyn, c-Fgr and Csk.

The effects of these substitutions in the very acidic peptide EDDEYEEV, an excellent substrate of Syk [6], are shown in Table 1. The phosphorylation of the L-Htc peptide, tested at 25 μM concentration, is almost identical to that of the parent peptide, while the D-Htc peptide is almost unaffected by Syk. The formation of a stable phosphoderivative of the L-Htc residue was confirmed by ESI–MS analysis of the phosphopeptide as specified in Section 2. The Hba-Gly substitution is quite well tolerated, albeit slightly less than the L-Htc one. In contrast, the L-Htc substitution suppresses the ability of the peptide EDNEYTA to undergo phosphorylation by the Src family kinases Lyn and c-Fgr (Table 1) and by Csk (not shown).

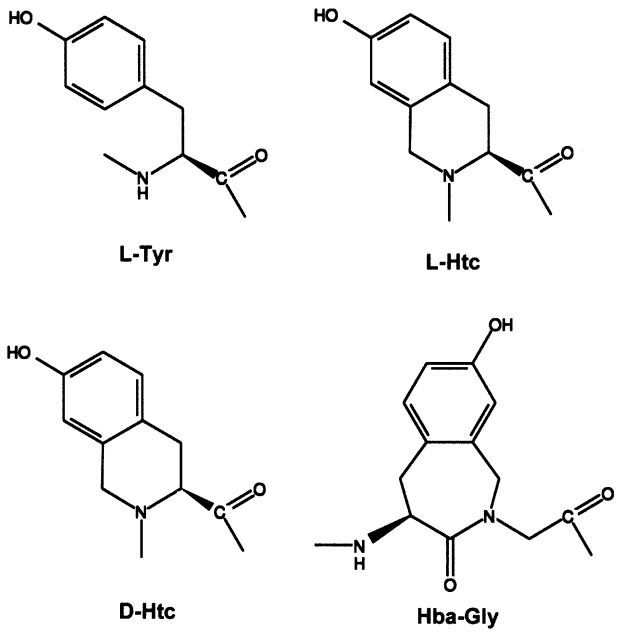

Fig. 1. Structure of tyrosine analogs.

To check whether the failure of Src family kinases to phosphorylate L-Htc peptides was due to their inability to bind the peptides or to catalyze the phosphate transfer to the Htc hydroxyl group, competition experiments were performed, where the Htc derivative of a tyrosyl peptide phosphorylatable by the different tyrosine kinases was tested as a potential inhibitor of the kinase reaction. As shown in Fig. 2A the phosphorylation of DRVYIHPF-R (angiotensin II containing an additional Arg residue at the C-terminus), a promiscuous peptide substrate for many tyrosine kinases, is inhibited by the L-Htc peptide in a dose dependent manner if Syk is the catalyst, while it is unaffected whenever the phosphorylating agents are Lyn, c-Fgr or Csk. The same is observed if the tyrosine kinase activities are monitored in terms of autophos-

Table 1
Phosphorylation rates of peptides containing either Tyr or its analogs by different tyrosine kinases

Protein kinase	Peptide substrate	Peptide phosphorylation (pmol ^{32}P incorporated/min)
Syk	EDDEYEEV	40
Syk	EDDE(L-Htc)EEV	44
Syk	EDDE(D-Htc)EEV	4
Syk	EDDE(Hba-Gly)EEV	33
Lyn	EDNEYTA	37
Lyn	EDNE(L-Htc))TA	<1
c-Fgr	EDNEYTA	32
c-Fgr	EDNE(L-Htc))TA	<1

Peptide and enzyme concentrations were 25 μM and 10 U, respectively. Data are the means of five separate experiments. S.E.M. values were always less than 18%.

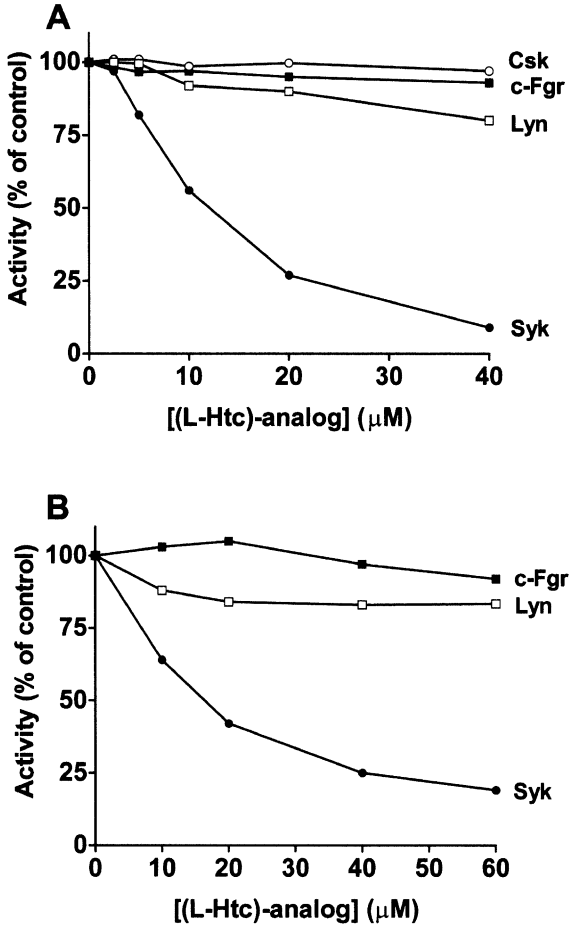

Fig. 2. Effect of L-Htc analog on either angiotensin phosphorylation (A) or autophosphorylation (B) activities of different tyrosine kinases. EDDE(L-Htc)EEV and EDNE(L-Htc)TA were used as inhibitors of either Syk or Lyn, c-Fgr and Csk, respectively. Enzyme activity on angiotensin-R was quantified by phosphocellulose papers, which cannot bind L-Htc derivatives. Data are the means of four separate experiments. S.E.M. values were always less than 16%.

phorylation (Fig. 2B). It can be concluded therefore that the replacement of Tyr by L-Htc abrogates the ability of the peptide to bind to the active site of the Src family kinases. Consequently, L-Htc peptides could also be exploited to probe the implication of Syk instead of Src family kinases in the phosphorylation of a given protein substrate whose phosphorylation will be inhibited only if Syk is the phosphorylating agent. On the other hand, the D-Htc derivative, which is not phosphorylated by Syk, is also unable to interfere with Syk mediated phosphorylation of angiotensin-R (not shown), indicating that the D conformation, unlike the L one, cannot be accommodated into the active site of Syk.

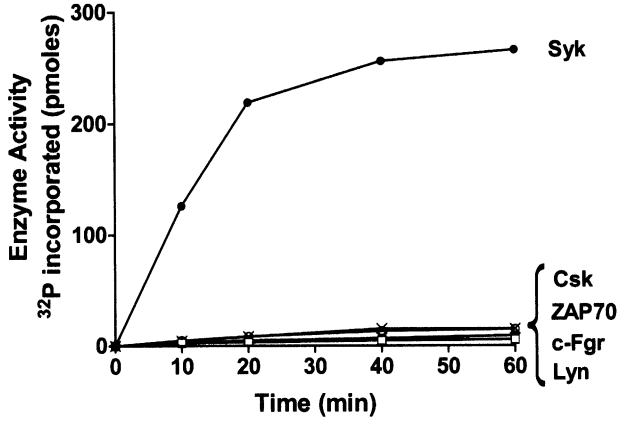

Fig. 3. Time courses of R_3A_2EDDE(L-Htc)EEV (syktide) phosphorylation by different tyrosine kinases. Syk (●), Csk (○), ZAP70 (×), c-Fgr (■) and Lyn (□). Peptide and enzyme concentrations were 25 μM and 10 U, respectively. Data are the means of four separate experiments except in the case of ZAP70, whose time course is the mean of two experiments.

The kinetic constants reported in Table 2 show that the efficient phosphorylation of EDDE(L-Htc)EEV by Syk is accounted for by both K_{cat}, which is even higher than that of the tyrosyl peptide, and K_m, whose value, albeit somewhat higher than that of tyrosyl peptide, remains nevertheless in the low μM range. Table 2 also shows that Tyr substitution by L-Htc is generally tolerated by Syk irrespective of the amino acid sequence surrounding the phosphorylatable residue, although some variability can be observed, mostly accounted for by differences in the increase of K_m values.

The data of Table 2 show that EDDE(L-Htc)EEV is by far the best phosphoacceptor substrate among the L-Htc peptides tested. We decided therefore to improve its assay by making applicable the fast and handy phosphocellulose paper procedure, which requires the presence of at least two positively charged side chains in the phosphorylated peptide. To this purpose an RRRAA tag was added to the N-terminal side of EDDE(L-Htc)EEV, giving the peptide RRRAAEDDE-(L-Htc)EEV, henceforth termed syktide, which is phosphorylated by Syk with an efficiency similar to that of the parent L-Htc peptide (see Table 2). As shown in Fig. 3, the phosphorylation of syktide by tyrosine kinases other than Syk, notably Lyn, c-Fgr, and Csk, is hardly detectable. Quite interestingly, syktide also discriminates between Syk and the related kinase ZAP70, which phosphorylates the syktide to a negligible extent as compared to Syk.

Table 2
Kinetic constants for Syk tyrosine kinase of peptides containing either Tyr or L-Htc

Peptide		K_{cat} (min^{-1})	K_m (μM)	Efficiency (K_{cat}/K_m)
1	EDNEYTA	72.5	58	1.25
2	EDNE(L-Htc)TA	61.3	185	0.33
3	EDNEYTAEDNEYTA	60.3	27	2.23
4	EDNE(L-Htc)TAEDNE(L-Htc)TA	65.2	60	1.08
5	c(EDNEYTAEDNEYTA)	46.0	6	7.66
6	c(EDNE(L-Htc)TAEDNE(L-Htc)TA)	54.9	31	1.77
7	EDDEYEEV	62.2	3	20.73
8	EDDE(L-Htc)EEV	70.3	7	10.04
9	R_3A_2EDDE(L-Htc)EEV	73.1	11	6.64

Synthesis and structure of cyclic peptides 5 and 6 are described elsewhere [27].

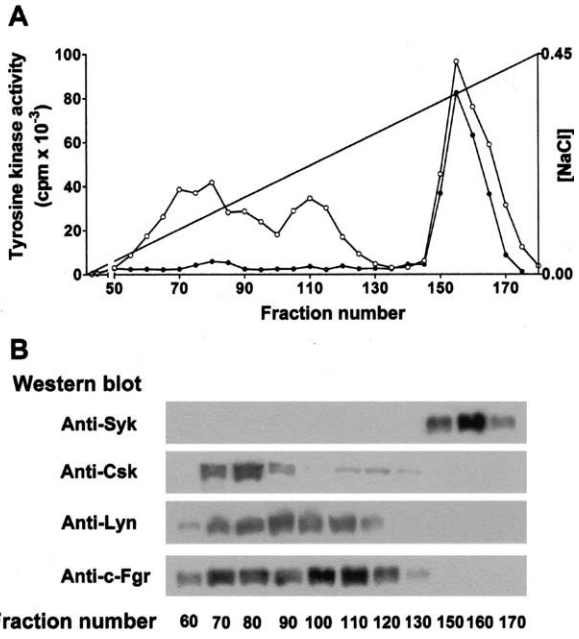

Fig. 4. Resolution of rat spleen PTKs by heparin-Sepharose column chromatography. A: Elution profile of tyrosine kinase activities monitored using either polyGlu$_4$Tyr (open circles) and syktide (solid circles) as phosphorylatable substrates. B: Immunostaining of the indicated fractions with antibodies raised against different tyrosine kinases.

To further assess the selectivity of the L-Htc peptide for just Syk among a variety of other kinases, the tyrosine kinase activities present in a crude spleen preparation were resolved by heparin-Sepharose column chromatography and monitored using either the non-specific substrate polyE$_4$Y or the newly developed syktide. As shown in Fig. 4, the polyE$_4$Y profile is highly heterogeneous, giving rise to several peaks accounted for by a variety of PTKs, which are especially abundant in spleen. In contrast, only one sharp peak eluted by 0.37 M NaCl was detected using the novel peptide substrate and its identification with Syk was corroborated by Western blot analysis with anti-Syk antibody. No reaction with anti-Syk antibody could be detected in the fractions eluted elsewhere, in which Lyn, c-Fgr, Fyn and Csk, still partially overlapping each other, were detectable by the specific antibodies. Additional validation of the syktide as a valuable tool for discriminating between Syk and kinases of the Src family was provided by showing that, while more than 60% of a spleen extract overall PTK activity assayed on angiotensin-R is abrogated by PP2, a specific inhibitor of Src kinases, the activity revealed by syktide was fully unaffected by PP2 treatment (not shown).

4. Discussion

The data presented show that, at variance with all the other PTKs tested, Syk tolerates quite well the replacement of tyrosyl residues by the conformationally constrained analogs Htc and Hba-Gly, whose phosphorylation efficiency remains similar to that of tyrosine. This behavior likely reflects unique features of the phosphoacceptor substrate binding site of Syk as compared to that of the other PTKs, since the constrained derivatives also fail to compete against the phosphorylation of tyrosyl peptides by tyrosine kinases unable to phosphorylate them, notably those belonging to the Src family. While the structural bases of such an unusual substrate specificity of Syk remain a matter of conjecture, advantage has been taken of this property to generate an optimal peptide substrate in terms of both phosphorylation efficiency and selectivity. This goal has been achieved by including the L-Htc phosphorylatable residue into an acidic context especially suited for Syk and by adding to its N-terminus a basic tag which makes applicable the quick and handy phosphocellulose paper procedure for the evaluation of incorporated radiolabeled phosphate. The resulting peptide, RRRAAEDDE(L-Htc)EEV, for which we have proposed the name 'syktide', is the first choice substrate available to date for the specific monitoring of Syk as judged both from its failure to undergo phosphorylation by a number of other PTKs and its ability to detect Syk alone amid a plethora of PTKs present in crude spleen extracts. Further investigation is required in order to assess how absolute the specificity of syktide for Syk is. It should be highlighted however that, somewhat surprisingly, syktide is only very poorly phosphorylated by the Syk-related kinase ZAP70. This finding suggests that, despite their relatedness, Syk and ZAP70 differentiate significantly in site specificity. This also provides a biochemical tool for readily distinguishing between Syk and ZAP70 activities in crude extracts. The ability of the syktide to reveal the activity of Syk among those of the other non-receptor PTKs, in particular those of the Src family, makes it a valuable tool for studies aimed at the identification of kinases implicated in signal transduction. It should be borne in mind in this connection that Syk, besides playing an essential role in hematopoietic cells and in particular in immunoreceptor signalling [22,23], has recently been shown to display anti-neoplastic [24,25] and anti-neurodegenerative potentials [26]. Therefore the availability of reagents, like the syktide, able to discriminate Syk activity from those of other kinases will prove helpful for the unraveling of the molecular mechanisms underlying such biological effects.

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