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# Phosphorylation of small peptides by spleen TPK-IIA, a tyrosine protein kinase stimulated by polylysine and by high ionic strength

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The specificity of TPK-IIA, a spleen tyrosine protein kinase whose activity is dramatically enhanced by either polylysine or very high ionic strength, has been inspected with the aid of a variety of very short peptide substrates. Unlike free tyrosine, which is not phosphorylated at all, and the dipeptides YA and AY, whose phosphorylation is hardly detectable, the tripeptides AYA, YYY and YYA, tetrapeptide AYAA and pentapeptide AAYAA are readily phosphorylated, the $V_{max}$ of AYA being 7- and 15-fold lower vs those of AYAA and AAYAA, respectively. In comparison to AYA, the tripeptides YAA, EYA, GYA and VYV are poorer substrates, while EYH is unaffected by TPK-IIA. The rate of phosphorylation of the pentapeptide EYAA is negligible as compared to AYAA; the hexapeptide EEYAA, however, is a substrate quite comparable to AAYAA, exhibiting a somewhat lower $V_{max}$ but also a 4-fold lower $K_m$ (3.9 vs 18.6 mM). Taken together, the data would indicate that although TPK-IIA does not exhibit any absolute requirement for a definite consensus sequence it is nevertheless endowed with peptide substrate specificity, mainly resulting from negative determinants that can be variably overcome by additional features of the sequence.

Protein-tyrosine kinase; Tyrosine phosphorylation; Peptide phosphorylation; (Spleen)

## 1. INTRODUCTION

The structural features determining the substrate specificity of tyrosine protein kinases (TPKs) are not as clearly understood as those of Ser/Thr protein kinases. In many instances, these latter have been shown to require definite consensus sequences, generally consisting of either basic or acidic residues at critical positions around the target amino acid (review [1]). The absolute requirement for such local structural determinants in the case of TPKs is not so evident. In particular, the putative role of acidic residues located upstream in determining the phosphorylation of tyrosine, is disputed. While such residues are found in most autophosphorylation sites of known TPKs [2], phosphorylation of tyrosyl residues lacking such a feature has also been reported (e.g. [3–5]). This latter finding as well as the capability of some TPKs to phosphorylate, albeit slowly, very small peptides and even tyrosine itself [6,7], gives one the impression that these enzymes are more promiscuous than Ser/Thr protein kinases. Consequently, it has been suggested that phosphorylation of neutral tetrapeptides by TPK from LSTRA cells could be favored by their $\beta$-turn conformation [5] rather than a definite amino acid sequence. On the other hand, using a set of peptides derived from the EGF-receptor sequence around Tyr-845 which is homologous to the $pp60^{v\text{-}src}$ autophosphorylation site, we have recently shown that 3 individual TPKs exhibit distinct specificities for being variably responsive to negative structural determinants [8].

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In order to address systematically the problem of site specificity and minimum structural requirements of TPKs we have employed here a new series of related di- to octa-peptides containing tyrosyl residues flanked by a variety of amino acids in different combinations. As phosphorylating enzyme we have chosen spleen TPK-IIA [9], whose activity is dramatically enhanced by polylysine and by very high ionic strength (up to 2 M NaCl). This peculiar property supports the relatedness of TPK-IIA with p40 [10], a tyrosine protein kinase proteolytically generated from higher molecular mass form(s), which is especially abundant in spleen, thymus and lung [11]. Our results are described in the present paper.

## 2. MATERIALS AND METHODS

Tyrosine protein kinase IIA (TPK-IIA) was extracted from the particulate fraction of bovine spleen and partially purified by a three-step procedure described previously [9]; an additional purification step consisting of Sephacryl S200 gel filtration was interposed between heparin sepharose and polylysine agarose. The final preparation exhibited a specific activity of 10500 Units/mg, one unit being defined as the amount of enzyme transferring 1 pmol phosphate/min to angiotensin II (2 mM) under the conditions detailed below. It was free of Ser/Thr protein kinase activity and behaved homogeneously upon a variety of chromatographic procedures [9].

The peptides AYA, AY, GYA, GAY, GGYR, YYY, VYV and angiotensin II were purchased from Sigma. All other tyrosyl peptides were synthesized by the traditional method in solution utilizing the standard procedure for peptide synthesis [12]. The final peptides were obtained as C-terminal ethyl ester derivatives and, when heterogeneous, were purified by chromatographic procedures, then converted to the hydrochloride salts by lyophilization from 5% HCl.

All peptides were >95% pure as judged by TLC on cellular plates and reversed-phase HPLC on a Viosfer C18 column.

Tyrosine protein kinase activity was routinely assayed using the polymer Glu/Tyr (4:1) as phosphorylatable substrate [9]. Phosphorylation of peptides was performed by incubation at 30°C in 50 $\mu$l of a medium containing 50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 2 M NaCl, 10 $\mu$M Na vanadate, 20 $\mu$M [$\gamma$-$^{32}$P]ATP (spec. act. 1000–1500 cpm/pmol) and TPK-IIA (15–20 U). Unless otherwise indicated the peptide concentration was 10 mM and the incubation time 10 min. The reaction was stopped by adding 0.45 ml of 30% acetic acid and $^{32}$P incorporated into the peptide was evaluated by combining ion-exchange [13] and isobutanol-benzene extraction [14], as detailed in [8]. In order to ascertain whether the radiolabeling of very poor peptide substrates was significant in extent, [$^{32}$P]phosphotyrosine was isolated by partial acid hydrolysis followed by high-voltage paper electrophoresis [9] and its radioactivity evaluated by autoradiography.

## 3. RESULTS

In table 1 the rates of phosphorylation by TPK-IIA for a series of small tyrosyl peptides are compared to that of angiotensin II, widely used as a peptide substrate for tyrosine protein kinases. While free tyrosine is not at all affected, very slow but detectable phosphorylation of the dipeptides AY and, to an even lesser extent, YA takes place. Both dipeptides, however, are very poor substrates compared to the tripeptide AYA, which is readily

Table 1

Phosphorylation rate of synthetic peptides by spleen tyrosine protein kinase IIA

| Peptides[a] | Phosphorylation rate (pmol) |
|---|---|
| Y | n.d. |
| YA | 1.5 |
| AY | 4.5 |
| AYA | 32.5 |
| AYAA | 150.0 |
| AAYAA | 238.4 |
| EAYAA | 208.5 |
| EEYAA | 222.0 |
| EEY | 40.5 |
| YAA | 7.3 |
| EYAA | 4.6 |
| EYA | 9.8 |
| EYH | n.d. |
| GYA | 3.7 |
| VYV | 3.3 |
| YYA | 64.5 |
| YYY | 94.5 |
| KEY | 10.5 |
| GAY | 46.5 |
| GGYR | 9.7 |
| KEYH | 4.1 |
| EYHAE | 1.2 |
| EKEYHAE | 6.5 |
| DRVYIHPF (angiotensin II) | 198.3 |
| Angiotensin II digested with trypsin | 12.0 |

[a] Peptides aligned with respect to their phosphorylatable tyrosine (underlined). Most (see section 2) were C-terminal ethyl ester derivatives

Phosphorylation rates are expressed as pmol $^{32}$P incorporated into the peptides (10 mM) during 10 min incubation with TPK-IIA under the conditions detailed in section 2. n.d., not detectable. The occurrence of YA and AY phosphorylation as opposed to free tyrosine was also assessed by high-voltage paper electrophoresis followed by autoradiography: radioactive spots comigrating with phosphotyrosine could be detected in the acid hydrolysates of the dipeptides but not with free tyrosine. Average values calculated from three or more experiments are shown. The standard error was less than 13%

phosphorylated by TPK-IIA. It is clear from the data of table 1 that the suitability of tripeptides as substrates for TPK-IIA is critically dependent on both the relative position of the tyrosine residue (cf. AYA and YAA) and the nature of the amino acids flanking it. The time courses for phosphorylation of those tripeptides containing tyrosine at the second position and varying combinations of amino acids at the other two positions are shown in fig.1. While AYA is a fairly good substrate, the substitution of either one or both alanines by a variety of different residues, including glycine, glutamic acid, histidine and valine, is detrimental, giving rise to peptides that are poorly phosphorylated or completely unaffected by TPK-IIA. In particular, the unfavorable effects of an N-terminal glutamic acid and of a C-terminal histidine are clearly illustrated by comparing the tripeptides AYA, EYA (which is a poorer substrate than AYA) and EYH, which is not a substrate at all (fig.1). The slow rate of phosphorylation of GGYR vs GAY, and of KEY vs EEY (table 1), also suggests that basic residues are hardly tolerated. Conversely, the inclusion of additional tyrosines in the tripeptide increases the phosphorylation rate (cf. YYY, YYA and AYA). This is probably not due to any preferential phosphorylation of the N-terminal tyrosine, since YAA is a much poorer substrate than AYA (table 1). On the other hand, the C-terminal tyrosine could account for the rapid phosphorylation of YYY, considering that some tripeptides with C-terminal tyrosine, like GAY and EEY, are readily phosphorylated (table 1).

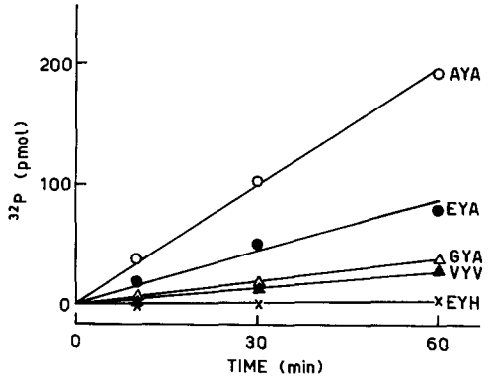


Fig.1. Time courses of tyrosyl tripeptide phosphorylation by TPK-IIA. Experimental conditions as in table 1.

Table 1 also shows that extension of the tripeptide AYA by additional alanines markedly increases the rate of phosphorylation, AYAA being a much better substrate than AYA, while AAYAA is even better than AYAA. Replacement of the N-terminal alanine(s) with glutamic acid(s), which has an adverse effect in the tri- and tetrapeptides, is almost without effect on the phosphorylation rate of the pentapeptide, EEYAA and EAYAA being substrates comparable to AAYAA.

The experiments of table 1 and fig.1 were performed with 10 mM peptides. In order to obtain more information on the factors affecting susceptibility to enzymatic phosphorylation, the kinetic constants of a number of representative peptide substrates were determined (table 2). Extension of the tripeptide AYA by two additional alanines to give AYAA and AAYAA does not affect the $K_m$ but does increase the $V_{max}$ by an order of magnitude. In contrast, the replacement of the N-terminal alanine of AYAA by glutamic acid causes both a dramatic drop in $V_{max}$ and a significant increase in $K_m$. Such a negative effect of glutamic acid adjacent to the N-terminal side of tyrosine, however, is suppressed if a second glutamyl residue is added: EEYAA is in fact an equally effective substrate to the case with AAYAA, its somewhat lower $V_{max}$ being compensated by a 4-fold lower $K_m$. The concept that negative determinants can be overcome by additional local features is also supported by the excellent kinetic parameters of angio-

Table 2

Kinetic constants of TPK-IIA for synthetic peptides

| | $V_{max}$ (pmol · min$^{-1}$) | $K_m$ (mM) |
|---|---|---|
| AYA | 3.0 | 15.0 |
| AYAA | 20.1 | 18.2 |
| AAYAA | 45.1 | 18.6 |
| EYAA | 1.2 | 35.0 |
| EAYAA | 29.4 | 16.0 |
| EEYAA | 20.9 | 3.9 |
| YAA | 0.8 | 33.0 |
| YYA | 12.0 | 12.5 |
| Angiotensin II (DRVYIHPF) | 22.5 | 1.6 |

$K_m$ and $V_{max}$ values were determined via double-reciprocal plots constructed from initial rate measurements fitted to the Michaelis-Menten equation. $V_{max}$ values are expressed as pmol transferred to peptide/min under the conditions described in section 2

tensin II, despite its motif around the tyrosyl residue (VYI) being nearly identical to the very poor substrate VYV. The positive feature in the case of angiotensin II seems to reside in the N-terminal doublet Asp-Arg, whose removal by trypsin seriously impairs subsequent phosphorylation (see table 1).

The negative effects of the residues flanking tyrosine, however, cannot be suppressed in every case by extending the peptide sequence: the motif EYH, for example, is barely susceptible to phosphorylation even when included in the peptide EKEYHAE whose length is comparable to that of AAYAA and angiotensin II, sharing with the latter an N-terminal acidic-basic doublet (EK vs DR) and a C-terminal histidine, albeit at a different position.

All attempts to correlate the suitability of a given peptide substrate with a definite secondary structure were unsuccessful. In particular, neither angiotensin II nor the peptides EEYAA and AAYAA, i.e. the best substrates for TPK-IIA, are likely to occur as $\beta$-turns, according to the predictive model of Chou and Fasman [15].

## 4. DISCUSSION

The experiments described here show that a polylysine- and NaCl-activated tyrosine protein kinase from spleen (TPK-IIA), probably related to a tyrosine protein kinase termed p40, detected in various tissues [10,11], can readily phosphorylate small, apparently featureless, peptides like AAYAA, AYAA, AYA and YYY. Phosphorylation of the dipeptides AY and YA, however, is barely detectable and free tyrosine is completely unaffected. In this respect, the behavior of TPK-IIA differs sharply from that of Ser/Thr-specific protein kinases which usually do not recognize peptide substrates unless they fulfil definite structural requirements generally consisting of either basic or acidic residues at given positions relative to the target amino acid (review [1]).

The apparent lack of specificity of TPK-IIA, however, is contradicted by the finding that the replacement of alanine(s) with different amino acids may fully compromise the phosphorylation of tri- and tetrapeptides, while the extension of these peptides with additional residues can restore to a variable extent or even improve their competence as phosphorylatable substrates.

Taken together, all of these observations would suggest that the peptide substrate specificity of TPK-IIA and possibly of other TPKs is largely determined by the balance between negative features, preventing tyrosine from being phosphorylated, and relieving factors that attenuate or overcome such structural hindrances. Consequently, any attempt to define exactly the role of an individual residue irrespective of the overall peptide structure is hazardous. Thus, an N-terminal glutamic acid appears variably deleterious in EYA and EYAA, when comparing these with their alanyl derivatives, AYA and AYAA, respectively, while it is very well tolerated in EEYAA, which is in fact a better substrate than AAYAA, having a 4-fold lower $K_m$ (though its $V_{max}$ is also somewhat lower). Likewise, two bulky hydrophobic residues flanking tyrosine completely prevent the phosphorylation of VYV (as compared to AYA), whereas they are apparently entirely harmless in angiotensin II (DRVYIHPF), which is an excellent substrate for TPK-IIA. In this case the relieving factor could be the N-terminal doublet DR whose tryptic removal dramatically impairs phosphorylation. A negative determinant, however, that seems to be refractory to local features is a histidine adjacent to the C-terminal side of tyrosine, as it occurs in EYH, EKEYHAE and five other related peptides which reproduce to variable extents the sequence around Tyr-845 of the EGF receptor, none of which is appreciably affected by TPK-IIA (table 1 and not shown). This finding is notable for two reasons, namely, (i) Tyr-845 is actually not phosphorylated in the EGF receptor, despite its homology with the main phosphorylation site of $pp60^{v\text{-}src}$ and other TPKs [16] and (ii) EYH and some of its derivatives are readily phosphorylated by TPKs other than TPK-IIA [8], thus strengthening the concept that structural requirements are quite variable among different members of the TPK family.

This observation is also pertinent to the vexed question of whether acidic residues upstream from tyrosine are needed for site recognition by TPKs. This seems not to be the case for TPK-IIA, whose activity toward a number of neutral peptides, such as YYY, YYA, AYAA and AAYAA, is quite remarkable, while homologous peptides with the

N-terminal alanines replaced by glutamic acid are either worse or barely comparable substrates. The opposite however is true of TPK-IIB, another spleen tyrosine protein kinase that strongly prefers EYAA over AYAA (unpublished). Moreover, while the suitability of neutral peptides for the protein tyrosine kinase from the LSTRA cell line has been related to their occurrence in the $\beta$-turn conformation [5], none of our most effective peptide substrates displays such a feature. Finally, it should be noted that the peptide substrate specificity of TPK-IIA itself is altered on assaying its activity in the absence of stimulatory concentrations of NaCl (unpublished). Apparently, therefore, the structural determinants for tyrosyl residue phosphorylation not only are dependent on the phosphorylating enzyme but can also be influenced by compounds that affect the catalytic activity.

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