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Phosphorylated residues as specificity determinants for an acidophilic protein tyrosine kinase

A study with src and cdc2 derived phosphopeptides

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Spleen TPK-IIB is an acidophilic protein tyrosine kinase, devoid of autophosphorylation activity, whose phosphorylation of the *src*-peptide NEYTA is crucially specified by Glu-2 [(1991) J. Biol. Chem. 266, 17798–17803]. We show that phosphothreonine, phosphotyrosine and phosphoserine are, in this order, specificity determinants even more effective than glutamic acid if they are replacing Glu-2, to give the phosphopeptides NTpYTA, NYpYTA, NSpYTA, respectively. Non-phosphorylated threonine, tyrosine and serine are conversely ineffective. Consequently also the heptapeptide GEGTYGV reproducing the phosphoacceptor and inhibitory site of $p34^{cdc2}$ is not appreciably affected by TPK-IIB, unless its threonyl residue is previously phosphorylated, the phosphoderivative GEGTpYGV being readily phosphorylated at its tyrosyl residue. Such a behaviour is unique for TPK-IIB among the protein tyrosine kinases tested (*lyn*-TPK, *fgr*-TPK and EGF-receptor, besides TPK-IIB). These data provide the first evidence that, in some instances, the targeting by protein tyrosine kinases can be specifically determined by the previous phosphorylation of the peptide substrate, thus extending the concept of 'hierarchal phosphorylation' [(1991) J. Biol. Chem. 266, 14139–14142] to tyrosyl residues as well.

Phosphopeptide; Protein tyrosine kinase; Specificity determinant; Hierarchal phosphorylation; Spleen

1. INTRODUCTION

A minority of Ser/Thr protein kinases recognize acidic residues as specificity determinants instead of being basofilic as most enzymes of this class (reviewed in [1,2]). Among such acidophilic Ser/Thr protein kinases, casein kinase-1 and glycogen synthase kinase-3 appear to be 'phosphate-directed', since their site specificity is determined in vitro by previously phosphorylated seryl residues whose effectiveness in this respect is much higher than that of carboxylic residues at the same position [3–6]. On the other hand the site specificity of at least one more acidophilic Ser/Thr protein kinase, casein kinase-2, though generally grounded on the carboxylic side chains of glutamic and aspartic acids, can also be determined, at least in vitro, by phosphotyrosine [7], and, to a lesser extent by phosphoserine [7,8].

The capability of phosphorylated residues to specify the site recognition by a number of protein kinases underlies the concept of 'hierarchal phosphorylation' [9] that implies the phosphorylation of a given residue(s) by a 'primary' kinase to be a pre-requisite for the subsequent targeting of the same protein substrate by a 'secondary' kinase.

It was not known whether the concept of hierarchal phosphorylation is also applicable to Tyr-specific protein kinases. This in principle is conceivable since protein tyrosine kinases are generally believed to be rather acidophilic, hence potentially susceptible to the generation of phosphoacceptor sites by the previous phosphorylation of other residue(s) nearby. In order to check this possibility we have synthesized a series of phosphopeptides derived either from the main phosphoacceptor site of $pp60^{c\text{-}src}$ (NEYTA) in which the glutamic acid adjacent to the N terminal side of tyrosine has been variably replaced by phosphorylated residues or from the down regulatory site of cdc2 (GEGTpYGV). These phosphopeptides as well as their unphosphorylated derivatives have been examined for

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Abbreviations: CK1, casein kinase 1; CK2, casein kinase 2; PKA, cAMP-dependent protein kinase; GSK3, glycogen synthase kinase 3; Fmoc, fluorenylmethoxycarbonyl; DCM, dichloromethane; DMF, *NN*-dimethylformamide; HOBt. 1-hydroxybenzotriazole; TBTU, 2-benzotriazolyl-tetramethyluronium tetrafluoroborate; RP-HPLC, reverse-phase high-performance liquid chromatography; MCPBA, *m*-chloroperoxybenzoic acid; PMSF, phenylmethylsulphonyl fluoride.

their capability to serve as substrates for four different protein tyrosine kinases.

2. MATERIALS AND METHOD

2.1. *Peptides*

Peptides were obtained by Fmoc solid-phase peptide synthesis using both manual (mod. Biolynx 4175, LKB) and automatic (Applied Biosytems 431A) peptide synthesizers. Tert-butyl acid labile protection was used, except for serine, threonine and tyrosine to be phosphorylated, which were used with free side chains. The N-terminal amino acids were introduced as Boc-derivatives. Manual synthesis was performed on Nova Syn KA (Novabiochem) resin using the continuous flow variant of the 'Fmoc-polyamidic' method [10]. Chain elongation was carried out via TBTU in situ activation of Fmoc amino acids (5 molar excess) in the presence of HOBt. Automatic synthesis was achieved on polystyrene resin according to the manufacture's instructions (Applied Biosystems).

Peptide phosphorylation was obtained following a global phosphorylation strategy [11]. After peptide synthesis, the resin was phosphinylated for 1 h in anhydrous DMF with di-tertbutyl-*N*,*N*-di-iso-propylphosphoramidite (10 eq.) in the presence of sublimed tetrazole (30 eq.). Oxidation was performed in anhydrous DMF with ter-butyl hydroperoxide 70% aqueous (20 eq., 1 h) or with MCPBA (85%, 10 min) in DCM solution [12].

Peptides were cleaved from the resin, and the side chain protecting groups removed by 95% trifluoroacetic acid (TFA) in the presence of 5% anisole for 1 h. Phosphorylation yields (> 70%) were evaluated by analytical RP-HPLC.

Peptides were purified by preparative RP-HPLC on a Waters Delta-pack C_{18} 300A 15 μm (3.9 mm × 30 cm) column, eluting with a linear gradient from 0.1% aqueous trifluoroacetic acid (TFA) to 20% acetonitrile containing 0.08% trifluoroacetic acid (TFA) in 20 min at 2.5 ml/min, monitoring at 220 and 280 nm. Purity (95% or more) was ascertained by amino acid analysis, analytical RP-HPLC and capillary electrophoresis. The presence of organic phosphate was demonstrated by phosphate analysis [13].

2.2. *Tyrosine protein kinases*

lyn-TPK, TPK-IIB and TPK-III/*fgr*-TPK were purified from the particulate fraction of rat spleen as previously described [14,15]. *fgr*-TPK has been further purified by FPLC on a Mono-Q HR 5/5 column connected to a Pharmacia LKB apparatus. The column equilibrated with 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 50 μM PMSF and 10% glycerol was washed with 15 ml of the same buffer and eluted with a linear NaCl gradient (0–0.3 M). *fgr*-TPK was eluted at 0.22 M NaCl. As source of EGF-receptor a crude membrane extract of A431 human epidermoid cells was used prepared as previously described [16] and kindly provided by Dr. P. De Witte (Leuven, Belgium).

2.3. *Tyrosine kinase assays*

Non-receptor-linked tyrosine protein kinase activities were assayed by incubating 10 units of enzyme as previously described [14]. One unit was defined as the amount of enzyme transferring 1 pmol phosphate/min to angiotensin II (2 mM).

EGF-Receptor tyrosine kinase activity was assayed in a final volume of 50 μl containing 20 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 100 μM sodium vanadate, 10 μM [γ-^{32}P]ATP (specific activity 2,000 cpm/pmol), 2.5 μg crude A431 membranes and 1 mM peptides. Incubations were initiated by the addition of [γ-^{32}P]-ATP after preincubation for 20 min at 0°C in the absence or in the presence of 1 μg/ml EGF.

Reactions were terminated after 10 min incubation (unless otherwise indicated) by the addition of 1 ml of 1 N HCl and labeled phosphopeptides were quantitated as previously described [17]. 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 the phosphomolybdic complex and extractions with isobutanol/toluene. The radioactivity, present in the aqueous phase, due to phosphopeptides, was then measured in a scintillation counter.

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

Among all peptides the only radiolabeled phosphoamino acid was Tyr-P; no radioactive Thr-P or Ser-P could be detected.

3. RESULTS

The pentapeptide NEYTA, reproducing the sequence around the main autophosphorylation site of c-*src* (Tyr-416), has been shown to behave as a good substrate for TPK-IIB, a protein tyrosine kinase unrelated to the *src* family and purified to homogeneity from spleen [15]. The efficient phosphorylation of NEYTA by TPK-IIB is crucially dependent on the glutamyl residue adjacent to the N-terminal side of tyrosine, its replacement with alanine giving rise to a much worse substrate [15]. As shown in Fig. 1A the three phosphorylated residues, Tyr-P, Ser-P and Thr-P, all behave more powerful as specificity determinants than glutamic acid, the phosphopeptides NYpYTA, NSpYTA and NTpYTA being phosphorylated by TPK-IIB at higher rates than the parent peptide, NEYTA. The beneficial effect of the phosphorylated residues is clearly due to their phosphorylated moiety, since the unphosphorylated peptides, NYYTA, NSYTA and NTYTA are phosphorylated very slowly. In particular, while NTYTA is a substrate as bad as NAYTA, its phosphorylated derivative, NTpYTA, displays the highest phosphorylation rate among the tested peptides.

Looking for a physiologically relevant example of a phosphoacceptor site including a phosphorylated residue which could act as specificity determinant for the phosphorylation of a tyrosine adjacent to its C terminal side, we synthesized two peptides reproducing the $p34^{cdc2}$ down-regulatory site either fully unphosphorylated (GEGTYGV) or singly phosphorylated at its threonine residue (Thr-14). It should be recalled that both Thr-14 and Tyr-15 became phosphorylated in the inactive form of $p34^{cdc2}$, although only the phosphorylation of Tyr-15 appears to strictly correlate with down-regulation [18]. As shown in Fig. 1B the unphosphorylated cdc2 peptide (tested at 1 mM concentration) is not significantly affected by TPK-IIB; however, it is converted into an excellent substrate by the previous phosphorylation of its threonyl residue.

The kinetic constants of Table I show that the superiority of phosphorylated vs. glutamyl residues is accounted for by lower K_m values, while the V_{max} values of the *src*-derived phosphopeptides are similar to that of the parent peptide, NEYTA. The phosphate group per se, however, exhibits a beneficial effect on both K_m and V_{max} values, as shown by comparing the kinetic constants of NTpYTA, GEGTpYGV and NYpYTA with those of their non-phosphorylated derivatives,

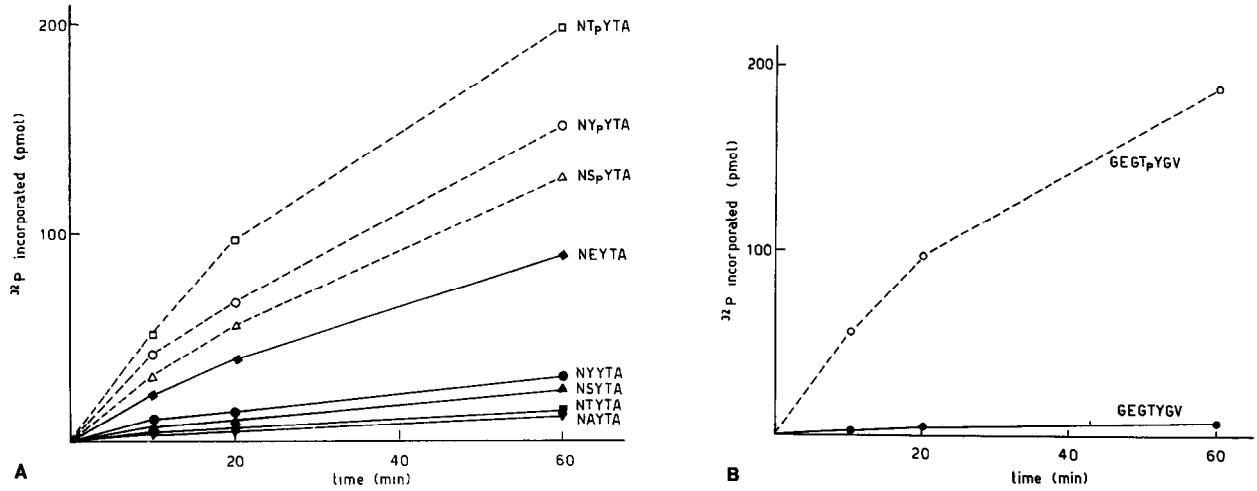

Fig. 1. Phosphorylation of *src*-related (A) and cdc2 (B) peptides by TPK-IIB. One-letter symbols are used for aminoacyl residues. Sp,Tp,Yp denote Ser-P, Thr-P and Tyr-P, respectively. Peptide concentration was 1 mM. Other experimental conditions are described in section 2.

NTYTA, GEGTYGV and NYYTA, respectively. The very high K_m value of GEGTYGV (21 mM) should be noted in this respect as compared to the relatively low K_m value of its phosphothreonyl derivative, GEGTpYGV (0.35 mM). Such a dramatic drop in K_m accounts for the finding that, when tested at 1 mM concentration, the unphosphorylated peptide is not significantly affected by TPK-IIB, while its phosphothreonyl derivative is readily phosphorylated.

Unlike TPK-IIB, which is a markedly acidophilic kinase [15,19,20], the protein tyrosine kinases of the *src* family do not appear to stringently require acidic residues upstream from the target tyrosine for the recognition of their peptide substrates [21]. Consequently neither the *lyn*-TPK nor spleen TPK-III, recently identified as the product of an oncogene either identical or very closely related for *fgr* [22], display any marked specificity toward peptides reproducing the Tyr-416 phosphoacceptor site of c-*src* [15]. It has been shown, however, that polylysine confers to the *lyn* kinase an accentuated affinity for acidic peptides with special reference to those reproducing the Tyr-416 c-*src* site [14]. As shown in Table II in fact polylysine subverts the activity ratio toward NEYTA and NAYTA, the former peptide being preferred over the latter only in the presence of polylysine, while the opposite is true if the enzyme is activated by NaCl. Roughly the same behaviour is observed by comparing the phosphorylated and non-phosphorylated congeners of NEYTA: each phosphopeptide is preferred over its unphosphorylated counterpart if *lyn*-TPK is activated by polylysine, while in the presence of NaCl the phosphate groups actually diminish the phosphorylation efficiency. It should be concluded therefore that in general phosphorylated side chains adjacent to the N terminal side of tyrosine are perceived by *lyn*-TPK like the carboxylic side chain of glutamic acid in that they play either a positive or negative role in the presence of polylysine and NaCl, respectively.

As also shown in Table II a rather detrimental effect of phosphorylated residues substituted for their unphosphorylated counterparts is constantly observed with *fgr*-TPK, a *src* related protein tyrosine kinase notable for its rather broad peptide substrate specificity [15,19,20]. The EGF-receptor protein tyrosine kinase on the other hand is almost insensitive to Ser-P for Ser and Thr-P for Thr substitutions at position −1 and it perceives a Tyr-P for Tyr substitution in that position as a detrimental feature (see Table II). It should be noted in this connection that the EGF-receptor kinase is unique in that it prefers the peptide with two adjacent tyrosines over all other *src* derivatives used in this study. Consequently a peptide substrate featured after the *syk* autophosphorylation site including the Tyr-Tyr motif [23], as several other protein tyrosine kinases [24], is also efficiently phosphorylated by the EGF-Receptor (see Table II), suggesting that indeed the motif YY could be especially suited for undergoing phosphorylation by the EGF-receptor.

4. DISCUSSION

The concept of hierarchal phosphorylation [9] developed from the observation that in some instances phosphoacceptor sites can be specified by the previous phosphorylation of other residues nearby. The first example of this event was provided by casein kinase-1, whose phosphorylation sites in casein are determined by N-terminal clusters of constitutively phosphorylated serines, the dephosphorylation of which was shown to slow down the subsequent targeting by CK1 [25]. Likewise the previous phosphorylation of a seryl residue by either CK2 [26] or PKA [27] is a pre-requisite for the efficient phosphorylation of another seryl residue lo-

Table I

Kinetic constants for TPK-IIB with synthetic peptide substrates

Peptide	K_{cat}	K_m (mM)	Efficiency $K_{cat} \cdot K_m^{-1}$
NEYTA	52.1	0.89	58.5
NAYTA	12.2	5.00	2.4
NSpYTA	30.9	0.36	85.8
NTpYTA	62.3	0.15	415.3
NTYTA	27.4	7.00	3.9
NYpYTA	46.3	0.26	178.1
NYYTA	24.1	4.00	6.0
GEGTpYGV	61.4	0.27	227.0
GEGTYGV	6.5	21.70	0.3

Assays were performed and the kinetic parameters determined, as described in section 2. The values shown are means of at least three independent experiments. For each of these means, the S.E. value was less than 10% of the mean.

cated up-stream, by GSK3. While the specificity of CK1 and GSK3, at least in vitro, appears to be crucially dependent on pre-phosphorylated residues, the acidic side chains of Glu and Asp being much less effective, the targeting by another acidophilic Ser/Thr protein kinase, CK2, is mostly determined by carboxylic residues; these can be however successfully replaced either by phosphoserine [8,28] or, even more efficiently, by phosphotyrosine [7], this latter being conversely ineffective in the case of CK1-mediated phosphorylation [29]. The behaviour of CK2 disclosed the possibility of *mixed*, hierarchal phosphorylation, that would allow a dialog between Ser/Thr and Tyr-specific protein kinases at the substrate level [7].

It was not known, however, whether protein tyrosine kinases could also be susceptible to hierarchal phosphorylation, although this possibility was consistent with the ordered phosphorylation occurring at the insulin-receptor autophosphorylation sites, whose Tyr-Tyr doublet is sequentially activated [30]. The data presented in this report unambiguously show that TPK-IIB, a spleen protein tyrosine kinase previously shown to be markedly acidophilic [15,19,20], can very efficiently phosphorylate peptide substrates in which a glutamyl residue crucially acting as specificity determinant, has been replaced by an *o*-phosphorylated residue. Phosphothreonine is most effective for this purpose, followed by phosphotyrosine and phosphoserine, the latter still being more effective than glutamic acid. It will be interesting to assess whether the additional acidic residues at positions − 3 and − 4, which also previously have been shown to facilitate the phosphorylation of peptide substrates by TPK-IIB [15], could be successfully replaced by phosphorylated residues, as in the case of the residue at position −1.

The behaviour of TPK-IIB supports the general concept that protein tyrosine kinases can indeed participate as 'secondary' kinases in hierarchal phosphorylation and shows that the motifs Ser-Tyr, Thr-Tyr and Tyr-Tyr may be converted into good targets for a protein tyrosine kinase by the previous phosphorylation of the N-terminal hydroxylic residue. It should be noted in this connection that the doubly phosphorylatable motif Thr-Tyr is recurrent at the catalytic site of several protein kinases belonging to the phylogenetic branch of cdc2, whose activity is actually suppressed by the phosphorylation of these residues [18]. The observation therefore that a heptapeptide reproducing the cdc2 phosphoacceptor site, GEGTYGV, is converted into an

Table II

Phosphorylation rate of synthetic phosphopeptides vs. peptides by three protein tyrosine kinases

Peptide	Phosphorylation rate (pmol · min^{-1})			
	lyn-TPK		*fgr*-TPK	EGF-receptor
	PolyLys (0.5 μM)	NaCl (2 mM)		
NEYTA	14.1	4.0	6.8	1.6
NAYTA	3.6	8.1	6.8	1.2
NSpYTA	2.6	0.3	0.8	0.2
NSYTA	0.8	1.9	2.8	0.2
NTpYTA	12.2	2.6	4.1	3.4
NTYTA	10.2	6.6	7.9	2.8
NYpYTA	17.2	1.7	3.0	2.0
NYYTA	9.5	9.4	7.2	4.5
GEGTpYGV	12.0	3.4	2.8	–
GEGTYGV	9.7	6.3	3.7	–
DENYYKA	–	–	–	5.2

Experimental conditions are described in section 2. The peptide concentration was 1 mM. The values shown are means of three different experiments. For each of these means, the S.E.M. value was less than 11% of the mean.

excellent substrate for TPK-IIB by the previous phosphorylation of its threonyl residue, is of special interest as it provides a physiologically relevant example of a site potentially susceptible to hierarchal phosphorylation involving TPK-IIB or another TPK with similar specificity.

Pertinent to this appears to be the observation that although an individual protein kinase with dual specificity, possibly wee 1, was originally supposed to be responsible for the phosphorylation of both Thr-14 and Tyr-15 of cdc2 [18], recent data support the view that these two residues could be targeted, at least in some instances, by distinct enzymes [31,32]. Our data would include TPK-IIB in the list of the kinases potentially involved in the phosphorylation of Tyr-15 alone and provide a plausible mechanism of regulation based on the previous phosphorylation of Thr-14 by another kinase. It should be mentioned in this respect that the phosphorylation of the cdc2 peptide by TPK-IIB is indeed entirely dependent on previous threonine phosphorylation. Such an absolute requirement could reflect a 'double key' control of cdc2, whose abrupt inactivation by TPK-IIB (or a TPK-IIB-like tyrosine kinase) could not occur unless Thr-14 is first phosphorylated by an as yet unidentified Ser/Thr protein kinase.

For its involvement in mixed hierarchal phosphorylation processes, TPK-IIB mirrors CK2, the former being a Tyr-specific kinase whose sites can be generated by Ser/Thr-specific kinases, while the latter is a Ser/Thr-specific enzyme whose sites can be generated by tyrosine kinases. In principle therefore both these protein kinases could create 'checkpoints' where kinases belonging to the two classes, Ser/Thr- and Tyr-specific, might influence each other and cross-talk through mixed hierarchal phosphorylation.

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