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Solid Phase Synthesis of pp60^{src}-Related Phosphopeptides via 'Global' Phosphorylation and Their Use as Substrates for Enzymatic Phosphorylation by Casein Kinase-2

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Abstract—The seven phosphopeptide derivatives based on the native -NEYTA- sequence of the pp60^{src} protein kinase family, Asn-Glu-Tyr(*P*)-Ser-Ala, Ala-Glu-Tyr(*P*)-Ser-Ala, Ala-Ser-Tyr(*P*)-Ser-Ala, Ala-Ser(*P*)-Tyr-Ser-Ala, Ala-Thr-Tyr(*P*)-Ser-Ala, Ala-Thr(*P*)-Tyr-Ser-Ala and Ala-Ser(*P*)-Tyr(*P*)-Ser-Ala, were prepared in good yield using the 'global' 'phosphite-triester' phosphorylation method. The peptide resins were assembled using the Fmoc mode of solid phase peptide synthesis (PyBOP® coupling method) with specific Ser-, Thr-, or Tyr-residues incorporated as their side chain free Fmoc-derivatives. The final 'global' phosphorylation of the peptide resins was accomplished using di-*tert*-butyl *N,N*-diethylphosphoramidite followed by *m*-chloroperoxybenzoic acid oxidation of the resultant di-*t*-butyl phosphite triester intermediate. Subsequent resin cleavage and deprotection of the phosphorylated peptide resins was effected by treatment with 5% anisole:TFA and gave the seven phosphopeptides in high yield and purity. The use of the seven synthetic phosphopeptides in enzymatic (casein kinase-2) phosphorylation studies showed that, (A) the change of the target Thr site to Ser resulted in markedly improved phosphorylation of the peptide substrates, (B) that the Tyr(*P*) residue in the −1 position was significantly more important than the Ser(*P*)/Thr(*P*) residue in the −2 position for efficient seryl phosphorylation, and (C) that an acidic residue in the −2 position relative to the target site facilitated phosphorylation of the downstream seryl residue irrespective of the nature of the acidic residue in the -Xxx-Tyr(*P*)-Ser- and -Xxx-Tyr-Ser- sequences {Xxx = Ser(*P*), Thr(*P*), Glu}. In addition to the Tyr(*P*) residue directing phosphorylation to the +1 position, the good phosphorylation of both ASY(*P*)SA and ATY(*P*)SA by casein kinase-2 indicated that the Tyr(*P*) residue was also able to direct phosphorylation to a Ser/Thr in the −1 position.

Introduction

In past work, it was shown that phosphorylation of the Tyr residue of Asn-Glu-Tyr-Thr-Ala (NEYTA), which reproduces the phosphorylated form of the auto-phosphorylation sequence of the *src*-protein kinase family,[1] resulted in an increase in the phosphorylation of the threonyl residue by casein kinase-2.[2,3] By the use of various synthetic peptides, it was found that the Tyr(*P*) residue acted as a specificity determinant for directing phosphorylation to the adjacent threonyl residue, this being unusual since the typical recognition sequence for CK-2 requires acidic residues on the *C*-terminal side of the target residue and preferably in the +3 position. As conversion of the Tyr-residue to the Tyr(*P*) residue produced an increase in enzymatic phosphorylation, we wished to examine whether a change of the target threonyl residue to the more favorable seryl residue would facilitate peptide phosphorylation and the effect of having phosphorylated residues in the −1 and −2 positions on the seryl phosphorylation rate. In this study, we describe the solid phase synthesis of several Ser(*P*)-, Thr(*P*)- and Tyr(*P*)-containing phosphopeptides by the use of di-*t*-butyl *N,N*-diethylphosphoramidite for the 'global' 'phosphite-triester' phosphorylation of assembled peptide resins and the subsequent use of the synthetic phosphopeptides in casein kinase-2 phosphorylation studies.

Key words: phosphopeptides; Fmoc/solid phase synthesis; di-*tert*-butyl *N,N*-diethylphosphoramidite; 'global' 'phosphite-triester' phosphorylation; casein kinase-2.

Results and Discussion

The selection of phosphorylated pentapeptide substrates for enzymatic phosphorylation studies were based on the simple -NEY(*P*)TA- sequence since we had established in an earlier study that the simple pentapeptide, NEY(*P*)TA, was phosphorylated with the same rate as RLIEDNEY(*P*)TARQG and thereby confirmed that the residues adjacent to the Thr residue were solely responsible for phosphorylation of the target site.[2] However, as earlier syntheses of NEYTA and NEY(*P*)TA were complicated due to minor side reactions of the N-terminal Asn residue and the low solubility of the peptides in water, we sought to replace the Asn residue with the simpler Ala residue with the proviso that this substitution did not significantly affect the phosphorylation rate. In order to establish the effect of changing the Thr residue to the Ser residue

and the importance of the N-terminal Asn residue, the first stage of this study required the generation of the two test peptides, NEY(*P*)SA (**1**) and AEY(*P*)SA (**2**), respectively. The preparation of the two target phosphopeptides was approached by the use of di-*t*-butyl *N,N*-diethylphosphoramidite[4] for the 'global' 'phosphite-triester' phosphorylation[5,6] of the pre-assembled Tyr-containing peptide resin.[7,8] Since the first description of this methodology appeared,[5,6] this approach has been used by many groups for the simple and rapid method of preparation for a wide range of phosphorylated peptides.[9–15]

In this synthetic approach, the two peptide resins were readily assembled by Fmoc solid phase synthesis[16] with the use of Fmoc-Ala-Wang Resin* as the polymer support and PyBOP® as the coupling reagent.[17] The phosphorylatable tyrosine residue was incorporated as Fmoc-Tyr-OH at the desired phosphorylation site[18,19] and the post-assembly phosphorylation of the peptide resins was accomplished using di-*tert*-butyl *N,N*-diethylphosphoramidite (10 equiv/hydroxyl group): 1*H*-tetrazole (60 min, 20 °C) followed by *m*-chloroperoxybenzoic acid oxidation[20] of the resultant 'phosphite-triester' intermediate. Final deprotection of the two phosphopeptide resins was readily effected by treatment with 5% anisole:TFA for 1 h and gave the crude phosphopeptides in high yield.† The two crude phosphopeptides were each found to be >95% pure by analytical C_{18}-HPLC and were readily purified to homogeneity using semi-preparative C_{18}-HPLC. The enzymatic phosphorylation data obtained for the treatment of NEY(*P*)SA and AEY(*P*)SA with casein kinase-2 (CK-2) confirmed that the N-terminal Asn residue was not critical for seryl phosphorylation since both NEY(*P*)SA and AEY(*P*)SA were phosphorylated with similar phosphorylation rates (see Fig. 1). In addition, the comparison of the phosphorylation rate data obtained for the four peptides, NEYTA, NEY(*P*)TA,[2,3] AEY(*P*)SA and AEYSA showed that the change of Thr to Ser as the target site resulted in a marked increase in phosphorylation efficiency with the rate increase being more pronounced when the Tyr residue in the −1 position was in its phosphorylated form (Table 1). While the above data established that the seryl residue was preferred over the threonyl residue in this atypical phosphorylation sequence and is consistent with the known preference of CK-2 for seryl target sites,[22] the phosphorylation of AEYSA is consistent with the previously observed phosphorylation of NEYTA[3] and corroborates our earlier finding that the Tyr residue can act as a weak specificity determinant for directing phosphorylation to an adjacent hydroxy amino acyl residue.

In the second stage of this study, we wished to establish the influence of phosphorylated residues in both the −1 and −2 positions of the target seryl site. While the preparation of Ala-Thr(*P*)-Tyr(*P*)-Ser-Ala (**8**) has been described previously,[8] the synthesis of phosphopeptides **3–7** was accomplished using the synthetic procedure described for the preparation of peptides **1** and **2** with side chain-free Fmoc-Ser-OH, Fmoc-Thr-OH, and Fmoc-Tyr-OH incorporated into peptide assembly at the desired phosphorylation site[18,19] (Fig. 2). While analytical C_{18} RP-HPLC of crude phosphopeptides **3**, **4**, **6** and **7** showed a major single product and confirmed that phosphorylation of Ser and Tyr residues proceeded smoothly, the analytical C_{18} RP-HPLC of crude phosphopeptide **5** showed the presence of 30% ATYSA and indicated that phosphorylation of the threonyl residue was hindered due to increased steric hindrance about its secondary hydroxyl group. The crude phosphopeptides were readily purified to homogeneity using semi-preparative or preparative C_{18}

Table 1. Kinetic data obtained for peptides and phosphopeptides

Peptide	K_m (mM)	V_{max} (mmol^{-1} min^{-1} mg^{-1})	Efficiency (V_{max}/K_m)
AS(*P*)Y(*P*)SA	4.3	41.8	9.7
AT(*P*)Y(*P*)SA	5.0	38.4	7.7
ASY(*P*)SA	3.1	28.1	9.1
ATY(*P*)SA	n.d.	n.d.	n.d.
NEY(*P*)SA	5.2	35.7	6.9
AEY(*P*)SA	4.5	27.5	6.1
AS(*P*)YSA	4.0	13.8	3.4
AT(*P*)YSA	5.0	14.2	2.8
AEYSA	n.d.	n.d.	n.d.
ASYSA	—	—	—
ATYSA	—	—	—

n.d. = not determined.

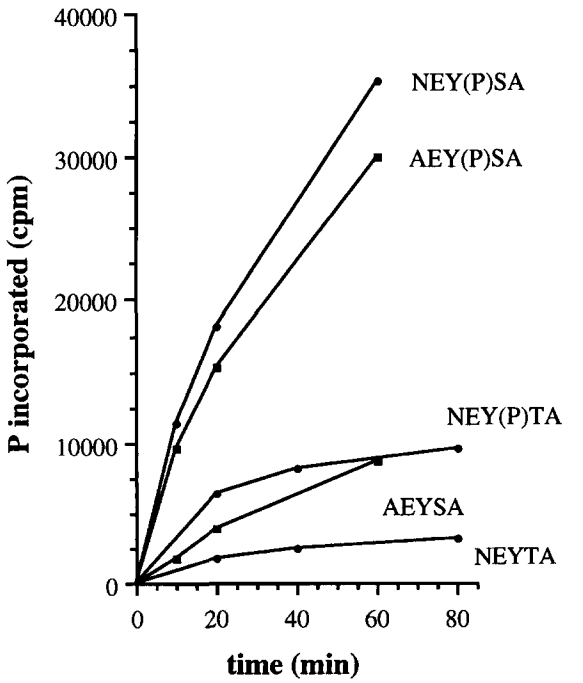

Figure 1. Time course data for the CK-2 phosphorylation of AEYSA, AEY(*P*)SA, NEY(*P*)SA, NEYTA and NEY(*P*)TA.

*Wang Resin = 4-hydroxymethylphenoxymethyl (HMP) linked polystyrene.

†The data obtained for phosphopeptide (**2**) were identical with those obtained for an authentic sample which had been previously prepared by the use of Fmoc-Tyr($PO_3^tBu_2$)-OH in Fmoc/solid phase peptide synthesis.[21]

RP-HPLC and were readily characterized by ^{13}C and ^{31}P NMR spectroscopy, FABMS and amino acid analysis.[‡] The identification of the phosphorylated amino acyl residues in the obtained peptides was readily ascertained from their ^{13}C NMR spectra which showed distinctive phosphorus-coupled doublet signals for the Cα- and Cβ-carbons of the Ser(*P*) or Thr(*P*) residues and for the aromatic C3- and C4-carbons of the Tyr(*P*) residue (see Experimental).

Ala-Ser(*P*)-Tyr-Ser-Ala (**3**)

Ala-Ser-Tyr(*P*)-Ser-Ala (**4**)

Ala-Thr(*P*)-Tyr-Ser-Ala (**5**)

Ala-Thr-Tyr(*P*)-Ser-Ala (**6**)

Ala-Ser(*P*)-Tyr(*P*)-Ser-Ala (**7**)

Ala-Thr(*P*)-Tyr(*P*)-Ser-Ala (**8**)

The ready preparation of phosphopeptides **3**–**7** in good overall yields demonstrates that the above synthetic procedure presents a simple method for the rapid generation of phosphopeptides which contain Ser(*P*), Thr(*P*) or Tyr(*P*) residues and simple combinations of these residues. A particular feature of this synthetic approach is that it is also suitable for the generation of Tyr(*P*)-containing phosphopeptides which also contain the Ser(*P*) and Thr(*P*) residue, the Fmoc/solid phase synthesis of such phosphopeptides being precluded by the base sensitivity of the Ser(PO_3R_2) and Thr(PO_3R_2) residue during peptide assembly (i.e., repetitive piperidine-mediated cleavage of the Fmoc group at each coupling cycle). Further more, the isolation of Ala-Ser(*P*)-Tyr(*P*)-Ser-Ala (**7**) in 62% yield confirms that the bis-phosphorylation of adjacent residues proceeds smoothly on the solid phase and is not hindered by the incorporation of the bulky di-*t*-butylphosphono groups.

Enzymatic phosphorylation studies

In the first instance, the CK-2 phosphorylation of the two Tyr(*P*) peptides, Ala-Ser-Tyr(*P*)-Ser-Ala and Ala-Thr-Tyr(*P*)-Ser-Ala, and their two Tyr peptide counterparts, were examined so as to establish the effect of substituting the acidic Glu residue in the Ala-Glu-Tyr(*P*)-Ser-Ala sequence with a non-acidic residue and the effect of tyrosyl phosphorylation of subsequent peptide phosphorylation. In the case of Ala-Ser-Tyr-Ser-Ala and Ala-Thr-Tyr-Ser-Ala, the negligible phosphorylation of both these peptides indicated that the hydroxy amino acyl residues adjacent to the Tyr residue were not targets for CK-2 phosphorylation when Tyr was in its native state. However, it was quite surprising that with the conversion of the Tyr residue to its Tyr(*P*) form, the time course data showed that both ASY(*P*)SA and ATY(*P*)SA were relatively good substrates for CK-2 with ASY(*P*)SA being a much better substrate than ATY(*P*)SA (Fig. 3). In consideration that the acidic Tyr(*P*) residue is the only site-directing residue in these two peptides and that both peptides contain two phosphorylatable target sites adjacent to the Tyr(*P*) residue, we therefore propose that the Tyr(*P*) residue is able to direct phosphorylation to the Ser/Thr residue in the −1 position in preference to the Ser residue in the +1 position. Indeed, phosphorylation of the upstream Ser/Thr residue is consistent with the observed kinetic rate difference observed for ASY(*P*)SA and ATY(*P*)SA, and reflects the known preference of CK-2 for seryl residues over threonyl residues.[22] This mode of phosphorylation is also consistent with our prior finding in which the Ser(*P*) peptide, Ser-Ser-Ser(*P*)-NHMe, was observed to be a good substrate for CK-2 with the Ser(*P*) residue directing phosphorylation to the seryl residue in the −1 position.[23]

In view that the above results showed that a Tyr(*P*) residue could direct phosphorylation to an upstream Ser/Thr residue and thereby convert a neutral residue to an acidic residue, the study of the Ala-Xxx-Tyr(*P*)-Ser-Ala peptide series {Xxx = Glu, Thr(*P*), Ser(*P*)} was now of particular interest in respect to the

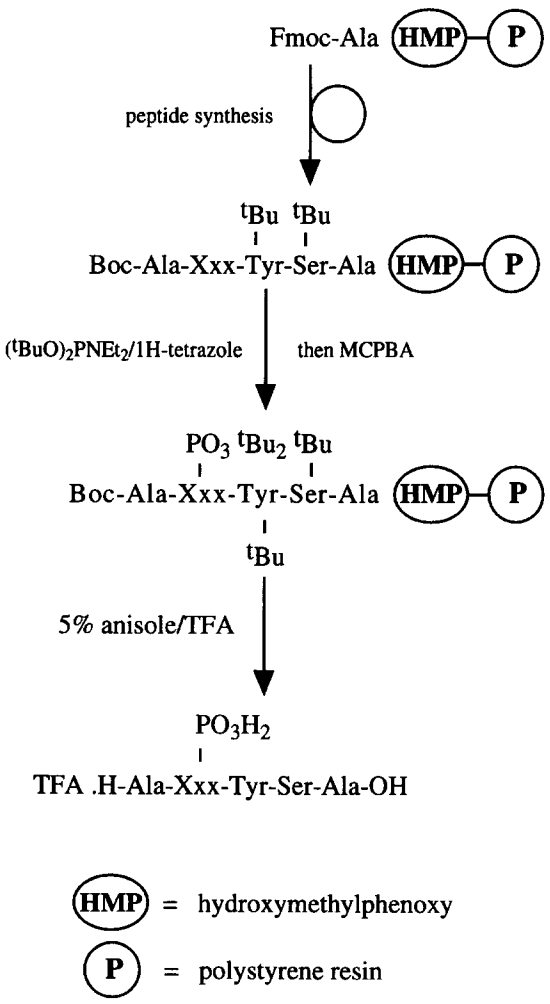

Figure 2. Solid phase synthesis of phosphopeptides (**3**) (Xxx = Ser) and (**5**) (Xxx = Thr).

[‡]The data obtained for phosphopeptide **6** were identical with those obtained for an authentic sample which had been previously been isolated as a by-product from the incomplete phosphorylation of Boc-Ala-Thr-Tyr-Ser(Bzl)-Ala-Wang Resin using di-*t*-butyl *N,N*-diethylphosphoramidite.[8]

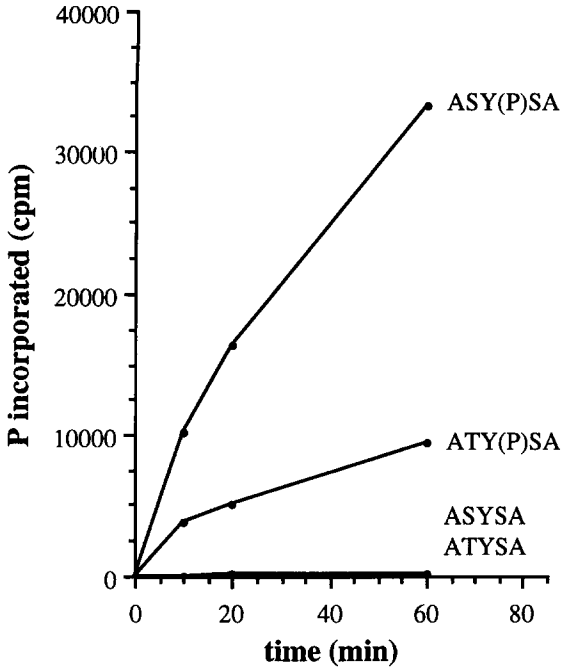

Figure 3. Time course data for the phosphorylation of AEY(*P*)SA, ATY(*P*)SA, ASY(*P*)SA, ATYSA and ASYSA.

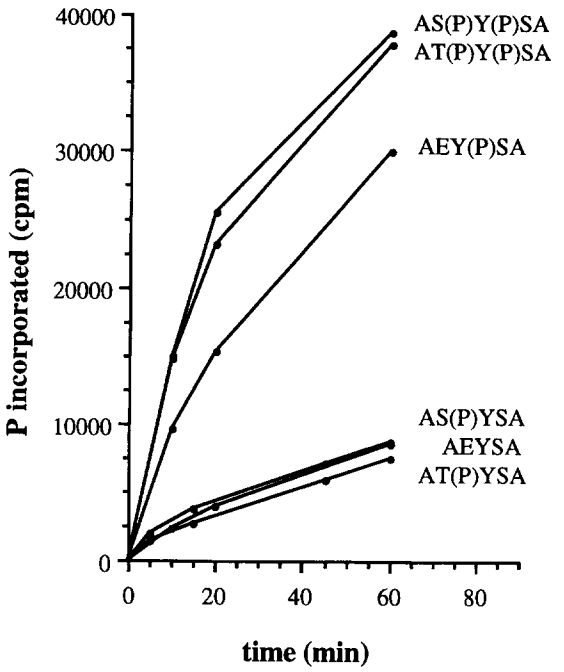

Figure 4. Comparative time course data for the phosphorylation of AS(*P*)Y(*P*)SA, AT(*P*)Y(*P*)SA, AEY(*P*)SA, AS(*P*)YSA, AT(*P*)YSA and AEYSA.

effect of multilevel peptide phosphorylation on the subsequent CK-2 phosphorylation of the downstream seryl residue. For the enzymatic phosphorylation of the Ala-Xxx-Tyr-Ser-Ala and Ala-Xxx-Tyr(*P*)-Ser-Ala peptide series {Xxx = Glu, Thr(*P*), Ser(*P*)}, the time course data showed that the three Ala-Xxx-Tyr(*P*)-Ser-Ala peptides were relatively good peptide substrates and were phosphorylated with markedly higher rates than the three Ala-Xxx-Tyr-Ser-Ala peptides (Fig. 4). While the two Tyr peptides, AS(*P*)YSA and AT(*P*)YSA, were determined to have phosphorylation efficiencies (V_{max}/K_m) of 3.4 and 2.8, respectively the three Tyr(*P*) peptides, AS(*P*)Y(*P*)SA, AT(*P*)Y(*P*)SA and AEY(*P*)SA, were determined to have phosphorylation efficiencies (V_{max}/K_m) of 9.7, 7.7 and 6.1, respectively. The comparison of the phosphorylation data obtained for these two peptide series shows that the Tyr(*P*) residue plays a major role in directing phosphorylation to its +1 position and that an acidic residue adjacent to the Tyr(*P*) residue (its −1 position) plays a minor role in promoting downstream seryl phosphorylation. On the basis of the above data both the ASY(*P*)SA and ATY(*P*)SA peptides present complex situations in which the hydroxy amino acyl residue in the −1 position to the Tyr(*P*) residue is a good phosphoacceptor site for CK-2 and that its phosphorylation generates the atypical -Ser(*P*)-Tyr(*P*)-Ser- and -Thr(*P*)-Tyr(*P*)-Ser-sequences (as in peptides **7** and **8**, respectively) in which secondary phosphorylation is then directed to the downstream hydroxy residue.

An interesting feature of the phosphorylation data was that AT(*P*)YSA was phosphorylated at the same rate as AS(*P*)YSA, and that AS(*P*)Y(*P*)SA was phosphorylated with the same rate as AT(*P*)Y(*P*)SA. The similar phosphorylation rates obtained for these two peptide sets were unexpected in consideration that the majority of past studies have generally shown the effect of acidic residues in directing site phosphorylation in typical consensus sequences to be Ser(*P*) ≫ Glu > Thr(*P*).[22–24] Thus, as the nature of the side chain acid group has little effect on the phosphorylation rate for these peptides, this indicates that the interaction of the acidic residue with casein kinase-2 is not related to the pK_a of the side chain carboxyl or phosphoryl group, but more a function of weak hydrogen bonding between the acidic proton with the kinase. While the threonyl residue is sterically more hindered than its seryl counterpart, the similar performance of the two Thr(*P*) peptides with their Ser(*P*) peptide counterparts suggests that in these two particular cases, the large polar phosphate group of the Thr residue is sufficiently displaced from the peptide backbone such that it has the necessary spatial flexibility to be involved in hydrogen bonding with the kinase.

In consideration that some protein tyrosine kinases can participate in hierarchical phosphorylation[25] and that CK-2 can recognize Tyr(*P*) as a specificity determinant, the results obtained in this study suggest that 'cross talk' between Ser/Thr and Tyr-specific tyrosine kinases may be important to the level of phosphorylation of a protein substrate at any given time and relevant to its activity. Of various protein substrates which were identified to possess a Ser or Thr residue on the *N*- or *C*-terminals of a known Tyr phosphorylation site (see Table 2), the KVEKIGEGTY(*P*)[15]GVVYK sequence of p34^{cdc2} is particularly interesting since the activity of Cdc2 when complexed with cyclins A or B is

Table 2. Potential Ser/Thr phosphorylation sites adjacent to a Tyr(*P*) residue

Src family kinases	NEY(*P*)TA
p34^{cdc2}	KVEKIGEGTY(*P*)GVVYK
S_6-kinase	YVVKETIGVGSY(*P*)SVCKRCVHK
FcγRIIB 1 receptor	EAENTITY(*P*)SLLKH
FcεRI-βITAM	DRLY(*P*)EELNHV
	Y(*P*)SPIY(*P*)SEL
MAP kinase	PEHDETGFLTEY(*P*)VATR

suppressed by phosphorylation of Tyr-15 and Thr-14.[26] While the kinase responsible for the phosphorylation of Tyr-15 has been identified as Wee-1 kinase, the kinase that phosphorylates Thr-14 has yet to be identified along with the order of the Thr and Tyr phosphorylations. In the cell cycle, activation of the Cdc2/cyclin complex at the appropriate time occurs via dephosphorylation of both Thr-14 and Tyr-15 by Cdc25 protein phosphatase and results in the onset of mitosis. While a past enzymatic phosphorylation study using NT(*P*)YTA as a substrate has shown that threonyl phosphorylation can precede Tyr phosphorylation,[25] the ready phosphorylation of both ASY(*P*)SA and ATY(*P*)SA by CK-2 in this study suggests a second pathway for the phosphorylation of the Cdc2-EGTYG-sequence may involve initial phosphorylation of the Tyr residue by Wee-1 kinase such that the Tyr(*P*) residue serves as a site recognition determinant for directing phosphorylation to the Thr residue.

While peptide sequences from the putative binding ATP-binding site of mouse ribosomal S_6-kinase,[27] the FcγRIIB1 receptor,[28] and FcεRI-βITAM[28] contain a seryl residue in the +1 position relative to the Tyr(*P*) residue and are potential phosphorylation sites, the respective -GSY(*P*)SV- and -ITY(*P*)SL- sequences of S_6-kinase and the FcγRIIB1 receptor may be suitable candidates for multilevel phosphorylation by virtue that both these contain Ser and Thr residues in the −1 and +1 positions about the Tyr(*P*) residue. In these cases, it is conceivable that phosphorylation of the Ser/Thr residue in the −1 position is one possible event and the generation of the atypical -GS(*P*)Y(*P*)SV- and -IT(*P*)Y(*P*)SL consensus sequences would permit phosphorylation of the downstream Ser residue as a second possible event.

The observed bis-phosphorylation of MAP-kinase at both Tyr-190 and Thr-188 by MAP-kinase kinase[29] presents an unusual case since the phosphorylation of the Thr residue may be directed via prior phosphorylation of the Tyr residue. Although the Thr residue is in the −2 position in this case, the -TEY(*P*)- sequence raises the question as to whether the Tyr(*P*) residue can also direct phosphorylation to the −2 position and is facilitated by the central Glu residue. The preparation of various phosphopeptide substrates based on this sequence for phosphorylation by various kinases may therefore be worthy of study so as to establish the mode of phosphorylation in this system.

In view of our previous finding that a Ser(*P*) residue can surrogate for a Tyr(*P*) residue in atypical consensus sequences, the phosphorylation of such protein sequences may provide a suitable pathway for the generation of phosphate clusters in specific protein systems. From an earlier data base search of proteins[24] which were phosphorylated by CK-2 and related the occurrence of acidic Glu, Asp and phosphorylated residues in the proximity of CK-2 phosphorylation sites, it was interesting that 26 out of 107 identified protein systems contained a seryl or threonyl residue in the +1 or −1 positions of a known phosphorylation site. Indeed, such atypical sequences may suggest that the wide occurrence of phosphate clusters in proteins may occur via 'hierarchical' phosphorylations[30] of atypical phosphorylated sequences and also provide a rationale for the phosphorylation of phosvitin and phosphophoryn by CK-2 despite their lack of typical consensus sequences.[23]

Conclusion

The above study demonstrates that phosphopeptides containing a mixture of Tyr(*P*) and Ser(*P*)/Thr(*P*) residues can be readily prepared in good yields via the 'global' 'phosphite-triester' phosphorylation of pre-assembled peptide resins. While past enzymatic phosphorylation studies have shown that the Tyr(*P*) residue is able to direct phosphorylation to a hydroxy amino acyl residue in the +1 position, the use of ASY(*P*)SA and ATY(*P*)SA in casein kinase-2 phosphorylation studies showed that the Tyr(*P*) residue is also able to direct phosphorylation to a Ser/Thr residue in its −1 position. While the comparison of NEY(*P*)SA with NEY(*P*)TA confirmed that a Ser residue was by far preferred over a Thr residue in the +1 position for phosphorylation by CK-2, the comparison of the phosphorylation data obtained from the two Ala-Xxx-Tyr-Ser-Ala and Ala-Xxx-Tyr(*P*)-Ser-Ala peptide series {Xxx = Glu, Thr(*P*), Ser(*P*)} established that the Tyr(*P*) residue was a major site recognition determinant for phosphorylation of the seryl residue in the +1 position and that an acidic residue in the −1 position relative to the Tyr(*P*) residue was favorable for downstream phosphorylation but was not influenced by the nature of the acidic side chain group. The marked CK-2 phosphorylation of the seryl residue in the three Ala-Xxx-Tyr(*P*)-Ser-Ala peptides {Xxx = Glu, Thr(*P*), Ser(*P*)} suggests that the -Xxx-Tyr(*P*)-Ser- {Xxx = Glu, Thr(*P*), Ser(*P*)} sequence constitutes an atypical consensus sequence which is recognized by CK-2 for the phosphorylation of the downstream seryl residue. Thus, the results of the study described herein suggest that a Tyr(*P*) can direct phosphorylation to its −1 position, and that phosphorylation of this site leads to the generation of an atypical consensus sequence and the possibility of a secondary phosphorylation if a Ser/Thr residue is also available in the +1 position. Indeed, it is conceivable that this enzymatic phosphorylation pathway may play a role in the bio-activity of particular protein/enzyme

systems through multilevel phosphorylation of the substrate.

Experimental

^{13}C NMR spectra of peptides **1**, **2**, **4**, **6** and **7** were obtained as D_2O solutions on a Jeol FX-90Q instrument operating at 22.5 MHz and were referenced to internal dioxan set to 66.5 ppm. The ^{13}C NMR spectra of peptides **3** and **5** were obtained as D_2O solutions on a Jeol GX-400 instrument operating at 100.0 MHz and were referenced to internal dioxan set to 66.5 ppm. ^{31}P NMR spectra of peptides **1–7** were obtained as D_2O solutions on a Jeol FX-100 instrument operating at 40.26 MHz and were referenced to external 85% H_3PO_4. FAB mass spectra were obtained on a Jeol AX-505H mass spectrometer equipped with a FAB source and peptide samples analyzed as aqueous acetic acid:glycerol mulls. Analytical and semi-preparative HPLC was performed on a Beckman System Gold instrument fitted with a semi-preparative Beckman Ultrasphere C_{18} ODS column (10 mm × 25 cm). Preparative HPLC of phosphopeptide **7** was performed on a high pressure Waters instrument (6000A dual pump system equipped with a 720 controller) fitted with a Whatman Partisil 10 ODS-3 Magnum 20 column (2.2 × 50.0 cm) and linked to a Waters 440 detector (254 nm) and a Cecil CE 2012 detector (214 nm).

All solvents were of AnalaR grade. Fmoc-Ala-Resin (0.5 mmol g^{-1}), Fmoc-amino acids, Boc-Ala-OH and PyBOP® were obtained from Novabiochem (Switzerland). Di-*tert*-butyl *N,N*-diethylphosphoramidite was prepared as described elsewhere.[4] 1*H*-Tetrazole, *m*-chloroperoxybenzoic acid and *N*-methylmorpholine were obtained from Aldrich Chemicals. Trifluoroacetic acid was obtained from SDS (France) and piperidine was obtained from Fluka Chemie AG and distilled. Amino acid analyses of the peptides were performed by vapor hydrolysis of the phosphopeptide with 5.7 M HCl (24 h at 110 °C) followed by analysis of the PTC-derivatized hydrolysate on a Waters HPLC instrument, the threonine and serine values being corrected on the basis of 5 and 10% degradation, respectively.

CK-2 was obtained as described elsewhere.[31] The enzymatic phosphorylation of the peptides was essentially performed and quantitated as described elsewhere[32] (procedure *a*) and involved incubating the peptides (1 mM) at 37 °C in a solution containing 50 mM Tris–HCl buffer (pH 7.5), 12 mM $MgCl_2$, 100 mM NaCl, 10 uM [γ-^{32}P]ATP (spec. act. 1000–1500 cpm $pmol^{-1}$) and the protein kinase. The phosphorylation was terminated by the addition of HCl (6 N final concentration), the phosphopeptides hydrolyzed at 110 °C for 4 h and the liberated phosphoserine and phosphothreonine (which co-migrates with phosphotyrosine) isolated by high-voltage paper electrophoresis at pH 1.9 and quantitated. Discrimination of phosphothreonine and phosphotyrosine was effected by elution of the radioactive spot and subsequent 2-D electrophoresis on cellulose plates.[33]

Solid phase peptide synthesis

Solid phase synthesis was performed manually with a solid phase reaction vessel (5.0 cm × 3.0 cm i.d.) containing Fmoc-Ala-Resin (0.5 mmeq g^{-1}) (0.20 g, 0.10 mmol) attached to a rotation instrument. The Fmoc group was cleaved from intermediate peptide resins during peptide growth by a 10 min treatment with 20% piperidine:DMF (5 mL), the solution removed by vacuum suction and the peptide resin washed with CH_2Cl_2 (3 × 10 mL). Amino acid couplings were performed by the addition of PyBOP® (3 equiv) and the N-protected amino acid (3 equiv) to the peptide resin followed by the addition of 1:1 DMF:DCM (5 mL). A solution of *N*-methylmorpholine (4.5 equiv) in DCM (1 mL) was then added and the vessel rotated (47 rev min^{-1}) for 20 min. The solution was removed by vacuum suction, the peptide resin washed with DCM (3 × 10 mL) and then dried under high vacuum.

Phosphorylation of peptide resins

A solution of freshly distilled di-*tert*-butyl *N,N*-diethylphosphoramidite (0.250 g, 1.00 mmol) in dry DCM (1 mL) was added to the peptide resin (0.10 mmol) swollen in dry DMF (4 mL). 1*H*-Tetrazole (0.210 g, 3.00 mmol) was then added in one portion and the vessel rotated for 1 h. The solution was removed by suction, the resin washed with DCM (5 mL) and a solution of 85% *m*-chloroperoxybenzoic acid (0.050 g, 0.25 mmol) in DCM (5 mL) added. The solution was rotated for 10 min, the solvent removed by suction, the peptide resin washed with DCM (5 × 10 mL) and then dried under high vacuum. The peptide resin was then suspended in 5% anisole:TFA (5 mL) and the reaction vessel rotated for 1 h at 20 °C. The solution was collected, the resin washed with TFA (4 mL) and the combined solution then evaporated under reduced pressure. The peptide was precipitated by the addition of diethyl ether (20 mL), triturated with diethyl ether (2 × 10 mL) and then dried under high vacuum to give a white solid. A portion (10.0 mg) of the crude peptide in water (0.25 mL) was applied to a semi-preparative C_{18} reversed-phase column (2 × 5.0 mg) and purified using a linear gradient elution of 0.1% TFA:0–15% CH_3CN over 40 min at a flow rate of 3.0 mL min^{-1}. Lyophilization of the major fraction gave phosphopeptides **1–6** as light, white fluffy solids.

H-Asn-Glu-Tyr(*P*)-Ser-Ala-OH.TFA (1). (55%); δ (^{13}C) (D_2O) (22.5 MHz) 16.1, Ala Me; 26.0, Glu Cβ; 29.6, Glu Cγ; 34.8, Asn Cβ; 36.1, Tyr(*P*) Cβ; 48.6, Ala Cα; 49.6, Asn Cα; 53.1, 54.8 and 55.2, Glu, Ser and Tyr(*P*) Cα; 61.1 Ser Cβ; 120.4 ($J_{P,C}$ 4.4 Hz), Tyr(*P*) Ar C3; 130.3, Tyr(*P*) Ar C2; 132.0, Tyr(*P*) Ar C1; 150.8 ($J_{P,C}$ 6.6 Hz), Tyr(*P*) Ar C4; 168.7, 170.7, 172.3, 172.5

and 172.6, Asn, Glu, Ser and Tyr(*P*) CO, Asn β-CO; 176.0 and 176.7, Ala CO and Glu δ-CO. δ (^{31}P) (D_2O) −4. 1.

H-Ala-Glu-Tyr(*P*)-Ser-Ala-OH.TFA (2). (76%); δ (^{13}C) (D_2O) (22.5 MHz) 16.1 and 16.4, Ala^{1,5} Me; 26.2, Glu Cβ; 29.7, Glu Cγ; 36.3, Tyr(*P*) Cβ; 48.6 and 48.8, Ala^{1,5} Cα; 52.9, 54.7 and 55.1, Glu, Ser and Tyr(*P*) Cα; 61.2, Ser Cβ; 120.4 ($J_{P,C}$ 4.4 Hz), Tyr(*P*) Ar C3; 130.3, Tyr(*P*) Ar C2; 131.6, Tyr(*P*) Ar C1; 150.8 ($J_{P,C}$ 6.6 Hz), Tyr(*P*) Ar C4; 170.6, 170.7, 172.5 and 172.6, Ala,^{1} Glu, Ser and Tyr(*P*) CO; 176.0 and 176.7, Ala^{5} CO and Glu δ-CO. δ (^{31}P) (D_2O) −4.2. FABMS (argon, positive mode)[§] *m/z* (rel. ratio) 658 (M+K, 4%), 642 (M+Na, 6). 620 (M+H, 35), 549 (7), 491 (7), 429 (3), 337 (14), 245 (8), 131 (32). Amino acid analysis: Ala 1.95 (2); Glu 0.95 (1); Ser 0.8 (1); Tyr 1.0 (1).

H-Ala-Ser-Tyr(*P*)-Ser-Ala-OH.TFA (4). (78%); δ (^{13}C) (D_2O) (22.5 MHz) 16.1 and 16.5, Ala^{1,5} Me; 36.3, Tyr(*P*) Cβ; 48.6 and 48.9, Ala^{1,5} Cα; 54.7, 55.1 and 55.2, Ser^{2,4} Cα and Tyr(*P*) Cα; 60.9 and 61.1, Ser^{2,4} Cα; 120.4 ($J_{P,C}$ 4.4 Hz), Tyr(*P*) Ar C3; 130.3, Tyr(*P*) Ar C2; 131.6, Tyr(*P*) Ar C1; 150.8 ($J_{P,C}$ 6.6 Hz), Tyr(*P*) Ar C4; 170.7 (×2), 171.0 and 172.6, Ala^{1}, Ser^{2,4} & Tyr(*P*) CO; 176.0, Ala^{5} CO. δ (^{31}P) (D_2O) −4.1. FABMS (argon, positive mode) *m/z* (rel. ratio) 616 (M+K, 4%), 600 (M+Na, 5). 578 (M+H, 32), 553 (2), 498 (8), 482 (4), 461 (7), 391 (6), 337 (44), 315 (13), 269 (28), 245 (40), 223 (>100), 216 (51), 207 (>100), 207 (>100). Amino acid analysis: Ala 2.1 (2); Ser 1.7 (2); Tyr 1.0 (1).

H-Ala-Thr-Tyr(*P*)-Ser-Ala-OH.TFA (6). (75%); δ (^{13}C) (D_2O) (22.5 MHz) 16.1 and 16.5, Ala^{1,5} Me; 36.4, Tyr(*P*) Cβ; 48.6 and 48.9, Ala^{1,5} Cα; 54.6, Tyr(*P*) Cα; 55.1, Ser Cα; 59.0, Thr Cα; 61.1, Ser Cβ; 67.0, Thr Cβ; 120.4 ($J_{P,C}$ 4.4 Hz), Tyr(*P*) Ar C3; 130.3, Tyr(*P*) Ar C2; 131.6, Tyr(*P*) Ar C1; 150.9 ($J_{P,C}$ 7.3 Hz), Tyr(*P*) Ar C4; 170.7 and 170.8, Ala^{1} and Ser CO; 172.5 and 172.6, Thr and Tyr(*P*) CO; 176.1, Ala^{5} CO. δ (^{31}P) (D_2O) −4.1. FABMS (argon, positive mode) *m/z* (rel. ratio) 630 (M+K, 8%), 614 (M+Na, 5), 592 (M+H, 15), 521 (8), 429 (32), 407 (8), 337 (>100), 315 (35), 245 (60), 233 (>100), 207 (>100), 151 (22), 131 (95), 115 (>100). Amino acid analysis: Ala 1.95 (2); Ser 0.8 (1); Thr 0.85 (1); Tyr 1.0 (1).

H-Ala-Ser(*P*)-Tyr-Ser-Ala-OH.TFA (3). (63%); δ (^{13}C) (D_2O) (100 MHz) 16.06 and 16.34, Ala^{1,5} Cβ; 36.23, Tyr Cβ; 48.61 and 48.87, Ala^{1,5} Cα; 54.06 ($J_{P,C}$ 7.3 Hz), Ser(*P*) Cα; 55.04 (×2), Ser Cα and Tyr Cα; 61.04, Ser Cβ; 64.14 ($J_{P,C}$ 4.4 Hz), Ser(*P*) Cβ; 115.39, Tyr Ar C2; 127.60, Tyr Ar C1; 130.54, Tyr Ar C3; 154.41, Tyr Ar C4; 170.14, 170.64, 170.81 and 172.61, Ala,^{1} Ser(*P*), Tyr and Ser CO; 176.04, Ala^{5} CO. δ (^{31}P) (D_2O) 0.2. FABMS (argon, positive mode) *m/z* (rel. ratio) 616 (M+K, 1%), 600 (M+Na, 2), 578 (M+H, 8), 498 (11), 480 (MH-98, 4), 337 (4), 304 (2), 223 (8), 207 (14), 136 (74), 115 (46), 75 (>100), 56 (>100). Amino acid analysis: Ala 2.05 (2); Ser 1.90 (2); Tyr 1.0 (1).

H-Ala-Thr(*P*)-Tyr-Ser-Ala-OH.TFA (5). (24%); δ (^{13}C) (D_2O) (100 MHz) 16.04 and 16.47, Ala^{1,5} Cβ; 17.77, Thr Cγ; 36.46, Tyr Cβ; 48.59 and 48.83, Ala^{1,5} Cα; 54.68 and 55.01, Ser Cα and Tyr Cα; 57.92 ($J_{P,C}$ 5.9 Hz), Thr(*P*) Cα; 61.04, Ser Cβ; 73.61 ($J_{P,C}$ 5.9 Hz), Thr(*P*) Cβ; 115.34, Tyr Ar C2; 127.64, Tyr Ar C1; 130.54, Tyr Ar C3; 154.35, Tyr Ar C4; 169.73, 170.68, 170.93 and 172.33, Ala,^{1} Thr(*P*), Tyr and Ser CO; 176.05, Ala^{5} CO. δ (^{31}P) (D_2O) −0.5. FABMS (argon, positive mode) *m/z* (rel. ratio) 592 (M+H, 3%), 512 (MH-80, 4), 494 (MH-98, 3), 337 (9), 315 (4), 245 (5), 223 (17), 207 (32), 136 (62), 115 (>100), 75 (>100), 56 (>100). Amino acid analysis: Ala 2.1 (2); Thr(*P*)/Thr 0.95 (1); Ser 0.9 (1); Tyr 1.0 (1).

H-Ala-Ser(*P*)-Tyr(*P*)-Ser-Ala-OH.TFA (7). The bis-phosphorylation of the peptide resin (0.10 mmol) was performed as described above except that the following amounts were used: di-*t*-butyl *N,N*-diethyl-phosphoramidite (0.50 g, 2.00 mmol), 1*H*-tetrazole (0.350 g, 5.00 mmol) and 85% *m*-chloroperoxybenzoic acid (0.101 g, 0.50 mmol). Preparative HPLC of the crude residue was performed using a linear gradient elution of 0.1% TFA:0–10% CH_3CN over 50 min at a flow rate of 10.0 mL min^{-1}. Lyophilization of the major fraction gave phosphopeptide **7** as a light, white fluffy solid. (0.048 g, 62%). δ (^{13}C) (D_2O) (22.5 MHz) 16.1 and 16.4, Ala^{1,5} Me; 36.3, Tyr(*P*) Cβ; 48.6 and 48.9, Ala^{1,5} Cα; 53.9 ($J_{P,C}$ 7.3 Hz), Ser(*P*) Cα; 54.8, Tyr(*P*) Cα; 55.1, Ser Cα; 61.0, Ser Cβ; 67.8 ($J_{P,C}$ 3.7 Hz), Ser(*P*) Cβ; 120.4 ($J_{P,C}$ 4.9 Hz), Tyr(*P*) Ar C3; 130.3, Tyr(*P*) Ar C2; 131.7, Tyr(*P*) Ar C1; 150.7 ($J_{P,C}$ 7.3 Hz), Tyr(*P*) Ar C4; 170.2, 170.7 and 170.7, Ala,^{1} Ser(*P*) and Ser CO; 172.6, Tyr(*P*) CO; 176.1, Ala^{5} CO. δ (^{31}P) (D_2O) 0.06 and −4.40. FABMS (argon, positive mode) *m/z* (rel. ratio) 696 (M+K, 1%), 680 (M+Na, 2), 658 (M+H, 17), 619 (4), 560 (MH−98, 5), 519 (10), 494 (5), 402 (27), 332 (11), 310 (>100). Amino acid analysis: Ala 2.1 (2); Ser 1.8 (2); Tyr 1.0 (1).

[§]For the reported FABMS data, the molecular ion M is defined as the non-protonated peptide.

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