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Phosphorylation of cyclin-dependent kinase 2 peptides enhances metal binding

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ABSTRACT

The cyclin-dependent kinase CDK2 is inactivated by phosphorylation on either of the two neighbouring residues Thr14 or Tyr15. The effect of phosphorylation on metal ion binding has been investigated with peptides incorporating residues 6–20 of CDK2. The stoichiometry of Ca^{2+} binding increased from 1 in the un- and singly-phosphorylated peptides to 2 in the doubly phosphorylated peptide, without large changes in the affinity (75–250 μM). In contrast although binding of ferric ions to the un-phosphorylated peptide was not detected, both singly- and doubly-phosphorylated peptides bound two Fe^{3+} ions. Binding of Ca^{2+} or Zn^{2+} ions to the doubly phosphorylated CDK2 peptide did not cause any change in absorbance, but increased the affinity of the peptide for Fe^{3+} ions. These results demonstrate that double phosphorylation of CDK2 peptides increases the stoichiometry of metal ion binding, and hence may contribute to the previously observed regulation of CDK2 activity by metal ions.

The cyclin-dependent kinases CDK1 (Cdc2) and CDK2 are key regulators of the eukaryotic cell cycle [1,2]. Human CDK1 has been implicated in the control of mitosis, while CDK2 is involved in the control of G1 and S phase events. CDK1 and CDK2 are themselves regulated by phosphorylation by several specific kinases. Phosphorylation of CDK1 on Thr14 within the ATP binding pocket by Myt1 [3] or on Tyr15 by Myt1 [3] or Wee1 [4] results in inactivation [5], and phosphorylation on Thr161 by Cdk-activating kinase (CAK) results in activation [6]. The closely related kinase CDK2 is also inactivated by phosphorylation of Thr14/Tyr15, and in this case the crystal structure reveals that the hydroxyl group of the Thr14 side chain is very close to the phosphate of ATP, so that phosphorylation would presumably disrupt the normal interaction between the enzyme and ATP [7,8]. The observation that several iron-selective chelators reduce CDK2 activity in 293 cells further suggests that the activity of the enzyme may be regulated by metal ions [9].

We have previously reported that phosphorylation of the sole tyrosine of the octapeptide hormone cholecystokinin (CCK_8) creates an additional binding site for Ca^{2+} or Fe^{3+} ions [10]. The proximity of the Thr14 and Tyr15 phosphorylation sites in CDK1 and 2 suggested that metal ion binding to the doubly phosphorylated peptide might be stronger than to either singly phosphorylated peptide. We have therefore investigated the binding of metal ions to un-phosphorylated (CDK2), singly threonine phosphorylated (pT-CDK2), singly tyrosine phosphorylated (pY-CDK2), and doubly phosphorylated (pTpY-CDK2) synthetic peptides based on residues 6–20 of the human CDK2 sequence, KVEKIGEGTYGVVYK. Binding of Ca^{2+} ions was measured with a calcium-selective electrode, and binding of Fe^{3+} ions was followed by changes in absorbance.

Materials and methods

Peptides. The CDK2 peptide (corresponding to residues 6–20 of the human CDK2 sequence, KVEKIGEGTYGVVYK) and pY-CDK2 (over 95% pure) were gifts from H.-C. Cheng (University of Melbourne). Both peptides were synthesized by Fmoc chemistry and purified by reverse-phase high performance liquid chromatography [11]. pT-CDK2 and pTpY-CDK2 (84% and 94% pure, respectively) were from Mimotopes (Clayton, Australia). The impurities consisted of water and salts.

Measurement of calcium binding. The change in free [Ca^{2+}] during addition of aliquots of calcium chloride to peptides (35–40 μM) in 10 mM Na^+ PIPES, pH 6.5, 100 mM NaCl, 0.005% Tween 20, 0.4% DMSO was measured at 20 °C with a uniPROBE calcium-selective electrode (TPS, Springwood, Australia) connected to a Hanna 8521 pH meter (Hanna Instruments, Tullamarine, Australia), as described by Park and coworkers [12]. The electrode was first calibrated with solutions of known [Ca^{2+}] in the range 1–1000 μM in the same buffer. The concentration of Ca^{2+} ion bound to each peptide was calculated by subtraction of the free [Ca^{2+}] from the total added [Ca^{2+}].

Absorbance spectroscopy. Absorbance of peptides at 275 nm (40 μM in 10 mM Na acetate (pH 4.0) or 10 mM Na PIPES (pH 6.5) containing 100 mM NaCl and 0.005% Tween 20) in the pres-

Abbreviations: CAK, Cdk-activating kinase; CCK_8, cholecystokinin; CDK, cyclin-dependent kinase

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ence of increasing concentrations of Fe^{3+} ions were measured against a buffer blank, in 1 ml quartz cuvettes thermostatted at 25 °C, with a Cary 5 spectrophotometer (Varian, Mulgrave, Australia).

Curve fitting and statistics. Data (expressed as means ± SEM) for the independent binding of Fe^{3+} or Ca^{2+} ions to CDK2 peptides were fitted to one-site or two-site ordered models with the program BioEqs [13,14].

Results

Binding of calcium ions to phosphorylated CDK2 peptides

To determine whether or not phosphorylation of CDK2 peptides increased their affinity for calcium ions, the changes in free [Ca^{2+}] during addition of aliquots of calcium chloride to CDK2, pT-CDK2, pY-CDK2 or pTpY-CDK2 were measured with a calcium-selective electrode. A pH of 6.5 was chosen to maximize the chance of detecting an interaction; data could not be obtained at pH 4.0 because that pH was outside the optimum range of the calcium-selective electrode. The binding curves (Fig. 1) indicate that CDK2, pT-CDK2 and pY-CDK2 each bound 1 mol calcium/mol peptide, with dissociation constants of 162, 253 and 187 μM, respectively. For pTpY-CDK2 the data were better fitted by a two-site (K_{d1} 97 μM, K_{d2} 75 μM, $\chi^2 = 3140$) than a one-site model (K_d 18 μM, $\chi^2 = 67{,}800$). We conclude that addition of a second phosphate group to either singly phosphorylated peptide generates a second calcium binding site.

Binding of ferric ions to phosphorylated CDK2 peptides

The effect of addition of ferric ions on the absorbance of CDK2, pT-CDK2, pY-CDK2 or pTpY-CDK2 was first investigated at pH 4.0. This pH value was chosen in order to avoid any problems with precipitation of ferric hydroxides. Although no change in the absorbance of CDK2 was observed at 275 nm, the absorbance of pT-CDK2, pY-CDK2 or pTpY-CDK2 increased to maxima of 201%, 260% and 324%, respectively, after the addition of 2 mol ferric chloride/mol peptide (Fig. 2A). A two-site model gave a reasonable fit to the experimental data for pTpY-CDK2 (Fig. 2A). The dissociation constants were 165 and 747 nM, and the maximum absorbances 274% and 335%, for the first and second sites, respectively. Although the titrations for pT-CDK2 or pY-CDK2 clearly indicated that the stoichiometry of ferric ion binding was two, the absorbance data could not be fitted satisfactorily with a two-site model with the program BioEqs, as was observed previously with pY-CCK_8 at the same pH [10]. We conclude that CDK2 does not bind ferric ions, but that pT-CDK2, pY-CDK2 and pTpY-CDK2 each bind two ferric ions. Hence addition of a single phosphate group to CDK2 creates two additional iron binding sites, but addition of a second phosphate group to either singly phosphorylated peptide does not generate any additional iron binding sites.

The titration experiments were then repeated at pH 6.5, to assess the contribution to ferric ion binding of ionization of the phosphoryl groups of the phosphothreonine (p*K*a 6.1 [15]) and phosphotyrosine (p*K*a 5.9 [15]) residues. Under these conditions,

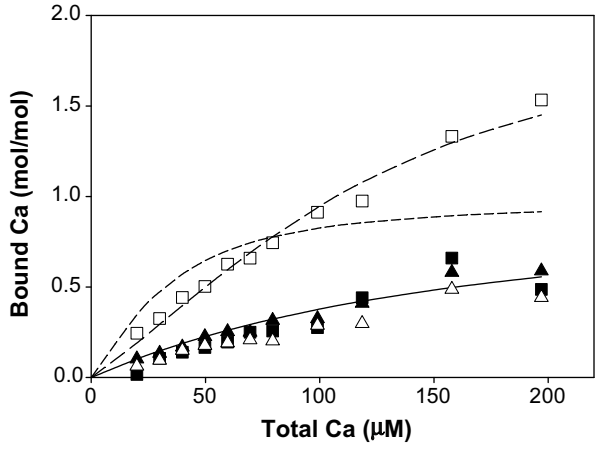

Fig. 1. Double phosphorylation enhances Ca^{2+} binding to CDK2 peptides. The change in free [Ca^{2+}] during addition of aliquots of calcium chloride to 40.0 μM CDK2 (▲), 37.4 μM pT-CDK2 (△), 40.0 μM pY-CDK2 (■) or 35.6 μM pTpY-CDK2 (□) in 10 mM Na^+ PIPES, pH 6.5, 100 mM NaCl, 0.005% Tween 20, 0.4% DMSO was measured at 20 °C with a calcium-selective electrode as described in Materials and methods. Points are means of at least three separate experiments; lines represent the best fits with the program BioEqs to one-site and two-site models, respectively. Binding of Ca^{2+} to CDK2, pT-CDK2 and pY-CDK2 was well fitted by a one-site model (solid line). The pTpY-CDK2 data were better fitted by a two-site (long dashed line) than a one-site model (short dashed line).

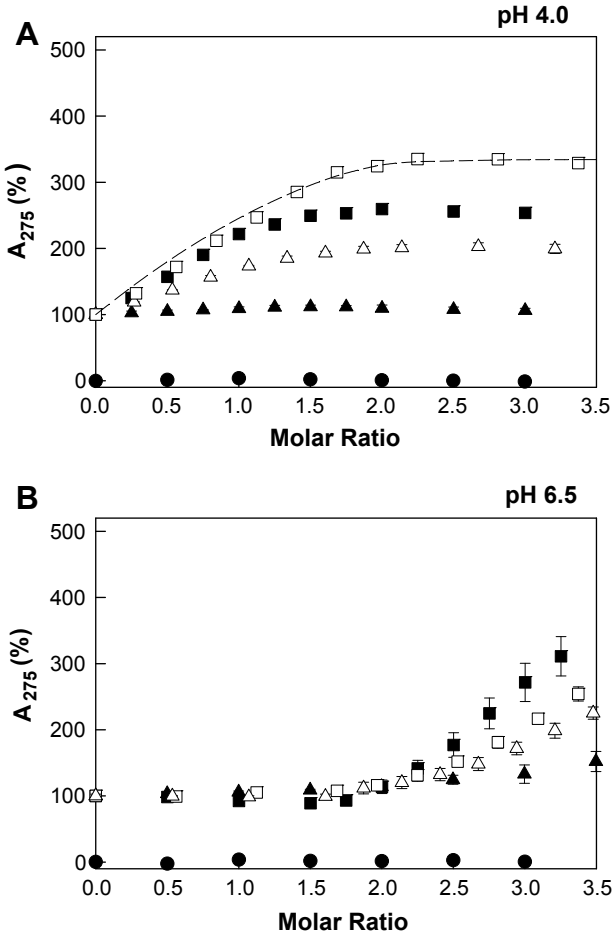

Fig. 2. Phosphorylation enhances Fe^{3+} binding to CDK2 peptides. At pH 4.0 (A), addition of aliquots of ferric chloride to 37.4 μM pT-CDK2 (△), 40.0 μM pY-CDK2 (■) or 35.6 μM pTpY-CDK2 (□) in 10 mM Na^+ acetate, 100 mM NaCl, 0.005% Tween 20, 0.4% DMSO resulted in an increase in the absorbance at 275 nm up to a molar ratio of 2 at 25 °C. Addition of aliquots of $FeCl_3$ to buffer alone (●) or to 40.0 μM CDK2 (▲) did not cause any change in absorbance. At pH 6.5 (B) in 10 mM Na^+ PIPES, 100 mM NaCl, 0.005% Tween 20, 0.4% DMSO, an increase in absorbance at 275 nm was observed on addition of ferric chloride to all four peptides. Data are expressed as a percentage of the absorbance of that peptide without ferric ions. Points are means of at least three separate experiments; bars represent the SEM. The long dashed line in (A) represents the best fit to a two-site ordered model with the program BioEqs; the appropriate K_d values and maximum absorbance values are given in the text.

no precipitation of iron hydroxides was observed during the course of the experiment provided the total ferric ion concentration did not exceed 100 μM (i.e., a molar ratio iron/peptide = 2.5). None of the peptide absorbances increased greatly during the addition of ferric ions to a molar ratio of two (Fig. 2B). Although the peptide absorbances increased on addition of further Fe^{3+} ions, and the changes differed between peptides and were not seen in the buffer only control (Fig. 2B), the data was not analysed further because the contribution of precipitation of iron hydroxides to the changes in absorbance was not known.

The effect of calcium or zinc ions on the binding of ferric ions to the doubly phosphorylated CDK2 peptide

To determine whether or not binding of other metal ions to pTpY-CDK2 enhanced the binding of ferric ions, the effects of prior addition of calcium or zinc ions on the changes in absorbance of pTpY-CDK2 in response to ferric ions were investigated. Neither of the above metal ions caused any increase in the absorbance maximum at 275 nm for pTpY-CDK2 (Fig. 3A and B) at pH 6.5. For pTpY-CDK2 with added Ca^{2+} (Fig. 3A), subsequent addition of ferric ions caused an increase in absorbance greater (2.3-fold at a molar ratio of 2 mol iron/mol peptide) than the 1.2-fold increase observed in the presence of ferric ions alone. A similar effect was observed in the presence of Zn^{2+} (Fig. 3B). An ordered two-site model gave a reasonable fit to the experimental data in both cases. The dissociation constants for binding of Fe^{3+} ions in the presence of Ca^{2+} ions were 220 and 2.56 μM, and the maximum absorbances 135% and 264%, for the first and second sites, respectively. The dissociation constants for binding of Fe^{3+} ions in the presence of Zn^{2+} ions were 2 and 7.14 μM, and the maximum absorbances 144% and 261%, for the first and second sites, respectively. The effect of Fe^{3+} ions on Ca^{2+} binding could not be measured with the calcium-selective electrode because ferric ions interfered with the electrode signal. We conclude that the first ferric ion binding site can also bind a Ca^{2+} or Zn^{2+} ion without any effect on peptide absorbance, but that binding of a Ca^{2+} or Zn^{2+} ion to the first site increases the changes in absorbance on, and enhances the affinity of, binding of ferric ions to the second site.

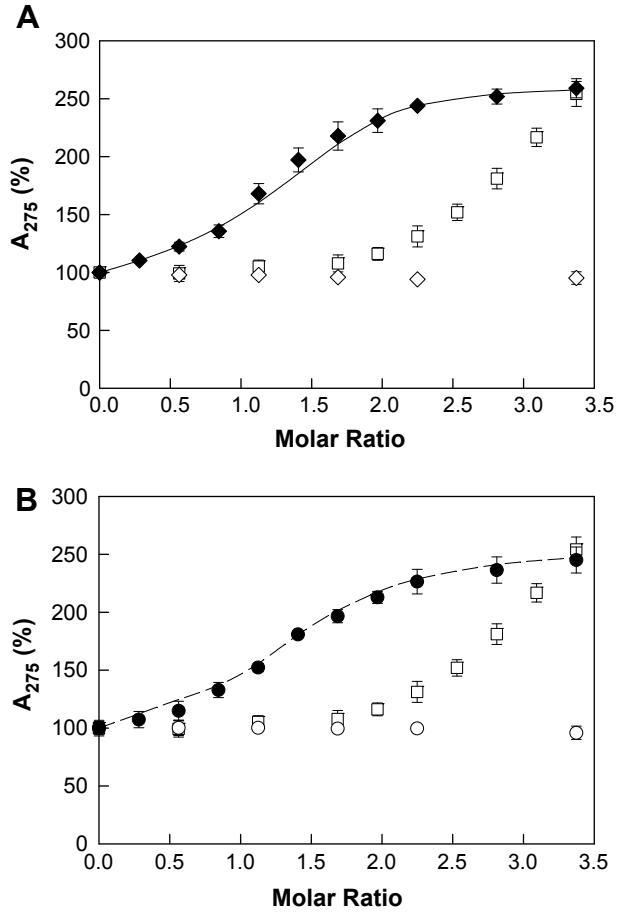

Fig. 3. Calcium or zinc ions enhance subsequent ferric ion binding to phosphorylated CDK2. Addition of aliquots of calcium chloride (A, ◇) or zinc sulfate (B,○) to 40 μM pTpY-CDK2 in 10 mM Na^+ PIPES, 100 mM NaCl, 0.005% Tween 20, 0.4% DMSO, did not result in any increase in the absorbance at 275 nm up to a molar ratio of 3.5. Subsequent addition of aliquots of ferric chloride to the solutions containing calcium (♦) or zinc (●) resulted in a greater increase in absorbance at molar ratios less than 3 than the increase seen when ferric ions were added to pTpY-CDK2 in the absence of calcium or zinc ions (□). Points are means of at least three separate experiments; bars represent the SEM. The lines represent the best fits to a two-site ordered model with the program BioEqs, with the K_d and A_{275} values given in the text.

Discussion

We previously reported that phosphorylation of the sole tyrosine of CCK_8 created an additional binding site for Ca^{2+} and Fe^{3+} ions [10]. Similarly phosphorylation of the sole tyrosine of a peptide derived from α-synuclein created an additional binding site for Tb^{3+} ions [16,17]. In the present paper these studies have been extended by investigation of the effect on metal ion binding of phosphorylation on the adjacent Thr14 and Tyr15 residues of a peptide corresponding to residues 6–20 of the human CDK2 sequence.

In the case of Ca^{2+} ions CDK2, pT-CDK2 and pY-CDK2 each bound 1 mol calcium/mol peptide, with dissociation constants of 162, 253 and 187 μM, respectively, while pTpY-CDK2 bound 2 mol calcium/mol peptide with similar dissociation constants for the two sites (K_{d1} 97 μM, K_{d2} 75 μM). In contrast, although CCK_8 also bound 1 mol calcium/mol peptide with a dissociation constant of 83 μM, pY-CCK_8 bound 2 mol calcium/mol peptide, with dissociation constants that differed by more than 10-fold (K_{d1} 46 μM, K_{d2} 580 μM). One possible explanation for the difference between pY-CDK2 and pY-CCK_8 is that other residues in the CCK_8 sequence are also involved in binding Ca^{2+} ions.

In the case of Fe^{3+} ions at pH 4.0, no binding was detected to the CDK2 peptide. For pT-CDK2 or pY-CDK2, although the titrations clearly indicated that the stoichiometry of ferric ion binding was two, the absorbance data could not be fitted satisfactorily with a two-site model with the program BioEqs. pTpY-CDK2 also bound 2 mol iron/mol peptide, and the dissociation constants for the two sites were similar (K_{d1} 0.17 μM, K_{d2} 0.75 μM), and 500-fold stronger than the dissociation constants for Ca^{2+} ion binding. Similarly no binding of Fe^{3+} ions to CCK_8 was detected at pH 4.0 and, while pY-CCK_8 bound 2 mol iron/mol peptide, the data could not be fitted satisfactorily with a two-site model. The binding of Fe^{3+} ions to pY-CDK2 and pY-CCK_8 thus appears to be similar.

In the case of Fe^{3+} ions at pH 6.5, no binding was detected to any CDK2 peptide on addition of up to 2 mol iron/mol peptide. Although the peptide absorbances increased on addition of further Fe^{3+} ions, and the changes differed between peptides, the commencement of precipitation of iron hydroxides in the buffer control at molar ratios greater than 2.5 (i.e., 100 μM Fe^{3+}) suggests that these changes should be treated with great caution. In contrast, CCK_8 bound 1 mol ferric ions/mol peptide with a dissociation constant of 0.6 μM, while pY-CCK_8 bound 2 mol ferric ions/mol peptide, with similar dissociation constants (K_{d1} 0.68 μM, K_{d2} 0.77 μM).

The effect of prior binding of Ca^{2+} or Zn^{2+} ions on the binding of Fe^{3+} ions at pH 6.5 also differed between the CDK2 and CCK_8 pep-

tides. With pY-CCK_8 in the presence of Ca^{2+} or Zn^{2+} ions the addition of Fe^{3+} ions caused an increase significantly greater (3.5-fold at a molar ratio of 1.5) than the 1.5-fold increase observed in the presence of Fe^{3+} ions alone. With pTpY-CDK2 the addition of Ca^{2+} or Zn^{2+} ions resulted in a leftward shift in the titration curve,with little alteration in the maximum change in absorbance. The data is consistent with the conclusion that the two ferric ion binding sites can also bind Ca^{2+} or Zn^{2+} ions without any effect on peptide absorption, but that binding of a Ca^{2+} or Zn^{2+} ion to the first site enhances the affinity of binding of a ferric ion to the second site. Further work will be required to define the precise mechanism of this effect.

In conclusion the results presented herein clearly demonstrate that phosphorylation of the CDK2 peptide increases the stoichiometry of Ca^{2+} ion binding from 1 in the un- and singly-phosphorylated peptides to 2 in the doubly phosphorylated peptide, without large changes in affinity. In contrast Fe^{3+} ions do not bind to the CDK2 peptide, but both singly- and doubly-phosphorylated peptides bind two Fe^{3+} ions. Since phosphorylation of CDK1 on Thr14 or Tyr15 inactivates kinase activity [5], the results of the present study imply that Ca^{2+} and/or Fe^{3+} ions may directly regulate CDK activity. This conclusion is consistent with the previous report that several iron-selective chelators have been shown to reduce CDK2 activity in 293 cells [9]. Calcium-dependent regulation of the activity of CDK1 and CDK2, which are key regulators of the eukaryotic cell cycle [1,2], may also be important in the development of the mammalian oocyte, in which Ca^{2+} spikes provide the necessary and sufficient trigger for fertilization [18].

Acknowledgments

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