Abstract
Protein kinases are an important class of substrate of the protein phosphatases. We have examined the mechanism of dephosphorylation of the activation segments of the insulin receptor kinase and cyclin-dependent kinase 2 by their respective phosphatases, namely the tyrosine specific phosphatase PTP1B and the dual specificity phosphatase KAP. These studies reveal that PTP1B and KAP utilize contrasting mechanisms in order to dephosphorylate their substrates specifically.

Introduction
The regulation of cellular processes by reversible protein phosphorylation is diverse and ubiquitous. The overall level of protein phosphorylation is controlled by the opposing and dynamic activities of protein kinases and phosphatases that catalyse protein phosphorylation and dephosphorylation events respectively. Insights into the regulation and specificity of the reactions catalysed by protein kinases and phosphatases are the key to understanding the process of protein phosphorylation. Many protein kinases are themselves regulated by phosphorylation, either resulting from autophosphorylation or by the actions of upstream kinases. Protein kinases therefore form an important class of substrates of the protein phosphatases. By dephosphorylating protein kinases, a protein phosphatase is capable of exerting both acute and global changes in the levels of protein phosphorylation. Similar to the regulation of other phosphoproteins, dephosphorylation of protein kinases results in either their activation or inactivation. A general mechanism for the regulation of protein kinases is the reversible phosphorylation of residues within the activation segment of the kinase [1]. Phosphorylation of residues within the activation segment is associated with an increase in kinase activity and therefore protein phosphatase-catalysed dephosphorylation at these sites is generally associated with negative regulation of a protein phosphorylation cascade.

The protein phosphatases are encoded by three major families, which include the serine/threonine protein phosphatases of the PPP and PPM families [2,3] and the protein tyrosine phosphatase (PTP) superfamily. The conventional PTPs are specific for the dephosphorylation of phosphotyrosine (pTyr)-containing proteins; however, a subfamily of the PTPs, termed the dual specificity protein phosphatases (DSPs), are characterized by their ability to dephosphorylate all three major phospho-amino acids of eukaryotic cells. To obtain insights into the mechanisms by which protein phosphatases regulate protein kinases, we have determined the structures of protein phosphatases in complex with phospho-protein kinase substrates. First, we have determined the structure of the protein tyrosine phosphatase PTP1B in complex with a pTyr-containing peptide derived from the activation segment of the insulin receptor (IR) kinase (IRK) and, secondly, we have determined the structure of a complex formed between the DSP called KAP (kinase-associated phosphatase; also termed Cdi1, Cip2) and phospho-cyclin-dependent kinase 2 (CDK2).

In this paper I compare and contrast the mechanism of dephosphorylation of pTyr-containing substrates by the PTPs, exemplified by the PTP1B-catalysed dephosphorylation of the activation segment of the IRK, with that of the DSP KAP-mediated dephosphorylation of pThr160 of CDK2.

PTP1B–IRK complex
Insulin signalling pathways initiate at the IR, which is comprised of two extracellular α-subunits and two transmembrane β-subunits. Activation of the receptor results in the autophosphorylation of both β-subunits on tyrosine residues. The major sites of autophosphorylation within the protein tyrosine kinase (PTK) domain of the β-subunit are Tyr1158, Tyr1162 and Tyr1163 [4], which are con-
tained within the PTK activation segment [5]. Autophosphorylation of all three sites is required for maximal activation [5]. Following activation, additional autophosphorylation reactions occur in the juxtamembrane segment and the C-terminal tail of the β-subunit [5].

Much preliminary data have implicated PTP1B in the regulation of IR signalling. Micro-injection of purified PTP1B into Xenopus oocytes inhibited insulin-induced activation of S6 kinase and transition through the G1/M-phase checkpoint. When overexpressed in Rat1 fibroblasts expressing the IR, PTP1B inhibited hormone-induced phosphorylation of the IR and downstream substrates, and inhibited hormone-induced incorporation of glucose into glycogen [6]. Furthermore, osmotic loading of neutralizing antibodies against PTP1B into hepatoma cells enhanced both insulin-induced receptor autophosphorylation and phosphorylation of the IR substrate IRS-1, suggesting a direct effect of the phosphatase on the IR [7]. Such direct effects were also supported by the demonstration of association between inactive Cys→Ser mutant forms of PTP1B, which may serve as substrate 'traps', and the IR [8]. The most compelling data implicating PTP1B as the IRK phosphatase were established from the studies of PTP1B knock-out mice. Disruption of the PTP1B gene yielded healthy mice that displayed enhanced sensitivity to insulin [9]. When fed a high-fat diet, the PTP1B knock-out mice were resistant to becoming obese and remained insulin-sensitive, in contrast with their normal littermates, who displayed a rapid gain of weight and insulin resistance. These effects were correlated with enhanced tyrosine phosphorylation of the IR in the PTP1B−/− animals. Together, these data illustrate that the IR is an important physiological substrate of PTP1B, and identify this phosphatase as a novel therapeutic target for the treatment of diabetes and obesity.

To understand how PTP1B dephosphorylates the IRK, we co-crystallized PTP1B in the presence of a series of pTyr peptides corresponding to the activation segment of the kinase [10]. The general features of all IRK phosphopeptides bound to PTP1B are similar to those of the previously determined epidermal growth factor receptor (EGFR) peptide–PTP1B complex [11]. The catalytic site of PTP1B is formed from a cleft on the protein’s molecular surface. The base of the cleft comprises residues of the conserved PTP signature motif (referred to as the PTP loop), with the phosphate group of the substrate co-ordinated by main-chain amide groups and the side chain of the Arg221 residue in this motif. Within the signature motif, the invariant cysteine residue (Cys215) is positioned to function in a nucleophilic attack on the phosphate moiety of the substrate. The depth of the cleft is determined by an invariant tyrosine, Tyr4′, from the pTyr-recognition loop, which is a major determinant of specificity for pTyr. Binding of substrate induces a conformational change in the protein in which the WPD loop (residues 179–187), containing the invariant aspartate residue (Asp185), closes around the substrate to create the pTyr recognition pocket and generates the catalytically competent form of the enzyme. Engagement of the pTyr residue in the substrate involves a number of hydrophobic interactions between the phenyl group of the pTyr residue and residues lining the cleft, which anchors the peptide to the substrate-binding site. The IRK peptide binds to PTP1B with an orientation perpendicular to the parallel region of the central β-sheet, with residues of the peptide flanking the substrate pTyr residue adopting an extended β-strand conformation (Figure 1).

A feature of the pTyr-substrate recognition by PTP1B is the nature of the extensive contacts formed between the enzyme, the pTyr-substrate residue and residues on the peptide that flank the pTyr residue. The bisphosphopeptide, which is phosphorylated on tyrosines corresponding to Tyr1162 and Tyr1163 in the IR activation segment sequence, interacts with PTP1B in a defined manner, such that pTyr1162 is located at the catalytic site, inducing a substrate selectivity for pTyr1162 relative to pTyr1163 (Figure 1). This mode of peptide recognition results from the extensive and specific PTP1B–peptide interactions involving residues both N- and C-terminal to the substrate pTyr residue, in particular Asp1161, pTyr1163 and Arg1164. The pTyr residue at the catalytic site (pTyr1162) interacts with PTP1B in a manner identical with that observed for the pTyr residue in the EGFR peptide–PTP1B complex [11]. The phenyl ring of pTyr1162 is sandwiched between the aromatic side chains of Tyr4′ and Phe162 (Figure 1). Bidentate hydrogen bonds between the carboxylate side chain of Asp48 and the peptide amide (-NH) groups of pTyr1162 and pTyr1163 (P + 1) are important in orienting the peptide.

The second pTyr residue of the peptide, pTyr1163 (P + 1), is located within a shallow groove on the protein surface connected via a channel to the catalytic pTyr-binding cleft. Interactions be-
between pTyr\textsuperscript{1163} and the protein are dominated by the salt bridges between its phosphate group and the side chains of Arg\textsuperscript{24} and Arg\textsuperscript{204} of PTP1B, an invariant residue in the PTP family. The phenyl ring of pTyr\textsuperscript{1163} participates in long contacts with the amide side chain of Gln\textsuperscript{362} and the side chain of Met\textsuperscript{388}. The specificity of this site for a pTyr residue C-terminal to the substrate pTyr, rather than the shorter pSer and pThr residues, is primarily due to the length of pTyr, which is the correct size to allow its phosphate group to reach Arg\textsuperscript{24} and Arg\textsuperscript{204}. The presence of a glycine residue at position 259 is a critical structural feature of this site in PTP1B that allows an interaction with a bulky pTyr residue. Amino acids with more bulky side chains would sterically hinder these interactions.

In addition to interactions involving the tandem pTyr residues, the bisphosphorylated peptide also makes several other interactions with PTP1B. First, N-terminal to the substrate pTyr residue, the carboxylate group of Asp\textsuperscript{1161} (P-1) of the IR peptide accepts a hydrogen bond from the guanidinium group of Arg\textsuperscript{47} of PTP1B. This interaction is responsible for the preference of PTP1B towards peptides with acidic residues N-terminal to the substrate pTyr residue. Similar interactions were observed between the glutamate (P-1) and aspartate (P-2) residues of the EGFR peptide in complex with PTP1B [11], although the conformation of the Arg\textsuperscript{47} side chain differs between the two complexes.

A distinguishing feature of the bisphosphopeptide–PTP1B complex is the presence of peptide–protein interactions involving residues C-terminal to the substrate pTyr. In addition to pTyr\textsuperscript{1163} (P+1), residues at P+2 and P+4 also interact with the protein. The P+2 residue is Arg\textsuperscript{116}, and its guanidinium side chain forms a classic \(\pi\)-cation interaction with the phenyl ring of Phe\textsuperscript{182} of the WPD loop (Figure 1). The guanidinium group of Arg\textsuperscript{116} is positioned parallel to the phenyl ring of Phe\textsuperscript{182}, a geometry that is preferred in protein structures. The Arg–Phe interaction illustrates the concerted character of the peptide–protein interactions, in that it requires

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**Figure 1**

PTP1B in complex with the IRK bisphosphopeptide

the WPD loop to adopt the closed conformation that is induced by the interaction of the substrate pTyr residue at the catalytic site.

The structural data presented here, which indicate that PTP1B recognizes tandem pTyr residues via interactions that principally involve the phosphate group of the P + 1 pTyr residue, are consistent with kinetic data showing that peptides phosphorylated on both Tyr1182 and Tyr1183 bind to PTP1B with ~100-fold higher affinity than peptides singly phosphorylated on Tyr1182 or Tyr1183 alone [10]. Examination of the sequences of other PTPs in the context of the PTP1B–IRK interactions defined by the PTP1B–IRK structure provides insights concerning the specificity of this interaction. As expected, residues of PTP1B that interact with the pTyr substrate are well conserved throughout tyrosine-specific phosphatases. However, residues of the peptide that flank the substrate pTyr residue interact with residues of PTP1B that are variable throughout the PTP family, and this finding supports the notion that the interactions between PTP1B and IRK will be specific to this phosphatase–peptide interaction.

**KAP–pCDK2 complex**

Progression through the cell cycle is co-ordinated by a family of CDKs composed of a protein kinase catalytic subunit and a regulatory cyclin subunit. The principal transitions of the cell cycle are triggered by oscillations of CDK activity, a complex process regulated by reversible protein phosphorylation and controlled synthesis and degradation of activator and inhibitor subunits [12].

The inactive monomeric CDK subunit becomes activated through protein conformational changes accompanied by its association with a specific cyclin molecule and phosphorylation of a conserved and essential threonine residue within the activation segment of the kinase (Thr140 of CDK2). Cyclin degradation and pThr dephosphorylation inactivate the kinase.

Genetic and biochemical studies have implicated KAP as one of the protein phosphatases responsible for dephosphorylating the activating pThr residue of human CDK1, CDK2 and CDK3 [13–16]. The enzyme, discovered using a yeast two-hybrid genetic screen as a CDK1-, CDK2- and CDK3-interacting protein [13–15], is a member of the DSP family, and is capable of dephosphorylating artificial protein substrates containing both pThr and pTyr residues. KAP associates with CDK1 and CDK2 in vivo and in vitro [15,16], and, in HeLa cells, there is evidence that KAP is present in complexes that include both phospho- and nonphospho-CDK2 and the CDK2-associated cyclins A and E [13]. Recognition that KAP dephosphorylates pThr140 of CDK2 was made by Poon and Hunter [16], who found that KAP purified either from HeLa cells or from overexpression in *Escherichia coli* dephosphorylates the pThr140 residue of monomeric CDK2, but is unable to dephosphorylate pTyr15. Dephosphorylation of pThr140 is dependent upon the tertiary structure of CDK2, a feature that distinguishes KAP from the non-specific PP2A catalytic subunit. Heat-denatured pCDK2 is not a substrate of KAP, and cyclin A association with pCDK2 prevents pThr140 dephosphorylation without blocking pCDK2–KAP interactions. The latter finding suggests that cyclin A controls the accessibility of pThr140 towards KAP, providing a mechanism of phosphatase regulation. The role of KAP in dephosphorylation of the activating pThr residue of pCDK2 explains the observation that overexpression of wild type, but not a catalytically inactive form of KAP, retards cell-cycle progression at G1 [13], and is consistent with the reduced levels of pCDK2 in HEK-293 cells overexpressing KAP [16].

In order to understand the mechanism of dephosphorylation of pThr140 of pCDK2 by KAP, and how this activity is modulated by the association of cyclin A, we have determined the crystal structures of KAP and of the pCDK2–KAP complex [17]. A Michaelis–Menten complex was formed between CDK2 phosphorylated on Thr140 and a catalytic site mutant of KAP (Cys148Ser). The essential features of the KAP structure are similar to those of PTP1B [10].

KAP has a compact structure comprising a central twisted five-stranded β-sheet surrounded by seven α-helices (Figure 2). The topology of KAP is almost identical with that of the DSPs VHR (‘*Vaccinia* H1-related phosphatase’) [18] and (the catalytic domain of) MKP-3 [‘mitogen-activated-protein kinase (MAPK) phosphatase-3’] [19], and represents the core secondary structure of the PTP superfamily. In common with both tyrosine-specific and dual specificity members of the PTP family, the catalytic site is located at the centre of the molecule, with the base of the site formed from the conserved PTP signature motif. The pTyr recognition segment that creates a deep catalytic site cleft specific for pTyr substrates is absent from the smaller DSPs, thereby creating a more open and shallow catalytic-site cleft suitable for the recognition of the shorter pSer and pThr...
residues (Figure 2). A structural feature of KAP not seen in other PTPs and DSPs is an eight-residue antiparallel β3/β4-hairpin inserted between the β2 strand and α2 helix. This hairpin structure projects above the surface of the catalytic face of the molecule, and plays a role in forming the pCDK2–KAP interface (Figure 2).

In the pCDK2–KAP complex, pCDK2 adopts its characteristic 'bilateral' structure comprising an N-terminal lobe rich in β-sheet and a C-terminal lobe that is mostly α helical (Figure 2). The catalytic site for ATP is located between the two lobes, whereas that for peptide substrate is associated with the activation segment of the C-terminal lobe. The striking feature of the pCDK2–KAP complex is the manner in which KAP interacts with the kinase through an extensive protein–protein interface. KAP is positioned on to the pCDK2 molecule so that the catalytic site of the phosphatase faces towards the catalytic site of the kinase, and critically towards the phosphorylated activation segment (Figure 2). Interactions between the two proteins involve three distinct recognition sites. First, the catalytic site of KAP recognizes the pThr169 residue from the activation segment of CDK2. The activation segment is pulled away from the CDK2 surface in order to make this contact. Secondly, residues from KAP from the C-terminal helix dock on to a site on CDK2 that includes residues from the start of the αG helix (which contains the motif GDSEID in CDK1, CDK2 and CDK3) and the L14 loop. Thirdly, the β3/β4 loop of KAP contacts CDK2 residue Tyr189, at the turn between β1 and β2 strands of the N-terminal lobe sheet (Figure 2). The conformation of KAP in the pCDK2–KAP complex is essentially identical with that of the sulphate-ligated molecule. In contrast, the conformation of pCDK2 in the complex differs significantly from that of monomeric pCDK2.

Structure of the pCDK2–KAP interface

The only significant interaction between KAP and pCDK2 at the catalytic site is the interaction between the phosphate group of the pThr169 residue of pCDK2 and the PTP loop of KAP. In contrast with the recognition of pTyr-containing peptides by PTP1B, where both the phospho-amino acid itself and its context in the sequence is important in defining the recognition process [10,11], KAP activity is not determined by either the identity of the phospho-amino acid or its sequence context. The affinity and specificity of the complex between KAP and CDK2 results from interactions at the second recognition site comprising CDK2 residues 205–210 (GDSEID of the αG helix) and 235–237 (DYK of the L14 loop)
and a region of KAP that is remote from the catalytic site. The KAP residues from the C-terminal helix, Ile<sup>188</sup> and Tyr<sup>186</sup>, dock into a non-polar pocket formed by the aliphatic groups of the CDK2 residues Leu<sup>174</sup>, Glu<sup>208</sup> and Ile<sup>208</sup>. There is a hydrogen bond between the main-chain nitrogen of Ile<sup>188</sup> (KAP) and side chain oxygen of Glu<sup>208</sup> (CDK2), and salt bridges between Lys<sup>184</sup> (KAP) and Asp<sup>235</sup> (CDK2) and between Glu<sup>191</sup> (KAP) and Lys<sup>237</sup> (CDK2). The structural and biochemical results suggest that this interaction site on CDK2 provides the dominant specificity site for recognition of the kinase by KAP. The GDSEID and DYK motifs are conserved in CDK1, CDK2 and CDK3, but not in CDK4 and CDK6. CDK4 does not interact with KAP, as defined by the yeast two-hybrid analysis [13,15], and we would also predict the same for CDK6.

**Conformational changes of pCDK2**

A surprising feature of the KAP-pCDK2 complex is that the conformation of pCDK2 is reminiscent of that of the activated CDK2 structure observed in the CDK2–cyclin A complex [20]. Similar motions of the N-terminal lobe, C-helix and melting of the L12 loop are observed in the two structures. This result suggests that KAP may interact more tightly with CDK2 when associated with cyclin A. The trigger for the conformational changes observed in pCDK2 in the KAP–CDK2 complex appear to originate from the induced unravelling of the kinase-activation segment that results on association of the pThr<sup>160</sup> residue at the catalytic site of KAP. In order for KAP to interact with pThr<sup>160</sup> of pCDK2, the activation segment is drawn away from the main body of the kinase, with the consequence that the L12 helix at the start of the activation segment is unfolded. Concomitant structural changes in the neighbouring C-helix and β-sheet of the N-terminal lobe result in an activated state of the kinase.

The study of Poon and Hunter [16] demonstrated that KAP-catalysed dephosphorylation of pCDK2 was dependent on the tertiary structure of CDK2. Heat denaturation of CDK2 rendered

![Diagram of a CDK2–cyclin A–KAP complex](image.png)

**Figure 3**

Model of a CDK2–cyclin A–KAP complex

CDK2 resistant to dephosphorylation by KAP, presumably because of a disruption of the major CDK2–KAP interface. In addition, it was found that the association of cyclin A with pCDK2 protected pThr<sup>140</sup> from dephosphorylation, although intriguingly KAP is capable of associating with the CDK2–cyclin A complex. Our structural results explain these findings. First, the sites on pCDK2 that interact with cyclin A and KAP are essentially non-overlapping, explaining the finding that KAP and cyclin A interact with CDK2 simultaneously (Figure 3). In the complex between pCDK2 and cyclin A, the pThr<sup>140</sup> residue is sequestered by three arginine residues of pCDK2 (Arg<sup>56</sup>, Arg<sup>126</sup> and Arg<sup>156</sup>). In the binary complex, these residues are in a position to interact with pThr<sup>140</sup> because of the cyclin A-induced shift of the C-helix and activation segment, which directly and indirectly alters the conformation of all three arginine residues. Moreover, the guanidinium side chain of Arg<sup>156</sup> is stabilized by direct hydrogen bonds to the cyclin A molecule. Degradation of cyclin A mediated by the ubiquitin–proteasome pathway causes a release of the pThr<sup>140</sup> residue, as seen in the monomeric pCDK2 structure, to allow the catalytic site of KAP to gain access to the pThr<sup>140</sup> residue.

**Concluding remarks**

Our studies of the structures of the PTP1B–IRK and KAP–pCDK2 complexes [10,17] have allowed us to compare and contrast the mechanism of dephosphorylation by a tyrosine-specific phosphatase with that of a DSP. PTP1B employs a catalytic site that selects for pTyr residues and excludes the shorter pSer/pThr residues. The conservation of catalytic-site residues of PTP1B throughout the PTP superfamily suggests that all PTPs will catalyse dephosphorylation reactions by a similar mechanism. The sequence context of the pTyr residue is also an important determinant of substrate selectivity, achieved by means of interactions involving variable residues of the phosphatase and residues of the peptide that flank the pTyr substrate residue. KAP, an example of a DSP, although employing a similar reaction mechanism, has a catalytic site that would not confer selection for a phospho-amino acid residue. Specificity for the dephosphorylation reaction is provided by interactions between the phosphatase and its kinase substrate that are remote from both the phosphatase's catalytic site and the site of dephosphorylation on the kinase.

I am grateful to my colleagues who have contributed to this work: Professor L. N. Johnson, A. Salmeen, H. Song (Laboratory of Molecular Biophysics, University of Oxford) and N. K. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.). The work has been funded by grants from the MRC and Wellcome Trust.

**References**


Received 12 April 2001