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Soufi, Abdenour; Noy, Peter; Buckle, M; Sawasdichai, A; Gaston, Kevin; Jayaraman, Padma-Sheela
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CK2 phosphorylation of the PRH/Hex homeodomain functions as a reversible switch for DNA binding

Abdenour Soufi¹, Peter Noy¹, Malcolm Buckle², Anyaporn Sawasdichai³, Kevin Gaston³ and Padma-Sheela Jayaraman¹,*

¹Institute for Biomedical Research, Birmingham University Medical School, Edgbaston, Birmingham, B15 2TT, UK, ²LBPA (UMR 8113 du CNRS), Ecole Normale Superieur de Cachan, 61 Avenue du President Wilson, 94235 Cachan, France and ³Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, UK

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ABSTRACT
The proline-rich homeodomain protein (PRH/Hex) regulates transcription by binding to specific DNA sequences and regulates mRNA transport by binding to translation initiation factor eIF4E. Protein kinase CK2 plays multiple roles in the regulation of gene expression and cell proliferation. Here, we show that PRH interacts with the β subunit of CK2 in vitro and in cells and that CK2 phosphorylates PRH. Phosphorylation of PRH by CK2 inhibits the DNA binding activity of this protein and dephosphorylation restores DNA binding indicating that this modification acts as a reversible switch. We show that phosphorylation of the homeodomain is sufficient to block DNA binding and we identify two amino acids within this domain that are phosphorylated by CK2: S163 and S177. Site-directed mutagenesis demonstrates that mutation of either of these residues to glutamic acid within this domain is sufficient to block DNA binding and we identify two amino acids within this domain that are phosphorylated by CK2: S163 and S177. Site-directed mutagenesis demonstrates that mutation of either of these residues to glutamic acid partially mimics phosphorylation but is insufficient to completely block DNA binding whereas an S163E/S177E double mutation severely inhibits DNA binding. Significantly, the S163E and S177E mutations and the S163E/S177E double mutation all inhibit the ability of PRH to regulate transcription in cells. Since these amino acids are conserved between many homeodomain proteins, our results suggest that CK2 may regulate the activity of several homeodomain proteins in this manner.

INTRODUCTION
Phosphorylation of transcription factors forms a link between signal transduction pathways and the expression of genes. Phosphorylation can control the activity of transcription factors by altering their DNA-binding affinity, sub-cellular compartmentalization, stability or ability to form protein–protein interactions (1). Protein kinase CK2 (formerly casein kinase II) is a ubiquitously expressed kinase that regulates multiple proteins involved in transcription, signalling, cell proliferation and DNA repair (2). In general, CK2 has growth promoting and oncogenic properties. CK2 is a tetramer consisting of α and α₀ subunits (forming the catalytic domain) and a β subunit dimer (forming the regulatory domain). CK2 usually phosphorylates serine and threonine residues and the consensus phosphorylation site is (S/T)XXD/E where X represents any amino acid (3). The CK2β subunit is important in the assembly of CK2, enzyme stability and enzyme activity. It can interact with modulators of CK2 activity as well as with CK2 substrates and is thought to be required for the selection of substrates. CK2β also has roles that are independent of CK2 enzymatic activity and these include the negative regulation of cell proliferation (4).

A number of homeodomain proteins that control transcription are regulated by CK2 including the Drosophila homeodomain proteins Antennapedia (5), Eve (6) and En (7,8) and the mammalian homeodomain proteins Hoxb-6 (9), Cut/CDP (10), Csx/Nk2.5 (11) and SIX1 (12). Phosphorylation of homeodomain proteins by CK2 at sites outside the homeodomain has been shown to influence protein–protein interactions (5,6), DNA-binding affinity (5,8) and protein trafficking (7). In some cases CK2 phosphorylation of these proteins has an effect on cell-cycle progression. For example, Six1 plays a role in regulation of the G2/M cell-cycle checkpoint (12). Phosphorylation of SIX1 by CK2 at sites located outside the homeodomain occurs during interphase and mitosis. Phosphorylation inhibits the DNA-binding activity of SIX1 and this may contribute to regulation of the G2/M checkpoint (12). Few homeodomain proteins are phosphorylated by CK2 at sites within the homeodomain. However, the Csx/Nk2.5 protein is phosphorylated at a consensus CK2 site located within the homeodomain and this site is conserved in the Nk and Six class homeodomain

*To whom correspondence should be addressed. Tel: +44 0121 414 6820; Email: p.jayaraman@bham.ac.uk

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proteins. CK2 phosphorylation at this site increases the DNA-binding activity of Nk2.5; however, the outcome of phosphorylation on transcriptional activity is not clear (11).

The proline-rich homeodomain (PRH) protein (also known as Hex) regulates both cell differentiation and cell proliferation (13,14). PRH is critical for many processes in embryonic development including embryonic patterning, formation of head, forebrain, thyroid and liver and heart and development of the vasculature (15–18). PRH also has a number of functions in the adult including that of a regulator of haematopoiesis (19–24). Exogenous expression of PRH inhibits cell proliferation and cellular transformation of haematopoietic cells of myeloid lineage (21,23). However, PRH can also function as an oncoprotein in haematopoietic cells of T-cell lineage (22,25). Interestingly, PRH can both activate and repress transcription and regulate mRNA translation in vitro (23,26). Our recent work has shown that PRH interacts with CK2 and in cells (27). The protein appears to consist of three domains: an N-terminal domain that is 20% proline, a central homeodomain that mediates binding to DNA, and an acidic C-terminal domain. The N-terminal domain is required for oligomerization and for the inhibition of myeloid cell proliferation and cell transformation by PRH (21,24). The N-terminal domain contains a region that interacts with the promyelocytic leukaemia (PML) RING-finger protein and a region that interacts with eIF4e in PML nuclear bodies (28). The interaction between PRH and eIF4E inhibits the transport of some mRNAs involved in growth regulation (23). The N-terminal domain also contains two regions that confer the ability to repress transcription (29). An E1 motif located within the first 46 amino acids of this domain mediates the binding of PRH to members of the Groucho/TLE family of co-repressor proteins and thereby allows PRH to recruit TLE co-repressor proteins (30). Binding of PRH to TLE proteins results in the increased phosphorylation and nuclear retention of TLE proteins resulting in transcriptional repression by the PRH-TLE complex (30,31). A second region within the N-terminal domain (amino acids 46–131) is able to repress transcription in the absence of the first 46 amino acids although with less efficiency (29,32). The PRH homeodomain can repress transcription independently of the N-terminal domain by binding to TATA box sequences and competing with the TATA box binding protein for these sites (29). PRH can also bind to TBP and this may also inhibit transcription by blocking assembly of the pre-initiation complex (29). In addition, PRH can repress transcription by binding to proteins that would otherwise activate transcription. For example, PRH binds to c-jun and blocks the ability of c-jun to activate transcription (33). Similarly, PRH binds to GATA-2 and prevents this protein from activating transcription although in this case, PRH inhibits the binding of GATA-2 to DNA (34). The C-terminal domain of PRH has been less intensively characterized but is reported to be required for the activation of transcription by PRH in haematopoietic cells (35).

Here we demonstrate that PRH interacts with CK2β. We show that CK2 phosphorylates PRH in vitro and in cells and that phosphorylation inhibits the DNA-binding activity of PRH in vitro. We map two sites of CK2 phosphorylation in the PRH homeodomain and we show that phosphorylation of these sites blocks the DNA-binding activity of PRH in vitro and inhibits transcriptional repression by PRH in cells.

**MATERIALS AND METHODS**

**Yeast two hybrid screen**

A human Erythroleukaemia Matchmaker cDNA library prepared from K562 cells in pACT2 was obtained from Clontech and amplified once before screening. pACT2-CK2β was isolated from the library and encodes the CK2βcDNA (amino acids 1–215) cloned in frame with GAL4 activation domain (1). pAS2-1-PRHN1–132 and pAS2-1-PRH N1–98 encode the full-length human PRH protein and truncated PRH proteins fused to GAL4 DBD and have been described previously (36). The plasmid pGAD-424-TLE1 encodes human TLE1 (amino acids 1–770) fused to GAL4 AD and has been described previously (37). The yeast two-hybrid screen was carried out in yeast strain CG1945 as described in the Clontech manual.

**Expression vectors used in this study**

The plasmid pTrcA-His-Myc-PRH expresses Myc and His-tagged human PRH (27) in bacterial cells. The plasmid pTrcAHisPRH expresses His-tagged avian PRH (38), whereas pTrcAHisPRH-HDC expresses His-tagged avian PRH lacking the N-terminal domain (29) and pTrcAHisPRH-HD expresses the His-tagged PRH homeodomain alone (39). The nucleotides encoding amino acids S163 and S177 within the pTrcAHisPRH HD construct were mutated using a QuikChange Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. This created pTrcAHisPRH-HD S163E, pTrcAHisPRH-HD S177E and the double mutant pTrcAHisPRH-HD S163E/S177E. pBSKS-CK2β was created by subcloning an EcoRI–XhoI fragment that encodes CK2β (amino acids 20–215) from pACT2-CK2β into pBSKS (Stratagene). The plasmid pMUG1-Myc-PRH expresses Myc-tagged human PRH protein (amino acid residues 7–270) under the control of the CMV promoter (30). The plasmid pCS2-HA-Hex expresses HA-tagged murine PRH and was a kind gift from Dr. Josh Brickman (University of Edinburgh). The pMUG1-Myc-PRH S163E plasmid was constructed by digesting pMUG1-Myc-PRH with Stul and PstI to release an Stul–PstI fragment that encodes CK2β and was a kind gift from Dr. Josh Brickman (University of Edinburgh). The pMUG1-Myc-PRH S163E plasmid was constructed by digesting pMUG1-Myc-PRH with Stul and PstI to release an Stul–PstI fragment that encodes CK2β and was a kind gift from Dr. Josh Brickman (University of Edinburgh). The pMUG1-Myc-PRH S163E plasmid was constructed by digesting pMUG1-Myc-PRH with Stul and PstI to release an Stul–PstI fragment that encodes CK2β and was a kind gift from Dr. Josh Brickman (University of Edinburgh).
introduce the S177E mutation into pMUG1-Myc-PRH and pMUG1-Myc-PRH S163E, respectively. All of the mutants were fully sequenced to confirm the sequence changes. Plasmids expressing human and avian GST-PRH fusion proteins have been described previously (30). The plasmids pRc/CMV-CK2α-HA (40), pRc/CMV-HA-CK2β (41) and pRc/CMV-CK2α-K68M-HA (42) express HA-tagged CK α and β subunits and a kinase-dead CK2α mutant respectively and were a kind gift from Professor David Litchfield (University of Ontario).

**Cell culture and transient co-transfections**

K562 cells were grown in glutamine-supplemented Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal calf serum at 37°C and 5% CO₂. The cells were transiently co-transfected by electroporation using a Bio-Rad GenePulser exactly as described previously (29). For repression assays K562 cells were co-transfected with a reporter plasmid containing the luciferase gene downstream of the minimal TK promoter and five PRH binding sites (pTK-PRH) and either pMUG1 vector or the pMUG1-Myc-PRH plasmids described above. Twenty-four hours post-transfection, the cells were harvested by centrifugation and luciferase activity assayed using the Promega Luciferase Assay System according to the manufacturer’s instructions. A plasmid expressing β-galactosidase was included in each transfection to control for transfection efficiency.

**Quantitative RT-PCR**

K562 cells were treated with DMAT (80μm) or DMSO for 24h at 37°C and 5% CO₂. RNA from 5×10⁶ cells was obtained using an RNAeasy kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed using Superscript III according to the standard protocol (Invitrogen). Quantitative PCR was performed by using the fluorescent dye SYBR Green Master Mix (SensiMix Quantase). The primers used for the PCR are as follows: neuropilin-1 forward 5′ ATT GG CGTGACTTCTCTGCATCG 3′ and neuropilin-1 reverse 5′ TGTGCTGAGGT TATCGCGGCTG 3′; GAPDH forward 5′ TGATGACATCAAGAAGGTGTTGAAG 3′ and GAPDH reverse 5′ TCCTTGGAGGC CATGTGGG 3′. All PCR reactions were performed in triplicate and the experiment was repeated three times. The data were analysed using Rotorgene 6 Software (Corbett Research, Rotorgene RG-3000). GAPDH was used as the internal control. Results for relative expression ratios were calculated according to the Efficiency Calibrated Mathematical Model (43).

**Protein purification**

Expression and purification of the GST-PRH fusion proteins and His-tagged PRH fusion proteins was carried out as described previously (30,39). The proteins are shown in Figure S1 and Figure 5B.

**Circular dichroism**

Circular dichroism (CD) spectra of the PRH HD proteins (0–2mg/ml in 25mM phosphate buffer pH 7.9) were obtained using a JY CD6 CD spectrometer with 2-mm path length cells. Spectra were collected between 190 and 260nm at 1-nm increments, using a 2-s integration time.

**Co-immunoprecipitation and western blotting**

Rabbit anti-PRH antibodies were prepared using full-length avian PRH purified from bacteria as antigen and bleeds collected from two animals. ELISA was used to detect high-affinity polyclonal antibodies to the PRH protein. In western blotting experiments antisera obtained after the third bleed were able to detect human PRH present in whole-cell extracts from K562 cells and Myc- and HA-tagged PRH in transiently transfected cells (Figure S2A). For western blotting, rabbit polyclonal antibodies were used at 1/2500 dilution with ∼50-μg whole-cell extract loaded on the gel. We have described previously the production and use of mouse polyclonal antisera that recognizes PRH in cell extracts (21). As expected, these antisera recognize human Myc-tagged PRH as well as murine HA-tagged PRH in transiently transfected cells (Figure S2B). The expression levels of the Myc-PRH and Myc-PRH mutant proteins used in this study were determined by western blotting with the Myc9E10 antibody (Santa Cruz Biotechnology). The western membranes were also probed with a Tubulin antibody (mouse monoclonal MS-581-P1 NeoMarkers) to confirm equal protein loading and transfer.

To examine PRH phosphorylation, whole-cell extracts were prepared from 5×10⁶ K562 cells incubated with 80-μM DMAT (Calbiochem) or 150-μM TBB (Calbiochem) in DMSO for 24h at 37°C or the equivalent volume of DMSO. Whole-cell extracts were then prepared by re-suspending the cells in lysis buffer (50mM Tris pH 8, 100mM NaCl, 20mM NaF, 10mM KH₂PO₄, 1mM DTT, 1% Triton, 10% glycerol) for 30min at 4°C. Protein concentration in the lysates was measured using a Bradford assay and equal amounts of protein were immuno-blotted onto Immobilon-P membrane and analysed by western blot using the mouse and rabbit PRH antisera described above.

For co-immunoprecipitation assays, K562 cells were co-transfected with pMUG1-Myc-PRH and the CK2 expression vectors described previously. Whole-cell extracts were then prepared and equal amounts of total protein added to an equivalent volume of binding buffer (50mM Tris pH 8, 100mM KCl, 0.1mM EDTA, 2mM DTT, 0.2% NP-40, 0.1% bovine serum albumin (BSA), 2.5% glycerol) before incubation with anti-HA antibodies (Clone 12CA5 from Roche) for 1h at 4°C with agitation. Protein G beads (Sigma) were incubated with the extracts for a further 2h at 4°C. The beads were then collected by centrifugation and washed three times in wash buffer (50mM Tris, pH 8, 200mM NaCl, 2mM DTT, 0.5% NP-40) at 4°C. All buffers and washes contained protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (50mM NaF, 1mM Na₃VO₄, 10mM Na...
Pull-down assays

*In vitro* pull-down assays were performed using GST-PRH fusion proteins and His-tagged PRH fusion proteins with *in vitro* transcribed and translated CK2β. Approximately 20 µg of each GST fusion protein or an equimolar amount of GST was bound to 50 µl glutathione-Sepharose 4B beads (Sigma) according to the manufacturer’s instructions. Approximately 20 µg of each His-tagged PRH protein was bound to 50 µl His-Select™ Nickel-Affinity gel (Sigma) according to the manufacturer’s instructions. CK2β was labelled with [35S]methionine was produced using pRC/CMV-HA-CK2β or pBSKS-CK2β,20–215 and a TNT Quick Coupled Transcription/Translation system (Promega) according to the manufacturer’s protocol. Labelled CK2β was incubated with the functionalized beads in binding buffer (20 mM HEPES pH 7.8, 200 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5% NP-40, 50 ng/µl BSA) for 3 h at 4°C. After extensive washing in binding buffer, bound proteins were eluted in boiling protein sample buffer containing 1% SDS and analysed by SDS–PAGE and fluorography.

Protein phosphorylation and dephosphorylation

Purified PRH protein, truncated PRH proteins and PRH mutants (10 ng) were incubated in CK2 phosphorylation buffer (50 mM KCl, 10 mM MgCl2, 20 mM Tris–HCl pH 7.5, 200 µM ATP) with 100 U CK2 enzyme (500 U/µl Calbiochem) for 30 min at 30°C. Dephosphorylation was brought about by incubation of the phosphorylated protein with 10 U of calf alkaline phosphatase (CAP) (6 U/µl Promega) in CAP buffer (50 mM Tris–HCl pH 9.3, 10 mM MgCl2, 1 mM ZnCl2, 10 mM spermidine) for 20 min at 25°C.

EMSA

The double-stranded PRH-binding site shown below (400 ng) was labelled with [α-32P]dATP using Klenow enzyme.

5′AGCTTCTGGGAAAGCAATTTAAAAATGGCTCGAGCT 3′
3′AGACCCTTCTGTTAATTTTTTACCGAGC 5′

After the removal of unincorporated [α-32P]dATP using a Micro Bio-Spin 6 column (Bio-Rad), the labelled oligonucleotide (100 pM) was incubated with His-tagged proteins in 20 mM Tris pH 8.0, 50 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, 0.5 mg/ml BSA and 10% glycerol at 4°C for 30 min. Free and bound DNA were separated on 5% non-denaturing polyacrylamide gels run in 1 x TAE and visualized using a PhosphorImager and ImageQuant 3.3 software (Molecular Dynamics).

Mass spectroscopy

Proteins were excised from SDS-PAGE gels and destained using three 30-min washes in 150 µl of 50:50 (v/v) acetonitrile (ACN):25 mM NH4CO3 at 22°C. The gel slices were then dried down before the addition of 5 µl trypsin (0.1 µg/µl Calbiochem in 1% acetic acid) and 25 µl of NH4CO3 pH 8. After incubation overnight at 37°C, peptides were extracted using three 30-min washes in 30 µl 50:50 (v/v) ACN:5% TFA at 22°C. After being dried down, the peptides were re-suspended in 5 µl 0.1% TFA/5%ACN before analysis. Proteins were initially identified using MALDI mass spectroscopy. Phosphopeptides were identified using an IMAC30 ProteinChip (CIPHERGEN) charged with gallium metal ions in a CIPHERGEN ProteinChip reader, controlled by ProteinChip software (version 3.2.1).

RESULTS AND DISCUSSION

PRH interacts with CK2β

We used a yeast two-hybrid screen to identify proteins that interact with PRH. This screen utilized a human erythroleukaemia matchmaker cDNA library derived from K562 cells and a plasmid that expresses PRH fused to the GAL4 DBD (36). Yeast cells that express both PRH and a protein that interacts with PRH were identified on the basis of β-galactosidase activity and histidine prototrophy (data not shown). The screen resulted in the isolation of five different cDNA clones. Sequence analysis identified one of the five positive clones as the regulatory β subunit of protein kinase CK2. In order to confirm that PRH interacts with CK2β, we made use of constructs in which amino acids 1–132 or 1–98 from CK2β are fused to the GAL4 DBD and a construct in which CK2β is fused to the GAL4 activation domain (AD) (Figure 1A). None of these constructs alone is capable of inducing β-galactosidase activity in yeast (Figure 1B, columns 1–3). However, co-expression of the constructs expressing PRH fusion proteins and CK2β fusion proteins in yeast cells produces significant levels of β-galactosidase activity (Figure 1B, columns 4 and 5). Similarly, the constructs expressing the GAL4 PRH fusion proteins produce significant β-galactosidase activity when co-expressed with a fusion protein consisting of the co-repressor protein TLE1 fused to GAL4 AD (Figure 1B, columns 7 and 8). We conclude that amino acids 1–98 within the N-terminal domain of PRH are sufficient to interact with CK2β in yeast cells and that this interaction is comparable to the previously characterized interaction between the PRH N-terminal domain and the co-repressor protein TLE1 (30).

PRH and CK2β interact *in vitro* and in cells

To determine whether CK2β interacts with PRH *in vitro* we carried out pull-down experiments. In order to determine whether any interaction is conserved, we made use of human and avian PRH-GST fusion proteins. The N-terminal domain of human PRH (amino acids 1–132) and avian PRH (amino acids 1–141) were expressed in β-glycerophosphate, 1 mM EDTA and 5 mM Na pyrophosphate). After SDS–PAGE, the proteins were immuno-blotted onto Immobilon-P membrane, and Myc-tagged PRH was detected using the Myc9E10 antibody (Santa Cruz Biotechnology).
bacteria as GST fusion proteins and partially purified on glutathione-Sepharose beads (Figure S1). The cDNA corresponding to CK2β was transcribed and translated in vitro to produce 35S-labelled CK2β Glutathione beads carrying GST or the GST-PRH N proteins were incubated with labelled CK2β and then washed extensively. Bound protein was eluted by boiling in SDS-PAGE loading buffer and analysed by SDS-PAGE. Figure 1C shows that labelled CK2β binds to the avian GST-PRH N 1-141 (lane 3) and human GST-PRH N 1-132 (lane 4) fusion proteins but binds only very weakly, if at all, to GST alone (lane 2). These data show that the N-terminal domains of human and avian PRH bind to CK2β in vitro.

In order to confirm that the GST tag is not responsible for the interaction with CK2β and to map any further CK2-interacting regions within PRH, we next performed pull-down assays with histidine-tagged full-length human PRH and histidine-tagged truncated PRH proteins (Figure S1). Nickel-agarose beads carrying histidine-tagged full-length PRH or histidine-tagged truncated PRH proteins were incubated with labelled CK2β and then washed extensively as described above. Bound CK2β was then eluted and analysed by SDS-PAGE as before. Figure 1D shows that as expected, labelled CK2β binds to the full-length human PRH protein (lane 3). However, labelled CK2β also binds to a truncated PRH protein lacking the N-terminal domain (lane 4) and to a truncated protein corresponding to the PRH homeodomain alone (lane 5). These data suggest that CK2β can bind independently to the PRH N-terminal domain and the PRH homeodomain. Removal of PRH sequences from the beads by protease digestion confirmed that CK2β does not bind to the His-tag sequence (data not shown).
Figure 2. PRH is a phosphoprotein in cells. Myc-tagged PRH was immunoprecipitated from K562 cells and run on an SDS–PAGE gel. (A) The precipitated proteins were stained using SYPRO Ruby (left panel). HC and LC indicate heavy and light antibody chains, respectively. Phosphorylated PRH was identified by staining with Pro-Q Diamond. (right panel). (B) Untreated PRH (1) and PRH treated with either CK2 and ATP (2) or CK2 and ATP and then CAP (3) were separated by SDS–PAGE and western blotted using mouse anti-PRH antisera (top). The membrane was first stripped and reprobed using rabbit anti-PRH antisera (middle) and then stripped and reprobed using a CK2 antibody as a control for protein loading (bottom). (C) K562 cells were treated with 80 µM DMAT or 150 µM TBB or equivalent volumes of DMSO and incubated for 24 h at 37°C. Whole-cell extracts were then prepared and examined for PRH by western blot using the mouse anti-PRH antisera (top). The membrane was first stripped and reprobed using rabbit anti-PRH antisera (middle) and then stripped and reprobed using a LaminA/C antibody as a control for protein loading (bottom). (D) K562 cells were treated with 80 µM DMAT or DMSO for 24 h. The cells were then harvested and RNA isolated for quantitative RT-PCR analysis. The data were normalized to GAPDH gene expression. The results presented are the average and standard deviation of three independent experiments.

Repeated attempts to co-immunoprecipitate endogenous PRH and CK2β from mammalian cells were unsuccessful possibly due to low protein levels (data not shown). However, we were able to co-immunoprecipitate epitope-tagged PRH and CK2β. We transiently co-transfected K562 cells with vectors expressing Myc epitope tagged PRH, HA-tagged CK2β and HA-tagged CK2α. Forty-eight hours post-transfection we immunoprecipitated CK2 using HA antibodies and then assayed for co-immunoprecipitation of Myc-PRH by western blotting with a Myc antibody (Figure 1E). As can be seen from the data, Myc-PRH co-immunoprecipitates when an HA antibody is added to lysates prepared from cells expressing HA-tagged CK2α and CK2β (Figure 1E, lane 3) or CK2β alone (lane 5), but is not immunoprecipitated from cells that are not expressing HA-tagged CK2 subunits (lanes 1 and 2). Interestingly, PRH is not immunoprecipitated from cells expressing CK2β and an HA-tagged kinase dead CK2α mutant CK2αK68M (lane 4). We conclude that PRH and CK2β interact in cells. In addition, these data suggest that the presence of kinase dead CK2α inhibits the PRH-CKβ interaction and/or that phosphorylation of PRH is important for the interaction.

PRH is a phosphoprotein in cells

To investigate the phosphorylation of PRH in cells, we first immunoprecipitated Myc-tagged PRH from transiently transfected K562 cells using a monoclonal Myc 9E10 antibody. The proteins in the immunoprecipitate were separated by SDS–PAGE and visualized by staining with Pro-Q Diamond phosphoprotein stain and subsequently with SYPRO Ruby protein stain (Figure 2A). The immunoprecipitated proteins were cut from a stained gel and identified using mass spectroscopy. Three bands contained peptides that originate from PRH. However, only the slowest migrating and most prominent PRH band is stained by Pro-Q Diamond, a reagent that binds specifically to phosphorylated proteins (Figure 2A). The immunoprecipitated proteins were cut from a stained gel and identified using mass spectroscopy. Three bands contained peptides that originate from PRH. However, only the slowest migrating and most prominent PRH band is stained by Pro-Q Diamond, a reagent that binds specifically to phosphorylated proteins (Figure 2A). We conclude that Myc-PRH is a phosphoprotein in cells.

To examine whether endogenous PRH is phosphorylated by CK2 we first generated polyclonal anti-PRH antibodies in rabbits. In western blotting experiments, the rabbit antiserum are able to detect a 37-kDa protein present in K562 nuclear extracts (Figure S2A). We confirmed that this protein is PRH by over-expressing HA- and Myc-tagged PRH proteins and showing that the tagged proteins are also detected by the rabbit antiserum (Figure S2A, lanes 2 and 3). Polyclonal mouse anti-PRH antiserum also detect endogenous PRH and the tagged PRH proteins in whole-cell extracts (Figure S2B). We then tested the ability of the rabbit and mouse antiserum to recognize PRH that has been incubated with CK2 and ATP or CK2 and ATP followed by CAP. As expected, both antiserum recognize purified PRH; this is shown in
Figure 2B where a membrane was first probed with the mouse antisera (Figure 2B, top panel), and then stripped and reprobed with the rabbit antisera (middle panel). However, the mouse antisera fail to bind, or bind very poorly, to PRH that has been incubated with CK2 and ATP (Figure 2B, top panel, lane 2). Incubation with CAP restores binding of the mouse antisera and induces a slight increase in the mobility of the PRH band (Figure 2B, top panel, lane 3). Conversely, the rabbit anti-PRH antisera bind to PRH that has been incubated with CK2 and ATP but does not bind to PRH that has been incubated with CAP (Figure 2B, middle panel, lanes 2 and 3). These data indicate that the mouse and rabbit anti-PRH antisera preferentially bind to different forms of PRH. The mouse antisera appear to bind preferentially to unphosphorylated PRH, whereas the rabbit antisera appear to bind preferentially to CK2-phosphorylated PRH. It would seem unlikely that the rabbit antisera are phosphospecific since it also recognizes PRH produced in bacterial cells. More likely, these antisera differentially recognize conformational states of PRH that are altered or produced by phosphorylation.

We next used the mouse and rabbit anti-PRH antisera to examine effects of two CK2 inhibitors on PRH in cells. We incubated K562 cells with the CK2 inhibitors DMAT and TBB for 24 h. The cells were then harvested and used to prepare whole-cell extracts for western blotting. Treatment with either inhibitor had no effect on the levels of PRH detected in these extracts using the mouse antisera (Figure 2C, top panel). However, both DMAT and TBB significantly reduced the amount of PRH detected after stripping the blot and reprobing with the rabbit antisera (Figure 2C, middle panel). Further stripping and reprobing with a LaminA/C antibody confirms equal protein loading in each lane (Figure 2C, bottom panel). Since we have shown that the rabbit antisera bind preferentially to CK2-phosphorylated PRH, we conclude that endogenous PRH is phosphorylated by endogenous CK2 in K562 cells.

To determine the functional consequence of the phosphorylation of PRH by CK2 in cells, we looked at the effect of DMAT on a PRH target gene. K562 cells were incubated with and without DMAT for 24 h and mRNA was extracted for analysis by quantitative RT-PCR. Figure 2D shows the relative mRNA levels of the PRH regulated gene, neuropilin-1 (20,44), under these conditions. Incubation of cells with DMAT results in a significant decrease in neuropilin-1 gene expression (Figure 2D, column 4). Since this gene is a target for repression by PRH (20,44), we conclude that the loss of PRH phosphorylation by CK2, results in more transcriptionally active PRH being present in the cell and, as a consequence, decreased expression of this PRH target gene.

Phosphorylation by CK2 inhibits PRH DNA-binding activity

To determine whether phosphorylation by CK2 has an effect on DNA binding by PRH, we purified full-length human and avian histidine-tagged PRH and performed EMSA after incubation of the proteins with CK2 and ATP. In the absence of CK2 and ATP, human PRH binds to DNA to form a complex with retarded mobility (Figure 3, lane 2). Incubation of PRH with CK2 alone or with ATP alone prior to EMSA, results in a slight decrease in the DNA-binding activity of PRH (Figure 3, lanes 3 and 4). In contrast, incubation of PRH with both CK2 and ATP prior to EMSA results in the almost complete loss of DNA-binding activity (Figure 3, lane 5). If, however, the PRH protein treated with CK2 and ATP is incubated with CAP prior to EMSA, full DNA-binding activity is restored (Figure 3, lane 6). Identical results were obtained for the avian PRH protein (data not shown). We conclude that phosphorylation by CK2 acts as a reversible switch for the DNA-binding activity of PRH. These data suggest that the interaction of CK2 with PRH in the absence of phosphorylation has little effect on DNA binding.

Phosphorylation of the PRH homeodomain is sufficient to inhibit DNA binding

Inspection of the amino acid sequence of PRH reveals several consensus CK2 sites that are conserved between the human and avian proteins. To investigate the importance of these or other phosphorylation sites in the inhibition of DNA binding, we made use of two truncated PRH proteins consisting of the PRH homeodomain and C-terminal domain (PRH-HDC) and the PRH homeodomain alone (PRH-HD), respectively (Figure 4A). The effects of phosphorylation on the DNA-binding activity
of these proteins were analysed using EMSA as described previously for the full-length protein. Figure 4B and C show that both PRH-HDC and PRH-HD are able to bind DNA. The DNA-binding activity of both of these proteins is somewhat reduced after incubation with CK2 alone. However, the DNA-binding activity of both proteins is abolished after incubation with both CK2 and ATP (Figure 4B and C, lane 4). Furthermore, in both cases incubation with CAP after treatment with CK2 and ATP, results in the restoration of DNA-binding activity (Figure 4B and C, lane 5). These data demonstrate that phosphorylation of the homeodomain alone is sufficient to inhibit the DNA-binding activity of PRH and that as in the case of the full-length protein, the effects of phosphorylation are readily reversible.

**Figure 4.** Phosphorylation of the homeodomain inhibits DNA binding. (A) A schematic representation of the full-length PRH protein (top line) and the truncated PRH proteins (second line) used in this study. Labels and numbering are as shown in Figure 1. The filled rectangle represents a histidine tag. The experiment shown in Figure 3 was repeated using the PRH-HDC (B) and PRH-HD (C) proteins.

**CK2 phosphorylates two serine residues within the PRH-HD**

To identify amino acids in PRH that are phosphorylated by CK2, we made use of surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS). Phosphorylated PRH was obtained by incubating purified full-length human PRH with CK2 and ATP. Samples of the phosphorylated and unphosphorylated PRH protein were then analysed by SDS–PAGE. Gel slices corresponding to the phosphorylated protein were excised from the gel and incubated with trypsin. In order to preferentially retain phosphopeptides, the tryptic peptides were extracted from the gel slice and applied to the active spot of an IMAC30 ProteinChip array charged with gallium ions. After on-spot washing, the IMAC30 array was placed in a ProteinChip reader and the masses of the retained peptides were obtained by SELDI-TOF-MS. A peptide with m/z 2829.5 was preferentially retained on the IMAC surface and is therefore probably phosphorylated. A theoretical trypsin digest performed on the PRH amino acid sequence using ProteinProspector did not identify this peptide. However, when the possible phosphorylation events are taken into account, the m/z 2829.5 peak corresponds to the peptide:

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P 161  -  YL  S  PPERKRLAKMLQL  S  ERQVK  -  182
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This phosphopeptide is from within the homeodomain of PRH and contains a consensus CK2 site at S163 and a non-consensus CK2 site at S177. These two serine residues (highlighted in bold) appear to be phosphorylated adding a mass of 160 Da (2 × 80 for the phosphoryl group) to the non-phosphorylated peptide (2672.21) and giving a total mass of 2832.2 Da, which is very close to the measured peak 2829.5 (with a deviation of 2.7).
Virtual phosphorylation of other potential tryptic peptides from PRH did not produce any masses close to the observed value of 2829.5 Da. Taken together, these data suggest that CK2 phosphorylates PRH on both S163 and S177. Phosphorylation of either or both of these amino acids could be responsible for the abrogation of DNA-binding activity by CK2.

**Phosphorylation of S163 and/or S177 inhibits DNA binding**

To examine the effects of phosphorylation at S163 and S177 on PRH, we mutated these positions to alanine to prevent phosphorylation, or glutamic acid to mimic phosphorylation, either individually or in combination (Figure 5A). The mutations were initially produced in the context of the PRH-HD protein construct using site-directed mutagenesis. These mutated proteins were then expressed in bacteria and purified as described previously (39). However, in each case, mutation to alanine resulted in very low yields of the homeodomain protein and consequently very low levels of DNA binding. We will therefore present data obtained using the glutamic acid mutants.

Equal amounts of the wild-type and mutated proteins were incubated with CK2 and ATP and then examined by SDS–PAGE (Figure 5B). The resulting gel was stained with Pro-Q Diamond Phosphoprotein Stain to assess protein phosphorylation followed by staining with Coomassie blue to assess the amount of protein loaded in each lane. Coomassie staining indicates that equal amounts of protein were loaded (Figure 5B, bottom panel). Staining for phosphoproteins reveals that the wild-type PRH-HD is phosphorylated by CK2 (Figure 5B, top panel, lane 2). However, the S163E and S177E proteins are less strongly stained suggesting that they are partially phosphorylated (Figure 5B, top panel, lanes 3 and 4). This implies that each single mutant is phosphorylated by CK2 at the unmutated site. In contrast, the S163E/S177E double mutant is very poorly stained suggesting that this protein is not phosphorylated. These data suggest that there are no additional CK2 sites within the homeodomain or that if any additional sites are present they are dependent on
phosphorylation of S163 and/or S177. To confirm that phosphorylation is reversible we incubated the wild-type and mutated proteins with CK2 and ATP and then treated the phosphorylated proteins with CAP. Treatment with CAP brings about dephosphorylation of the wild-type PRH HD protein (Figure 5B, top panel, lane 7). Interestingly the phosphorylated S163E and S177E mutants appear to be only partially dephosphorylated after treatment with CAP (Figure 5B, top panel, lanes 8 and 9). It is possible that in each case the introduced glutamic acid residue might impair the ability of CAP to dephosphorylate the neighbouring serine residue.

To investigate the DNA-binding activity of the mutated proteins, increasing equivalent amounts of each were added to a labelled DNA carrying a PRH-binding site and the free and bound DNA separated as described above. As expected, the wild-type protein binds to the labelled DNA (Figure 5C, lanes 2–4). In contrast, there was poor binding of PRH HD S163E protein to this DNA (Figure 5C, lanes 5–7). The PRH HD S177E protein and the double mutant PRH HD S163E/S177E did not bind DNA under the conditions used in these experiments (Figure 5C, lanes 8–13). These data suggest that phosphorylation at S177 abolishes DNA binding while phosphorylation at S163 only partially blocks DNA binding. However, it is possible that the S163E mutation does not accurately mimic the effects of phosphorylation due to the flexibility of the glutamic acid side chain. Furthermore, it is also possible that the S177E mutant and the S163E/S177E double mutant are unstable or unfolded. In order to discount the latter possibility, we examined the folding of all four proteins using CD. The CD spectra obtained were identical indicating that each protein has similar secondary structure (data not shown).

In order to determine whether the DNA-binding activity of the mutated proteins could be stabilized we increased the protein concentration, formed the complexes at 4°C and ran the EMSA gels at low temperature. Under these conditions, the S163E and S177E protein are able to bind to the PRH site albeit less efficiently than the wild-type protein (Figure 5D, lanes 5 and 8). These data confirm that the S177E mutation is not sufficient to completely block DNA binding and suggest that phosphorylation at this position also results in the partial inhibition of DNA binding. These data also show that the S163E mutation does not totally abolish DNA binding suggesting that phosphorylation at S163 is also not sufficient to completely block DNA binding. In each case, treatment with CK2 and ATP significantly reduces this residual DNA-binding activity (Figure 5D, lanes 6 and 9). This experiment confirms that phosphorylation at both positions contributes to the inhibition of DNA binding and that phosphorylation at both positions is required to completely block DNA binding.

**Mutation of S163 and/or S177 inhibits the repression of transcription by PRH**

We next set out to examine the effects of the S163E and S177E mutations on the ability of PRH to repress transcription. In order to do this, the S163E and S177E mutations and the S163E/S177E double mutation were introduced into the full-length PRH protein in the context of Myc-PRH fusion proteins (Figure 6A). The wild-type and mutated full-length proteins were then expressed in...
K562 cells along with a reporter plasmid carrying the luciferase gene under the control of the TK promoter and five PRH-binding sites. Western blotting shows that wild-type and mutated proteins are expressed at equivalent levels in the transfected cells (Figure 6A). However, although wild-type PRH represses reporter activity to around 30% of the unrepressed level (Figure 6B, column 2), the S163E and S177E mutants and the S163E/S177E double mutant have greatly reduced repression activity (Figure 6B, columns 3–5). We conclude that these mutations inhibit the DNA-binding activity of PRH in cells resulting in a reduction in the ability of these proteins to repress transcription.

CONCLUSIONS AND IMPLICATIONS

Here, we have shown that human and avian PRH bind to the β subunit of CK2 and that PRH is phosphorylated by CK2 in vivo. Moreover, we have demonstrated that there is a decrease in PRH target gene expression in the presence of an inhibitor of CK2. This suggests that a functional consequence of phosphorylation of PRH by CK2 is the inhibition of transcriptional repression by PRH. We have shown that CK2 phosphorylates PRH at two sites within the homeodomain. Phosphorylation at these sites inhibits the DNA-binding activity of PRH in vitro and inhibits the transcription repression function of this protein in cells. Although several other homeodomain proteins are substrates for CK2, in these cases phosphorylation generally occurs outside the homeodomain. The cooperative binding to DNA of homeodomain proteins Antennapedia and Extradenticle, for example, is regulated by CK2 phosphorylation of Antennapedia at multiple sites outside the homeodomain (5). Similarly, Engrailed is phosphorylated by CK2 at two sites outside the homeodomain but in this case phosphorylation results in increased DNA-binding activity (8) and the inhibition of inter-cellular trafficking (7). In contrast, phosphorylation of Csx/Nk2.5 by CK2 occurs within the homeodomain at a consensus CK2 site ([S/T]XX[E/D]) conserved among all NK class homeodomain proteins and identified here as a phosphorylation site S163 in PRH (11). However, in the case of Csx/Nk2.5 phosphorylation results in increased DNA binding (11), S163 is located at the start of the recognition helix of the PRH homeodomain and this residue is not thought to be involved in binding to DNA. However, model building suggests that this amino acid in PRH could be in close approach to the phosphate backbone in the PRH–DNA complex (Figure 7A). Phosphorylation at this position in PRH would thus place a negative charge close to the DNA phosphate backbone and this would be expected to result in decreased DNA binding. Our mutagenesis data shows that the replacement of S163 with glutamic acid to mimic phosphorylation decreases the DNA-binding activity of PRH but is not sufficient to completely block DNA binding. Surface exposed glutamic acid residues can occupy several positions due to the flexibility of the side chain, and this might explain why DNA binding is reduced but not abolished by this substitution.

Phosphorylation site S177 in PRH has not been identified previously in any other homeodomain protein. This site occurs within the motif SERQ and this sequence is present at the same position in a number of other homeodomain proteins, including HoxD4, HoxD3, FTZ, HoxA6 and others. Model building suggests that S177 is located at the start of the recognition helix in close proximity in space to Q180 and allowing the formation of two hydrogen bonds (Figure 7B). The OH group of the S177 side chain can donate a hydrogen to the O group of Q180 side chain, the NH group of the S177 main chain to contact the phosphate backbone of the opposing DNA. Together, these interactions keep the side chain of Q180 in place to contact the phosphate backbone of DNA and form a hydrogen bond network that links S177 to DNA via Q180. In both images, the pink shading indicates the volume that would be occupied by phosphoserine.

Figure 7. Molecular models of S163 and S177. A molecular model of the PRH homeodomain–DNA complex based on a 3D alignment of the PRH homeodomain structure (PDB 1WQ1) and the homeodomain protein–DNA complexes (PDB 1ENH, 1HDP and 1IG7) was made using 3D-superimpose in Strap (45) and visualized using PyMOL (46). (A) The figure highlights the proximity of S163 to the DNA backbone (thick red line). (B) The hydrogen bond network that links S177 to the DNA backbone via Q180. In both images, the pink shading indicates the volume that would be occupied by phosphoserine.


