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Purification of the proline-rich homeodomain protein

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Abstract

The proline-rich homeodomain protein (PRH), also known as Hex, is a transcriptional repressor expressed in a variety of cell types. The PRH protein contains a proline-rich N-terminal domain that can repress transcription when attached to a heterologous DNA binding domain, a central homeodomain that mediates sequence-specific DNA binding, and an acidic C-terminal domain of unknown function. Although individual domains of PRH have been expressed in bacterial cells as GST- and histidine-tagged fusion proteins, attempts to express and purify the full-length protein have met with little success. Here we describe the purification of a histidine-tagged full-length PRH fusion protein. The protein described here will allow us to determine the mechanisms whereby PRH represses transcription.

Keywords: Purification; Proline-rich homeodomain protein

1. Introduction

The proline-rich homeodomain protein (PRH) was originally identified in avian haematopoietic cells [1]. PRH was subsequently shown to be conserved between avian, amphibian, and mammalian species, where it has also been called Xhex and Hex, respectively [2–4]. The PRH protein is a member of the homeodomain family of transcription factors. The homeodomain is a 60 amino acid motif that mediates sequence-specific DNA binding and can also mediate protein–protein interactions [5,6]. Many members of the homeodomain family regulate embryonic development and cellular differentiation and more recently, some of these proteins have been shown to be involved in the regulation of cell proliferation [5,7]. PRH is expressed in a number of tissues during development and homozygous prh−/prh− knockout mice display an embryonic lethal phenotype [8]. However, PRH expression persists in the liver and thyroid and in haematopoietic precursors suggesting that this protein is also important at later stages of development [1,3,9]. Recent work has shown that PRH is a transcriptional repressor protein in haematopoietic, liver, and thyroid cells and in embryonic stem cells [10,3,9,11] and that this protein can influence the differentiation of haematopoietic cells [12].

The PRH protein appears to be divided into three domains: a proline-rich N-terminal domain, an acidic C-terminal domain, and a central homeodomain. The proline-rich N-terminal domain can repress transcription when attached to a heterologous DNA binding domain [10]. The PRH homeodomain can also repress transcription, even in the absence of the proline-rich repression domain. The PRH homeodomain binds to TATA box sequences in vitro and can

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repress transcription from TATA box-dependent promoters in vivo [10]. Thus, PRH can repress transcription by at least two independent mechanisms. As yet, no function has been assigned to the acidic C-terminal domain.

The individual domains of PRH have been expressed in bacterial cells as GST- and histidine-tagged fusion proteins [1,10]. However, attempts to express and purify the full-length PRH protein have met with little success. Here we describe the purification of a histidine-tagged full-length PRH fusion protein and we use electrophoretic mobility shift assays to show that this protein binds DNA.

2. Experimental

2.1. Protein purification

The histidine-tagged full-length PRH expression plasmid pTrcHisA-PRH was created by cloning the human PRH cDNA between the *Xho*I and *Eco*RI sites of pTrcHisA (Invitrogen). The His-PRH fusion protein was expressed in *Escherichia coli* BL21 pLysS cells (Novagen). Cells containing pTrcHisA-PRH were grown to an absorbance at 600 nm, $A_{600\ nm}$, of 0.6. Protein expression was then induced using 1 m*M* IPTG. After incubation at 37 °C for 4 h, the cells were harvested by centrifugation, resuspended in lysis buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole, pH 8.0) containing protease inhibitor cocktail (Roche). The cells were then lysed using 1 mg/ml lysozyme (4 °C for 30 min) followed by sonication (two 15-s bursts on ice). After incubating with 5 μg/ml DNase and 10 μg/ml RNase at 4 °C for 30 min, the cell extract was clarified by centrifugation and filtered (0.44 μm), before being applied to a 1 ml metal-chelated affinity nickel HiTrap column (Amersham Pharmacia Biotech) using an Äkta fast protein liquid chromatography (FPLC) system and UNICORN 3.10 software (Amersham Pharmacia Biotech). After washing with 40 column volumes of lysis buffer, bound protein was eluted using an imidazole gradient (10 to 150 m*M*). His-PRH elutes at 25 m*M* imidazole and was dialysed at 4 °C overnight against Tris buffer (50 m*M* Tris, pH 8.5) containing protease inhibitor cocktail (Roche). This protein was applied to a 5 ml HiTrap Heparin column (Amersham Pharmacia Biotech). After washing with 10 column volumes of Tris buffer, bound protein was eluted using a salt gradient (0 to 2 *M* NaCl). His-PRH elutes at 800 m*M* NaCl and was snap frozen in 20% glycerol and stored at −70 °C. Protein concentrations were determined from the absorbance at 280 nm ($A_{280\ nm}$) using the molar extinction coefficient and molecular mass was confirmed using a VG Quattro triple quadrupole mass spectrophotometer with electrospray ionization.

2.2. Electrophoretic mobility shift assays

A PRH binding site was produced by annealing the complementary single-stranded oligonucleotides shown below (90 °C for 1 min and then slow cooling to 20 °C):

5′ AGCTTCTGGGAAGCAATTAAAAAATGG-CTCGAGCT 3′

3′ AGACCCTTCGTTAATTTTTTACCGAGC 5′

This double-stranded PRH binding site (400 ng) was labelled with [α^{32}]P dATP using Klenow enzyme. Unincorporated label was removed using a Micro Bio-Spin 6 column (Bio-Rad). Labelled oligonucleotides (20 000 cpm) were incubated with purified protein in 20 m*M* Tris, pH 8.0, 50 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 80 ng/ml poly(dI.dC)(dI.dC), 0.5 μg/μl bovine serum albumin, and 10% glycerol. After 30 min at 4 °C, the free and bound DNA were resolved on 6% non-denaturing polyacrylamide gels run in 0.5×TBE. The free and bound labelled DNA was then visualised and quantified using a PhosphorImager with Molecular Dynamics ImageQuant software (version 3.3).

3. Results and discussion

Previous attempts to purify the full-length PRH protein have met with no success and, as a consequence, the DNA binding properties of PRH have been investigated using truncated proteins that lack the N-terminal repression domain [1,10]. To enable studies using the full-length PRH protein, we expressed a histidine-tagged full-length PRH fusion protein (His-PRH) in bacterial cells and purified the

protein to homogeneity using a metal-chelated affinity nickel column (Fig. 1A) followed by a Heparin column (Fig. 1B and C). The identity of the purified protein was confirmed using mass spectrophotometry. The overall yield of His-PRH is 0.5 mg per litre of bacterial culture and the protein is soluble up to at least 2 mg/ml.

To assay the DNA binding activity of the purified protein, increasing amounts of His-PRH were added to labelled oligonucleotides carrying a PRH binding site. After 30 min at 4 °C, free and bound labelled DNA were separated by non-denaturing polyacrylamide gel electrophoresis and visualised using a PhosphorImager (Fig. 2). As can be seen from the data, the full-length PRH protein binds to the labelled PRH site resulting in the formation of a retarded complex. Shorter or longer incubation times did not result in the appearance of more bound DNA suggesting that equilibrium had been reached (data not shown). When the equilibrium constant (K) is much higher than the concentration of labelled DNA, an apparent equilibrium constant can be estimated from the protein concentration at half maximum DNA binding. In this case, although the concentration of labelled DNA is less than 1 nM, less than 50% of the labelled DNA is bound at 2 μM His-

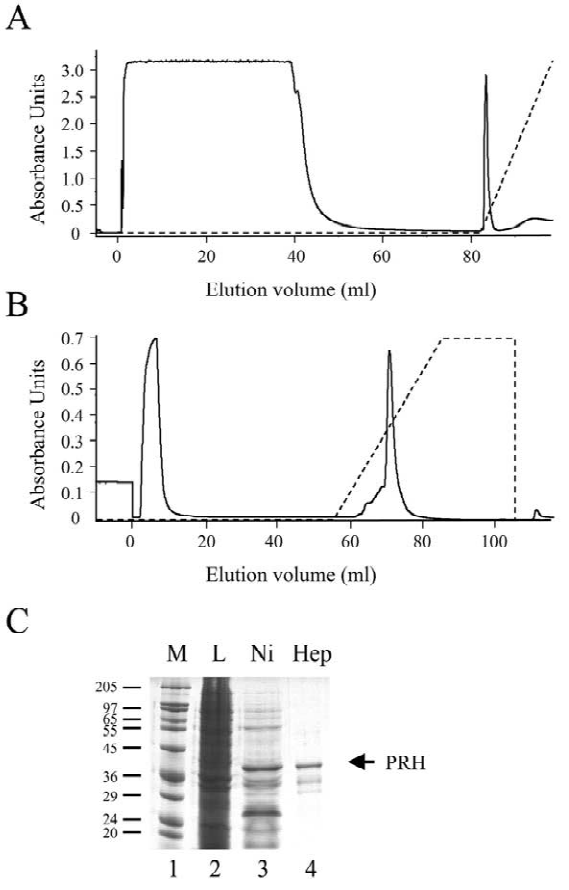

Fig. 1. Purification of a His-PRH fusion protein. (A) Purification of His-PRH on a 1 ml metal-chelated affinity nickel HiTrap column. Showing the absorbance at 280 nm (solid line) and a 10–150 mM imidazole gradient (dashed line). (B) Purification of the peak fractions from (A) on a 5 ml HiTrap Heparin column. Showing the absorbance at 280 nm (solid line) and a 0–2 M NaCl gradient (dashed line). (C) Samples of purified His-PRH fusion protein were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The lanes marked L, Ni, and Hep show cell lysate and peak fractions from the nickel HiTrap and Heparin columns, respectively. The sizes (kM_r) of the markers (lane M) are indicated in the figure.

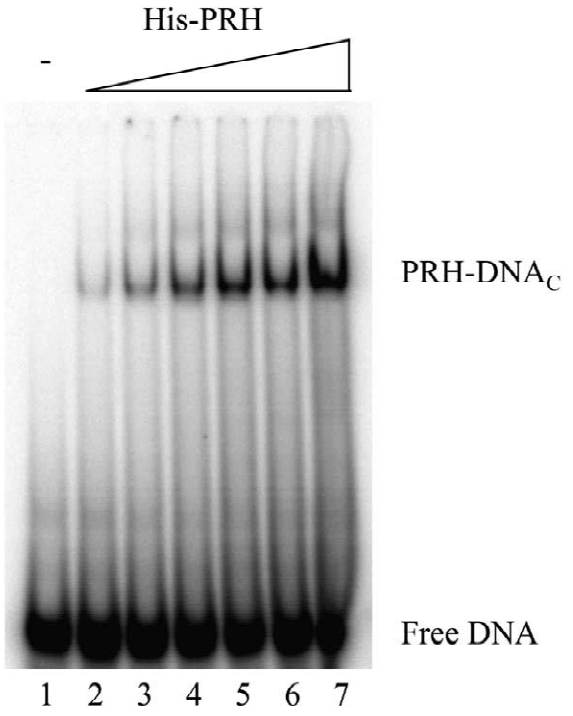

Fig. 2. The DNA binding activity of His-PRH. Increasing amounts of the His-PRH fusion protein were incubated with a labelled PRH binding site. Lanes 1 to 7 contain 0, 60, 120, 250, 500, 1000, and 2000 nM protein. After 30 min at 4 °C, free and bound labelled DNA were separated on a 6% polyacrylamide gel and visualised using a PhosphorImager.

PRH. Thus, the apparent equilibrium constant must be higher than 2 μM. The labelled PRH binding site is capable of being fully bound by a truncated PRH protein that lacks the N-terminal domain (data not shown, but see Ref. [10]). Therefore, the failure of His-PRH to bind all of the labelled DNA is not due to the presence of oligonucleotides that are incapable of being bound or to a problem with the assay. There are several possible explanations for the relatively poor DNA binding activity of the full-length PRH protein. One possibility is that a fraction of the protein might be incorrectly folded and therefore non-functional. However, this is very unlikely given that the purified protein is soluble even at high concentrations. Alternatively, amino acid sequences in the N-terminal domain of PRH might inhibit the DNA binding activity of the PRH homeodomain. This type of autoinhibition has been observed in several other transcription factors, for example, sequences flanking ETS domain can inhibit the DNA binding activity of ETS proteins [13]. It is also possible that PRH might require post-translational modification to enable DNA binding or that PRH might require a partner protein in order to bind DNA with high affinity. Clearly much further work will be needed to address this question in full. However, this purified protein will allow us to study the repression of transcription by PRH in an in vitro transcription system with mammalian nuclear extract and in reconstituted systems with purified components. Hopefully this protein will also allow us to perform detailed structural and biochemical studies.

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