The transcriptional repressor protein PRH interacts with the proteasome

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PRH (proline-rich homeodomain protein)/Hex is important in the control of cell proliferation and differentiation. We have shown previously that PRH contains two domains that can bring about transcriptional repression independently; the PRH homeodomain represses transcription by binding to TATA box sequences, whereas the proline-rich N-terminal domain can repress transcription by interacting with members of the Groucho/TLE (transducin-like enhancer of split) family of co-repressor proteins. The proteasome is a multi-subunit protein complex involved in the processing and degradation of proteins. Some proteasome subunits have been suggested to play a role in the regulation of transcription. In the present study, we show that PRH interacts with the HC8 subunit of the proteasome in the context of both 20 and 26 S proteasomes. Moreover, we show that PRH is associated with the proteasome in haematopoietic cells and that the proline-rich PRH N-terminal domain is responsible for this interaction. Whereas PRH can be cleaved by the proteasome, it does not appear to be degraded rapidly *in vitro* or *in vivo*, and the proteolytic activity of the proteasome is not required for transcriptional repression by PRH. However, proteasomal digestion of PRH can liberate truncated PRH proteins that retain the ability to bind to DNA. We discuss these findings in terms of the biological role of PRH in gene regulation and the control of cell proliferation.

Key words: haematopoiesis, Hex, transcriptional repression.

INTRODUCTION

The 26 S proteasome is a large multimeric enzyme that degrades polyubiquitinated proteins. It is made up of a 19 S regulatory subunit and a 20 S particle. The 19 S particle contains six related ATPases and is thought to denature and unfold substrates and translocate them to the 20 S particle for degradation [1]. The proteolysis of ubiquitinated proteins is essential for many cellular processes, including cell division, apoptosis, cell differentiation and gene regulation [2]. The turnover of a number of transcription factors [3–5] and the large subunit of RNA polymerase II are known to be regulated by ubiquitylation and proteasomal degradation [6]. However, some transcription factors are not fully degraded by the proteasome, but rather undergo limited degradation or processing [7]. In addition, several subunits of the proteasome have been implicated in the activation of transcription initiation and in transcription elongation. For example, the 19 S complex plays a non-proteolytic role in transcription elongation [8] and several of the 19 S ATPases bind to the TATA-box-binding protein, a central component of the transcription initiation machinery [9]. Furthermore, chromatin immunoprecipitation assays demonstrate that a subset of the 19 S ATPases is recruited to GAL4-regulated promoters in yeast by the GAL4 activator protein [10]. Interestingly, the activation domains (ADs) of some transcription factors are also the regions that are targeted for ubiquitylation and degradation [11]. It appears that the more potently the ADs activate transcription, the more rapidly the activator is degraded [12]. Recent work has shown that, in some cases, mono-ubiquitylation of transcription ADs is important for transcription activation and it has been suggested that polyubiquitylation promotes degradation [13].

PRH (proline-rich homeodomain protein) was originally identified in avian haematopoietic and liver cells [14] and was subsequently found to be conserved in humans, *Xenopus*, mice and rats where it has also been called XHex or Hex [15–17]. PRH is a transcriptional repressor protein in haematopoietic cells [18], liver cells [17], thyroid cells [19] and embryonic stem cells [20]. PRH is expressed in a number of different tissues during development [20–22] and homozygous prh^{-}/prh^{-} knockout mice display an embryonic lethal phenotype [23]. PRH expression is associated with both cell differentiation [17,19,24] and cell proliferation [21]. Recent experiments have shown that PRH interacts directly with the growth control protein and transcriptional co-repressor PML (promyelocytic leukaemia protein) [25]. PRH also interacts with the translation initiation factor eIF4E [26].

We have demonstrated previously that PRH can regulate transcription in haematopoietic cells by more than one mechanism [18]. The PRH homeodomain binds to TATA box sequences and represses promoter activity by competing with the TATA-box-binding protein. In addition, the proline-rich N-terminus of PRH is a transferable repression domain that represses transcription, at least partially, by interacting with co-repressor proteins of the Groucho/TLE (transducin-like enhancer of split) family (T. E. Swingler, K. L. Bess, J. Yao, S. Stifani and P.-S. Jayaraman, unpublished work). To characterize further the role of PRH in gene regulation, we screened a cDNA library derived from K562 haematopoietic cells to identify proteins that interact with PRH. In the present study, we show that PRH interacts with the HC8 subunit of the proteasome and we investigate the biological significance of this interaction.

EXPERIMENTAL

Yeast two-hybrid screening

A human erythroleukaemia matchmaker cDNA library prepared from K562 cells in pACT2 was obtained from Clontech Lab Inc.

Abbreviations used: AD, activation domain; CHX, cycloheximide; DBD, DNA-binding domain; DTT, dithiothreitol; GST, glutathione S-transferase; MCS, multiple cloning sequence; MG132, Cbz-Leu-Leu-leucinal; NB, nuclear body; NLS, nuclear localization signal; PRH, proline-rich homeodomain protein; HPRH, human PRH; PRHC, PRH homeodomain plus C-terminus; TK, thymidine kinase; TLE, transducin-like enhancer of split.

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(Palo Alto, CA, U.S.A.) and amplified once before screening. The cDNA encoding HPRH (human PRH) was kindly provided by Dr G. Manfioletti (University of Trieste, Trieste, Italy) as a pBlueScript clone (pBSK-HPRH) [14]. To create pAS2-1-HPRH (amino acids 1–271), an *Eco*RI fragment carrying the HPRH cDNA was inserted into the *Eco*RI site of pAS2-1 (Clontech Lab Inc.). To create pAS2-1-$PRH_{N1–132}$, an *Eco*RI–*Stu*I fragment from pBSK-HPRH encoding the N-terminal 132 amino acids of PRH was ligated between the *Eco*RI and *Sma*I sites of pAS2-1. In each construct, the PRH coding sequence was placed in frame with the GAL4 DBD (DNA-binding domain) by inserting an oligonucleotide 5′-CATGCAGTACCCGCACCCC-3′ between the *Eco*RI site and the internal *Sma*I site in the HPRH cDNA. To create pAS2-1-$PRH_{N1–98}$, pAS2-1-$PRH_{N1–132}$ was digested with *Bam*HI and partially digested with *Apa*I. An *Apa*I–*Bam*HI oligonucleotide (5′-CGCCGCGCCCACG-3′) was then ligated between the *Apa*I site located at amino acid 98 within the PRH amino acid sequence and the unique *Bam*HI site in the vector pAS2-1. The yeast two-hybrid screening was performed in yeast strain CG1945 essentially as described by Fields and Song [28] and following the manufacturer's instructions (Clontech Lab Inc.).

Bacterial expression plasmids

The plasmids pTrc-His-PRH (amino acids 1–277) and pTrc-His-$PRHC_{137–277}$ (where PRHC represents PRH C-terminus) express full-length avian PRH and a truncated PRH construct respectively and have been described previously [18,29]. A GST (glutathione S-transferase)-tagged chicken PRH N-terminus (GST–$PRH_{N1–141}$) expression vector was created by cloning the DNA sequence encoding the avian PRH N-terminus (amino acids 1–141), as a *Sal*I–*Spe*I fragment into pGEX20T, which had been cut with *Xho*I and *Spe*I, creating pGEX20T-$PRH_{N1–141}$. pGEX20T is a derivative of pGEX2T (Amersham Biosciences) and contains unique *Xho*I and *Spe*I restriction sites in the polylinker downstream of the GST moiety. The GST-tagged HPRH N-terminal expression vector pGEX-$HPRH_{N1–132}$ was a gift from Dr G. Manfioletti. Briefly, DNA sequences encoding the HPRH N-terminus (amino acids 1–132) were cloned as an *Eco*RI fragment into pGEX3X (Amersham Biosciences). The DNA sequence of these plasmids and the plasmids described below were verified by DNA sequencing.

Expression and purification of PRH and GST–PRH proteins

The expression and purification of full-length His-tagged avian PRH and His-$PRHC_{137–277}$ proteins have been described previously [18,29]. The human and avian GST–PRH N-terminal fusion proteins were expressed in BL21 pLysS cells (Novagen, Madison, WI, U.S.A.). The fusion protein expression was induced with 1 mM isopropyl β-D-thiogalactoside. Cells were harvested and lysed by incubation with 100 μl of lysozyme (1 mg/ml) for 20 min followed by sonication in PBS and 1 % Triton X-100. GST–PRH N-terminal fusion proteins were partially purified over glutathione–Sepharose 4B beads (Sigma) according to the manufacturer's instructions and snap-frozen in liquid nitrogen. Aliquots of these proteins were eluted with 10 mM glutathione and assayed for purity by SDS/PAGE followed by staining with Coomassie Blue. Proteins were quantified using the Bio-Rad phosphoric acid protein assay.

Proteolysis studies *in vitro*

Purification of the 20 and 26 S proteasomes was performed as described previously [30,31]. His-tagged PRH (4 μg) [29] was either incubated with an equal amount of purified 26 S proteasome in Buffer A [50 mM Tris (pH 7.0)/0.25 mM ATP] or incubated in Buffer A alone. Aliquots were taken after 0, 2, 4 and 8 h and stopped by the addition of acetic acid. The proteins were then separated by SDS/PAGE and, after immunoblotting, PRH was detected with an anti-PRH mouse polyclonal antibody [24] and an ECL® kit (Amersham Biosciences).

EMSA (electrophoretic mobility-shift assay)

A PRH-binding site was produced by annealing the following complementary single-stranded oligonucleotides:

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5′AGCTTCTGGGAAGCAATTAAAAAATGGCTCGAGCT 3′
   3′ AGACCCTTCGTTAATTTTTTACCGAGC 5′
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This double-stranded PRH-binding site (400 ng) was labelled with [α-^{32}P]dATP using Klenow enzyme. The unincorporated label was removed using a Micro Bio-Spin 6 column (Bio-Rad Laboratories). Labelled oligonucleotides (20 000 c.p.m.) were incubated with purified protein in 20 mM Tris (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 80 ng/ml poly-(dI-dC) · (dI-dC), 0.5 μg/μl BSA and 10 % (v/v) glycerol. After 30 min at 4 °C, the free and bound DNAs were resolved on 6 % non-denaturing polyacrylamide gels run in 0.5 × TBE (0.045 M Tris-borate/0.001 M EDTA). The free and bound labelled DNAs were then visualized and quantified using a PhosphorImager with Molecular Dynamics ImageQuant software (version 3.3). The apparent equilibrium constant $K_{eq(apparent)}$ was obtained using the Grafit4 software and the following equation:

$$[\text{bound DNA}] = \frac{[\text{maximum bound DNA}][\text{protein}]}{\{[\text{protein}] + K_{eq(apparent)}\}}$$

When $[DNA] = K_{eq}$, $K_{eq(apparent)}$ is equal to the protein concentration at half-maximum DNA binding. All experiments were repeated three times.

Mammalian expression plasmids and reporter plasmids

The pTK and pTK-Gal, and pSV-lacZ reporter plasmids have been described previously [18]. The pSV-lacZ reporter is available from Promega.

The mammalian expression plamid pMUG1-Myc-PRH expresses full-length HPRH and was created as follows: pUHD15-1 [31a] was modified by replacing the sequence between the unique *Bam*HI and *Eco*RI sites with a linker which destroys these two restriction sites and contains a MCS (multiple cloning sequence). The sequence of the linker is as follows: 5′-AATTG-GATCC**ATG**GGAATTCGAGGTCGACAGTGA-3′. The linker contains a translational start signal (boldface) and *Bam*HI, *Nco*I, *Eco*RI and *Sal*I restriction sites. The resulting pMUG1 plasmid contains the cytomegalovirus promoter with a MCS downstream. A *Bam*HI–*Sma*I double-stranded oligonucleotide encoding a Myc tag (Myc 9E10 epitope) (5′-GATCCATGGAACAAAAA-CTCATCTCAGAAGAGGATCTG-3′) and a *Sma*I–*Eco*RI fragment from pBSK-HPRH carrying the HPRH coding sequence from amino acid 7 was inserted between the *Bam*HI and *Eco*RI sites in pMUG1. This results in an expression construct where the PRH coding sequence from amino acid 7 was placed in frame with the Myc tag and the ATG in the MCS.

pMUG1-GAL4-HC8 is a mammalian expression vector that contains the HC8 cDNA, obtained from the yeast plasmid pACT2-HC8, in frame with an SV40 NLS (where SV40 stands for Simian virus 40 and NLS for nuclear localization signal) and the GAL4 DBD. Expression of the GAL4–HC8 fusion protein is under the

control of the cytomegalovirus promoter. pMUG1-GAL4-HC8 was constructed by inserting an *Eco*RI–*Xho*I fragment obtained from pACT2-HC8 between the unique *Eco*RI and *Sal*I sites of pMUG1. The *Eco*RI–*Xho*I fragment contains the HC8 cDNA and 52 bp of 5′-untranslated sequence to the HC8 cDNA. Subsequently, an oligonucleotide encoding the SV40 NLS [32], namely 5′-AATTGCTCCTCCTAAAAAGAAGAGGAAGGG-3′, was inserted into the unique *Eco*RI site and, finally, a *Bam*HI PCR fragment encoding the GAL4 DBD (amino acids 1–147) was cloned into the unique *Bam*HI site in pMUG1. The resulting GAL4–HC8 fusion protein thus carries the SV40 NLS between the GAL4 DBD and the HC8 coding sequence. The DNA sequence of this plasmid and the plasmids described above were verified by DNA sequencing.

Cell culture and transient transfections

K562 cells were grown in glutamine-supplemented Dulbecco's modified Eagle's medium (Sigma) with 10 % foetal calf serum at a density of approx. 1×10^6 cells/ml. The cells were collected by centrifugation and then resuspended in media plus 10 % foetal calf serum to a density of 5×10^7 cells/ml. Cells (1×10^7) were transiently transfected with 5 μg each of the luciferase and β-galactosidase reporter plasmids described above, and the amount of expressor plasmids indicated in the Results section by electroporation using a Bio-Rad Genepulser (200 V, 975 μF). After electroporation, the cells were left undisturbed for 10 min and then incubated overnight in 10 ml of supplemented media at 37 °C and 5 % CO_2. After 24 h, the cells were harvested and luciferase activity was assayed using the Promega luciferase assay system according to the manufacturer's instructions. β-Galactosidase assays were performed as an internal control for transfection efficiency. After subtraction of the background, the luciferase counts were normalized against the β-galactosidase value.

Whole cell and nuclear extracts

Whole cell extract was made from 2×10^8 K562 cells as follows. The cell pellet was collected by centrifugation for 5 min at 200 ***g*** in a Centurion bench-top centrifuge. The cell pellet was washed twice in PBS and then resuspended in 1 ml of high-salt lysis buffer [500 mM NaCl/50 mM Tris (pH 7.5)/0.1 % SDS/0.1 % Nonidet P40]. The cell suspension was drawn up and down six times through a 3 × Monojet needle (1.1 mm × 50 mm, 19-guage × 2 s), incubated on ice for 5 min, and then centrifuged at maximum speed (6000 ***g***) for 5 min at 4 °C in an Eppendorf microcentrifuge. Nuclear extracts were prepared from 3×10^7 K562 cells as follows. The cells were pelleted as described above. All subsequent manipulations were performed at 4 °C unless otherwise stated. The cell pellet was washed in PBS, re-pelleted and then resuspended in Buffer A [20 mM Tris (pH 7.5)/5 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT], containing 0.05 % Triton X-100. After 10 min on ice, the nuclei were collected by centrifugation for 10 min at 2300 ***g*** in an Eppendorf refrigerated centrifuge. The nuclei were then washed twice in 0.5 ml of Buffer A. The pellet was then resuspended in 50 μl of Buffer B [20 mM Tris (pH 7.5)/5 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT/400 mM NaCl] and incubated on ice for 30 min. The lysate was then centrifuged at 20 124 ***g*** for 30 min in an Eppendorf refrigerated centrifuge. The supernatant was snap-frozen in liquid nitrogen. Nuclear extract containing approx. 100 μg of total protein resulted from 3×10^7 cells.

Pull-downs and Western-blot analysis

Whole cell extract was added to approx. 10 μg of GST–$HPRH_{N1-132}$ protein or 10 μg of GST protein bound to glutathione resin, and the samples were incubated at 4 °C for 2 h with tumbling. After this time, the resin was collected by centrifugation, washed three times in 1 ml of RIPA buffer [150 mM NaCl/50 mM Tris (pH 7.5)/0.1 % SDS/0.1 % Nonidet P40] and resuspended in 50 μl of 2 × SDS loading buffer. All operations were performed at 4 °C and in the presence of protease inhibitors (Roche Diagnostics). After SDS/PAGE, the proteins were immunoblotted on to an Immobilon-P membrane. HC8 protein was detected using a mouse monoclonal anti-HC8 antibody (Affiniti, Mamhead Castle, Mamhead, Exeter, Devon, U.K.) and visualized using an ECL® kit (Amersham Biosciences).

Co-immunoprecipitation assays

A nuclear extract was prepared from untransfected K562 cells or from cells that had been transfected with Myc-tagged HPRH. The nuclear extract was incubated with the 9E10 anti-Myc mouse monoclonal antibody (Santa Cruz Biotechnology) and 40 μl of Protein G beads (Sigma) for 3 h at 4 °C. The beads were then washed three times with 1 ml of RIPA buffer and resuspended in 50 μl of 2 × SDS-loading buffer. After SDS/PAGE, the proteins were immunoblotted on to an Immobilon-P membrane and the HC8 protein was detected as described above. Co-immunoprecipitation experiments with endogenous proteins were performed as described above, except that whole cell extracts were used and the extracts were incubated either with a mouse monoclonal anti-HC8 antibody (Affiniti) and Protein A beads (Sigma) or with Protein A beads alone. After SDS/PAGE, the proteins were immunoblotted on to an Immobilon-P membrane and the PRH protein was detected using a mouse polyclonal anti-PRH antibody.

RESULTS

PRH and HC8 interactions in yeast

We screened for proteins that interact with PRH using the yeast two-hybrid system [28]. Briefly, a human erythroleukaemia matchmaker cDNA library derived from K562 cells was constructed in the GAL4 AD vector pACT2. The HPRH cDNA was placed in frame with GAL4 DBD in the vector pAS2-1 to create pAS2-1-HPRH (see the Experimental section). The cDNA library in pACT2 and pAS2-1-HPRH was co-transformed into yeast strain CG1945, which contains integrated copies of the LacZ and His reporter genes under the control of GAL4-dependent promoters. A functional interaction between the two hybrids in this strain would be expected to produce β-galactosidase activity and His prototrophs. To inhibit leaky expression of the His gene, transformants were assayed for growth on dropout medium containing 1 mM 3-amino-triazol. Transformants that grew well in the absence of His and in the presence of 3-amino-triazol were assayed for β-galactosidase activity (Figure 1A). After eliminating any false positive transformants, the cDNAs from the positive colonies were isolated and sequenced. The screen resulted in the isolation of five different cDNA clones. Sequence analysis identified one of the five positive clones as HC8.

PRH consists of three regions: a proline-rich N-terminal domain, an acidic C-terminal domain [18] and a central homeodomain [14]. The PRH N-terminus has been shown to act as a transferable transcriptional repression domain and to interact with TLE1 (T. E. Swingler, K. L. Bess, J. Yao, S. Stifani and P.-S. Jayaraman, unpublished work) and PML [25]. To determine whether the PRH N-terminus is responsible for the interaction with HC8, we co-transformed yeast strain MaV203 (Invitrogen, Carlsbad, CA, U.S.A.) with pACT2-HC8 and two plasmids

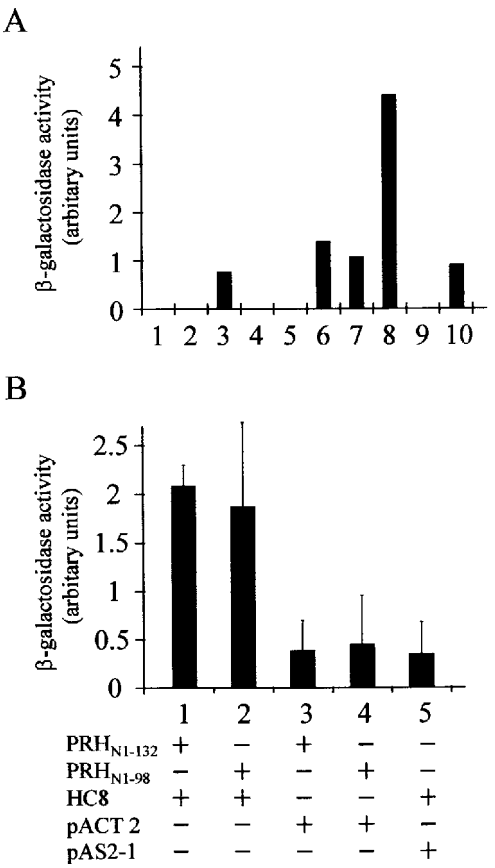

Figure 1 PRH and HC8 interact in yeast cells

(**A**) Ten candidates isolated in a yeast two-hybrid screening for proteins that interact with PRH were assayed for β-galactosidase activity. Five of the candidates (3, 6–8 and 10) produce significant β-galactosidase activity. DNA sequencing revealed candidate 7 to be the proteasome subunit HC8. (**B**) Yeast strain MaV203 was transformed with: pAS2-1-derived plasmids expressing the GAL4 DBD fused to amino acids 1–132 or 1–98 of PRH (PRH$_{N1-132}$ and PRH$_{N1-98}$ respectively), a pACT2-derived plasmid expressing the GAL4 AD fused to HC8 (HC8) or the pACT2 and pAS2-1 empty vectors. The transformants were assayed for β-galactosidase activity and the results represent the means $\pm$ S.E.M. from two experiments performed in triplicate.

expressing GAL4 DBD–PRH N-terminal fusion proteins. pAS2-1-HPRH$_{N1-132}$ and pAS2-1-HPRH$_{N1-98}$ encode the N-terminal 132 and 98 amino acids of PRH respectively, fused to the GAL4 DBD. Yeast cells expressing HC8 and either HPRH$_{N1-132}$ or HPRH$_{N1-98}$ produce β-galactosidase activity (Figure 1B). In contrast, yeast cells expressing either partner alone and transformed with the respective expressing vector without insert do not produce β-galactosidase activity. Taken together, these results show that PRH and HC8 interact in yeast cells and that the N-terminal 98 amino acids of PRH are sufficient for this interaction.

PRH interacts with intact proteasomes *in vitro*

The HC8 subunit is an integral component of the 20 S proteasome. Although HC8 is important for the assembly of the 20 S particle, it does not play a role in proteolysis [33]. To determine whether HC8 and PRH can interact *in vitro* when the HC8 subunit is part of the 20 or 26 S proteasome, we performed pull-down experiments. The N-terminal domain of HPRH (amino acids 1–132) was expressed in bacteria as a GST fusion protein and partially purified on glutathione–Sepharose beads. Purified 20 S proteasomes

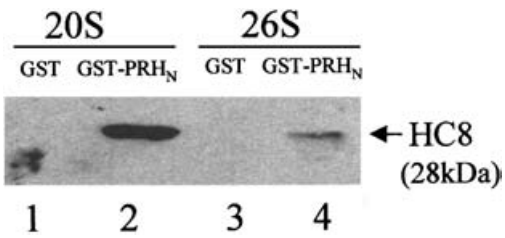

Figure 2 PRH binds to 20 and 26 S proteasomes *in vitro*

Glutathione–Sepharose beads coated with GST (lanes 1 and 3) or glutathione–Sepharose beads coated with a GST–PRH$_{N1-132}$ fusion protein (lanes 2 and 4) were incubated with purified 26 or 20 S proteasomes. Bound proteasomes were detected by Western-blot analysis using an anti-HC8 monoclonal antibody.

(1 μg) or the equivalent molar amount of 26 S proteasomes (3 μg) were incubated with glutathione–Sepharose beads carrying equal amounts of GST or GST–PRH$_{N1-132}$. After extensive washing, the bound proteins were separated by SDS/PAGE and subjected to Western-blot analysis using a mouse monoclonal anti-HC8 antibody. Figure 2 (lanes 1 and 3) shows that GST alone is unable to pull-down intact 20 or 26 S proteasomes. In contrast, GST–PRH$_{N1-132}$ is able to pull-down purified 20 S (lane 2) and 26 S (lane 4) proteasomes. These results demonstrate that the PRH N-terminal repression domain binds to purified 20 and 26 S proteasomes *in vitro*.

PRH interaction with HC8 *in vivo*

To investigate the biological significance of the interaction between PRH and HC8 in K562 cells, we first examined the expression and intracellular localization of PRH and HC8. We have shown previously that PRH is expressed in the K562 cell line in the nucleus [24]. To examine the intracellular localization of PRH and HC8 in K562 cells, we used confocal laser microscopy and immunofluorescence. PRH and HC8 are expressed in both the nucleus and the cytoplasm of K562 cells; however, the proteins do not appear to co-localize to any particular subcellular structure (results not shown).

To determine whether PRH and HC8 interact in K562 cells, whole cell extract was incubated with glutathione–Sepharose beads carrying equal amounts of either GST–PRH$_{N1-132}$ or GST. After extensive washing, the bound proteins were separated by SDS/PAGE and probed for HC8 using a mouse monoclonal anti-HC8 antibody. Figure 3(A) shows that the GST–PRH$_{N1-132}$ protein is able to pull-down HC8 present in K562 cells (lane 3). In contrast, the GST control protein is unable to pull-down HC8 (Figure 3A, lane 2). These results suggest that the PRH N-terminal domain is capable of interacting with endogenous HC8 proteins present in these cells. To confirm this result, we performed co-immunoprecipitation assays. K562 cells were transiently transfected with plasmid expressing Myc–PRH. After 24 h, nuclear extracts were prepared from the transfected and untransfected control cells and used for immunoprecipitation using a monoclonal anti-Myc-9E10 antibody. After extensive washing, the immunoprecipitates were subjected to SDS/PAGE and Western-blot analyses using a monoclonal anti-HC8 antibody. The mobility of the 28 kDa HC8 protein present in nuclear extracts from cells transfected with Myc–PRH and from untransfected cells is shown in Figure 3(B), lanes 1 and 2 respectively. A band corresponding to the HC8 protein was detected in co-immunoprecipitates from Myc–PRH-transfected cells (Figure 3B, lane 4) but not in untransfected cells (lane 3). In addition, a smaller protein of slightly faster

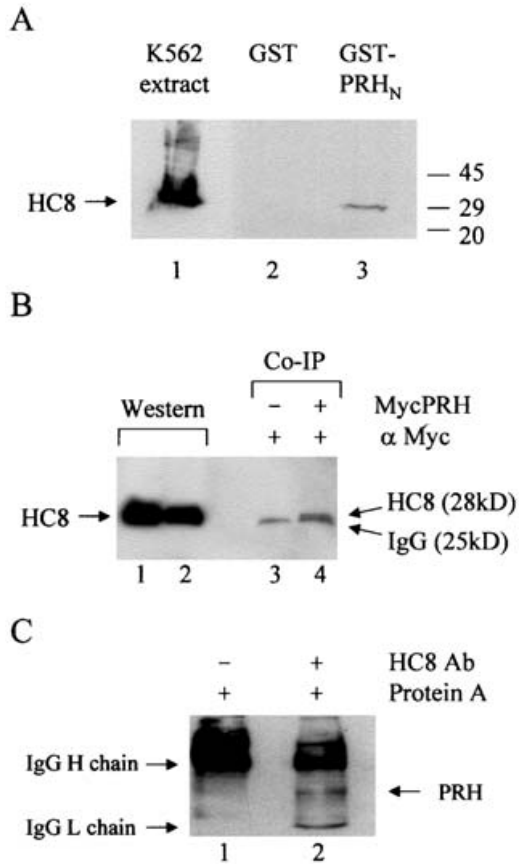

Figure 3 PRH interacts with proteasomes *in vivo*

(**A**) Pull-down assay. Lane 1, Western-blot analysis of HC8 in K562 cell nuclear extract. Lanes 2 and 3, Western-blot analysis of HC8 binding to glutathione–Sepharose beads coated with GST (lane 2) or to glutathione–Sepharose beads coated with the GST–PRH$_{N1–132}$ fusion protein (lane 3). The molecular masses of marker proteins are indicated. (**B**) Co-immunoprecipitation assay. Lanes 1 and 2, Western-blot analysis of HC8 in nuclear extracts prepared from untransfected K562 cells or K562 cells transfected with pMUG1-Myc-HPRH. Lanes 2 and 3, Western-blot analysis of HC8 after co-immunoprecipitation from the same nuclear extracts using Protein G beads incubated with a monoclonal anti-Myc-9E10 antibody (αMyc). (**C**) Co-immunoprecipitation assay. Lane 1 shows a Western blot of PRH after incubation of Protein A beads alone with nuclear extracts from K562 cells. Lane 2 shows a Western blot of PRH after incubation of Protein A beads and the anti-HC8 monoclonal antibody with nuclear extracts from K562 cells. H chain, heavy chain; L chain, light chain.

mobility (25 kDa) was detected in co-immunoprecipitates from both the transfected and the untransfected cells (Figure 3B, lanes 3 and 4). This protein corresponds in molecular mass to the IgG light chain present in the anti-Myc monoclonal antibody used for the co-immunoprecipitation assays.

Co-immunoprecipitation assays were also performed with endogenous PRH and HC8 proteins. Whole cell extracts were prepared from K562 cells and used for immunoprecipitation with Protein A beads alone (Figure 3C, lane 1) or Protein A beads and a monoclonal antibody raised against HC8 (lane 2). After extensive washing, the immunoprecipitates were subjected to SDS/PAGE and Western-blot analyses using a mouse polyclonal anti-PRH antibody. A protein of approx. 35 kDa that corresponds in molecular mass to PRH is detected when the HC8 antibody is present and HC8 is immunoprecipitated (Figure 3C, lane 2). Thus pull-down assays and co-immunoprecipitation assays with both overexpressed and endogenous proteins demonstrate that PRH and HC8 interact in K562 cells.

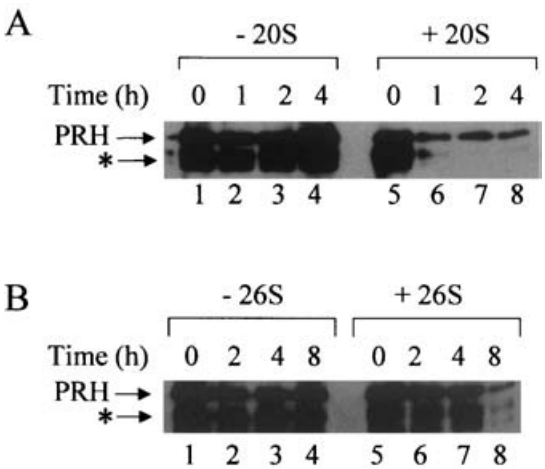

Figure 4 PRH is not rapidly degraded by the proteasome *in vitro*

(**A**) A full-length His-tagged PRH fusion protein was incubated in the absence (lanes 1–4) or presence (lanes 5–8) of purified 20 S proteasomes for the time periods indicated. The proteins were then separated by SDS/PAGE and subjected to Western-blot analysis using an anti-PRH polyclonal antibody. (**B**) A full-length His-tagged PRH fusion protein was incubated in the absence (lanes 1–4) or presence (lanes 5–8) of purified 26 S proteasomes for the time periods indicated. The proteins were then separated by SDS/PAGE and subjected to Western-blot analysis using an anti-PRH polyclonal antibody. The asterisk in (**A**) and (**B**) indicates partially degraded PRH.

PRH can be cleaved by the proteasome *in vitro*

The cyclin-dependent kinase inhibitor p21$^{WAF/CIP1}$ interacts directly with HC8 and the interaction is essential for the rapid ubiquitin-independent degradation of the p21 protein by the 20 S proteasome *in vitro* [34]. Many transcription factors with potent ADs can be rapidly degraded by the proteasome [12]. However, it is not known whether repressor proteins are degraded in a similar fashion. Since PRH can interact with intact 20 and 26 S proteasomes *in vitro* and *in vivo*, we next investigated whether PRH is a substrate for the proteasome. The purification of full-length His-tagged avian PRH (His-PRH) has been described previously [29]. To determine whether PRH is a substrate for the 20 S proteasome *in vitro*, 4 μg of PRH protein that had been purified over a nickel–agarose column was incubated in 50 mM Tris buffer (pH 7.0) or was incubated with an equal amount of purified 20 S proteasomes in the same buffer (Figure 4A). The incubations were stopped with acetic acid at the time intervals shown in the Figure and the proteins were loaded on to an SDS/polyacrylamide gel and subjected to Western-blot analysis using a mouse anti-PRH polyclonal antibody raised against the N-terminus of PRH. In the absence of the proteasome, Western-blot analysis using the anti-PRH antibody detects full-length PRH and partially degraded PRH. Presumably, this is truncated PRH protein produced in bacteria. Incubation of PRH protein with 20 S proteasomes for 1 h results in a decrease in the intensity of the partially degraded PRH and a lesser decrease in the intensity of the full-length PRH band (Figure 4A, cf. lanes 5 and 6). This suggests that PRH can undergo ubiquitin-independent degradation by the 20 S proteasome. However, full-length PRH appears to be a poor substrate for the 20 S proteasome *in vitro* as it is not fully degraded even after 4 h incubation. To determine whether PRH is degraded by the 26 S proteasome *in vitro*, PRH protein (4 μg) was incubated with 26 S proteasomes as described above in Tris buffer containing 0.25 mM ATP (Figure 4B). Incubation of PRH protein with 26 S proteasomes results in a decrease in the intensity of the PRH bands but only after 8 h (Figure 4B, cf. lanes 1–4 with 5–8). This

suggests that PRH can also be cleaved by the 26 S proteasome in a ubiquitin-independent process. However, full-length PRH that is not partially degraded during purification does not appear to be rapidly degraded by either the 20 or 26 S proteasome *in vitro*.

Some transcription factors such as nuclear factor κB [35], SPT23 (multicopy suppressor of Ty-induced mutations) and MGA2 [multicopy suppressor of gam1 (snf2)] [36] are proteolytically cleaved by the proteasome, but they are not fully degraded by the proteasome. To determine whether the proteasome might have a limited proteolytic activity on PRH, we performed EMSAs with purified full-length His-tagged PRH and a His-tagged truncated PRH protein comprising the homeodomain and C-terminus of PRH (PRHC). To assay the DNA-binding activities of the purified protein, increasing amounts of PRH or $PRHC_{137–277}$ were added to labelled oligonucleotides carrying a PRH-binding site. After 30 min at 4 °C, free and bound labelled DNAs were separated by non-denaturing PAGE and visualized using a PhosphorImager (Figure 5A). As can be seen from the results, the full-length PRH protein has a much lower affinity for DNA than PRHC (Figure 5A, cf. lanes 1–7 with lanes 8–14); the full-length protein has a $K_{eq(apparent)}$ higher than 2 μM, whereas PRHC has a K_{eq} of approx. 200 nM.

To examine the effect of proteasome activity, PRH (200 nM) or PRHC (200 nM) was preincubated with the 26 S proteasome (0.2 μM) in the absence or presence of ATP for 3 h before the EMSA was performed. Figure 5(B, lane 2) shows that full-length PRH binds to DNA and produces a retarded protein–DNA complex. However, when PRH is preincubated with 26 S proteasome in the presence or absence of ATP, PRH no longer forms a protein–DNA complex with the same mobility; instead, a new protein–DNA complex of faster mobility results (Figure 5B, lanes 3 and 4 respectively). This faster mobility protein–DNA complex probably corresponds to a truncated fragment of PRH that is able to bind to DNA. Preincubation of PRH with ATP in the absence of the proteasome has no significant effect on DNA binding (Figure 5B, lane 5). In contrast, preincubation of PRHC with either the 26 S proteasome or ATP did not result in any change in complex mobility (Figure 5C, cf. lanes 2, 3 and 5). Preincubation of PRHC with the 26 S proteasome and ATP results in a small decrease in the amount of the protein–DNA complex (Figure 5C, lane 4) and we infer that the 26 S proteasome is capable of degrading PRHC in the presence of ATP. In summary, these results suggest that the 26 S proteasome can bring about limited proteolysis of full-length PRH *in vitro*, which generates a truncated PRH protein that retains DNA-binding activity. In contrast, the homeodomain and C-terminus of PRH are more resistant to proteolysis and proteolysis does not result in the formation of a protein–DNA complex containing a further truncated protein.

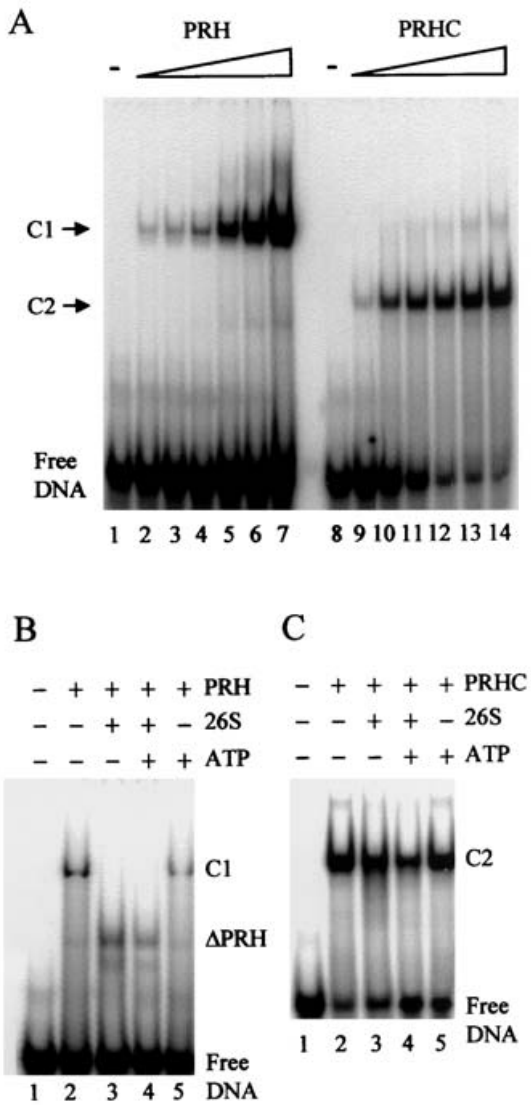

Figure 5 PRH can be cleaved by the proteasome *in vitro*

(**A**) Increasing amounts of the full-length His-tagged PRH or His-tagged PRHC (amino acids 137–277) were incubated with a labelled PRH-binding site. Lanes 1–7 contain 0, 60, 120, 250, 500, 1000 and 2000 nM His-PRH protein. Lanes 8–14 contain the same concentrations of PRHC. After 30 min at 4 °C, free and bound labelled DNAs were separated by PAGE (6 % gel) and visualized using a PhosphorImager. C1 and C2 indicate the PRH–DNA and PRHC–DNA complexes respectively. (**B**) His-PRH (250 nM) was incubated with 26 S proteasomes (3 μM), 26 S proteasomes and ATP (10 mM) or ATP (10 mM) alone for 3 h before EMSA. (**C**) The experiment described in (**B**) was repeated using 250 nM PRHC.

No rapid degradation of PRH by the proteasome in K562 cells

To determine whether PRH is rapidly turned over by the proteasome in K562 cells, we examined the stability of PRH in these cells. PRH levels in K562 cell nuclear extracts were assayed by Western-blot analysis using a mouse anti-PRH polyclonal antibody, at various time points after the addition of the protein synthesis inhibitor CHX (cycloheximide). As a control, an established substrate of the proteasome, namely cyclin E, was also assayed by Western-blot analysis using an anti-(cyclin E) monoclonal antibody. As can be seen from the results shown in Figure 6(A), both cyclin E and PRH levels decline with time in the presence of CHX (cf. lanes 1 and 4). However, the decline in PRH levels is not rapid; the half-life ($t_{1/2}$) of PRH in these cells appears to be >8 h. To determine whether the decline in PRH levels seen in the presence of CHX is due to proteasome activity, this experiment was repeated in the presence of CHX and MG132 (Cbz-Leu-Leu-leucinal), a reversible inhibitor of the proteasome. Figure 6(B) shows that in the presence of both CHX and MG132, PRH levels do not decline over 18 h (cf. lanes 1 and 4). Similar results were obtained for PRH and cyclin E in the presence of the proteasomal inhibitor PSI [Cbz-Ile-Glu(*O*-t-Bu)-Ala-leucinal] (results not shown). Thus inhibition of the proteasome appears to increase the stability of PRH. Under these conditions, cyclin E levels accumulate in the first 3 h (Figure 6B, cf. lanes 1 and 2), consistent with previous work that has shown that cyclin E is rapidly turned over by the proteasome. After the initial accumulation of cyclin E, there is a slow decline in cyclin E levels, presumably by a non-proteasomal mechanism. In conclusion, PRH does not appear to

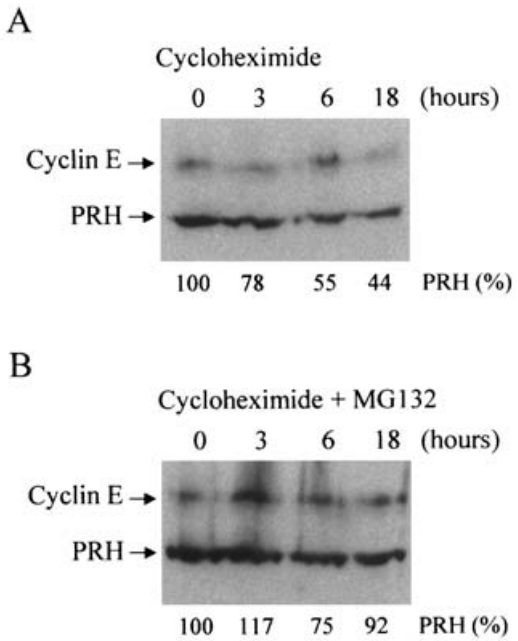

Figure 6 PRH is not rapidly turned over *in vivo*

(**A**) Western-blot analyses of cyclin E and PRH in K562 cell nuclear extracts prepared at the time points indicated after the addition of the protein synthesis inhibitor CHX (20 μg/ml). The percentage of PRH remaining at each time point was estimated by densitometry and is indicated in the Figure. (**B**) Western-blot analyses of cyclin E and PRH in K562 cell nuclear extracts prepared at the time points indicated after the addition of 20 μg/ml CHX and 10 μM proteasome inhibitor MG132.

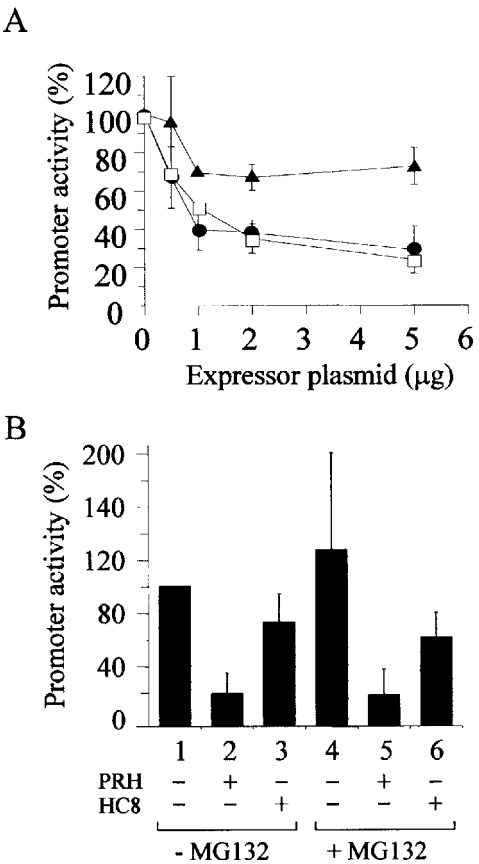

Figure 7 Repression by HC8 and PRH does not require proteasome activity

(**A**) K562 cells were transiently transfected with a reporter plasmid containing the minimal TK promoter and five GAL4-binding sites upstream of the firefly luciferase gene and with increasing amounts of a plasmid expressing the GAL4 DBD (▲) or a GAL4–HC8 fusion protein (●). As a control, K562 cells were also transiently transfected with a reporter plasmid containing the minimal TK promoter alone upstream of the firefly luciferase gene and with increasing amounts of a plasmid expressing GAL4–HC8 (□). In each case, the results are presented as promoter activity relative to the reporter alone. The luciferase activity was normalized for transfection efficiency using a co-transfected plasmid expressing β-galactosidase and the results represent the means ± S.E.M. from at least three experiments. (**B**) K562 cells were transiently transfected with 5 μg of the pTK-Gal luciferase reporter plasmid, a plasmid expressing β-galactosidase, and with 0.5 μg of a plasmid expressing a GAL4–PRH fusion protein (PRH) or an equal amount of a plasmid expressing GAL4–HC8. The cells were treated 24 h after transfection with 10 μM MG132 for 3 h (lanes 4–6) or were left untreated and then assayed for luciferase and β-galactosidase activity. Luciferase activity was normalized and presented exactly as in (**A**).

be rapidly turned over by the proteasome in K562 cells. However, inhibition of the proteasome does result in some stabilization of the protein. We did not observe the presence of truncated PRH proteins in K562 cells; however, our antibodies cannot detect the presence of N-terminally deleted PRH proteins and so it is possible that PRH cleavage products exist that are biologically active.

Overexpression of HC8 can repress transcription

Given that a number of proteasome subunits play a role in transcription, we next set out to investigate whether the interaction of HC8 with PRH is connected with the role of PRH as a transcriptional repressor. To this end, we constructed a GAL4–HC8 fusion protein by placing the HC8 coding region in frame with the GAL4 DBD in the mammalian expression vector pMUG1. This construct was transiently transfected into K562 cells together with a reporter plasmid containing the firefly luciferase gene under the control of the minimal thymidine kinase (TK) promoter and five GAL4-binding sites (TK-Gal). In addition, we co-transfected a reporter plasmid expressing β-galactosidase as a control for transfection efficiency [18]. Figure 7(A) shows that GAL4–HC8 (filled circles) causes a dose-dependent repression of TK-Gal reporter activity relative to that seen in the presence of an equal amount of a plasmid expressing the GAL4 DBD (filled triangles). These results suggest that GAL4–HC8 is capable of binding to the GAL4 sites within this reporter and repress transcription. However, we also examined the ability of GAL4–HC8 to repress transcription from a TK reporter construct lacking GAL4-binding sites (pTK). Somewhat surprisingly, GAL4–HC8 is also able to repress the activity of this reporter (Figure 7A, empty squares). Thus HC8 is able to repress transcription from the TK promoter even when not tethered to the DNA via the GAL4 DBD. Interestingly, overexpression of HC8 has no effect on the activity of the strong SV40 enhancer/promoter present in the β-galactosidase control reporter.

To establish whether HC8 can function as a co-repressor for PRH in transient transfection assays, we co-transfected a vector expressing full-length HC8 together with a vector expressing a GAL4 DBD–$PRH_{N1–132}$ fusion protein (GAL4–PRH) and reporter plasmids described above. Co-expression of GAL4–PRH and HC8 did not result in increased repression of the TK promoter (results not shown). Similar co-transfection experiments were performed with vectors expressing GAL4–HC8 and a fusion protein between the PRH N-terminal domain and the LexA DBD. A LexA–$PRH_{N1–132}$ fusion protein was used, as this protein does not repress transcription unless it is tethered to DNA at LexA binding sites. Co-expression of GAL4–HC8 and LexA–$PRH_{N1–132}$ fusion proteins at a promoter containing both GAL4- and LexA-binding sites did result in greater repression than that seen with expression of LexA–$PRH_{N1–132}$ alone (results not shown). However,

co-expression of GAL4–HC8 and LexA–$PRH_{N1–132}$ with a reporter that contains only GAL4-binding sites did not result in more repression than that seen with GAL4–HC8 alone (results not shown). Taken together, these experiments suggest that although HC8 is able to repress transcription, PRH and HC8 do not act synergistically. These results also suggest that HC8 is not a co-repressor for PRH.

Proteasome activity not required for transcriptional repression by PRH or HC8

HC8 plays an important role in the 20 S proteasome [33,34,37]. Thus the repression seen when HC8 is overexpressed might simply be a consequence of increasing the amount of functional proteasome within the cell, with the result that there is increased degradation of RNA polymerase II or other proteins. Alternatively, the proteolytic activity of the proteasome could be important for transcriptional repression by PRH. Therefore we set out to determine whether there is a connection between proteasome activity and transcriptional repression by PRH or HC8. Transient transfection assays were performed in K562 cells in the presence of MG132. Plasmids expressing either Myc–PRH or GAL4–HC8 were transiently co-transfected into K562 cells along with the TK reporter plasmid and the control reporter plasmid. MG132 was added directly to the growth media 24 h after transfection, and the cells were left to grow for a further 3 h before being harvested and assayed for luciferase and β-galactosidase activity. Under these conditions, proteolytic degradation of cyclin E is completely inhibited (see Figure 7B). In keeping with our previously published results, Myc–PRH brings about the repression of TK promoter activity to approx. 20 % of the unrepressed reporter activity (Figure 7B, cf. columns 1 and 2). Similarly, GAL4–HC8 represses TK promoter activity. However, since in this experiment we were looking for enhanced repression in the presence of MG132, we used only 0.5 μg of the GAL4–HC8 expression plasmid and the level of repression is therefore weak (Figure 7B, cf. columns 1 and 3). The addition of MG132 has little effect on TK promoter activity and has no significant effect on transcriptional repression by PRH or HC8 (Figure 7B, columns 4–6). We conclude that the inhibition of proteasome activity for 3 h does not alter transcriptional repression by PRH or HC8. A longer period of incubation of transfected K562 cells with MG132 (6 h) also failed to show any significant effect on PRH- or HC8-dependent repression (results not shown). However, 6 h incubations with MG132 are cytotoxic to K562 cells. Avian BM2 haematopoietic cells are more tolerant to treatment with MG132. However, incubation of these cells with MG132 for 15 h also failed to bring about any significant change in PRH- or HC8-dependent repression (results not shown). These results suggest that proteasome activity is not required for repression of the TK promoter by PRH or HC8.

DISCUSSION

In the present study, we have demonstrated that the transcriptional repressor protein PRH and the HC8 subunit of the proteasome can interact in yeast and that this interaction occurs in the context of intact proteasomes *in vitro* and in mammalian haematopoietic cells. We have shown that PRH can be degraded by the 20 S proteasome in an ubiquitin-independent process. However, it does not appear to be rapidly degraded by the 26 S proteasome *in vitro* or *in vivo*. PRH protein levels are stabilized in cells where proteasome activity has been inhibited for 18 h; however, there does not appear to be a rapid stabilization of PRH protein when proteasome activity is inhibited. Moreover, we did not observe any enhanced transcriptional repression by PRH after 3 or 6 h incubation with a proteasome inhibitor. Thus there is no significant increase in repression by PRH or PRH levels after inhibition of the proteasome. Conversely, inhibition of the proteasome does not block transcriptional repression by PRH, suggesting that proteasome activity is not required for transcriptional repression by PRH. This is in line with other studies which have shown that the proteasome or proteasome subunits are important for transcription activation and transcription elongation but that proteasome activity is not required [8].

What then is the biological significance of the interaction between PRH and HC8 ? One possibility is that transcriptional repression by PRH involves HC8 and the proteasome but does not require proteasome activity. Our observations that the HC8 subunit of the proteasome can itself repress TK promoter activity and that this repression is also independent of proteasome activity may support this idea. In addition, our findings do not rule out the possibility that PRH levels in various nuclear subcompartments are rapidly altered by proteasome activity. PRH interacts with both HC8 and Groucho/TLE proteins (T. E. Swingler, K. L. Bess, J. Yao, S. Stifani and P.-S. Jayaraman, unpublished work) in the same cells and TLE1 is associated with the nuclear matrix [38]. It is possible that PRH associated with TLE1 in the nuclear matrix might be rapidly turned over by the proteasome. Additionally, the interaction of PRH with the proteasome may be important in regulating interactions between PRH and TLE1 or other co-repressors. Another possibility is that PRH undergoes processing by the proteasome. Although PRH is not rapidly degraded by the proteasome *in vitro* or *in vivo*, PRH can undergo a limited proteasomal cleavage *in vitro*, which results in the formation of a truncated fragment that retains DNA-binding activity. Interestingly, studies have shown that expression of a truncated PRH protein, consisting of the PRH homeodomain and C-terminal domain, in haematopoietic and epidermal cells has significant effects on proliferation and differentiation [24,39]. Although the effects observed might be dominant-negative activities, it is also possible that truncated PRH proteins are produced *in vivo*. However, we have no evidence to suggest that truncated PRH proteins are present in cells and further experiments will be needed to investigate this possibility.

Finally, the proteasome might be important for activities of PRH that are not related to the regulation of transcription. PRH can influence the proliferation and differentiation of haematopoietic cells [24]. PRH has been shown to interact with the multifunctional growth control protein and transcription regulator PML [25,40,41]. PML is found in the nucleoplasm and also in the nuclear-matrix-associated structures known as PML nuclear bodies (NBs) or PML oncogenic domains [41–43]. Two recent findings suggest that PML and the proteasome are also associated in cells. First, mature PML NBs have been shown to contain proteasomes. These bodies are thought to accumulate in response to viral infection and hormone stimulus to allow the rapid degradation of target proteins [44]. Secondly, a regulatory subcomplex of the proteasome was shown to be recruited to PML NBs by PML [45]. It is probable that PRH, PML and HC8 co-exist in PML NBs. It is possible that the interaction of PRH with HC8 regulates proteasome activity and thereby plays a role in the turnover or localization of PML. Clearly, further experiments are needed to elucidate the role played by the PRH–proteasome interaction in the regulation of gene expression and the control of cell proliferation and differentiation.

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