Domain mapping on the human metastasis regulator protein h-Prune reveals a C-terminal dimerization domain

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The human orthologue of the *Drosophila* prune protein (h-Prune) is an interaction partner and regulator of the metastasis suppressor protein NM23-H1 (non-metastatic protein 23). Studies on a cellular breast-cancer model showed that inhibition of the cAMP-specific PDE (phosphodiesterase) activity of h-Prune lowered the incidence of metastasis formation, suggesting that inhibition of h-Prune could be a therapeutic approach towards metastatic tumours. H-Prune shows no sequence similarity with known mammalian PDEs, but instead appears to belong to the DHH (Asp-His-His) superfamily of phosphoesterases. In order to investigate the structure and molecular function of h-Prune, we expressed recombinant h-Prune in a bacterial system. Through sequence analysis and limited proteolysis, we identified domain

boundaries and a potential coiled-coil region in a C-terminal corticillin homology domain. We found that this C-terminal domain mediated h-Prune homodimerization, as well as its interaction with NM23-H1. The PDE catalytic domain of h-Prune was mapped to the N-terminus and shown to be active, even when present in a monomeric form. Our findings indicate that h-Prune is composed of two independent active sites and two interaction sites for the assembly of oligomeric signalling complexes.

Key words: dimerization, metastasis, non-metastatic protein 23 (NM23-H1), phosphodiesterase (PDE), protein–protein interaction, prune.

INTRODUCTION

Many cancer deaths are caused as a result of metastasis formation, rather than by the primary tumour. Several metastasis-suppressor proteins have been characterized, including human NM23-H1 (non-metastatic protein 23), which is also known as NDPK (nucleoside diphosphate kinase)-A [1]. NM23-H1 is a protein with NDPK activity which plays a major role in cellular motility, although other activities have been identified, including DNase and protein kinase activity [2,3]. NM23-H1 overexpression has been shown to exert an anti-metastatic effect in several tumour types, such as sarcoma and breast carcinoma, whereas for other tumour cells, e.g. those involved in prostate cancer and neuroblastomas, overexpression of NM23-H1 leads to an increased metastatic potential [1,4–6]. These different effects are mainly explained by NM23-H1 activity being regulated by a multitude of mechanisms and molecules. One of these regulators is h-Prune, the human orthologue of the *Drosophila* prune protein [7,8]. h-Prune was found to be overexpressed and NM23-H1 was down-regulated in a variety of metastatic cancers, and h-Prune appears to decrease the NDPK activity of NM23-H1 [9], and this is likely to be mediated through the physical interaction detected between these two proteins [7,10]. Studies on a cellular breast-cancer model revealed that inhibition of the cAMP-specific PDE (phosphodiesterase) activity of h-Prune can lower the incidence of metastasis formation, suggesting that h-Prune inhibition could potentially play a role in therapeutic intervention [7]. More recently, h-Prune was also shown to bind to GSK3 β (glycogen synthase kinase 3 β) [11]. These two proteins appear

to co-operatively regulate the disassembly of focal adhesions, thereby promoting cell migration. Modulation of the interaction and mutual regulation of h-Prune and its partners, NM23-H1 and GSK3 β , appears to provide several approaches for the development of novel drugs, but the molecular details of these physical and functional interactions remain to be elucidated.

Despite its PDE activity, h-Prune shows no sequence similarity to the known mammalian PDE families 1–11. Sequence comparison analysis instead indicated that h-Prune belongs to the DHH (Asp-His-His) superfamily of phosphoesterases [12]. DHH proteins possess four conserved motifs, with the third motif carrying the Asp-His-His sequence. The DHH superfamily can be divided into two groups on the basis of an additional, more variable C-terminal motif. The first group (group I) is composed mainly of nucleases, whereas h-Prune and polyphosphatases appear to form a second group (group II). The structures of RecJ [13], as a representative of group I, and of a bacterial PPase (pyrophosphatase) [14] from group II confirmed that the N-terminal domains from both groups share the same β -sheet topology, and that the C-terminal domains show larger structural variations. Therefore the overall structure of the N-terminal section of the h-Prune catalytic domain is likely to resemble the topology of these proteins. However, the large structural variations in the C-terminal section, the low overall sequence conservation (24% identity between h-Prune and the structurally characterized bacterial PPase), and the different substrates, e.g. inorganic pyrophosphate and cyclic nucleotides for PPases and h-Prune respectively, indicate large differences in structural details and the active sites, even between proteins of the same group. Furthermore, h-Prune

Abbreviations used: CHD, corticillin homology domain; CK1, casein kinase 1; DHHA2, Asp-His-His family-associated motif 2; DTT, dithiothreitol; GSK3 β , glycogen synthase kinase 3 β ; MGC, mammalian gene collection; NDPK, nucleoside diphosphate kinase; NM23-H1, non-metastatic protein 23; PDE, phosphodiesterase; PPase, pyrophosphatase; SPA, scintillation proximity assay; TEV, tobacco etch virus.

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contains a further C-terminal extension which is not present in PPases. This additional domain was shown previously to mediate the binding of h-Prune to GSK3 β [11].

In the present study, we performed biochemical studies on the structural and functional domain arrangement of h-Prune. We established the recombinant expression of h-Prune and h-Prune fragments in a bacterial system. Through sequence analysis and limited proteolysis, we identified the domain boundaries in h-Prune, as well as a potential coiled-coil region. We found that h-Prune forms a homodimer in solution, and this is mediated by a C-terminal domain which includes the putative coiled-coil region. The C-terminal domain was shown to mediate binding to NM23-H1. The PDE catalytic domain of h-Prune was mapped to the N-terminus and shown to be active, even as a monomer. Our findings suggest a model for the h-Prune architecture with two independent active sites and two interaction sites for signalling complex assembly.

MATERIALS AND METHODS

Sequence analysis

BLAST analysis was performed on the EXPASY server (<http://www.expasy.org/tools/blast>). Analysis of the physicochemical sequence properties, i.e. hydrophobicity, flexibility and accessibility, was performed using the Domainator program (C. Steegborn, unpublished work). Coiled-coil detection was performed with the COILS server (http://www.ch.embnet.org/software/COILS_form.html) [15] and the predicted secondary structure was determined using the Jpred program (<http://www.compbio.dundee.ac.uk/~www-jpred/>) [16].

Cloning of h-Prune and NM23-H1

Full-length h-Prune (amino acid residues 1–453) and h-Prune deletion constructs were amplified by PCR from an MGC (mammalian gene collection) human-verified full-length cDNA clone [RZPD (German Resource Center for Genome Research), Berlin, Germany; clone ID IRAUp969B0982D6]. The constructs were cloned using TOPO technology into pET151/D-TOPO (Invitrogen), resulting in constructs with a His-tag fused to the N-terminus of h-Prune via a linker consisting of a TEV (tobacco etch virus) protease cleavage site. The human full-length NM23-H1 gene was PCR amplified from MGC cDNA clone IRAUp969A091D6 (RZPD) and inserted into the pET151/D-TOPO vector by TOPO ligation, yielding an N-terminally His-tagged NM23-H1 protein with a specific TEV cleavage site.

Protein expression and purification

Full-length NM23-H1, h-Prune, and h-Prune deletion constructs were expressed in *Escherichia coli* Rosetta2 cells (Merck). Cells were grown at 37°C in Luria–Bertani broth containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol until the attenuation (D_{600}) reached 0.6–0.8. Expression of the target gene was then induced by the addition of 0.5 mM IPTG and cells stored on ice for 15 min. Subsequently, cells were grown at 20°C overnight. After harvesting, cells were disrupted with an Emulsiflex homogenizer (Avestin) and cell debris was removed by centrifugation at 36 000 g for 40 min at 4°C in a HFA22.50 rotor. The supernatant was supplemented with 15 mM imidazole and incubated with talon resin (Clontech) for 1 h at 20°C. The resin was transferred into a column, washed with 10 volumes of buffer A (50 mM Tris/HCl, pH 7.8 and 500 mM NaCl) and 10 volumes of buffer B

(50 mM Tris/HCl, pH 7.8, 200 mM NaCl and 5 mM imidazole), and the fractions were eluted using 50 mM Tris/HCl, pH 7.8, 20 mM NaCl and 150 mM imidazole. The fractions were supplemented with 0.5 mM EDTA and 2 mM DTT (dithiothreitol) and analysed by SDS/PAGE. The fractions containing the protein were then pooled, concentrated using a Centricon®-10 centrifugal filter unit (Millipore) and further purified using a Superdex 200 gel-filtration column (GE Healthcare) in buffer C (20 mM Tris/HCl, pH 7.8, 50 mM NaCl and 2 mM DTT). The protein was analysed by SDS/PAGE, re-concentrated, and snap-frozen in liquid nitrogen for storage at –80°C.

Limited proteolysis

A 12 µg protein aliquot in buffer C was supplemented with 50 ng of elastase or 50 ng of trypsin respectively, using stock solutions of 0.1 mg/ml protease in 20 mM Tris/HCl, pH 7.8. After 30 min incubation at either 4°C or 25°C, the reaction was stopped by the addition of SDS/PAGE sample buffer (pre-heated to 80°C) and was immediately boiled for 5 min. Samples were analysed by SDS/PAGE and Coomassie Brilliant Blue staining for approximate size determination with Quantity One (Bio-Rad). For N-terminal microsequencing, samples were electroblotted on to PVDF membranes and analysed by Edman degradation performed by Toplab (Martinsried, Germany). The C-termini of fragments were estimated from the N-terminus and fragment size, and could mostly be determined precisely owing to the specificity of trypsin for positively-charged residues.

Activity assay

PDE activity was measured using an SPA (scintillation proximity assay) (GE Healthcare). Samples were diluted as required and incubated at 30°C in 100 µl assay buffer (50 mM Tris/HCl, pH 7.4, 8.3 mM MgCl₂ and 1.7 mM EGTA) containing the desired concentrations of cAMP as substrate [3:1 ratio of unlabelled cAMP/[³H]cAMP (GE Healthcare)]. All reactions, including buffer-only blanks, were conducted in triplicate and allowed to proceed for an incubation time which yielded < 25 % substrate turnover (empirically determined). Reactions were terminated by the addition of 50 µl yttrium silicate SPA beads (GE Healthcare). Enzyme activities were calculated for the amount of radiolabelled product detected according to the manufacturer's protocol. K_m and V_{max} values were determined by measuring the initial hydrolysis rates in the presence of a range of substrate concentrations (0.1–2.5 µM) and a fixed amount of purified enzyme (800 ng).

Pull down experiments

Phosphorylation of 10 µg of NM23-H1 was performed using 1000 units of CK1 (casein kinase 1) (New England Biolabs) in 1 × CK1 buffer (New England Biolabs) supplemented with 200 µM ATP. The sample was incubated for 1 h at 30°C. Phosphorylated His-tagged NM23-H1 (50 µg) was mixed with 50 µl of talon resin and then incubated at 20°C for 1 h. Subsequently, 80 µg of h-Prune (without the His-tag) was added and the incubation was continued for a further 15 min. Samples were then centrifuged at 5200 g for 5 min, and the pellet was washed twice with 500 µl of buffer A containing 5 mM imidazole. Elution was performed using 50 µl of a buffer containing 50 mM Tris/HCl, pH 7.8, 200 mM NaCl and 200 mM imidazole. Wash samples and elution samples were then analysed by SDS/PAGE. For competition experiments, 5 µg of His-tagged h-Prune was incubated for 1 h at 4°C with up to 37 µg of phosphorylated, untagged NM23-H1 (up to 20-fold molar excess over

Figure 1 Domain architecture of h-Prune and protein fragments used in this study

(a) Domain arrangement in h-Prune. A well-defined DHH domain is followed by a less well-conserved DHHA2 domain and a C-terminal CHD with a putative coiled-coil region and a proline-rich region. The constructs of h-Prune and its fragments which were expressed and purified in this study are indicated. (b) Alignment of h-Prune (370–453) with the cortaxillin I coiled-coil region. The best-aligned region of h-Prune covers the predicted coiled-coil in the N-terminal section of the CHD subdomain, and a secondary structure prediction for h-Prune is indicated on top of its sequence. (c) Limited proteolysis of full-length h-Prune at 4°C and 25°C respectively. The protease used is detailed on top, and the fragments which were further analysed are indicated on the right-hand side.

GSK3 β) and 50 μ l of Ni-NTA (Ni²⁺–nitrilotriacetic acid) resin (Qiagen) in the presence of 10 mM imidazole. Subsequently, 5 μ g of GSK3 β (Biomol) was added, and the incubation allowed to continue for a further 1 h, followed by washing with buffer A and with buffer B with 15 mM imidazole respectively. Protein elution and analysis was performed as described above.

RESULTS

Sequence analysis, recombinant expression and limited proteolysis

The N-terminus of the h-Prune sequence (UniProtKB/TrEMBL ID: Q86TP1; residues 10–180) displayed homology with DHH phosphoesterases and contained DHH-specific motifs [12]. The protein was grouped into the polyphosphatase subfamily on the basis of weaker similarity within the C-terminal neighbouring domain (h-Prune residues 215–350), which carries a Pfam DHHA2 (Asp-His-His family-associated motif 2) signature [7]. This subdomain, approx. 120 amino-acid residues in size, is much less conserved. It has a conserved N-terminal Asp-Xaa-Lys motif and appears to be involved in substrate selection of the respective protein. Comparison of this C-terminal subdomain region of h-Prune (residues 180–400) with proteins with known crystal

structures revealed only short stretches of moderate similarity. Residues 215–280 contain a short stretch of similarity to β -lactamases, and residues 320–390 show similarity to reverse gyrase domains H2 and H3, which are proposed to mediate DNA strand separation. Analysis of the physicochemical properties (hydrophobicity and accessibility) indicated a likely domain border in h-Prune around residue 325. We therefore cloned fragments of h-Prune (cloned fragments ranged in length from between residues 1–330 and 1–420) for bacterial expression (Figure 1a).

We then analysed the C-terminal extension in h-Prune (residues 361–453), which is absent from other DHH proteins, by performing a BLAST search on structurally characterized proteins. Initially, a pronounced similarity was found between h-Prune residues 375–430 and the coiled-coil dimerization domain in cortaxillin I from *Dictyostelium discoideum* (PDB ID: 1D7M) [17]. Closer inspection of this similarity showed that h-Prune residues 375–405 align well with cortaxillin I, followed by a stretch of about 20 residues which is proline-rich, followed by another stretch (425–453) which can be aligned with the cortaxillin coiled-coil (Figure 1b). Secondary structure prediction for the h-Prune C-terminus indicated a high probability of α -helix formation, interrupted between positions 405 and 430 due to the presence of the proline residues. Consistently, the COILS algorithm [15] predicts a coiled-coil structure, starting at position

Table 1 Oligomerization states of h-Prune constructs

—, no soluble protein; +, small amounts of soluble protein; ++, larger amounts of soluble protein; +++, high yields of soluble protein. n.a., not applicable.

h-Prune construct (amino acid residues)	Solubility/yield	Oligomerization state
1–453	+++	Dimer
1–420	+	Dimer
1–400	—	n.a.
1–393	+++	Monomer
1–379	++	Monomer
1–365	+	Monomer
1–330	No expression	n.a.

370–375 and ending at approximately residue 405. We therefore termed the C-terminus of h-Prune CHD (cortexillin homology domain), and assumed this domain is composed of a bipartite structure with a coiled-coil subdomain, followed by a C-terminal extension. For use in further experiments, we then assumed that full-length h-Prune possessed the following three-domain structure (Figure 1a): a DHH domain, followed by a DHHA2-like or specificity-determining domain, followed by a tripartite CHD with a putative coiled-coil structure, a proline-rich region, and a C-terminal helical extension.

In order to study the structural and functional domains present in h-Prune, we cloned and recombinantly expressed various h-Prune fragments in *E. coli* (Figure 1a). Efficient expression of full-length h-Prune could only be achieved using *E. coli* Rosetta2 cells, which harbour additional tRNA genes for codons which are rarely used in *E. coli*. Fragments consisting of only the C-terminus of h-Prune could not be expressed in detectable amounts, whereas several C-terminal deletion constructs gave good protein yields (Table 1). We then subjected purified full-length h-Prune to limited proteolysis in order to determine experimentally the structural domain boundaries. Major proteolytic fragments obtained with elastase digestion corresponded to residues 1–427 (ending behind the proline-rich region) and residues 1–approx. 352 (the C-terminus could not be determined exactly), which lies within the DHHA2 domain. Additional fragments obtained with trypsin digestion corresponded to residues 221–453 and 221–427 (Figure 1c), which start at the beginning of the DHHA2 domain and end at the native C-terminus and after the proline-rich region respectively. Together with our expression results (Table 1), these results imply the following domain arrangement in h-Prune: the catalytic domain, consisting of two large subdomains, the DHH domain, which is well defined in sequence alignments, and a second subdomain starting around residue 221, which is a major site for proteolysis. This second subdomain seemed to extend approximately to residues 380–390 on the basis of our expression experiments (Table 1), with a flexible region present around residue 352. The flexible region was indicated by proteolysis and the region was larger than expected after analysis of the DHHA2 amino acid sequence. The C-terminal CHD is also well-ordered in its non-helical proline-rich region, with the only protease-sensitive region present around residue 427 at the end of the non-helical region.

h-Prune contains an explicit dimerization domain

The phosphoesterases of the DHH family form homodimers through the association of two catalytic domains. Therefore we tested the oligomerization state of purified h-Prune by size-exclusion chromatography using a Superdex 200 column. Full-length h-Prune (1–453) eluted at the position expected for a homodimeric

Figure 2 Mapping of dimerization and catalytic domains

(a) Behaviour of full-length h-Prune and of h-Prune (1–393) in size-exclusion experiments. A logarithmic graph of a molecular-mass standards (MW) and the elution volumes of these proteins is shown. At the elution positions of the two h-Prune constructs, labels indicate the molecular masses for a monomeric and a dimeric species respectively. (b) Comparison of specific activities of full-length h-Prune (□) and the monomeric h-Prune (1–393) construct (▲). Shown is a double reciprocal plot used for the determination of K_m and V_{max} values [fit for full-length h-Prune, solid line; h-Prune (1–393), dotted line]. Both proteins show almost identical activity despite their different oligomeric states.

species (Figure 2a). Surprisingly, h-Prune (1–365) and h-Prune (1–379) fragments, which cover the h-Prune region which has homology with the DHH family, were resolved as monomers in size-exclusion experiments (Figure 2a and Table 1). Testing of further constructs showed that the h-Prune (1–393) fragment still runs as a monomer, whereas h-Prune (1–420) is capable of forming dimers (Table 1). Size-exclusion chromatography depends not only on the molecular-mass of the molecule, but also on the shape. We therefore verified by blue native gel electrophoresis that h-Prune (1–393) is monomeric (results not shown).

Our results show that the region between residues 393 and 420 is essential for dimerization, and therefore the CHD is a dimerization domain. The putative coiled-coil region in the CHD appears to be involved in dimerization, but the non-helical region is essential for dimer formation. This result is different from other DHH family members, which use their catalytic domains for dimerization instead of an explicit dimerization domain. It also demonstrates that the five putative leucine zipper repeats between residues 157 and 185 of h-Prune are not responsible for dimer formation.

The N-terminus of h-Prune carries a complete and monomeric catalytic domain

The closest structurally characterized homologue identified for h-Prune is the soluble inorganic type-C PPase from *Streptococcus*

Figure 3 Interaction of h-Prune with NM23-H1

Pull down experiments were performed using His-tagged and phosphorylated NM23-H1 and either full-length h-Prune or h-Prune (1–393). **(a)** Lane 1, molecular-mass standards. Lanes 2–5, pull down with full-length h-Prune: lane 2, flow-through; lane 3, first wash; lane 4, second wash; lane 5, elution. Lanes 6–9, pull down with h-Prune (1–393); lane allocation is the same as for lanes 2–5. **(b)** As a control for non-specific interactions, NM23-H1 was incubated with BSA. Lane 1, molecular-mass standards; lane 2, flow-through; lane 3, wash; lane 4, elution. **(c)** Pull down experiment with tagged full-length h-Prune (lanes 1–5). GSK3 β binds h-Prune in the presence of excess NM23-H1, indicating a non-competitive interaction. Lanes 6–9: control, showing that there is no non-specific binding of GSK3 β to the resin. Lane 1, molecular-mass standards; lanes 2 and 6, flow-through; lanes 3 and 7, first wash; lanes 4 and 8, second wash; lanes 5 and 9, elution.

gordonii (Swissprot ID P95765). This protein shows reasonable homology with the N-terminal 360 residues of h-Prune. Crystal structures of this PPase (PDB entries 1WPP [18] and 1K20 [14]) reveal a dimeric arrangement. Dimerization is mediated by parts of the catalytic domain not involved in formation of the active site, and each monomer within the dimer contains a complete active site. The N-terminal fragment of h-Prune, consistent with the section homologous to the PPase, possesses a high level of PDE activity. Full-length h-Prune degrades cAMP with a $V_{\max}$ of 19 nmol/min per mg, and the truncated form h-Prune (1–393) degrades cAMP with a $V_{\max}$ of 20 nmol/min per mg (Figure 2b). Both proteins also show comparable affinity for this substrate, with the K_m^{cAMP} for full-length h-Prune being 1.4 μM , and the K_m^{cAMP} for h-Prune (1–393) being 1.5 μM , indicating that the active site is indeed complete. Interestingly, we find that this fully-active deletion mutant forms a monomer in solution. We therefore conclude that the catalytic domain of h-Prune alone is not able to dimerize, which is different to other PPases of this family.

In order to refine further the domain boundaries in the N-terminal catalytic part of h-Prune, we subjected purified h-Prune (1–393) to limited proteolysis. After elastase treatment, a major fragment covered residues 149–393, whereas trypsin digestion yielded a dominant fragment containing residues 221–393. This result indicates that there is no flexible region at the C-terminus of the catalytic domain, as determined by limited proteolysis. On the basis of the solubility and purification yield of h-Prune fragments (Table 1), we mapped the domain end in the region of amino acids 379–393. The cleavable site at residue 221 is consistent with the digest of full-length h-Prune, and indicates the beginning of the DHHA2 domain. The cleavage site at residue 149 is inside the DHH homology domain (as determined by a previous modelling exercise [7]), however, it would map to an exposed loop within this domain. We therefore assume that the DHH domain of h-Prune covers the whole homologous region of amino acids 10–180.

The C-terminal h-Prune domain mediates NM23-H1 binding

h-Prune interacts with GSK3 β , and this binding was shown to be mediated by the C-terminal portion of h-Prune between residues 330 and 453 [11]. Previously, h-Prune was also shown to interact with the metastasis suppressor protein NM23-H1 [7,10], but the h-Prune domain mediating this interaction has not yet been identified. We used our full-length and the C-terminal truncated h-Prune constructs in order to test which part of h-Prune mediates NM23-H1 binding. In pull down experiments with His-tagged NM23-H1, phosphorylated through treatment with CK1 (L. Garzia, A. D'Angelo, A. Amoresano, S.K. Knauer, C. Cirulli, C. Campanella, R.H. Stauber, C. Steegborn, A. Iolascon and M. Zollo, unpublished work), full-length h-Prune bound specifically to this interaction partner (Figures 3a and 3b). However, extensive washing (> 30 column volumes) leads to some leakage of the bound h-Prune, indicating that its interaction with NM23-H1 is not very strong. Analogous experiments using the C-terminally truncated h-Prune (1–393) fragment showed no binding to immobilized NM23-H1 (Figure 3a), indicating that the interaction is mediated by the CHD of h-Prune, located between residues 393 and 453. Further pull down experiments with full-length h-Prune and GSK3 β in the presence of excess NM23-H1 showed that both interacting partners do not compete for h-Prune binding, but instead can bind simultaneously (Figure 3c), indicating different interaction surfaces for GSK3 β and NM23-H1 within the h-Prune C-terminal region.

DISCUSSION

h-Prune is a protein involved in cellular motility and tumour metastasis formation [7,19]. The PDE activity of h-Prune can be inhibited with dipyrindamole, and this inhibition was shown to reduce cellular motility in the MDA-MB-435 breast-cancer cell

Figure 4 Model for the architecture of h-Prune-containing complexes

Homodimers of h-Prune are created, containing two separate and independent catalytic cores formed by the DHH and DHHA2 subdomains (blue). They are held together through interactions with the C-terminal CHD (green), which consists of a putative coiled-coil region (left), a proline-rich region (centre), and a helical extension (right). The two CHDs of the dimer also enable the binding of at least two partner molecules, such as NM23-H1 (yellow) and GSK3β (red), which can lead to the assembly of higher-order oligomeric structures. The various parts of h-Prune, as well as its interaction partners, are not drawn to scale, and the exact binding sites for GSK3β and NM23-H1 within the CHD are not yet known.

line [7]. However, dipyradamole is a compound which inhibits a variety of mammalian PDEs as well as other targets, such as nucleoside transporters [20]. More specific inhibitors would not only be useful for further *in vivo* studies on the function of h-Prune, but also as lead compounds for the development of novel cancer therapies. Therefore we started to characterize h-Prune and have described here the functional and structural assignment of its domains.

h-Prune was initially characterized as containing an N-terminal DHH domain and an adjacent DHHA2 domain [7,12]. Due to the low conservation within the DHHA2 domain, the domain end is difficult to determine. On the basis of sequence comparisons, the domain end of DHHA2 was mapped to amino acid residue 350 of h-Prune. Our experimental data indicates that this domain extends beyond this position up to residues 379–393. A similar effect can be observed for Class III nucleotidyl cyclases, where the low conservation in the C-terminal part of the catalytic domain leads to a C-terminal-truncated protein family signature [21]. In the DHH family, the catalytic residues are contained within the DHH domain, whereas the DHHA2 domain provides the substrate-binding residues and therefore influences the specificity of the interactions. Including the complete DHHA2 domain is therefore important for studies on possible substrates of h-Prune. The DHHA2 domain was shown previously to display PDE activity. A glycosyl hydrolases family 5 signature (I²⁸⁷FFNTHNEPV²⁹⁶) [22] indicates that h-Prune can display hydrolase activity against other substrates. The association with NM23-H1, which has DNA endonuclease and exonuclease activity [2,23] in addition to its NDPK activity, might suggest other reactions on phosphorylated nucleotides or polynucleotides. The region of h-Prune which is similar to the DNA strand-separating gyrase domains and the nuclear localization of fractions of h-Prune and NM23-H1 make it tempting to speculate that both proteins might form a DNA-modifying protein complex.

On the basis of its homology with a bacterial PPase, it might be expected that the catalytic portion of h-Prune could form dimers with two separate active sites, each completely contained in one h-Prune polypeptide chain. We found that h-Prune can dimerize and that this homodimeric interaction is not important for the activity of the individual catalytic domains. However, we found that the catalytic domain of h-Prune is monomeric in solution and requires a separate domain for dimerization, which is a major difference from other PPases of the same family. This observation indicates that, when compared with other DHH PPases, h-Prune contains a distinct structure in the second subdomain of the catalytic core and has a different mode of association with its interacting partners. The catalytic domain might still contribute to the dimerization of h-Prune, but the additional C-terminal domain, which is not present in other DHH family members, is essential for efficient h-Prune dimerization. Our findings also indicate that a putative five-repeat leucine zipper region between residues 157 and 185 does not mediate h-Prune oligomerization. The stretch of similarity to β-lactamase found in the h-Prune DHHA2 domain could indicate a structural relationship to PdeD-like PDEs, which appear to have evolved from the metallo-β-lactamase fold [24].

Coiled-coil regions normally mediate the oligomerization of proteins [25], and this is consistent with our results that the CHD of h-Prune, which covers a putative coiled-coil region, mediates homodimerization. Surprisingly, the proline-rich region C-terminal to the putative coiled-coil is essential for the formation of a soluble dimeric protein. The arrangement with two predicted helical regions, interrupted by a short non-helical section, could also indicate an intramolecular antiparallel coiled-coil, which would then associate with the coiled-coil of the second monomer to form a four-helix bundle. However, our finding that the second helical region (residues 420–453) is dispensable for dimer formation renders such a dimerization architecture unlikely. More detailed studies on the CHD were not possible owing to the

C-terminal h-Prune fragments not being expressed successfully. The comparison of full-length h-Prune and C-terminally truncated variants, however, showed that the CHD not only mediates dimerization, but also controls the interaction with NM23-H1. We demonstrated previously that the hexameric form of NM23-H1, but not the dimeric or trimeric forms, binds to h-Prune *in vitro* (L. Garzia, A. D'Angelo, A. Amoresano, S.K. Knauer, C. Cirulli, C. Campanella, R.H. Stauber, C. Steegborn, A. Iolascon and M. Zollo, unpublished work), possibly indicating that the dimeric h-Prune interacts with two of the three NM23-H1 dimers within the hexamer. For further characterization of the regulation of this interaction, such as through post-translational modifications, experiments conducted in a more physiological environment will be informative in the future, like immunoprecipitation experiments from mammalian cells or yeast two-hybrid experiments. The C-terminal region of h-Prune was also found to be responsible for the interaction with GSK3 β [11], and we found that GSK3 β and NM23-H1 can bind simultaneously to h-Prune, suggesting that they use different parts of the h-Prune C-terminus for binding. A model for the architecture of h-Prune, such that the h-Prune dimer has two separate and independent catalytic cores as implied by these results, is shown in Figure 4. They are kept together through the interactions of the CHD coiled-coil domain and the neighboring proline-rich region. The two CHDs of the dimer also enable the binding of at least two partner molecules, such as NM23-H1 or GSK3 β , thereby enabling the assembly of higher-order oligomeric complexes.

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