Characterization of PREP2, a paralog of PREP1, which defines a novel sub-family of the MEINOX TALE homeodomain transcription factors

C. Fognani, C. Kilstrup-Nielsen1, J. Berthelsen, E. Ferretti, V. Zappavigna1 and F. Blasi*

Unit of Molecular Genetics and 1Unit of Transcriptional Regulation of Development, DIBIT, Department of Molecular Biology and Functional Genetics, Università Vita Salute San Raffaele, Via Olgettina 58, 20132 Milano, Italy

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ABSTRACT

TALE (three amino acid loop extension) homeodomain proteins include the PBC and the MEINOX sub-families. MEINOX proteins form heterodimer complexes with PBC proteins. Heterodimerization is crucial to DNA binding and for nuclear localization. PBC–MEINOX heterodimers bind DNA also in combination with HOX proteins, thereby modulating their DNA-binding specificity. TALE proteins therefore play crucial roles in multiple developmental and differentiation pathways in vivo. We report the identification and characterization of a novel human gene homologous to PREP1, called PREP2. Sequence comparisons indicate that PREP1 and PREP2 define a novel sub-family of MEINOX proteins, distinct from the MEIS sub-family. PREP2 is expressed in a variety of human adult tissues and displays a more restricted expression pattern than PREP1. PREP2 is capable of heterodimerizing with PBC proteins. Heterodimerization with PBX1 appears to be essential for nuclear localization of both PREP2 and PBX1. A comparison between the functional properties of PREP1 and PREP2 reveals that PREP2–PBX display a faster DNA-dissociation rate than PREP1–PBX heterodimers, suggesting different roles in controlling gene expression. Like PREP1, PREP2–PBX heterodimers are capable of forming ternary complexes with HOXB1. The analysis of some PREP2 in vitro properties suggests a functional diversification among PREP and between PREP and MEIS MEINOX proteins.

INTRODUCTION

The homeodomain is a three-helix DNA-binding domain formed by 60 amino acid residues and is present in a large number of transcription factors. Within this large group, the TALE (three amino acid loop extension) family of homeodomain proteins is characterized by the presence of three additional amino acids between the first and second helix of the homeodomain. The TALE family is divided into two sub-families, the PBC and the MEINOX. The PBC sub-family includes PBX1, 2, 3 and 4, and the Caenorhabditis elegans CEH-20 and the Drosophila EXTRADENTICLE (EXD) gene products. The MEINOX sub-family includes the mammalian MEIS1, 2 and 3, the human and mouse PREP1, the C.elegans CEH-25, the Drosophila HOMOTHORAX (HTH) and other MEIS orthologous gene products from Dario rerio, Xenopus laevis and yeast (1). This sub-division has both a structural and functional basis, since members of one sub-family form stable DNA-binding heterodimers with members of the other. The interaction of MEINOX and PBC proteins is DNA independent, requires specific conserved regions in the respective N-termini, and is compatible with the interaction of the PBC homeodomain with anterior HOX proteins (2–6). The use of different interaction surfaces allows PBC proteins to act as a bridge between MEINOX and HOX proteins and to form transcriptionally active ternary complexes (4,7–9). In addition, some TALE proteins can also interact directly with posterior HOX (10,11).

Heterodimerization between MEINOX and PBC proteins is required to prevent the active export of the PBX1 and EXD proteins from the nucleus (12–14). However, while the MEIS gene product is able to directly reach the nucleus, the PREP1 protein is missing a nuclear localization signal and is only translocated to the nucleus by interaction with PBX (7).

The sequence similarities within the MEINOX sub-family are essentially confined to the homeodomain and to the two N-terminal regions, involved in protein–protein interactions with PBC proteins, termed MH or HR domains (3,5,7,15). PREP1 and PBX form a stable complex in solution, interacting through sequences within the N-terminal part of both proteins; the interaction does not require the respective homeodomains. In addition, complex formation precedes DNA binding, as deletion of the N-terminal part of either protein disrupts co-operative DNA binding of the PREP1–PBX complex (5). Due to the different interaction surfaces used in the PBX–HOX and PREP1–PBX complexes, interaction of PREP1 with PBX does not exclude further interaction with HOX proteins and, indeed, a ternary PREP1–PBX–HOX complex is observed (7).
complex can be observed to bind a PBX–HOX DNA target. This ternary complex has a higher transcriptional activity compared to a PBX–HOX heterodimer (4,8). In contrast to PREP1, MEIS1 can also directly bind to some of the most posterior HOX proteins, like HOXA9 (10,11), through its C-terminal sequence. This apparently results in a functional difference between PREP1 and MEIS1, since only the latter is able to accelerate HOXA9-dependent acute myeloid leukemia in mice (16).

PREP1 is ubiquitously expressed in adult tissues, and is expressed in the embryos throughout development (6). Conversely, the MEIS-family proteins seem to have a specific pattern of expression during development. MEIS2 is expressed in the developing nervous system, limbs and face and in various viscera (17,18).

Here we describe the identification and cloning of a novel MEINOX family member displaying a high degree of similarity with PREP1, which we have termed PREP2. PREP2 is located on chromosome 11 and is expressed in a variety of human adult tissues and displays a more restricted expression pattern in mice (16).

The analysis of the protein sequence of PREP2 indicates that PREP1 and PREP2 form a distinct structural group within the MEINOX family defining a novel sub-family of proteins different from that including MEIS and HTH. PREP2 is capable of forming heterodimers with PBC proteins and heterodimerization with PBX1 appears to be essential for nuclear localization of both PREP2 and PBX1. PREP2, like PREP1, can form ternary complexes with PBX1 and HOX1. A comparison between PREP1 and PREP2 functional properties reveals that PREP2–PBX complexes display a faster DNA-dissociation rate than PREP1–PBX heterodimers, suggesting different roles in controlling gene expression. Our results indicate the existence of two functional groups within the MEINOX family of TALE proteins, represented by the PREP and MEIS sub-families. Furthermore, our analysis of some of the PREP2 functional properties in vitro suggests a diversification of activities among PREP proteins.

MATERIALS AND METHODS

Cloning of human PREP2

PREP2 cDNA was cloned by PCR using oligonucleotide primers generated as described in the Results section. Oligo 2 and oligo 3 were used to amplify (94°C 30 s, 60°C 1 min, 72°C 3 min for 35 cycles) a fragment of 740 bp from a double-stranded cDNA reverse-transcribed from a human fetal mRNA (Clontech 7438-1) in a 50 μl reaction containing 0.5 ng cDNA, 10 pmol each primer, 0.2 mM dNTPs and 1.5 U high fidelity Taq polymerase (Boehringer-Mannheim). The 740 bp fragment was cloned directly in a T-vector (Invitrogen K4500-01) and sequenced. Based on this sequence we designed two specific primers (oligos 4 and 5) to clone the full length PREP2 cDNA by 5′- and 3′-rapid amplification of cDNA ends (5′- and 3′-RACE) according to the manufacturer’s protocol (Clontech 7438-1). The 3025 bp long PREP2 cDNA was cloned into pCR2.1 expression vector for transcription–translation studies and into vectors containing a C-terminal FLAG for transfection in mammalian (pSG-FLAG-Prep2) and Drosophila cells (pPAC-FLAG-Prep2). Primers list:

oligo 2: 5′-TGT GAA CAG GCC ACC CAG GGC-3′
oligo 3: 5′-ACT AAG CAT GGG CTG CAG GAT-3′
oligo 4: 5′-TGG CGG AGG TGA TGC ACT CAG-3′
oligo 5: 5′-CAA CTG GTI CAT CAA TGC CCG-3′

In vitro transcription–translation

All pCR2.1 expression vectors were translated in vitro using the coupled TNT transcription/translation system (Promega) as described previously (5). PREP2 and PBX or PREP1 and PBX were co-translated (plasmids in equimolar amounts).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (5). One micro-liter of nuclear extract (5 μg of proteins) or 2 μl of reticulocyte lysate, containing a combination of in vitro translated PREP1, PREP2, PBX1 and HOXB1, were mixed with 18 μl of PPH binding buffer (10 mM Tris–HCl pH 7.5, 75 mM NaCl, 1 mM EDTA, 6% glycerol, 3 mM spermidine, 1 mM DTT and 0.5 mM PMSF) in the presence of 0.5 μg poly(dI–dC), 30 000 c.p.m. 32P-labeled oligonucleotides and, when necessary, antibody or unlabeled competitors. After 30 min on ice, the reactions were separated by a 5% polyacrylamide gel in 0.5× TBE. For the analysis of the dissociation rate, labeled probe was incubated with in vitro translated proteins for 10 min on ice, then a 500-fold molar excess of unlabeled competitor oligonucleotide was added to the reactions. Samples from the same binding reactions were taken at different times and immediately loaded on native gels. The oligonucleotide probes used in EMSA were: O1, 5′-CACCTGAGGTACAGAGAAGGCGGAGGAGGAG-3′; b2PP2, 5′-GGAGCTTGCAGGGGCTAAGATTGGATCGCTCTA-3′. O1 contains one binding site for PREP1–PBX complex from the urokinesin enhancer (19). b2PP2 contains both a PREP–MEIS and a PBX–HOX site from the Hoxb2 r4 enhancer (18).

Northern blot analysis

Two commercially available human northern blots (Clontech 7759-1 and 7760-1) were hybridized with a 32P-labeled probe generated by PCR, from the expression vector pCR2.1 containing the PREP2 full length cDNA. The probe included only the 3′-untranslated region from nucleotide 1380 to 3025. Hybridization was carried out according to the manufacturer’s protocol.

Transfections

NIH 3T3 fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn calf serum and antibiotics. Cells were transfected with 5 or 10 μg of pSG-Pbx1a and pSG-FLAG-Prep2 by CaPO4 precipitation in 10 cm dishes. Cells were reseeded after 24 h on Chambers Slides (Nunc) and fixed after 16 h for immuno-cytochemistry. Drosophila SL-2 Schneider cells were grown in Drosophila medium (Gibco) at 25°C and transfected with calcium phosphate, using 5 μg each of pPAC-FLAG-Prep2 and pPAC-Pbx1a. The BUX1a vectors have been described before (13). COS-7 cells were maintained in DMEM, supplemented with 10% fetal calf serum (Gibco), 100 IU/ml of penicillin and 100 μg/ml streptomycin. In a typical transfection experiment, 8 μg of reporter plasmid, 4–8 μg of expression construct and 0.2 μg of pCMV-β-gal (Clontech) as an internal control, were used per 10 cm dish. Forty-eight hours after transfection, cells were washed and lysed directly on the plate with

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RESULTS

Identification and structural characterization of a novel PREP1 paralogous gene

We have searched for PREP1 homologous genes in the human genome databank. To avoid finding a member of the MEIS sub-family, we looked for homologies not confined to specific domains, but extending throughout the entire length of PREP1. The PREP1 gene maps on the sequenced chromosome 21, and its exon–intron structure is known (21) and conserved in the mouse gene (our unpublished data). This information allowed us to circumvent the problem represented by the possible presence of introns in the genomic sequence of putative PREP1 homologs. We searched the data bank for genomic sequences having a high level of identity to individual exons of PREP1. The similarities found covered the entire PREP1 cDNA, with the exception of the C-terminus, and were all present on a sequenced, but not totally mapped, 200 000 bp fragment of human chromosome 11. This putative gene was called PREP2. Interestingly, all the homologies were compatible with the exon–intron structure of the PREP1 gene. We reconstructed a putative PREP2 cDNA sequence that we used to synthesize oligonucleotide primers (see Materials and Methods) to amplify a double stranded cDNA reverse transcribed from human fetal mRNA. We next isolated the full-length PREP2 cDNA by 5’- and 3’-RACE. The PREP2 cDNA was completely sequenced and found to be 3025 bp long (deposited in the EMBL data bank, accession no. HX1003667103). In parallel, we also found that a 3175 bp cDNA sequence, AK023136, had been deposited in the EMBL data bank. This sequence is almost identical to that of our PREP2 cDNA, except for a significant sequence divergence in the coding region, since it lacks a CT dinucleotide (position 895–896) causing a premature termination of the open reading frame (data not shown).

The amino acid sequence of PREP2 deduced from the cDNA is reported in Figure 1A in comparison with PREP1. PREP2 is longer than PREP1 (461 versus 436 residues), mainly because of small insertions at different positions. Overall, the amino acid residues in the two proteins are 52% identical with some conservative substitutions raising the overall similarity to 60% (Table 1). The homeodomains and the two regions of homology with MEIS (HR1 and HR2) share the highest level of similarity. The N-terminal 50 residues share 36% identity, whereas the extreme C-termini are largely divergent. Interestingly, there is a 10 amino acid insertion in PREP2 between residues 172 and 176 of PREP1. Since this general region is involved in the formation of heterodimers with PBX proteins, this difference might lead to qualitative and/or quantitative differences in heterodimer formation.

Alignment of the PREP2 cDNA sequence with the previously identified 200 kb chromosome 11 region allowed us to define the intron–exon organization and to determine the size of most human PREP2 introns (Fig. 1B). As expected, PREP1 and PREP2 share the same exon–intron organization. In Figure 1B, in some cases the size of the introns in the PREP2 gene is not indicated because the sequence of some subfragments of the 200 kb region lacked overlapping verification.

The two PREP proteins share amino acid sequence similarity with MEIS1, MEIS2, MEIS3 and HTH. However, PREP1 and PREP2 lack similarity to any MEIS family members in the 55 most N-terminal residues, in the region between HR2 and the homeodomain (residues 244–264 and 328–407 of PREP1), and in the C-terminus from amino acid 339 to the end. Conversely, in these same three regions, PREP1 and PREP2 display a high degree of similarity. Generation of a phylogenetic tree using the CLUSTALX and NJPLOT software (22) confirms that PREP1 and PREP2 define a novel different sub-family distinct from that including MEIS and HTH (Fig. 1C).

The PREP2 gene is expressed in a variety of human adult tissues

We next tested whether PREP2 mRNA was expressed in human tissues by performing a northern blot hybridization on a commercially available (Clontech) array of RNAs extracted from several different human tissues as indicated in Figure 2. A single band of ~4 kb was observed in some, but not all tissues (Fig. 2). In particular, the expression was strong in mRNA extracted from liver, kidney, spleen, thymus, colon and peripheral blood leukocytes. The amount of poly(A)+ RNA on this blot was adjusted by the manufacturer (Clontech) so that the β-actin hybridization signal was of comparable intensity in every lane.

PREP2 displays similarities and differences in DNA-binding and transcriptional regulating activity compared to PREP1

In vitro synthesized PREP2 has a migration rate corresponding to an apparent molecular weight of 70 kDa, thus somewhat slower than PREP1, in agreement with its higher molecular weight (463 versus 436 amino acids of PREP1), but also slower than expected from its calculated molecular weight (data not shown). PREP2 behaves in this respect as PREP1, displaying an apparent molecular weight higher than expected from its amino acid composition (5).

To assess the DNA-binding activity of PREP2 and to compare it with PREP1, we used the O1 oligonucleotide. We previously showed that O1 contains a single binding site for PREP1–PBX complexes (5). We performed binding studies in the presence and absence of PBX1a. Neither PREP2 nor
**Figure 1.** Protein sequence and exon–intron comparisons highlight the fact that PREP1 and PREP2 belong to the same sub-family of TALE proteins. (A) Amino acid sequence comparison between PREP1 (upper line) and PREP2 (lower line). The N-terminal arm is underlined; the extra three amino acids of the TALE homeodomain are indicated in bold; the positions of the α-helices are indicated. (B) Structural organization of the human PREP1 and PREP2 (PKNOX1 and PKNOX2) genes. The numbers above refer to the amino acid residues in the cDNA. The numbers at the bottom indicate the intron sizes in nucleotides. The data are taken from a comparison of the sequences as present in the human genome data bank and compared with the cDNAs. Human PREP1 maps on chromosome 21, whereas the 200 kb fragment containing PREP2 is annotated in the human genome data bank as mapping on chromosome 11. The size of some PREP2 gene introns is not included because the DNA sequence in the data bank lacked double stranded sequence verification or overlap verification. Gray boxes, 5′-untranslated region; white boxes, 3′-untranslated region; black boxes, coding region; hatched boxes, homology regions 1 and 2; solid bars boxes, homeodomain. (C) Phylogenetic tree derived from the comparison of the protein sequences of *Drosophila* HTH, human MEIS 1, MEIS2, MEIS3 and human PREP1 and PREP2, using the Clustalx and Njplot applications. Numbers indicate branch lengths.
PBX1a bound DNA alone. However, PREP2 formed retarded bands with the O1 DNA when co-translated with PBX1a (Fig. 3A). The PBX1–PREP2 complex ran slightly slower than the PBX1–PREP1 included as a control, in accordance with the higher molecular weight of PREP2. The binding was competed by a 500-fold molar excess of cold oligonucleotide and was inhibited by anti-PBX1a and partially by anti-PREP1 antisera. PREP1 antibodies totally inhibited the binding of the PREP1–PBX1a heterodimer as described previously (5). A pre-immune serum had no effect on either PREP1–PBX or PREP2–PBX binding. The high amino acid similarity between PREP1 and PREP2 and the fact that the antiserum was raised against the full-length PREP1 protein justify the cross-inhibition of the anti-PREP1 antiserum on PREP2. The anti-PREP1 antiserum recognized PREP2 also in immunoprecipitation of the in vitro translated protein (data not shown).

Since the sequences of the PREP1 and PREP2 homeodomains somewhat diverge in the first helix and in the first loop, but the two proteins seem to bind equally well to the O1 site, we tested whether we could point out differences in the binding dissociation rate of PREP1–PBX1a versus PREP2–PBX1a complexes. The labeled O1 oligonucleotide was incubated with the in vitro co-translated PBX1a–PREP1 or PBX1a–PREP2 protein complexes for 10 min at 4°C. Subsequently, an excess of unlabeled O1 oligonucleotide was added and the incubation was continued for various times as indicated in Figure 3B. Aliquots of the incubation mixture

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**Table 1. Sequence comparison of PREP1 and PREP2 proteins**

<table>
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<tr>
<th>Region of PREP1 considered</th>
<th>% identity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% similarity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Total protein</td>
<td>225/436 (52)</td>
<td>262/436 (60)</td>
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<tr>
<td>HR1 (50–83)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24/34 (70.5)</td>
<td>29/34 (85)</td>
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<tr>
<td>HR2 (108–153)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39/45 (87)</td>
<td>41/45 (91)</td>
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<tr>
<td>HR1+HR2 (50–153)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82/103 (80)</td>
<td>91/103 (89)</td>
</tr>
<tr>
<td>Homeodomain (222–285)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54/63 (86)</td>
<td>56/63 (89)</td>
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<tr>
<td>N-terminus (1–50)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18/50 (36)</td>
<td>34/50 (62)</td>
</tr>
<tr>
<td>C-terminus (285–415)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75/130 (58)</td>
<td>104/130 (80)</td>
</tr>
<tr>
<td>Extreme C-terminus (416–436)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4/21 (19)</td>
<td>5/21 (23)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers refer to the number of amino acid residues of PREP2 identical or similar to those of PREP1. Numbers in parentheses express percentage identity or similarity.

<sup>b</sup>Numbers in parentheses indicate the amino acid residues in PREP1 and the regions considered.
were loaded on a non-denaturing polyacrylamide gel at different times. As shown in Figure 3B, the PREP2–PBX1a complex dissociated faster than the PREP1–PBX1a complex, being completely abolished in <5 min of incubation with the cold competitor. These results suggest that the sequence divergence between PREP2 and PREP1 within the homeodomain determines different functional properties of the two proteins at the DNA-binding level.

A characteristic of the PREP or MEIS proteins is to form specific ternary complexes with PBX and HOX family members on given DNA binding sites which are functionally relevant (7–9). We therefore analyzed whether PREP2 was capable of forming a ternary complex with PBX1 and HOXB1. For this, we used the b2PP2 oligonucleotide from the Hoxb2 gene enhancer, which contains both a PBX–HOX and a PREP1–MEIS binding site and was previously shown to specifically bind a ternary PREP1–PBX–HOXB1 complex (8). As shown in Figure 4A, incubation of PREP2, HOXB1 and PBX1a with the b2PP2 oligonucleotide formed a retarded band, corresponding to a PREP2–PBX–HOXB1 ternary complex. Binding was totally competed by the unlabelled oligonucleotide. In addition, formation of the ternary complex was inhibited by antisera against PREP1, PBX1a or HOXB1. As shown in Figure 4B, PREP2 was also able to form a ternary complex with HOXB1 and PBX1b, the shorter alternatively spliced form of PBX1. Ternary complexes with PREP2 (lane 9) co-migrated with the endogenous ternary complexes found in retinoic acid treated P19 cells (lane 11) (8).

To verify possible differences between PREP1 and PREP2 in modulating the transcriptional activity of a HOX–PBX heterodimer, we chose the transactivation of the HOXB1–PBX1 complex on the Hoxb1 autoregulatory element (Hoxb1-ARE) as a model system (20). We have previously shown that co-expression of PREP1 was able to further enhance the transcriptional activation of the HOXB1–PBX1 heterodimer on the Hoxb1-ARE (5). We therefore analyzed the effect of co-expression of PREP2 on the HOXB1–PBX1-dependent transactivation through the Hoxb1-ARE enhancer in transient transfections of COS cells. HOXB1 and PBX1a co-expression induced a 5–6-fold activation of transfection (20). Co-expression of PREP1 with PBX1 or HOXB1, as previously reported (5), significantly further stimulated the Hoxb1-ARE reporter activity (Fig. 4C). However, co-expression of PREP2 did not enhance the activation by HOXB1–PBX1, but instead led to a slight, although consistent, reduction of the HOXB1–PBX1 activity (Fig. 4C). These results further suggest the existence of differences in the functional properties of these two closely related MEINOX proteins.

PREP2 is localized to the cytoplasm and requires PBX1 for its nuclear translocation

We have previously reported that PREP1, if exogenously expressed in cultured cells, is found mainly in the cytoplasm. We found that PREP1 lacks a nuclear localization signal, and is carried into the nuclei of expressing cells by heterodimerization with PBX1 (13). We have tested for the subcellular localization of PREP2 by expressing it exogenously in NIH 3T3 mouse fibroblast and in SL2 Drosophila cells. As previously reported, PBX1 is found in the nuclei of exogenously expressing NIH 3T3 cells, whereas it is cytoplasmic in SL2 cells and is transported to the nucleus only upon co-expression with PREP1 (13). We expressed a FLAG epitope-tagged PREP2 and used the anti-FLAG™ antibody to follow the distribution of the protein by immunofluorescence upon cell transfection. As shown in Figure 5, in cells transfected with a FLAG-Prep2 expression vector, PREP2 was mostly found in the cytoplasm of both NIH 3T3 and SL2 transfected cells. However, when cells were co-transfected with an expression vector for the PBX1a protein, both PBX1a and PREP2 were found exclusively in the nucleus in both cell lines. These data indicate that as in the case of PREP1, PREP2 is capable of forming heterodimer complexes with PBX1a, to be transported to the cell nucleus, and to be able to induce nuclear localization of PBX1a in SL2 cells.

DISCUSSION

In the presence of DNA, PBC and MEINOX TALE sub-class of homeoproteins interact with HOX proteins to modulate their functions (20,23–28). HOX–PBC interaction occurs only in the presence of the target DNA and requires the homeodomains and the YPWM peptide stretch N-terminal to the homeodomain (29). On the other hand, the interaction between MEIS and HOX requires the C-terminus of the MEIS proteins (11). In addition to these DNA-dependent interactions, MEINOX proteins can form heterodimers with PBC proteins (4,30,31) that are stable enough to be purified from cell extracts (19). MEINOX–PBC interactions have functional consequences since dimerization prevents the nuclear export of PBX and EXD PBC proteins (12–15). Moreover, PBX can bind at the same time both HOX proteins and TALE members, forming complexes like the heterotrimeric HOXB1–PBX1–PREP1/MEIS1 complex which appears to be essential for HOXB1-dependent HOXb2 expression in the mouse hindbrain (rhombomere 4) (7–9).

Here we report the identification and cloning of a novel MEINOX homeodomain family member which we have named PREP2. A CLUSTALX (22) comparison of the amino acid sequence of PREP2, PREP1, MEIS1, MEIS2, MEIS3 and Drosophila HTH reveals that PREP1 and PREP2 belong to a sub-family of MEINOX proteins different from MEIS (Fig. 1C). From now on we therefore name these sub-families PREP and MEIS, respectively. PREP1 and PREP2, in addition to the similarities in HR1, HR2 and the homeodomain, shared with MEIS, also share three more regions of homology: one at the N-terminus, one between HR2 and the homeodomain and a third downstream of the homeodomain. PREP1 and PREP2 sequences mainly differ in the extreme C-terminus.

Northern blot analysis of PREP2 mRNA expression in human tissues reveals that PREP2 is expressed at higher levels in heart, brain, skeletal muscle and ovary, at lower levels in placenta, lung, pancreas, prostate, testis and small intestine, and is absent in liver, kidney, spleen, thymus, colon and peripheral blood leukocytes (Fig. 2). Comparison with published data shows that the expression domains of PREP1 and PREP2 are similar, but not superimposable, PREP2 displaying a more restricted tissue and organ expression (2). Moreover, in testis an additional band of 7–8 kb is observed, which could represent a tissue-specific transcript arising from alternative splicing and/or from a different tissue-specific transcription start site.

The molecular analysis of PREP2 has shown properties that are similar, but not identical to those of PREP1. PREP2 shares
Figure 4. PREP2 can form a heterotrimeric complex with HOXB1 and PBX1. (A) EMSA analysis of combinations of the PREP1 or PREP2, PBX1a and HOXB1 in vitro translated proteins, as indicated, using b2PP2 oligonucleotide as a probe. The probe contains both a PREP1–MEIS and a PBX–HOX binding site from the Hoxb2 r4 enhancer. The binding was competed by a 500-fold molar excess of cold oligonucleotide and inhibited by anti-PREP1, anti-PBX or anti-HOXB1 antisera. Endogenous activity is indicated (Lys) and the arrows show the ternary complexes. (B) EMSA analysis of combinations of the PREP1 or PREP2, PBX1b and HOXB1 in vitro translated proteins, as indicated. b2PP2 oligonucleotide was used as probe. The EMSA was performed under similar conditions as in (A). The dimers and the ternary complexes are indicated by arrows. (C) Luciferase activity assayed from extracts of transiently transfected COS cells. Cells were transfected with 8 µg of the pAdMLARE reporter construct, with 4 µg of the pSGHOXB1 and 8 µg of the pSGPbx1a expression constructs as indicated. Cells were also co-transfected with 8 µg of pSOPrep1 or pSOPrep2 as indicated. An aliquot of 0.2 µg of the pCMVb-gal plasmid was co-transfected as an internal standard. Bars represent the mean luciferase activity ± SEM of at least four independent experiments.
with PREP1 the ability to form complexes in solution with PBX1a and 1b, to bind DNA in association with PBX proteins and to form ternary complexes with PBX1 and HOXB1 on a Hoxb2 promoter regulatory element. Apart from these similarities, PREP1 and PREP2 display some functional differences at the level of DNA-binding and transcriptional regulation. PREP2–PBX1 complexes show a significantly faster dissociation rate than PREP1–PBX complexes on a classical MEINOX–PBX binding consensus. This difference in DNA binding might be related to the sequence differences between PREP1 and PREP2 within the homeodomain. The third helix of the PREP1 and PREP2 homeodomains are essentially identical and in fact the two proteins recognize the same TGACAG sequence. The main differences are found in the N-terminal arm, in the first helix and in the first loop including the three extra amino acids characteristic of TALE proteins. Residues within the N-terminal arm of the homeodomain make contacts with DNA in the minor groove, whereas the three extra amino acids in the loop between the first and second helix in TALE proteins are usually required for protein–protein interactions with other homeodomain proteins (e.g. the HOX) (32,33).

These sequence differences between PREP1 and PREP2 may therefore in different ways affect the stability of the DNA-bound protein complex, thus explaining the difference in dissociation rates between PREP2– and PREP1–PBX complexes. Moreover, we observed that while PREP1 increased the transcriptional activation of a HOXB1–PBX1 complex, PREP2 did not stimulate, but instead led to a slight, reproducible, reduction of the HOXB1–PBX1 heterodimer activity. Since we could not detect differences between PREP2 and PREP1 in the efficiency of PREP–PBX or HOX–PREP–PBX complex formation in vitro, we believe it is unlikely that the differential capabilities of PREP1 versus PREP2 to enhance HOXB1–PBX1 activity reside on the lack of heterotrimer formation by PREP2. The observed different behaviour may rather depend on a different capability to stabilize HOX–PBX complexes on DNA, or on the lack of a transactivation domain in PREP2 which could be conversely present in PREP1. These results, together with the observation that PREP2 expression displays a narrower tissue distribution than the expression of PREP1, which was previously reported to be nearly ubiquitous (2,6), suggest that PREP2 and PREP1 may perform different functions. Further experiments, including the targeted inactivation of the two genes, are needed to clarify this issue.

We previously reported that in cultured cells exogenously expressed PREP1 is found mainly in the cytoplasm. Formation of a heterodimeric complex with PBX is required to induce the nuclear localization of PREP1, which lacks a nuclear localization signal (13). We have observed that upon transfection of NIH 3T3 mouse fibroblast and in SL2 Drosophila cells PREP2 also remains largely cytoplasmic. Only upon co-expression with PBX1, PREP2 is relocalized to the nucleus, as observed for PREP1 (13). As previously reported, PBX1 is found in the nuclei of exogenously expressing NIH 3T3 cells, whereas it is cytoplasmic in SL2 cells and is transported to the nuclei of these only upon co-expression with PREP1 (13). Similarly PREP2 expression in SL2 cells induces nuclear localization of PBX1, indicating that as in the case of PREP1, PREP2 is capable of forming heterodimer complexes with PBX1, which are subsequently transported to the cell nucleus.

Since PREP and MEIS belong to separate sub-families of the MEINOX proteins, they may play different functional roles. The molecular analysis revealed that both MEIS and PREP proteins form heterodimers with PBX, form ternary complexes with HOXB1 and are required to prevent PBX export from the nucleus. In Drosophila, mutations that alter the homeodomain of the Drosophila MEIS paralog, HTH, cause only partial homeotic transformation, whereas mutations that alter the HR1/HR2 site modify the nuclear localization of EXD, prevent the formation of ternary complexes and have more profound effects on fly development (15,34). In Drosophila, the function of HTH can be complemented by mammalian MEIS1 (15,31). The same experiment has not been carried out with PREP; however, overexpression of PREP1 in Drosophila induces an homeotic phenotype, similar but not superimposable to HTH overexpression. The latter is characterized by the absence of the eye and by leg and antenna alterations, whereas in the overexpression of PREP1 the effect on the eye is weaker (14).
Furthermore, a major functional difference between MEIS and PREP proteins observed so far is in the acceleration of HOX-dependent leukemogenesis, as PREP1 was found unable to substitute for MEIS1 to reinforce HoxA9 transforming activity in bringing about leukemic transformation (16). This result was interpreted to be due to the inability of PREP1 to bind posterior HOX proteins, even though this has not yet been directly tested. In fact, PREP proteins have a totally divergent sequence at the C-terminus, which in MEIS1 is involved in the binding of the HOX9 protein (11). Further experiments in vitro and in vivo are required to fully understand the different functional roles played by the two MEINOX sub-families.

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