The family of hepatoma-derived growth factor proteins: characterization of a new member HRP-4 and classification of its subfamilies

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Hepatoma-derived growth factor (HDGF)-related proteins (HRPs) comprise a family of polypeptides named after HDGF, which was identified by its mitogenic activity towards fibroblasts. In the present study, we describe a hitherto unknown HRP, termed HRP-4. The cDNA of bovine HRP-4 (bHRP-4) predicts a polypeptide of 235 amino acids. Northern- and Western-blot analyses of various bovine tissues demonstrated that HRP-4 is only expressed in the testis. Recombinantly produced bHRP-4 and murine HDGF (mHDGF) histidine-tagged polypeptides display growth-factor activity for cultured primary human fibroblasts at an optimum concentration of 1 ng/ml in serum-free medium. The growth-factor activity declines with increasing concentrations to reach background levels at 1 µg/ml. The expression of the fusion proteins, bHRP-4–green fluorescent protein and mHDGF–green fluorescent protein, in HEK-293 cells demonstrates nuclear localization of the proteins. bHRP-4 and mHDGF

bind to the glycosaminoglycans heparin and heparan sulphate, but not to chondroitin sulphate. Affinity constants determined for these interactions are between 6 and 42 nM. Comparison of the bHRP-4 amino acid sequence with HRP-1–3 and p52/75/lens epithelium-derived growth factor (LEDGF) shows that these proteins share a conserved N-terminal part of 91 amino acids but have C-termini of different lengths and charge. This demonstrates the modular structure of these proteins and allows its classification into three groups based on charge, size and sequence comparison. HRP-4, HRP-1 and HDGF are small acidic proteins, HRP-3 is a small basic protein, and HRP-2 and p52/75/LEDGF are larger basic proteins.

Key words: bacterial protein expression, glycosaminoglycan, mitogen, nuclear localization, surface plasmon resonance.

INTRODUCTION

Hepatoma-derived growth factor (HDGF) has been independently purified from conditioned medium of a human hepatoma cell line [1] and rat metanephrotic mesenchymal cells [2]. Recently (F. Dietz, unpublished work), we have purified HDGF from the bovine testis, where it co-purified with an RNA-binding activity. It has mitogenic activity towards Swiss 3T3 cells [1], endothelial cells [2] and smooth muscle cells [3], and it has recently been demonstrated that this activity depends on the nuclear localization of HDGF [4,5].

The human HDGF cDNA predicts a polypeptide of 240 amino acids and a molecular mass of approx. 27 kDa. The primary sequence of HDGF is homologous with the high-mobility group (HMG) proteins [1,6], but it lacks the typical HMG box, which is the DNA-binding domain of these proteins. HMG polypeptides comprise a group of nuclear proteins involved in chromosomal replication [7], transcription [8] and chromatin structure [9]. HDGF is localized in various compartments. The purification from media of cultured cells and Western-blot analysis demonstrate its presence in secretions [1,2]. In metanephrogenic mesenchymal cells of rat it is found in the cytoplasm [2], whereas in smooth-muscle cells its localization is nuclear [3]. However, HDGF seems to act primarily in the nucleus, because nuclear targeting of HDGF is essential for its growth-promoting activity [4,5]. Except for its mitogenic activity, the function of HDGF is

unknown, although it has been suggested to play a role in nephrogenesis [2] and vascular development [3]. HDGF may also play a role in tumorigenesis, since its reduction in human esophageal cancer cells is associated with decreased sensitivity to irradiation [10]. HDGF is a member of a growing family of HDGF-related proteins (HRPs).

The cDNAs of two proteins, termed HRP-1 and HRP-2, have been isolated by screening a library with an HDGF cDNA under low-stringency conditions [11]. Another member of this family co-purified with a transcriptional activator and it binds to heat shock and stress-related genes [12,13], which supports the view that proteins of the HRP family may function in the nucleus. Owing to differential splicing, this protein occurs as a 52 or 75 kDa polypeptide, hence it has been termed p52/75. In an independent study [14], this protein was found to have growth- and survival-promoting activity on lens epithelial cells, keratinocytes and fibroblasts, hence it has been termed lens epithelium-derived growth factor (LEDGF).

Yet another member of the HRP family, termed HRP-3, has been identified [15]. Like HDGF, HRP-3 also seems to have growth-promoting activity. Whereas the function of HRP-2 and HRP-3 is unknown, HRP-1, which is expressed only in the testis, seems to have a regulatory function in defined developmental stages during spermatogenesis [16]. The alignment of amino acid sequences reveals a high degree of homology of these proteins with HDGF in the N-terminal part of the polypeptides [11]. In

Abbreviations used: FCS, foetal calf serum; FGF, fibroblast growth factor; GAG, glycosaminoglycan; GFP, green fluorescent protein; HDGF, hepatoma-derived growth factor; hath, homologous with the amino terminus of HDGF; HMG, high-mobility group; HRP, HDGF-related protein; LEDGF, lens epithelium-derived growth factor; ORF, open reading frame; SPR, surface plasmon resonance.

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the present study, we present a new member of this family, termed HRP-4, and describe its proliferative activity and nuclear localization.

EXPERIMENTAL

Materials

The chemicals used in the present study were purchased from Serva (Heidelberg, Germany), Invitrogen (Paisley, U.K.), Roche (Basel, Switzerland) and Merck (Darmstadt, Germany). α -[32 P]-dCTP (specific radioactivity approx. 110 TBq/mmol) and [3 H]thymidine (specific radioactivity approx. 4.19 TBq/mmol) were from Amersham Pharmacia Biotech. The pcDNA1/Amp plasmid used for library construction was purchased from Invitrogen, and the pGEX vector for GST fusion was from Pharmacia (Uppsala, Sweden). The pQE80L plasmid used for construction of histidine-tagged fusion proteins was from Qiagen (Hilden, Germany) and the pEGFP-C3 plasmid used for construction of fusion proteins of green fluorescent protein (GFP) was from BD Bioscience ClonTech (Palo Alto, CA, U.S.A.).

Construction of a bovine testis cDNA library

Total RNA was isolated by homogenization of bovine testis in guanidine isothiocyanate and subsequent centrifugation through a cesium chloride cushion [17]. The RNA pellet was redissolved and subjected to 2 cycles of enrichment with polyadenylated [poly(A)⁺] RNA via chromatography on an oligo(dT) column. Poly(A)⁺ RNA (5 μ g) was reverse-transcribed with Super Script TM reverse transcriptase (Invitrogen) according to the supplier's protocol. cDNA synthesis was primed with a primer consisting of 18 T and a *NotI* restriction site at the 5'-terminus (5'-AACC-CGGCTCGAGCGGCGCT₁₈-3'). After completion of cDNA synthesis according to standard protocols [17], an adapter with a *BstXI* restriction site was ligated.

Subsequently, cDNA was digested with *NotI*, generating fragments with a *NotI* restriction site at the 3'-terminus and a *BstXI* site at the 5'-terminus. This allowed unidirectional cloning into the pcDNA1/Amp expression plasmid digested with *BstXI* and *NotI*. Transfection of an aliquot of the cDNA/plasmid ligation into *E. coli* DH5 α yielded a non-amplified library of approx. 120 000 independent colonies.

Screening of the bovine testis cDNA library

A human HDGF cDNA [1] was labelled with [α - 32 P]dCTP by random priming. The labelled cDNA was hybridized to the bovine testis cDNA library. After hybridization, filters were washed at 45 °C in 0.2 $\times$ SSC for 30 min, and 33 double-positive clones were identified (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate). These clones were isolated and the respective plasmids were analysed by Southern-blot analysis with the human HDGF probe [1] to select for clones harbouring plasmids with the largest inserts. The inserts of the isolated plasmids were initially sequenced with T7 and SP6 primers and then by internal primer walking.

Sequencing of plasmid containing bovine HDGF (bHDGF) and bovine HRP-4 (bHRP-4) cDNA

Primer walking was applied to plasmids having HDGF cDNA inserts. The following primers were used for sequencing: primer bHDGF/1 (sense, 5'-CATGTCGCGATCCAACCGGC-3'), primer bHDGF/2 (sense, 5'-AGAGCGGGAGACCTACTG-GA-3'), primer bHDGF/3 (sense, 5'-GAGATCACGAGAGC-CTGTAG-3'), primer bHDGF/4 (antisense, 5'-CTCCCATA-

ACACCTGGGGTC-3') and primer bHDGF/5 (antisense, 5'-CACCCAGACAGCAGCAGGAAC-3'). Sequence analysis was performed with an automatic ABI Prism 310 genetic analyser. Sequencing reactions were performed according to the manufacturer's protocol.

One of the plasmids analysed by sequencing with T7 and SP6 primers had an insert that was not identical with the HDGF cDNA. This insert was further sequenced by primer walking using the following oligonucleotides: primer HRP-4/1 (sense, 5'-AGGCGGGGCTTCAGCGAAG-3'), primer HRP-4/2 (sense, 5'-GCCGCTGTCGCCGAGGAGGCC-3'), primer HRP-4/3 (antisense, 5'-AACATCCCGTGAGCCCCT-3'), primer HRP-4/4 (antisense, 5'-CGGTTACACATCAGATCTG-3').

Northern-blot analysis

Total RNA from different bovine tissues was prepared as described for the testis cDNA library. Total RNA (5 μ g per tissue) was separated on a denatured agarose gel electrophoresis and subjected to Northern-blot analysis as described previously [18]. Digoxigenin-labelled RNA, corresponding to the coding region of bHRP-4, was prepared according to the manufacturer's instructions (digoxigenin RNA labelling kit; Roche).

Production of recombinant proteins and HRP-4-specific antisera

The GST-bHRP-4 fusion protein was produced in *E. coli* DH5 α and purified via glutathione-affinity chromatography. The eluted fraction was applied to a preparative SDS/PAGE and the proteins were visualized via KCl precipitation. The band corresponding to GST-bHRP-4 was cut from the gel and the fusion protein was electroeluted. Six aliquots of approx. 100 μ g each were used to immunize a rabbit. For affinity purification of antisera and proliferation assays, mHDGF and bHRP-4 were produced as N-terminal histidine-tagged fusion proteins (mHDGF-His and bHRP-4-His respectively) in BL21 Pro bacteria (BD Bioscience ClonTech). The mHDGF and bHRP-4 open reading frame (ORF) was amplified from cDNA prepared from murine or bovine testis mRNA, with two primers located at the 5'-end and 3'-end of the ORF. The primers provided endonuclease restriction sites, which allowed ligation into the correspondingly digested pQE80L vector. The sequences of primers were 5'-CGGGATCCATGTCGCGATCCAACCGGCAG-3' (sense) and 5'-ACGCGTCGACCTACAGGCTCTCTCATGATCTC-T-3' (antisense) for mHDGF, and 5'-CGGGATCCATGTCGCGATTCACCGTCGC-3' (sense) and 5'-CCCAAGCTTCTACTGGCTCTCGTGGCC-3' (antisense) for bHRP-4. Proteins were purified on Ni²⁺-nitrilotriacetate agarose (Qiagen) as recommended by the supplier. Purified bHRP-4-His was coupled with Affigel 10 according to the manufacturer's protocol (Bio-Rad, München, Germany), and the resulting matrix was used for immunoaffinity purification of α GST-bHRP-4 antibodies. The affinity-purified antibody was tested for cross-reaction with HDGF by Western blotting with recombinant bHDGF. For GFP fusion, ORFs of the mHDGF cDNA and bHRP-4 cDNA were cloned into pEGFP-C3 vectors (BD Bioscience ClonTech). DNA was prepared by PCR using the primers 5'-CTCGAGCTCAA-GCTTGTCATGTCGCGATCCAAC-3' (sense) and 5'-GAAG-AGCCCTGGATCCCCAGGCTCTCATGATC-3' (antisense) for mHDGF, and 5'-CTCGAGCTCAAGCTTGTCATGTCGCGATTCTAC-3' (sense) and 5'-GAAGAGCCCTGGATCCCCTGGCTCTCGTGGCC-3' (antisense) for bHRP-4. The primers provided endonuclease restriction sites, which allowed in-frame ligation to the GFP ORF of the vector.

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1  tgagccctgtccgcagtcattctgcgattctacgctcgcaagtcagtcggggacctg
(1)  M S R F Y R R K Y K C G D L
61  gtgttttgcgaattaaagggctacgcccactggcccagcagcagacggccgag
(15) V P A K L K G Y A H W F A R I E O T A E
121  gccaacgcgtaccaggtgtttttctcgggacccacgagacggccttctcgggcccagg
(35) A N R Y Q V F F G T H E T A F L G P R
181  cactcttctccgtagcagggagtcacaggagaagtttggcaaacgaataagaggcgggg
(55) H L F P Y E E S K E K F G K P N K R R G
241  ttacgaggaagcctgtgggagattgagaaacacccacgctccaggcttccgattaccag
(75) F S E G L W E I E N N P T V Q A S D Y Q
301  tgcgccctggagaagagctgcccctgaggagcccagcccagggtcggcggggcgag
(95) C A L E K S C P E E P E P E V A E G G E
361  gaccggaagagccacacacacgcgccgacgacgatgacaaagggaagctggcgctcgac
(115) D P K S H T N G G D D D D Q G K L G V D
421  ctgcccagctgaggaagagaacagaaggagaccctgaaggagccgagcagccgcca
(135) L P A E E E N K K E T L K R T A E D P P
481  gaggacatcccaagcgtcccaaggaggtgatcctgaagaaggagaggaaggag
(155) E D I P K R P K E A D P E E G E R K E
541  gcggccgctgtcgcggaggagccgaggatgcgaggccgctccttgggaggtggagaac
(175) A A A V A E E A E D A R P L L V E V E N
601  gaccgcccgccctcctgctgctggcctggcctgggggctgccctgatggagcaggagcca
(195) D P A A S V L G L A W G L P V M E Q E P
661  gaggaaagagtcgctgagagagagggccagggcccgaggtggccacgagagc
(215) E E E S A E R E A Q A P G V G G G H E S
721  cagtgtaccacaaagtctccagaagagccccccacccttctgctgagggcctggctgt
(235) Q *
781  cacagggggaactggccatggcctgcaaaactgggacgcctccccacccttgcctc
841  ccaccgcccctctctgcccctcccgcccgccctcgggattgaccggagagggcagccc
901  accagggccctcaccctctgcgccccacacccctgggatctgagtcagcgcctctgctccct
961  gggcgaggttgaaaaccacccgctggccctccctgctggaactgttttgcaaggctct
1021  gttggagccagaccggccgttttccacctcctgatgccccctgctccctccccaccccc
1081  gccccgcccctcttgagtcacacgttggaatcatagccgggagtttcggaagggtgaag
1141  gataagagccggggggcacttccagggacgtggcagaggaacccctcaagctgtgtgtg
1201  tgggaatggcaggaggttggtgtattgctgagctcgctccactcctccaggccctacgg
1261  tatttgaggcctagattctgagaaagtgaacggccttgccggaggccctcttctgctg
1321  acccagggtgtatgggagttgtagttttcatcagataagtggtgctcttccaggtgtg
1381  agcgagagcggaaagcttagcttcgcccagcttcttggttgtaaggctgaggaacatctg
1441  tcccatcttagatctttaggggctcaggggatgttctgggaagacccctggccaccctt
1501  tccctcctccccagcctcaaggccaccgcaagtataaacatcaccaggtataagag
1561  aactgggtacctcactctcctagttttaccgttcagttaccgtttctgagatgaact
1621  aggtggttccctttctacttgaggtttcaggtcgtttatgtagtttaagtttcgtgttc
1681  ctttttccaccgcatccctggacgtggtccctcagagttgtaccagatctgatgt
1741  gtaacagactaagttaggaaacaaagggggccctccccacattcaatggtgtctat
1801  ctgctctcctccatcttgggtctcaccatgccttctccttgggctctgaggaatat
1861  tgttgacagtagcttctgaagtttagattgaatatttcagttcctaaggaaataaaccc
1921  gttgattacttaaaaaaagt

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Figure 1 Nucleotide and cDNA-predicted amino acid sequence of bHRP-4

Underlined amino acid sequences represent the conserved N-terminal hath region. The sequence is available under GenBank® accession no. AJ237666.

Immunocytochemistry

HEK-293 cells (30000) were seeded on to 12 mm coverslips in a 24-well plate. They were then transfected with bHRP-4–GFP or mHDGF–GFP fusion proteins or GFP vector cDNA by using Exgen® (Fermentas, Heidelberg, Germany) according to the manufacturer's protocol. After 1 day in culture, the cells were fixed by incubation in 4% paraformaldehyde and 10% sucrose for 10 min at room temperature. After permeabilization with 0.2% (v/v) Triton X-100 in PBS, the cells were incubated with rabbit anti-GFP antibody (1:50 in PBS; Abcam Ltd, Cambridge, U.K.). After washing with PBS, the bound antibody was visualized by incubation with FITC-labelled goat anti-rabbit antiserum (1:50; Jackson IR). Cell nuclei were counter-stained with 4,6-diamidino-2-phenylindole.

Cell proliferation assay

The effects of recombinant bHRP-4-His and mHDGF-His on human skin fibroblasts were determined by measuring the incorporation of tritium-labelled thymidine into DNA of proliferating cells [19]. Fibroblasts were seeded on to 24-well plates at a density of 7000 cells/cm² and cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS). After 8 days, the cells were nearly confluent, and the medium was replaced with Dulbecco's modified Eagle's medium containing no FCS but different concentrations of the respective re-

Figure 2 Tissue distribution of bHRP-4

Seven different bovine tissues were tested for their expression of bHRP-4 mRNA (A) or protein (B). mRNA (5 µg) or 30 µg of protein from different tissues were loaded and separated by gel electrophoresis. bHRP-4 mRNA was detected by incubation with a digoxigenin-labelled probe, whereas bHRP-4 protein was detected by an antibody raised against recombinant bHRP-4. Only testis contained significant amounts of bHRP-4 mRNA and protein. The lower part of Figure 2(A) shows the agarose gel for similar mRNA loading in each lane.

combinant proteins. In addition, each well received 8.9 kBq of [³H]thymidine (specific radioactivity 365 MBq/mmol) and cells were further incubated for 28 h. Cells were harvested and the incorporation of radioactivity was measured in a 1900 CA liquid-scintillation counter (Packard, Meriden, CT, U.S.A.). In a separate experiment, the medium containing 10% FCS was not changed after 8 days, but recombinant proteins and labelled thymidine were directly added to the medium.

Surface plasmon resonance (SPR) experiments

SPR experiments were performed on a Biacore 3000 instrument. Heparin, heparan sulphate and chondroitin sulphate were

Figure 3 Cell growth activity of bHRP-4 and mHDGF on human skin fibroblasts

The growth-stimulating effects of bacterially expressed bHRP-4-His and mHDGF-His on human fibroblasts cultivated either without FCS (**A**) or with FCS (**B**) are shown. Both bHRP-4-His and mHDGF-His increase [³H]thymidine incorporation in a concentration-dependent manner. Medium without growth factor and medium with insulin (1 μ g/ml) as a strong mitogen were used as negative and positive controls respectively. Error bars refer to the maxima and minima of three independent measurements.

biotinylated as described previously [20]. Biotinylated glycosaminoglycans (GAGs) were adsorbed on to a streptavidin-coated biosensor chip to approx. 160 resonance units (RU). Qualitative binding analysis of mHDGF and bHRP-4 on different GAG surfaces was performed at a flow rate of 30 μ l/min. Association and dissociation were followed for 600 s each. Regeneration of the surfaces was obtained by 1 min pulses of 0.5% SDS and 1.5 M NaCl. As a control, the streptavidin surface without biotinylated GAG was used. For quantitative analysis, association time was extended to 1200 s at a flow rate of 15 μ l/min. Since equilibrium was not reached during this measurement, the curve was extrapolated using BIA evaluation software 3.0. Values of the affinity constant K_D were derived from a standard Scatchard plot (the amount of bound material versus the amount of bound material divided by the free ligand concentration) and compared with the values calculated by the instrument's software.

RESULTS

Isolation of a bHRP-4 cDNA

We initially screened a bovine testis cDNA library to isolate HDGF cDNA-containing plasmids. Several double-positive clones were isolated. A plasmid isolated from one of these clones contained an insert of 1932 bp, of which 243 bp represent the 5' untranslated region, followed by an ORF of 717 bp and a 3' untranslated region of 944 bp. A polyadenylation signal is found at position 1904, followed by a brief poly(A)⁺ tail. Comparison of the amino acid sequence predicted by the ORF with human HDGF led to the identification of the cDNA as the bovine orthologue of HDGF (GenBank[®] accession no. AJ237996; results not shown).

Furthermore, we isolated a plasmid in the course of the screening, which contained an insert with a sequence homologous but not identical with HDGF. This insert has a size of 1942 nt, of

Figure 4 Nuclear localization of bHRP-4 expressed in HEK-293 cells

Cellular localization of bHRP-4 and mHDGF was examined by the expression of GFP fusion proteins in HEK-293 cells. In contrast with GFP alone (A), bHRP-4-GFP (B) and mHDGF-GFP (C) mainly locate to the nuclear compartment of the cells.

which 18 nt represent the 5' untranslated region, followed by an ORF of 705 nt and a 3' untranslated region of 1218 nt (Figure 1). The ORF predicts a protein of 235 amino acids with a calculated molecular mass of 26.3 kDa. The cDNA sequence predicts 30 basic amino acids and 54 acidic amino acids. Thus at neutral pH the protein is negatively charged, and the pI is predicted to be 4.54. We term this protein as HRP-4 (GenBank® accession no. AJ237666).

Expression of HRP-4 in bovine tissue

To investigate whether mRNA expression of bHRP-4 is limited to the testis, we isolated RNA from seven different bovine tissues. Figure 2(A) shows that bHRP-4 mRNA could not be de-

tected in other tissues than the testis. To test whether the mRNA message in the testis is translated to bHRP-4 protein, we developed an antibody raised against the recombinantly expressed GST-bHRP-4 fusion protein. The affinity-purified antibody showed no cross-reactivity to recombinant bHDGF in Western-blot analysis (results not shown). The Western-blot results obtained for this antibody corresponded to the Northern-blot analysis. Only a single band at 36 kDa was detected in the tissue (Figure 2B).

Cellular localization and growth-factor activity of bHRP-4

To investigate a potential growth-promoting activity of bHRP-4, we expressed this protein as well as mHDGF with a histidine-tag attached to the N-terminus in bacteria. bHRP-4-His and mHDGF-His were purified by Ni-chelating chromatography and the purified proteins were used at different concentrations to condition the media of primary human skin fibroblasts (Figure 3). In the absence of FCS, 1 ng/ml of bHRP4-His and mHDGF-His reached 62 and 33 % of the proliferating activity of 1 μ g/ml insulin respectively (Figure 3A). Interestingly, increasing the growth-factor concentration up to 1 μ g/ml resulted in proliferation activities declining to control levels for both factors. To investigate the influence of serum on the growth-factor activity, we repeated the experiment in the presence of medium containing 10 % FCS. Here the growth-promoting activity of both proteins was enhanced. Already 0.1 ng/ml of the two factors were sufficient to reach 78 % (bHRP-4-His) and 45 % (mHDGF-His) of insulin activity (Figure 3B). This difference might be due to the additive effect of the stimulating factors supplied with the serum.

Cellular localization of bHRP-4

To examine whether the nuclear localization that was shown for other members of the HRP family also applies to bHRP-4, we constructed cDNAs that code for bHRP-4 and mHDGF fusion proteins, in which GFP was fused to the N-terminus of both polypeptides. Examination of the cells for localization of GFP revealed a nuclear localization for the bHRP-4 and mHDGF fusion proteins. In contrast, in cells that were transfected with GFP cDNA alone, GFP protein was found to be present in the cytoplasm (Figures 4A–4C).

Binding of bHRP-4 and mHDGF to GAG

Since several growth factors have been described to bind to heparin and heparan sulphate [21], we investigated by SPR studies whether this feature is also shared by bHRP-4 and mHDGF. Biotinylated GAGs were immobilized on a streptavidin sensor surface at 150 pg/mm². bHRP-4-His and mHDGF-His were expressed in bacteria and purified via Ni-chelating chromatography from bacterial lysates. mHDGF and bHRP-4 showed strong binding to immobilized heparin and heparan sulphate, whereas no binding could be detected to the structurally related polyanionic sugar, the GAG chondroitin sulphate (Figure 5). To obtain quantitative data about this interaction, we analysed the binding of different growth-factor concentrations to the GAG sensor chip (Figure 6). Mathematical analysis of the interaction of heparin and mHDGF was performed by fitting a one-site Langmuir binding model to the experimental curves. The mean K_D value for the seven different concentrations used was 37 nM (Table 1). Equilibrium was not reached during the measurement,

Figure 5 Binding of bHRP-4-His and mHDGF-His to different GAGs

Binding was detected by SPR. The sensorgrams show the association and dissociation of recombinant mHDGF-His (A) and bHRP-4-His (B), at a concentration of 500 nM, to heparin, heparan sulphate and chondroitin sulphate. The association interval (600 s) is indicated by the black part of the bars above the sensorgrams, and the dissociation interval (600 s) is indicated by the white part of the bars. RU given on the y-axis show the amount of growth factor bound to the sensor surface.

therefore RU of the steady state (R_{eq}) were calculated by the instrument's software and plotted against the concentration of the analyte (Figure 6B). Non-linear regression of this plot suggests a single class of binding site. A K_D value of 42 nM was calculated from the slope of the linear regression of a Scatchard plot (Figure 6C). Table 1 summarizes all affinity constants obtained by different kinds of mathematical analyses for the interaction of both the growth factors with heparin and heparan sulphate.

DISCUSSION

HDGF and HRP comprise a family of polypeptides. So far, cDNAs of six members have been isolated. These include HDGF itself [1,2], HRP-1 [16], HRP-2 [11], p52/75/LEDGF [12,14] and HRP-3 [15], and bHRP-4 has been identified in the present study. bHRP-4, like mouse HRP-1, seems to be testis-specific. In reducing SDS/PAGE, the protein runs at a molecular mass of 36 kDa, which differs from the one calculated from the amino acid se-

Figure 6 Quantitative analysis of the interaction of mHDGF with heparin

Various concentrations of mHDGF (10, 25, 50, 75, 100, 150 and 200 nM) were flushed over a heparin sensor chip surface and allowed to bind for 1200 s (**A**). From these sensorgrams, values of the affinity constant K_D were calculated separately for each concentration by BIA evaluation software. For comparison, RU of the steady state (Req) were calculated and plotted against the growth-factor concentration (**B**). The regression coefficient of the non-linear regression of this plot is given as R^2 in the sensorgram. (**C**) The K_D value (given in the sensorgram) was calculated from the negative slope of the linear regression of a Scatchard plot (Req versus Req/conc.).

quence (26.3 kDa; see Table 2B). The reason for this discrepancy is not clear, but recombinantly produced bHRP-4 showed the same running behaviour in SDS/PAGE (results not shown) and this difference was described for mHRP-1 [16]. Although HDGF has been implicated in renal and vascular development, the biological function of this growth factor is largely unknown. The capability to stimulate proliferation of cultured cells has so far been demonstrated for HDGF, HRP-3 and LEDGF [1,2,22–24]. In the present study, we provide evidence that bHRP-4 also has growth-promoting activity. bHRP-4-His and mHDGF-His polypeptides were expressed in bacteria and purified to apparent homogeneity. They stimulate the growth of cultured primary human fibroblasts. In the concentration range

tested, both growth factors reached the highest activity at 1 ng/ml in medium without FCS. In the presence of FCS, similar values for [3 H]thymidine incorporation were obtained at 10-fold lower concentrations, i.e. 0.1 ng/ml, indicating that factors supplied with the medium enhance the stimulating effect of the recombinant proteins. We used the strong growth-factor activity of insulin as a positive control. In the presence (absence) of FCS, bHRP-4 and mHDGF respectively reached 78 % (62 %) and 45 % (33 %) of the insulin growth-promoting activity, indicating a substantial growth-factor activity of bHRP-4 and mHDGF expressed in bacteria. Surprisingly, increasing the growth-factor concentration to 1 μ g/ml resulted in a decrease in [3 H]thymidine incorporation.

Table 1 Summary of the quantitative binding analysis of mHDGF and bHRP-4 to heparin and heparan sulphate

Values of the affinity constant K_D for the binding of mHDGF and bHRP-4 to heparin and heparan sulphate were calculated either by fitting a one-site Langmuir binding model to the experimental curves or from the negative slope of the linear regression of a Scatchard plot.

mHDGF concentration (nM)	K_D (nM)	
	Heparin	Heparan sulphate
200	33.4	17.6
150	36.7	15.1
100	38.1	14.9
75	32.6	13.8
50	35.2	14.5
25	45.8	18.3
10	37.4	16.5
Mean	37 ($\pm$ 4.4)	15.8 ($\pm$ 1.7)
Scatchard	42	17.5
bHRP-4 mean	8.7 ($\pm$ 5.2)	6.1 ($\pm$ 2.3)

Table 2 Summary of the amino acid identities and properties of HDGF and HRP

(A) Percentages of amino acid identities of HDGF, HRP-1, HRP-2, HRP-3, HRP-4 and p52/75/LEDGF for the 100 N-terminal amino acid residues. (B) Predicted molecular masses and pI. The bold Roman numerals indicate the classification of different subfamilies.

(A)						
	HDGF	mHRP-1	mHRP-2	hHRP-3	bHRP-4	p52/75/ LEDGF
HDGF	*	80	72.5	79	86	75.5
mHRP-1		*	67	62	89	66
mHRP-2			*	72	70	80
hHRP-3				*	70	75
bHRP-4					*	69
p52/75/LEDGF						*

(B)			
	Molecular mass (kDa)	pI	Subfamily
mHDGF	26.3	4.77	I
bHRP-4	26.3	4.54	
mHRP-1	31.7	4.2	
hHRP-3	22.6	8.39	II
mHRP-2	74.3	8.66	III
p52/75/LEDGF	64.3	9.18	

Transient transfection of bHRP-4-GFP and mHDGF-GFP fusion proteins into HEK-293 cells showed that both proteins localize to the nucleus. For HDGF, it was recently shown that its growth-promoting activity depends on its nuclear localization [4,5]. An 18-amino-acid nuclear localization signal in the C-terminal part of the protein is responsible for this nuclear targeting. Sequence comparison of bHRP-4 with mHDGF reveals that the localization and sequence of this nuclear localization sequence/signal is conserved among the two proteins. Thus it seems that HRP proteins fulfil important functions in the nucleus. So far, functional studies supporting this assumption have only been provided for p52/75/LEDGF, which has been shown in an *in vitro* assay to function as a transcriptional co-activator activating stress-related genes [12,13].

Figure 7 Amino acid sequence alignment of the N-terminal part of HDGF and HRP

Alignment of the sequences of the 100 N-terminal amino acids of bHDGF, bHRP-4, mHRP-1, mHRP-2, hHRP-3 and p52/75/LEDGF are shown (GenBank® accession nos. AJ237996, AJ237666, D63663, D63850, AB029156 and AF098483 respectively). * Indicate amino acid residues identical with bHDGF. In the case of non-identity, the * is omitted and the amino acid residue occurring in the respective protein is indicated. Deletions and insertions are indicated by dashes. The PWPP domain is shaded in light grey and the central four amino acids of this domain are shaded in dark grey. The region in between amino acids 31 and 42 of bHDGF is boxed.

Both HDGF and LEDGF, however, have been shown to be secreted proteins, and it has been demonstrated in the case of both proteins that on addition to cells, they were taken up and transported to the nucleus [5,25]. In addition, all HRP lack a signal sequence for targeting across the membrane of the endoplasmic reticulum, and they are released by an unknown delivery pathway. These are features that HRP share with the basic fibroblast growth factor (FGF), which too has no signal sequence, is secreted and is believed to act in the nucleus [26–28].

Moreover, similar to FGF and vascular endothelial growth factor [21], HDGF, HRP-4 and LEDGF also bind to heparin and heparan sulphate, which suggests that they are able to interact with the cell surface. For LEDGF and other growth factors, this interaction promotes its nuclear transport and its mitogenic activity [25,29]. The binding of recombinant bHRP-4-His and mHDGF-His to heparin and heparan sulphate is specific and cannot be attributed simply to the negative charge of these GAGs, since neither of the two proteins binds to chondroitin sulphate. The K_D value of this interaction was calculated to be between 6 and 42 nM. These data correspond to those obtained for the affinity of other growth factors, e.g. FGF-2, to heparin [30]. This observation suggests that heparin and heparan sulphate in particular may promote the interaction of HRP with the cell surface and facilitate their internalization and intracellular targeting [29].

Based on sequence comparisons, the HRP protein family can be subdivided into three subgroups. Alignment of the cDNA-predicted amino acid sequence of bHRP-4 to the amino acid sequences of human HDGF and other HRP reveals a high degree of amino acid residue identity, in particular among N-terminal 91 amino acids (Table 2A, Figure 7). Interestingly, in all HRP, this region contains a PWPP domain (Figure 7), which is suspected to play a role in cell growth and differentiation and in protein–protein interactions in general [31]. This N-terminal region has been termed homologous with the amino terminus of HDGF (hath) region [11]. In this region, bHRP-4 shares 86 and 89 % of amino acid residues with HDGF and mHRP-1 respectively, but it shares only 70 and 69 % of amino acid residues with mHRP-2/human HRP-3 (hHRP-3) and p52/75/LEDGF respectively. Between amino acids 31 and 42 (numbers refer to the HDGF sequence) of the hath region, bHRP-4 is different from HDGF. Compared with HDGF, bHRP-4 lacks six amino

acids in this region and four are non-identical with HDGF. Alignment of mHRP-1 to HDGF reveals that these six amino acids are also missing in mHRP-1 and that the remaining five amino acids are almost identical with bHRP-4. Thus in the hath region mHRP-1 and bHRP-4 share a deletion of six amino acids, which is not found in HDGF. Interestingly, in this area the gap of six amino acids does not occur in mHRP-2, hHRP-3 or p52/75/LEDGF and the degree of amino acid identity to HDGF in this area is low. Thus it may be speculated that this area serves different purposes in HDGF, mHRP-1/bHRP-4 and mHRP-2/hHRP-3/p52/75/LEDGF. When the C-terminal region (amino acid residues 91–235) of bHRP-4 is compared with HDGF, bHRP-4 shares more amino acid residues with HDGF than with mHRP-1 (results not shown). In summary, because of the similarity in the hath region and the identical tissue distribution, bHRP-4 might have a similar function as mHRP-1 [16], but the higher similarity to HDGF in its C-terminal part suggests that it is not the orthologue of mHRP-1. bHRP-4 bears no similarity to mHRP-2, hHRP-3 and p52/75/LEDGF in the C-terminal part.

mHRP-1, bHRP-4 and mHDGF are acidic proteins with a molecular mass of 26–31 kDa. In contrast, mHRP-2 and p52/75/LEDGF are basic proteins with a molecular mass of approx. 70 kDa (see Table 2B). The N-terminal hath region is equally basic in all HRP. Thus the C-terminal part determines the pI of the various HRP. The C-termini of HDGF, mHRP-1 and bHRP-4 are strongly acidic and those of hHRP-3 are weakly acidic, whereas those of mHRP-2 and p52/75/LEDGF are strongly basic. Thus based on size, pI and amino acid identities in the N-terminal domain, HDGF, mHRP-1 and bHRP-4 constitute one subfamily of HRP proteins, whereas mHRP-2 and p52/75/LEDGF constitute another. hHRP-3 cannot be grouped into these subfamilies. Despite the basic pI, it is smaller than other HRP. Comparison of the amino acid sequences reveals a modular character of these proteins, suggesting that the N-terminal hath region may have a function common to all HRP, whereas the C-terminal part may confer specific functions to the HRP of the respective subfamilies.

Comparison of the number of amino acids of human HDGF, mHDGF and bHDGF reveals that human HDGF and bHDGF are more closely related to each other than human HDGF and mHDGF (results not shown). bHDGF and human HDGF display 96% amino acid identity, whereas mHDGF and human HDGF display only 88%.

The differences in the amino acid sequence among the HDGFs of different species occur only in the C-terminal part, whereas the N-terminal hath region is fully conserved. This strong evolutionary conservation suggests an important function of this region.

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