Expression patterns and different subcellular localization of the growth factors HDGF (hepatoma-derived growth factor) and HRP-3 (HDGF-related protein-3) suggest functions in addition to their mitogenic activity

Mekky M. ABOUZIED*, Stephan L. BAADER†, Frank DIETZ‡, Joachim KAPPLER*, Volkmar GIESELMANN* and Sebastian FRANKEN*[1]

*Institut für Physiologische Chemie, Rheinische Friedrich-Wilhelms Universität, Nussallee 11, 53115 Bonn, Germany, †Anatomisches Institut, Anatomie und Zellbiologie, Rheinische Friedrich-Wilhelms Universität, Nussallee 10, 53115 Bonn, Germany, and ‡Institut für Physiologische Biochemie, Universität Bremen, Leobener Strasse NW2/B2, 28359 Bremen, Germany

HDGF (hepatoma-derived growth factor) and the HRPs (HDGF-related proteins) comprise a family of six proteins which display high identity in their N-terminus, but differ at the C-terminus. Here we investigate the patterns of expression of HDGF and HRP-3, by generating antisera specifically recognizing each growth factor. Whereas HRP-3 protein is expressed only in brain, HDGF can be found in a broad range of tissues, with highest levels in brain, testis, lung and spleen. The expression of HDGF and HRP-3 was found to be regulated during brain development, with highest levels around birth, followed by a decline until postnatal day 9. Interestingly, expression of HRP-3 increases again in adult brain. *In situ* hybridization and immunohistochemistry of cerebellar, cerebral and hippocampal brain slices showed that expression of both growth factors is not limited to areas of high proliferative activity. Both mRNAs and proteins are expressed in neuronal as well as glial cells. Immunocytochemistry of cultured neocortical neurons revealed that HDGF and HRP-3 can be found in the nucleus as well as the cytoplasm. HDGF is restricted to the neuronal soma, whereas HRP-3 can also be found in neurites. Thus the expression of HDGF and HRP-3 in differentiated cells, post-mitotic neurons and primary cultures of rat neocortex points to functions in brain that might not be limited to proliferation. In addition, their simultaneous expression in the same cell and their different subcellular localization in cultured neurons suggest different functions of HDGF and HRP-3 within single cells.

Key words: brain development, cell proliferation, HDGF-related protein-3 (HRP-3), hepatoma-derived growth factor (HDGF), nuclear localization, survival factor.

INTRODUCTION

HDGF (hepatoma-derived growth factor) was initially purified from the supernatant of human hepatoma cell lines [1–3]. It has been shown to have growth factor activity for hepatoma cells, fibroblasts, smooth muscle cells and endothelial cells [1,3–5]. Subsequently, five related proteins have been identified by different approaches [4,6–8]. Four of these proteins have been termed HRP-1 (HDGF-related protein-1) to HRP-4; the fifth is called p52/75 or LEDGF (lens epithelium-derived growth factor). HDGF and the HRPs, including LEDGF, display between 54 % and 78 % sequence identity among their 91 N-terminal amino acids [4,7,8]. Because of this similarity, the N-terminal region has been termed the HATH [homologue to N-terminus ('amino-terminus') of HDGF] region [8]. In contrast, the lengths and amino acid sequences of the C-terminal regions of HRPs vary, suggesting a modular structure of these proteins. Except for their growth factor activity, the functions of these proteins are largely unknown. LEDGF has been shown to function as a transcriptional activator, and it has been speculated that HDGF plays a role in renal, lung and heart development, but the precise function has not been elucidated for any of these proteins [9–14].

The main cellular localization of HDGF is nuclear, although in some cells HDGF can be found in the cytosol [2,5,13]. HDGF has two nuclear localization signals, one in the conserved HATH region and the other in the C-terminal region that is specific for the different family members. Nuclear localization has been shown to be a prerequisite for the mitogenic activity of HDGF [15,16].

Description of the patterns of expression of the different HRPs has so far relied mostly on Northern blot analysis. Thus mRNA expression in mice shows a broad tissue distribution for HDGF and HRP-2, whereas HRP-1 and HRP-4 mRNAs are restricted to testis [4,8]. In contrast, the distribution of HRP-3 mRNA has so far been examined only in human tissues. Here expression is prominent in brain, and low amounts of mRNA are also found in heart, testis and kidney [7]. These data point to a particular role for HRP-3 in the nervous system. Currently there are no data about the developmental expression or cellular distribution of HRP-3 and HDGF proteins in the central nervous system in particular. Such information is a prerequisite for understanding the function of these members of the HRP growth factor family in brain tissue. Therefore we have developed HDGF- and HRP-3-specific antisera and used them to examine protein expression patterns in murine brain and neuronal cell cultures.

EXPERIMENTAL

Production of recombinant proteins and polyclonal antisera

Production of anti-HDGF antibody was performed as described previously for HRP-4 [4]. Briefly, the HDGF coding region was cloned into the pGEX vector (Amersham Pharmacia Biotech, Freiburg, Germany) using *Bam*HI and *Eco*RI as restriction sites. The GST (glutathione S-transferase) fusion protein (HDGF–GST) was produced in *Escherichia coli* DH5α and purified via glutathione affinity chromatography.

Abbreviations used: EGL, external granule cell layer; GST, glutathione S-transferase; HATH, homologous to N-terminus ('amino-terminus') of HDGF; HDGF, hepatoma-derived growth factor; HRP, HDGF-related protein; LEDGF, lens epithelium-derived growth factor; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time of flight MS.

[1] To whom correspondence should be addressed (e-mail franken@institut.physiochem.uni-bonn.de).

For production of anti-HRP-3 antiserum, a truncated murine HRP-3 lacking the first 100 amino acids was produced in BL21 Pro bacteria (BD Bioscience Clontech, Palo Alto, CA, U.S.A.) as an N-terminal histidine-tagged fusion protein. The open reading frame of the truncated protein was cloned into a pQE80L vector (Qiagen, Hilden) using a sense primer containing a *Bam*HI restriction site (5′ AACAGAGGATCCGAAACT GAGGGAGAA 3′) and an antisense primer containing a *Hin*dIII restriction site (5′ GCAGCCAAGCTTAGGTCCCTTCACCGG 3′). Homogenates of bacteria were purified on Ni^{2+}-nitrilotriacetate agarose (Qiagen) as recommended by the supplier.

The purified proteins were used to immunize New Zealand White rabbits (Lammers, Euskirchen, Germany). For the first immunization, 250 μg of protein dissolved in Freund's complete adjuvant (Sigma, Deisenhofen, Germany) was injected subcutaneously. Each animal was boosted twice at intervals of 4 weeks with the same amount of antigen in incomplete Freund's adjuvant (Sigma). Immunization was performed according to local governmental regulations.

Serum was collected after 12 weeks and polyclonal antibodies were purified by immunoaffinity chromatography. For this purpose, mouse HDGF and HRP-3 were produced in bacteria as histidine-tagged and GST fusion proteins respectively. After affinity chromatography, the purified proteins were coupled to Affigel 10 using the protocol supplied by the manufacturer (Bio-Rad, München, Germany) and the resulting matrices were used for immunoaffinity purification of anti-HDGF and anti-HRP-3 antibodies.

Affinity-purified antibodies were tested for cross-reaction with other HRP family members by Western blot against Strep-tag® fusion proteins produced in COS-7 cells. Therefore oligonucleotides (sense, 5′ AATTCGATATCGGTACCTGGAGCCACCCG-CAGTTCGAAAAATAAGC 3′; antisense, 5′ GGCCGCTTA-TTTTTCGAACTGCGGGTGGCTCCAGGTACCGATATCG 3′) coding for Strep-tag® II (WSHPQFEK; IBA, Göttingen, Germany) were cloned into the multi-cloning site of the pCDNA3 vector (Invitrogen, Paisley, U.K.), and coding regions of the different HRP family members lacking the stop codons were cloned 5′ to the Strep-tag® oligonucleotide. COS-7 cells were transfected using DEAE-dextran and lysed after 48 h in buffer containing 50 mM Tris/HCl, pH 7.4, 1 % Nonidet P40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 μg/ml each of aprotinin, leupeptin, and pepstatin.

MALDI-TOF (matrix-assisted laser-desorption ionization–time of flight) MS

For molecular mass determination, recombinant histidine-tagged HDGF and HRP-3 were purified by nickel-chelating chromatography and dialysed against PBS. Buffer was changed against organic solvent by using a C18 matrix containing ZipTip (Millipore, Schwalbach, Germany) according to the protocol supplied by the manufacturer. After elution in 75 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid, proteins were mixed 1:1 (v/v) with sinapic acid (Fluka, Deisenhofen, Germany) and spotted on a MALDI-TOF MS target. Molecular mass was determined using a Voyager-DE STR (Applied Biosystems, Darmstadt, Germany) instrument in the linear mode.

Preparation of protein extracts and Western blot analysis

Unless stated otherwise, chemicals for protein preparation were from Merck (Darmstadt, Germany), and those for gel electrophoresis were from Serva (Heidelberg, Germany).

Protein extracts from various tissues or from the brains of mice of different ages were prepared by homogenization in TBS (Tris-buffered saline; 20 mM Tris/HCl, pH 7.0, and 150 mM NaCl) containing 1 % Nonidet P40 and protease inhibitors (5 mM EDTA, 2 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin). After homogenization, samples were centrifuged at 20 000 ***g*** for 20 min at 4 °C to remove unhomogenized material. Protein content in the supernatants was determined by a detergent-compatible assay (DC-assay; Bio-Rad). Equal amounts of protein for each tissue were loaded on to a SDS/PAGE gel (12.5 % acrylamide) and resolved according to the method of Laemmli [17].

After electrophoresis, proteins were transferred to nitrocellulose and stained with Ponceau S to ensure proper loading and blotting, as recommended by the supplier (Roth, Karlsruhe, Germany). Free protein binding sites on the membrane were blocked by incubation in 3 % (w/v) skimmed milk in TBST (TBS containing 0.05 % Tween 20). Primary antibodies against HRP-3 (1:500) or HDGF (1:1000) and peroxidase-labelled secondary antibody against rabbit (1:10 000; Jackson, West Grove, PA, U.S.A.) were incubated in TBST containing 0.3 % skimmed milk for 1 h at room temperature. After three washings in TBST, bound antibodies were visualized by the ECL® system (Amersham Pharmacia Biotech).

In situ hybridization

In situ hybridization was performed on paraffin-embedded sections using digoxigenin-labelled RNA probes. The basic procedure described by Wanner and co-workers [18] was adapted to paraffin sections; this will be described in detail elsewhere (M. Holst and S. L. Baader, unpublished work; details available on application to S. L. B.). Briefly, mice of different ages were deeply anaesthetized by intraperitoneal injection of 2.5 % avertin in 0.9 % saline (0.8 ml/100 g body weight). Subsequently, they were perfused transcardially with 0.4 ml/g body weight of Ringers solution for mammals, followed by 2 ml/g body weight of 4 % (w/v) freshly prepared formaldehyde in PBS (150 mM NaCl and 10 mM NaH_2PO_4, pH 7.2). Following perfusion, brains were removed from the skulls and post-fixed for at least 24 h at room temperature in the same fixative. After several washings in tap water, tissues were dehydrated and embedded in Histovax. Sagittal sections were cut at 10 μm thickness on a microtome (Leica, Bensheim, Germany).

Digoxigenin-labelled riboprobes specific for HDGF or HRP-3 were obtained by *in vitro* transcription in the presence of digoxigenin-11-UTP. After deparaffinization and hydration of sections, tissue was post-fixed again to ensure attachment of sections to slides and incubated in 1 % H_2O_2 to block endogeneous peroxidase. Tissues were permeabilized by proteinase K digestion and Triton X-100 and HCl treatments. Following triethanolamine treatment, samples were hybridized with 0.1 mg of probe/ml of hybridization buffer at 70 °C overnight. Specimens were then subjected to stringent post-hybridization washings. Bound probes were detected with a chromogenic substrate (Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) using anti-digoxigenin antiserum and alkaline phosphatase. No staining was found with sense controls for HDGF and HRP-3 prepared in parallel.

Immunostaining

For immunocytochemistry, neuronal cultures from embryonic day 15 rat cortex were prepared as described elsewhere [19,20]. Approx. 40 000 cells were seeded on to D-lysine-coated 12 mm coverslips, and were fixed the next day by incubation in 4 % (v/v) paraformaldehyde/10 % (w/v) sucrose for 10 min at room temperature. After permeabilization with 0.02 % Triton X-100

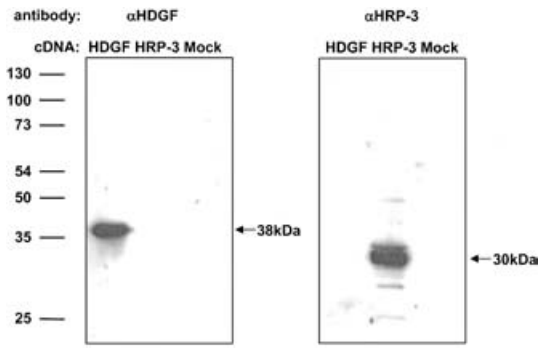

Figure 1 Specificity of antisera against HDGF and HRP-3

cDNAs of murine HDGF and HRP-3 and a control cDNA (mock) were transiently expressed in COS-7 cells. At 48 h after transfection, cell lysates were subjected to Western blot analysis with antisera against HDGF and HRP-3 as indicated. Each antiserum reacted only with lysates from cells transfected with the respective cDNA. No cross-reaction with the other family member or with cells transfected with the control cDNA could be detected. Bars on the left indicate the position and apparent molecular mass of standard proteins (in kDa).

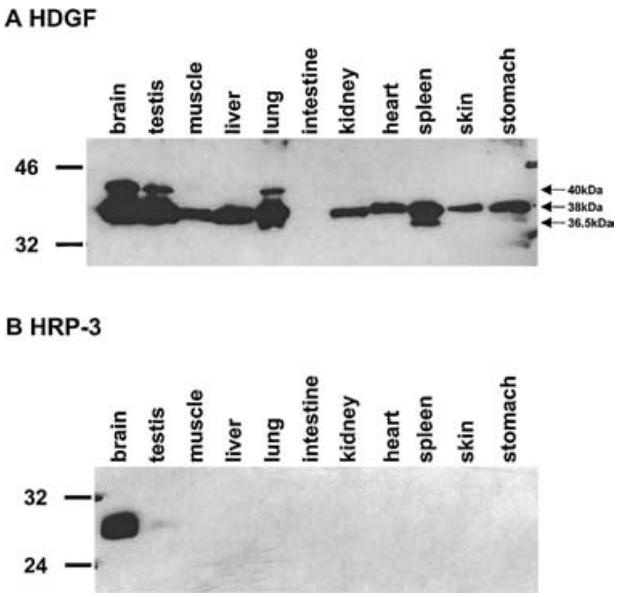

Figure 2 Examination of HDGF and HRP-3 expression

Equal amounts (50 μg of protein/lane) of protein extracts of different tissues prepared from 3-month-old mice were subjected to Western blot analysis with the anti-HDGF (**A**) and anti-HRP-3 (**B**) antibodies. Whereas HDGF is widely expressed and appears in three different molecular mass forms (sizes given on the right), HRP-3 is found almost exclusively in brain tissue. Bars on the left indicate the positions of molecular mass marker polypeptides (in kDa).

in PBS, cells were incubated with affinity-purified antibodies against HRP-3 or HDGF at a concentration of 4 μg/ml, and for double staining with a monoclonal antibody against mouse microtubule-associated protein-2 (Sigma, 1:100) for 1 h at room temperature. Bound antibodies were visualized using Cy3-conjugated goat anti-rabbit or Cy2-conjugated goat anti-mouse antibodies (Dianova, Hamburg, Germany). As a control, the antibodies were preincubated for 1 h at room temperature with 1 mg/ml HDGF–GST or HRP-3–GST, and staining was performed as described above. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole. Coverslips were mounted in 50 % (v/v) glycerol/PBS and analysed by epifluorescence microscopy on an Axiovert M instrument (Zeiss, Jena, Germany).

For immunohistochemistry, C57Bl6 mice were perfused transcardially exactly as described for the *in situ* hybridization procedure. For fixation, 4 % (v/v) paraformaldehyde was used. After post-fixation for 7 h, tissue was kept in PBS overnight. Then 40 μm-thick sagittal sections were cut in PBS using a Leica vibratome (VT1000S; Leica, Wetzlar, Germany). Sections were stained as floating tissue according to the protocol described above. Pictures were analysed by epifluorescence microscopy on an Axioskop 2 (Zeiss).

RESULTS

Specificity of anti-HDGF and anti-HRP-3 antisera

To investigate and compare the protein expression patterns of mouse HDGF and HRP-3, we developed specific antisera against the recombinant bacterially expressed proteins (see the Experimental section). Due to the high degree of identity of HRP proteins, it was necessary to examine possible cross-reactions of the antisera between HDGF and HRP-3. For that purpose, COS cells were transiently transfected with the HDGF, HRP-3 or a control cDNA (mock). Cell lysates were prepared 48 h after transfection and analysed by Western blot analysis. Figure 1 demonstrates that the anti-HDGF and anti-HRP-3 antisera recognized polypeptides of 38 and 30 kDa respectively. Neither antiserum displayed cross-reactivity with the other HRP or with cell lysates transfected with a control cDNA. Both antisera reacted only with polypeptides from COS-7 cells transfected with the respective cDNAs (Figure 1).

Expression and molecular mass determination of HDGF and HRP-3

After confirmation of the specificity of the antisera, we performed Western blot analysis of different mouse tissue homogenates to investigate HDGF and HRP-3 expression. Figure 2 shows a Western blot of various mouse tissues performed with either the anti-HDGF (Figure 2A) or the anti-HRP-3 (Figure 2B) serum. In all tissues, except intestine, the anti-HDGF serum detected a major polypeptide of 38 kDa. In brain, testis and lung, a minor additional higher-molecular-mass polypeptide of 40 kDa was observed. In addition, in lung and spleen a smaller polypeptide of 36.5 kDa was detected. HDGF protein expression was highest in brain and testis, followed by lung and spleen. In contrast, the anti-HRP-3 serum detected a single polypeptide of 30 kDa in brain only (except for very low expression in testis). As expected from the data shown in Figure 1, the antisera showed no cross-reactivity with other HRPs or any other proteins.

The predicted molecular mass of mouse HDGF is 26.3 kDa and that of HRP-3 is 22.4 kDa. The discrepancy between the predicted molecular masses and those observed by SDS/PAGE may be explained either by post-translational modifications or by abnormal running behaviour of these proteins on SDS/PAGE. To address this question, we compared bacterially expressed HDGF and protein homogenates prepared from brain tissue in Western blot analysis. The apparent molecular mass on SDS/PAGE of recombinant or brain HDGF was 38 kDa. The slightly higher apparent molecular mass of recombinant HDGF is likely to be due to the addition of six histidines in this protein (predicted molecular mass 27.1 kDa). In contrast with SDS/PAGE, MALDI-TOF MS analysis of the recombinant protein gave the predicted molecular mass of 27.1 kDa (Figure 3). A similar observation was made for HRP-3 (results not shown). Thus the size discrepancy is due to abnormal electrophoretic behaviour of HDGF and HRP-3.

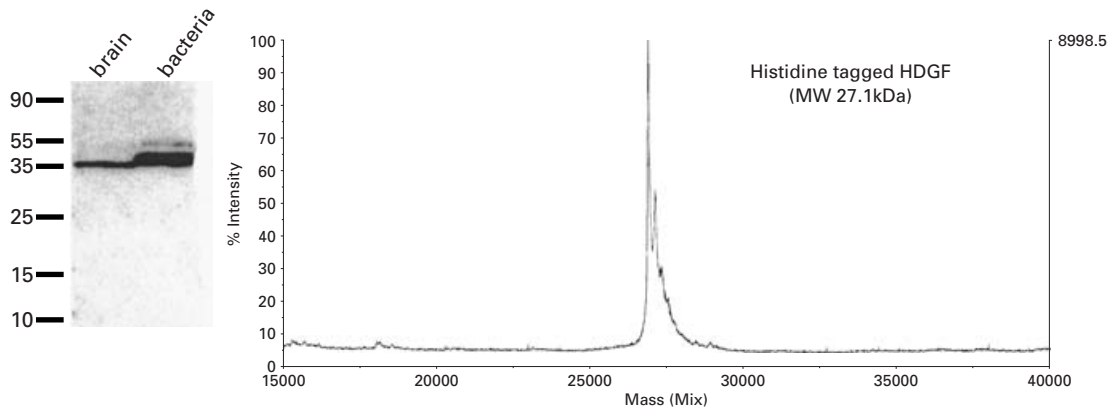

Figure 3 Determination of the molecular mass of HDGF

Recombinant mouse HDGF produced in bacteria and mouse brain extracts were subjected to Western blot analysis with the anti-HDGF antiserum (left panel). Bars on the left indicate the positions of molecular mass marker polypeptides (in kDa). In addition, molecular mass of recombinant murine HDGF was determined as 27.1 kDa by MALDI-TOF MS analysis (right panel).

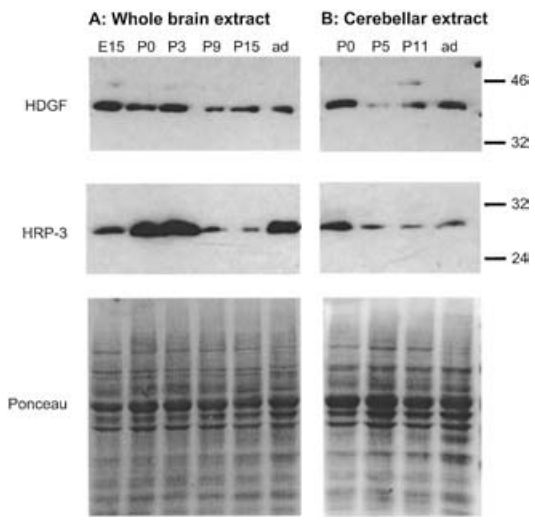

Figure 4 Expression of HDGF and HRP-3 polypeptides during brain development

Equal amounts (10 μg of protein/lane) of protein extracts from whole brain (**A**) or cerebellum (**B**) were subjected to Western blot analysis with the specific anti-HDGF and anti-HRP-3 antibodies. Ponceau S staining was used as a control for correct loading and blotting. Note that HRP-3 is re-expressed in whole brain extracts from adult animals. E, embryonic day; P, postnatal day; ad, adult: Bars on the right indicate the positions of molecular mass marker polypeptides (in kDa).

Expression of HDGF and HRP-3 in brain

In view of the high (HDGF) or exclusive (HRP-3) expression of these proteins in brain, we focused on the nervous system and examined the developmental expression of both growth factors in whole brain and in cerebellar homogenates prepared from mice of different ages.

HDGF was expressed at the first time point examined, i.e. embryonic day 15. This level of expression remained virtually unchanged until postnatal day 3, and then decreased rapidly until postnatal day 9 to lower levels that were maintained until adulthood (Figure 4A). In contrast HRP-3 was expressed to a lower extent at embryonic day 15, but expression increased strongly until birth and remained at this level for the first few postnatal days. Then, as for HDGF, the level of expression decreased rapidly until postnatal day 9. Interestingly, expression of HRP-3 was shown to increase again in the adult brain (Figure 4A).

When we focused on the cerebellum, where cellular development is comparably less complex and quite well understood, we found both similarities and differences with regard to the developmental regulation of HDGF/HRP-3 expression when compared with whole brain extracts. Again, HDGF and HRP-3 were expressed at the time of birth, and both were down-regulated until postnatal day 5. In contrast with the whole brain, expression of HRP-3 in cerebellum remained low until adulthood, whereas HDGF expression increased (Figure 4B).

In order to identify the cell populations that express HDGF and HRP-3, *in situ* hybridizations were performed using probes specific for HDGF and HRP-3. Expression of HDGF and HRP-3 was observed within the cerebellar cortex of newborn mice (Figures 5B and 5C). Purkinje cells stained for both HDGF and HRP-3. HDGF was also expressed within the EGL (external granule cell layer), located just beneath the pia of the cerebellar cortex (arrowhead in Figure 5B). This EGL staining could be observed to a much lower extent in tissue slices hybridized with the HRP-3 probe. At postnatal day 9, HDGF was still clearly visible within the Purkinje cell layer, albeit overlaid by strong staining within the internal granule cell layer (arrow in Figure 5E). Again, prominent staining could be found within the EGL (arrowhead in Figure 5E). More specifically, upon close examination, this signal could be located to the outer part of the EGL. In contrast, HRP-3 expression was only weak within the Purkinje cell layer, and much lower in the EGL and internal granule cell layer at postnatal day 9 (Figure 5F). In the adult animal, HDGF was expressed in all neurons, including basket/stellate cells within the molecular layer (Figure 5H). In contrast, HRP-3 was expressed within the internal granule cell layer, and weakly within the Purkinje cell layer, but not within basket/stellate cells (Figure 5I). Occasionally, cells staining for both probes could be observed within the white matter, most probably representing oligodendrocytes or astroglia.

We also investigated other regions of the central nervous system, and found a wide distribution of HDGF and HRP-3 in differentiating neurons. Within the hippocampus, the cornu ammonis and the subiculum were heavily stained by the HDGF and HRP-3 probes, whereas in the dentate gyrus HDGF expression was stronger than that of HRP-3. HDGF was additionally found within a cell layer close to the fissura hippocampi (arrow in Figure 6A), which was negative for HRP-3. Based on the specific

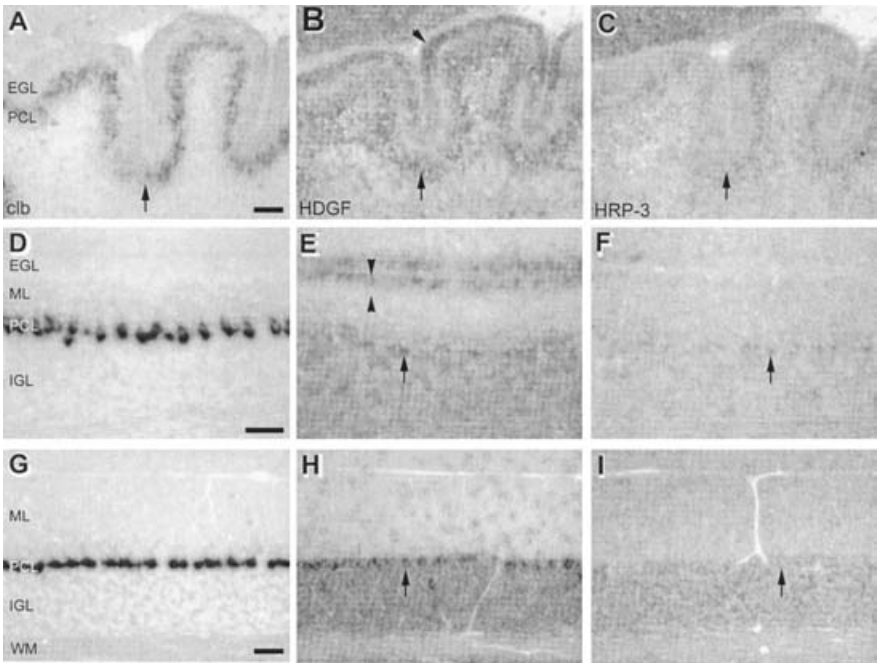

Figure 5 Expression of HDGF and HRP-3 mRNAs during cerebellar development

Cerebellar slices from postnatal or adult mice were hybridized with riboprobes for calbindin-D28k (clb; **A**, **D**, **G**), HDGF (**B**, **E**, **H**) and HRP-3 (**C**, **F**, **I**) at different developmental stages: (**A**–**C**) day of birth; (**D**–**F**) postnatal day 9; (**G**–**I**) adult. The clb probe was used to demonstrate the position of the Purkinje cell layer (PCL) and to facilitate the identification of the cerebellar layering. Arrows depict the PCL, and arrowheads indicate the EGL. IGL, internal granule cell layer; WM, white matter.

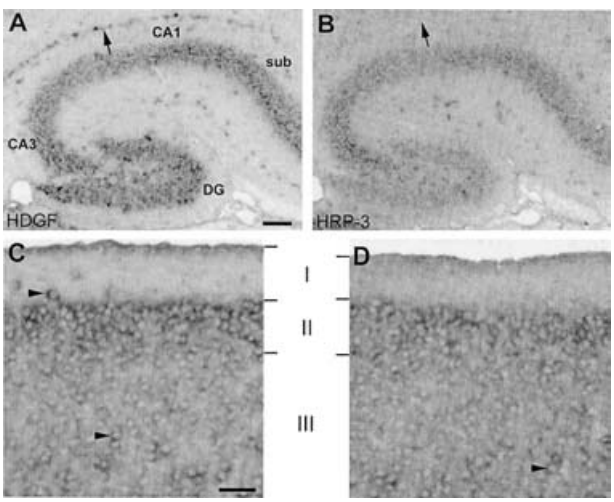

Figure 6 Expression of HDGF and HRP-3 mRNAs in differentiated neurons of the hippocampus and the cerebral cortex

Whole brain slices from 9-day-old mice were hybridized with riboprobes for HDGF (**A**, **C**) and HRP-3 (**B**, **D**). Pictures were taken from the hippocampus (**A**, **B**) and the cerebral cortex (**C**, **D**). HDGF and HRP-3 are strongly expressed within differentiated neurons of the hippocampus [cornu ammonis (CA), dentate gyrus (DG) and subiculum (sub) in **A** and **B**] and of all layers of the cerebral cortex. Shown are layers I–III (arrowheads point to selected neurons in **C** and **D**). Differential expression was found within cells close to the fissura hippocampi (arrows in **A** and **B**). (**A**, **B**) Bar = 100 μm; (**C**, **D**) bar = 50 μm.

position, this signal might represent either Cajal–Retzius or glial cells. The cerebral cortex revealed comparable patterns of expression of HDGF and HRP-3.

The expression of HDGF and HRP-3 protein on postnatal day 9 basically resembled the distributions described for their mRNAs. Whereas HDGF was highly expressed in all cortical regions of the central nervous system, HRP-3 protein expression was more restricted. It was expressed mainly within the Purkinje cell layer and the Golgi cells of the cerebellar cortex (Figure 7B), but not in cerebellar granule cells. Similarly, only small amounts of HRP-3 could be found in hippocampal granule cells. As for the mRNA, cells of the dentate gyrus were only weakly stained by the anti-HRP-3 antibody (Figure 7D). The distributions of the two proteins in the cerebral cortex seemed to be similar, although a more widely distributed pattern of expression of HDGF was apparent (see layer I in Figures 7G and 7H). At the subcellular level, both HDGF and HRP-3 proteins were localized within the cell nuclei. In contrast with HDGF, HRP-3 protein could also be detected in axonal projections of all cortical regions (arrows in Figures 7B and 7F). Whether small amounts of the growth factors are also present in cell somata could not be clarified by this immunohistochemical approach. In summary, whereas HDGF is more widely distributed at the cellular level, HRP-3 can be found in axonal projections throughout the different brain regions.

Expression of HDGF and HRP-3 in neocortical cell culture

To analyse the expression of HDGF and HRP-3 at the cellular level in more detail, we prepared neocortical neurons from rats and performed immunocytochemistry with the affinity-purified antibodies against HDGF and HRP-3 (Figures 8A and 8B). Preabsorption with HDGF–GST or HRP-3–GST reduced the intensity of staining significantly (Figures 8E and 8F). To identify neuronal cells, double staining with an antibody against microtubule-associated protein-2 was performed (Figures 8C and 8D). The results clearly show that both HDGF and HRP-3 are expressed in neurons (Figures 8A–8D, stars). Staining was not restricted to the nuclei, but was also cytoplasmic. In particular, HDGF and HRP-3 showed different distributions with respect to neurites. Whereas HDGF was mostly restricted to the neuronal soma, strong HRP-3 immunoreactivity was found also in neurites.

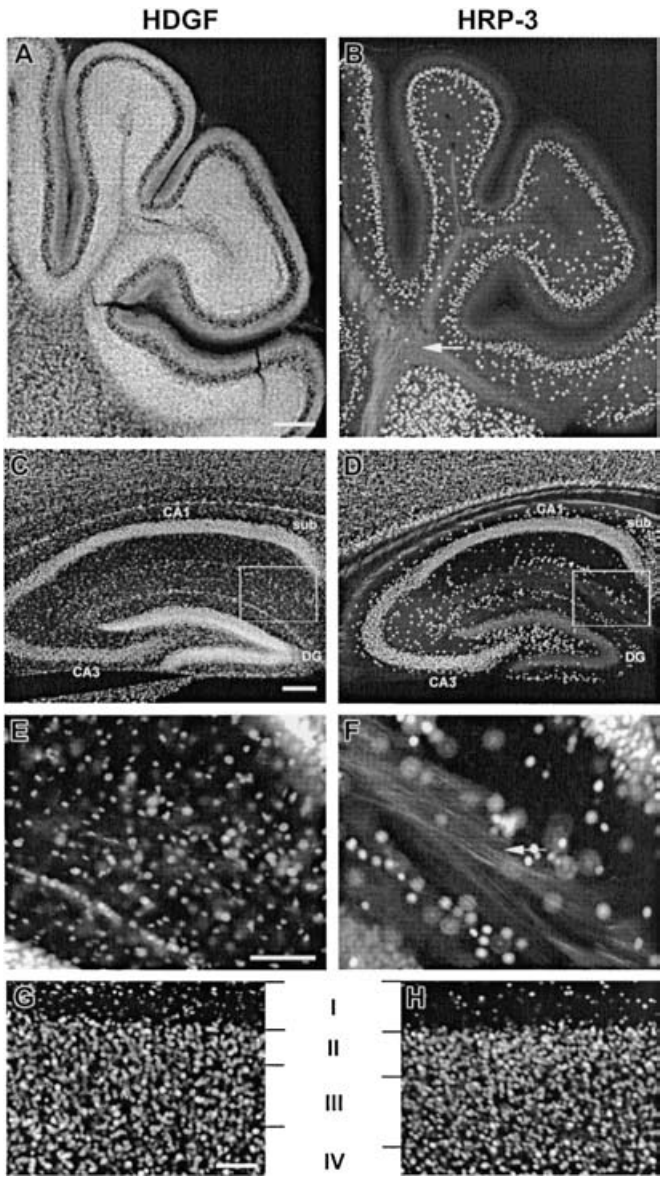

Figure 7 Differential localization of HDGF and HRP-3 proteins in cell nuclei and axonal projections

Whole brain vibratome slices from 9-day-old mice were immunostained with antibodies against HDGF (left panels) or HRP-3 (right panels). Pictures were taken from the cerebellum (**A**, **B**), the hippocampus (**C**, **D**; boxes are magnified in **E**, **F**) and the cerebral cortex (**G**, **H**). The cellular distributions of HDGF and HRP-3 proteins are comparable with the results obtained by *in situ* hybridization indicating expression in differentiating neurons. Whereas both proteins were localized to the cell nuclei, only HRP-3 protein could be detected in axons of all cortical regions (arrows in **B** and **F**). Bar = 100 μm. CA, cornu ammonis; DG, dentate gyrus; sub, subiculum.

In addition, cells that were not positive for microtubule-associated protein-2, and thus most probably represented glial cells, also expressed HDGF or HRP-3 (Figures 8A–8D, white arrows).

DISCUSSION

mRNA expression patterns in mice have been shown to differ between HRP family members. HDGF and HRP-2 mRNAs show prominent expression in most tissues tested, whereas HRP-1 and HRP-4 are restricted to testis [4,8]. Except for HRP-4, no data on HRP protein expression have been available, because of a

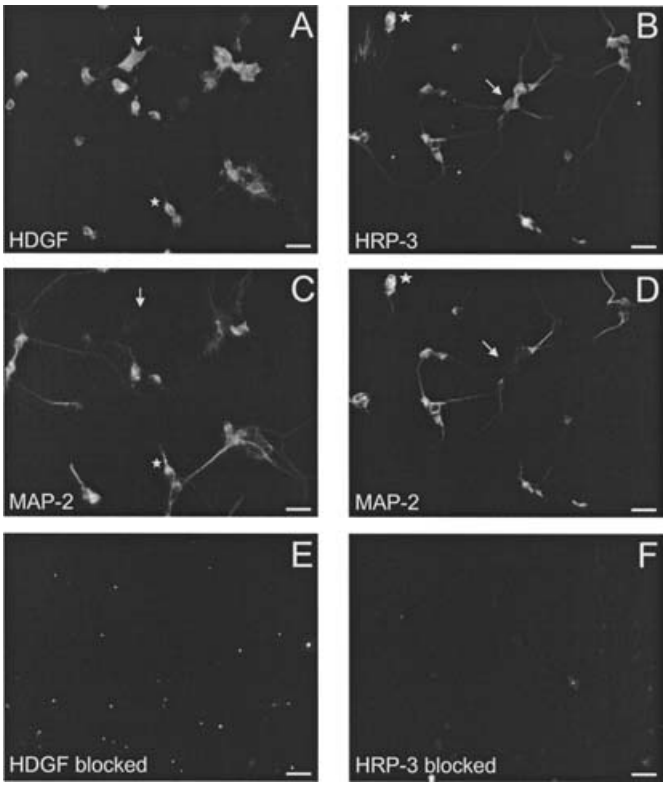

Figure 8 Expression of HDGF and HRP-3 in cultures of neocortical neurons

HDGF (**A**) and HRP-3 (**B**) expression was examined in neuronal cultures prepared from embryonic rat cortices. For comparison, neurons were stained with an antibody against MAP-2 (**C**, **D**). HDGF and HRP-3 are expressed in neuronal cells (stars) as well as non-neuronal cells (arrows). The specificity of the antibodies was confirmed by preabsorption with HDGF–GST (**E**) and HRP-3–GST (**F**). Bars = 20 μm.

lack of specific antibodies for the different family members. Here we present data obtained with antibodies specific for HDGF and HRP-3. Both antisera were highly specific; they did not react with other members of the HRP protein family or with any other protein in COS-7 cell lysates or tissue homogenates. In the case of HRP-3 this may not be surprising, since we used the recombinant C-terminus for immunization, and the C-terminal part of HRPs differs among members of this protein family. In case of HDGF, however, we used the whole protein, including the highly conserved HATH region. The high degree of conservation observed not only among different HRP family members, but also among different species (100 % between mouse, human, rat, and bovine), most probably leads to low immunogenicity of the HATH region [4], which could be the reason for the surprising specificity of the anti-HDGF serum obtained.

Our results for HDGF protein expression in various murine tissues are in accordance with those obtained by Northern blot analysis [8]. However, a comparison of mRNA levels and protein expression reveals that they do not correlate for different tissues. For example, skeletal muscle and testis show the highest signals in Northern blot [8], but protein expression in muscle is low when compared with that in brain or testis. mRNA and protein data for HDGF also seem to be divergent for spleen: whereas in comparison with other organs HDGF mRNA is only weakly expressed in spleen, the Western blot signal is higher than in most other tissues tested. The lack of correlation between HDGF mRNA and protein levels suggest post-transcriptional and/or post-translational modifications of HDGF polypeptide expression.

Nakamura and co-workers [7] found a strong HRP-3 mRNA signal in brain; in contrast, only low mRNA levels were detected in heart and testis. In accordance with this, we detected immunoreactivity mainly in brain, with a very low level in testis, but not in heart tissue. The latter might reflect a difference in expression between mice and humans; alternatively, HRP-3 protein levels in heart may be too low to be detected in Western blots.

Both HDGF and HRP-3 show a substantial discrepancy between the molecular mass predicted by the amino acid sequence and the apparent molecular mass on SDS/PAGE (see also [21]). To reveal the molecular basis of this discrepancy, we determined the molecular masses of bacterially expressed recombinant HDGF and HRP-3 in MALDI-TOF MS analysis. The masses determined by MALDI-TOF were in accordance with those predicted from the amino acid sequences. Since the same protein preparations displayed apparent molecular masses for HDGF and HRP-3 of 38 kDa and 30 kDa respectively on SDS/PAGE, it can be concluded that the discrepancy is not due to post-translational modifications, but to abnormal electrophoretic behaviour of the proteins on SDS/PAGE. Since the C-terminus of HRP-3 expressed in bacteria also showed the same behaviour, it can be assumed that the structure responsible for the abnormal apparent molecular mass is not located in the HATH region, but rather in the C-terminal part of the proteins.

In tissues, HRP-3 appears as a single polypeptide of 30 kDa, whereas HDGF is present in three different molecular mass forms of 40, 38 and 36.5 kDa. These size differences may be due to post-translational modifications, alternative splicing or limited proteolysis. Oliver and Al-Awqati [13] found HDGF polypeptides of different molecular masses in cell homogenates (40 kDa) or cell culture supernatants (25 and 35 kDa) of metanephrotic mesenchymal cells. They speculated that the smaller secreted forms of 25 and 35 kDa are due to extracellular proteolytic processing of the intracellular 40 kDa form of HDGF. Thus our results may reflect different secretion and/or proteolytic processing rates in different tissues.

To date, the only known biological activity of HDGF and HRP-3 is the proliferative effect that they have on various cells [1,3,5,10,13]. Thus it is not surprising that Western blots of whole brain extracts showed prominent expression of both growth factors during embryonic and neonatal brain development, when the rate of proliferation is high. After birth, HDGF and HRP-3 expression in whole brain declined but, surprisingly, levels of HRP-3 in whole brain and of HDGF in cerebellar extracts rose again in adult animals, pointing to expression of both growth factors in non-proliferative cells as well. We performed *in situ* hybridization to identify cells expressing HDGF and HRP-3 in brain. At early postnatal stages, HDGF in particular was found in proliferative brain regions, and at postnatal day 9 staining of the outer part of the EGL, which contains proliferating granule cells, also suggested mitogenic activity of HDGF. Expression in mitotically active granule cells was not observed to the same extent for HRP-3, suggesting a somewhat different function of this family member in cerebellar development. The possibility of functions of HDGF and HRP-3 in addition to their growth factor activity is supported by the expression of both growth factors in post-mitotic cells, such as granule cells of the inner part of the EGL, or neocortical and hippocampal neurons in the adult brain. In addition, the existence of cells expressing both growth factors suggests different functions for HDGF and HRP-3 on the cellular level, which is supported by the presence of HRP-3 (but not HDGF) protein in axonal projections.

The results of immunocytochemistry performed with cultured neocortical neurons also showed co-expression of HDGF and HRP-3. In addition, the differences in subcellular localization support the hypothesis that the two growth factors have different functions within a single cell, and that they both have functions other than growth factor activity. Interestingly, the intracellular localization of both factors was nuclear as well as cytoplasmic. Extranuclear localization of HDGF has been shown previously for other cell types [2,13]. Whether HDGF has a cytoplasmic localization in cortical neurons *in vivo* could not be demonstrated by immunohistochemistry. Nevertheless, the observed axonal staining pattern of HRP-3 is in accordance with the cell culture data, whereby prominent HRP-3 staining of cellular processes could be detected. Extranuclear staining of differentiated cells supports the hypothesis of additional functions of HDGF and HRP-3 other than proliferation, since the proliferative activity of HDGF has been shown to be dependent on nuclear localization [15,16]. The cellular localization of HDGF in cell somata and the strong staining of HRP-3 in cell processes support the suggestion of different functions for the two growth factors at the cellular level. Taken together, the maintained or even increased expression of HRP-3 in adult brain, the clear localization of HDGF and HRP-3 mRNAs in post-mitotic neurons, and the extranuclear expression of both growth factors in cultured differentiated neurons point to functions of these growth factors that are not limited to proliferative activity.

In this respect, it is interesting to note that LEDGF, which is also a member of the HRP family, enhances the survival of skin fibroblasts, keratinocytes, photoreceptor and retinal pigment epithelial cells [22–25], in addition to its proliferative activity. Thus HDGF and HRP-3 might also function as survival factors in adult brain.

The idea of a different or additional function of HDGF in brain is supported by the fact that HDGF is structurally related to high mobility group-1 protein, also known as amphoterin because of its highly dipolar sequence [2]. This protein, which like HDGF has heparin-binding activity, is also developmentally regulated, and its expression is clearly lower in adult than in developing brain [26]. Functionally, amphoterin has adhesive and neurite outgrowth-promoting activity in central nervous neurons [27,28].

Dual roles of proteins purified initially as growth-promoting molecules are well known. For example, it was shown that neurotrophins regulate processes as diverse as cell growth, survival, axonal and dendritic growth, and even synapse formation and activity-dependent plasticity during development [29]. In addition, the morphogen Sonic hedgehog has been shown recently to switch from cell proliferative to axon guidance activity during development [30]. These functional changes can be induced by different receptors or by manipulation of the signal transduction pathways through cross-talk with other ligand/receptor systems [31–33].

Taken together, our data show that HDGF and HRP-3 are expressed by neurons of the central nervous system, and that their expression is highly regulated during brain development. These observations indicate that the functions of the two family members differ with regard to cells and brain regions, and that these functions are not limited to proliferation.

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