Expression and Functional Interaction of Hepatocyte Growth Factor-Scatter Factor and its Receptor c-met in Mammalian Brain

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Abstract. Hepatocyte growth factor-scatter factor (HGF-SF) is a pleiotropic cytokine with mito-, morpho-, and motogenic effects on a variety of epithelial and endothelial cells. HGF-SF activity is mediated by the c-met protooncogene, a membrane-bound tyrosine kinase. Here, we demonstrate that both genes are expressed in developing and adult mammalian brains. HGF-SF mRNA is localized in neurons, primarily in the hippocampus, the cortex, and the granule cell layer of the cerebellum, and it is also present at high levels in ependymal cells, the choroid plexus, and the pineal body. c-met is expressed in neurons, preferentially in the CA1 area of the hippocampus, the cortex, and the septum, as well as in the pons. In the embryonic mouse, brain HGF-SF and c-met are expressed as early as days 12 and 13, respectively. Neuronal expression of HGF-SF is evolutionary highly conserved and detectable beyond the mammalian class. Incubation of septal neurons in culture with HGF-SF leads to a rapid increase of c-fos mRNA levels.

The results demonstrate the presence of a novel growth factor-tyrosine kinase signaling system in the brain, and they suggest that HGF-SF induces a functional response in a neuronal subpopulation of developing and adult CNS.

Materials and Methods

Preservation and Preparation of Tissues and Cells

Tissues were obtained by autopsy and were immediately snap frozen in liq-
uid nitrogen and continuously stored at −70°C. Animal experiments were performed according to an officially accepted experimental animal protocol.

Adult and 4-d-old (P4) Wistar rats were killed after receiving ether anesthesia, and various brain areas were rapidly excised and frozen. Fetal mice were obtained after controlled mating for one night and their age (designated Ex in days after conception) was additionally controlled by histological examination in comparison with established standards (Thieler, 1972). Expression analysis was performed on sagittal, 12-μm thick cryostat sections of adult brain, and in the case of the fetal brain, on parasagittal sections of whole fetuses and horizontal sections of the brain.

**Plasmids and Probes**

Hybridization for HGF-SF mRNA was partly carried out with a 1.4-kb EcoRI-fragment covering the 3′ translated part of the rat HGF-SF cDNA (Tashiro et al., 1990), subcloned into pBluescript SK(+) kindly supplied by T. Nakamura (Stratagene, La Jolla, CA) and also in part with rfPCR-amplified and sequenced rat, mouse, and human HGF-SF cDNA fragments subcloned into pSK(+). The hybridization for c-met mRNA was performed with a 1.3-kb fragment, spanning the tyrosine kinase domain of the human c-met cDNA (Park et al., 1987), subcloned into pGEM3Z(+)(Promega Corp., Madison, WI). For Northern and Southern hybridization experiments, the purified HGF-SF and c-met cDNA fragments were labeled by primer extension, either with 32P or digoxigenin-coupled dCTP (Feinberg and Vogelstein, 1983). Single-stranded, [35S]-UTP-labeled riboprobes were prepared by linearizing the vector downstream of the inserted cDNA fragment and subsequent in vitro transcription using the respective phage polymerase (Sambrook et al., 1989).

**In Situ Hybridization**

In situ hybridization was carried out by adapting a previously described protocol (Schimacher et al., 1992). Frozen tissues were embedded in OCT (Miles Inc., Elkhart, IN). 12-μm cryostat sections were postfixed in 4% paraformaldehyde, 5 mM MgCl2, dehydrated in graded ethanol, acetylated (0.1 M triethanolamin, pH 8, 0.25% [vol/vol] acetic anhydride), and prehybridized (50% formamide, 2.5× Denhardt’s solution, 150 μg/ml salmon sperm DNA, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8, 0.1% SDS, and 0.05 ng/ml wheat germ RNA). Hybridization was performed at 45°C in humid chambers using [35S]UTP-labeled rat HGF sense and antisense probes (106 cpm/ml). The hybridization buffer consisted of prehybridization buffer with 10% dextrane sulfate and 10 mM dithiotreitol. Subsequently, the sections were washed several times under stringent conditions (0.2 × SSC at 50°C), treated with RNaseA (20 μg/ml; Behring Diagnostics, Somerville NJ), dehydrated, and exposed to films (X-OMAT-AR; Eastman Kodak, Rochester, NY) at −70°C overnight. Afterwards, the slides were coated with emulsion (NTB-2; Eastman Kodak), and the exposure time, typically between 1 and 2 wk, was evaluated based on the signal intensity on the autoradiography film. After development and fixation, the sections were stained with Harris’s hematoxylin and eosin, and were then mounted afterwards. The sections were observed under bright and dark field, and photomicrographs were taken with a microscope (Labophot; Nikon Corp., Tokyo, Japan).

**Northern Hybridization**

Tissue RNA was prepared by guanidium isothiocyanate extraction and cesium chloride ultracentrifugation (Chirgwin et al., 1979), while total RNA from primary cultures of fetal central nervous system neurons was obtained according to a method described by Chomczynski and Sacchi (1987). 10 μg of total RNA were separated on denaturing 1.2% agarose gels and transferred onto nylon membranes (Amersham Buchler, Braunschweig, Germany) by capillary transfer using 20 × SSC and immobilized by 5 min of shortwave UV treatment (Sambrook et al., 1989). The filters were prehybridized for 2 h and then hybridized overnight with 2 × 106 cpm of the respective [32P]dCTP-labeled cDNA fragment in 20 ml hybridization buffer. The hybridization buffer consisted of 50% formamide, 12.5% dextran sulfate, 5 × SSC, 1 × Denhardt’s solution, 50 mM NaH2PO4, and 0.25 mg/ml salmon sperm DNA. After the hybridization reaction, the filters were washed under stringent conditions (0.1 × SSC at 65°C) and exposed to Kodak X-OMAT-AR films at −70°C using intensifying screens.

RT-PCR was essentially carried out under conditions described by Gilliland et al. (1990) using the following primers:

**HGF-SF:** sense 5'-GGGGAATGGGAAATGACGAGC-3' (1920-1941)
antisense 5'-CCCTGTATCAAGGGAGCCT-3' (2215-2234)

**c-met:** sense 5'-ACAGTGCTGATGCAACATCGCT-3' (2413-2434)
antisense 5'-GCTCGTAGCTCACAAGATTC-3' (3049-3068)

In all cases, the amplification products were separated in 1.8% agarose gels, transferred onto nylon membranes (Amersham Buchler) after documentation, hybridized to digoxigenin-labeled HGF-SF or c-met cDNA fragments spanning the amplified sequence, washed under stringent conditions, and exposed to Kodak X-OMAT-AR films using a chemiluminescent detection kit (Boehringer Mannheim, Mannheim, Germany). Expected sizes of the amplified fragments were 314 bp in the case of rat HGF-SF and 655 bp in the case of human c-met. In addition, bands of the expected size from the different species were eluted from the gels, subcloned into pSK(+)(Stratagene) or pCRI000 (Clontech, Palo Alto, CA), and submitted to automated dideoxy chain termination sequencing (PRISM Ready Reaction DyeDeoxy Termination Cycle Sequencing Kit; Applied Biosystems, Foster City, CA) on a sequencer (373A; Applied Biosystems, Inc.).

**Cell Cultures and Effect of HGF-SF**

Neurons from septum and hippocampus of fetal rats (E17) were prepared as previously described (Zafra et al., 1990; Lindholm et al., 1994). The cells were taken up in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and plated on poly-d-ornithine-precoated 24-well dishes (Costar Corp., Cambridge, MA) at a density of 0.5 × 106 cells/dish. 3 h after plating, the cultures were switched over to a serum-free medium as described previously (Zafra et al., 1990). Cultures were maintained for 7 d

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**Figure 1.** Northern hybridization analysis of HGF-SF (A) and c-met (B) expression in the adult and developing rat brain. 10 μg of total RNA obtained from different regions of adult (lanes 2–5) and P4 rat brain (lanes 6–9) were analyzed: (lane 1) adult rat liver; (lanes 2 and 9) hippocampus; (lanes 3 and 8) cortex; (lanes 4 and 7) cerebellum; (lanes 5 and 6) midbrain; (lane 10) liver from P4 rat. The positions of 18S and 28S ribosomal RNA are indicated (shade between the positions of 18S and 28S rRNA of lane 9 in A is a non-reproducible artifact).
Figure 2. Expression of HGF/SF and c-met mRNAs in embryonic mouse brain. Dark fields images (left panels) show the hybridization signal and bright field pictures (right panels) the histology. Bars, 200 μm. (A and B) HGF-SF expression in the E14 telencephalon (parasagittal). Note expression in the periventricular neuroepithelium and in the preferentially frontal part of the cortical plate. (C and D) HGF-SF expression in the E17 telencephalon (parasagittal). Expression is still present in the neuroepithelium and the cortical plate. (E and F) c-met expression in the E17 telencephalon (parasagittal). Expression is located in the cortical plate and in the cortical subplate, preferentially in the posterior areas. (G and H) c-met expression in the E17 septum (arrows, horizontal section).
at 37°C in a 95% air/5% CO2 humidified atmosphere. Human recombinant HGF (30 ng/ml; Yoshiyama et al., 1991) was then added to the cultures for 30 min and total cellular RNA was extracted by the method of Chomczynski and Sacchi (1987). Control cultures received either 30 ng/ml of nerve growth factor (NGF) purified from mouse submandibular gland (Berninger et al., 1993) or an equal volume of bovine serum albumin. RNA electrophoresis and hybridization were performed as described earlier (Lindholm et al., 1993) using a v-fos probe (Lindholm et al., 1988) labeled by random priming. For control purposes, all filters were also hybridized with a rat β-actin cDNA probe as described previously (Lindholm et al., 1988). After washing, the filters were exposed to x-ray films, and the relative intensity of the c-fos mRNA signals were determined using a laser densitometer (Ultra Scan XL; Pharmacia-LKB, Uppsala, Sweden). Four independent stimulation experiments were analyzed using autoradiography films with signal intensities in the linear range. Signal intensities at the site of specific c-fos hybridization were determined and normalized for the intensity of the respective β-actin hybridization signals. Relative signal intensities of the different stimulation experiments were obtained by arbitrarily setting the c-fos signal intensity of the respective nonstimulated neurons at 1, and afterwards determining mean and standard deviation of the four independent experiments.

Results

HGF-SF and c-met Transcripts Are Expressed in the Mammalian Brain

In a preliminary screening of rat tissues using rTPCR, we have detected expression of HGF-SF and c-met in developing and adult rat brain (data not shown). To further localize these transcripts, microdissection of adult and developing (P4) rat brain was performed, and total RNA from hippocampus, cortex, cerebellum, and midbrain was analyzed by Northern hybridization.

HGF-SF transcripts were present in all fractions from adult and developing brain (Fig. 1A). The transcript size of ~6 kb corresponds well to the known, major HGF-SF mRNA species coding for full-length HGF-SF (Tashiro et al., 1990). In the adult brain, HGF-SF transcript levels were consistently highest in the hippocampus and significantly lower in cortex, cerebellum, and midbrain. In developing rat brain (P4), the signal was also present in the hippocampus, but the levels were higher in the cortex fraction.

Northern hybridization analysis for c-met transcripts demonstrated the presence of a transcript of ~7 kb in all fractions from adult and developing rat brain (Fig. 1B). The expression was highest in the hippocampus of the adult and in the cortex and hippocampus of the developing brain (P4).

In Situ Localization of HGF-SF and c-met Transcripts in Adult and Developing Brains

To exactly localize the HGF-SF– and c-met–expressing cells in the brain, in situ hybridization with specific [35S]UTP-
labeled riboprobes was performed and analyzed by film autoradiography (see Fig. 3), as well as by dark and bright field microscopy (see Figs. 2, 4-7). As demonstrated before by rtPCR and Northern hybridization, expression of both genes was found in both adult and developing brains.

During the development, HGF-SF signals were first detected in E12 mouse brain. At that time and throughout further development, HGF-SF mRNA was prominently expressed in the neuroepithelial layer of the telencephalic vesicle. Furthermore, expression was seen in the developing cortical plate, most prominently in the frontal part (Fig. 2). At P4, there was prominent expression in the ependymal layer with a weaker expression in the hippocampus, the outer layers of the cortex, and the medial habenula (Fig. 3).

In the adult, low levels of HGF-SF mRNA were detected widespread in neurons of many brain areas. The strongest signals were found in the hippocampus (Fig. 4), where they were present in all hippocampal areas with highest levels in the dentate gyrus. Lower signals were detected in all cortical areas, most prominently in the superficial layers II-III and layer V. As in the developing brain, a strong signal was seen in the ependymal cells lining the brain ventricles, as well as in the choroid plexus (Fig. 5). A strong signal was also present in the pineal body (not shown). In the hindbrain, HGF-SF mRNA was expressed in the pontine nuclei and in the deep cerebellar nuclei. A relatively strong signal was seen in cerebellar granule neurons in the adult (Fig. 5), where the expression was very low at P4. No signal above background was present in Purkinje cells.

c-met expression in the developing brain was detected from E13 forward, and was low up to E14, but E17 (Fig. 2), a relatively strong signal was detected in the developing cortical plate, as well as in the neurons of the cortical subplate. Posterior parts of the cortex, such as occipital and temporal areas, expressed higher levels of c-met mRNA compared to the frontal cortex. In addition, a clear signal was found in septal neurons at this age. At P4 (Fig. 3), there was a relatively strong expression in the outer layers of the cortex, especially in the parietal, occipital, and temporal areas. In the hippocampus, the c-met expression was confined to the CA-1 area, while no signal was detected in the dentate gyrus or the CA-3 area. Neurons in the septum continued to express c-met mRNA, and a specific signal was also detected in the developing facial nucleus. In the adult brain, the distribution of c-met mRNA resembled that found in P4. Thus, expression was found in the posterior cortical areas and in the CA-1 area of the hippocampus. A clear signal was again present in septal neurons (Fig. 6; see Table I).

Expression of HGF-SF in the Brain is Evolutionary Conserved

If brain HGF-SF is involved in important biological functions, its expression should have been conserved during evolution. Therefore, we analyzed HGF expression in the central nervous system of different mammalian species, as well as in avian brain.

rtPCR amplifications using specific primers derived from
conserved regions of known HGF-SF and c-met cDNA sequences (Tashiro et al., 1990; Park et al., 1987) demonstrated that both transcripts are present in mouse and human brains. The identity of the amplification products was characterized by Southern blot hybridization and partial sequencing (data not shown). In situ hybridization analysis using [35S]UTP-labeled rat HGF-SF riboprobes (Fig. 7) detected specific signals in hippocampal neurons of mouse and human brains. Furthermore, HGF-SF expression was demonstrated in cortical neurons of duck brain.

c-met transcripts were found in hippocampal neurons of adult human and mouse brains, but could not be detected in avian brain by in situ hybridization using human c-met probes, possibly because of lower homology of the different c-met mRNA sequences compared to HGF-SF (data not shown).

HGF-SF Stimulates c-fos Expression in Cultured Septal Neurons

To further investigate whether c-met-expressing neurons are able to respond to stimulation with HGF-SF, fetal septal neurons (E17) expressing c-met (see Fig. 2) were prepared and cultured for 1 wk (Lindholm et al., 1994). Since the activity of HGF-SF is conventionally assayed by functional effects elicited by epithelial cells, we decided to monitor for a potential effect of HGF-SF on neurons by analyzing early gene response. It has been demonstrated in rat fetal hepatocytes that stimulation with HGF-SF leads to an immediate activation of c-fos expression (Fabregat et al., 1992). As shown in Fig. 8, the addition of 30 ng/ml recombinant human HGF-SF (Yoshiyama et al., 1991) to fetal neuronal cultures from the septum induced reproducibly an approximately fourfold increase in c-fos mRNA levels in these cultures. Control experiments using recombinant NGF also led to a comparable increase of c-fos mRNA levels (sixfold) in the septal neurons. An increase in c-fos mRNA levels after stimulation with HGF-SF was also observed in cultured hippocampal neurons (E17), although the response varied between different primary cultures (data not shown). This was probably caused by differences in cell sampling because c-met-expressing neurons in the hippocampus are restricted to the CA-1 area (see Figs. 3 and 6; Table I). Taken together, the data demonstrate that stimulation with HGF-SF activates an immediate early gene response in c-met-expressing neurons.
Discussion

In this paper, we have demonstrated that HGF-SF and c-met are expressed in defined parts of the mammalian brain, and that, to a significant extent, both transcripts localize to neurons. HGF-SF immunolocalization to the central nervous system neurons and in the fetal brain has been described (Wolf et al., 1991; De Frances et al., 1992). Localization differences, especially in neuronal cell populations, are likely to result from the fact that previous studies could not distinguish between synthesis, uptake, and storage. While neuronal c-met expression has not been reported, immunolocalization of c-met to microglia has been reported (De Renzo et al., 1993). So far, we have been unable to demonstrate c-met transcripts above background in microglia. Although this does not exclude expression, c-met mRNA levels in microglia must be very low. Taken together, HGF-SF and c-met have to be added to the family of neurotrophins and growth factors that are expressed together with their receptor tyrosine kinases in the developing and/or adult brain. These include the neurotrophic factors NGF/trk (Martin-Zanca et al., 1986), BDNF/trk (Leibrock et al.,

Figure 6. Expression of c-met mRNA in adult rat brain. (A) Hippocampus (CA1/CA3, CA-1 and CA-3 areas; DG, dentate gyrus) with c-met expression only in the CA-1 area. (B) Respective bright field (Bar, 200 μm). (C) c-met-positive cells in the medial septum. Bar, 100 μm.
Table 1. Semiquantitative Analysis of HGF-SF and c-met Transcripts in Adult and Developing Rat Brain

|                     | HGF, adult | HGF, P4 | c-met, adult | c-met, P4 |
|---------------------|------------|---------|--------------|-----------|
| Cortex              | ++         | +       | +            | ++        |
| (II, III, V)        |            |         |              |           |
| Hippocampus         |            |         |              |           |
| DG                  | ++++       | (+)     | –            | –         |
| CA-1                | +++        | +       | ++ + +       | ++ + +    |
| CA-3                | +++        | (+)     | –            | –         |
| Olfactory bulb      | ++         | –       | +            | –         |
| Septum              | +          | –       | +            | +         |
| Thalamus            | +          | + + (+) (Medial habenula) | –         | –         |
| Pons                | + (Pontine nuclei, deep cerebellar nuclei) | (+) | – | – |
| Cerebellum          |            |         |              |           |
| Granule cells       | ++++       | (+)     | –            | –         |
| Purkinje cells      | –          | –       | –            | –         |
| Pineal body         | ++++       | n.d.    | –            | n.d.      |
| Ependyma            | +++++      | +++++   | –            | –         |

DG, dentate gyrus; n.d., not done.

Figure 7. Evolutionary conservation of HGF-SF expression in neurons of adult brain. (A–C) HGF-SF expression in adult mouse hippocampus (A, antisense probe, dark field; B, corresponding bright field; C, control hybridization with respective HGF-SF sense probe). (D and E) HGF-SF expression in cortical neurons of adult duck brain (D, dark field; E, corresponding bright field). (F and G) HGF-SF expression in human hippocampus (F, dark field; G, corresponding bright field) (Bars, 200 μm in B, 100 μm in E, and 1 mm in G).
pressed in the ependyma and the chorioid plexus, indicating the participation of four different experiments. C, control; P < 0.01 for NGF and BDNF (Gospodarowicz et al., 1984; Rail et al., 1985; Gomez-Pinilla et al., 1988). Recombinant protein experiments, H. J. Schlicht (Institute of Virology, Humboldt University, Berlin) from the Deutsche Forschungsgemeinschaft, and H. J. Schlicht (Institute of Genetics, University of Malnz, Germany) for supply of duck brain tissues, and T. Nakamura (Department of Biology, Kyushu University Fukuoka, Japan) for plasmid pBRC-1.

The function of HGF-SF in the brain differs from the conventional HGF-SF–induced, c-met–mediated mesenchymal–epithelial and –endothelial interaction, at least with respect to the cell types involved. Interestingly, melanocytes, cells of neural crest origin, are stimulated to grow by HGF-SF (Matsumoto et al., 1991). In the adult, both genes are expressed in neurons in a way that paracrine, interneuronal signaling is possible. Furthermore, in some parts of the brain (CA-1 area of the hippocampus and cortical layers II–III), both HGF-SF and c-met mRNAs, are expressed, which suggests the potential for an autocrine cycle, although we currently cannot provide direct evidence for colocalization of both messages in single neurons.

In analogy to the neurotrophins, such as NGF and BDNF, both of which are highly expressed in the hippocampus, HGF-SF may possibly represent a target-derived neurotrophic factor. For example, HGF-SF–expressing neurons in the hippocampus may either act on c-met–expressing neurons in the CA-1 area of the hippocampus or c-met–positive neurons in the septum that project into the hippocampus. This hypothesis is further supported by the fact that HGF-SF can induce an immediate early gene response in c-met–expressing neurons derived from the septum to an extent comparable to the known stimulatory effect of NGF. In addition, activation of hybrid c-met receptors in PC12 cells induces neurite outgrowth (Lithrumberger, T., N. Inagaki, P. Schirmacher, and D. Lindholm, manuscript in preparation). Our data using septal neurons provide strong evidence for the HGF-SF/c-met interaction to constitute a novel signaling system active in the developing brain and possibly also in adult mammalian brain. The observed effects suggest a trophic role of HGF-SF for a subpopulation of central nervous system neurons. Whether HGF-SF has additional effects on responsive neurons in the brain remains to be analyzed.

We thank S. Oppelt and B. Gilberg for excellent assistance in analyzing the fetal brain tissues, P. Pulkowski and T. Böhmi for photography work, T. Hinkel (Institute of Genetics, University of Mainz, Germany) for automated sequencing of rPCR subclones, W. Müller-Esterl (Institute of Physiological Chemistry, University of Mainz) for helpful suggestions in some recombinant protein experiments, H. J. Schlicht (Institute of Virology, University of Ulm, Germany) for supply of duck brain tissues, and T. Nakamura (Department of Biology, Kyushu University Fukuoka, Japan) for plasmid pBRC-1.

The work presented in this paper was supported by research grants Schi 111/3-1 (P. Schirmacher) and Sonderforschungsbereich 302/Z30 (H.-P. Dienes and P. Schirmacher) from the Deutsche Forschungsgemeinschaft, and it also contains part of the Ph.D. thesis of W. Jung. This research was sponsored in part by the National Cancer Institute, Department of Health and Human Services, under contract no. NOI-CO-74101 with Advanced BioScience Laboratories Inc. (G. F. Vande Woude).

Received for publication 11 January 1994 and in revised form 8 April 1994.

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