A Novel Role for Protein Tyrosine Phosphatase SHP1 in Controlling Glial Activation in the Normal and Injured Nervous System

Andrea Horvat,1 Franz-Werner Schwaiger,1 Gerhard Hager,1 Frank Bröcker,1 Robert Streif,1 Pjotr G. Knyazev,2 Axel Ullrich,2 and Georg W. Kreutzberg1

1Department of Neuromorphology, Max-Planck-Institute of Neurobiology and 2Department of Molecular Biology, Max-Planck-Institute of Biochemistry, D-82152 Martinsried, Germany

Tyrosine phosphorylation regulated by protein tyrosine kinases and phosphatases plays an important role in the activation of glial cells. Here we examined the expression of intracellular protein tyrosine phosphatase SHP1 in the normal and injured adult rat and mouse CNS. Our study showed that in the intact CNS, SHP1 was expressed in astrocytes as well as in pyramidal cells in hippocampus and cortex. Axotomy of peripheral nerves and direct cortical lesion led to a massive upregulation of SHP1 in activated microglia and astrocytes, whereas the neuronal expression of SHP1 was not affected. In vivo experiments revealed that in astrocytes, SHP1 associates with epidermal growth factor (EGF)-receptor, whereas in microglia, SHP1 associates with colony-stimulating factor (CSF)-1-receptor. In postnatal and adult moth-eaten viable (me+/me) mice, which are characterized by reduced SHP1 activity, a strong increase in reactive astrocytes, defined by GFAP immunoreactivity, was observed throughout the intact CNS, whereas neither the morphology nor the number of microglial cells appeared modified. Absence of [3H]-thymidine-labeled nuclei indicated that astrocytic proliferation does not occur. In response to injury, cell number as well as proliferation of microglia were reduced in me+/me mice, whereas the posttraumatic astrocytic reaction did not differ from wild-type littermates. The majority of activated microglia in mutant mice showed rounded and ameboid morphology. However, the regeneration rate after facial nerve injury in me+/me mice was similar to that in wild-type littermates. These results emphasize that SHP1 as a part of different signaling pathways plays an important role in the global regulation of astrocytic and microglial activation in the normal and injured CNS.

Key words: tyrosine phosphorylation; signal transduction; protein tyrosine phosphatase SHP1; peripheral nerve axotomy; facial nerve; hypoglossal nerve; sciatic nerve; direct cortical lesion; microglial proliferation; mutant moth-eaten viable mice

Activation of astrocytes and the macrophage-related microglia, as a ubiquitous hallmark of different neuropathological states, is important in forming an environment that contributes to successful repair of damaged brain tissue (for review, see Norenberg, 1994; Rothwell and Hopkins, 1995; Masliah et al., 1996; Ebadi et al., 1997; Schwaiger et al., 1998; Tacconi, 1998). In various pathological states, the nervous tissue becomes a source and a target for signaling molecules that mediate the coupling between the cytokine receptors and modulation of gene expression in activated microglia and astrocytes in vivo are poorly characterized.

Binding of cytokines to specific receptors triggers tyrosine phosphorylation of different signaling molecules within the target cells (for review, see Karnitz and Abraham, 1995). Such modifications of protein tyrosine residues have also been implicated in the activation of microglial cells in response to brain injury (Karp et al., 1994). The state of tyrosine phosphorylation depends on the balance between the antagonistic activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). PTPs have been reported to be involved in both positive (signal enhancing) and negative (signal terminating) regulation of cytokine signaling (for review, see Hunter, 1995). For example the cytosolic SH2 domain-containing protein tyrosine phosphatase SHP1 (also known as SH-PTP1, PTP-1C, HCP, or PTPN6) appears to be a negative regulator of receptors for c-kit, colony stimulating factor-1 (CSF-1), interleukin (IL)-3, IL-4, and interferon (IFN)-α/β in hematopoietic cells as well as of IFN-γ-receptor in cultured astrocytes (Yi and Ihle, 1993; Yi et al., 1993; Klingmuller et al., 1995; Chen et al., 1996; Massa and Wu, 1996, 1998; Paulson et al., 1996). In contrast, in epithelial cells SHP1 is positively involved in epidermal growth factor (EGF) and IFN-γ signaling (Su et al., 1996; You and Zhao, 1997). A physiological role of SHP1 has been elucidated in the investigations of so-called moth-eaten (me/me) and moth-eaten viable (me+/me+) mice, which display multiple hematopoietic abnormalities. Both mice strains carry a single base mutation in the SHP1 gene resulting in splicing defects. In the me/me mice no SHP1 protein is detectable, whereas the me+/me+ mutation leads to the production of two variant forms of SHP1 protein with reduced biological activity (for review, see Tsui and Tsui, 1994; Schultz et al., 1997). Although the role of SHP1 in hematopoietic cells is well defined, little is known about SHP1 expression in the CNS and after brain injury. In the present study we examined the expression and regulation of SHP1 in the intact and injured adult rat and mouse brain and the signaling pathways, where SHP1 may be involved. Because...
of a longer viability of me/me mice in comparison to me/me, these mice have been used to study the function of SHP1 in the injured nervous system in vivo using two models of CNS trauma: the facial motor nucleus after nerve lesion and the direct cortical lesion.

**MATERIALS AND METHODS**

**Animals.** Adult male Wistar rats (200-250 gm) were housed under standard laboratory conditions in the animal house of the Max-Planck-Institute of Biochemistry (Martinsried, Germany). Wild-type C57BL/6J and SHP1 null (C57BL/6J-Hsp2c-null) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous and heterozygous mice were further bred in our animal house. All animal experiments and care protocols were approved by the Regierung von Oberbayern (AZ 211-2531-10/93 and AZ 211-2531-37/97).

**Facial, hypoglossal, and sciatic nerve transaction.** Under ether anesthesia, the facial nerve was transected at the stylo-mastoid foramen; the hypoglossal nerve was cut where it passes the carotid artery, and the sciatic nerve was cut at the level of the sciatic notch. In all experiments the unoperated contralateral side served as a control. After the indicated time intervals (3, 7, 14, 28, 42, and 84 d), animals were overdosed with chloral hydrate. The brains and lumbar spinal cords were quickly removed and frozen at -80°C.

**Direct cortical lesion (cortical stab wound).** Adult rats and mice (normal littersates and me/me mice) were deeply anesthetized by intraperitoneal injection (150 µl/100 gm body weight of a mixture containing 6% xylazine and 0.7% xylazinhydrochlorid) and placed in a stereotaxic apparatus. After the scalp was reflected, burr holes were positioned over the cerebral cortex at 6 mm caudal to bregma and 3.5 mm lateral to the tip of the corpus callosum. A 20-gauge needle, mounted in the stereotaxic apparatus, was angled 10° away from the midline and inserted to a depth of 3.0 mm from the surface of the brain. After surgery, animals were allowed to survive for different time intervals (1, 3, 7, and 14 d).

**Cell culture.** For astroglial primary cultures the cortices of newborn rats were homogenized, and the suspension was kept in a 75 cm² uncoated culture flask in the incubator and fed on days 7 and 12 with DMEM supplemented with 10% FCS, whereas microglia were cultured in the same medium supplemented with 10% FCS. After washing in distilled water, sections were dehydrated in an ascending ethanol series, air-dried, and dipped in photographic emulsion (NTB-2, Kodak, Rochester, NY). After drying the sections for 4°C, the sections were developed, counterstained with hematoxylin, dehydrated in a graded series of ethanol, and coverslipped with DePeX (Gurr, BDH, Poole, UK).

**Semi-quantitative RT-PCR.** Semi-quantitative analysis of SHP1 mRNA expression in facial nuclei at different time points (0, 3, 7, 14, 28, and 56 d) after nerve axotomy was performed as described previously (Hager et al., 1999; Hol et al., 1999). For each time point, cDNA was analyzed from six rats in triplicate. PCRs were performed in a final volume of 12.5 µl containing 1 µM sense and 1 µM antisense primer, 1× PCR buffer, 1.5 mM MgCl₂ (Eurobio), 200 µM dNTPs (MBI Fermentas), 1 µCi (13 mCi) α-32P-dATP, 1 µCi (13 mCi) [α-32P]-dCTP (Amersham), and 1 µl cDNA (unoperated or operated rat facial nuclei). The SHP1 fragment was amplified with the sense primer 5’-CCAGGCGAGTACTGCAGTCGTT-TGAGCA-3’ and antisense primer 5’-GGGCTGGTGACTTCTGCTCAACTT-3’ for 32 cycles; for cyclophilin amplification, the conditions were 30 sec at 94°C (with a 1 sec time increment for each subsequent cycle), 30 sec at 64°C, and 1 min at 74°C.

**Cloning of the rat SHP1-cDNA.** To obtain an in situ probe, the rat SHP1-cDNA (775 bp) was amplified with the sense (5’-CGCAAAAGACCAGAGT ACTCTT3’- C) and antisense primers (5’-GCAGGACATCTGGCCTGTT-GTC-3’) based on the European Molecular Biology Laboratory accession number RNU77038 (U77038). Amplification performed in a final volume of 12.5 µl containing 1 µM sense and 1 µM antisense primer, 200 µM dNTPs (MBI Fermentas), Vilnius, Lithuania), 1× PCR buffer [16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, pH 8.8, 0.1% Tween-20], 3 mM MgCl₂, 1 U Taq polymerase (all from Eurobio, Les Ulies, France), and 1 µl cDNA (operated rat facial nuclei as). The PCR was performed for 35 cycles under the following conditions: 10 sec at 94°C, 20 sec at 64°C, 40 sec at 74°C, 5 min at 72°C. The PCR product was blunted with Pfu polymerase (Stratagene, La Jolla, CA), ligated into pZErO-1-vector (Invitrogen, Groningen, The Netherlands), and sequenced in the sequencing facility of the Max-Planck-Institute of Neurobiology.

**In situ hybridization.** The cloned rat SHP1 fragment was PCR amplified using T7 or SP6 primers specific for pZErO-vector (Invitrogen). The PCR was performed for 25 cycles at 94°C denaturing for 10 sec, 65°C annealing for 20 sec, and 74°C elongation for 30 sec. The template was purified using the QIAquick purification kit (Qiagen), and labeling was performed by transcription using either T7 or SP6 RNA-polymerase (Boehringer Mannheim, Mannheim, Germany) and α-32P-UTP (Amer sham Life Sciences, Braunschweig, Germany). To allow diffusion of probe into the tissue, the labeled SHP1 RNA probe (775 bp) was lyophilized in carbonate buffer (60 mM Na₂CO₃, 40 mM NaHCO₃, pH 10.2) for 30 min at 60°C according to manufacturer’s protocol (Boehringer Mannheim). The hybridization was stopped by adding the neutralization buffer (200 mM NaAc, 1% (v/v) CH₃COOH, pH 6.0). The labeled RNA probe was precipitated and reconstituted in bidistilled water.

**Western blotting and immunoprecipitation.** Western blotting and immunoprecipitation was performed as described previously (Hager et al., 1999; Hol et al., 1999). The signal for SHP1 was normalized for the cyclophilin A signal, as described previously (Hager et al., 1999; Hol et al., 1999). PCR products of SHP1 and cyclophilin were cloned into pZErO-vector (Invitrogen), and their identity was confirmed with sequencing. For each experiment and cDNA synthesis, the number of PCR cycles for optimal PCR conditions was determined independently for each primer pair to obtain a logarithmic increase of PCR product. The optimal cycle conditions for SHP1 were as follows: 10 sec at 94°C (with a 1 sec time increment for each subsequent cycle), 20 sec at 64°C, 40 sec at 74°C for 32 cycles; for cyclophilin A, the conditions were 30 sec at 94°C (with a 1 sec time increment for each subsequent cycle), 10 sec at 59°C, and 40 sec at 74°C for 25 cycles. Semi-quantitative and statistical analyses (a paired, two-tailed Student’s t test) for SHP1 mRNA expression were further performed as described previously (Hager et al., 1999; Hol et al., 1999).

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Schuell, Dassel, Germany). The blots were incubated overnight at 4°C with rabbit anti-SHP1 (1:2000, Santa Cruz Biotechnology) or the mouse anti-actin antibodies (1:1000, Amersham) in 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 containing 1% BSA.

For immunoprecipitation, the microglial or astrocytic cultured cells (~5 × 10^6 cells) were lysed in lysis buffer as described above. After centrifugation (12,000 × g, 20 min, 4°C) of cell lysates, rabbit anti-SHP1 antibodies (Santa Cruz Biotechnology) and protein A-Sepharose 4B (Pharmacia Biotech, Freiburg, Germany) were added. The mixtures were rotated at 4°C overnight, and the precipitates were washed in cold lysis buffer four times before SDS-electrophoresis analysis and Western blotting. For Western blotting, protein samples, separated on SDS-PAGE gels, were blotted to nitrocellulose membrane (Schleicher & Schuell). The membranes were then incubated with mouse anti-phosphotyrosine antibody (1:5000, Upstate Biotechnology, Lake Placid, NY), mouse anti-SHP1 antibodies (1:1000, Transduction Laboratories, San Diego, CA), rabbit anti-EGF-R antibodies (1:1000, Santa Cruz Biotechnology), or rabbit anti-CSF-1-R antibodies (a gift from Dr. Axel Ullrich, Department of Molecular Biology, Max-Planck Institute for Biochemistry, Martinsried, Germany).

In all Western blotting experiments, detection of primary antibodies was performed by incubating the membrane for 1 hr at room temperature with secondary goat anti-rabbit or anti-mouse IgGs coupled to horseradish peroxidase (Pierce, Rockford, IL). Specific antibody signals were detected using the enhanced chemiluminiscence kit (ECL, Amersham).

Immunohistochemistry. For all immunohistochemical studies, cryostat sections from fresh material were air-dried and fixed with 4% formaldehyde in 0.01 PBS for 10 min, 50% acetone for 2 min, 100% acetone for 2 min, and 50% acetone for 2 min. After repeated washing in 0.01 M PBS, the sections were incubated overnight at 4°C with primary antibodies. Thereafter the slices were rinsed in 0.01 M PBS and incubated with secondary antibodies for 2 hr at room temperature.

Immunofluorescence/confocal laser microscopy. Titration of the polyclonal rabbit anti-human SHP1 antibody (Santa Cruz Biotechnology) on cryostat tissue sections, 3 d after facial nerve axotomy, from 1:50 to 1:40,000 dilution revealed optimal staining intensity at 1:5000 dilution, which was used throughout the following study. Thereafter the slices where incubated with goat anti-rabbit IgGs coupled to indocarbocyanin (Cy3, 1:400; Dianova, Hamburg, Germany). Astrocytes were identified using antibodies directed against GFAP (Zymed, San Francisco, CA). Microglia in rat brain slices were identified using antibodies against OX42 (Serotec, Oxford, UK). For colocalization studies (triple staining), the rabbit anti-SHP1/goat anti-rabbit Cy3 brain sections were first incubated with mouse anti-OX42 antibodies (1:2000), which were visualized by goat anti-mouse IgGs coupled to indodicarbocyanin (Cy5, 1:400; Dianova) and additionally with rat anti-GFAP antibodies (1:100) detected by donkey anti-rat IgGs coupled to dichlorotriazinylamino-fluorescein (DTAF, 1:200; Dianova). In the direct cortical lesions section for colocalization of SHP1 we used the following: (1) with T-lymphocytes, hamster antibody against CD3-antigen (1:1000, PharMingen, San Diego, CA) detected by goat anti-hamster DTAF (1:200, Dianova); (2) with macrophages/monocytes, mouse antibody against ED1-antigen (1:100, Serotec) detected by goat anti-mouse DTAF (1:200, Dianova). Galanin was detected by rabbit antibody against galanin (1:5000, Peninsula, Heidelberg, Germany). The sections were evaluated using a confocal laser scanning microscope in an inverted configuration (Leica TCS 4D, Leica, Bensheim, Germany). Consecutive optical sections up to 20 μm depth in the slices were taken in two-channel mode, and each channel was projected into a two-dimensional micrograph using the maximum intensity projection algorithm. To avoid cross-talk between the red, green, and infrared channels, all fluorescence images were recorded in sequential mode.

Light microscopic immunohistochemistry. Mouse brain sections (20 μm) from normal littermates and mutant me/me mice were incubated with either rat monoclonal antibodies against α5β1-integrin (1:6000, Serotec) or rat monoclonal antibodies against GFAP (Zymed). Longitudinal sections (10 μm) of the facial nerve were incubated with either rabbit polyclonal antibodies against galanin (1:400, Penninsula) or calcitonin gene-related peptide (CGRP) (1:1000, Penninsula). Primary antibodies were detected using appropriate biotinylated goat antibodies (1:100, Camon, Wiesbaden, Germany) and ABC peroxidase complex (Vector, Burlingame, CA) followed by diaminobenzidine kit (Pierce).

Quantification of cell proliferation and microglial cell number using bright-field microscopy. To quantify cell proliferation (2 and 3 d after facial nerve axotomy), both me/me mice and wild-type littermates were injected intraperitoneally with a single dose of [3H]-thymidine (Amersham) (10 μCi/gm body weight) 2 hr before they were killed (Raivich et al., 1994). After direct cortical lesion, each animal received 2 d after lesion) four consecutive intraperitoneal injections of [3H]-thymidine (10 μCi/gm body weight per injection) separated by 3 hr intervals (Janeczko, 1993). Autoradiography with NTB2 photoemulsion (Kodak) was performed after overnight 4% formaldehyde (in 10 mm PBS) fixation of fresh cryostat sections as described previously (Raivich et al., 1994). To determine the type of proliferating glial cells, immunohistochemistry (α5β1-integrin labeling of microglia and GFAP labeling of astrocytes) (see Immunohistochemistry) was performed on fresh tissue sections from animals treated with [3H]-thymidine, followed by autoradiography. The number of [3H]-thymidine-positive cells and α5β1-immunopositive microglia were scored under a 40× objective in bright field. The total number of cells in 20 random fields per section was...
Regeneration rate in the facial nerve. The facial nerve regeneration rate in me/me mice and wild-type littermates was performed as described previously (Werner et al., 2000). Briefly, the facial nerve was crushed near the stylomastoid foramen for 30 sec. Four days after facial nerve crush, the animals were killed, followed by a 5 min perfusion with 4% FA-PBS and then by slow 60 min perfusion with 1% FA-PBS. The facial nerves were then dissected, frozen on dry ice, and cut longitudinally (10 μm), and the regenerating axons were visualized by immunostaining for galanin or CGRP. Every fifth section was used per antibody, and the distance between the crush side and the most distal-labeled growth cone of a method described previously (Sanner et al., 1993). The micrographs were analyzed using an image analysis program (OPTIMAS 6.2, Optimas Corporation, Bothell, WA). Regions of interest were built by the selection of the astrocytic cells, applying a threshold that was set to mean + SD of the mean (mean = average pixel intensity of the picture). This threshold, defined on the axotomized side, was also used for the respective control side. To test significance, a paired, two-tailed Student’s t test was used for statistical analyses.

Confocal microscopy/quantitative fluorescence immunohistochemistry. To quantify the GFAP immunoreactivity (IR) in astrocytes, digital micrographs of the DTAF fluorescence (1024 × 1024 pixels, 0–255/8 bit gray scale) were recorded subsequently with a Leica TCS 4D confocal laser scanning microscope, 10× objective, and constant ArKr laser power. Fifteen consecutive equidistant levels were scanned per slice (total vertical span = 20 μm). From this stack, two-dimensional projections (1-Mbyte Tiff files) were made using the maximum intensity projection algorithm provided by the microscope software. Quantification of GFAP immunoreactivity was performed with a slight modification of a method described previously (Sanner et al., 1993). The micrographs were analyzed using an image analysis program (OPTIMAS 6.2, Optimas Corporation, Bothell, WA). Regions of interest were built by the selection of the astrocytic cells, applying a threshold that was set to mean + SD of the mean (mean = average pixel intensity of the picture). This threshold, defined on the axotomized side, was also used for the respective control side. To test significance, a paired, two-tailed Student’s t test was performed between control sides of normal and moth-eaten littermates or between axotomized sides of normal and moth-eaten viable littermates. The counts from six sections from each animal were averaged, and the means of four animals per time point were graphed.

RESULTS

SHP1 is constitutively expressed in astrocytes and pyramidal neurons in the normal CNS

To determine whether SHP1 is expressed in the normal rat CNS, tissue sections of brain and cervical spinal cord were processed for in situ hybridization and immunohistochemistry. Strong SHP1 hybridization signal was found in CA1, CA2, and CA3 regions of hippocampus and in dentate gyrus (Fig. 1A1, A2), in ependymal and subependymal layers along the third ventricle (Fig. 1A1), the fourth ventricle (Fig. 1B1, B2), and the central canal of the spinal cord (data not shown) as well as in cortical layers (II–IV) (Fig. 1C1, C2). In addition, a weak hybridization signal, slightly above the background, was evidently dispersed throughout the whole gray matter of brain parenchyma. Controls performed with sense cRNA transcripts did not show any specific cellular signal (data not shown). Immunohistochemistry showed that SHP1 immunoreactivity colocalized with GFAP-positive astrocytes (Fig. 1A3, B3). It appeared that most, if not all, astrocytes were labeled, whereas Bergmann glia in cerebellum remained negative. No SHP1 labeling on microglial cells was observed. In hippocampus and in cortical layers (II–IV), SHP1 immunoreactivity was also localized on the pyramidal neurons and their apical dendrites (Fig. 1A3, C3).

Regulation of SHP1 is associated with glial cell activation in response to injury in the nervous system

SHP1 mRNA and protein expression in response to brain injury was studied in peripheral (facial, hypoglossal, and sciatic) nerve axotomy models and after direct cortical lesion. Hybridization with the SHP1-specific cRNA probe on brain slices 3 d after operation revealed an increase of silver grains over the axotomized facial (Fig. 2A) and hypoglossal motor nuclei (Fig. 2B) as well as over ventral and dorsal gray matter of the lumbar spinal cord after sciatic nerve axotomy (C). In response to direct cortical lesion (Dcl), upregulation of SHP1 mRNA is observed only within and around the brain wound (D). In all injury models, weak hybridization signals, slightly above the background, are present on the control, unoperated side (Con). Scale bars, 200 μm.

Figure 2. Regulation of SHP1 mRNA expression after peripheral (facial, hypoglossal, sciatic) nerve axotomy and direct cortical lesion 3 d after injury. The dark-field micrographs illustrate massive induction of the SHP1 mRNA expression in the axotomized (Ax) facial (A) and hypoglossal (B) motor nuclei. The SHP1 mRNA is also upregulated in ventral and dorsal gray matter of the lumbar spinal cord after sciatic nerve axotomy (C). In response to direct cortical lesion (Dcl), upregulation of SHP1 mRNA is observed only within and around the brain wound (D). In all injury models, weak hybridization signals, slightly above the background, are present on the control, unoperated side (Con). Scale bars, 200 μm.
SHP1 mRNA declined after 14 d, and no significant difference between the facial nucleus of the axotomized side and the control side could be measured 28 and 56 d after axotomy. The time course of SHP1 mRNA regulation in the PCR analysis paralleled the data obtained by in situ hybridization experiments (Fig. 3B).

To determine the cell types that upregulate SHP1 mRNA after peripheral and CNS lesions, the in situ hybridization slides were counterstained with hematoxylin (Fig. 4A). Silver grains strongly accumulated over non-neuronal, small cells on the injured side, whereas neurons remained unlabeled. On the control side, a faint accumulation of silver grains was also found over some non-neuronal cells. Triple-labeling immunohistochemistry revealed a strong increase in the IR for SHP1 (shown in red) on the injured side, which was found on Ox42-positive microglia (Fig. 4B).
positive astrocytes showed colocalization with SHP1 immunoreactivity. In the facial nerve axotomy model, the microglial and astrocytic SHP1-IR reached maximum intensity within 3–14 d after operation. At later time points (up to 84 d), a faint staining for SHP1-IR became restricted to astrocytes (data not shown). After a direct cortical lesion, SHP1-IR was not only present in microglia and astrocytes but also in invading macrophages and T-lymphocytes (Fig. 4D). Preincubation of primary antibody with SHP1 peptide completely abolished specific SHP1 labeling (data not shown).

The specificity of the applied SHP1 antibody was characterized using Western blot analysis of total protein performed from lysates of facial nuclei (Fig. 3C). Facial motor nuclei homogenates from different time points after axotomy revealed a major band at 68 kDa, which corresponds to the molecular weight of the SHP1 protein in Jurkat T cells. The band intensity was markedly higher 3–14 d after axotomy and decreased at the later time points (150 d) but stayed elevated in comparison to the nonoperated controls. This was consistent with the time course of SHP1 immunohistochemistry.

**Cellular reactions in SHP1 mutant mice (me+/me−) in the intact brain and after peripheral or central lesion**

To elucidate functional aspects of SHP1 in the nervous system, we analyzed me+/me− mice, which carry a defect in the phosphatase domain of SHP1 resulting in reduction of SHP1 activity. Immunostaining with anti-GFAP antibody, as a marker of early astrocytic activation, revealed a statistically significant increase in GFAP immunoreactivity (p < 0.05 nonaxotomized facial nucleus; p < 0.001 random brainstem area) throughout the whole intact brain in adult me+/me− mice when compared with wild-type littermates (Figs. 5B, 6A). No incorporation of [3H]-thymidine was found, indicating that in the normal adult brain GFAP-positive astrocytes do not proliferate in me+/me− mice (data not shown). In addition, increased GFAP immunoreactivity through different intact brain regions was found in me+/me− mice at postnatal days 4 and 14 compared with wild-type littermates (Fig. 5A). In contrast to the prominent increase in GFAP immunoreactivity in astrocytes, immunostaining with anti-α3β1-integrin antibody revealed that in intact brain regions of adult me+/me− mice, the morphology (Fig. 5C) and number (Fig. 6B1) of labeled microglia were not conspicuously modified compared with wild-type littermates.

In response to peripheral nerve injury, posttraumatic increase of GFAP-IR in the axotomized facial nucleus was similar in me+/me− mice and wild-type littermates (Fig. 6A). In contrast, the number of α3β1-positive microglia (p < 0.001) and the number of proliferating [3H]-thymidine-labeled cells (p < 0.001) in me+/me− mice were statistically significantly reduced by 40–60% compared with wild-type littermates 2 and 3 d after facial nerve axotomy (Fig. 6B3). The combination of [3H]-thymidine autoradiography and immunohistochemistry showed that the proliferating cells in me+/me− mice were α3β1-integrin-positive microglia, whereas no GFAP-positive astrocytes were labeled (Fig. 5D). After direct cortical lesion, the number of proliferating ([3H]-thymidine-labeled) α3β1-positive microglia was also statistically significantly reduced in me+/me− mice compared with wild-type littermates (p < 0.001) (Fig. 6B2). In the axotomized facial nucleus, only a small subset of α3β1-positive microglia achieved characteristic ramified morphology, as detected by light microscopy, whereas most activated microglia of mutant mice were rather rounded and ameboid (Fig. 5C). Hence, the ramification of microglia seemed to be delayed in me+/me− mice at days

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Figure 5. Astrocytic and microglial reaction in the intact brain during adulthood, in the intact (Con) and axotomized (Ax) facial nucleus in normal, wild-type littermates (WT) and SHP1 mutant, moth-eaten viable mice (MEV). A. GFAP-immunoreactivity in the brainstem of WT and MEV mice at postnatal days 4 (P4) and 14 (P14). Note that GFAP-IR is increased in MEV mice compared with the same brain regions of WT littermates. B. GFAP-IR in the brainstem of WT and MEV mice 3 d after facial nerve transection. Note that GFAP-IR is increased on the control, unoperated side in MEV mice (Con-MEV) (as well as in the whole brain) compared with the same region of normal littermates (Con-WT). C. The morphology of microglia, detected by α3β1-integrin immunoreactivity 2 and 3 d after facial nerve axotomy. Note that after facial nerve axotomy, the microglial cells in MEV mice are characterized by rounded, ameboid morphology with delayed and decreased ramification. D. Combination of [3H]-thymidine autoradiography (white points) with either GFAP-immunoreactivity (green) or α3β1-integrin-IR (red) 3 d after facial nerve axotomy. Note that the proliferating cells in MEV mice are exclusively α3β1-integrin-positive microglia. Scale bars, 100 μm.

Ox42-IR shown in green) as well as on GFAP-positive astrocytes (Fig. 4C, GFAP-IR shown in green). No change in SHP1-IR was observed for neurons. On the unoperated side, some GFAP-
3 (Fig. 5C) and 7 (data not shown) after facial nerve axotomy. At the ultrastructural level, the intracellular structures of neurons, microglia, or astrocytes did not show differences between mev/mev and wild-type mice (data not shown). When the neuronal responses in the axotomized facial nuclei between wild-type littermates and mev/mev mice were compared, no difference could be detected for staining with an anti-galanin antibody (data not shown).

To examine the functional role of SHP1 during nerve regeneration, we compared the regeneration rate in mev/mev mice and wild-type littermates using a facial nerve crush model. The facial nerve was crushed near the foramen stylomastoideum and allowed to regenerate for 4 d. The longitudinal nerve sections were stained for neuropeptides galanin or CGRP (Fig. 6C), and the distance between the crush side and the most distal-labeled growth cone was measured using light microscopic grid scaling. At day 4, the regeneration distance for the wild-type littermates was 6.45 ± 0.76 mm for galanin and 6.30 ± 0.63 mm for CGRP immunoreactivity (Fig. 6C). A similar distance was also found for mev/mev mice (6.41 ± 0.56 mm for galanin and 6.27 ± 0.43 mm for CGRP-positive axons).

In microglia and astrocytes, SHP1 is involved in different signaling transduction pathways

After brain injury, growth factors such as CSF-1 and EGF as well as their receptors (CSF-1-R and EGF-R) are upregulated in microglial and astrocytic cells (for review, see Ferrer et al., 1996; Raivich et al., 1998). To determine whether SHP1 is involved in the signal transduction pathways of these receptor protein tyrosine kinases, we performed in vitro experiments. Microglial cells were stimulated with CSF-1 at different time intervals, and SHP1 was immunoprecipitated from nonstimulated and stimulated cells (Fig. 7A). Stimulation of microglial cells with CSF-1 induced increase in tyrosine phosphorylation of SHP1 and CSF-1-R, reaching a maximum after 30 min, and declined after 60 min. CSF-1 had no effects on the levels of SHP1 as shown in the blots probed with anti-SHP1 antibodies. To determine whether SHP1 might associate with CSF-1-R, blots of immunoprecipitates of SHP1 were probed with antisera against CSF-1-R. As illustrated in Figure 7A, an immunoreactive protein corresponding to CSF-1-R was detected in SHP1 immunoprecipitates of both nonstimulated and stimulated cells.

**Figure 6.** Quantitative effects of reduced SHP1 activity on astrocytic (A) and microglial reaction (B) in the intact and injured brain as well as on axonal outgrowth (C). A. Quantification of GFAP-IR (pixel number) in the intact brain (random brainstem area, nonaxotomized facial nucleus) and 3 d after facial nerve axotomy in wild-type (WT) littermates and MEV mice. Note that only in the uninjured brain regions there is a statistically significant increase for GFAP-IR in MEV mice compared with WT mice (6 sections per each animal; n = 4 animals). B1. Quantification of αβ2-integrin-positive microglia in intact brain regions (random brainstem area and nonaxotomized facial nucleus). Note that the number of resting microglia is similar in WT and MEV mice. B2. Reduction in the number of proliferating ([3H]-thymidine-labeled) αβ2-positive microglia in MEV mice 2 d after direct cortical lesion. B3. Reduction in the number of αβ2-positive microglia and the proliferation rate ([3H]-thymidine-labeled cells) in MEV mice 2 and 3 d after facial nerve axotomy compared with wild-type littermates. The microglial reaction was always quantified in six sections per animal (n = 4). C. Four days after crush near the foramen stylomastoideum, the facial nerve was cut longitudinally and stained for galanin or CGRP, which accumulate in the terminals of the elongating neurites. The average distance between the most distal-labeled growth cone and the crush side was determined for each axonal marker in five tissue sections per animal (n = 6). Both the galanin- and CGRP-positive axonal populations show the same regeneration distance of ~6 mm at day 4 in the WT littermates and MEV mice. All statistical analyses were performed using a paired, two-tailed Student’s t test. Statistically significant changes are indicated by asterisks (*p < 0.05, **p < 0.001).
The potential association of SHP1 with EGF-R was examined in cultured astrocytes stimulated with EGF. SHP1 immunoprecipitates showed that EGF stimulation induced an increase in tyrosine phosphorylation of SHP1 and EGF-R. Tyrosine phosphorylation of both proteins reached a maximum after 30 min and declined after 60 min. EGF did not have an effect on the level of SHP1, whereas the amount of EGF-R detected in SHP1 immunoprecipitates of stimulated astrocytes was increased and paralleled the level of EGF-R tyrosine phosphorylation.

**DISCUSSION**

**SHP1 expression and its possible role in the nervous system**

In hematopoietic cells, the role of protein tyrosine phosphatase SHP1 in signal transduction pathways of different cytokine receptors is well defined (Imboden and Koretsky, 1995). Although SHP1 has been detected by Northern blot analysis in the brain (Plutzky et al., 1992) and in cultured astrocytes (Massa and Wu, 1996), the expression and function of SHP1 in the normal and injured adult nervous system *in vivo* has not been investigated. In the present study we show that SHP1 is strongly expressed in astrocytes in ependymal and subependymal layers along the third and fourth ventricles and the central canal of the spinal cord as well as in CA1, CA2, and CA3 regions of hippocampus and in dentate gyrus. A weak SHP1 expression is found in astrocytes dispersed in the gray matter of brain parenchyma, whereas Bergmann glia in cerebellum remain negative. In addition, SHP1 is also expressed in the pyramidal cells and their apical dendrites in the hippocampus and cortical layers II–IV.

In the adult SHP1 mutant mev/mev mice, which are characterized by a reduction in SHP1 activity (for review, see Tsui and Tsui, 1994; Schultz et al., 1997), we found a strong increase in reactive astrocytes, evident by an increase in GFAP-immunoreactivity, throughout the whole intact brain. Neither the morphology nor the number of microglial cells appeared modified. Absence of [3H]-thymidine-labeled nuclei indicated that the increase of GFAP-positive astrocytes did not result from astrocytic proliferation but rather from enhanced GFAP expression. In addition, the finding that GFAP immunoreactivity is increased through different intact brain regions in mev/mev mice at postnatal days 4 and 14 suggests that this is rather an early event that develops during embryogenesis. Reactive astrocytes have also been noted in the transgenic mice overexpressing the IL-6 or CNTF cytokines, but in contrast to our results, an additional activation of resident microglial cells was observed (Chiang et al., 1999; Fattori et al., 1995; Winter et al., 1995). The lack of marked microglial activation in the intact CNS of mev/mev mice indicates that the GFAP induction in mev/mev mice is unlikely to be the result of elevated serum levels of cytokines such as IL-6, tumor necrosis factor, and IFN-γ (Khaled et al., 1998), which affects both astroglial and microglial cell populations (for review, see Raivich et al., 1999). Thus, the astrocytic localization of SHP1 in the intact CNS in wild-type animals and the increase in GFAP immunoreactivity in postnatal and adult mev/mev mice suggest that SHP1 is critical for the normal development of astrocytes as well as for initiation and maintenance of the reactive astrocytic phenotype.

Similar to the mev/mev mice, reactive astrocytes without activation of microglia occur in the intact CNS of transgenic mice overexpressing TGFα (Rabchevsky et al., 1998), a growth factor that mediates its biological actions through EGF receptor (EGF-R) (for review, see Lee et al., 1995). Additionally, astrocytic localization of EGF-R in the same CNS regions as SHP1 (Nieto-Sampedro et al., 1988) as well as the appearance of EGF-R in reactive astrocytes (Ferrer et al., 1996) suggested that SHP1 might be involved in the EGF-R signaling cascade. This hypothesis was supported by our in vitro results, which demon-
strated that in astrocytes after EGF stimulation, SHP1 was tyrosine-phosphorylated and associated with EGF-R. The interaction of SHP1 with the EGF-R transduction pathway has been already shown in human cervical carcinoma HeLa cells and embryonic kidney 293 cells (Su et al., 1996; You and Zhao, 1997). In astrocytes, the association with EGF-R occurred within the same period during which tyrosine phosphorylation of EGF-R was detected, indicating that SHP1 does not participate in dephosphorylation of EGF-R. This suggests that in astrocytes, SHP1 might act as a mediator to other intracellular signaling molecules in the EGF-R signaling cascade.

**A role for SHP1 in the glial cells after injury of the nervous system**

To investigate the expression and regulation of SHP1 after brain injury, we used two different models: transection of the peripheral nerves and direct cortical lesion. Both injury models have different functional outcomes in terms of restitution and neuronal survival. The peripheral motor nerve lesion causes a remote challenge to the motoneuron, which is accompanied by a controlled gial reaction. In contrast to most central neurons, motoneurons survive and regenerate their axons after peripheral nerve axotomy. The neural tissue remains intact, allowing the observation of the astrocytic reaction without scar formation (Streit et al., 1988). Furthermore, the blood–brain barrier is not disturbed, and therefore the microglial reaction can be studied in the complete absence of infiltrating hematogenous cells (for review, see Kreutzberg, 1996).

In both lesion models in wild-type animals, we found strong upregulation of SHP1 in microglia and moderate SHP1 upregulation in astrocytes on the injured side during the first week after injury. This time period coincides with the microglial proliferation, characterized by a four- to sixfold increase in microglial cell number (Graeber et al., 1988). In addition, in SHP1 mutant me<sup>−/−</sup>me<sup>−</sup> mice, a strong and selective inhibition of microglial proliferation after injury was observed, whereas the posttraumatic astrocytic reaction was similar to the wild-type littermates. This points to the question of whether SHP1 might be necessary for a stimulatory effect on microglial proliferation after brain injury. Similar to our data in the me<sup>−/−</sup>me<sup>−</sup> mice, an inhibition of microglial proliferation after facial nerve axotomy was observed in mice with a genetic deficiency in microglial mitogen CSF-1 (Raivich et al., 1994). These results, together with the previous data demonstrating that CSF-1-R is upregulated on microglia after brain injury (Raivich et al., 1998), suggested that in microglia SHP1 might be a part of the CSF-1-R signaling pathways. In our in vitro experiments we showed that SHP1 was constitutively tyrosine-phosphorylated and associated with CSF-1-R in microglia. After CSF-1 stimulation there was an increase in tyrosine phosphorylation of SHP1 and an increase in the amount of coprecipitated CSF-1-R. Tyrosine phosphorylation CSF-1-R was increased during the whole period of stimulation, suggesting that SHP1 does not participate in the dephosphorylation of CSF-1-R. Thus, our in vitro results suggest that in microglia SHP1 might have a role as an adaptor molecule in CSF-1-R signaling cascades.

On the other hand, the reduction of microglial proliferation after brain injury in me<sup>−/−</sup>me<sup>−</sup> mice contradicts the observations that the cells of monocyte/macrophage lineage hyperproliferate in these mutant mice (Imboden and Koreskyy, 1995). Although microglia and monocyte/macrophages show the antigenic and morphological similarities and are assumed to be of the same origin (for review, see Kreutzberg, 1996), our observation suggests that SHP1 might play a stimulatory role in microglial proliferation in response to injury. Such a stimulatory role for SHP1 in the mitogenic responses has already been shown for EGF-activated human embryonic kidney cells (Su et al., 1996). Furthermore, reduced proliferation of embryonic carcinoma cells after treatment with retinoic acid correlates with a reduction of SHP1 expression (Mizuno et al., 1997).

The reduced microglial response after injury in me<sup>−/−</sup>me<sup>−</sup> mice could also be indirectly influenced by changes in the reactive astrocytes, which have been shown to produce several microglial mitogens such as CSF-1, CSF-2, or IL-3 (Frei et al., 1985; Malipiero et al., 1990). These results, together with our observation that SHP1 was upregulated in activated astrocytes in the wild-type animals after injury, suggest that SHP1 might be involved in the synthesis of microglial mitogens. Thus, if the SHP1 activity is reduced, as in me<sup>−/−</sup>me<sup>−</sup> mice, the synthesis and release of microglial mitogens from astrocytes might be disturbed, resulting in an aberrant microglial reaction to brain injury.

In contrast to glial cells, the reduction of SHP1 activity in me<sup>−/−</sup>me<sup>−</sup> mice did not appear to affect a process of facial nerve regeneration. Although it has been thought that glial activation supports nerve regeneration (Streit, 1993), recent studies showed that cytostatic ablation of proliferating microglia with cytosine-arabinoside does not affect the speed of axonal regeneration in the hypoglossal nerve (Svensson and Alskogius, 1993). A similar absence of effect on the regenerating facial nerve is observed in mice with a genetic deficiency in CSF-1 (G. Raivich, personal communication). These results together with our data support the hypothesis that glial activation after nerve injury does not have a major influence on the neuronal regeneration program. On the other hand, it has been suggested that glial response has a rather anti-infectious role protecting the damaged neurons from possible infection (Raivich et al., 1999).

In summary, the present results emphasize that the intracellular signaling molecule SHP1 is an important mediator of different signaling pathways in astrocytes and microglia. In astrocytes, SHP1 might be involved in the regulation and maintenance of astrocytic activation in the intact brain. After injury, SHP1 seems to be involved in a complex of different glial activation processes in addition to its role in the immune reaction at the nerve lesion. The lack of SHP1 suppresses microglial proliferation similar to what can be found in CSF-1 knock-outs. This stresses the idea that SHP1 has a different function in signal transduction depending on the cell type and tissue environment.

**REFERENCES**


