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SHP-1 EXPRESSION IN AVIAN MIXED NEURAL/GLIAL CULTURES

by

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B.A. Chemistry 1995
Eastern Washington University

Presented in partial fulfillment of the requirements for the
Master of Science degree

The University of Montana

1998

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9-4-98

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Jeffrey Duane Sorbel, M.S., 1998 Pharmacology
 SHP-1 EXPRESSION IN AVIAN MIXED NEURAL/GLIAL CULTURES
 Director: Diana I. Lurie, Ph.D.

Diana I. Lurie

Thesis Abstract

One of the major hallmarks of central nervous system injury is astrocyte proliferation. However, not all astrocytes enter the cell cycle following damage, and the mechanism that determines which astrocytes will proliferate and which will remain quiescent has not been elucidated. Protein tyrosine phosphorylation has been shown to play an important role in the regulation of the cell cycle in a number of different systems, and has been implicated in astrocyte proliferation and differentiation. The protein tyrosine phosphatase SHP-1 modulates cellular proliferation in the hematopoietic system and is involved in various growth factor signal transduction cascades. Our laboratory has previously shown an increase in SHP-1 immunoreactivity in a subpopulation of astrocytes following deafferentation of the chick auditory brainstem. These SHP-1-positive cells appear to be those that do not enter the cell cycle following deafferentation. The present study examined whether manipulation of cellular proliferation *in vitro* modifies the expression of SHP-1 immunoreactivity and attempted to identify factors that may contribute to the increase in SHP-1 expression following CNS injury.

Mixed neural/glial cultures were prepared from E14 chick brainstems (8-30 animals/run). SHP-1 immunoreactivity was examined when the cultures were growing, growth arrested, and growing again after the reintroduction of serum into the cultures. In actively proliferating cultures, SHP-1 immunoreactivity is localized to the cytoplasm of a subset of cells, while in growth arrested cultures, SHP-1 immunoreactivity appears to be localized to the nucleus of some cells. Cultures which have been induced to divide again following growth arrest contain numerous that once again exhibit a cytoplasmic distribution of SHP-1.

The protein tyrosine phosphatase (PTPase) inhibitor sodium orthovanadate was then introduced into growing cultures and the rate of proliferation was examined after PTPase activity was inhibited. Sodium orthovanadate was found to cause an increase in proliferation but does not appear to dramatically alter either SHP-1 intensity or distribution in these cultures.

Neuronal debris as well as various growth factors have been shown to be mitogenic for glial cells and are thought to play a role in the glial response to injury. SHP-1 has been shown to interact with several growth factors, including epidermal growth factor (EGF), interleukin-3 (IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF). All three growth factors are mitogenic in our culture system and also resulted in slightly differing patterns of SHP-1 expression. Addition of EGF to growth arrested cultures results in a nuclear distribution of SHP-1, while addition of GM-CSF or IL-3 produces both a cytoplasmic and nuclear distribution of SHP-1 immunoreactivity.

Finally, the role of neuronal debris on SHP-1 expression was examined by selectively killing neurons by excitotoxicity in confluent cultures. While SHP-1 positive cells can be found next to healthy neurons, dead or dying neurons are found in areas devoid of SHP-1 positive cells. This is in contrast to the pattern observed for glial fibrillary acidic protein (GFAP) positive astrocytes, which are often located directly under, or adjacent to, dying neurons.

These results demonstrate that SHP-1 expression can be altered by modulating both cellular proliferation and by manipulating the extracellular environment. Significantly, inhibition of tyrosine phosphatase activity results in increased proliferation, indicating that tyrosine phosphatases such as SHP-1 may play a key role in regulating the glial response to injury.

ACKNOWLEDGMENTS

I must extend my thanks to the Department of Pharmaceutical Sciences, the School of Pharmacy, and the Graduate School at The University of Montana for allowing me the opportunity to better my education. In the last two years here, I have worked with and learned from numerous people who I have relied upon for insight and help with this project.....

.....Dr. Diana Lurie, as my advisor, you allowed me to take a project and make it my own. You gave the encouragement and advise on this project when I needed it the most. You showed me what being a scientist is all about and I thank you for it.

.....Dr. Vernon Grund, for serving on my committee and allowing me to be a part of this department when I didn't think that I had a chance.

.....Dr. Keith Parker, for providing inspiration and a kind word every time I saw you. You had a great influence on my life, career and in this project

.....Dr. Scott Samuels, for serving on my committee and for your insight into this project. Your honest commentary helped make this project a better one.

.....Sarj Patel, Richard "Sparky" Bartlett, and Thomas Ortiz, for helping me when I had questions, problems or just needed someone to go out with; Veronica Karpiak, for introducing me to culture work when I started out in the lab; Hans, Wallace, Liping, Dan, and Brett for their helpful hands when I needed them; Darla and Kasey for the constant help in the lab and their insightful knowledge about my moods.

.....My brother and sister, for giving me the encouragement and advise about the everyday things when I need it.

.....My fiancée Kasey Cochran, thank you for putting up with me on those long weeks and for being there when I need you. I Love You.

.....Lastly, I would like to thank my parents, Duane and Kathy Sorbel. Thanks for all the encouragement and the love that you have given me over the years, I couldn't have made it without it. You allowed me to follow my dreams and you have supported all one of them. Thanks.

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INTRODUCTION

The astrocyte response to central nervous (CNS) injury has been traditionally characterized in terms of morphological changes, including proliferation, process extension, and increased levels of the glial intermediate fibrillary acidic protein (GFAP; Reier and Houle 1988; Reier et al. 1983; Graeber and Kreutzberg 1986; Graeber and Kreutzberg 1988; Tetzlaff et al. 1988; Condorelli et al. 1990; Steward et al. 1990). Astrocytic proliferation following injury results in an astroglial scar and these events are referred to as “reactive gliosis” (Reier and Houle 1988). While it is generally believed that the formation of the neuro/glial scar is an impediment to axonal regeneration (Reier and Houle 1988), many reactive astrocytes appear to engage in activities that appear to promote rather than inhibit neuronal regeneration. Such activities include the phagocytosis of neuronal debris, reconstruction of a functional neural tissue, reconstitution of glial limitans, synthesis of extracellular matrix proteins, and the synthesis of numerous neurotrophic factors (which are thought to enhance neuronal survival and induce neuronal growth and differentiation; Reier and Houle 1988; Vaca and Wendt 1992; Carmen-Krzan et al. 1991; Banker 1980; McCafferty 1984). While the end result is well characterized, neither the signals that regulate astrocyte proliferation following injury, nor the basis on which certain subsets of astrocytes enter the cell cycle are well understood. The process, however, must be well controlled to allow for the formation of a limited glial scar, as massive astrocyte proliferation leading to large glial masses (i.e. tumors) at the site of injury does not occur. It is clear that reactive astrocytes have

developed control mechanisms that limit both the window of time in which astrocytes proliferate following CNS damage, as well as the number of cells that actually progress through the cell cycle. The role that proliferating vs. non-proliferating astrocytes play following CNS damage remains to be elucidated and it may be that those signals that send certain populations of astrocytes into the cell cycle also influence the degree of functional recovery that can occur after damage.

Previous studies in our laboratory have found a subset of astrocytes in the chick auditory brainstem that upregulate the expression of the protein tyrosine phosphatase SHP-1 following CNS injury. It may be that this enzyme plays a role in regulating which astrocytes enter the cell cycle following damage.

Cell Cycle Regulation

The phases of the cell cycle are classified as follows: mitosis (M), first gap (G1), DNA synthesis (S) and second gap (G2). There is also a resting stage (Go) of variable duration from early G1. The phases are defined by cytologic criteria and different protein complexes are activated in order to trigger progression through the cell cycle (Grana and Reddy 1995). The advance through checkpoints (in particular G1-S and G2-M) is regulated by protein complexes composed of a regulatory protein (cyclin) and a catalytic protein (cyclin-dependent kinase; CDK) (Cavalla and Schiffer 1997; Schiffer et al. 1997). These protein complexes form the core

regulatory machinery for the cell cycle and loss of these regulatory mechanisms will result in uncontrolled proliferation.

Protein Tyrosine Phosphorylation

The phosphorylation state of proteins and enzymes is critical for many cellular functions including cell division. The phosphorylation of tyrosine residues has been shown to regulate both cellular proliferation as well as differentiation (Bishop 1991; Cantley et al. 1991; Hunter 1991). This phosphorylation is regulated by both protein tyrosine kinases (PTKs), which phosphorylate tyrosine residues, and protein tyrosine phosphatases (PTPs), which dephosphorylate tyrosine residues. However, it is clear that the balance between the activities of PTKs and PTPs is critical for both cellular proliferation and differentiation.

Protein tyrosine phosphatases (PTPs) have been shown to both decrease as well as indirectly increase tyrosine phosphorylation. The PTPs are obviously able to dephosphorylate proteins, including kinases, which in some circumstances leads to an increase in kinase activity with a resulting increase in phosphorylation. While there is evidence that PTPs can reverse the effect of PTKs, there is also increasing evidence that PTPs are involved in a synergistic effect with PTKs (Walton and Dixon 1993; Saito and Streuli, 1991; Fischer et al. 1991; Brautigan 1992).

Of particular interest is the fact that protein tyrosine phosphorylation has also been implicated in both astrocyte proliferation and differentiation. The differentiation of O2A progenitor cells into type 2

astrocytes results in a decrease in several tyrosine phosphorylated proteins (Ingraham and Maness 1990). In addition, the phorbol ester phorbol 12-myristate-13-acetate (PMA), which has been shown to induce synthesis of the protein tyrosine phosphatase SHP-1 (Zhao et al. 1994), also induces changes in astrocyte morphology (Harrison and Mobley 1991). Growth factors that activate protein tyrosine kinase receptors, including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, interleukin-1, and insulin-like growth factor, are mitogenic for astrocytes *in vitro* (Huff and Schrier 1990; Malhotra et al. 1990; Langan et al. 1994).

Finally, modulation of tyrosine phosphorylation mediated by PTPs, participates in both cell cycle regulation and differentiation. Whether these enzymes activate or inhibit cell division appears to be dependent upon the individual phosphatase and the cellular environment in which it is expressed (Lau and Baylink 1993; Neel 1993).

Protein Tyrosine Phosphatase SHP-1

SHP-1 (also known as PTP1C, SHPTP-1, SHP, and HCP) is a cytoplasmic tyrosine phosphatase. It contains 2 *src*-homology 2 (SH2) domains, an uninterrupted PTP region, and a C-terminal extension that has several potential phosphorylation sites. The SH2 domain is a conserved region of about 100 amino acids and was originally identified in multiple members of the *src* subfamily of non-transmembrane PTKs. The SH2 domains fall within a non-catalytic region of the cytoplasmic protein tyrosine kinases and are thought to be involved the regulation of the

kinase's activity (Sadowski et al. 1986). The structure of the SH2 domain has been identified in other cytoplasmic signaling proteins (Feng and Pawson 1994) and it is this structure that promotes protein-protein interaction by binding to specific phosphorylated tyrosine residues (Koch et al. 1991; for review see Schlessinger and Ullrich 1992). The SH2 domains of PTPs exert an inhibitory influence on the activity of these enzymes when not engaged. When the SH2 domains are engaged by protein substrates, the enzyme becomes activated (Neel 1993). Recent studies have also shown that recombinant SHP-1 lacking its SH2 domains is enzymatically activated against phosphotyrosyl peptides (Pei et al. 1993; Townley et al. 1993).

The C-terminal has also been shown to be important in the activation of the phosphatase. The C-terminus of the phosphatase serves as an auto-inhibitor of this enzyme. Limited trypsinolysis of SHP-1, which results in the removal of the C-terminal 41 amino acids, leads to substantial activation (Zhao et al. 1993a; 1993b). The C-terminal domain of SHP-1 may serve both to fold the enzyme into proper conformation to bind protein substrates and also inhibit catalysis. Finally, it has been shown that when SHP-1 is activated, it translocates to a lipid membrane (Zhao et al. 1994). The specific cellular membrane has yet to be identified.

SHP-1 has been most thoroughly characterized in hematopoietic cells, where it is expressed strongly (Plutzky et al. 1992; Yi et al. 1993; David et al. 1995; Krautwald et al. 1996). However, SHP-1 has also been found in a variety of cells including: human epithelial carcinoma cells (Tomic et al. 1995), human cervical carcinoma cells (You and Zhao 1997),

human embryonic kidney cells (Bouchard et al. 1994), rat liver cells (Ram and Waxman 1997), rat pancreatic tumor cells (Cambillau et al. 1995) and mouse astrocytes (Massa and Wu 1996).

In general, SHP-1 phosphatase activity is inversely related to growth and proliferation of cells. Recently, several groups have studied SHP-1 expression in different systems. The consensus has been that SHP-1 is playing a negative role in cellular growth and proliferation. For example somatostatin inhibits cellular proliferation and stimulates SHP-1 activity in Chinese hamster ovary cells (Lopez et al. 1996; 1997). The glucocorticoid dexamethasone inhibits proliferation of rat pancreatic tumor acinar cells and selectively increases SHP-1 expression and activation several-fold (Cambillau et al. 1995). Phorbol esters inhibit proliferative activity in human leukemia cells and stimulate expression and activity of SHP-1 (Uchida et al. 1993; Zhao et al. 1994). In a related observation, the interleukin-3-induced growth stimulation of hematopoietic cells is suppressed by the induction of SHP-1 expression (Yi et al. 1993). However, a negative role for SHP-1 in cellular proliferation is not universal. Overexpression of SHP-1 in epidermal growth-factor-activated human embryonic kidney cells suggests a positive role for the phosphatase in proliferative activity (Su et al. 1996). It has become clear that the role of SHP-1 in growth, proliferation, and differentiation of cells is apparently dependent on context: including cell type, signaling molecules, and perhaps other factors.

The signal transduction pathway of SHP-1 has yet to be fully elucidated. However, SHP-1 has been shown to play a role in growth

factor mediated signal transduction in a variety of different cells. For example, SHP-1 binds to the interleukin 3 (IL-3) receptor following ligand binding and can dephosphorylate the B chain of the receptor *in vitro* (Yi and Ihle 1993). SHP-1 also associates with activated EGF receptors (You and Zhao 1997; Tenev et al. 1997) and may also dephosphorylate novel substrates critical for GM-CSF signaling in mouse macrophages (Yang et al. 1998). However, the *in vivo* target substrates and resulting signal transduction cascades activated by SHP-1 have not been determined for most cells, and probably differ among various cell types.

As mentioned above, the role of SHP-1 *in vivo* remains to be elucidated. The best characterized *in vivo* model is a mouse knockout for SHP-1; the *Motheaten* (*me*) mouse, named after the motheaten appearance of their coats. Classically viewed as a severe combined immunodeficiency disease. Motheaten mice do have profound abnormalities in immune function and survive only a few weeks to a few months (Bignon and Siminovitch 1994; Shultz and Sidman 1987). There is also a profound auto-immunity, marked by dramatic macrophage hyperproliferation.

SHP-1 Expression in Astrocytes of the Deafferented Auditory Brainstem

Our laboratory has previously described a subset of astrocytes in the chick auditory brainstem that express SHP-1 (Lurie et al. 1993). SHP-1 immunoreactivity increases strongly in response to deafferentation, and this is correlated with increased astrocyte proliferation. However, SHP-1

immunoreactivity appears to be upregulated in astrocytes that fail to enter the cell cycle following injury (Lurie et al. 1997; Lurie et al. 1998). Thus, SHP-1 may play a role in regulating the number of astrocytes that proliferate in response to deafferentation, thereby limiting the size of the glial scar. A related observation has been in the rat hippocampus, where SHP-1 immunoreactivity in glial cells increases following either excitotoxic or metabolic-induced injury (Willis et al. 1997). Taken together, these results clearly indicate that SHP-1 is an important component of the CNS response to injury.

We hypothesize that once a signal for proliferation has been initiated (such as deafferentation or other CNS injury) the affected glial cells can take one of two different pathways. One pathway is to divide in response to the signal, the other pathway is to actively prevent cell division in the presence of a signal to divide. SHP-1 may be part of the intracellular cascade that is turned on to prevent some glial cells from proliferating in the presence of an extrinsic signal to divide. When there is no such signal to divide, i.e. in the uninjured brain, there is no need to upregulate SHP-1, and its expression remains low. Only when there is an extrinsic signal to divide does a regulatory mechanism become important, and SHP-1 is upregulated. Obviously, some sort of regulatory mechanism must be activated in order to modulate the glial response to injury. If every astrocyte or microglia entered the cell cycle following damage, they would overrun each other with potentially negative consequences.

Specific Objectives of the Current Project

The overall goal of this project was to determine whether SHP-1 played a role in regulating glial proliferation *in vitro*. Specifically, we asked three questions: 1) Does manipulation of the cell cycle change either the amount and/or distribution of SHP-1?, and 2) Does inhibition of protein tyrosine phosphatase activity alter glial proliferation?, and 3) What extracellular signals lead to changes in both SHP-1 expression and glial proliferation? In order to address these questions, the following series of experiments were performed using mixed neural/glial cultures from the chick brainstem.

1. The cell cycle was manipulated *in vitro* in order to observe changes in SHP-1 expression.
2. The tyrosine phosphatase inhibitor sodium orthovanadate was added to cultures and changes in both SHP-1 expression and proliferation were monitored.
3. The growth factors EGF, IL-3 and GM-CSF were added to cultures in order to determine whether these factors played a role in upregulating SHP-1 expression following injury *in vivo*.
4. Neurons in mixed cultures were killed using the excitotoxin NMDA in order to determine whether neuronal death plays a role in upregulating SHP-1 expression following injury *in vivo*.

MATERIALS AND METHODS

Subjects

Embryonic chicks (white leghorn; Truslow Farms Chestertown, MD) were used for this investigation. Fertilized eggs were incubated at 37-38°C in a humidified environment in the University of Montana AAALAC-approved animal care facilities. Brainstems were removed from stage 41 (embryonic day E-14) animals using the Hamburger and Hamilton's (1951) staging scheme. Eight to thirty embryos were used for each experiment. All procedures were approved by the University of Montana Institutional Animal Care and Use Committee.

In vitro preparation

Embryonic day-14 chicks were sacrificed by decapitation. The brainstems were dissected from the embryos and freed of the meninges (performed in calcium/magnesium free buffer: CMF). The tissue was then transferred to a 35 ml petri dish, minced with a sterile razor blade in Earle's Balanced Salt Solution (EBSS), and then placed in a vial containing 20 units papain/500 units DNase in 0.5 mM EDTA (Worthington Biochemical Corp.) The vial containing the cells was placed in a 37°C water bath for 20 minutes with sterile 95% O₂/5% CO₂ bubbled through the solution. Cells were then gently triturated with a flame constricted glass pipette for 5 minutes. The suspension was next placed in a 15 ml centrifuge tube and centrifuged at 300 x g for five minutes at room temperature. The

supernatant was then discarded and 2.7 mls EBSS/333 units DNase and 1.665 units ovomucoid protease inhibitor was added to the cells. The solution was next centrifuged at 70 x g for six minutes (Worthington Biochemical Corp.; Papain Dissociation System). The pellet was resuspended in 4 mls of EMEM (minimum essential medium with EBSS; GibcoBRL) containing 10% horse serum (HS; GibcoBRL), 10% fetal bovine serum (FBS) (Hyclone), 1% penicillin-streptomycin (GibcoBRL), 2 mM L-glutamine and 1% Fungizone (GibcoBRL). Cells were counted using a hemacytometer (Fisher Scientific), and then plated in 12 well plates with poly-L-lysine coated coverslips at a seeding density of 5×10^5 cells/well. After plating, the cells were placed in an incubator at 37°C under an atmosphere of 5% CO₂ in air.

Culture Experiments

Manipulation of the cell cycle

Once the cultures were placed in the incubators they were allowed to grow for 6 days. At day 6, one third of the plates were pulsed for 6 hours with the thymidine analog, BrdU (1µg/ml; Sigma), to label dividing cells. After 6 hours, the cultures were fixed in ice-cold methanol for 2 minutes or 4% paraformaldehyde for 15 minutes, rinsed in 0.1M Tris buffer, pH 7.4 and then processed for BrdU, SHP-1, GFAP, OX42, CNP and Neurofilament immunohistochemistry. The remaining plates were then gradually serum depleted over the next 3 days in order to growth arrest the cultures. This was accomplished by gradually reducing the concentrations of fetal bovine serum and horse serum from 10% (days 1-6)

to 5% (day 6) to 3% (day 7), and finally 1% (day 8). On the 9th day the medium was changed to a Neuralbasal medium with B-27 supplement (GibcoBRL). This medium contains no serum or proliferative growth factors but has been shown to sustain cells *in vitro* (Brewer et al., 1993; 1995). The cultures were then left in the Neuralbasal medium for 6 days (with a media change at day 3). At day 15, half of the cultures were pulsed with BrdU for 6 hours, fixed in ice-cold methanol and processed for the antibodies stated above. Serum was added to the remaining cultures at day 15 (EMEM 10% FBS/10% HS) and the cells were allowed to grow for an additional 3 days. At day 18 the remaining plates were pulsed with BrdU for 6 hours, fixed, and processed for immunocytochemistry.

Addition of the phosphatase inhibitor Sodium Orthovanadate

Cultures were allowed to grow for 7 days in EMEM 10% FBS/10% HS (at day 6 the media was changed). On day 7, sodium orthovanadate was added to the cultures at concentrations of 10, 20, 30, 40 and 50 μ M. Twenty-four hours after the addition of sodium orthovanadate, the cultures were pulsed with BrdU for 6 hours. The cells were then fixed and processed for BrdU and SHP-1 immunohistochemistry.

Addition of growth factors

For some experiments, growth factors instead of serum were added back to the cultures at day 15. These growth factors included EGF, IL-3 and GM-CSF (Becton Dickinson; see results for specific concentrations). The cultures were then pulsed with BrdU at day 16 (24 hours after

addition of growth factors or serum) and day 18 (72 hours after addition of growth factors or serum). After each pulse the cells were fixed with either ice-cold methanol or 4% paraformaldehyde for 15 minutes and then processed for BrdU and SHP-1 immunohistochemistry.

Addition of NMDA

Cultures were allowed to grow in EMEM 10% FBS/10% HS for 11 days. The media was changed on days 6, 9 and 11. N-methyl-D-aspartate (NMDA; Tocris) was introduced on day 11 at concentrations of 25, 50, 75 and 100 μ M. Twenty-four and 48 hours after the addition of NMDA, the cultures were pulsed for 6 hours with BrdU. The cells were then fixed and processed for BrdU, SHP-1, GFAP, and Neurofilament immunohistochemistry.

Immunohistochemical procedures

Cultures were first rinsed twice for 5 minutes with 0.1 M Tris-HCl buffer, pH 7.4. Cells which were labeled for CNP and OX42 were fixed in 4% paraformaldehyde for 15 minutes, all others were fixed in ice-cold methanol for 2 minutes. Cells were next rinsed in 0.1 M Tris-HCl buffer, pH 7.4 and Tris-HCl buffer, pH 7.4 with 1% bovine serum albumin (BSA; Sigma). Two additional steps were performed when labeling for BrdU; a 5 minute rinse in 0.01M HCl followed by an additional Tris-HCl, pH 7.4 rinse. The cells were then incubated for 20 minutes in 4% normal horse serum for monoclonal antibodies and 4% normal goat serum for polyclonal antibodies. All immunohistochemical reagents were prepared in 1%

BSA/1% sodium azide in Tris-HCl buffer, pH 7.4 [except for the avidin biotin complex (ABC) reagent]. The cultures were incubated in the primary antibody overnight; anti-BrdU (1:2000; Becton Dickinson); 205 (1:2000; a generous gift from Dr. Shen, Montreal Canada); anti-GFAP (1:1500; DAKO); anti-Neurofilament (1:800; courtesy of Dr. Virginia Lee, Philadelphia, PA.); anti-CNP (1:100; Promega); anti-OX42 (1:500; Serotec). The next day, the cells were washed in Tris-HCl buffers, pH 7.4, incubated in either biotinylated horse anti-mouse or biotinylated goat anti-rabbit (monoclonal and polyclonal, respectively) at a concentration of 1:400. The cells were then washed in Tris buffers, incubated in ABC (1:6 dilution; Vectastain Elite ABC kit; Vector Labs) for 1 hour. The cells were rinsed for 10 minutes in Tris and developed with diaminobenzidine as the chromagen (0.5 mg/ml; Sigma) with 0.1% H₂O₂ and 1 mM imidazole in Tris-HCl buffer, pH 7.4. For BrdU the diaminobenzidine was intensified with 0.08% nickel chloride to create a black rather than a brown reaction product. The coverslips were then dehydrated and coverslipped using DPX (BDH Laboratories).

Double-label immunohistochemistry

Cultures were rinsed twice for 5 minutes with 0.1M Tris-HCl, pH 7.4 and then fixed. Cells were then rinsed three times for 5 minutes with phosphate buffer saline (PBS), pH 7.4. Cells were blocked with either 4% normal horse serum (NHS) or 4% normal goat serum for 20 minutes. This and all other immunocytochemical reagents were prepared in 1% BSA in PBS. The cells were next incubated in monoclonal antibodies (CNP-1:5

OX42-1:10 and Neurofilament-1:10) or polyclonal antibodies (SHP-1-1:400, GFAP-1:10) overnight at room temperature. The next day the cells were rinsed three times for 5 minutes each with PBS, then incubated for 1 hour in 1:200 biotinylated goat anti-rabbit linked to FITC or Cy3 or 1:200 biotinylated horse anti-mouse linked to FITC or Cy3 (Vector Labs). Cells were then rinsed three times for 15 minutes in PBS, rinsed in ddH₂O and coverslipped with vectashield mounting media (Vector Labs).

Covalent Coupling of Fluorescein to Antibody

The polyclonal antibody glial fibrillary acidic protein (GFAP) was directly linked to fluorescein to allow for double labeling with two polyclonal antibodies. This protocol is modified from Schreiber and Haimovich (1983). Briefly, in the absence of light fluorescein isothiocyanate (FITC) was dissolved in absolute ethanol, at a concentration of 5 mg/ml. 250 μ l 1M sodium carbonate buffer (pH 9.0) and the appropriate amount of FITC (25 μ g FITC/mg IgG) was added to the GFAP antibody solution (Dako), vortexed and allowed to come to ambient temperature. The FITC/antibody solution was passed over a G-50 Sephadex column. The FITC/antibody solution which passed through the column was collected and the concentration of fluorescein-conjugated IgG (mg/ml) and molar ratio of dye/protein was determined by absorbance at 280 nm and 493 nm.

Immunohistochemical analysis

Quantification of BrdU labeled cells

Cultures were viewed with a Nikon Eclipse E800 microscope at magnifications ranging from 4-60X. BrdU positive cells within the cultures were counted using NIH Image Analysis Program 1.61.

With a random start, nine random fields of 1.78 mm² were analyzed for each well. Slides were viewed with a microscope that was attached to a COHU high performance CCD video camera connected to a Power Macintosh 8600/200 computer. NIH Image 1.61 (image analysis software) allowed the analysis of the digitized images. Once the image was digitally captured the NIH Image 1.61 program macros were used to count the number of BrdU positive cells. The total number of labeled cells were calculated and averaged over the controls. Significant differences were determined by a one-way ANOVA.

Quantification of phosphatase immunoreactivity

The density of the phosphatase immunoreactivity within the cultures was determined by using NIH Image Analysis Program 1.61. As stated above, nine random fields were selected and the image captured on the viewing screen. Once captured a density threshold was set for each culture. The mean density was measured and averaged over control density. Significant differences were determined by a one-way ANOVA.

RESULTS

Neural/Glial Culture

Mixed neural/glial cultures were prepared from E14 chick brainstems. Cultures contained both neural and glial cells as demonstrated by immunocytochemical labeling for various cell types. All cultures contained a small number of neurons that were immunopositive for neurofilament (Fig. 1A). Neurons were observed to be present at all times, even through day 18 in culture. The neurons appeared to be viable and healthy with intact cell bodies and many long processes. In some cases, neurons appeared to be contacting each other (Fig. 1A). In addition, the cultures contained astrocytes, oligodendrocytes, and microglia as demonstrated by immunostaining for GFAP, CNP, and OX42 respectively (Fig. 1B, C, D). Finally, a subset of cells were immunopositive for SHP-1 *in vitro* (Fig. 1E). Staining was largely confined to the cytoplasm and the initial segment of some glial processes. Thus, the *in vitro* system contains neurons, all glial sub-types, and SHP-1+ cells.

Identification of SHP-1 positive cells

A subset of all three glial cell types appear to express SHP-1 *in vitro*. Control cultures (day 12) were double-labeled for SHP-1 and either Neurofilament, GFAP, OX42, and CNP. A few astrocytes, microglia, and oligodendrocytes were found to be SHP-1+ (Fig. 2A, B, C). Neurons did not appear to express SHP-1 (Fig. 2D). The presence of SHP-1 in all three

Fig. 1. Mixed neural glial cultures contain neurons, astrocytes, oligodendrocytes, microglia, and SHP-1+ cells (Day-18 in culture). A) Neurons immunostained for neurofilament (RM0 270). Two large healthy neurons (large arrows) with intertwined processes (small arrow). B) The cultures contain many GFAP+ astrocytes (arrows). Bar=50 μ m (A and B). C) CNP+ oligodendrocytes are found throughout the culture (arrows). D) OX42+ microglia are present in the culture (arrows). E) Many SHP-1+ cells are found in day 6 growing cultures. Note the cytoplasmic localization of the immunoreactivity (arrows). Bar=30 μ m (C, D, and E)

Fig. 2. SHP-1 positive cells (red) at day 13 in culture. A) Few differentiated GFAP+ astrocytes (green) are also SHP-1+ (arrows). B) Many less differentiated GFAP+ (green) cells are SHP-1+ (arrows). C) Several CNP+ oligodendrocytes (green) are SHP-1+ (arrow). D) Several OX42+ microglia (green) are SHP-1+ (arrows). Bar=20 μ m E) No neurons (green; arrow) appear to express SHP-1. Bar=80 μ m

glial cell types differs from previous findings *in vivo*, where only astrocytes appear to become immunopositive for SHP-1 following deafferentation. In the avian brainstem, astrocytes appear to be the only type of glial cell that proliferates following deafferentation (Lurie and Rubel 1996; Lurie et al. 1998). In this system, the onset of astrocyte proliferation is correlated with significant increases in SHP-1 immunoreactivity. However SHP-1+ cells do not appear to enter the cell cycle. Therefore, it has been hypothesized that the expression of SHP-1 plays a role in regulating the mitotic behavior of these astrocytes (Lurie et al. 1998). Presumably all types of glial cells proliferate in culture, and thus it is not unexpected that SHP-1 is expressed by a sub-population of all three glial types.

Cellular proliferation.

In order to determine whether SHP-1 is playing a role in glial proliferation, the expression of SHP-1 was examined when cultures were either growing or growth arrested. To establish the amount of glial proliferation during the various growth conditions, 6 hour pulses of the thymidine analogue 5'-Bromodeoxyuridine (BrdU) was introduced into the cultures in order to label dividing cells. Visual inspection of BrdU-labeled cells at three times points, growing (Day-6), growth arrested (Day-15) and serum addition/growing (Day-18), revealed that manipulation of glial proliferation could be accomplished by serum deprivation (Fig. 3). When cultures were growing towards confluence, there were many BrdU positive cells (Day-6, growing; Fig. 3A). The number of proliferating cells decreased after serum deprivation (Day-15, growth arrested; Fig. 3B) and

Fig. 3. Brdu-positive cells during manipulation of the cell cycle. A) Note the many BrdU+ cells at day 6 in culture (growing) (arrows). B) The number of BrdU+ cells dramatically decreases after 6 days of serum deprivation (Day-15 in culture; growth arrested) (arrows). C) Reintroduction of serum for 3 days (Day-18 in culture) results in increased numbers of BrdU+ cells (arrows). Bar=50 μ m

Figure 4: Quantification of proliferation of cultures during different growing conditions as described in methods. The Y-axis represents the average number of BrdU-labeled cells in nine random fields with each culture. Serum depletion results in a significant decrease in proliferation. Re-introduction of serum results in a significant increase in proliferation when compared to growth arrested cultures. Significant differences among growing, growth arrested and serum addition cultures were determined using a one-way ANOVA, * $p \leq 0.0001$.

Fig. 5 SHP-1 immunoreactivity of cultures during manipulation of the cell cycle. A) Growing cultures (Day-6) contain numerous SHP-1+ cells. Note the cytoplasmic staining (arrows). B) Growth arrested cultures (Day-15) contain areas devoid of any immunoreactivity. C) Some areas of the growth arrested cultures contain clusters (approximately 30%) of SHP-1+ cells with a nuclear distribution of immunoreactivity (arrows). D) Re-introduction of serum results in growing cultures (Day-18) with scattered SHP-1+ cells. The immunoreactivity is once again localized to the cytoplasm (arrows). Bar=30 μ m

then increased again after serum was re-introduced into the cultures for three days (Day-18, serum addition; Fig. 3C). The number of BrdU-labeled cells was then quantified using image analysis techniques. Serum deprivation reduced the number of proliferating cells by 2.5 fold (Fig. 4); however, we were unable to completely eliminate all cellular division by serum depletion. The addition of serum back into the cultures increased proliferation to within 73% of the original growing cultures (Fig. 4).

SHP-1 expression was then correlated to the different growth conditions. At the earliest time point (Day-6), SHP-1 immunoreactivity was distributed within the cytoplasm and initial segments of the processes in some glial cells (Fig. 5A). However, when the cultures were growth arrested (Day-15), there was a distinct change in the pattern of SHP-1 immunoreactivity which fell into one of two distributions. Some areas of the culture contained very little immunostaining for SHP-1 (Fig. 5B) while in other areas the immunoreactivity for SHP-1 was localized to the nucleus of clusters of cells (Fig. 5C). When cultures were induced to divide again by re-introduction of serum, SHP-1 immunoreactivity once again appeared to have a cytoplasmic distribution (Fig. 5D).

Addition of Sodium Orthovanadate

If SHP-1 plays a role in negatively regulating the glial cell cycle *in vitro*, then inhibiting this phosphatase should result in increased glial proliferation. In order to test this hypothesis, the nonselective tyrosine phosphatase inhibitor sodium orthovanadate was added to growing cultures at day 7 for 24 hr. Some cultures were then pulsed with BrdU for

the last 6 hours of the 24 hr vanadate treatment and other cultures were immunostained for SHP-1. Vanadate was found to be toxic to the cultures at concentrations higher than 100 μ M, and caused the cells to lift off of the coverslips and the cultures to become patchy (data not shown). However, at concentrations below 100 μ M the cells appeared healthy. Adding vanadate to growing mixed neural/glial cultures resulted in a dose dependent increase in cellular proliferation. Increases in cell division were observed with the addition of as little as 10 μ M of vanadate to the cultures and significant increases were observed with 20 μ M of vanadate (Fig. 6A, C, E and Fig. 7). The highest dose of vanadate which did not produce cell toxicity (50 μ M) resulted in an almost 3-fold increase in cellular proliferation over controls (Fig. 7).

Interestingly, the expression of SHP-1 did not change significantly with the addition of vanadate. While concentrations of 30 and 50 μ M of sodium orthovanadate appeared upon visual inspection to result in slightly darker SHP-1 immunolabeling, quantification of the density of staining throughout the cultures did not reveal significant increases over control (Fig. 6B, D, F and Fig. 8).

Fig. 6 Effects of sodium orthovanadate on proliferation and SHP-1 immunoreactivity. A and B- control cultures. C and D- 24 hr. of 30 μ M vanadate. E and F- 24 hr. of 50 μ M vanadate. (A, C, and E) Cultures are labeled for BrdU-immunohistochemistry. Note the increase in the number of BrdU+ cells with increasing concentrations of vanadate. Bar=50 μ m. (B, D, and F) Immunostaining for SHP-1 does not appear to change following the addition of vanadate. A similar number of cells continue to display a cytoplasmic distribution of SHP-1 (arrows). Bar=30 μ m

24 hours after addition of sodium orthovanadate

All concentrations are μM .

Figure 7: The ratio of the number of BrdU+ cells after treatment of sodium orthovanadate over control. The Y-axis represents the ratio of BrdU-labeled cells over the control in nine random fields within each culture. There is a significant increase in proliferation at concentrations of vanadate $> 20 \mu\text{M}$. Significant differences between control and vanadate concentrations were determined using a one-way ANOVA, * $p \leq 0.01$.

All concentrations are in μM .

Figure 8: Quantification of density of immunoreactivity of SHP-1 after the addition of vanadate to the cultures. The Y-axis represents the ratio of density of SHP-1 immunoreactivity over the control cultures. There was no significance difference among the control, 30 μM , and 50 μM vanadate groups, determined using a one-way ANOVA.

Addition of Growth Factors

Growth factors are thought to play a key role in regulating the glial response to injury. We therefore investigated whether a variety of growth factors influenced SHP-1 expression *in vitro*. We chose to examine three growth factors that have been shown to interact with either the SHP-1 protein itself and/or lead to increased glial proliferation in other CNS culture systems.

EGF addition

Epidermal growth factor (EGF) has been demonstrated to be mitogenic for a variety of astrocytes *in vitro* (Langan et al. 1994; Huff and Scheier 1990). Our cultures also responded to EGF with an increase in cellular proliferation. To establish the amount of glial proliferation induced by the addition of EGF, BrdU was introduced into the cultures during the last 6 hours of either 24 or 72 hr. of EGF treatment in order to label dividing cells. Parallel control cultures received serum rather than EGF and were also pulsed with BrdU. The number of BrdU-labeled cells within nine random fields per culture were then counted.

EGF significantly increased proliferation at all concentrations (20-320 ng/ml) when compared to growth arrested cultures at 24 hr. (Fig. 9). In addition, there was a significant increase in proliferation ($\geq 20\%$) over the serum controls for concentrations ≥ 40 ng/ml (Fig. 9).

The expression of SHP-1 after the addition of EGF differs from that of the serum controls. Unlike the cytoplasmic distribution of SHP-1 in serum cultures (Fig. 10B), the distribution of SHP-1 after the addition of

24 hours after addition of EGF

All concentrations are in ng/ml.

Figure 9: The ratio of the number of BrdU+ cells after EGF treatment for 24 hr. over control cultures. The Y-axis represents the ratio of BrdU-labeled cells over controls in nine random fields within each culture. The significance was determined using a one-way ANOVA, $p \leq 0.0001-0.05$. EGF significantly increased proliferation at all concentrations (20-320 ng/ml) when compared to growth arrested cultures. In addition, there was a significant increase in proliferation ($\geq 20\%$) over the serum controls for concentrations ≥ 40 ng/ml.

Fig. 10. SHP-1 immunoreactivity 24 hr. following the addition of growth factors. A) SHP-1 immunostaining in growth arrested cultures (Day-16). SHP-1 immunoreactivity is localized to the nucleus (arrows). B) SHP-1 immunostaining in cultures after the reintroduction of serum (Day-16). Note the cytoplasmic distribution of SHP-1 staining (arrows). C and D- SHP-1 immunostaining after the addition of EGF (Day-16). C) Many cells display a dark area of staining located just below the nucleus (arrows). D) Other cells exhibit a nuclear distribution of immunoreactivity (arrows). E) SHP-1 immunostaining after the addition of GM-CSF (Day-16). Many large, vacuolated cells are darkly stained for SHP-1 (arrows). Some cells have both cytoplasmic and nuclear staining (arrow). F) SHP-1 immunostaining after the addition of IL-3 (Day-16). Note the many large, vacuolated immunopositive cells (arrows) that resemble those seen following GM-CSF treatment. Bar=30 μ m

EGF appears to be largely nuclear (Fig. 10C, D). This is a similar staining pattern to that of the growth arrested cultures (Fig. 10A) with strong immunopositive staining in or around the nucleus. Small, dense areas of immunostaining located just below the nuclear membrane can also be seen in approximately 80% the EGF treated cultures (Fig. 10C).

GM-CSF addition

GM-CSF treatment has been shown to regulate mitogenic signaling in mouse macrophages as well as potentially dephosphorylating novel substrates critical for GM-CSF signaling (Yang et al. 1998), making it a potential candidate for influencing SHP-1 expression following injury. GM-CSF was therefore introduced to growth arrested cultures on day 15 for 24 hours and the cultures were then pulsed with BrdU as described above. GM-CSF increased cellular proliferation by at least two-fold over serum depleted cultures at all concentrations (10-640 ng/ml; Fig. 11). GM-CSF also induced a 36% increase in proliferation over serum addition at concentrations ≥ 40 ng/ml (Fig. 11).

Addition of GM-CSF results in a subset of cells that display dark cytoplasmic as well as nuclear staining (Fig. 10E). This contrasts with the expression pattern seen in growth arrested or serum treated cultures, which contain a nuclear or cytoplasmic distribution, respectively (Fig. 10A, B). In addition, many of the SHP-1+ cells appear to have vacuolated staining in the cytoplasm, which may reflect immunostaining around mitochondrial membranes (Fig. 10E).

24 hours after addition of GM-CSF

All concentrations are in ng/ml.

Figure 11: The ratio of the number of BrdU+ cells after GM-CSF treatment for 24 hr. over control cultures. The Y-axis represents the ratio of BrdU-labeled cells over controls in nine random fields within each culture. The significance was determined using a one-way ANOVA, $p \leq 0.0001-0.05$. GM-CSF significantly increased proliferation at all concentrations (10-640 ng/ml) when compared to growth arrested cultures. In addition, there was a significant increase in proliferation ($\geq 36\%$) over the serum controls for concentrations ≥ 40 ng/ml.

IL-3 addition

SHP-1 has been found to bind to, and dephosphorylate interleukin-3 (IL-3) in myeloid cells. However, its effects on avian neural and glial cells are not known. Therefore, IL-3 was the third type of growth factor introduced into growth arrested cultures. Addition of IL-3 resulted in a 1.8-fold increase in proliferation at all concentrations (2-44 ng/ml) over growth arrested cultures. Interestingly, unlike EGF and GM-CSF, IL-3 did not cause a significant increase in proliferation over serum addition (Fig. 12).

The pattern of SHP-1 immunoreactivity following the addition of IL-3 is similar to that induced by GM-CSF. Some cells contained very darkly labeled cytoplasm and nucleus and some areas of the cultures contained cells with nuclear staining only (Fig. 10F). In addition, the vacuolated pattern of immunostaining seen with GM-CSF as also seen with IL-3 (Fig. 10F).

Finally, no additional increase in cellular proliferation was observed when growth factors were added to cultures for 72 rather 24 hours (data not shown). In fact, BrdU incorporation decreased slightly at 72 hours, presumably because the cultures were confluent by this time. Likewise, cultures which had been treated for 24 and 72 hours did not differ in SHP-1 expression or distribution.

24 hours after addition of IL-3

All concentrations are in ng/ml.

Figure 12: The ratio of the number of BrdU+ cells after IL-3 treatment for 24 hr. over control cultures. The Y-axis represents the ratio of BrdU-labeled cells over controls in nine random fields within each culture. The significance was determined using a one-way ANOVA, $p \leq 0.0001$. IL-3 significantly increased proliferation at all concentrations (2-44 ng/ml) when compared to growth arrested cultures.

NMDA

Injury to the CNS *in vivo* often results in degeneration of neural cell bodies and their processes. It may be that neuronal debris plays a role in signaling the upregulation of SHP-1 expression in the brain following damage. In order to investigate this possibility, NMDA was added to confluent cultures presumably to excitotoxically kill neurons but leave glial cells intact. Both control cultures and NMDA-treated cultures were then examined for the distribution of SHP-1+ and GFAP+ glia within the entire culture as well as relative to intact and damaged neurons.

In control cultures, SHP-1+ and GFAP+ cells were found distributed throughout the culture as well as underneath or directly adjacent to the neurons (Figs. 13A, 14A). The addition of NMDA to cells at day 11 in culture resulted in neuronal degeneration at concentrations of 75-100 μ M after 48hrs. At these toxic concentrations, SHP-1+ cells were now found to be located some distance away from dying neurons and neuronal debris (Fig. 13B). While clusters of SHP-1+ cells could be observed in areas of the culture that did not contain neurons (data not shown), cells underneath and/or directly adjacent to dying neurons and neuronal fragments were SHP-1 negative (Fig. 13B). In contrast, GFAP immunoreactivity increased both beneath and surrounding dying neurons and neuronal debris (Fig. 14B). In addition, there appears to be an overall increase in GFAP+ astrocytes throughout the cultures 48hrs following the addition of toxic concentrations of NMDA.

It is important to note that this increase in GFAP immunoreactivity does not appear to be correlated with an increase in glial proliferation. The

addition of NMDA (25-100 μ M) did not result in a significant increase in proliferation at either 24 (Fig. 15) or 48 hours (Fig. 16).

Fig. 13 Mixed neural/glial culture immunostained for neurofilament and SHP-1. A) Control culture (Day-13). The SHP-1+ cells (arrows) surround the neuron and its processes (shown in green). B) Following 48 hr. of 100 μ M NMDA, there is a decrease in SHP-1+ cells (arrows) around the dying neuron. Bar=80 μ m

Fig. 14. Mixed neural/glial culture immunostained for neurofilament and GFAP. A) Control culture (Day-13). The GFAP+ cells (arrows) surround the neuron and its processes (shown in green). B) Following 48 hr. of 100 μ M NMDA, there is a dramatic increase in GFAP+ cells (arrows) around and beneath the dying neuron. Bar=80 μ m

24 hours after addition of NMDA

All concentration are in μM .

Figure 15: The ratio of the number of BrdU+ cells after NMDA treatment for 24 hr. over control cultures. The Y-axis represents the ratio of BrdU-labeled cells over controls in nine random fields within each culture. NMDA did not significantly increase proliferation at any concentrations (25-100 μM).

48 hours after addition of NMDA

All concentration are in μM .

Figure 16: The ratio of the number of BrdU+ cells after NMDA treatment for 48 hr. over control cultures. The Y-axis represents the ratio of BrdU-labeled cells over controls in nine random fields within each culture. NMDA did not significantly increase proliferation at any concentrations (25-100 μM).

DISCUSSION

A subset of astrocytes, oligodendrocytes, and microglia within mixed neural/glial cultures of the chick auditory brainstem are immunopositive for SHP-1. Neurons do not appear to express SHP-1. This distribution differs from previous *in vivo* findings where deafferentation of the avian auditory brainstem results in an increase in SHP-1 immunoreactivity within astrocytes only (Lurie et al. 1998). In this injury model, the onset of increased SHP-1 immunoreactivity is correlated temporally with an increase in astrocyte proliferation, and astrocytes are the only class of glia that proliferate following deafferentation in the chicken auditory brainstem. SHP-1+ astrocytes do not appear to be those which enter the cell cycle and it has been hypothesized that the expression of SHP-1 plays a role in negatively regulating the mitotic behavior of astrocytes following deafferentation or other injury (Lurie et al. 1998) perhaps in order to prevent a pathological formation of large glial masses at the lesion site. The hypothesis that SHP-1 expression may negatively regulate astrocyte proliferation is in agreement with observations in other systems where SHP-1 activity has been found to be inversely related to growth and proliferation of cells (Lopez et al. 1996; 1997; Cambillau et al. 1995; Uchida et al. 1993; Zhao et al. 1994; Yi et al. 1993).

Our present finding that SHP-1 is present in microglia and oligodendrocytes as well as astrocytes *in vitro*, may reflect the fact that all three glial subtypes would be expected to proliferate in culture. If SHP-1 is expressed in those populations of cells that are dividing in order to regulate the extent of cell division, then it is not unexpected that a subset

of all glial cells are immunopositive for SHP-1 while neurons do not label for the enzyme. However, we have not yet been able to directly show that SHP-1+ cells are not BrdU positive. Studies are currently underway using other cell cycle markers such as cyclin antibodies to confirm that SHP-1+ glial cells do not proliferate. Interestingly, to date, astrocytes have been the only glial cell type that have been described to contain SHP-1. The present study is the first report of SHP-1 expression in microglia and oligodendrocytes (Lurie et al. 1998; Massa and Wu. 1996).

Manipulation of the cell cycle

Manipulating the cell cycle of the mixed neural/glial cultures alters both the number of immunoreactive cells and the distribution of SHP-1 staining. Specifically, growth arrest of the cultures results in large areas that contain no SHP-1 immunoreactivity. However, there are clusters of SHP-1+ cells that are widely scattered throughout the culture. These immunopositive cells show a nuclear rather than a cytoplasmic distribution of SHP-1 staining, indicating that the enzyme has translocated to the nucleus following growth arrest. This observation is in agreement with published reports in other systems, where the redistribution of SHP-1 in response to signaling has been noted. For example, the phorbol ester, phorbol 12-myristate 13-acetate (PMA) has been shown to induce SHP-1 expression as well as produce a translocation of SHP-1 from the cytoplasm to a plasma membrane (Zhao et al. 1994). In addition, growth hormone has been shown to both increase SHP-1 activity and induce a translocation of most of the enzyme from the cytoplasm to the nucleus in rat liver cells

(Ram and Waxman 1997). Finally, localization of SHP-1 may reflect its activity. When the enzyme is localized to the cytosol of HL-60 cells, its activity is at a minimum, but when a lipoprotein such as PMA is added, the enzyme translocates to a lipid membrane and its activity is increased 10^3 to 10^4 fold (Zhao et al. 1994). It may be that the translocation we observe during growth arrest reflects the activity of SHP-1. Our laboratory is in the process of developing a SHP-1-specific activity assay that should yield insight into the enzymatic activity of SHP-1 during localization to the cytoplasm vs. the nucleus.

One factor that should be considered in these studies is the fact that we were unable to completely growth arrest the cultures. Serum deprivation for 6 days was found to significantly growth arrest the cultures, but not completely eliminate all cell division. We attempted to extend the time in the serum free-medium (B-27) but were never able to completely stop all cell division. In addition, when the time in serum-free medium was extended, the cultures became slightly more confluent, thus decreasing the amount of inducible proliferation after serum was re-introduced. We also found that the amount of proliferation after re-introduction of serum was slightly less than the proliferation observed during the initial growth stage. This was presumably due to the fact that the cultures were slightly more confluent following the 6 days of serum deprivation and contact inhibition may have played a role in reducing the amount of glial proliferation. Ara-C was also used in our initial experiments in an attempt to completely stop all cell division. However, concentrations of Ara-C that were not toxic to the cells did not

successfully stop all proliferation, while higher concentrations resulted in cellular degeneration.

It is clear, though, that the expression of SHP-1 immunoreactivity changes during the three different growth conditions: serum (initial growth), serum-deprivation (growth arrested), and reintroduction of serum (growing). The observation that the immunoreactivity of SHP-1 changes when the cultures are actively dividing versus growth arrested, supports the hypothesis that SHP-1 may be involved in those cellular cascades that regulate the cell cycle. Its role is likely to be very complex in light of the fact that SHP-1 immunostaining is not eliminated during growth arrest and areas of the culture contain groups of cells that are SHP-1+. These clusters of SHP-1+ cells do not appear to be correlated with areas of dividing cells, those few remaining BrdU+ cells are widely scattered throughout the growth arrested cultures and are not localized to any particular region. The finding that the SHP-1 enzyme appears to be located in different cellular compartments (i.e. cytoplasmic vs. nuclear) when cells are proliferating versus when they are quiescent is intriguing, and may reflect functional properties of this enzyme.

Sodium orthovanadate

The hypothesis that SHP-1 plays a role in negatively regulating the glial cell cycle was tested by the addition of sodium orthovanadate. Vanadate is a general PTP inhibitor (Swarup et al. 1982) whose mechanisms of actions have not been investigated in detail, but have been shown to inhibit SH2 domain containing tyrosine phosphatases

(Pugazhenthir et al. 1996; Huyer et al. 1996). Vanadate is a phosphate analog and is generally thought to bind as a transition state analog to the phosphoryl transfer enzymes that it inhibits, since it can easily adopt a trigonal-bipyramidal structure (Goldfine et al. 1995). Vanadate has a wide array of effects on biological systems (Shechter 1990). It is an insulin-mimetic and has been shown in human clinical trials to be potentially useful in treating both insulin- and non-insulin-dependent diabetes and it has been suggested that part of vanadate's insulin-mimetic effect may be due to its inhibition of PTPs (Shechter 1990). However, vanadate is a non-specific tyrosine phosphatase inhibitor and will thus inhibit the activity of SHP-1 as well as other tyrosine phosphatases.

The addition of vanadate into growing cultures resulted in an increase in cellular proliferation over serum control. Thus, inhibition of phosphatase activity did increase the number of BrdU labeled cells, indicating a strong correlation between tyrosine phosphatase activity and inhibition of cellular proliferation. Vanadate has also been shown to alter the regulation of IFN-gamma-signaling in mouse astrocytes in similar ways to astrocytes that do not contain SHP-1 (Massa and Wu 1996), implicating a role for SHP-1 in this signaling cascade. However, it is clear that a specific inhibitor for SHP-1 will have to be developed in order to definitively show that SHP-1 is a key player in these systems and is the phosphatase that negatively regulates glial proliferation in the avian brainstem.

The addition of vanadate did not appear to significantly alter SHP-1 expression and/or distribution within the cultures. Quantification of the

amount of immunostaining throughout the entire culture did not reveal any significant differences at any of the concentrations of vanadate. Specifically, vanadate neither increased nor decreased the total amount of SHP-1 expressed within the culture. Visual inspection revealed that perhaps a slight increase in the density of SHP-1 staining within some individual glial cells occurred with the addition of vanadate, but this did not seem to be the case for every SHP-1+ cell. Finally, SHP-1 immunoreactivity remained localized to the cytoplasm following vanadate treatment and did not appear to translocate to the nucleus. Thus, inhibition of tyrosine phosphatase activity does not seem to alter either the amount, or localization, of SHP-1 immunoreactivity within our culture system.

Growth factors

Both the signals that activate SHP-1 *in vivo*, as well as its substrates, are as yet unknown and the signal transduction pathway of SHP-1 has not been fully elucidated. However, SHP-1 is involved in growth factor mediated signal transduction in a variety of different cells. For example, SHP-1 binds to the interleukin 3 (IL-3) receptor following ligand binding (Yi and Ihle 1993) and also associates with, and can dephosphorylate, activated EGF receptors (You and Zhao 1997; Tenev et al. 1997; Tomic et al. 1995). In addition, SHP-1 has been shown to play an important role in downregulating GM-CSF mitogenic signaling in motheaten macrophages (Yang et al. 1997). Our finding that the addition of these three growth factors (EGF, IL-3, and GM-CSF) to our serum depleted cultures resulted in

increased cellular proliferation confirms that these growth factors are mitogenic for avian CNS cells. All three of these factors have been shown to be mitogenic for astrocytes *in vitro* in other systems (Langan et al. 1994; Huff and Scheier 1990; Malhotra et al. 1990) and have also been demonstrated to interact with SHP-1.

Growth factors and their receptors are excellent candidate signaling molecules for SHP-1 and other tyrosine phosphatases due to the fact that growth factor receptors of the tyrosine kinase-type undergo rapid autophosphorylation upon ligand stimulation (Schlessinger and Ullrich 1992; Fantl et al. 1993). This autophosphorylation generates binding sites for SH2 domain containing intracellular proteins. Membrane recruitment of these molecules by binding to the autophosphorylated growth factor receptors is considered an important step in the activation of several intracellular signaling cascades which ultimately lead to cellular responses such as cellular division, differentiation, or locomotion (Schlessinger and Ullrich 1992). Dephosphorylation of the autophosphorylated growth factor receptors by PTPases presents a major mechanism of negative regulation of tyrosine kinase receptor signaling. SHP-1 has been shown to interact with multiple growth factors and their receptors and to modulate their activity (Schlessinger and Ullrich 1992; Feng and Pawson 1994), which could presumably lead to alterations in cell division and differentiation.

EGF

SHP-1 has been shown to associate with and dephosphorylate epidermal growth factor receptors (Tomic et al. 1995; Tenev et al. 1997; You and Zhao. 1997). Inactivation of SHP-1 by a point mutation of the C-terminus SH2-domain slightly reduces its association with the EGF receptor, but a point mutation at the N-terminus SH2-domain leads to a large reduction in association, indicating that the SH2-domain responsible for binding of SHP-1 to the EGF receptor is the N-terminus SH2 domain (Tenev et al. 1997). Thus, the catalytic domains of SHP-1 are thought to provide specificity for the interaction with the EGF receptor although functional interactions of SHP-1 with the EGF receptor requires association that is mediated by both SH2 domains (Tenev et al. 1997).

The addition of EGF for 24 hours to our serum deprived cultures led to at least a two-fold increase in BrdU-incorporation at all concentrations examined. EGF also induced significantly more proliferation than serum at concentrations of 40 ng/ml and higher. This result was not unexpected given that EGF has been shown to be mitogenic for astrocytes in other *in vitro* systems (Langan et al. 1994; Huff and Scheier 1990; Malhotra et al. 1990). In addition to increased proliferation, EGF resulted in a nuclear distribution of SHP-1 in those cells that were immunoreactive. This dark nuclear immunostaining of SHP-1 resembled the nuclear staining of SHP-1 when the cultures were growth arrested. Dark areas of perinuclear immunoreactivity could also be seen following EGF treatment. The relationship between this pattern of SHP-1 immunoreactivity and its

activity following EGF stimulation remains to be elucidated. Intriguingly, SHP-1 is thought to play a positive role in EGF-activated mitogenesis in human embryonic kidney cells (Su et al, 1996). Whether SHP-1 plays a positive or negative role in EGF-activated mitogenesis in avian CNS cultures will require further studies.

GM-CSF

SHP-1 has been shown to be involved in downregulating GM-CSF mitogenic signaling in motheaten macrophages and may also dephosphorylate novel substrates critical for GM-CSF signaling in these macrophages (Yang et al. 1997). GM-CSF has also been shown to be secreted by astrocytes and can stimulate the *in vitro* proliferation of simian astrocytes (Guillemin et al. 1996), as well as induce IL-6 production by mouse microglia (Suzumura et al. 1996). Finally, GM-CSF has also been demonstrated to result in microglial proliferation and can also facilitate the ramification of rat microglia *in vitro* (Fujita et al. 1996; Lee et al. 1994).

The addition of GM-CSF to our cultures caused an increase in proliferation similar to that seen with the addition of EGF, indicating that GM-CSF is mitogenic for avian CNS cells. GM-CSF also induced a 36% increase in proliferation over serum addition at concentrations ≥ 40 ng/ml. However, the immunostaining for SHP-1 differed from that seen with EGF in that GM-CSF induced a cytoplasmic as well as a nuclear distribution. The cytoplasmic staining often appeared vacuolated, which may reflect immunoreactivity around mitochondrial membranes. It is interesting that both EGF and GM-CSF result in a similar increase in proliferation but have

very different patterns of SHP-1 immunoreactivity. It may be that GM-CSF and EGF act on different types of cells within our mixed neural/glia cultures or that the differential staining pattern reflects different functional states of SHP-1.

IL-3

IL-3 has been detected in hippocampal and septal murine neurons (Konishi et al, 1994) as well as rat microglial cells (Gebicke-Haerter et al. 1994; Ganter et al. 1992). Microglia have also been found to express mRNA for IL-3 receptors (Sawada et al. 1993). SHP-1 has been shown to associate with the IL-3 receptor and may also regulate mitogenic signaling by IL-3 (Yang et al. 1998; Yi et al. 1993; Bone et al. 1997). IL-3 is a potent mitogen for microglia both *in vivo* and in culture (Gebicke-Haerter et al. 1994; Ganter et al. 1992) and SHP-1 may play a role in this signaling cascade. SHP-1 has been shown to bind to IL-3 in the cytosol of the cells after it has been internalized and may regulate IL-3 mediated signal transduction pathways (Bone et al. 1997).

The addition of IL-3 to our growth arrested cultures resulted in an increase in proliferation similar to that seen with EGF and GM-CSF. However, no concentration of IL-3 tested produced a significant increase in proliferation over the addition of serum. The pattern of SHP-1 immunoreactivity following the addition of IL-3 was similar to that induced by GM-CSF rather than serum or EGF.

This similarity in SHP-1 immunoreactivity between GM-CSF and IL-3 may reflect similarities in 1) the structure of GM-CSF and IL-3 receptors

and 2) the mediation of mitogenic signal transduction. The receptors for GM-CSF and IL-3 share a common beta chain and both growth factors, as well as EGF use tyrosine phosphorylation to control proliferation (Mire-Sluis et al. 1995). Of particular interest is the finding that GM-CSF and IL-3-induced proliferation is stimulated by a tyrosine phosphatase inhibitor in a human erythroleukemia cell line while IL-5 induced proliferation is inhibited (Mire-Sluis et al. 1995). Both growth factors also induce microglial proliferation. The large, darkly stained and vacuolated SHP-1+ cells that are present in our cultures following GM-CSF and IL-3 treatment resemble the OX42-labeled microglia. In contrast, the SHP-1+ cells that are present following EGF treatment appear to be much smaller cells and more closely resemble astrocytes. It may be that EGF induces SHP-1 expression primarily in astrocytes while GM-CSF and IL-3 induce SHP-1 expression in microglia. Further studies are needed to confirm this hypothesis. It would also be of interest to determine whether EGF results in astrocyte proliferation while GM-CSF and IL-3 results in microglial proliferation.

NMDA

Following deafferentation of the chick auditory brainstem, SHP-1 immunoreactivity increases to a peak three days following cochlea removal (Lurie et al. 1998). At this time, there is a 30% neuronal loss with accompanying axonal degeneration in the primary auditory nucleus. It may be that this neuronal cell death plays a role in the upregulation of SHP-1 immunoreactivity *in vivo*.

Therefore, NMDA was added to the avian brainstem cultures in order to kill neurons and leave the glial cells intact. NMDA is a known selective glutamate receptor agonist and is found on neuronal post synaptic terminals. Application of NMDA results in excitotoxic neuronal death due to excessive activation of NMDA receptors. To date no known NMDA receptors have been found on glial cells and therefore NMDA is thought to induce excitotoxicity in neurons only. Over-activation of NMDA receptors leads to an increase of Ca^{2+} into the cell (Ferreira et al. 1996), which in turn activates several cascades ultimately leading to neuronal death. These cascades include activation of phospholipases and proteases, cellular breakdown, and DNA fragmentation (Lynch and Dawson 1994).

Application of NMDA to our cultures resulted in neuronal degeneration but did not appear to induce any increases in cellular proliferation. However, due to technical considerations, we have not yet been able to double-label cultures for both BrdU and neurofilament in order to determine whether glial proliferation increased around degenerating neurons. Careful examination of BrdU-labeled cultures that have been treated with neurotoxic concentrations of NMDA reveals a fairly uniform distribution of proliferative cells rather than clusters of BrdU+ cells.

In contrast to undamaged neurons, most degenerating neurons are located in areas that are devoid of SHP-1 immunostaining. In untreated cultures, SHP-1+ cells are found throughout the culture, and many neurons are surrounded by SHP-1 immunoreactivity. Following NMDA treatment,

clusters of SHP-1+ cells are located at a distance from the dead or dying neurons, but few SHP-1+ cells remain directly adjacent to the neuronal fragments. This differs from the pattern of GFAP immunoreactivity, where many GFAP+ astrocytes are located around and beneath the dying neurons. These results are particularly intriguing given the fact that our *in vivo* findings indicate that at least initially following deafferentation, SHP-1+ astrocytes represent a different population of cells than the GFAP+ astrocytes (Lurie et al. 1998). It may be that neuronal degeneration induces the activation of GFAP+ astrocytes while other factors are involved in signaling an increase in SHP-1+ glia.

Conclusions

Based upon the information presented for this thesis, several conclusions can be drawn:

1. Manipulation of the cell cycle in chick brainstem cultures was successfully accomplished and this manipulation changed the distribution of SHP-1 immunoreactivity..
2. The cell types that contain SHP-1 are of glial origin demonstrating that SHP-1 is expressed in those cells which are able to proliferate after injury *in vitro*.

3. Introduction of the protein tyrosine phosphatase inhibitor, sodium orthovanadate, increased the proliferation of the cultures indicating a relationship between phosphatase activity and the regulation of proliferation.
4. Addition of specific growth factors (IL-3, EGF and GM-CSF) resulted in different expression patterns of SHP-1. It may be that SHP-1 is involved in more than one signal transduction cascade regulating cellular division.
5. Neuronal degeneration results in little SHP-1 immunoreactivity in the immediate vicinity of dead and dying neurons. This contrasts with GFAP+ astrocytes, which are located around and beneath areas of neural degeneration.

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