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SHP-1 is involved in neuronal differentiation of P19 embryonic carcinoma cells

Kazuya Mizuno, Tatsuo Katagiri, Etsuko Maruyama, Kiminori Hasegawa, Mami Ogimoto, Hidetaka Yakura*

Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183, Japan

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Abstract Accumulating evidence suggests that tyrosine phosphorylation plays an important role in the development of the central nervous system and in the differentiation of neuronal cells. To identify protein tyrosine phosphatases (PTPs) that might regulate signaling events leading to neuronal cell differentiation, we cloned PTP genes from the murine P19 embryonic carcinoma cell line and examined the change of their expression during differentiation. P19 cells are known to be pluripotent and the aggregate formation and subsequent replating in the presence of retinoic acid (RA) induce growth arrest and neuronal differentiation. The results demonstrated that among several PTP genes expressed in P19 cells, a cytosolic Src homology region 2 domain-containing PTP, SHP-1, is expressed highly in undifferentiated P19 cells, but is reduced to an undetectable level at day 3 after replating in the presence of RA. Further, SHP-1 was tyrosine-phosphorylated and activated at day 1 after replating. When ectopic SHP-1 was constitutively expressed, P19 cells continued to proliferate and failed to differentiate upon stimulation with RA. Collectively, these results suggest that the regulated expression and activity of SHP-1 may be involved in the neuronal differentiation of P19 cells.

Key words: Protein tyrosine phosphatase; SHP-1; Neuronal differentiation

1. Introduction

Protein tyrosine phosphorylation is one of the main mechanisms controlling cell proliferation and differentiation [1–3]. The level of phosphorylation on tyrosine residues of intracellular proteins is tightly regulated by the balance between the activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Since the first cytosolic PTP (PTP1B) was cloned from human placenta [4], a number of receptor-type (RPTP) and cytosolic PTP genes have been cloned in several species. However, their roles in specific cellular processes are largely unclarified.

In the brain, cytosolic PTKs such as Src, Fyn and Yes are abundantly expressed in a developmental stage-specific manner [5–7], and are localized at high levels in growth cones [8], especially in growth cone particles [9]. Several growth factors in the nervous system such as fibroblast growth factor, epidermal growth factor (EGF) and nerve growth factor (NGF) can promote cell proliferation and neurite outgrowth by binding to and activating their receptors with PTK activities. Recent studies suggest that both cytosolic [10,11] and receptor-type PTKs [12] function downstream of cell adhesion molecules, controlling neurite outgrowth. All these examples indicate important roles of tyrosine phosphorylation in neuronal cell differentiation and neural development.

There have been many reports suggesting that PTPs are major regulators in the nervous system. For example, several RPTPs, such as PTPδ [13,14], PTPσ [15–19], LAR [15,20] and RPTPβ [21], are highly expressed in the neuronal tissues of the vertebrates. The carbonic anhydrase domain of RPTPβ/PTPζ is a ligand for the axonal recognition molecule contactin [22]. Further, four out of five RPTPs identified in *Drosophila* are expressed predominantly on axons in the developing nervous system [23–26]. Recent studies provide evidence that three such RPTPs, DLAR [27], DPTP69D and DPTP99A [28], are required for the guidance of specific motor axons during *Drosophila* embryonic development.

In this study, we investigated the possible involvement of PTPs in in vitro neuronal cell differentiation of murine P19 embryonic carcinoma (EC) cells. P19 cells are widely used as a model system for murine pre-implantation development [29–31]. For the induction of neuronal differentiation, P19 cells are allowed to form aggregation for 3 days and replated for 3–4 days in the presence of retinoic acid (RA). P19 cells can also differentiate into mesodermal and endodermal cells with dimethylsulfoxide (DMSO) stimulation and without any inducers, respectively. Here we demonstrate that undifferentiated P19 cells express Src homology region 2 (SH2) domain-containing phosphatase 1, SHP-1 [32–35], and the expression of SHP-1 is reduced to a background level at the terminal differentiation stage into neuroectodermal cells. Constitutive overexpression of ectopic SHP-1 in P19 appeared to suppress neuronal differentiation and induce continuous proliferation. Thus SHP-1 may play a critical role in the neuronal differentiation and growth control of P19 cells.

2. Materials and methods

2.1. Cells

P19 EC cell line was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in α-modified minimum essential medium supplemented with 20 mM L-glutamine and 10% fetal bovine serum. Aggregation of the P19 cells was done by culturing cells on poly(2-hydroxyethyl methacrylate) (poly-HEMA)-coated Petri dishes for 3 days. Subsequently, the aggregates were replated on tissue culture dishes for 3 days. Endoderm-like cells were induced in this culture. For the induction of neuroectoderm- and mesoderm-like cells, aggregate formation and replating into monolayer cultures were performed in the presence of 0.1 μM RA and 1% DMSO, respectively.

2.2. Antibodies and reagents

Polyclonal rabbit anti-human SHP-1 antibody (Ab), which cross-reacts with the mouse product, was purchased from Santa Cruz Bio-

*Corresponding author. Fax: (81) (423) 21-8678.
E-mail: yakura@tmin.ac.jp

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technology (Santa Cruz, CA). Anti-phosphotyrosine (PY) monoclonal antibody (mAb) 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG and AP-conjugated mouse anti-rabbit IgG were obtained from Bio-Rad Laboratories (Richmond, CA) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively. Poly-HEMA was purchased from Sigma (St. Louis, MO).

2.3. RT-PCR and cDNA cloning

RT-PCR was performed as described previously [36]. The first strand cDNA was synthesized using oligo (dT) primer. The synthesized cDNA was directly used as a template for PCR amplification. To obtain genes for PTPs, two sets of PCR were performed using the following degenerative primers which correspond to highly conserved catalytic domain amino acid sequences DFWRM(I/V)W (upstream), KC(D/A)(K/E/Q)YWP (internal) and HCSAGVG (downstream): 5′-GA(C/T)TC(T/C)TGG(A/C)G(A/G)ATGA(C/T)CTGG-3′ (sense primer for the first PCR), 5′-AA(A/G)TG(C/T)(C/G)(A/C)(C/T)CA(A/G)TA(C/T)TGGCC-3′ (sense primer for the second PCR), and 5′-CC(A/T)TC(G/T)CC(A/C)GC(A/G)CT(A/G)CA(A/G)TG-3′ (antisense primer for the first and second PCRs). The PCR product of about 260 bp after the second PCR was blunt-ended, cloned in pUC18 using SureClone Ligation Kit (Pharmacia Biotech, Uppsala, Sweden) and sequenced by the dideoxy chain termination method. The SHP-1 and SHP-2 gene expression was examined by RT-PCR using oligo (dT)-primed cDNA and the following primers were used for SHP-1: 5′-TGGTTTCACCGGGACCTCAGC-3′ (sense primer) and 5′-AGTAAGGGTGCCGCAGGTAGA-3′ (antisense primer), which amplify cDNA fragments encoding amino acids 4–213, and for SHP-2: 5′-TGGTTTCACCCAAATATCACT-3′ (sense primer) and 5′-GGGCTGCTTGAGTTGTAGTAC-3′ (antisense primer), which amplify cDNA fragments encoding amino acids 6–215. For the internal control, synthesized cDNA was amplified with glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers (Clontech, Palo Alto, CA).

2.4. Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analysis were performed as described previously [37]. Cells were harvested, washed twice with ice-cold PBS containing 1 mM Na_3VO_4 and 2 mM EDTA, and lysed in TNE lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, and 0.15 U/ml aprotinin). After centrifugation, protein concentration was adjusted and each sample was immunoprecipitated with protein G-Sepharose coupled with anti-SHP-1 Ab. Total cell lysates or immunoprecipitates were subjected to 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane. The membranes were incubated overnight with anti-PY mAb or anti-SHP-1 Ab, followed by incubating AP-conjugated goat anti-mouse IgG or mouse anti-rabbit IgG, respectively. The blots were visualized by developing with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.5. PTP assay

PTP activity was assayed in 200 μl citrate buffer (100 mM, pH 5.0) containing immunoprecipitated SHP-1 and *p*-nitrophenyl phosphate (*p*NPP) as a substrate and at 37°C for 20 min. The reaction was stopped by the addition of 200 μl 1 N NaOH, and the absorbance was measured at 405 nm.

2.6. Plasmid and transfection

To generate an expression construct for SHP-1, a full-length SHP-1 cDNA was inserted in pcDNA3 vector (Invitrogen, San Diego, CA). Stable transfectants were obtained using the calcium phosphate precipitation method, followed by selection with 300 μg/ml geneticin (Gibco BRL, Gaithersburg, MD) for 14 days. Colonies were picked up and clones were established by limiting dilution.

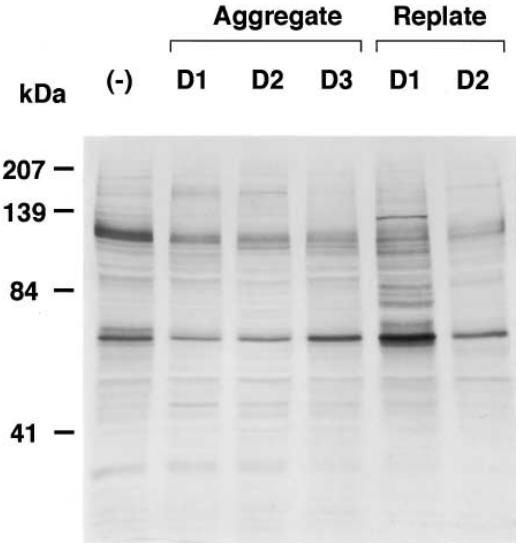

Fig. 1. Altered tyrosine phosphorylation of cellular proteins during in vitro differentiation of P19 cells. Western blot analysis using anti-PY mAb of cell lysates from P19 cells during neuronal differentiation. For the induction of neuroectoderm-like cells, P19 cells were allowed to form aggregate by culturing cells on poly-HEMA-coated Petri dishes for 3 days in the presence of 0.1 μM RA, and then the aggregates were replated on tissue culture dishes for 3 days. 20 μg of cell lysate was separated on a 10% SDS-PAGE gel. After transfer to a nitrocellulose membrane, Western blot was performed with anti-PY mAb.

3. Results

3.1. Tyrosine phosphorylation of cellular proteins in neuronally differentiated P19 cells

P19 cells are known to differentiate in vitro into mesodermal and neuroectodermal cells upon DMSO and RA stimulation, respectively, or endodermal cells without any inducers. As a first step, we investigated tyrosine phosphorylation of cellular proteins during neuronal differentiation of P19 cells. For the induction of neuroectoderm-like cells, P19 cells were allowed to form aggregate by culturing cells on poly-HEMA-coated Petri dishes for 3 days in the presence of 0.1 μM RA, and then the aggregates were replated on tissue culture dishes for 3 days. Western blot analysis of total cellular proteins using anti-PY mAb demonstrated that in the presence of RA tyrosine phosphorylation of 110 kDa and 60 kDa proteins was reduced at days 1–3 and days 1–2 of aggregate formation, respectively (Fig. 1). After replating of aggregates, tyrosine phosphorylation was markedly induced at day 1 in many species of proteins, e.g. 60 kDa, 75 kDa, 80 kDa, 100 kDa and 150 kDa, but returned to the baseline level at day 2 (Fig. 1).

Table 1
PTP genes expressed in neuronally differentiated P19 cells

	PTPα	P19-PTP	PTPε	PTPζ	PTP-MEG	CD45	SHP-1
Aggregate day 1	16	4	3	3	0	3	5
Replate day 2	9	1	0	0	2	2	3

RNA isolated at 1 day after aggregation or at 2 days after replating in the presence of RA was used as a template for cDNA synthesis. The number of isolated clones corresponding to each PTP gene is indicated.

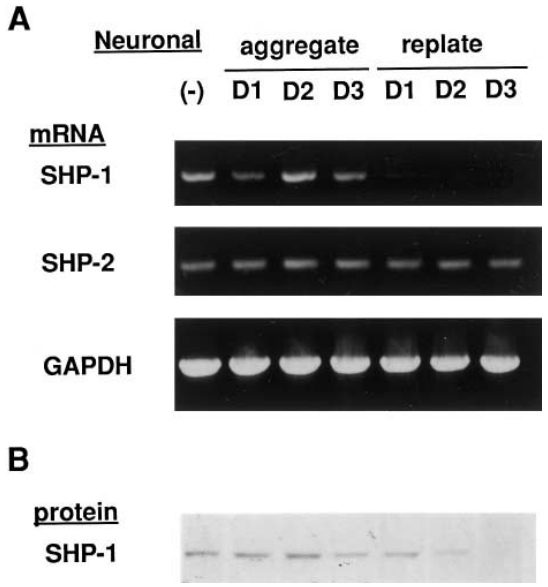

Fig. 2. Expression of SHP-1 and SHP-2 in the neuronally differentiating P19 cells. A: Gene expression of SHP-1 and SHP-2 was examined by RT-PCR. Oligo (dT)-primed cDNA was subjected to PCR reaction with each set of primers as described in Section 2. B: 20 μg of total cell lysate was size-fractionated on a 10% SDS-PAGE gel. After transfer to a nitrocellulose membrane, Western blot was performed with anti-SHP-1 Ab.

These changes were not detected in the mesodermal or endodermal differentiation process (data not shown).

3.2. *PCR cloning of PTP genes expressed in differentiated P19 cells*

To investigate PTPs involved in the regulation of neuronal differentiation of P19 cells, we cloned PTPs expressed in P19 cells by RT-PCR with degenerative primers specific for the conserved regions in the PTP domains. RNA isolated at 1 day after aggregation or at 2 days after replating was used as a template for cDNA synthesis. PCR products were blunt-ended and cloned in pUC18. Sequence analysis and subsequent GenBank database search revealed that all clones display significant homology to catalytic domains of known PTP genes (Table 1). No novel PTP sequences were found. The most frequently isolated clones (16 clones from aggregates and nine clones from replate cultures) corresponded to either the first or the second catalytic domain of PTPa, which was previously shown to be expressed in P19 cells [38]. The second group consisted of five clones (four clones from aggregates and one clone from replate cultures) with sequences identical to P19-PTP, which was originally cloned from P19 cells [39]. In addition to PTPα and P19-PTP, PTPε, PTPζ, PTP-MEG, CD45 and SHP-1 were identified (Table 1). Among these PTPs, we focused on SHP-1 that was reported to be expressed in neuronal tissues or cell lines [40,41].

3.3. *SHP-1 expression is reduced after replating during neuronal differentiation*

SHP-1 is a cytosolic PTP that contains two tandem SH2 domains at the N-terminus and is preferentially expressed in hematopoietic cells [32–35]. In mammals, another SH2 domain-containing PTP, SHP-2, is known to be present in a wide range of tissues [42–45]. To gain insight into possible involvement of SHP-1 in neuronal cell differentiation, we determined the changes of SHP-1 gene expression (Fig. 2A). RT-PCR analysis revealed that undifferentiated P19 cells express SHP-1 mRNA, which was maintained during aggregate formation in the presence of RA. However, upon replating of aggregates, the expression of SHP-1 was reduced to an almost undetectable level. The pattern of SHP-1 protein expression was correlated well with that of SHP-1 gene expression (Fig. 2B). In contrast, such alteration was not observed during endodermal or mesodermal differentiation which was induced as mentioned in Section 2 (Fig. 3). SHP-2 expression was not

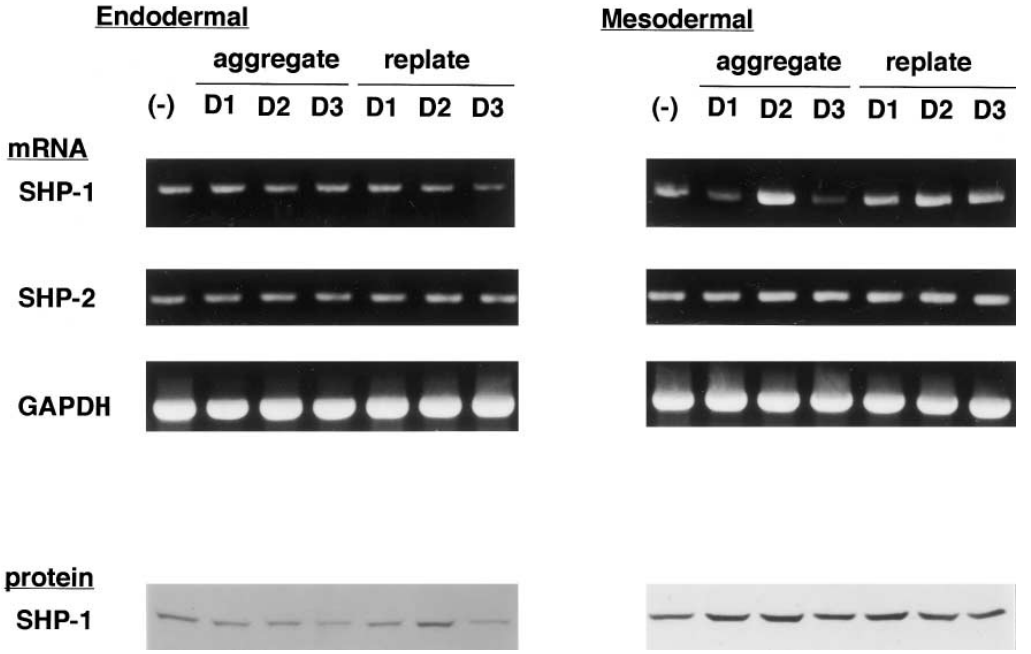

Fig. 3. SHP-1 and SHP-2 expression in P19 cells during endodermal or mesodermal differentiation, which are induced as described in Section 2. Total RNA was prepared from P19 cells during endodermal or mesodermal differentiation and cDNA was synthesized with oligo (dT) primer. PCR reaction was performed as described in the legend to Fig. 2. 20 μg of cell lysate was used for anti-SHP-1 Western blotting.

changed significantly during differentiation into derivatives of three germ layers (Fig. 2AFig. 3). Thus SHP-1 may play a unique regulatory role selectively in neuronal differentiation of P19 cells.

3.4. SHP-1 is transiently tyrosine-phosphorylated and activated during neuronal differentiation

Since tyrosine phosphorylation of SHP-1 has been reported to induce the enzymatic activity [41,46], we examined whether the state of tyrosine phosphorylation in SHP-1 is altered during cultures of P19 cells in the presence of RA. Total cell lysates from RA-treated cells were immunoprecipitated with anti-SHP-1 Ab and then subjected to Western blot analysis with anti-PY mAb. This experiment revealed that tyrosine phosphorylation of SHP-1 is transiently enhanced at day 1 after replating (Fig. 4). No other phosphotyrosine-containing proteins were present in the SHP-1 immunoprecipitates, although GST-SHP-1-SH2 fusion proteins could precipitate several phosphotyrosine-containing proteins in vitro (data not shown). We further tested the PTP activity of SHP-1 immunoprecipitated from undifferentiated or differentiated P19 lysates. The results shown in Fig. 5 clearly demonstrated that the PTP activity of SHP-1 is transiently upregulated on day 1 after replating, when tyrosine phosphorylation of SHP-1 is enhanced. This upregulation of PTP activity was proved to be significant ($P < 0.01$) by paired t-test.

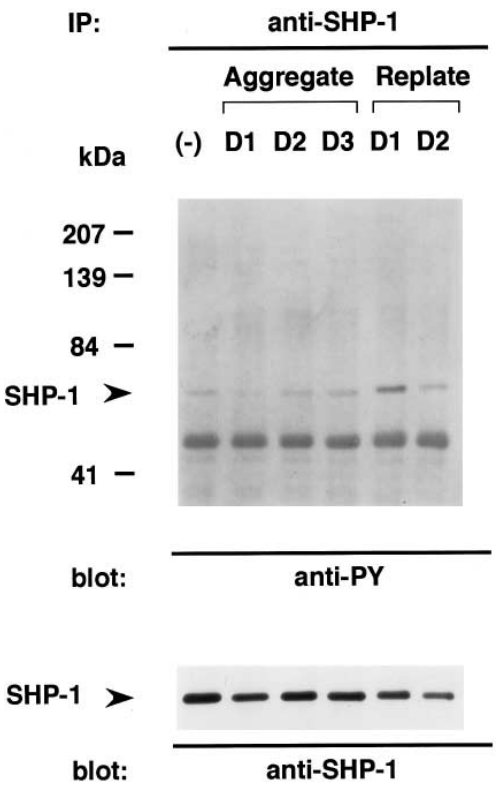

Fig. 4. SHP-1 is transiently tyrosine-phosphorylated after replating RA-treated aggregates. P19 cells were treated with RA to induce neuronal differentiation. SHP-1 was immunoprecipitated from 2 mg of total cellular proteins, subjected to SDS-PAGE and immunoblotted with anti-PY mAb. The lower panel shows the relative amount of SHP-1 in each immunoprecipitate.

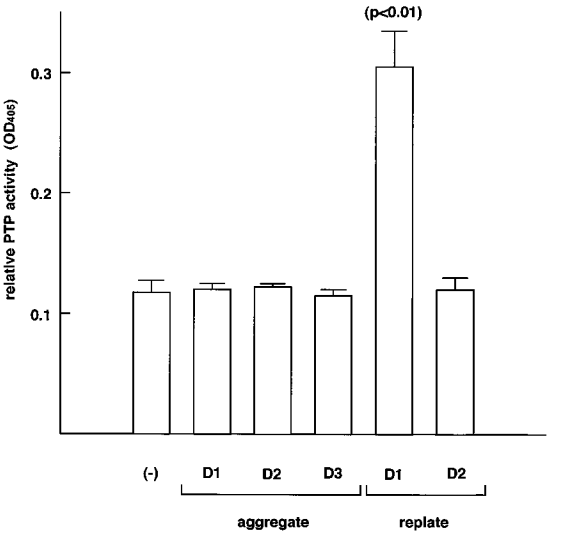

Fig. 5. PTP activity of SHP-1 is upregulated on day 1 after replating. SHP-1 immunoprecipitates were suspended in 200 μl citrate buffer (100 mM, pH 5.0) containing pNPP and incubated at 37°C for 20 min. The reaction was stopped by adding 1 N NaOH. The absorbance was measured at 405 nm. Three independent experiments were performed and mean absorbance ± S.E.M. was shown.

3.5. Effect of ectopic SHP-1 expression on neuronal differentiation of P19 cells

Finally, to investigate whether reduced SHP-1 expression is required for neuronal differentiation, exogenous SHP-1 was stably expressed in P19 cells. CMV promoter-driven expression vector for SHP-1 was constructed by insertion of a full-length SHP-1 cDNA into the mammalian expression vector pcDNA3. After geneticin selection for 2 weeks, several clones were established by limiting dilution. SHP-1 gene and protein expression in two representative clones (10 and 16) is shown in Fig. 6A,B, respectively. Basal PTP activity of SHP-1 immunoprecipitates from P19, mock-transfected P19 cells, clone 10 and 16 was 1.0, 1.0, 1.16 and 1.20, respectively (data not shown). SHP-1 remained to be highly expressed in the two clones until day 3 after replating (data not shown). To determine the effect of constitutive expression of SHP-1 on the fate of differentiation of P19 cells, we examined the ability of the SHP-1-transfected clones to extend neurite outgrowth in response to RA. In contrast to P19 cells transfected with vector alone which showed growth arrest and significant neurite extensions, SHP-1-transfected clones continued to proliferate even after replating and had less neurite extensions even at day 3 after replating (Fig. 6C).

4. Discussion

In this report, we investigated the role of PTPs in neuronal cell differentiation using P19 cells as a model of in vitro differentiation. P19 cells are murine pluripotent EC cells that are known to differentiate into derivatives of all three germ layers by different stimulations [29–31]. In endodermal or mesodermal differentiation of P19 cells, tyrosine phosphorylation of cellular proteins was almost unchanged (data not shown). In contrast, slightly reduced tyrosine phosphorylation of a few proteins (110 kDa and 60 kDa) was obtained during RA-induced aggregate formation, and enhanced tyrosine phosphorylation was observed in several protein species (60 kDa,

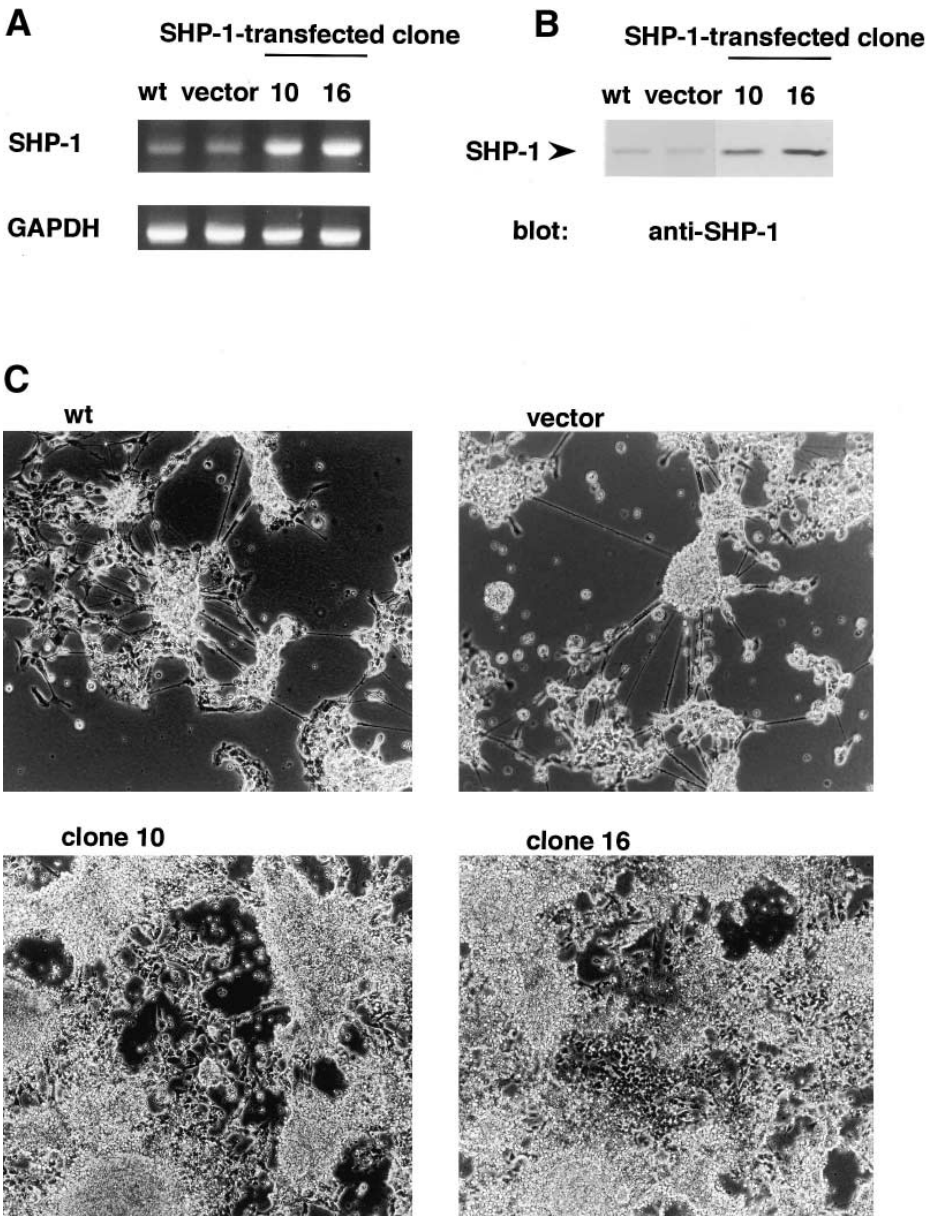

Fig. 6. Constitutive expression of ectopic SHP-1 inhibits RA-induced neurite extensions and growth arrest in P19 cells. A: Gene expression of SHP-1 was examined by RT-PCR analysis in 2 SHP-1 transfectants, a clone transfected with empty vector, and wild-type P19 cells. B: Relative amount of SHP-1 protein expression in each clone was examined by Western blot analysis. 20 μg of total cell lysate was subjected to SDS-PAGE and immunoblotted with anti-SHP-1 Ab. C: Each clone was allowed to form aggregates, replated in the presence of RA and photographed at 3 days after replating. Note that SHP-1 transfectants continue to proliferate and have fewer neurite extensions.

75 kDa, 80 kDa, 100 kDa and 150 kDa) at day 1 after replating (Fig. 1). When a specific PTP inhibitor Na_3VO_4 was added to cultures, neurite outgrowth and MAP-2-positive cells were strongly reduced, suggesting a possible involvement of PTPs in this process (data not shown).

Previously, P19 cells were shown to express two PTP genes, PTPa [38] and P19-PTP [39]. P19-PTP mRNA was reported to be detected upon aggregation of P19 cells either in the absence of chemical inducers or in the presence of DMSO or RA and to be reduced to an undetectable level upon replating of aggregates [39]. P19-PTP showed the highest expression during the period of corticogenesis [40], implying a role in the regulation of proliferation and determination of neuronal fate. den Hertog et al. demonstrated that endogenous PTPα expression is enhanced during neuronal differentiation of P19 cells and that overexpression of PTPα alters the differentiation fate of P19 cells in favor of neuronal differentiation [38]. In addition, c-Src was shown to be activated in PTPα-transfected cells, suggesting PTPα-mediated activation of c-Src. Studies on knockout mice for c-Src and c-Fyn demonstrated that L1-dependent neurite outgrowth is impaired in vitro in neurons from Src-deficient mice [47] and that NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn^- mice [48]. Taken together, it is possible that individual PTK functions downstream of specific CAMs, whose activity is in turn regulated by a specific PTP. In this study, enhanced tyrosine phosphorylation was observed in 60 kDa proteins at 1 day after replating (Fig. 1). It remains to be determined whether this molecule is c-Src or other Src-family PTKs.

The present study further identified PTPε, PTPζ, PTP-MEG, CD45 and SHP-1 in P19 cells (Table 1). We focused on cytosolic PTPs containing two SH2 domains at the N-terminus, SHP-1 and SHP-2 for further analysis. Recent data have demonstrated that the gene encoding SHP-1 is mutated in motheaten and viable motheaten mice resulting in autoimmune disease and immunodeficiency [49,50] and that SHP-1 negatively regulates B cell antigen receptor-initiated signaling [51,52]. In contrast to ubiquitous SHP-2, SHP-1 is preferentially expressed in hematopoietic cells [32–35,53], but is also found in cells of epithelial origin [53] and in the brain [40]. We found that SHP-1 but not SHP-2 expression is decreased after replating RA-treated P19 aggregates (Fig. 2) and that the constitutive expression of ectopic SHP-1 inhibits strongly neuronal cell differentiation (Fig. 6). Furthermore, SHP-1 was tyrosine-phosphorylated and activated transiently during neuronal differentiation (at 1 day after replating) (Figs. 4 and 5). Since protein expression of SHP-1 has already been decreased at 1 day after replating (Fig. 4), the degree of tyrosine phosphorylation and activation per SHP-1 molecule should be more prominent. These results suggest that timely activation of SHP-1 and downregulation of SHP-1 may be critical to the final differentiation into neuronal cells and the continuous expression of SHP-1 has negative effect on this process.

The mechanisms whereby SHP-1 regulates neuronal differentiation in P19 cells are currently open. SHP-1 may act on a molecule that is specifically expressed at or selectively required for this particular step of differentiation. To define the role of SHP-1, it will be important to identify the physiological substrate in neuronal cells. There was no difference in tyrosine phosphorylated proteins in anti-SHP-1 immunoprecipitates between SHP-1-activated and -non-activated phases (Fig. 4). If a putative substrate is present at all stages of differentiation, this result suggests that a putative substrate may not be in anti-SHP-1 immunoprecipitates. Together with characterization of SHP-1-binding proteins, we are in the process of performing 'substrate trapping' experiments using a catalytically inactive form of SHP-1.

Sahin et al. demonstrated that the expression level of SHP-1 transcript remains constant during cortical development [40]. However, they failed to detect in situ hybridization signals in the developing cortex of the E17 embryo, leading them to conclude that SHP-1 expression in the brain might be due to the contamination of the blood cells. In contrast, Vambutus et al. [41] showed that SHP-1 may play a role in neuronal differentiation in NGF-induced differentiation of PC12 cells. In PC12 cells, SHP-1 was shown to be tyrosine-phosphorylated and activated after stimulation with NGF but not EGF and to bind NGF receptor TrkA in vitro in an NGF-dependent manner. Our results provide further evidence that SHP-1 is a crucial regulator in neuronal differentiation. It is of interest where in the brain SHP-1 is expressed and whether SHP-1-deficient motheaten mice display any neurological disorders.

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