Receptor protein tyrosine phosphatase α activates pp60c-src and is involved in neuronal differentiation

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Here we report that protein tyrosine phosphatases (PTPases), like their enzymatic counterpart the protein tyrosine kinases, can play an important role in cell differentiation. Expression of the transmembrane PTPase receptor protein tyrosine phosphatase α (RPTPα) is transiently enhanced during neuronal differentiation of embryonal carcinoma (EC) and neuroblastoma cells. Retinoic acid induces wild type P19 cells to differentiate into endoderm- and mesoderm-like cells. By contrast, retinoic acid treatment leads to neuronal differentiation of P19 cells, ectopically expressing functional RPTPα, as illustrated by their ability to generate action potentials. Endogenous pp60c-src kinase activity is enhanced in the RPTPα-transfected cells, which may be due to direct dephosphorylation of the regulatory Tyr residue at position 527 in pp60c-src by RPTPα. Our results demonstrate that RPTPα is involved in neuronal differentiation and imply a role for pp60c-src in the differentiation process. 

Key words: embryonal carcinoma cells/neuronal differentiation/pp60c-src/protein tyrosine phosphatase/regulation

Introduction

Protein phosphorylation on tyrosine residues is one of the main cell signalling mechanisms and is of profound importance for cell proliferation and differentiation. Cellular phosphotyrosyl levels are regulated by the activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). Whereas the PTKs constitute a well-characterized family of proteins (Hunter and Cooper, 1985; Schlessinger and Ullrich, 1992), relatively little is known about the biological function of PTPases. The PTPases have been recognized as a family of enzymes with high specificity for phosphotyrosine containing substrates (Tonks et al., 1988a,b; Streuli et al., 1989, 1990; Charbonneau et al., 1989). Molecular analysis of the PTPases resulted in the identification of a common homology domain (for reviews see Alexander, 1990; Fischer et al., 1991; Saito and Streuli, 1991), containing a conserved Cys residue that is absolutely required for PTPase activity (Guan and Dixon, 1990; Streuli et al., 1989, 1990; Guan et al., 1991) and which constitutes at least part of the catalytic site of these enzymes (Guan and Dixon, 1991). Both cytoplasmically localized (non-receptor) as well as transmembrane (receptor-like) PTPases, comprising one and two PTPase domains, respectively, have been identified (Cool et al., 1989; Brown-Shimer et al., 1990; Chernoff et al., 1990; Krueger et al., 1990). Mutagenesis of the catalytic Cys residue in the PTPase domain, closest to the membrane, leads to loss of PTPase activity, indicating that this domain contains the actual activity of transmembrane PTPases (Streuli et al., 1990). However, the second domain of the receptor-like PTPase α (RPTPα) has been demonstrated to display PTPase activity towards some substrates in vitro (Wang and Pallen, 1991).

Clear evidence has been obtained that PTKs play an important role in development. For instance fibroblast growth factors (FGFs) and their receptors have been shown to be capable of triggering mesoderm induction in the early Xenopus embryo (Kimelman and Kirschner, 1987; Slack et al., 1987; Kimelman et al., 1988). Furthermore, several PTKs have been implicated in neural development. The trk family, including trk, trkB and trkC, encodes PTK receptors for neurotrophic factors that are expressed predominantly in the nervous system (Klein et al., 1989; Martin-Zaica et al., 1989; Bothwell, 1991; Lamballe et al., 1991; Middlemass et al., 1991). In addition, the cytoplasmically localized PTK pp60c-src has been implicated in neuronal differentiation both in vitro and in vivo. c-src mRNA expression and pp60c-src kinase activity are upregulated during neuronal differentiation (Sorge et al., 1984; Brugge et al., 1985; Lynch et al., 1986; Boulter and Wagner, 1988a; Bjelfman et al., 1990) and ectopic expression of v-src, the viral constitutively active homologue of c-src, leads to neuronal differentiation of pre-neuronal cells (Alema et al., 1985; Haltmeier and Rohrer, 1990; Cox and Maness, 1991; Thomas et al., 1991).

Several reports indicate that PTPases might also regulate crucial steps during development. Microinjection of purified protein preparations of the non-receptor PTPase PTP-1B severely inhibits insulin-induced Xenopus oocyte maturation (Tonks et al., 1990). Furthermore, three transmembrane PTPases, including LAR (Streuli et al., 1989), have been implicated in neural development, based on their expression patterns in Drosophila ( Tian et al., 1991; Yang et al., 1991). Recently Howard et al. (1992) identified a yeast PTPase that may play a crucial role in Dictyostelium development.

We investigated the involvement of the transmembrane PTPase RPTPα [Sap et al., 1990; also called LCA-related phosphatase (Matthews et al., 1990)] in in vitro differentiation of murine P19 embryonal carcinoma (EC) cells. EC cells are widely used as a model system for murine pre-implantation development (Graham, 1979; Martin, 1980). These pluripotent stem cells can be differentiated in vitro and
differentiated derivatives of all three germ layers have been obtained (Jones-Villeneuve et al., 1982; McBurney et al., 1982; Mummery et al., 1986). We demonstrate that RPTPα mRNA is expressed during neuronal, but not endodermal or mesodermal differentiation of P19 EC cells. Furthermore, RPTPα mRNA expression is enhanced during neuronal differentiation of C1003 EC cells and N1E-115 neuroblastoma cells. Overexpression of functional RPTPα in P19 EC cells alters the differentiation fate of these cells in favour of neuronal differentiation. Following retinoic acid treatment in monolayer the differentiated derivatives of RPTPα-transfected P19 cells morphologically resemble neuronal cells and they exhibit electrical excitability. In search of putative RPTPα substrates that are involved in neuronal differentiation, we investigated the non-transmembrane PTK pp60c-src. Recently, Zheng et al. (1992) demonstrated that pp60c-src is activated in fibroblasts that overexpress RPTPα. Similarly, the RPTPα-transfected P19 cells display enhanced pp60c-src kinase activity, but not expression, which is correlated with decreased phosphorylation of the regulatory site (Tyr527) of pp60c-src. In addition, we demonstrated that bacterially expressed RPTPα can dephosphorylate Tyr527 of pp60c-src in vitro. Our results demonstrate that RPTPα plays an important role in neuronal differentiation which may involve activation of pp60c-src.

Results

RPTPα expression is enhanced during neuronal differentiation

Recently we cloned full-length murine RPTPα from a P19 aggregates cDNA library, using probes generated by PCR with degenerative oligonucleotides (den Hertog et al., 1992). Analysis of RPTPα mRNA expression during in vitro differentiation of P19 cells by Northern blotting analysis indicates that RPTPα is not expressed in undifferentiated P19 EC cells (which resemble the pluripotent stem cells of the inner cell mass), nor in RA-treated P19 cells growing in monolayer (a mixed population of endoderm- and mesoderm-like cells) (Figure 1A). However, upon aggregation of P19 cells RPTPα is expressed independently of the presence of RA or dimethyl sulfoxide (DMSO) (Figure 1B). After replating of the aggregates for 3 days, only the RA-treated P19 derivatives maintain expression of detectable levels of RPTPα mRNA, which diminishes to undetectable levels after 6 days (Figure 1B). Taken together, RPTPα expression is observed in embryoid bodies (aggregated P19 cells) and neuroectoderm-like cells, obtained by aggregation and replating in the presence of RA.

Since RPTPα is expressed in neuroectoderm-like derivatives of P19 cells, we further investigated RPTPα mRNA expression during neuronal differentiation of serum deprived C1003 EC cells (Darmon et al., 1992) and DMSO-treated N1E-115 neuroblastoma cells (Kimhi et al., 1976). RPTPα is induced transiently in both cell lines with maximal levels of expression after 1–2 days following treatment for neuronal differentiation (Figure 2). Undifferentiated N1E-115 cells grown under normal serum conditions do not express detectable RPTPα mRNA levels (data not shown). For both C1003 EC cells as well as N1E-115 cells, hardly any differentiated cells can be detected after 1–2 days’ treatment, while a high percentage of neuronal cells is observed after 5 days (Kimhi et al., 1976; Darmon et al., 1982). These results demonstrate that RPTPα is induced transiently during neuronal differentiation of three cell types with maximal levels of RPTPα expression preceding the neuronal phenotype.

Ectopic expression of RPTPα in P19EC cells

In order to investigate the involvement of RPTPα in neuronal differentiation, RPTPα and a non-functional mutant of RPTPα, derived by site-directed mutagenesis of the active site Cys residue to an Ala residue in the first PTPTase domain (RPTPαC433A), were stably expressed in P19 EC cells. SV40 early promoter driven expression vectors for RPTPα and RPTPαC433A were constructed by insertion of the complete cDNA in the expression vector pSG5 (Green et al., 1988). PSG-RPTPα or pSG-RPTPαC433A were co-transfected with pSV2neo (Southern and Berg, 1982) into P19 EC cells. Following genetin selection for 14 days, colonies were picked and established as stable cell lines.
RNA was isolated from these stable cell lines and probed for RPTPa mRNA expression. Exogenous and endogenous transcripts could be discerned since pSG-RPTPa and pSG-RPTPaC433A gave rise to a smaller transcript (2.7 kb) than endogenous RPTPa transcripts (3.5 kb) (data not shown). None of the stably transfected P19 cells expressed endogenous RPTPa mRNA. Several clones, expressing exogenous RPTPa or RPTPaC433A mRNA, were selected for further analysis (Table I). To investigate whether these selected clones express RPTPa protein, nearly confluent cells (except P19-RPTPa69, ~50% confluent) were labelled with $^{35}$S)methionine and $^{35}$S)cysteine and subsequently RPTPa was immunoprecipitated, using polyclonal anti-RPTPa antisemur 2A (Sap et al., 1990). As a positive control RPTPa was immunoprecipitated from $^{35}$S-labelled human embryonic kidney 293 cells, transiently transfected with pSG-RPTPa. Using this effective transient expression system, RPTPa could be demonstrated as a 130 kDa protein in 293 cells transiently transfected with pSG-RPTPa, but not in control pSG5-transfected 293 cells (Figure 3). Wild type P19 EC cells as well as P19 EC cells, stably transfected with empty expression vector (P19-N-2) do not express RPTPa protein (Figure 3), as expected from the Northern blotting analysis (Table I). Several clones were isolated, expressing mutant RPTPaC433A protein at different levels (P19-RPTPaC433A-54 and -59). In addition, stable cell lines were isolated, including P19-RPTPaC433A-5, 17, 69 and 75, expressing RPTPa as a 130 kDa protein, with P19-RPTPaC433A-69 and 75 expressing the highest levels.

Morophologically the stably transfected cells resemble wild type P19 EC cells. The differentiation state of these clones was investigated, using common markers, including SSEA-1, ECMA-7 and TROMA-1. SSEA-1 and ECMA-7 are specific for undifferentiated EC cells (Solter and Knowles, 1978; Kemler, 1980), while TROMA-1 only stains differentiated cells (Kemler et al., 1981). As demonstrated in Table I, the P19-RPTPa and RPTPaC433A transfectants stain positive with SSEA-1 and ECMA-7, while no staining was observed with TROMA-1. Another marker for undifferentiated EC cells is their ability to grow in soft agar. The P19-RPTPa transfectants retain their ability to grow in soft agar, albeit some clones do not grow as effectively in soft agar as wild type P19 EC cells (Table I). No influence of stable RPTPa or RPTPaC433A expression could be detected on cell proliferation, as determined by $^{3}H$thymidine incorporation and cell count assays (data not shown). The isolation of several undifferentiated P19 transfectants indicates that ectopic expression of RPTPa or a non-functional mutant, RPTPaC433A, in P19 EC cells, in itself, does not induce differentiation.
Functional RTP\(\alpha\) is expressed by the P19-RTP\(\alpha\) transfectants

The PTPase activity of exogenous RTP\(\alpha\) in the P19-RTP\(\alpha\) transfectants was investigated by an in vitro PTPase assay. RTP\(\alpha\) or mutant RTP\(\alpha\)C433A was immunoprecipitated from the transfectants using anti-RTP\(\alpha\) antiserum 2A and subsequently the immunocomplexes were incubated with \(\text{[32P]}\)phosphotyrosine containing myelin basic protein (MBP). Dephosphorylation was allowed to proceed for 5 min after which the amount of released \(\text{[32P]}\)phosphate was assayed. Immunoprecipitates from wild type P19EC cells contain some background PTPase activity (Figure 4A). The level of PTPase activity is not enhanced in P19 cells stably transfected with pSG5 expression vector (P19-N-2), nor in cells stably expressing mutant RTP\(\alpha\)C433A (Figure 4A). Immunoprecipitates from the P19-RTP\(\alpha\) transfectants show a 2- to 4-fold increase in PTPase activity relative to wild type P19 cells. The RTP\(\alpha\) protein levels correspond directly to the absolute levels of PTPase activity with P19-RTP\(\alpha\)-5, 69 and 75 displaying the highest substrate dephosphorylation activities (cf. Figures 3 and 4A). P19-RTP\(\alpha\)-2 and 38 show only background PTPase activity, reflecting the low level of RTP\(\alpha\) protein expression (Figures 3 and 4A).

Dephosphorylation by RTP\(\alpha\) immunoprecipitated from the different cell lines, as depicted in Figure 4A, was assayed after 5 min at 37°C. To ascertain that this time point lies in the initial linear range of the time course, time dependency of RTP\(\alpha\) PTPase activity was determined. RTP\(\alpha\) was immunoprecipitated from P19-RTP\(\alpha\)-75 and, as a control, from P19-N-2 cells. The PTPase activity of these immunocomplexes was assayed after different periods of time. As shown in Figure 4B, dephosphorylation by RTP\(\alpha\) is linear for at least 6 min and reaches a plateau after 18 min.

The enzymatic activity of purified human RTP\(\alpha\) has been characterized (Daum et al., 1991). In order to compare the \(K_m\) of immunoprecipitated murine RTP\(\alpha\) with purified human RTP\(\alpha\), MBP dephosphorylation by immunoprecipitated RTP\(\alpha\) was assayed at different substrate concentrations. The \(K_m\) of murine RTP\(\alpha\), immunoprecipitated from P19-RTP\(\alpha\)-75, was calculated from a Lineweaver−Burk plot (Figure 4C) and is \(\sim 17 \mu M\), which is in the same range as the \(K_m\) of purified human RTP\(\alpha\) (10 \mu M; Daum et al., 1991). Although PTPase activity could be detected in vitro by RTP\(\alpha\) immunocomplex dephosphorylation assays (Figure 4), Western blotting analysis using anti-phosphotyrosine antibodies and whole cell lysates showed no gross differences in overall tyrosine phosphorylation between wild type and RTP\(\alpha\)-75 cells (data not shown). In conclusion, the in vitro substrate dephosphorylation assays illustrate that the P19-RTP\(\alpha\) transfectants express functional PTP\(\alpha\) protein. In addition, a single amino acid substitution (Cys to Ala at position 433) renders the otherwise intact enzyme inactive under these assay conditions.

Neuronal differentiation is induced in P19-RTP\(\alpha\) transfectants by RA

The ability of P19-RTP\(\alpha\) and RTP\(\alpha\)C433A transfectants to differentiate in vitro was assayed in order to determine whether ectopic expression of RTP\(\alpha\) influences the differentiation fate of P19 EC cells. Wild type P19 cells and the control cells, P19-N-2, give rise to a mixed population of endoderm- and mesoderm-like cells after differentiation by RA for 5 days in monolayer (Figure 5). Similarly, P19-RTP\(\alpha\)C433A cells give rise to a mixed population of endoderm- and mesoderm-like cells, following treatment with RA. By contrast, a high incidence of neuronal differentiation was observed upon RA-induced differentiation of the P19-RTP\(\alpha\) transfectants growing in monolayer. A significant proportion of the cells (20-40%) had neurite extensions and morphologically resembled neuronal cells (Figure 5). Whereas the different stably transfected P19-RTP\(\alpha\) cell lines express varying amounts of RTP\(\alpha\) protein and activity, there was no apparent qualitative or quantitative difference in the extent of neuronal differentiation. The differentiated cells stained positive with neurite-specific immunofluorescence markers (anti-NF-70 and anti-N-CAM) (data not shown). No apparent differences could be observed in differentiation fate between wild type and RTP\(\alpha\)-transfected P19 cells upon aggregation and replating either in the absence of chemical agents or in the presence of RA or DMSO, indicating that the observed effects are specific for differentiation in monolayer.
Development of electrical excitability in RA-differentiated P19-RPTPa-75

One of the most striking and characteristic features of neuronal cells is their capacity to generate action potentials. Therefore we studied, using whole-cell patch clamp, the electrical properties of P19-N-2, P19-RPTPaC433A-54 and P19-RPTPa-75 cells after RA-induced differentiation for 5 days. It appeared that the P19-N-2 cells show a high input resistance (5 GΩ) and did not show any sign of voltage dependent currents (Figure 6). Similarly, the P19-RPTPaC433A-54 cells did not show any electrical excitability (data not shown). By contrast, P19-RPTPa-75 cells exhibited an ∼10-fold lower input resistance (200–800 MΩ) and displayed large inward currents upon depolarization followed by slower outward currents (Figure 6). Such electrical properties clearly illustrate that P19-RPTPa-75 cells have acquired electrical excitability (i.e. the ability to generate action potentials). Whereas all morphologically neuronal P19-RPTPa-75 cells exhibited electrical activity (n ~ 50), none of the P19-N-2 nor P19-RPTPaC433A-54 cells showed any electrical excitability (n ~ 25). The development of electrical excitability in P19-RPTPa-75, but not in P19-N-2 nor P19-RPTPaC433A-54 cells, demonstrates that upon RA-induced differentiation P19-RPTPa-75 cells evolve fully to mature neurons, which can participate in electrical neuronal signalling and that this neuronal differentiation is dependent on PTPase activity of RPTPα.

pp60c-src activity is enhanced in RPTPα-expressing P19 cells

In search of possible mechanisms, underlying the observed neuronal differentiation, we investigated pp60c-src expression and activity in P19-N-2, P19-RPTPaC433A-54 and P19-RPTPa-75 cells. During neuronal differentiation of a number of cell lines, including P19 and neuroblastoma cells, pp60c-src expression and kinase activity are enhanced (Brugge et al., 1985; Lynch et al., 1986; Boulter and Wagner, 1988a; Bjelfman et al., 1990). pp60c-src was immunoprecipitated from 35S-labelled P19-N-2, P19-RPTPaC433A-54 and P19-RPTPa-75 cells, using monoclonal anti-pp60c-src antibodies, MAb327 (Lipsich et al., 1983). Equal amounts of 35S-labelled proteins, as determined by liquid scintillation counting of aliquots of the cell lysates, were used for the different immunoprecipitations. pp60c-src activity was determined by an immunocomplex kinase reaction, using MAb327, and as a substrate acid denatured enolase. There is no significant difference in pp60c-src protein levels between P19-N-2, P19-RPTPaC433A-54 and P19-RPTPa-75 cells (Figure 7A), indicating that ectopic expression of RPTPα does not affect pp60c-src expression. By contrast, pp60c-src auto- and substrate phosphorylation activity is enhanced 4- to 6-fold in P19-RPTPa-75 as compared with P19-N-2 and P19-RPTPaC433A-54 (Figure 7B). In the RPTPa-transfected as well as the control cell lines pp60c-src protein levels and kinase activity is enhanced 2-fold in response to RA-induced differentiation (Figure 7A,B). Apparently, pp60c-src kinase activity, but not expression, is enhanced in the RPTPa-transfected P19 cells, as compared with the control cells.

Dephosphorylation of the regulatory site of pp60c-src (Tyr527) in P19-RPTPa transfectants

It has been reported that dephosphorylation of pp60c-src in vitro leads to a 10- to 20-fold increase in kinase activity (Courtemeige, 1985; Cooper and King, 1986). A C-terminal Tyr residue (Tyr527) has been identified to be the regulatory site, inhibiting pp60c-src activity upon phosphorylation (Cooper and King, 1986; Cartwright et al., 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987;
Reynolds et al., 1987). In order to investigate the phosphorylation state of pp60<sub>c-src</sub>, both undifferentiated and 5 day RA-treated P19-N-2, P19-RPTPαC433A-54 and P19-RPTPα-75 cells were metabolically labelled with [32P]orthophosphate. pp60<sub>c-src</sub> was immunoprecipitated using MAb327 and subsequently 32P-labelled pp60<sub>c-src</sub> was digested with cyanogen bromide (CNBr). The major phosphorylation sites of pp60<sub>c-src</sub> can be discerned following CNBr cleavage as a 31 kDa fragment containing the N-terminal Ser phosphorylation sites, a 10 kDa peptide containing the autophosphorylation site (Tyr416) and the regulatory Tyr527 as a 4 kDa peptide (Bolen et al., 1987). Figure 8A and B show that the 4 kDa peptide is less phosphorylated in the undifferentiated and in the differentiated RPTPα-transfected cells, as compared with control and RPTPαC433A-transfected cells. Typically a 50–75% reduction in Tyr527 phosphorylation, relative to N-terminal Ser phosphorylation, was observed in the 5 day RA-treated P19-RPTPα-75 cells (Figure 8B). The effect in undifferentiated P19-RPTPα-75 cells is smaller, which is conceivably caused by enhanced expression of RPTPα in RA-treated cells (data not shown), due to RA-induced transcriptional activation of the SV40 promoter in P19 cells (den Hertog and Kruijer, unpublished). A chase of 1 h with regular growth medium, lacking 32P, enhances the observed effect on Tyr527 phosphorylation in undifferentiated cells (Figure 8A), indicating that the turnover rate of Tyr527 phosphorylation is higher in RPTPα-transfected cells than in control cells. These results indicate that in RPTPα-transfected cells Tyr527 is less phosphorylated than in control cells, which may result from direct dephosphorylation of this site by RPTPα.

The ability of RPTPα to dephosphorylate Tyr527 directly was investigated by an in vitro dephosphorylation reaction using pp60<sub>c-src</sub> 32P-labelled in vivo or in vitro as a substrate. For these in vitro dephosphorylation reactions the complete intracellular portion of RPTPα was expressed in bacteria as a GST fusion protein. Bacterially expressed RPTPα (bRPTPα) but not mutant bRPTPαC433A dephosphorylated Tyr527, present in the 4 kDa peptide of in vitro labelled pp60<sub>c-src</sub> (Figure 8C). Phospho-Tyr416, present in in vitro labelled pp60<sub>c-src</sub> can be dephosphorylated by bRPTPα also (Figure 8C). In conclusion, pp60<sub>c-src</sub> Tyr527 phosphorylation is reduced in RPTPα-transfected P19 cells. Furthermore, Tyr527 is dephosphorylated in vivo by bRPTPα, which may
electrophoresis, PTKs
Fig. 8. pp60±src Tyr527 dephosphorylation by RPTPa in vivo and in vitro. (A) Undifferentiated P19-N-2 (N-2), P19-RPTPaC433A-54 (C433A) and
P19-RPTPa-75 (PTPa) cells were labelled with [-32P]orthophosphate for 16 h. Subsequently the medium was replaced with regular cell growth
medium and the cells were incubated for another hour (1 h chase). Following immunoprecipitation, pp60±src was eluted from the gel, digested with
cyanogen bromide (CNBr) and run on a 20% SDS–polyacrylamide gel. The positions of the three major pp60±src phosphopeptides are indicated in
kDa on the left. (B) P19-N-2 (N2), P19-RPTPaC433A-54 (CA) and P19-RPTPa-75 (α) cells were treated with RA (10−6 M) for 5 days and
labelled with [-32P]orthophosphate. pp60±src was immunoprecipitated, digested with CNBr and electrophoresed on a 20% gel as described in panel A.
(C) Immunoprecipitated in vivo labelled pp60±src (V), or in vitro labelled pp60±src (R) was incubated with 100 ng bacterially expressed PTPα (α),
mutant bacterially expressed PTPαC433A (CA) or control bacterial extract (−) in M6 buffer for 30 min at 37°C. Following polyacrylamide gel
electrophoresis pp60±src was eluted from the gel, digested with CNBr and analysed by SDS–PAGE.

indicate that RPTPa directly dephosphorylates pp60±src
Tyr527 in vivo as well. Reduced Tyr527 phosphorylation
may account for the observed enhanced activity of pp60±src.
No enhanced Tyr416 phosphorylation is detected in vivo
in the RPTPa-transfected cells (Figure 8A and B), although
pp60±src is activated in these cells. However, bPTPa
readily dephosphorylates Tyr416 in vitro, which may indicate that the autophosphorylation site of pp60±src is
dephosphorylated by RPTPa in vivo as well.

Discussion
The PTPases have been recognized as a separate family of
enzymes, distinct from the Ser/Thr protein phosphatases
(Fischer et al., 1991 and references therein). Since it has
been reported that PTKs and PTK signal transduction play
crucial roles in vertebrate and invertebrate development, it
is tempting to speculate that PTPases, the enzymes that
antagonize PTK signalling, are equally important in this
process. Here we demonstrate that endogenous RPTPa
expression is enhanced during neuronal differentiation and
that overexpression of RPTPa alters the differentiation fate of
pluripotent P19 EC cells in favour of neuronal differentiation.
Furthermore, we show that pp60±src, a non-receptor PTK
which has previously been shown to be involved in neuronal
differentiation, is activated in RPTPa-transfected cells. We
propose that RPTPa-mediated activation of pp60±src triggers
neuronal differentiation.

Expression of RPTPa during neuronal differentiation
Analysis of endogenous RPTPa mRNA expression during
in vitro differentiation of cell lines, capable of neuronal
differentiation, shows that RPTPa is transiently induced in
the differentiation process, with maximal RPTPa mRNA
levels preceding the morphological appearance of the
neuronal phenotype. The transient induction is independent
of cell type and inducer, as it is apparent in aggregated P19,
serum-deprived C1003 and DMSO-treated N1E-115 cells.
It is noteworthy that although RPTPa is widely expressed
in adult murine tissues, highest levels of RPTPa mRNA
expression have been observed in the brain (Sap et al.,
1990). Moreover, recent in situ hybridization experiments
show highest RPTPa mRNA levels in the central nervous
system of 16 day old rat embryos (J. Sap and J. Schlessinger,
personal communication), indicating that RPTPa may play
a role during neurogenesis in vivo. The RPTPa expression
patterns during in vitro neuronal differentiation of three
distinct cell lines, with maximal levels at an early stage in
the differentiation process, may indicate that RPTPa is
involved in neuronal differentiation rather than neuronal
outgrowth.

Neuronal differentiation in RPTPa-transfected P19
cells
Several P19 EC cell lines were derived expressing mutant,
inactive RPTPaC433A or functional RPTPa protein with similar enzymatic characteristics as previously described
human RPTPa (Daum et al., 1991). It has been speculated
that PTPases might have growth inhibitory effects, since
PTPases conceivably counteract the mitogenic effects
of PTKs. However, no significant effects on cell proliferation,
as determined by cell count and [3H]thymidine incorporation
assays were observed in the P19-RPTPa transfecants,
indicating that RPTPa is not involved in growth inhibition
of these cells (data not shown).

Ectopic expression of functional RPTPa in P19 EC cells
does not induce differentiation, since several undifferentiated
RPTPa-transfected cell lines could be isolated. However,
a high percentage of neuronal cells was obtained upon RA-
induced differentiation of P19-RPTPa transfecants, while
wild type and control transfected P19 cells give rise to
dendormph- and mesoderm-like cells under these conditions.
The RA-treated RPTPa-transfected P19 cells, but not control
P19 cells show electrical excitability, illustrating that in
response to RA the RPTPα-transfected P19 cells evolve fully to mature neurons, capable of participating in electrical neuronal signalling. It has been reported that PTases might play an important role in Drosophila neural development. Three transmembrane PTases were identified to be expressed selectively on central nervous system axons of the developing Drosophila embryo and are thought to be involved in axon outgrowth and guidance (Tian et al., 1991; Yang et al., 1991). We show that the differentiation rate of pluripotent P19 cells is altered in favour of neuronal differentiation by ectopic expression of RPTPα, indicating that PTases, like PTKs, play an important role in neuronal development.

**pp60^c-src is activated in RPTPα-transfected P19 cells**

Recently it was reported that ectopic expression of human PTases in rat embryo fibroblasts leads to transformation of these cells (Zheng et al., 1992). Zheng et al. demonstrate that pp60^c-src is activated in these cells and that Tyr527 is totally dephosphorylated in cells overexpressing PTPα, suggesting that PTPα exerts its effects on transformation through activation of pp60^c-src. We demonstrate that pp60^c-src kinase activity is enhanced 4- to 6-fold in RPTPα-transfected P19 cells, relative to control transfected and RPTPαC433A-transfected cells, while pp60^c-src expression is similar in RPTPα-transfected and control P19 cells. Analysis of the phosphorylation state of pp60^c-src by CNBr cleavage of in vivo 32P-labelled pp60^c-src indicated that Tyr527 is less phosphorylated in the RPTPα-transfected P19 cells. Furthermore, we demonstrate that bPTPα, but not functionally mutant bPTPαC433A can dephosphorylate pp60^c-src Tyr527 in vitro. Reduced phosphorylation of Tyr527 may account for the observed increase in pp60^c-src activity, since it is well-established that this site plays a key role in regulation of pp60^c-src activity (Courtneidge, 1985; Cooper and King, 1986; Cartwright et al., 1987; Kmiecik and Shalloway, 1987; Piwica-Worms et al., 1987; Reynolds et al., 1987). Transmembrane PTase-mediated activation of cytoplasmically localized PTKs is not unprecedented, since it has been demonstrated that the Src family member pp56^c-src is activated by the transmembrane PTase CD45 (Mustelin et al., 1989; Ostergaard et al., 1989). In T cells CD45 specifically dephosphorylates the regulatory Tyr residue of pp56^c-src (Tyr505), which is functionally similar to Tyr527 of pp60^c-src. Recently it was reported that overexpression of CD45 can activate another member of the Src family, pp59^fyn also (Shiroo et al., 1992).

**RPTPα and neuronal differentiation**

Protein tyrosine phosphorylation has been shown to be capable of triggering neuronal differentiation and several PTKs have been implicated in this process. The family of *trk* proto-oncogenes is expressed exclusively in the central nervous system of both embryonic as well as adult mice and their transmembrane PTK gene products constitute proteins for neurotrophic factors (Klein et al., 1989; Lamballe et al., 1991; Martin-Zanca et al., 1991; Middlemass et al., 1991). Furthermore, we recently demonstrated that ectopic expression of the human epidermal growth factor receptor in P19 EC cells alters the differentiation fate of these cells in favour of neuronal differentiation (den Hertog et al., 1991). In addition, both expression and activity of the non-receptor PTK pp60^src are enhanced during *in vitro* neuronal differentiation of several cell lines, including P19 EC cells and neuroblastoma cells (Brugge et al., 1985; Lynch et al., 1986; Boulter and Wagner, 1988a; Bjelfman et al., 1990) and during *in vivo* neuronal differentiation of chick neural retina cells (Sorge et al., 1984). Endogenous pp60^src is essential for nerve growth factor induced neuronal differentiation of P12 pheochromocytoma cells (Kremers et al., 1991), indicating that pp60^src plays a crucial role in the differentiation process. Overexpression of highly active pp60^src induces neuronal differentiation of pre-neuronal cells, including avian sympathetic neurons and PC12 cells (Alema et al., 1986; Haltmeier and Rohrer, 1990; Cox and Maness, 1991; Thomas et al., 1991). pp60^src overexpression studies in P19 EC cells indicate that the role of pp60^src in neurogenesis is complex (Boulter and Wagner, 1988b; Schmidt et al., 1992). Differential effects have been reported for overexpression of the highly potent pp60^src and the moderately active pp60^src*, the neuronal variant of pp60^src (Schmidt et al., 1992). Overexpression of pp60^src induces epithelial differentiation of P19 cells, while pp60^src*-transfected cells retain the undifferentiated phenotype. The pp60^src*-transfected cells have lost their capacity to differentiate into neuronal cells, while pp60^src + overexpression inhibits neuronal differentiation to some extent. The correlation of higher pp60^src kinase activity of pp60^src with more extensive effects on P19 EC cell differentiation indicates that the level of pp60^src kinase activity plays a crucial role in P19 EC cell differentiation. In addition, proper timing of elevation of pp60^src kinase activity seems to be essential, since endogenous pp60^src activity is enhanced during neuronal differentiation of P19 cells, while prior elevation of pp60^src activity by overexpression of pp60^src or pp60^src* inhibits neuronal differentiation (Brugge et al., 1985; Schmidt et al., 1992).

Here we demonstrate that RPTPα is likely to be involved in neuronal differentiation. Ectopic expression of RPTPα in P19 EC cells leads to activation of endogenous pp60^src, conceivably through direct RPTPα-mediated dephosphorylation of Tyr527. Activated endogenous pp60^src, in turn, leads to specific enhanced tyrosine phosphorylation of pp60^src substrates, which may account for the observed RA-induced neuronal differentiation. It is noteworthy that only subtle modulations of overall phosphoryrosine content could be detected in RPTPα-transfected P19 cells, as compared with control cells (data not shown). This may indicate that pp60^src is specifically dephosphorylated by RPTPα and that endogenous pp60^src kinase activity is only moderately enhanced. Whereas we identify pp60^src as a possible RPTPα substrate, additional, as yet unidentified RPTPα substrates (e.g. the Src family members Yes and Fyn) may contribute to the observed neuronal differentiation, following RPTPα-mediated activation.

Neuronal differentiation of RPTPα-transfected P19 cells, growing in monolayer, is induced only following treatment with RA, while control cells yield a mixed population of endoderm- and mesoderm-like cells under these conditions. RA exerts its cellular effects through nuclear hormone receptors, acting as ligand inducible transcription activators (reviewed by Chambon et al., 1991). It is therefore possible that RA treatment results in the expression of one or more substrates whose tyrosine phosphorylation is essential for neuronal differentiation to occur. In this respect it is noteworthy that neuronal differentiation of wild type P19
cells can be induced by aggregation in the presence of RA, indicating that RA-responsive genes are required for neuronal differentiation. Similarly, these substrates could be induced during RA treatment of RPTPa-transfected P19 cells in monolayer. These substrates may become phosphorylated on tyrosine as targets of activated pp60^{src}, thereby altering the differentiation rate of these cells in favour of neuronal differentiation. Although several pp60^{src} substrates have been identified, it is presently not known what the key substrates are that mediate neuronal differentiation. Identification of pp60^{src} and RPTPa substrates and analysis of their phosphorylation states will facilitate the study of the antagonistic or cooperative activities of PTKs and PTPases in neuronal differentiation.

Materials and methods

Cells

P19 EC cells (McBurney and Rogers, 1982) were cultured in bicarbonate buffered DF medium supplemented with 7.5% fetal calf serum (FCS). C1003, NIE-115 and 293 cells were cultured as described (Kimhi et al., 1976; Graham et al., 1977; Darmon et al., 1982). Differentiation of P19 cells, growing in monolayer, was done by addition of 10^{-6} M (final concentration) RA to the medium. C1003 cells were induced to differentiate by replacing the medium with serum-free SATO medium (Barnes and Sato, 1980). Neuronal differentiation of NIE-115 cells was induced by replacing the medium with DMEM supplemented with 2% FCS and 1% DMSO (Kimhi et al., 1976). Aggregation of the P19 cells was done by culturing them on a layer of agarose (1%) for 5 days. Subsequently the aggregates were replated on gelatinized dishes and cultured for 3–6 days. Aggregation and replating was done in the absence of chemical agents (rendering endoderm-like cells), in presence of 10^{-7} M RA (rendering neuroectoderm-like cells) or in the presence of 1% DMSO (rendering mesoderm-like cells).

Northern blotting analysis

RNA was isolated from the cells using a phenol extraction method (Chomczynski and Sacchi, 1987). Fifteen micrograms of total RNA were fractionated on an 0.8% agarose gel and subsequently transferred to nitrocellulose filters using standard protocols. The nitrocellulose filters were hybridized to 32P-labelled probes in 50% formamide, 5 × SSC, 50 mM NaH_2PO_4, pH 6.9, 0.1% SDS, 0.1 mg/ml sonicated salmon sperm DNA and 2 × Denhardt’s solution at 42°C overnight. Subsequently the filters were washed three times with 2 × SSC–0.1% SDS, once with 1 × SSC–0.1% SDS at room temperature and once with 0.1× SSC–0.1% SDS. The 32P-labelled probes were obtained, using a multiprime labelling kit (Amersham) routinely with 50 ng DNA fragment and 50 μCi [α-32P]dCTP (Amersham). As probes for the Northern blotting analysis a 0.4 kb HindIII—PstI fragment, located in the extreme 3′ part of the RPTPa cDNA (Sap et al., 1990; den Hertog et al., 1992) and a 1.4 kb fragment of the rat glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Fort et al., 1985) were used.

Plasmids and transfections

PSG-RPTPa was generated by insertion of the complete cDNA of RPTPa (den Hertog et al., 1992) in EcoRI/BamHI cut PSG (Green et al., 1988). PSG5 is an SV40 early promoter driven expression vector. The functional mutant of RPTPa (RPTPaC433A) was derived by site-directed mutagenesis of the Cys residue at position 433 to an Ala residue, using an Altered Sites kit (Promega) and an oligonucleotide (5′ CTACACCTGCAGCTGGGTGGGACCGACGATG 3′) exactly as described by the manufacturer. Stable transfectants of P19 cells were derived by co-transfection of pSV2neo (Southern and Berg, 1982) and pSG-RPTPa or pSGRPTPaC433A, using a slightly modified calcium phosphate precipitation method as described previously by den Hertog et al. (1991). After selection with geneticin (GIBCO BRL) 200 μg/ml for 14 days, colonies were picked and established as stable cell lines. Transient transfections were done essentially as described previously by den Hertog et al. (1991) and cells were harvested 48 h after transfection.

Immunofluorescence and soft agar growth assay

Cells were grown on cover slips coated with laminine and at the appropriate time the cells were fixed with ice-cold methanol—acetone 95:5 (v/v). Incubation of the cover slips with antibodies and FITC-conjugated second antibodies was done exactly as described previously by den Hertog et al. (1991). The antibodies that were used in these studies are: anti-SSEA-1 (Soelter and Knowles, 1978), ECMA-7 (Kemler et al., 1980), TRIMA1 (Kemler et al., 1980), anti-A260 NF 70 (directly coupled to a 70 kDa anti-human serum component) and anti-N-CAM (Thiery et al., 1982). Soft agar growth assays were performed as described previously by Todaro et al. (1981).

Labelling and immunoprecipitation

Cells were seeded in 60 cm² dishes and after 24 h the cells were labelled in media/Cys-free medium supplemented with 7.5% dialysed FCS and 25 μCi (final concentration) Translabel (ICN), containing [35S]methionine and [32S]cysteine for 16 h at 37°C. After washing twice with ice-cold PBS, the cells were lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100 and 10 mM PMSF for 20 min on ice. After preclearance the cell lysates were incubated with antibodies and protein A—Sepharose (Pharmacia) on a rotating wheel for 2–16 h at 4°C. For RPTPa immunoprecipitations polyclonal anti-RPTPa antiseraum 2A (Sap et al., 1990) and for pp60^{src} immunoprecipitations monoclonal antibodies MAB 327 (Lipsich et al., 1983) were used.

After extensive washing (twice with 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol; twice with 10 mM Tris, pH 7.5 and 0.1% Triton X-100), the Sepharose beads were resuspended in 50 μl of Laemml sample buffer containing SDS and boiled for 5 min. The supernatants were electrophoresed on a 10% SDS—polyacrylamide gel.

PTPase assay

As a substrate for the in vitro PTPase assay, 32P-labelled MBP (Sigma) was used. MBP (120 μM, final concentration) was labelled on Tyr residues by incubation with labelling mix (20 mM HEPES, pH 7.5, 5 mM MnCl₂ and 50 μCi/ml [γ-32P]ATP) and immunoprecipitated epidermal growth factor receptor (EGF-R) for 4–6 h at 37°C. EGF-R was immunoprecipitated from A431 membranes using anti-EGF-R MAB 528 (Onconase Science) essentially as described above. After labelling MBP was precipitated using 25% trichloroacetic acid (TCA) in order to remove free [γ-32P]ATP. Using this protocol 80–90% of the 32P-label was incorporated in phospho-Tyr as determined by phosphoamino acid analysis (data not shown).

For PTPase assays RPTPa was immunoprecipitated as described above. After washing of the Sepharose beads, the immunoprecipitates were washed twice in M6 assay buffer (20 mM MES, NaOH, pH 6.0, 5% glycerol, 0.1% mercaptoethanol and 0.1% Triton X-100) (Daum et al., 1991). Subsequently the immunoprecipitates were incubated with 32P-labelled MBP (4 μM final concentration unless otherwise indicated), containing 3–6 × 10⁵ c.p.m., at 37°C in 60 μl M6 buffer. At the appropriate times the samples were removed from 37°C and placed on ice. Immediately 750 μl of PTPase stop mix (0.9 M HCl, 90 mM pyrophosphate, 2 mM NaHPO₄ and 4% activated charcoal (Norit A)) were added and the radioactivity in the supernatant determined by Cerenkov counting.

Electrical recording

Electrophysiological experiments were performed using the tight seal whole-cell configuration of the patch clamp technique at 33°C (amplifier designed and built by our laboratory). The bath medium contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.3 with NaOH. The patch pipette contained 140 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA and 10 mM HEPES, adjusted to pH 7.3 with KOH. Currents were sampled at 250 kHz by a HP3565 digital signal analyser and digitized at 0.9 s. The data were processed with the help of an HP-apollo 400T computer.

pp60^{src} kinase assay

For pp60^{src} kinase assays, pp60^{src} was immunoprecipitated with MAB 327, as described above. After extensive washing of the Sepharose beads, the beads were washed twice in ice-cold buffer (20 mM HEPES, pH 7.2). Subsequently the immunocomplexes were incubated in 30 μl kinase buffer (20 mM HEPES, pH 7.2 and 10 mM MgCl₂), containing 2 μCi [γ-32P]ATP and as a substrate 3 μg acid denatured enolase (Boehringer). After 10 min at room temperature Laemml sample buffer was added, the samples were boiled for 5 min and electrophoresed on a 10% SDS—polyacrylamide gel.

CNBr cleavage of 32P-labelled pp60^{src}

Cells were metabolically labelled with [32P]orthophosphate (ICN Radiochemicals; 1.0 mCi/ml) for 16 h and lysed in RIPA buffer. Labelling of the undifferentiated P19 cells and transfectants was chased by incubation in fresh growth medium for 1 h. pp60^{src} was immunoprecipitated and electrophoresed on a 10% SDS—polyacrylamide gel as described above. pp60^{src} was cut out from the dried gel, eluted and digested with CNBr (300 mg/ml in 70% formic acid for 1.5 h at room temperature)
essentially as described by Bolen et al. (1987). Following lysisophosphatization, the samples were run on a 20% SDS–polyacrylamide gel. For the in vitro dephosphorylation, reactions were performed in vitro as described above. The complete intracellular portion of PTPx was expressed in bacteria as a GST fusion protein using bacterial expression vectors for PTPx (kind gift of Dr S. Tracy) or PTPx-C433A. Approximately 100 ng bPTPx were incubated with 2P-labeled ppT60 in 30 min at 37°C. Polyacrylamide gel electrophoresis and CNBr cleavage were done as described above.

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