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Receptor protein tyrosine phosphatase α activates pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$ 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 pp60$^{c\text{-}src}$ by RPTPα. Our results demonstrate that RPTPα is involved in neuronal differentiation and imply a role for pp60$^{c\text{-}src}$ in the differentiation process.

Key words: embryonal carcinoma cells/neuronal differentiation/pp60$^{c\text{-}src}$/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 kinsases (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-Zanca *et al.*, 1989; Bothwell, 1991; Lamballe *et al.*, 1991; Middlemass *et al.*, 1991). In addition, the cytoplasmically localized PTK pp60$^{c\text{-}src}$ has been implicated in neuronal differentiation both *in vitro* and *in vivo*. c-*src* mRNA expression and pp60$^{c\text{-}src}$ 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 $pp60^{c\text{-}src}$. Recently, Zheng *et al.* (1992) demonstrated that $pp60^{c\text{-}src}$ is activated in fibroblasts that overexpress PTPα. Similarly, the RPTPα-transfected P19 cells display enhanced $pp60^{c\text{-}src}$ kinase activity, but not expression, which is correlated with decreased phosphorylation of the regulatory site (Tyr527) of $pp60^{c\text{-}src}$. In addition, we demonstrate that bacterially expressed PTPα can dephosphorylate Tyr527 of $pp60^{c\text{-}src}$ *in vitro*. Our results demonstrate that RPTPα plays an important role in neuronal differentiation which may involve activation of $pp60^{c\text{-}src}$.

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.

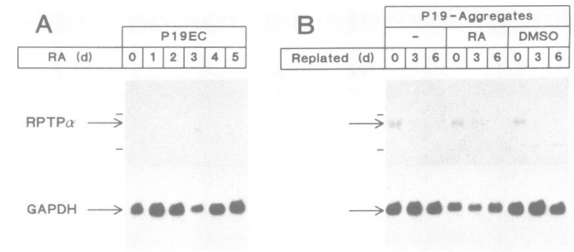

Fig. 1. Differential expression of RPTPα during differentiation of P19 EC cells. (**A**) P19 EC cells were differentiated in monolayer with RA (10^{-6} M, final concentration) for 0–5 days. Subsequently RNA was isolated and fractionated on a 0.8% formaldehyde–agarose gel (15 μg of total RNA per lane), transferred to nitrocellulose and hybridized to a probe, specific for RPTPα, as described in Materials and methods. (**B**) P19 cells were allowed to aggregate by culturing in suspension on a layer of agarose (1%) for 5 days. Subsequently the aggregates were either replated in tissue culture dishes for the times indicated or RNA was immediately isolated and processed as described in panel A (0 day replated). Aggregation and replating were done in the absence of chemical agents (–), in presence of RA (10^{-7} M, final concentration) (RA) or in presence of dimethyl sulfoxide (1%, final concentration) (DMSO). Expression of GAPDH was monitored to correct for possible differences in the amount of RNA loaded per lane.

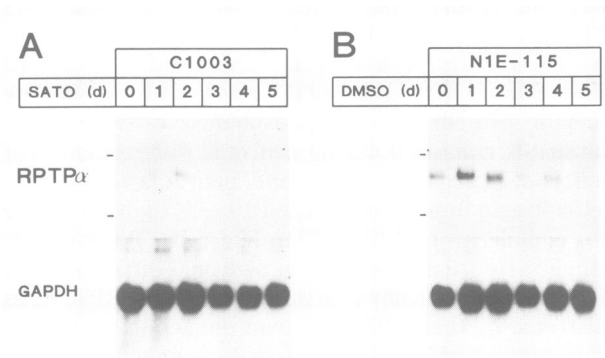

Fig. 2. Transient expression of RPTPα mRNA during neuronal differentiation. (**A**) C1003 cells were cultured in serum-free SATO medium for 0–5 days, as indicated. (**B**) N1E-115 cells were induced to differentiate in medium, supplemented with 2% FCS and 1% DMSO for 0–5 days, as indicated. Subsequently RNA was isolated and processed as described in the legend to Figure 1. Nitrocellulose filters were sequentially hybridized to probes specific for RPTPα and GAPDH, as described in Materials and methods.

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 PTPase 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 geneticin selection for 14 days, colonies were picked and established as stable cell lines.

Table I. Properties of P19-RPTPα transfectants

Cell line	Immunofluorescence markers[a]				Soft agar growth[c]
	mRNA[b]	SSEA-1	ECMA-7	TROMA1	
P19 EC WT	−	++	++	−	++++
P19 RA WT[d]	−	−	−	++	−
P19 AG WT	++++	ND	ND	ND	ND
P19-N-2	−	++	+	−	+++
P19-RPTPαC433A-54	++	++	++	−	ND
P19-RPTPαC433A-59	++	++	++	−	ND
P19-RPTPα-2	+	ND	ND	ND	ND
P19-RPTPα-5	++	++	++	−	+
P19-RPTPα-17	++	++	++	−	++
P19-RPTPα-38	+++	++	++	−	+++
P19-RPTPα-69	++	++	++	−	++
P19-RPTPα-75	++	++	++	−	++++

[a] − and ++ indicate relative degrees of immunofluorescence intensity.
[b] −, +, ++, +++ and ++++ indicate relative levels of exogenous RPTPα mRNA expression, except for P19 aggregates (P19 AG WT) where endogenous RPTPα expression is indicated.
[c] −, +, ++, +++ and ++++ indicate the ability to grow in soft agar, relative to wild type P19 cells.
[d] P19 RA WT indicates wild type P19 cells after RA-induced differentiation for 5 days for columns 1−4 and soft agar growth in the presence of RA for column 5.
ND, not determined.

RNA was isolated from these stable cell lines and probed for RPTPα mRNA expression. Exogenous and endogenous transcripts could be discerned since pSG-RPTPα and pSG-RPTPαC433A give rise to a smaller transcript (2.7 kb) than endogenous RPTPα transcripts (3.5 kb) (data not shown). None of the stably transfected P19 cells expressed endogenous RPTPα mRNA. Several clones, expressing exogenous RPTPα or RPTPαC433A mRNA, were selected for further analysis (Table I). To investigate whether these selected clones express RPTPα protein, nearly confluent cells (except P19-RPTPα-69, ~50% confluent) were labelled with [^{35}S]methionine and [^{35}S]cysteine and subsequently RPTPα was immunoprecipitated, using polyclonal anti-RPTPα antiserum 2A (Sap *et al.*, 1990). As a positive control RPTPα was immunoprecipitated from ^{35}S-labelled human embryonic kidney 293 cells, transiently transfected with pSG-RPTPα. Using this effective transient expression system, RPTPα could be demonstrated as a 130 kDa protein in 293 cells transiently transfected with pSG-RPTPα, 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 RPTPα protein (Figure 3), as expected from the Northern blotting analysis (Table I). Several clones were isolated, expressing mutant RPTPαC433A protein at different levels (P19-RPTPαC433A-54 and -59). In addition, stable cell lines were isolated, including P19-RPTPα-5, 17, 69 and 75, expressing RPTPα as a 130 kDa protein, with P19-RPTPα-5, 69 and 75 expressing the highest levels.

Morphologically the stably transfected cells resemble wild type P19 EC cells. The differentiation state of these clones was investigated, using immunofluorescence 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-RPTPα and RPTPαC433A 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-RPTPα 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 RPTPα or RPTPαC433A 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 RPTPα or a non-functional mutant, RPTPαC433A, in P19 EC cells, in itself, does not induce differentiation.

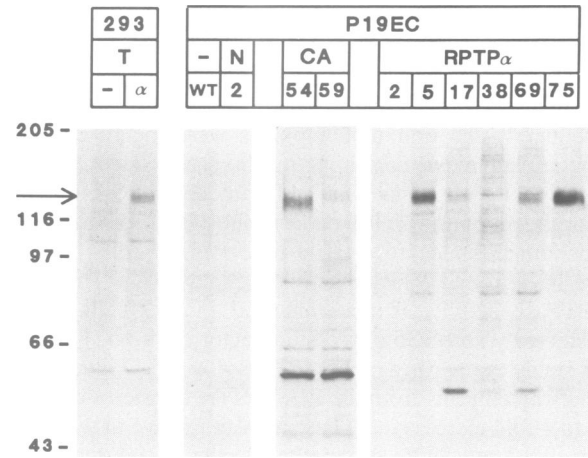

Fig. 3. Stably transfected P19 EC cells express RPTPα as a protein of 130 kDa. 293 cells (lanes T) were transiently transfected with pSG5 (−) or pSG-RPTPα (α). The transiently transfected 293 cells, wild type P19 EC cells (WT), P19 EC cells stably transfected with pSG5 (N2), P19 EC cells stably transfected with pSG-RPTPαC433A, a functional mutant of RPTPα (CA-54 and -59) and six different stable P19-RPTPα transfectants (clones 2−75) were labelled with [^{35}S]methionine and [^{35}S]cysteine (25 μCi/ml) for 16 h. Subsequently the cells were lysed and RPTPα was immunoprecipitated, using the polyclonal anti-RPTPα antiserum 2A. After extensive washing, the Sepharose beads were resuspended in Laemmli sample buffer containing SDS and the supernatants were electrophoresed on a 10% SDS−polyacrylamide gel, together with a molecular weight marker. The size of the molecular weight marker is indicated in kDa on the left. The position of the 130 kDa RPTPα is indicated with an arrow.

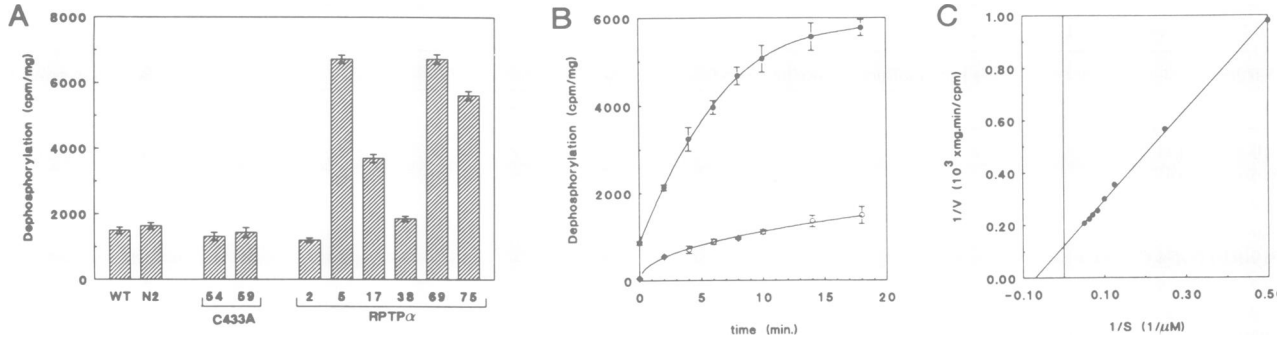

Fig. 4. Stably transfected P19 cells express functional RPTPα. (**A**) RPTPα was immunoprecipitated, using polyclonal anti-RPTPα antiserum 2A, from wild type P19 EC cells (WT), P19 cells stably transfected with expression vector, P19-N-2 (N2), P19 cells expressing functionally mutant RPTPα (C433A-54 and -59) and six P19-RPTPα transfectants (clones 2–75). The immunoprecipitates were incubated with [^{32}P]Tyr labelled MBP (4 μM) for 5 min at 37°C. Subsequently the amount of released free [^{32}P]phosphate was determined by Cerenkov counting. The values thus obtained were normalized against the amount of protein used for the immunoprecipitation. (**B**) Time curve of [^{32}P]MBP dephosphorylation by immunoprecipitates from P19-N-2 (open symbols) and P19-RPTPα-75 (closed symbols) cells. Incubations with [^{32}P]MBP were done as in panel A. The dephosphorylation reactions were stopped after the times indicated. (**C**) Lineweaver–Burk plot of P19-RPTPα-75 PTPase activity. Dephosphorylation reactions using different concentrations of [^{32}P]MBP (2–16 μM) were allowed to proceed for 5 min at 37°C, after which the released free [^{32}P]phosphate was determined. Reciprocal velocities of reaction (1/V) is given as a function of reciprocal substrate concentration (1/S).

Functional RPTPα is expressed by the P19-RPTPα transfectants

The PTPase activity of exogenous RPTPα in the P19-RPTPα transfectants was investigated by an *in vitro* PTPase assay. RPTPα or mutant RPTPαC433A was immunoprecipitated from the transfectants using anti-RPTPα antiserum 2A and subsequently the immunocomplexes were incubated with [^{32}P]phosphotyrosine containing myelin basic protein (MBP). Dephosphorylation was allowed to proceed for 5 min after which the amount of released [^{32}P]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 RPTPαC433A (Figure 4A). Immunoprecipitates from the P19-RPTPα transfectants show a 2- to 4-fold increase in PTPase activity relative to wild type P19 cells. The RPTPα protein levels correspond directly to the absolute levels of PTPase activity with P19-RPTPα-5, 69 and 75 displaying the highest substrate dephosphorylation activities (cf. Figures 3 and 4A). P19-RPTPα-2 and 38 show only background PTPase activity, reflecting the low level of RPTPα protein expression (Figures 3 and 4A).

Dephosphorylation by RPTPα, 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 RPTPα PTPase activity was determined. RPTPα was immunoprecipitated from P19-RPTPα-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 RPTPα is linear for at least 6 min and reaches a plateau after 18 min.

The enzymatic activity of purified human RPTPα has been characterized (Daum *et al.*, 1991). In order to compare the K_m of immunoprecipitated murine RPTPα with purified human RPTPα, MBP dephosphorylation by immunoprecipitated RPTPα was assayed at different substrate concentrations. The K_m of murine RPTPα, immunoprecipitated from P19-RPTPα-75, was calculated from a Lineweaver–Burk plot (Figure 4C) and is ~17 μM, which is in the same range as the K_m of purified human RPTPα (10 μM; Daum *et al.*, 1991). Although PTPase activity could be detected *in vitro* by RPTPα 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 RPTPα-75 cells (data not shown). In conclusion, the *in vitro* substrate dephosphorylation assays illustrate that the P19-RPTPα transfectants express functional RPTPα 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-RPTPα transfectants by RA

The ability of P19-RPTPα and RPTPαC433A transfectants to differentiate *in vitro* was assayed in order to determine whether ectopic expression of RPTPα 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-RPTPαC433A 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-RPTPα 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-RPTPα cell lines express varying amounts of RPTPα 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 RPTPα-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.

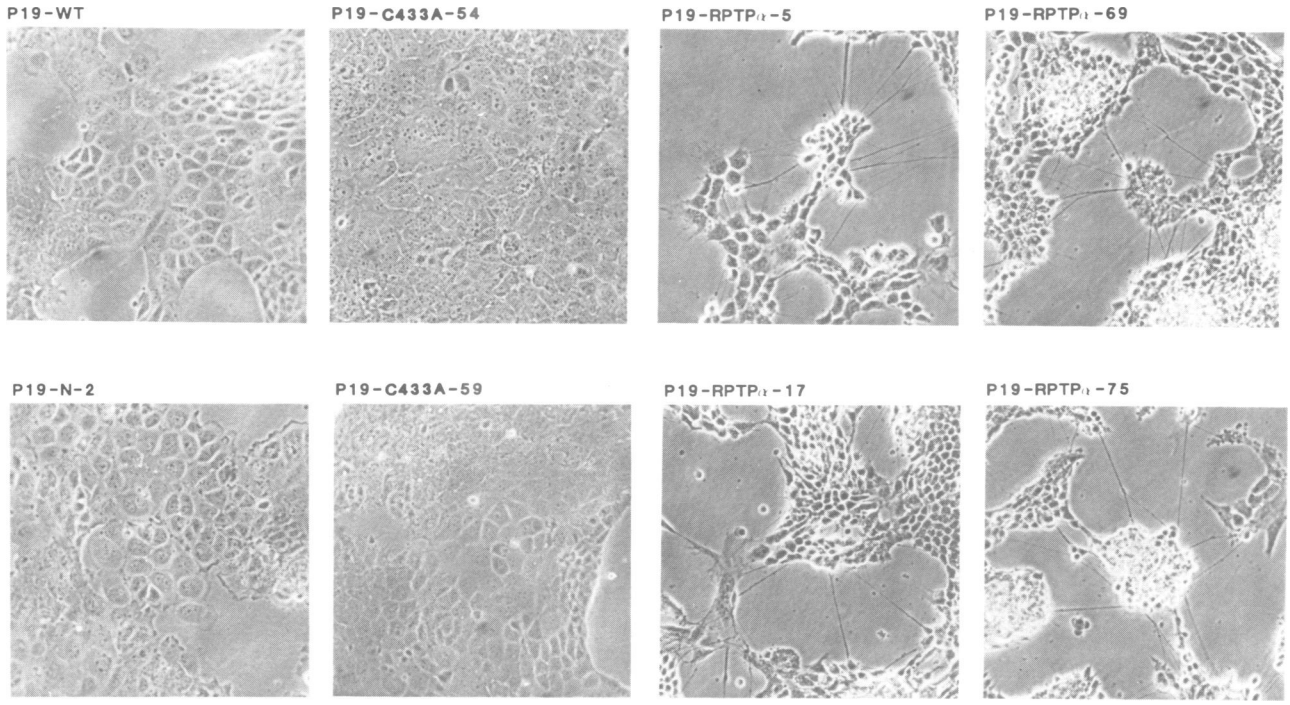

Fig. 5. Retinoic acid induces neuronal differentiation of P19-RPTPα transfectants. Differentiated wild type P19 EC cells (P19-WT), control transfected (P19-N-2), RPTPαC433A-expressing (54 and 59) and four different P19-RPTPα transfectants (P19-RPTPα-5 through -75) were photographed after 5 days of RA treatment in monolayer (10^{-6} M). Note the neurite extensions of the differentiated RPTPα transfectants which are not detected in the cultures of differentiated wild type, P19-N-2 or P19-RPTPαC433A cells.

Development of electrical excitability in RA-differentiated P19-RPTPα-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-RPTPαC433A-54 and P19-RPTPα-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-RPTPαC433A-54 cells did not show any electrical excitability (data not shown). By contrast, P19-RPTPα-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-RPTPα-75 cells have acquired electrical excitability (i.e. the ability to generate action potentials). Whereas all morphologically neuronal P19-RPTPα-75 cells exhibited electrical activity (n ~50), none of the P19-N-2 nor P19-RPTPαC433A-54 cells showed any electrical excitability (n ~25). The development of electrical excitability in P19-RPTPα-75, but not in P19-N-2 nor P19-RPTPαC433A-54 cells, demonstrates that upon RA-induced differentiation P19-RPTPα-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.

pp60$^{c\text{-}src}$ activity is enhanced in RPTPα-expressing P19 cells

In search of possible mechanisms, underlying the observed neuronal differentiation, we investigated pp60$^{c\text{-}src}$ expression and activity in P19-N-2, P19-RPTPαC433A-54 and P19-RPTPα-75 cells. During neuronal differentiation of a number of cell lines, including P19 and neuroblastoma cells, pp60$^{c\text{-}src}$ expression and kinase activity are enhanced (Brugge *et al.*, 1985; Lynch *et al.*, 1986; Boulter and Wagner, 1988a; Bjelfman *et al.*, 1990). pp60$^{c\text{-}src}$ was immunoprecipitated from ^{35}S-labelled P19-N-2, P19-RPTPαC433A-54 and P19-RPTPα-75 cells, using monoclonal anti-pp60$^{c\text{-}src}$ antibodies, MAb327 (Lipsich *et al.*, 1983). Equal amounts of ^{35}S-labelled proteins, as determined by liquid scintillation counting of aliquots of the cell lysates, were used for the different immunoprecipitations. pp60$^{c\text{-}src}$ activity was determined by an immunocomplex kinase reaction, using MAb327, and as a substrate acid denatured enolase. There is no significant difference in pp60$^{c\text{-}src}$ protein levels between P19-N-2, P19-RPTPαC433A-54 and P19-RPTPα-75 cells (Figure 7A), indicating that ectopic expression of RPTPα does not affect pp60$^{c\text{-}src}$ expression. By contrast, pp60$^{c\text{-}src}$ auto- and substrate phosphorylation activity is enhanced 4- to 6-fold in P19-RPTPα-75 as compared with P19-N-2 and P19-RPTPαC433A-54 (Figure 7B). In the RPTPα-transfected as well as the control cell lines pp60$^{c\text{-}src}$ protein levels and kinase activity is enhanced 2-fold in response to RA-induced differentiation (Figure 7A,B). Apparently, pp60$^{c\text{-}src}$ kinase activity, but not expression, is enhanced in the RPTPα-transfected P19 cells, as compared with the control cells.

Dephosphorylation of the regulatory site of pp60$^{c\text{-}src}$ (Tyr527) in P19-RPTPα transfectants

It has been reported that dephosphorylation of pp60$^{c\text{-}src}$ *in vitro* leads to a 10- to 20-fold increase in kinase activity (Courtneidge, 1985; Cooper and King, 1986). A C-terminal Tyr residue (Tyr527) has been identified to be the regulatory site, inhibiting pp60$^{c\text{-}src}$ activity upon phosphorylation (Cooper and King, 1986; Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987;

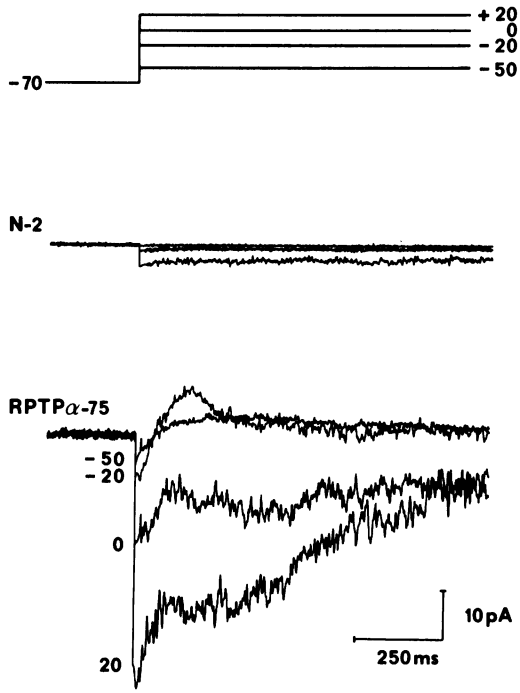

Fig. 6. Electrical excitability of RA-differentiated RPTPα-transfected P19 cells. P19-N-2 (N-2) and P19-RPTPα-75 (RPTPα-75) cells were treated with RA (10^{-6} M) for 5 days. Representative whole cell patch clamp traces demonstrate that in P19-RPTPα-75 cells depolarization from a holding potential of −70 mV to more positive values evokes large inward currents. The P19-N-2 cells do not display any sign of voltage dependent currents. The voltage protocol is depicted at the top of the figure (values in mV). The traces have been corrected for leak and capacitative currents.

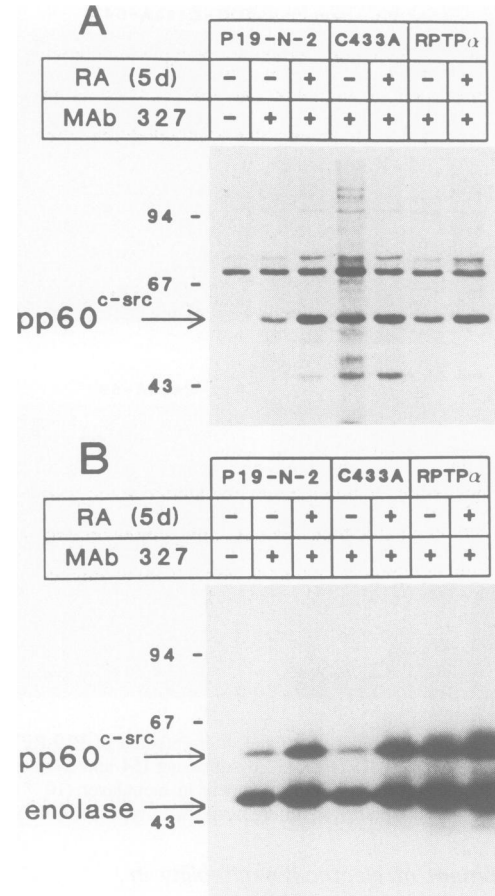

Fig. 7. pp60$^{c\text{-}src}$ kinase activity is enhanced in RPTPα-transfected P19 cells. (**A**) Immunoprecipitation of pp60$^{c\text{-}src}$ from ^{35}S-labelled P19-N-2 (N-2), P19-RPTPαC433A-54 (C433A) and P19-RPTPα-75 (RPTPα) cells, using monoclonal anti-pp60$^{c\text{-}src}$ antibody MAb 327. pp60$^{c\text{-}src}$ was immunoprecipitated from undifferentiated (0 d RA) as well as RA-differentiated (5 d RA) cells. Equal amounts of ^{35}S-labelled proteins, as determined by liquid scintillation counting of an aliquot of the cell lysates, were used for the immunoprecipitation in each lane. The molecular weights of markers that were co-electrophoresed with the immunoprecipitates on an SDS−polyacrylamide gel (10%) are indicated in kDa on the left. (**B**) pp60$^{c\text{-}src}$ kinase activity was determined by immunoprecipitation of pp60$^{c\text{-}src}$ (1 mg of total cell lysate protein per sample), using MAb 327, from undifferentiated (0 d RA) or RA-differentiated (5 d RA) P19-N-2 P19-RPTPαC433A-54 (C433A) and P19-RPTPα-75 (RPTPα) cells. Subsequently immunocomplex kinase reactions were done as described in Materials and methods with acid denatured enolase as a substrate. As a negative control, MAb 327 was omitted from the immunoprecipitation protocol.

Reynolds *et al.*, 1987). In order to investigate the phosphorylation state of pp60$^{c\text{-}src}$, both undifferentiated and 5 day RA-treated P19-N-2, P19-RPTPαC433A-54 and P19-RPTPα-75 cells were metabolically labelled with [^{32}P]orthophosphate. pp60$^{c\text{-}src}$ was immunoprecipitated using MAb327 and subsequently ^{32}P-labelled pp60$^{c\text{-}src}$ was digested with cyanogen bromide (CNBr). The major phosphorylation sites of pp60$^{c\text{-}src}$ 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 ^{32}P, 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$^{c\text{-}src}$ ^{32}P-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 PTPα (bPTPα) but not mutant bPTPαC433A dephosphorylated Tyr527, present in the 4 kDa peptide of *in vivo* labelled pp60$^{c\text{-}src}$ (Figure 8C). Phospho-Tyr416, present in *in vitro* labelled pp60$^{c\text{-}src}$ can be dephosphorylated by bPTPα also (Figure 8C). In conclusion, pp60$^{c\text{-}src}$ Tyr527 phosphorylation is reduced in RPTPα-transfected P19 cells. Furthermore, Tyr527 is dephosphorylated *in vitro* by bPTPα, which may

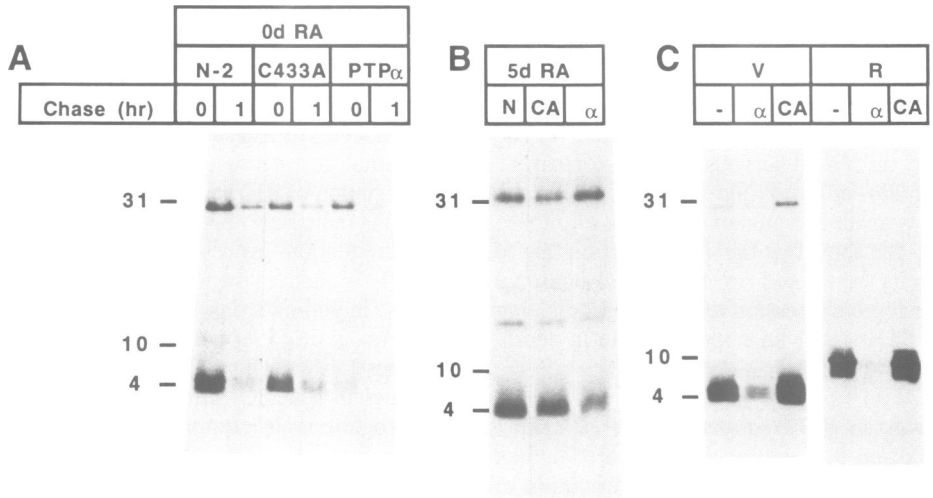

Fig. 8. pp60$^{c\text{-}src}$ Tyr527 dephosphorylation by RPTPα *in vivo* and *in vitro*. (**A**) Undifferentiated P19-N-2 (N-2), P19-RPTPαC433A-54 (C433A) and P19-RPTPα-75 (PTPα) cells were labelled with [^{32}P]orthophosphate for 16 h. Subsequently the medium was replaced with regular cell growth medium and the cells were incubated for another hour (1 h chase). Following immunoprecipitation, pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ phosphopeptides are indicated in kDa on the left. (**B**) P19-N-2 (N2), P19-RPTPαC433A-54 (CA) and P19-RPTPα-75 (α) cells were treated with RA (10^{-6} M) for 5 days and labelled with [^{32}P]orthophosphate. pp60$^{c\text{-}src}$ was immunoprecipitated, digested with CNBr and electrophoresed on a 20% gel as described in panel A. (**C**) Immunoprecipitated *in vivo* labelled pp60$^{c\text{-}src}$ (V), or *in vitro* labelled pp60$^{c\text{-}src}$ (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$^{c\text{-}src}$ was eluted from the gel, digested with CNBr and analysed by SDS−PAGE.

indicate that RPTPα directly dephosphorylates pp60$^{c\text{-}src}$ Tyr527 *in vivo* as well. Reduced Tyr527 phosphorylation may account for the observed enhanced activity of pp60$^{c\text{-}src}$. No enhanced Tyr416 phosphorylation is detected *in vivo* in the RPTPα-transfected cells (Figure 8A and B), although pp60$^{c\text{-}src}$ is activated in these cells. However, bPTPα readily dephosphorylates Tyr416 *in vitro*, which may indicate that the autophosphorylation site of pp60$^{c\text{-}src}$ is dephosphorylated by RPTPα *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 RPTPα expression is enhanced during neuronal differentiation and that overexpression of RPTPα alters the differentiation fate of pluripotent P19 EC cells in favour of neuronal differentiation. Furthermore, we show that pp60$^{c\text{-}src}$, a non-receptor PTK which has previously been shown to be involved in neuronal differentiation, is activated in RPTPα-transfected cells. We propose that RPTPα-mediated activation of pp60$^{c\text{-}src}$ triggers neuronal differentiation.

Expression of RPTPα during neuronal differentiation

Analysis of endogenous RPTPα mRNA expression during *in vitro* differentiation of cell lines, capable of neuronal differentiation, shows that RPTPα is transiently induced in the differentiation process, with maximal RPTPα 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 RPTPα is widely expressed in adult murine tissues, highest levels of RPTPα mRNA expression have been observed in the brain (Sap *et al.*, 1990). Moreover, recent *in situ* hybridization experiments show highest RPTPα mRNA levels in the central nervous system of 16 day old rat embryos (J.Sap and J.Schlessinger, personal communication), indicating that RPTPα may play a role during neurogenesis *in vivo*. The RPTPα 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 RPTPα is involved in neuronal differentiation rather than neuronal outgrowth.

Neuronal differentiation in RPTPα-transfected P19 cells

Several P19 EC cell lines were derived expressing mutant, inactive RPTPαC433A or functional RPTPα protein with similar enzymatic characteristics as previously described human RPTPα (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 [^{3}H]thymidine incorporation assays were observed in the P19-RPTPα transfectants, indicating that RPTPα is not involved in growth inhibition of these cells (data not shown).

Ectopic expression of functional RPTPα in P19 EC cells does not induce differentiation, since several undifferentiated RPTPα-transfected cell lines could be isolated. However, a high percentage of neuronal cells was obtained upon RA-induced differentiation of P19-RPTPα transfectants, while wild type and control transfected P19 cells give rise to endoderm- and mesoderm-like cells under these conditions. The RA-treated RPTPα-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 PTPases might play an important role in *Drosophila* neural development. Three transmembrane PTPases 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 fate of pluripotent P19 cells is altered in favour of neuronal differentiation by ectopic expression of RPTPα, indicating that PTPases, like PTKs, play an important role in neural development.

pp60$^{c\text{-}src}$ is activated in RPTPα-transfected P19 cells

Recently it was reported that ectopic expression of human PTPα in rat embryo fibroblasts leads to transformation of these cells (Zheng *et al.*, 1992). Zheng *et al.* demonstrate that pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$. We demonstrate that pp60$^{c\text{-}src}$ kinase activity is enhanced 4- to 6-fold in RPTPα-transfected P19 cells, relative to control transfected and RPTPαC433A-transfected cells, while pp60$^{c\text{-}src}$ expression is similar in RPTPα-transfected and control P19 cells. Analysis of the phosphorylation state of pp60$^{c\text{-}src}$ by CNBr cleavage of *in vivo* ^{32}P-labelled pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ Tyr527 *in vitro*. Reduced phosphorylation of Tyr527 may account for the observed increase in pp60$^{c\text{-}src}$ activity, since it is well-established that this site plays a key role in regulation of pp60$^{c\text{-}src}$ activity (Courtneidge, 1985; Cooper and King, 1986; Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987). Transmembrane PTPase-mediated activation of cytoplasmically localized PTKs is not unprecedented, since it has been demonstrated that the Src family member pp56^{lck} is activated by the transmembrane PTPase CD45 (Mustelin *et al.*, 1989; Ostergaard *et al.*, 1989). In T cells CD45 specifically dephosphorylates the regulatory Tyr residue of pp56^{lck} (Tyr505), which is functionally similar to Tyr527 of pp60$^{c\text{-}src}$. Recently it was reported that overexpression of CD45 can activate another member of the Src family, p59^{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$^{c\text{-}src}$ 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$^{c\text{-}src}$ is essential for nerve growth factor induced neuronal differentiation of P12 pheochromocytoma cells (Kremer *et al.*, 1991), indicating that pp60$^{c\text{-}src}$ plays a crucial role in the differentiation process. Overexpression of highly active pp60$^{v\text{-}src}$ 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$^{v\text{-}src}$ and the moderately active pp60$^{c\text{-}src+}$, the neuronal variant of pp60$^{c\text{-}src}$ (Schmidt *et al.*, 1992). Overexpression of pp60$^{v\text{-}src}$ induces epithelial differentiation of P19 cells, while pp60$^{c\text{-}src+}$-transfected cells retain the undifferentiated phenotype. The pp60$^{v\text{-}src}$-transfected cells have lost their capacity to differentiate into neuronal cells, while pp60$^{c\text{-}src+}$ overexpression inhibits neuronal differentiation to some extent. The correlation of higher pp60^{src} kinase activity of pp60$^{v\text{-}src}$ 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$^{c\text{-}src}$ activity is enhanced during neuronal differentiation of P19 cells, while prior elevation of pp60^{src} activity by overexpression of pp60$^{v\text{-}src}$ or pp60$^{c\text{-}src+}$ 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$^{c\text{-}src}$, conceivably through direct RPTPα-mediated dephosphorylation of Tyr527. Activated endogenous pp60$^{c\text{-}src}$, in turn, leads to specific enhanced tyrosine phosphorylation of pp60$^{c\text{-}src}$ substrates, which may account for the observed RA-induced neuronal differentiation. It is noteworthy that only subtle modulations of overall phosphotyrosine content could be detected in RPTPα-transfected P19 cells, as compared with control cells (data not shown). This may indicate that pp60$^{c\text{-}src}$ is specifically dephosphorylated by RPTPα and that endogenous pp60$^{c\text{-}src}$ kinase activity is only moderately enhanced. Whereas we identify pp60$^{c\text{-}src}$ 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 RPTPα-transfected P19 cells in monolayer. These substrates may become phosphorylated on tyrosine as targets of activated pp60$^{c\text{-}src}$, thereby altering the differentiation fate of these cells in favour of neuronal differentiation. Although several pp60$^{c\text{-}src}$ substrates have been identified, it is presently not known what the key substrates are that mediate neuronal differentiation. Identification of pp60$^{c\text{-}src}$ and RPTPα 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, N1E-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 N1E-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 ^{32}P-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 0.1 × SSC–0.1% SDS at room temperature and once with preheated (55°C) 0.1 × SSC–0.1% SDS. The ^{32}P-labelled probes were obtained, using a multiprime labelling kit (Amersham) routinely with 50 ng DNA fragment and 50 μCi [α-^{32}P]dCTP (Amersham). As probes for the Northern blotting analysis a 0.4 kb *Hin*dIII–*Pst*I fragment, located in the extreme 5′ part of the RPTPα cDNA (Sap *et al.*, 1990; den Hertog *et al.*, 1992) and a 1.4 kb fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Fort *et al.*, 1985) were used.

Plasmids and transfections

PSG-RPTPα was generated by insertion of the complete cDNA of RPTPα (den Hertog *et al.*, 1992) in *Eco*RI/*Bam*HI cut pSG5 (Green *et al.*, 1988). PSG5 is an SV40 early promoter driven expression vector. The functional mutant of RPTPα (RPTPαC433A) 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′ CTACACCTGCACTGGCGTGGACCACGATAG 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-RPTPα or pSGRPTPαC433A, 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 seeded on gelatinized glass coverslips and at the appropriate time the cells were fixed with ice-cold methanol–acetic acid 95:5 (v/v). Incubation of the coverslips 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 (Solter and Knowles, 1978), ECMA-7 (Kemler *et al.*, 1980), TROMA1 (Kemler *et al.*, 1981), anti NF 70 (directed against a 70 kDa neurofilament 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 cm^2 dishes and after 24 h the cells were labelled in Met/Cys-free medium supplemented with 7.5% dialysed FCS and 25 μCi/ml (final concentration) Translabel (ICN), containing [^{35}S]methionine and [^{35}S]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 $MgCl_2$, 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 RPTPα immunoprecipitations polyclonal anti-RPTPα antiserum 2A (Sap *et al.*, 1990) and for pp60$^{c\text{-}src}$ 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 Laemmli 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, ^{32}P-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 $MnCl_2$ and 50 μCi/ml [γ-^{32}P]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 (Oncogene Science) essentially as described above. After labelling MBP was precipitated using 25% trichloroacetic acid (TCA) in order to remove free [γ-^{32}P]ATP. Using this protocol 80–90% of the ^{32}P-label was incorporated in phospho-Tyr as determined by phosphoamino acid analysis (data not shown).

For PTPase assays RPTPα 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 ^{32}P-labelled MBP (4 μM final concentration unless otherwise indicated), containing 3–6 × 10^4 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 Na_2HPO_4 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 $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.3 with NaOH. The patch pipette contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 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$^{c\text{-}src}$ kinase assay

For pp60$^{c\text{-}src}$ kinase assays, pp60$^{c\text{-}src}$ 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 $MgCl_2$), containing 2 μCi [γ-^{32}P]ATP and as a substrate 3 μg acid denatured enolase (Boehringer). After 10 min at room temperature Laemmli sample buffer was added, the samples were boiled for 5 min and electrophoresed on a 10% SDS–polyacrylamide gel.

CNBr cleavage of ^{32}P-labelled pp60$^{c\text{-}src}$

Cells were metabolically labelled with [^{32}P]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 regular growth medium for 1 h. pp60$^{c\text{-}src}$ was immunoprecipitated and electrophoresed on a 10% SDS–polyacrylamide gel as described above. pp60$^{c\text{-}src}$ 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 lyophilization the samples were run on a 20% SDS–polyacrylamide gel. For the *in vitro* dephosphorylation reactions $pp60^{c\text{-}src}$ was labelled *in vitro* or *in vivo*, as described above. The complete intracellular portion of PTPα was expressed in bacteria as a GST fusion protein using bacterial expression vectors for PTPα (kind gift of Dr S.Tracy) or PTPαC433A. Approximately 100 ng bPTPα were incubated with ^{32}P-labelled $pp60^{c\text{-}src}$ for 30 min at 37°C. Polyacrylamide gel electrophoresis and CNBr cleavage were done as described above.

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