Receptor protein tyrosine phosphatase α activates Src-family kinases and controls integrin-mediated responses in fibroblasts

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Background: Fyn and c-Src are two of the most widely expressed Src-family kinases. Both are strongly implicated in the control of cytoskeletal organization and in the generation of integrin-dependent signalling responses in fibroblasts. These proteins are representative of a large family of tyrosine kinases, the activity of which is tightly controlled by inhibitory phosphorylation of a carboxy-terminal tyrosine residue (Tyr527 in chicken c-Src); this phosphorylation induces the kinases to form an inactive conformation. Whereas the identity of such inhibitory Tyr527 kinases has been well established, no corresponding phosphatases have been identified that, under physiological conditions, function as positive regulators of c-Src and Fyn in fibroblasts.

Results: Receptor protein tyrosine phosphatase α (RPTPα) was inactivated by homologous recombination. Fibroblasts derived from these *RPTPα*$^{-/-}$ mice had impaired tyrosine kinase activity of both c-Src and Fyn, and this was accompanied by a concomitant increase in c-Src Tyr527 phosphorylation. *RPTPα*$^{-/-}$ fibroblasts also showed a reduction in the rate of spreading on fibronectin substrates, a trait that is a phenocopy of the effect of inactivation of the *c-src* gene. In response to adhesion on a fibronectin substrate, *RPTPα*$^{-/-}$ fibroblasts also exhibited characteristic deficiencies in integrin-mediated signalling responses, such as decreased tyrosine phosphorylation of the c-Src substrates Fak and p130^{cas}, and reduced activation of extracellular signal regulated (Erk) MAP kinases.

Conclusions: These observations demonstrate that RPTPα functions as a physiological upstream activator of Src-family kinases in fibroblasts and establish this tyrosine phosphatase as a newly identified regulator of integrin signalling.

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Received: 5 January 1999
Revised: 7 April 1999
Accepted: 22 April 1999

Published: 5 May 1999

Current Biology 1999, 9:505–511
http://biomednet.com/elecref/0960982200900505

 ISSN 0960-9822

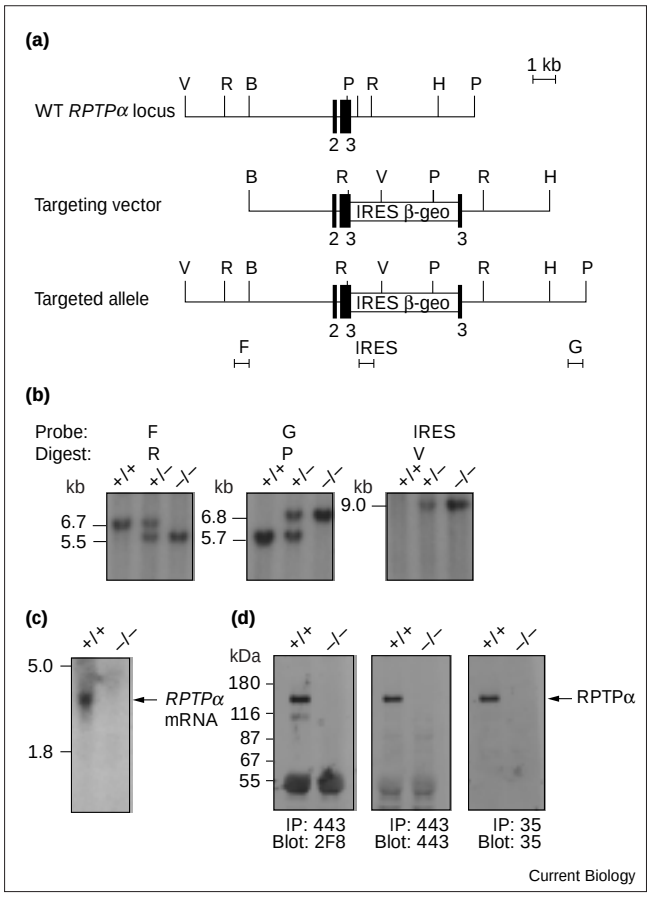
Background

Genetic inactivation of c-Src or Fyn, or transformation by v-Src — a gain-of-function mutation — affect cell adherence and cytoskeletal organization; these effects correlate with altered levels of tyrosine phosphorylation of proteins thought to mediate integrin signalling or control cytoskeletal architecture [1–3]. Src-family kinases clearly mediate integrin-dependent adhesive responses [4–8] because, during cell attachment on fibronectin matrices, c-Src is required for normal cell spreading [9], whereas Fyn is required for extracellular signal regulated kinase (Erk) activation [10].

A key mechanism controlling the activity of Src-family kinases is the reversible phosphorylation of a carboxy-terminal tyrosine residue — Tyr527 in chicken c-Src —that is characteristic of this enzyme family. This phosphorylation event, which is mediated by separate kinases (such as Csk and Chk [11,12]), favours the adoption of a conformational state associated with reduced catalytic activity, increased detergent solubility, and altered intracellular localization. Loss of this phosphorylation-based inhibitory switch, for example, by deletion or mutation of the Tyr527 phosphorylation site, is a common mechanism that leads to oncogenic activation of c-Src [13]. Conversely, dephosphorylation of Tyr527 by a specific protein tyrosine phosphatase (PTPase) may be a mechanism favouring the adoption of the 'open' kinase-active state. Such a mechanism appears to account for the requirement for the PTPase CD45 in antigen-receptor signalling pathways involving Lck and Fyn in lymphocytes [14,15]. CD45 may function in a different manner with respect to other Src-family kinases in macrophages, however [16].

RPTPα is a widely expressed transmembrane receptor protein tyrosine phosphatase [17] that can dephosphorylate Tyr527 of c-Src *in vitro*, and that can lead to increased c-Src kinase activity and transformation when overexpressed [18,19]. It has also been reported that RPTPα overexpression can modulate cell adhesion [20,21]. By relying on drastic overexpression, however, such studies have failed to provide reliable insight into the role of endogenous RPTPα in these processes or in signalling pathways involving the normal function of Src-family

Figure 1



Generation of an *RPTPα* null allele by gene targeting. (a) Schematic representation of the targeting vector and the structure of the wild-type (WT) *RPTPα* locus surrounding exons 2 and 3 [38] before (top) and after (bottom) homologous recombination. β-geo, β-galactosidase–neomycin-resistance fusion gene [39]; B, *Bam*HI; H, *Hind*III; P, *Pst*I; R, *Eco*RI; V, *Eco*RV; IRES, internal ribosomal entry site. The regions detected by probes F, G and IRES are also indicated. (b) Genotyping of wild-type (+/+), heterozygous $RPTP\alpha^{+/-}$ (+/–), and homozygous $RPTP\alpha^{-/-}$ (–/–) mice by Southern hybridization using probes F, G, or IRES. (c) Northern hybridization of RNA from embryonic fibroblasts derived from $RPTP\alpha^{+/+}$ or $RPTP\alpha^{-/-}$ embryos, using as probe a 0.7 kb *Xba*I fragment corresponding to the membrane-proximal PTPase domain of RPTPα. The 3 kb *RPTPα* mRNA is indicated. (d) Absence of RPTPα protein from $RPTP\alpha^{-/-}$ mouse brain. Mouse brain lysates derived from $RPTP\alpha^{+/+}$ or $RPTP\alpha^{-/-}$ animals were subjected to immunoprecipitation followed by immunoblotting using various combinations of antibodies recognizing different domains in RPTPα. The domains recognized by each antibody are as follows: 443, the entire intracellular domain; 2F8, the ectodomain; and 35, the carboxyl terminus. The 130 kDa RPTPα protein is indicated.

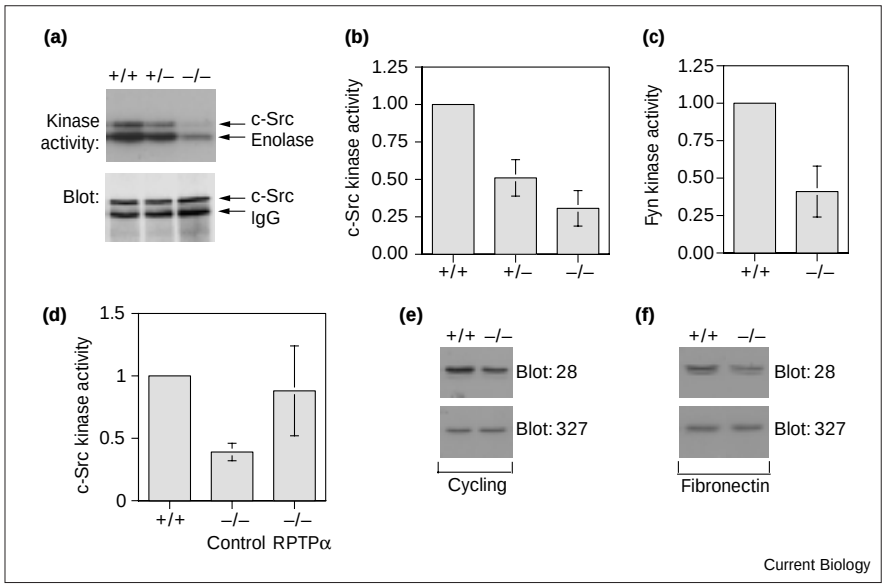
kinases. A deficiency in the kinase Csk can be partly rescued by null mutations of c-Src, thus firmly establishing the epistatic relationship between c-Src and its upstream modulator Csk [1]. By contrast, no definite functional link has thus far been apparent between c-Src and individual members of the PTPase family. We reasoned that the criteria for the identification of the phosphatase that acts as a physiological upstream activator of c-Src in a particular lineage or process would be twofold. First, deletion of the phosphatase should cause decreased c-Src catalytic activity. Second (and most importantly), loss of the PTPase should generate a phenotype that mimics, or at least has some characteristics in common with, that resulting from a null mutation of c-Src. We therefore used homologous recombination in mouse embryonic stem (ES) cells to generate a null allele at the *RPTPα* locus. The phenotype observed in $RPTP\alpha^{-/-}$ fibroblasts derived from these mice included impaired c-Src and Fyn kinase activity and reduced cellular responses to adhesion on fibronectin, consistent with RPTPα acting as an upstream activator of Src-family kinases during this process.

Results

A β-galactosidase–neomycin-resistance (β-geo) fusion gene, preceded by an internal ribosomal entry site (IRES), was introduced into exon 3 (corresponding to the RPTPα extracellular domain) of the mouse *RPTPα* locus by homologous recombination (Figure 1a,b). Homozygous RPTPα mutant progeny were born in accordance with expected Mendelian ratios (data not shown). RNA analysis, using a probe corresponding to the region encoding the RPTPα membrane-proximal phosphatase domain, revealed no detectable levels of *RPTPα* mRNA in homozygous mutant embryonic fibroblasts (Figure 1c). Furthermore, protein analysis, using a battery of antibodies to various domains in the RPTPα protein, failed to detect any cross-reactive protein species in various tissues and cells from homozygous mutant animals, demonstrating the generation of a null allele (Figure 1d). This analysis also failed to detect evidence for the secretion of a small soluble RPTPα extracellular-domain fragment that might still be produced from the mutant locus upstream of the insertion site (data not shown). $RPTP\alpha^{-/-}$ animals survived to adulthood and were fertile, but displayed gender-specific changes in body size, as well as nurturing defects. A full and detailed characterization of these whole-animal phenotypes will be reported at a later date.

To characterize the effects of *RPTPα* gene inactivation, we performed *in vitro* kinase activity measurements of c-Src and Fyn proteins immunoprecipitated from explanted, exponentially growing, primary fibroblasts derived from wild-type mice (+/+), $RPTP\alpha^{+/-}$ heterozygous mice, or $RPTP\alpha^{-/-}$ homozygous mice. These experiments revealed dose-dependent control of the activity of these kinases by RPTPα: the activities of c-Src and Fyn towards enolase —provided as an exogenous substrate — were reduced by 70% and 60%, respectively, in $RPTP\alpha^{-/-}$ cells compared with wild-type cells (Figure 2a–c). This change in activity occurred in the absence of changes in c-Src or Fyn protein levels (Figure 2a). The c-Src kinase deficiency in cells derived from $RPTP\alpha^{-/-}$ embryos was

Figure 2



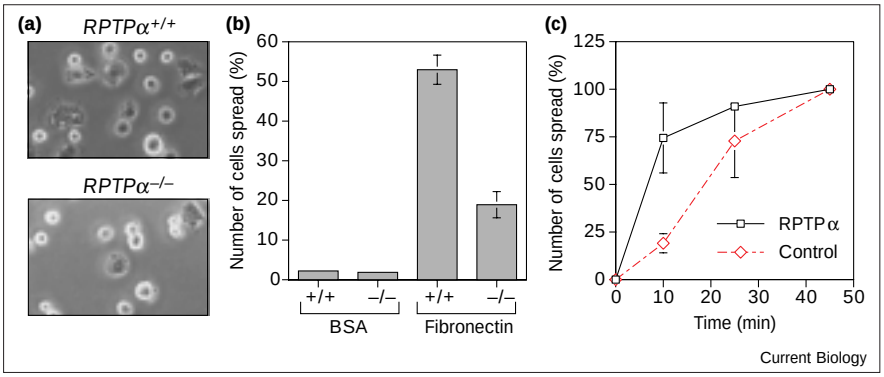
RPTPα gene dosage affects c-Src and Fyn kinase activity in fibroblasts. The (a,b) c-Src or (c) Fyn proteins were immunoprecipitated from cells of the indicated genotypes at the *RPTPα* locus and their activity was assayed by measuring the amount of ^{32}P incorporated into an exogenous substrate (enolase) *in vitro*. (a) Autoradiograph of a typical c-Src *in vitro* kinase assay, showing the incorporation into enolase, as well as autophosphorylation of c-Src (top panel), and a control anti-c-Src immunoblot of an aliquot of the immunoprecipitate (bottom panel) to show that equal amounts of protein were used in each assay. (b) Quantitation of c-Src kinase activity. Bars represent the average ± standard error (SE) of three separate experiments. (c) Quantitation of Fyn kinase activity. Bars represent the average ± SE of four separate experiments. Data in (a–c) were confirmed using several independently isolated primary or established fibroblast cultures. (d) Restoration of c-Src kinase activity by reintroduction of RPTPα into *RPTPα*$^{-/-}$ fibroblasts. *RPTPα*$^{-/-}$ cells were transfected with empty vector (control) or an RPTPα expression plasmid, and individual clones assayed for c-Src activity using the *in vitro* kinase reaction described in (a–c). Data are an average of three clones for cells transfected with control vector and two for the RPTPα-expressing clones. In (b–d) the c-Src or Fyn kinase activity in *RPTPα*$^{+/+}$ cells was taken as 1. (e,f) Difference in reactivity of c-Src protein from primary wild-type (+/+) and *RPTPα*$^{-/-}$ (–/–) fibroblast cells to an antibody (clone 28) specific for the Tyr527-dephosphorylated (active) form of Src. (e) c-Src protein was precipitated from equal amounts of lysate from the respective cycling cells, using an antibody (327) against the SH3 domain of Src; the precipitate was then halved and each half immunoblotted with either clone 28 (top) or clone 327 (bottom) antibodies. The increased reactivity to clone 28 in *RPTPα*$^{+/+}$ cells compared with *RPTPα*$^{-/-}$ cells averaged 1.7 (as revealed by densitometry) over four experiments, performed with different pairs of primary cells or established lines. (f) Equal amounts of total lysate from wild-type (+/+) or *RPTPα*$^{-/-}$ (–/–) primary fibroblasts allowed to adhere for 15 min on a fibronectin-coated surface were subjected to immunoblotting with clone 28 (top) or clone 327 (bottom) antibodies.

rescued by reintroduction of exogenous RPTPα (Figure 2d), and rescue of c-Src kinase activity by RPTPα was dependent on the dimerization state of the RPTPα protein (G. Jiang, J. den Hertog, J. Su, J. Noel, J. Sap, and T. Hunter, unpublished observations). Reduced c-Src kinase activity was also observed in tissue lysates from *RPTPα*$^{-/-}$ animals (data not shown).

To determine whether the effect of RPTPα ablation on c-Src kinase activity was associated with changes in phosphorylation of Tyr527 in c-Src, we made use of a monoclonal antibody, clone 28, that specifically recognizes the active (non-Tyr527-phosphorylated) form of c-Src. This antibody recognizes the motif surrounding Tyr527 in a manner that is hindered by phosphorylation of Tyr527 [22]. Equal amounts of lysate from exponentially growing wild-type or *RPTPα*$^{-/-}$ cells were subjected to immunoprecipitation with antibody 327, which recognizes an epitope in the Src homology 3 (SH3) domain of c-Src. The immunoprecipitate was subsequently halved and each half immunoblotted either with antibody 327 (as a control to normalize for the total amount of c-Src protein), or with clone 28. These experiments revealed a higher reactivity of c-Src with the activation-specific antibody (clone 28) in wild-type cells compared with their *RPTPα*$^{-/-}$ counterparts (Figure 2e). Higher reactivity to clone 28 in wild-type cells compared with *RPTPα*$^{-/-}$ cells was also observed in total cell lysates during short-term adhesion assays on fibronectin (Figure 2f).

c-Src$^{-/-}$ fibroblasts have previously been shown to display a delay in adhesion-dependent spreading on fibronectin-coated substrates [9]. This effect can be rescued by kinase-negative versions of c-Src, demonstrating that physiological c-Src functions rely on adaptor-like mechanisms as well as on kinase activity. We explored whether c-Src and RPTPα might be linked in a common signalling cascade by determining whether the absence of RPTPα would mimic the fibroblast-spreading-deficiency phenotype characteristic of c-Src ablation [9]. Lack of RPTPα did not affect the efficiency of cell attachment to a fibronectin-coated surface (data not shown), suggesting that the integrin-adhesive function *per se* was not detectably affected. By contrast, following the attachment phase, *RPTPα*$^{-/-}$ cells displayed a clear but transient delay in cell spreading, which was most prominent between

Figure 3



RPTPα ablation delays the integrin-mediated cell spreading response of fibroblasts. (a) *RPTPα*$^{+/+}$ and *RPTPα*$^{-/-}$ cells 15 min after plating onto fibronectin-coated surfaces. (b) *RPTPα*$^{+/+}$ and *RPTPα*$^{-/-}$ cells were allowed to adhere for 15 min to bovine serum albumin (BSA)- or fibronectin-coated surfaces, and cell spreading expressed as a fraction of total adherent cells. (c) Accelerated *RPTPα*$^{-/-}$ cell spreading upon reintroduction of wild-type RPTPα. *RPTPα*$^{-/-}$ cells were infected with a retrovirus expressing RPTPα or with empty control vector and cell spreading was monitored over time after plating onto a fibronectin-coated surface.

10 and 20 minutes (Figure 3a,b), similar to that reported for *c-src* gene ablation [9]. In a rescue experiment, infection of *RPTPα*$^{-/-}$ cells with an RPTPα-expressing retrovirus (but not with a control virus) enhanced the spreading rate (Figure 3c), supporting the notion that the spreading deficit in RPTPα$^{-/-}$ cells is reversible, and a relatively direct result of the lack of RPTPα.

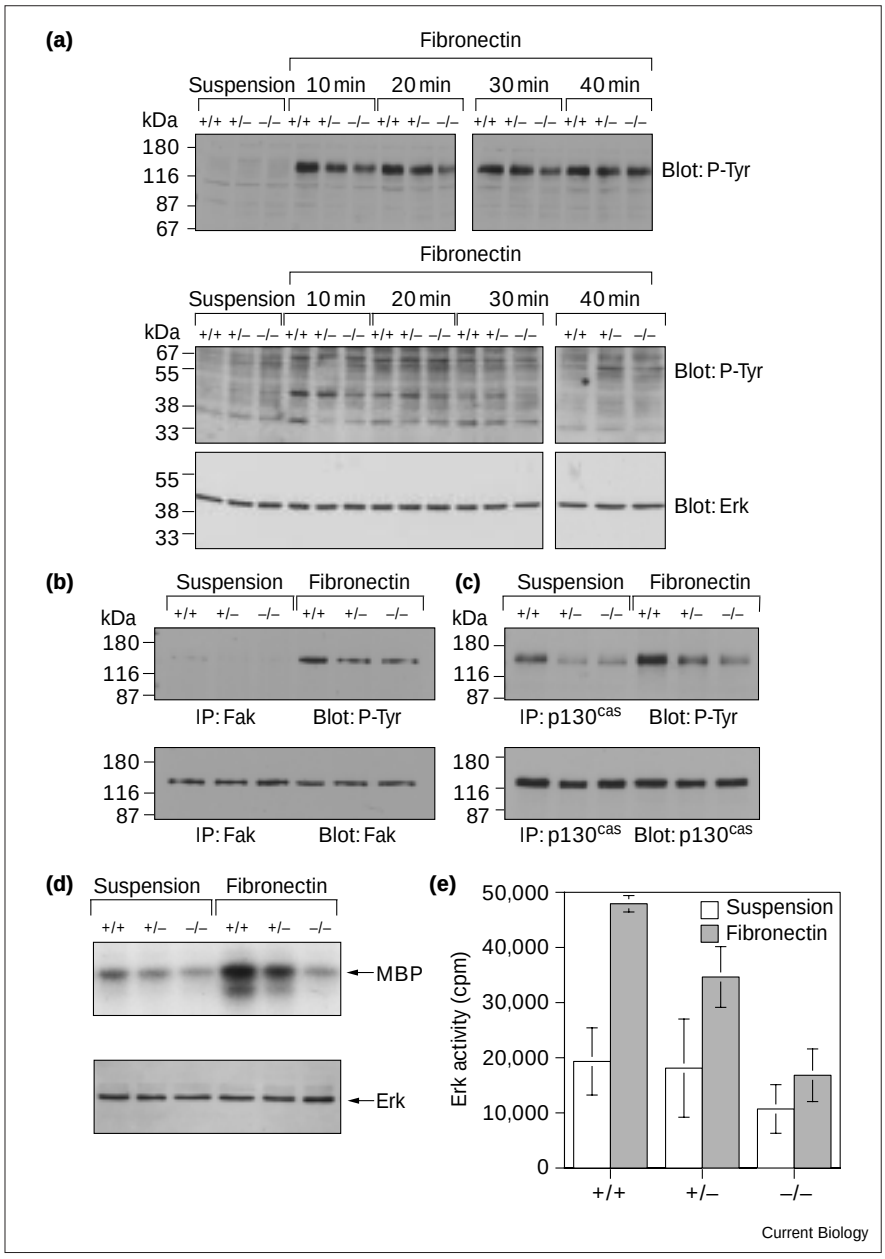
To determine whether deficiencies in proximal integrin-dependent signal transduction could account for the reduced spreading rate of *RPTPα*$^{-/-}$ fibroblasts, we monitored the levels of protein phosphorylation on tyrosine residues during the spreading process. Anti-phosphotyrosine immunoblotting analysis of total cellular lysates prepared at various stages of spreading revealed reduced tyrosine phosphorylation of proteins in the 130 kDa (Figure 4a, upper panel) and 40 kDa (Figure 4a, middle panel) range in cells lacking RPTPα, supporting the notion that RPTPα functions as an upstream activator of a tyrosine kinase during this process. Focal adhesion kinase (Fak), a protein tyrosine kinase, and the Crk-associated substrate p130^{cas}, a multi-adaptor docking protein, were both identified as proteins the phosphorylation of which is markedly enhanced in v-Src-transformed cells; in non-transformed cells, both proteins are phosphorylated by, and associate with, c-Src in response to integrin-dependent cell adhesion, probably mediating some of the integrin-dependent events [2–4]. Immunoprecipitation with the respective specific antibodies revealed reduced tyrosine phosphorylation of p130^{cas} and Fak in *RPTPα*$^{-/-}$ cells, consistent with impaired c-Src function (Figure 4b,c). Concomitant with tyrosine kinase activation, integrin-dependent fibroblast responses also include activation of the Erk arm of the mitogen-activated protein (MAP) kinase cascade [5,7], either through recruitment of the Grb2 adaptor to Fak–c-Src complexes [8,23], or through Fyn-mediated tyrosine phosphorylation of the adaptor protein Shc [10,24]. In accordance with this, attachment and spreading of wild-type fibroblasts on a fibronectin substrate was accompanied by enhanced Erk activity, as assessed by phosphorylation of exogenously supplied myelin basic protein (MBP) in anti-Erk immunoprecipitates. By contrast, Erk activation was significantly reduced as a consequence of RPTPα ablation (Figure 4d,e).

Discussion

Although we cannot exclude additional mechanisms — such as the involvement of an intermediate phosphatase, a role for RPTPα in regulating Csk, or the ability of other PTPases to contribute to the control of c-Src tyrosine phosphorylation or activity [25–28] — the most economic interpretation of our observations is that RPTPα directly dephosphorylates c-Src Tyr527 *in vivo*, consistent with its ability to do so *in vitro* [18,19]. This dephosphorylation event would then either initiate, or maintain, the open conformation of c-Src. Such a direct relationship would also be consistent with the reported association of RPTPα with c-Src or Fyn [21,29]. We propose that our observations that RPTPα inactivation reduces c-Src and Fyn kinase activity, while also generating a phenocopy of c-Src ablation in fibroblasts, strongly suggest that this phosphatase acts as a physiological upstream regulator of Src-family kinases in this lineage. Our data clearly do not, however, rule out the possible existence of additional targets for RPTPα.

The control of c-Src function by RPTPα is reminiscent of the contribution of CD45 towards Lck or Fyn activity in lymphocytes [30], but contrasts with the apparent negative control of Src-family kinase function by CD45 in macrophages [16]. The cytosolic PTPase SHP-1 has been implicated in the activation of c-Src in platelets and thymocytes [25], although it may also inactivate the Src-family kinases Lck and Fyn [26]. The PTPase SHP-2 has been shown to associate with c-Src in fibroblasts and HT-29 colon carcinoma cells, and to be capable of dephosphorylating Tyr527 *in vitro* [27]. Intriguingly, *SHP-2*$^{-/-}$ fibroblasts display a phenotype very similar to that of

Figure 4



Effect of RPTPα deletion on phosphorylation events following adhesion on fibronectin. (a) Equal amounts of protein lysates derived from wild-type (+/+), *RPTPα*$^{+/-}$ (+/–) and *RPTPα*$^{-/-}$ (–/–) fibroblasts, prepared at various times after adhesion to a fibronectin substrate, as indicated, were analyzed by anti-phosphotyrosine (P-Tyr; top and middle panels) or anti-Erk (lower panel) immunoblotting. (b) Anti-Fak and (c) anti-p130^{cas} immunoprecipitates from cells in suspension or cells allowed to adhere on fibronectin for 20 min were analyzed by immunoblotting with anti-phosphotyrosine antibodies (top panels) or, as control, with the respective p130^{cas} or Fak antisera (bottom panels). (d) Anti-Erk immune complexes, prepared from cells in suspension or cells allowed to adhere on fibronectin for 15 min, were halved, with one half being subjected to an *in vitro* Erk kinase reaction using myelin basic protein (MBP) as substrate (top panel), and the other half to anti-Erk immunoblotting (lower panel). (e) The Erk activities from three independent experiments such as those presented in (d) were quantitated and are shown as the average ± SE.

RPTPα$^{-/-}$ cells, that is, delayed spreading on fibronectin-coated substrates, and impaired activation of integrin-induced signalling events such as activation of Src-family kinases, phosphorylation of focal adhesion proteins, and Erk activation [28,31]. The involvement of both RPTPα and SHP-2 in the regulation of c-Src activity and in fibroblast spreading clearly calls for a more precise dissection of the specific roles of these two phosphatases in integrin signalling, and in the complex processes that constitute focal adhesion formation and turnover, and cytoskeletal remodelling. One range of possibilities may involve both PTPases independently (possibly directly) controlling Src Tyr527 phosphorylation levels. Under such a scenario, both would be essential, as a result of, for instance, their abilities to act on different Src populations, in different intracellular locations, or at a different stage of the spreading process. Fibroblast adhesion is indeed known to involve a c-Src translocation event [9]. Alternatively, SHP-2 and RPTPα may act sequentially (but not necessarily in this order), with only one of these PTPases (or possibly even yet another PTPase) directly dephosphorylating Tyr527. In this case, regulatory interactions between RPTPα and SHP-2 must be postulated, and these might involve direct dephosphorylation of one

PTPase by the other, or more indirect mechanisms involving intermediate proteins. At any rate, the positive roles of RPTPα and SHP-2 contrast with the recently proposed negative role of the tumor suppressor PTEN in cell spreading and in signalling responses downstream of integrin engagement by extracellular matrix molecules [32,33]. Fibroblasts lacking the PTPase PTP-PEST were also recently shown to display enhanced spreading rates when plated on fibronectin, together with enhanced tyrosine phosphorylation of Fak and p130^{cas} [34].

An additional consequence of c-Src ablation in mice is osteopetrosis due to a functional deficit in the osteoclast lineage [35,36]. X-ray analysis of *RPTPα*$^{-/-}$ mice up to 9 month old has thus far failed to detect signs of osteopetrosis (data not shown). This apparent paradox may suggest that, in osteoclasts, the role of RPTPα in c-Src function is performed by other PTPases. For instance, RPTPε, the PTPase most similar to RPTPα, displays a much more restricted expression pattern than RPTPα, but is actually abundant in differentiated osteoclasts [37]. Similarly, in T lymphocytes, activation of Fyn has been suggested to be largely mediated by the PTPase CD45 [14]. By analogy, phosphorylation of Tyr527 in c-Src can be mediated by at least two kinases with different expression patterns, Csk and Chk [11,12]. Alternatively, given the large number of processes in which c-Src has been implicated [6,7], the requirement for RPTPα in c-Src function may be stimulus-specific, indicating that fibroblast spreading on fibronectin and bone resorption by osteoclasts involve different regulators of c-Src. Taken together, these considerations suggest a testable model, in which coupling between particular PTPases and Src-family kinases may convey a measure of cell- and stimulus-specificity to signalling pathways.

Materials and methods

Homologous recombination

A replacement-type targeting vector, consisting of an IRES–β-geo cassette [38] (I. Chambers, Edinburgh) inserted at a *Pst*I site present in exon 3 of the mouse *RPTPα* locus [39], was electroporated into the mouse ES cell line W4 (A. Joyner, NYU). Positive clones were identified by Southern hybridization, chimeric founders mated with 129SvJ animals, and the mutated allele maintained on this background.

Immunoprecipitations and antibodies

Cells were lysed in RIPA buffer (50 mM HEPES pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin. Equal amounts of protein from cleared extracts were subjected to standard immunoprecipitation or immunoblotting procedures. Antibodies used were: clone 327 against c-Src (Oncogene Science); anti-Erk 1 and 2 (Santa Cruz); F15020 (Transduction Laboratories) against Fak; antiserum B against p130^{cas} (T. Parsons, University of Virginia); anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology); anti-RPTPα 443 and 35, raised against the entire RPTPα intracellular domain or a carboxy-terminal peptide, respectively; antibody 2F8, against the RPTPα ectodomain, was a gift from M. Thomas (Washington University, St. Louis); clone 28 antibody was most generously donated by H. Kawakatsu (UCSF).

Src and Fyn kinase assays

Immune complexes of c-Src or Fyn were washed three times with RIPA lysis buffer followed by two washes with kinase buffer (20 mM PIPES pH 7.0, 5 mM $MnCl_2$), and reacted in a volume of 50 μl kinase buffer containing 5 μCi [γ-^{32}P]ATP and 12.5 μg acid-denatured enolase at 30°C for 5 min. After electrophoresis, phosphorylation of enolase was quantified with a Phosphor-Imager (Molecular Dynamics).

Erk assay

Triton-buffer lysates were subjected to immunoprecipitation with anti-Erk 1 and 2 antisera, and the immune complexes washed twice with lysis buffer and twice with kinase buffer (10 mM Tris pH 7.4, 10 mM $MgCl_2$). An *in vitro* kinase reaction was performed in 50 μl kinase buffer containing 5 μCi [γ-^{32}P]ATP and 12.5 μg MBP. After incubation for 30 min at 30°C, the reaction was stopped by boiling in SDS–PAGE sample buffer, and the reaction products separated by SDS–PAGE. The radioactive content of ^{32}P-labeled MBP was determined using liquid scintillation counting.

Cell culture

Primary embryonic fibroblasts were isolated from E13–E15 day old embryos, with *RPTPα*$^{+/+}$ and *RPTPα*$^{-/-}$ embryos and cells always being derived in parallel from the same pregnancy. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. For spontaneous immortalization, cells were trypsinized and reseeded every 3 days at a density of 10^6/100 mm dish until crisis had been overcome. Alternatively, a retroviral vector expressing polyoma large T antigen was used to immortalize the cultures. All experiments shown were performed on several independently isolated lines as well as on primary isolates. For reintroduction of RPTPα, an *RPTPα*$^{-/-}$ culture was infected as a pool with an RPTPα-expressing or control retrovirus. Quantitative infection was established by fluorescence of a green fluorescent protein (GFP) marker coexpressed from the same vector.

Cell spreading

Cells were trypsinized briefly, and the digestion stopped using 0.5 mg/ml trypsin inhibitor (Gibco). They were then resuspended (125,000 cells/ml) in DMEM, and plated in 4 ml adhesion medium on 6 cm tissue-culture dishes coated with fibronectin (5 μg/ml of human fibronectin (Gibco) overnight at 4°C). Plates were incubated at 37°C for the designated periods of time, chilled on ice for 15 min, and five microscope fields were photographed and counted.

Acknowledgements

The authors thank I. Chambers, T. Hunter, G. Jiang, A. Joyner, H. Kawakatsu, T. Parsons and M. Thomas for reagents, A. Auerbach, S. Ortega and N. Shalts for help with ES cell manipulation and C. Jarvis for technical assistance. We highly appreciated comments on the manuscript by F. Giancotti, T. Hunter, G. Jiang, and J. Schlessinger. This research was supported by the NIH (R29 CA68365 to J. Sap, and NCI core support grant P30CA16087), the American Heart Association (New York City Affiliate), Sugen, and the U.S. Department of Defense (DAMD17-98-1-8136).

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