Protein phosphatase 1 binds to phospho-Ser-1394 of the macrophage-stimulating protein receptor

Massimo M. SANTORO*, Giovanni GAUDINO* and Emma VILLA-MORUZZI†[1]

*Department of Medical Sciences, University of Piemonte Orientale 'A. Avogadro', 28100 Novara, Italy, and †Department of Experimental Pathology, University of Pisa, via Roma 55, 56126 Pisa, Italy

The tyrosine kinase Ron, receptor for MSP (macrophage-stimulating protein), displays several serine residues of unknown functions. Using [^{32}P]H_3PO_4 metabolic labelling, we found that Ron is serine-phosphorylated and dephosphorylated *in vitro* by PP1 (protein phosphatase 1). PP1 associates with Ron obtained from cells of different origins. The association is stimulated by MSP or serum and is prevented by wortmannin, an inhibitor of the Akt/PKB (protein serine/threonine kinase B) pathway. Akt/PKB phosphorylates Ron Ser-1394, thus providing a docking site for 14-3-3 (scaffold proteins binding to phosphoserine/phosphothreonine-containing sequences). In living cells, PP1 binds to the Ron mutant S1394A, but the association is no longer regulated by serum, MSP or wortmannin. The role of PP1 association with Ron is highlighted by (1) Ser-1394 dephosphorylation by PP1 *in vitro* and in living cells, (2) loss of 14-3-3 association with Ron after Ser-1394 dephosphorylation by PP1 *in vitro* and (3) an increase in 14-3-3 association after PP1 inactivation in living cells. These results suggest that PP1 can modulate the downstream Ron signalling generated by MSP via Akt/PKB and 14-3-3 binding. This is the first report on ligand-regulated association of PP1 with a growth factor receptor.

Key words: Akt/protein serine/threonine kinase B (PKB), macrophage-stimulating protein (MSP), protein phosphatase, protein phosphorylation, receptor tyrosine kinase, Ron.

INTRODUCTION

Receptor tyrosine kinases (RTKs) can be regulated by PTPs (protein tyrosine phosphatases [1]) in many ways. PTPs may inactivate the RTKs, thus terminating stimulation by the ligand [2]. Alternatively, PTPs may modulate receptor kinase activity or its signalling pathway in ligand-dependent or -independent ways [1,2]. One example is PTP-S, the phosphatase that we found to interact with the HGF (hepatocyte growth factor) receptor Met [3,4]. The association was stimulated by HGF and involved a juxtamembrane tyrosine residue which contributed to the negative Met regulation [4].

RTKs also contain several serine and threonine residues, some of which are located within consensus sequences for known kinases. There is increasing evidence that serine/threonine phosphorylation plays a relevant role in modulating RTK functions [5–7]. For instance, phosphorylation inhibits the tyrosine kinase activity of insulin [5], epidermal growth factor [6] and HGF [7] receptors. Alternatively, phosphorylated serine residues may provide docking sites for binding proteins mediating the downstream receptor signalling [8].

In contrast, nothing is known about the serine/threonine phosphatases that dephosphorylate the RTKs. Two enzymes that are probably involved are PP1 (protein phosphatase 1) and PP2A [9,10]. These are two phosphatase families that regulate a variety of signalling pathways (see below). To tackle this problem, we used the MSP (macrophage-stimulating protein) receptor Ron, an RTK displaying several serine and threonine residues in its cytosolic domain [11,12]. Ron belongs to the HGF receptor family and is involved in a wide spectrum of biological activities, including mitogenesis, apoptosis and cell motility [11,13]. Ron signalling occurs after phosphorylation of a multifunctional tyrosine docking site and association of multiple effectors [13]. It has been demonstrated recently that MSP stimulation induces phosphorylation of Ser-1394 [14]. This serine residue is located at the C-terminus of Ron and is highly conserved in human, rat and mouse homologues, but not in Met. Phosphorylation of Ser-1394 occurs via MSP-dependent activation of the phosphoinositide 3-kinase–Akt pathway [14]. Akt/PKB (protein serine/threonine kinase B), a kinase involved in cell proliferation, survival and migration [15], recognizes Ser-1394 and its surrounding sequence as an optimal phosphorylation site (RXRXXS) [14]. After phosphorylation, Ser-1394 becomes a docking site for 14-3-3 (scaffold proteins binding to phosphoserine/phosphothreonine-containing sequences) [14]. These are scaffold proteins and co-factors that bind to phosphoserine/phosphothreonine-containing sequences and thus regulate the subcellular localization or functions of target proteins [16]. The interaction of 14-3-3 with Ron induces a multiprotein complex, containing Ron, 14-3-3 and the $\alpha6\beta4$ integrin [14]. This complex, in turn, down-regulates the $\alpha6\beta4$-dependent adhesion and favours migration. Such a mechanism is involved in keratinocyte migration and re-epithelization during wound healing [14].

PP1 is present in virtually all cell compartments, suggesting its involvement in several different cellular functions [9,10,17–20]. The holoenzyme consists of a catalytic subunit bound to a regulatory subunit [10]. Various unrelated regulatory subunits have been described previously [10]. The regulatory subunits modulate the enzymic activity and target the enzyme to specific functions [10,17]. In mammalian cells, three highly homologous isoforms of the PP1 catalytic subunit exist, namely α, $\gamma1$ and δ (also called β) [9,19]. Isoform-specific antibodies (see e.g. [18]) were used to study the differential association and localization of the isoforms [18,20–22].

In the present study, we show that PP1 associates with and dephosphorylates Ron Ser-1394, both *in vitro* and in living cells.

Abbreviations used: Akt/PKB, protein serine/threonine kinase B; FCS, foetal calf serum; HGF, hepatocyte growth factor; LB broth, Luria–Bertani broth; PP2A, protein phosphatase 2A; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase; MSP, macrophage-stimulating protein; GST, glutathione S-transferase.

[1] To whom correspondence should be addressed (e-mail villa@biomed.unipi.it).

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This association is ligand- or serum-dependent and modulates the interaction of 14-3-3 with Ron in a negative way.

EXPERIMENTAL

Antibodies, purified proteins and chemicals

PP1 isoform-specific antibodies were raised in rabbits injected with peptides derived from the C-termini of the three PP1 isoforms [18]. Anti-PP2A polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Ron polyclonal antibodies were raised as described previously [11]. Phospho-Ser-1394-specific antibodies were raised to the Ron-derived phosphopeptide (N-NVRRPRPLpSEPPRPT-C; Eurogentec, Seraing, Belgium) coupled with keyhole-limpet (*Diodora aspera*) haemocyanin (Sigma) and were affinity-purified [14]. Akt phospho-Ser-473-specific polyclonal antibodies and anti-Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.) and anti-pan-14-3-3 monoclonal antibodies from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Recombinant MSP was obtained from R&D Systems (Minneapolis, MN, U.S.A.). PP1 and PP2A were purified from rabbit muscle as isolated catalytic subunits, as described by Resink et al. [23]. Wortmannin and okadaic acid were obtained from Sigma.

DNA constructs and cell expression

Full-length Ron [11] and Tpr-Ron [24,25] were subcloned into the pMT2 vector and expressed transiently in COS-1 cells, using the CellPhect transfection kit (Amersham Biosciences). NIH-3T3-fibroblast stable cell lines, expressing either wild-type Ron or the S1394A mutant full-length Ron, were obtained by co-transfection of each recombinant plasmid cDNA [14] with pSV2-neo plasmid (in the ratio 15:1) using the CellPhect transfection kit. Subsequent selection was performed in growth medium supplemented with 0.4 mg/ml geneticin sulphate (G418; Sigma). Stable cell-culture clones expressing the same level of the respective recombinant proteins were selected by Western-blot analysis using specific C-terminal Ron antibodies [11]. GST-fusion proteins of C-terminal Ron (residues 1350–1400) and of its S1394A mutant were described previously [14].

Cell culture and extracts

NIH-3T3 fibroblasts, GTL-16 gastric carcinoma cell line and COS-1 cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10 % (v/v) FCS (foetal calf serum; Invitrogen) at 37 °C in a 5 % CO_2 water-saturated atmosphere [20]. Cells were lysed in cell lysis buffer [25 mM Hepes, pH 7.4/10 % (v/v) glycerol/100 mM NaCl/5 mM $MgCl_2$/1 mM EGTA/1 % Triton X-100], containing 7.5 mM 2-mercaptoethanol, 5 μg/ml L-1-*p*-tosylamino-2-phenethyl chloromethane, 4 μg/ml leupeptin, 10 μg/ml soya-bean trypsin inhibitor, 40 μg/ml PMSF and 20 μg/ml benzamidine. The extracts were clarified by centrifugation at 10 000 ***g*** for 10 min and stored at − 80 °C.

Metabolic labelling of cells

The culture medium was changed to H_3PO_4-free medium (Sigma) containing 10 % H_3PO_4-free FCS. After 1 h, the medium was changed to the same medium supplemented with $[^{32}P]H_3PO_4$ (1 mCi/150 mm plate in 1 ml medium; Amersham Biosciences) and left for an additional 5 h [20]. To preserve serine and threonine phosphorylations, the extraction buffer also contained 1 μM okadaic acid (Sigma), whereas tyrosine phosphatase inhibitors were absent, to allow tyrosine dephosphorylation [20]. In some experiments, the cells were additionally treated with 20 or 200 nM okadaic acid during the last 30 min of labelling.

Immunoprecipitation and immunoblotting

Immunoprecipitates were prepared with anti-Ron rabbit serum (5 μl/ml cell extract) or anti-PP1 (α, γ1 or δ) rabbit sera (10 μl/ml) and Protein A–Sepharose (Sigma) [18]. After incubation at 4 °C for 90 min with shaking and three washes with wash buffer (50 mM Hepes, pH 7.4/100 mM NaCl/300 mM sucrose/5 mM $MgCl_2$/5 mM EGTA), containing 20 μg/ml PMSF and 20 μg/ml benzamidine, the immunocomplexes were boiled in Laemmli buffer. For further PP1 treatment, the immunocomplexes were washed once again with 25 mM imidazole (pH 7.5), resuspended in 25 μl of the same buffer containing 2 mM 2-mercaptoethanol, incubated with purified PP1 [23] as specifically indicated, and boiled in Laemmli buffer. Electrophoresis was on 8.5 or 12.5 % SDS/polyacrylamide gels and Immobilon-P (Millipore, Billerica, MA, U.S.A.) was used for transblotting. The membranes were probed with the indicated antibodies diluted in 2.5 % (w/v) BSA/Tris-buffered saline, followed by Protein A–peroxidase or anti-mouse IgG–peroxidase and the enhanced chemiluminescence system (Amersham Biosciences). For reprobing with a second antibody, the membranes were incubated previously in 5 mM phosphate buffer, 2 % (w/v) SDS and 2 mM 2-mercaptoethanol at 60 °C for 30 min [18]. For autoradiography, the membranes were dried and exposed to Hyperfilms-MP (Amersham Biosciences) at − 80 °C, using intensifying screens.

Two-dimensional phospho-amino-acid analysis

Phosphoproteins, transferred on to Immobilon-P, were hydrolysed in 6 M HCl at 110 °C for 1 h and analysed as described by Kamps [26].

Bacterial culture, extracts and *in vitro* association

Bacteria were grown at 37 °C in LB (Luria–Bertani) broth containing 50 μg/ml ampicillin, induced with 0.1 mM isopropyl β-D-thiogalactoside for 2 h and extracted as described previously [20]. The GST (glutathione S-transferase)-fusion proteins from bacterial extracts were bound to 25–50 μl of glutathione–Sepharose beads (Amersham Biosciences). After 90 min rotation at 4 °C and three washes with 50 mM Tris (pH 7.5), supplemented with 0.25 % Triton X-100, 20 μg/ml benzamidine and 20 μg/ml PMSF, the beads were Akt-phosphorylated (see below) and incubated with purified PP1 (diluted in cell-lysis buffer). Further processing was the same as described for immunoprecipitation and blotting.

In vitro phosphorylation of Ron by Akt

C-terminal Ron (either GST-fusion protein bound to glutathione–Sepharose beads or after removal of GST by thrombin [14]) or Ron immunoprecipitated from cell extract was resuspended in 25 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 400 μM [γ-^{32}P]ATP (3000 c.p.m./pmol) and 7.5 mM 2-mercaptoethanol and incubated with 2 units of purified Akt (Cell Signaling Technology) at 30 °C for 20 min. This was followed by the addition of 10 mM EDTA (final concentration) and processing as specifically indicated in the Figure legends.

In vitro dephosphorylation of Ron by PP1

Ron immunocomplexes prepared from *in vivo*-labelled cells or Ron phosphorylated *in vitro* by Akt were washed with 25 mM

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imidazole (pH 7.5), resuspended in 25 μl of the same buffer and incubated with 1–5 units of purified PP1 [23] at 30 °C for 30 min. This was followed by boiling in Laemmli buffer and processing as described for immunoblotting and autoradiography.

Phosphatase assay

Immunocomplexes were prepared as indicated above, washed with 25 mM imidazole (pH 7.5) and resuspended in 25 μl of 25 mM imidazole and 50 mM 2-mercaptoethanol. The reaction was started by adding 25 μl of 3 mg/ml [^{32}P]phosphorylase *a*, run for 20 min at 30 °C and stopped by adding cold 10 % (w/v) trichloroacetic acid [20]. Okadaic acid (5 nM) was present in the assays [20], a concentration that did not affect PP1, but inhibited PP2A to > 95 %, according to a preliminary test using muscle-purified phosphatases. The phosphatase activity was calculated from the [^{32}P]H_3PO_4 released from the substrate, after subtracting the c.p.m. of control immunocomplexes, which were prepared using preimmune serum and run in parallel. The phosphatase activity is given as (fmol of P_i released from the substrate) $\cdot$ min^{-1} $\cdot$ (mg of cell extract used for immunoprecipitation)$^{-1}$.

RESULTS

Ron serine phosphorylation and dephosphorylation by PP1

Ron displays several serine and threonine residues that may undergo phosphorylation [11,12], thus becoming potential targets for PP1. To investigate the serine/threonine phosphorylation of Ron, cells were metabolically labelled with [^{32}P]H_3PO_4 and lysed in the absence of tyrosine phosphatase inhibitors (thus favouring tyrosine dephosphorylation). Ron was found to be phosphorylated (Figure 1A) and phospho-amino-acid analysis detected only phosphoserine (Figure 1B). The phosphorylation decreased after incubation of the Ron immunocomplex with a PP1 catalytic subunit (purified from muscle [23]; Figure 1A). These results indicate that Ron represents a potential PP1 substrate.

Detection of the Ron-associated PP1

The presence of an associated PP1 was investigated in Ron immunoprecipitated from epithelial and fibroblast cell lines. The associated phosphatase activity was assayed with the exogenous substrate phosphorylase *a*. The assay was selective for PP1, because it was run under the following conditions: (i) in the presence of 5 nM okadaic acid (a concentration that did not affect PP1, but inhibited PP2A to > 95 %, according to a preliminary test on muscle-purified phosphatases) and (ii) in the absence of Mg^{2+} or Ca^{2+} (ions required by other PP2 types). Moreover, the same activity values were obtained in assays run in the presence or absence of okadaic acid. This indicated that no PP2A was present in the immunocomplexes (results not shown). The Ron-associated PP1 activity was detected in Ron-expressing NIH-3T3 fibroblasts (Figure 2A). The activity was also detected in COS-1 cells transiently expressing Ron (either as an entire receptor or as constitutively active chimaera Tpr-Ron [25]; results not shown) and in the gastric carcinoma GTL-16 cells (expressing endogenous Ron; results not shown).

To confirm the identity of PP1, Ron immunocomplexes from GTL-16 cells were probed with the isoform-specific anti-PP1 antibodies [18]. PP1α and PP1γ1 were detected, whereas PP1δ was absent (Figure 2B). This result may apply also to the other cell lines examined, since the three PP1 isoforms are present in all mammalian cells. On the other hand, the use of anti-PP2A antibodies confirmed the absence of PP2A from the Ron immunocomplex (results not shown).

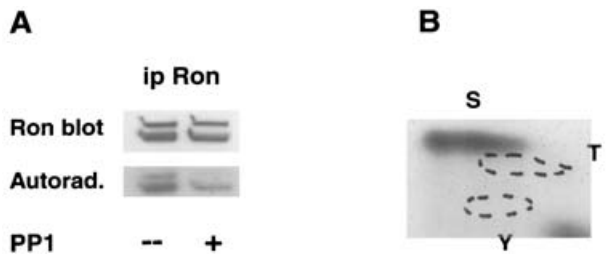

Figure 1 Ron serine phosphorylation and dephosphorylation by PP1

(**A**) GTL-16 cells were metabolically labelled with [^{32}P]H_3PO_4 and Ron was immunoprecipitated (ip Ron) from the cell extract (3 mg/immunoprecipitation) in duplicate. One immunocomplex was treated with 1 unit of muscle-purified PP1 catalytic subunit at 30 °C for 20 min (PP1 +). Both samples were subjected to SDS/PAGE and transblotted on to Immobilon-P. The blot was first probed with the anti-Ron antibodies (Ron blot), and then exposed to detect the radioactivity incorporated into Ron (Autorad.). (**B**) The Ron protein (ip Ron, PP1 −) was excised from the blot, hydrolysed and subjected to two-dimensional phospho-amino-acid analysis, to detect the radioactivity incorporated into phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y).

PP1 association with Ron is induced by MSP

The next step was to investigate whether the Ron–PP1 association was constitutive or functionally correlated with Ron. Stimulation of Ron by MSP activates the tyrosine kinase and its downstream signalling [27]. To verify whether MSP influences the PP1 association, Ron-expressing NIH-3T3 fibroblasts were serum-starved and then exposed to MSP. Serum-starvation for 12 h decreased the Ron-associated PP1 activity to the level of non-transfected cells. Subsequent exposure to MSP for 15 min restored the associated PP1 activity (Figure 2A). The association of PP1 in response to MSP was confirmed also by detection of the PP1 protein (Figure 2C). We conclude that PP1 associates with Ron after serum or MSP stimulation.

To test whether the interaction is transient or sustained, Ron-expressing NIH-3T3 fibroblasts were serum-starved and exposed to MSP for up to 90 min. The cells were collected as indicated and lysed. Ron was immunoprecipitated and the associated PP1 activity was assayed on the immunocomplexes. Results indicated that the association was maximal at 15 min and then decreased (Figure 3A). The association of PP1 in response to MSP and the subsequent dissociation were confirmed by immunoblotting. In most of the experiments (one of which is shown in Figure 3B), the PP1 protein was clearly detected only at 15 and 30 min, the time points when the maximal PP1 activity was assayed. This indicates that the changes induced by MSP in associated PP1 activity were due to reversible association of PP1 rather than to changes in the specific activity of a constitutively bound PP1.

MSP stimulation induces phosphorylation of Ser-1394

Among the Ron serine residues, Ser-1394 was recently shown to be a substrate for Akt [14]. Therefore Ser-1394 phosphorylation and Akt activation were analysed by immunoblotting in Ron-expressing NIH-3T3 cells. In serum-starved cells, Akt was not active (based on the absence of Akt Ser-473 phosphorylation) and Ron Ser-1394 was not phosphorylated. MSP stimulation induced both Ser-1394 phosphorylation and Akt activation.

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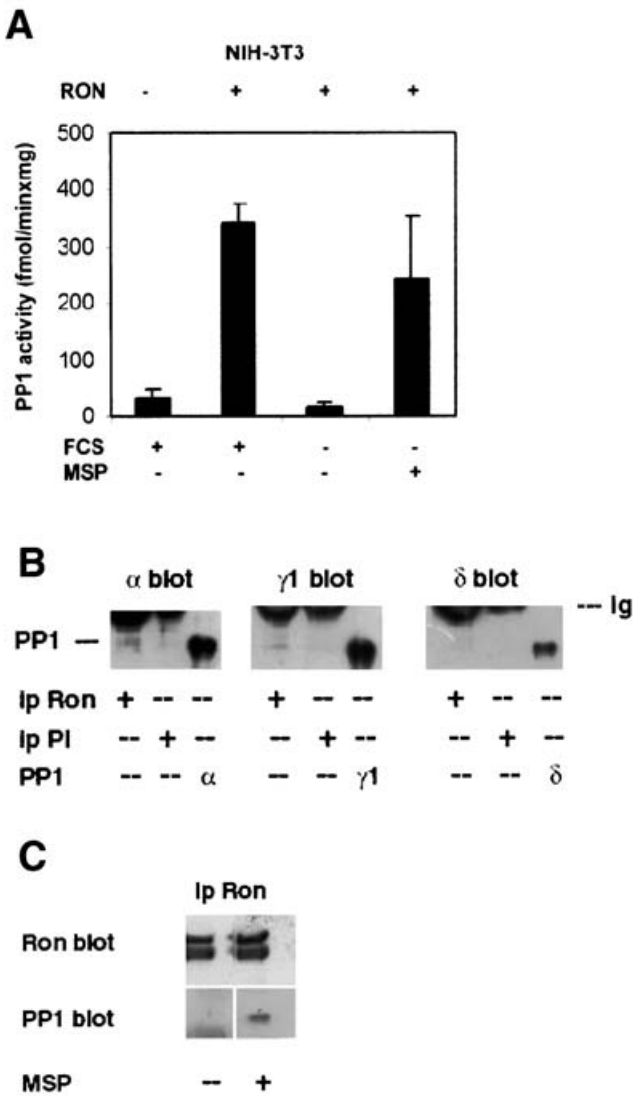

Figure 2 Association of PP1 with Ron and stimulation by MSP

(**A**) Ron-expressing NIH-3T3 fibroblasts (Ron +) were either grown in a medium containing 10 % FCS (FCS +) or serum-starved for 12 h (FCS −), and further treated with 100 ng/ml recombinant MSP for 15 min (MSP +). Mock-transfected NIH-3T3 fibroblasts were used as controls (Ron −). Ron was immunoprecipitated from the cell extracts (3 mg/immunoprecipitation) and the associated PP1 activity was assayed. PP1 activity is given as (fmol of P_i released) · min^{-1} · (mg of cell extract)$^{-1}$. Control immunoprecipitates (prepared with unrelated IgGs) were run in parallel with each sample and assayed for PP1. The activity assayed on each control (between 0 and 30 fmol · min^{-1} · mg^{-1}) was subtracted from the activity assayed on the corresponding anti-Ron immunoprecipitate, to obtain the indicated activities. Results are means ± S.E.M. from five determinations. (**B**) Three sets of immunoprecipitates were prepared with anti-Ron (ip Ron) or preimmune serum (ip PI) from GTL-16 cell extracts. The three blots obtained were probed with anti-α, anti-γ1 and anti-δ antibodies, as indicated. Lanes α, γ1 and δ, 20 ng of recombinant PP1 catalytic subunits. After electrophoresis and transblotting, the blots were probed with the indicated anti-PP1 isoform antibodies. (**C**) Ron was immunoprecipitated from Ron-expressing NIH-3T3 cells. The cells were either serum-starved (MSP −) or MSP-treated (MSP +), as described in (**A**). The blots were probed to detect PP1 (PP1 blot) using a mixture of the three isoform-specific antibodies. This was followed by removal of the antibodies and re-probing with the anti-Ron antibodies (Ron blot). Other experimental details are as described in (**B**).

Phosphorylation of both sites was maximal at 15 min and then decreased (Figure 4A). This kinetics parallels the kinetics of PP1 interaction with Ron (Figure 3), suggesting the involvement of Ser-1394 phosphorylation in the interaction.

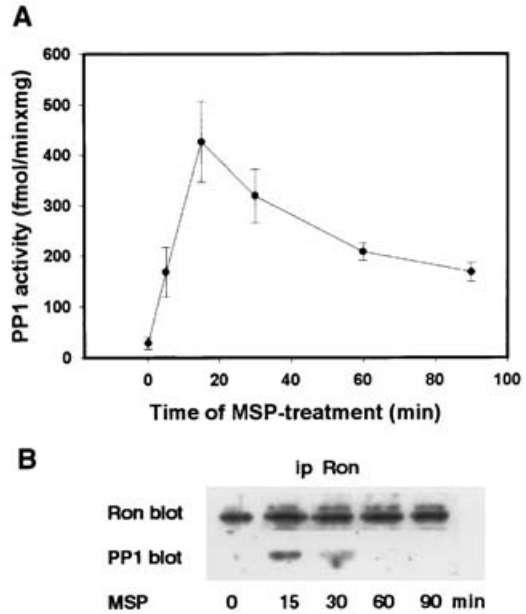

Figure 3 Time course of the MSP-stimulated association of PP1 with Ron

(**A**) Ron-expressing NIH-3T3 cells were serum-starved for 12 h (zero time) and further treated with MSP, as described in the legend to Figure 2(A). Cells were collected at the indicated time points. PP1 activity was assayed on anti-Ron and control immunocomplexes, as described in the legend to Figure 2(A). Results are means ± S.E.M. from five determinations. (**B**) Cells were treated as in (**A**) and collected at the indicated time points. Ron was immunoprecipitated from the cell extracts and the blot obtained was probed to detect PP1 and Ron, as described in the legend to Figure 2(C).

To characterize the correlation between Akt activation and Ser-1394 phosphorylation, Ron-expressing NIH-3T3 cells were treated with wortmannin, a phosphoinositide 3-kinase inhibitor. Wortmannin prevented both Akt activation and Ser-1394 phosphorylation (Figure 4B). Most significantly, a total loss of Ron-associated PP1 occurred after wortmannin treatment (Figure 4C; see also Figure 6C, which displays detection of PP1). This result further supported the hypothesis that phospho-Ser-1394 is involved in PP1 association.

Role of Ser-1394 in Ron–PP1 interaction *in vitro*

The role of Ser-1394 in PP1 binding was first tested in an *in vitro* binding assay, using purified PP1 catalytic subunit and GST–Ron (a fusion protein reproducing the last 50 C-terminal residues of wild-type Ron) or its S1394A mutant. GST–Ron was able to bind the PP1 catalytic subunit, whereas GST–Ron S1394A failed to do so (Figure 5). The binding was performed also with purified PP2A, which failed to associate with GST–Ron, whether wild-type or S1394A mutant. These results indicate that Ser-1394 is required for the *in vitro* interaction between C-terminal Ron and the isolated PP1 catalytic subunit.

Role of Ser-1394 in Ron–PP1 interaction in living cells

The subsequent step was to test whether Ser-1394 is also involved in the interaction with PP1 in living cells, where PP1 is generally targeted to substrates by a regulatory subunit and Ron is expressed as an entire receptor. For this purpose, Ron carrying the S1394A

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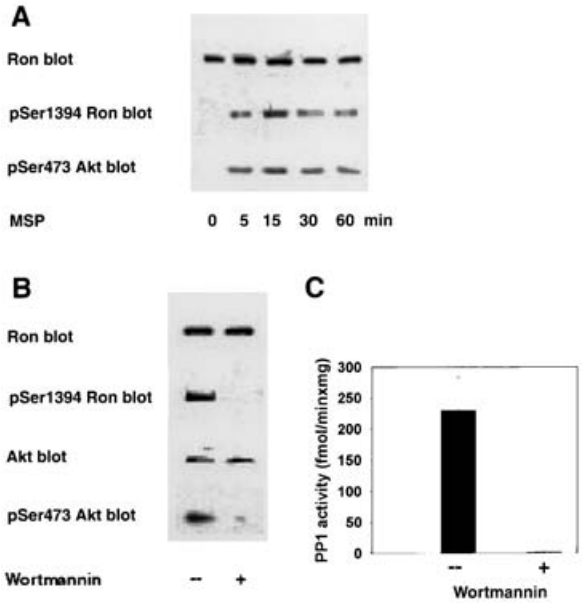

Figure 4 Effects of MSP and wortmannin on Ron Ser-1394 phosphorylation, Akt activation and Ron–PP1 interaction

(**A**) Ron-expressing NIH-3T3 cells were serum-starved and subsequently treated with MSP for a maximum of 60 min (as in Figure 3). Extracts were obtained at each time point and were used to prepare three separate blots to detect Ron (Ron blot), Ron phospho-Ser-1394 (pSer1394 blot) and Akt phospho-Ser-473 (which indicates Akt activation; pSer473 Akt blot). (**B**) Ron-expressing NIH-3T3 cells were treated with or without 100 nM wortmannin for 30 min in the absence of serum. The extracts obtained were used to prepare four separate blots to detect Ron, Ron phospho-Ser-1394, Akt protein (Akt blot) and the Akt phospho-Ser-473. (**C**) Cell extracts obtained as in (**B**) were used to immunoprecipitate Ron and assay the associated PP1 activity (as in Figure 2). Results are means ± S.E.M. from three determinations.

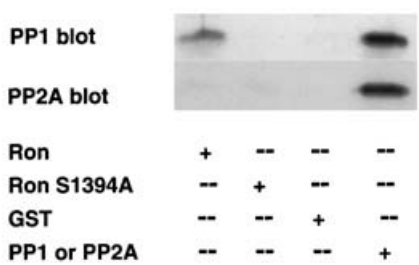

Figure 5 Loss of Ron–PP1 interaction *in vitro* induced by S1394A mutation

GST–C-terminal Ron (residues 1350–1400), either wild-type (Ron) or S1394A mutant (Ron S1394A) or GST alone, were bound to glutathione–Sepharose beads, phosphorylated *in vitro* by Akt (2 units during 20 min incubation at 30 °C; see the Experimental section) and were used to associate either PP1 or PP2A catalytic subunits. The blots obtained were probed with either anti-PP1 (PP1 blot) or anti-PP2A (PP2A blot) antibodies. Lane PP1+ or PP2A+, 30 ng of each catalytic subunit.

mutation was stably expressed in NIH-3T3 fibroblasts. Ron and Ron S1394A from cells grown in the serum were tested for PP1 binding. Ron S1394A was still associated with PP1 activity, although less than wild-type Ron (Figure 6A). This was bona fide PP1 activity, since it was totally inhibited by 0.5 μM okadaic acid (a concentration that inhibits PP1 completely; results not shown). Moreover, the presence of PP1 was confirmed by immunoblotting (Figure 6B). To test whether PP1 was still regulated, serum-starved cells expressing the S1394A mutant were exposed to

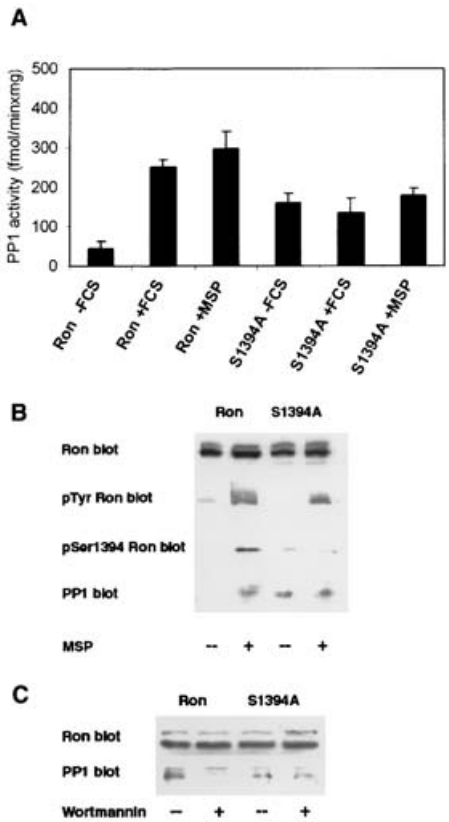

Figure 6 Association of PP1 with the S1394A Ron mutant in living cells

(**A**) Ron or the S1394A Ron mutant were stably expressed in NIH-3T3 fibroblasts. Cells were either serum-starved for 12 h (Ron − FCS and S1394A − FCS) or grown in medium containing 10 % FCS (Ron + FCS and S1394A + FCS) or serum-starved for 12 h and further treated with 100 ng/ml recombinant MSP for 15 min (Ron + MSP and S1394A + MSP). Ron was immunoprecipitated from the cell extracts and the associated PP1 activity was assayed. PP1 was assayed also on control immunoprecipitates, as described in the legend to Figure 2. Results are means ± S.E.M. from four determinations. (**B**) Cells expressing Ron or the S1394A Ron mutant were either serum-starved (MSP −) or serum-starved and further treated with MSP (MSP +), as described in (**A**). The extracts were used to immunoprecipitate Ron and the blot obtained was probed to detect the Ron-associated PP1 (PP1 blot). Aliquots of these extracts were also used to prepare three additional blots to detect Ron (Ron blot), Ron phosphotyrosine (pTyr Ron blot) and Ron phospho-Ser-1394 (pSer1394 blot). Other experimental details are as described in the legend to Figure 2. (**C**) Cells expressing Ron or the S1394A Ron mutant were treated with or without wortmannin, as in Figure 4(B). Ron was immunoprecipitated from the cell extracts and the blot obtained was probed to detect PP1 and Ron, as described in the legend to Figure 2(C).

MSP. The mutant-associated PP1 activity was neither decreased by serum starvation nor increased by MSP treatment (Figure 6A). The loss of response to serum starvation and MSP treatment was confirmed by immunoblotting (Figure 6B). The results could not be attributed to differential expression of the receptor, because the two cell lines expressed similar receptor levels (see the Experimental section). Moreover, both Ron forms were activated by MSP, as shown by the increase in tyrosine phosphorylation observed after ligand exposure (Figure 6B). Also the sensitivity to wortmannin was lost, since the Ron-S1394A-associated PP1 did not dissociate after cell treatment with wortmannin (Figure 6C).

Altogether, the results indicate that, in living cells, PP1 still interacts with the Ron mutant S1394A, but no longer responds to the regulatory signals. This, in turn, indicates the involvement of Ser-1394 in PP1 binding and suggests a functional link between PP1 and Ron within the ligand-stimulated Ron signalling.

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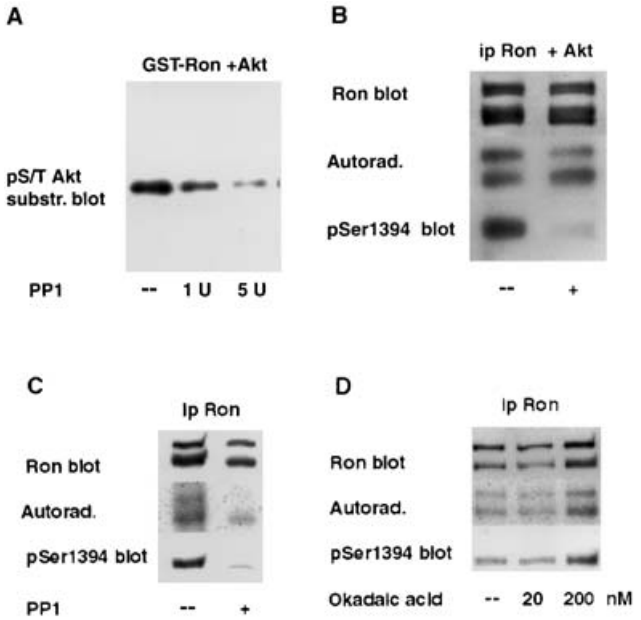

Figure 7 Dephosphorylation of Ron by PP1 *in vitro* and in living cells

(**A**) C-terminal Ron was phosphorylated *in vitro* in triplicate by Akt (2 units) during 20 min incubation at 30 °C (Ron + Akt). After the addition of 10 mM EDTA (final concentration) to all the samples and 1 or 5 units of muscle-purified PP1, as indicated, the samples were further incubated at 30 °C for 20 min. The blot obtained was probed to detect the site phosphorylated by Akt using anti-phospho(Ser/Thr) Akt substrate antibodies. (**B**) Ron, immunoprecipitated in duplicate from serum-starved NIH-3T3 cell extract, was phosphorylated *in vitro* by Akt (ip Ron + Akt) and treated with or without 1 unit of PP1, as described in (**A**). The blot obtained was first probed with the anti-Ron antibodies (Ron blot). This was followed by re-probing with the anti-pSer-1394 antibodies (pSer1394 blot) and exposure to detect the radioactivity incorporated into Ron (Autorad.). (**C**) Ron was immunoprecipitated in duplicate from metabolically labelled NIH-3T3 cells (as in Figure 1) and treated with or without 1 unit of PP1. The blot obtained was probed to detect Ron, phospho-Ser-1394 and radioactivity, as described in (**B**). (**D**) Metabolically labelled cells were exposed to the indicated amounts of okadaic acid during the last 30 min of labelling. Ron was immunoprecipitated and the blot obtained was probed to detect Ron, pSer-1394 and radioactivity.

Dephosphorylation of Ser-1394 by PP1

The consequent step was to investigate the ability of PP1 to dephosphorylate Ser-1394. This was first tested *in vitro*, using either C-terminal Ron or cellular Ron. C-terminal Ron was phosphorylated *in vitro* by Akt and subsequently treated with 1 or 5 units of purified PP1. Phosphorylation was detected using phospho-specific antibodies recognizing phosphorylated Akt substrates. Of the two PP1 amounts used, 5 units of PP1 dephosphorylated Ser-1394 almost completely (Figure 7A). Alternatively, Ron was immunoprecipitated from serum-starved cells and phosphorylated *in vitro* by Akt, in the presence of [γ-^{32}P]ATP. As expected, Ron was phosphorylated at Ser-1394. Subsequent treatment with muscle-purified PP1 removed the phosphate from Ser-1394 almost completely (Figure 7B). In another experiment, Ron was phosphorylated at Ser-1394 during [^{32}P]H_3PO_4 metabolic labelling, performed in the presence of 10 % serum. The subsequent PP1 treatment *in vitro* dephosphorylated Ser-1394 completely (Figure 7C).

The involvement of PP1 in Ser-1394 dephosphorylation was then tested in living cells. For this purpose, the Ron-expressing NIH-3T3 cells were treated with the phosphatase inhibitor okadaic acid. This was used at 20 nM, a concentration that inhibits PP2A only, and at 200 nM, a concentration that inhibits both PP1 and PP2A [28]. Cells were metabolically labelled with [^{32}P]H_3PO_4 and exposed to okadaic acid during the last 30 min of labelling. Ron immunoprecipitated from cells treated with 200 nM okadaic acid was phosphorylated more when compared with Ron immunoprecipitated from cells untreated or treated with 20 nM okadaic acid. Specifically, the increase in Ron phosphorylation involved Ser-1394 (Figure 7D). This last result indirectly indicates the involvement of PP1 in Ron Ser-1394 dephosphorylation in living cells.

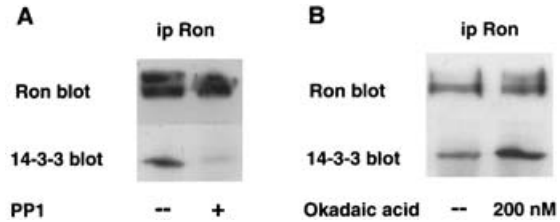

Figure 8 Modulation of the Ron–14-3-3 association by PP1

(**A**) GTL-16 cell extract was used to immunoprecipitate Ron in duplicate (ip Ron). One immunocomplex was further treated with 1 unit of PP1 catalytic subunit, as in Figure 1. After electrophoresis and transblotting, the blot was probed with anti-pan-14-3-3 antibodies (14-3-3 blot), followed by re-probing with the anti-Ron antibodies (Ron blot). (**B**) Ron was immunoprecipitated from GTL-16 cells treated with or without 200 nM okadaic acid for 60 min. Other experimental details are as described in (**A**).

PP1 influences the association of 14-3-3 with Ron

Once phosphorylated by Akt, Ser-1394 provides a docking site for 14-3-3. Consequently, we investigated the ability of PP1 to influence such binding *in vitro* and in living cells. As expected, Ron immunoprecipitated from cells grown in the serum displayed associated 14-3-3 (Figure 8A; [14]). Treatment of the Ron immunocomplex with PP1 dissociated 14-3-3 almost completely. This indicated the ability of PP1 to compete with 14-3-3 for the Ser-1394 site, at least *in vitro*. On the other hand, treatment of the cells with 200 nM okadaic acid, a concentration that was able to inhibit PP1, increased the amount of associated 14-3-3 (Figure 8B). This confirmed the ability of PP1 to recognize this site as a substrate and indicated that PP1 may be the enzyme counteracting the effects of Akt on Ser-1394 phosphorylation in living cells.

DISCUSSION

Several tyrosine phosphatases may associate with RTKs, either in response to ligand stimulation or in a ligand-independent manner [1–4]. In the present study, we have shown that a serine/threonine phosphatase associates with an RTK, namely the tyrosine kinase Ron. The interaction occurs after receptor activation through both MSP-dependent and -independent mechanisms.

The finding that PP1 associates with Ron in cells of different origins prompted us to characterize this association. We found that, in cells expressing wild-type Ron, PP1 binding is not constitutive, but transient. The interaction occurs after ligand activation of Ron or after Akt kinase-activating signals. Very little PP1 co-immunoprecipitates with Ron from serum-starved cells, when little or no Ser-1394 phosphorylation is detected. MSP treatment rescues PP1 to the same level observed in cells grown in the serum. Furthermore, the association is transient and its kinetics parallels the kinetics of Ser-1394 phosphorylation. To our knowledge, this is the first report on the association of PP1 with a growth factor receptor and of its regulation by the ligand.

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Ser-1394 has been characterized recently as a specific phosphoserine docking site, generated after Ron activation by MSP [14]. This is in addition to the well-known phosphotyrosine multifunctional docking site, surrounding Tyr-1353 and Tyr-1360 [13,27]. This new serine site resides within a consensus sequence for Akt [14]. Most remarkably, phosphorylated Ser-1394 binds proteins of the 14-3-3 family [14]. In turn, 14-3-3 may form a multiprotein complex with the $\alpha 6\beta 4$ integrin, which down-regulates the $\alpha 6\beta 4$-dependent adhesion [14]. These results and their implication in cell migration and in re-epithelization during wound healing were reported previously [14].

The involvement of Ser-1394 in the interaction with PP1 is suggested by the fact that, *in vitro*, the association between isolated catalytic subunit and C-terminal Ron occurs only when Ser-1394 is preserved. The specificity is further highlighted by the fact that PP2A fails to interact with C-terminal Ron. The picture is more complex in living cells, where Ron is expressed as an entire receptor. Ron mutation does not prevent PP1 interaction, but its regulation is completely lost. PP1 neither dissociates from S1394A-Ron after serum starvation or wortmannin treatment nor responds to MSP, as it does with wild-type Ron. PP1 seems to be trapped on the mutated receptor in an active, de-regulated state. A possible interpretation of these results assumes the presence of a regulatory subunit, as it is generally the case with PP1 [10]. The function of the regulatory subunits is to target PP1 to substrates and to modulate the enzymic activity [10]. It is possible that, for the S1394A-Ron, the regulatory subunit still anchors PP1 to the mutated receptor. However, the absence of the target serine residue would prevent a proper response to the regulatory signals. Identification of the regulatory subunit of the Ron-directed PP1 may help to clarify this mechanism. Within this picture, the association may also involve a Ron site that binds the PP1-regulatory subunit. Such a site might be located outside the C-terminus of Ron and it might play a role only in living cells, where the entire receptor is expressed. This hypothesis may help to explain the discrepancy between the results obtained *in vitro* and in living cells.

The loss of PP1 function is only one of the consequences of the S1394A mutation. The mutation was also reported to induce the loss of MSP- and Akt-dependent association of 14-3-3 with Ron, thus hampering the Ron–$\alpha 6\beta 4$ integrin interaction and the consequent modulation of cell adhesion [14].

PP1 binding is presumably followed by Ser-1394 dephosphorylation. This hypothesis is supported by the increase in phospho-Ser-1394 after cell exposure to an okadaic acid concentration that inhibits PP1. This result indicates that the Ser-1394-directed PP1 is active in cells grown in the serum. PP1 inhibition by okadaic acid shifts the balance towards Ser-1394 phosphorylation. In principle, under such conditions, the binding of 14-3-3 to Ser-1394 should also be favoured [14]. Indeed, we showed that okadaic acid treatment increased also the Ron-associated 14-3-3. Altogether, the results define a role for PP1 in balancing the effects of Ser-1394 phosphorylation by Akt within the serum- and MSP-dependent signalling.

Phosphorylation of specific serine and threonine sites of RTKs has been linked to the negative regulation of the receptors. For instance, phosphorylation of Ser-654 of the epidermal growth factor receptor [6] or Ser-985 of the HGF receptor [7] inhibited the receptor kinase activity and its biological effects. In this case, the function of the phosphatase was to remove the inhibitory effect of phosphorylation, thus contributing to receptor activation. Alternatively, serine phosphorylation may generate docking sites for effectors, thus sustaining the downstream signalling of specific pathways. For Ron, Ser-1394 phosphorylation by Akt generates a docking site for 14-3-3 [14]. Within this picture, the role of the phosphatase might be to modulate or terminate the downstream signalling generated at this site.

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