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The MAP-kinase ERK2 is a specific substrate of the protein tyrosine phosphatase HePTP

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HePTP is a tyrosine specific protein phosphatase that is strongly expressed in activated T-cells. It was recently demonstrated that in transfected T-cells HePTP impairs TCR-mediated activation of the MAP-kinase family members ERK2 and p38 and it was suggested that both ERK and p38 MAP-kinases are substrates of HePTP. The HePTP gene has been mapped to human chromosome 1q32.1. Abnormalities in this region are frequently found in various hematopoietic malignancies. HePTP is highly expressed in acute myeloid leukemia and its expression in fibroblasts resulted in transformation. To address a possible involvement of HePTP in hematopoietic malignancies we sought to identify HePTP substrate(s) in leukemic cells. Using substrate trapping mutants we have identified the MAP-kinase ERK2 as a specific target of HePTP in the myelogenous leukemia cell line K562. Tyrosine phosphorylated ERK2, but not ERK1, p38, or JNK1, efficiently bound to catalytically inactive HePTP mutants in which the active site cysteine (HePTP-C/S) or the conserved aspartic acid residue (HePTP-D/A) had been exchanged for serine and alanine, respectively. Moreover, the interaction of ERK2 with HePTP trapping mutants was dependent on ERK2 tyrosine phosphorylation, indicating that HePTP is specifically targeted to activated ERK2. Using a deletion mutant of HePTP (HePTP-dLD), in which 14 amino acid residues within the N-terminus are missing, we show that regions outside the catalytic domain are also required for the interaction. Furthermore, overexpression of HePTP in K562 cells and fibroblasts interfered with PMA or growth factor induced MAP-kinase activation and HePTP efficiently dephosphorylated active ERK2 on the tyrosine residue in the activation loop *in vitro*. Together, these data identify ERK2 as a specific and direct target of HePTP and are consistent with a model in which HePTP negatively regulates ERK2 activity as part of a feedback mechanism. *Oncogene* (2000) **19,** 858–869.

Keywords: signal transduction; phosphatase; HePTP; MAP-kinase

Introduction

Protein tyrosine phosphorylation is an important mechanism through which multicellular organisms regulate cell proliferation and differentiation. This tightly regulated process requires the combined action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Neel and Tonks, 1997; Byon *et al*., 1997). Aberrant regulation of signaling pathways that are controlled by tyrosine phosphorylation has been associated with various malignancies and many PTKs have been identified as the products of protooncogenes (Aaronson, 1991; Patarca, 1996).

Like the PTKs, the PTPs constitute a large family of transmembrane receptor-like as well as cytoplasmic enzymes (Fischer *et al*., 1991). Transmembrane PTPs frequently contain Ig-like or fibronectin type III domains within their extracellular domain and one or two catalytic domains within their intracellular portion. Cytoplasmic PTPs have one catalytic domain and one or more additional sequence motifs required for subcellular localization, substrate recognition or regulation of activity (Mauro and Dixon, 1994). Based on expression studies and chromosomal localization several PTPs (i.e. PTPβ, PTP-PEST, PTPδ) have been suggested to function as tumor suppressors (Gaits *et al*., 1994; Takekawa *et al*., 1994; Urushibara *et al*., 1998). Conversely, a small number of PTPs have been implicated in cell transformation or the development of cancer (reviewed in Zhang, 1998; Radha *et al*., 1997).

The identification of relevant substrates is crucial for the understanding of the physiological function of PTPs as well as their possible involvement in various malignancies. All PTPs contain a conserved 11 amino acid residue sequence motif ([I/V]HCXAGXXR[S/TG) within the catalytic domain which constitutes the active site of the phosphatase (Fischer *et al*., 1991). Within this motif the invariant cysteine is absolutely required for catalysis. N-terminal to this region is a conserved aspartic acid residue which functions as a general acid during catalysis (reviewed in Fauman and Saper, 1996). Mutation of the cysteine or aspartic acid residue to serine or alanine, respectively, results in enzymes which are catalytically inactive but retain their ability to bind tyrosine phosphorylated substrates and have successfully been used for the identification of candidate PTP substrates (Flint *et al*., 1997; Garton *et al*., 1996).

The hematopoietic PTP HePTP (also known as LC-PTP) is a cytosolic enzyme with a single catalytic domain (Adachi *et al*., 1992; Zanke *et al*., 1992) and is most similar to the brain specific PTPs STEP and PTP-SL (also termed PTPBR7, PCPTP1, and PC12-PTP) (Ogata *et al*., 1995; Hendriks *et al*., 1995; Shiozuka *et al*., 1995; Lombroso *et al*., 1991; Sharma *et al*., 1995; Bult *et al*., 1997). In T-cells, its mRNA is rapidly induced in response to IL2, PHA, LPS, ConA and T-cell antigen receptor (TCR) crosslinking, suggesting that it may play an important role in the regulation of T-cell proliferation and activation (Zanke *et al*., 1992; Adachi *et al*., 1994, 1995). HePTP protein is also

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detectable in resting cells with a moderate increase in expression after IL2 stimulation (Adachi *et al*., 1995). The HePTP gene has been mapped to human chromosome 1q32.1 (Adachi *et al*., 1992; Zanke *et al*., 1994). Abnormalities in this region are frequently found in various hematopoietic malignancies. Deletions of 1q32 have been detected in non-Hodgkin lymphomas and chronic lymphoproliferative disorders (Mitelman *et al*., 1990). Patients with myelodysplastic syndrome, a preleukemic condition, are frequently characterized by partial trisomies of this region. Zanke *et al*. (1994) identified a patient with myeloid dysplasia coincident with triplication of the HePTP gene and highly elevated HePTP protein levels. Importantly, overexpression of HePTP in NIH3T3 fibroblasts resulted in dramatic morphological changes, anchorage independent growth, and loss of contact inhibition (Zanke *et al*., 1994). Taken together, these data suggest that aberrant expression of HePTP may contribute to the development of hematopoietic malignancies.

Saxena *et al*. (1998) analysed the effect of HePTP overexpression on TCR-induced transcriptional activation. In these experiments transfection of Jurkat T-cells with wild type HePTP, but not a catalytically inactive mutant (HePTP-C270S), resulted in a block of TCR induced activation of a reporter gene driven by a NFAT/AP-1 element taken from the IL2 gene promoter, and also reduced activation of the MAP-kinase ERK2. Subsequently the same group demonstrated that in transiently transfected T-cells HePTP also impaired TCR mediated activation of the MAP-kinase family member p38. In *in vitro* phosphatase assays, HePTP was able to dephosphorylate ERK2 and p38 (Saxena *et al*., 1999). Further, wild type HePTP and HePTP-C270S associated with ERK2 and p38. This interaction required the non-catalytic N-terminus of HePTP and was independent of ERK2 or p38 phosphorylation.

To further investigate a possible function of HePTP in hematopoietic malignancies we sought to identify candidate HePTP substrates in leukemic cells. To this end we made use of a substrate trapping approach. Using lysates of pervanadate treated K562 myelogenous leukemia cells a single tyrosine phosphorylated protein of 43 kD bound specifically to HePTP substrate trapping mutants (HePTP-C/S and HePTP-D/A), but not to the wild type phosphatase. Using specific antisera we were able to identify this protein as the MAP-kinase family member ERK2. Consistent with the findings of Saxena *et al*. (1999), activated ERK2 was efficiently dephosphorylated by HePTP *in vitro* and expression of wild type HePTP in tissue culture cells impaired the activation of ERK2 by PMA or growth factor treatment. However, we found the interaction of HePTP with MAP-kinase family members to be specific for ERK2. Binding of activated p38 to HePTP-C/S was only observed under less stringent low-salt conditions. Further, we demonstrate that this association requires a 14 amino acid sequence within the N-terminus of HePTP and also involves the catalytic domain. The formation of a stable complex between ERK2 and HePTP-C/S was dependent on ERK2 tyrosine phosphorylation and could be disrupted by tyrosine phosphorylated peptides modeled from the ERK2 activation loop sequence. Our data suggest that ERK2 is the predominant HePTP target in K562 cells and are consistent with a model in which HePTP get recruited to activated ERK2, thereby modulating its activity in the cytosol as part of a feedback mechanism.

Results

A tyrosine phosphorylated 43 kD protein interacts with HePTP substrate trapping mutants

To generate HePTP substrate trapping mutants we exchanged the active site cysteine residue (C269) and the conserved aspartic acid residue (D235) to serine and alanine, respectively. The resulting mutants, HePTP-C/S and HePTP-D/A, as well as wild type HePTP were used to generate Gst-fusion proteins for pull down experiments. We analysed the binding of these fusion proteins to tyrosine phosphorylated proteins from K562 chronic myelogenous leukemia cells which had been treated with the PTP inhibitor pervanadate in order to induce protein tyrosine phosphorylation (Figure 1a). K562 cells constitutively

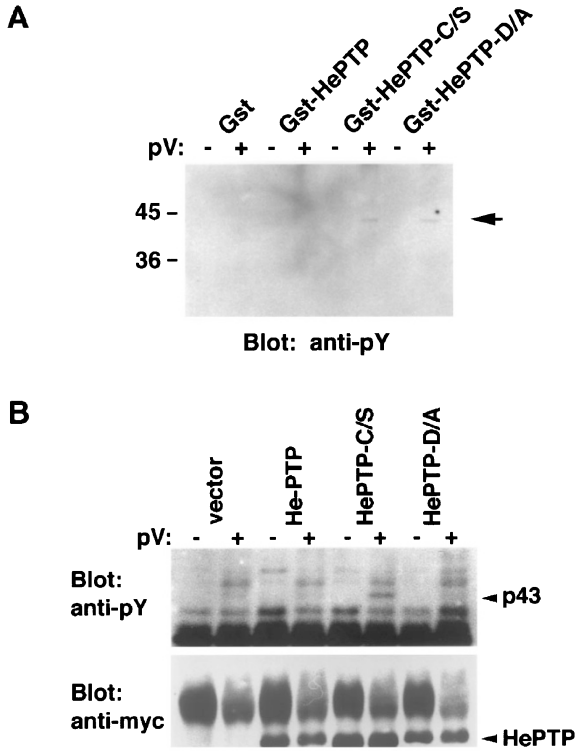

Figure 1 A 43 kD tyrosine phosphorylated protein interacts with catalytically inactive HePTP mutants. (**a**) K562 cells were either left untreated or were treated with pervanadate (pV) as indicated and then lysed. The cleared lysates were incubated with Gst or Gst-HePTP fusion proteins and bound proteins were detected by SDS–PAGE and anti-phosphotyrosine (anti-pY) immunoblot using mAb PY20. (**b**) Stable K562 cell lines expressing *myc*-tagged wild type HePTP, HePTP-C/S or HePTP-D/A, and a vector transfected control line were left untreated or were treated with pervanadate as indicated. The cells were lysed and the cleared lysates subjected to immunoprecipitation with mAb 9E10 (anti-*myc*). Tyrosine phosphorylated proteins coprecipitating with HePTP or HePTP mutants were detected by immunoblot with mAb PY20 (upper panel). The blot was stripped and reprobed with mAb 9E10 to demonstrate efficient precipitation of wild type HePTP and HePTP mutants (lower panel)

express HePTP (SM Pettiford and R Herbst (1998), unpublished observation) and therefore should be expected to also contain relevant HePTP substrates. We consistently observed binding of a single tyrosine phosphorylated protein of 43 kD from pervanadate treated K562 cells to Gst-HePTP-C/S and Gst-HePTP-D/A, but not to Gst-HePTP or Gst controls, suggesting that this protein is a likely HePTP substrate. To confirm these results *in vivo* we established K562 cell lines stably expressing *myc*-tagged versions of wild type HePTP, HePTP-C/S or HePTP-D/A, respectively. The cell lines were treated with pervanadate and association of tyrosine phosphorylated proteins with HePTP or HePTP mutants was detected by coimmunoprecipitation (Figure 1b). Consistent with the results of the Gst pull down experiments, a single tyrosine phosphorylated protein of 43 kD coprecipitated with HePTP-C/S from pervanadate treated K562 cells. The failure of phosphorylated p43 to bind to HePTP-D/A from pervanadate treated cells is most likely explained by the mechanism through which this compound inhibits PTPs. In contrast to vanadate itself, which is a competitive PTP inhibitor and binds to the active site, pervanadate treatment results in a modification of the catalytic pocket by oxidizing the active site cysteine residue (Huyer *et al*., 1997). In HePTP-C/S the cysteine residue is missing and, consequently, this mutant should be insensitive to the oxidizing effect of pervanadate. In light of this interpretation, the results shown in Figure 1b suggest that at least part of the interaction of tyrosine phosphorylated p43 with HePTP is mediated by the catalytic pocket of the phosphatase.

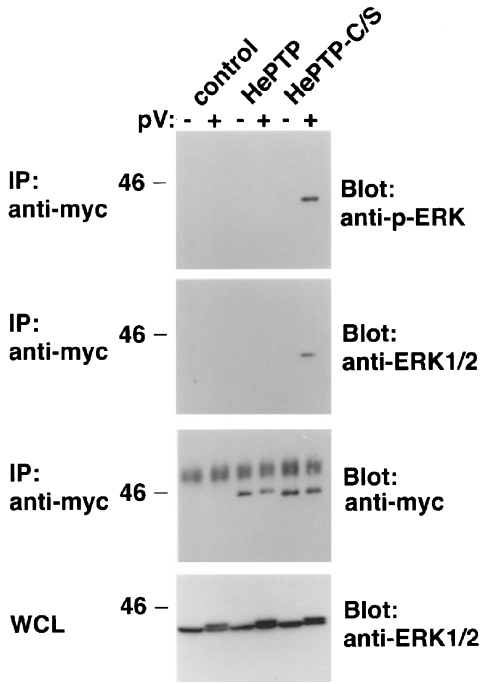

Figure 2 Identification of p43 as the MAP-kinase family member ERK2. K562 cells stably expressing *myc*-tagged wild type HePTP or HePTP-C/S or vector transfected control cells were left untreated or were treated with pervanadate prior to lysis. Lysates were subjected to immunoprecipitation with mAb 9E10 (anti-*myc*) and the precipitates analysed by SDS–PAGE and immunoblot with phospho-p44/p42 antiserum (anti-p-ERK; upper panel). The blot was stripped and reprobed with the antiserum anti-ERK1/2 (panel two from top). Probing the blot with mAb 9E10 demonstrated efficient precipitation of both wild type HePTP and HePTP-C/S (panel 3). To demonstrate efficient activation of ERK2 in all pervanadate treated samples whole cell lysates were immunoblotted with antiserum anti-ERK1/2 (bottom panel)

Identification of p43 as the MAP-kinase family member ERK2

We next sought to determine the identity of the putative HePTP substrate p43. To this end, we performed a series of coimmunoprecipitations and probed the resulting immunoblots with antibodies specific for various tyrosine phosphorylated signaling molecules in the molecular weight range of 40–50 kD, including MAP-kinase. Of the antibodies tested, only antiserum that specifically recognizes the MAP-kinase family members ERK1 and ERK2 was able to detect p43 coprecipitating with HePTP-C/S from pervanadate treated K562 cells (Figure 2). Its mobility on SDS–PAGE and its cross-reactivity with anti-ERK antibodies (see also Figures 4a,b, 6a,b) demonstrate that p43 is in fact ERK2 MAP-kinase. Probing the blot with a phospho-specific anti-MAP-kinase antiserum which specifically recognizes activated ERK1 and ERK2 revealed that it is the active form of ERK2 that coprecipitates with HePTP-C/S (Figure 2, top panel). Probing whole cell lysates with anti-ERK antiserum demonstrated efficient activation of ERK2 in all cell lines upon pervanadate treatment, as indicated by its shift towards a higher molecular weight on SDS–PAGE (Figure 2, bottom panel). ERK1 is expressed at relatively low levels in these cells and could not be detected.

To test if the interaction of ERK2 with HePTP could be detected in other cell lines we performed Gst pull down experiments from a panel of different cell lines. At the same time we asked if the observed interaction of HePTP with MAP-kinase family members was specific for ERK2 or if we could also detect binding of ERK1 from cell lines that express comparable amounts of both MAP-kinase family members. As shown in Figure 3, phosphorylated ERK2 bound exclusively to Gst-HePTP-C/S in all cell lines tested. We could detect weak binding of ERK2 to Gst-HePTP for two of the cell lines analysed (NIH3T3 and RBL-2H3). In contrast, only with lysates from RBL-2H3 were we able to detect significant amounts of ERK1 binding to Gst-HePTP-C/S (Figure 3, top panel). Probing the blot with a phospho-specific anti-ERK antiserum, however, did not reveal binding of active ERK1 from these cells to Gst-HePTP-C/S (Figure 3, middle panel).

As shown in Figure 4, the observed interaction of HePTP with ERK2 is not limited to pervanadate treated cells, but can also be detected in response to other stimuli that result in MAP-kinase activation. In Figure 4a, coprecipitation of phosphorylated ERK2 with HePTP and HePTP-C/S from PMA treated stable cell lines was analysed. Again, we could only detect coprecipitation of activated ERK2 from PMA treated cells with HePTP-C/S. In addition, we tested the binding of HePTP fusion proteins to ERK2 from growth factor treated U373 glioblastoma cells (Figure 4b). U373 cells express receptors for epidermal growth factor (EGF) and platelet derived growth factor

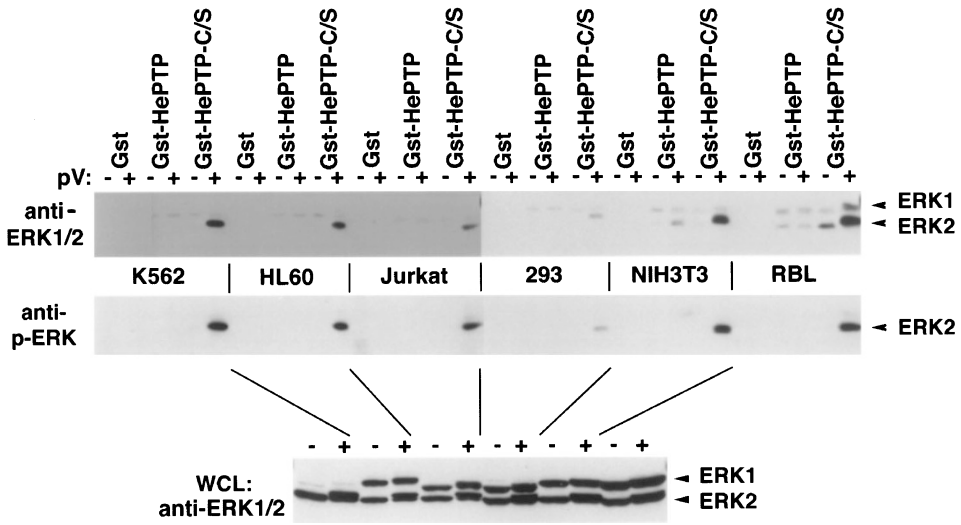

Figure 3 HePTP interacts with activated ERK2 from multiple cell lines. K562, HL60, Jurkat, 293, NIH3T3, and RBL-2H3 cells were treated with pervanadate (pV) or vehicle, and the lysates were then incubated with Gst, Gst-HePTP, or Gst-HePTP-C/S bound to glutathione agarose, as indicated. Binding of MAP-kinase family members ERK1 and ERK2 was determined by immunoblot with anti-ERK1/2 (upper panel). Probing the blot with a phospho-specific anti-ERK antiserum (anti-p-ERK) confirmed that only activated ERK2 was bound to Gst-HePTP-C/S (middle panel). To confirm that both ERK1 and ERK2 were activated upon pervanadate treatment in the cell lines tested, whole cell lysates (WCL) were immunoblotted with the antiserum anti-ERK1/2 (bottom panel)

(PDGF) and show increased ERK phosphorylation upon treatment with the respective ligands (not shown). Binding of ERK2 to Gst-HePTP-C/S was only detected if the cells had been treated with EGF or PDGF. In this experiment, we also observed weak binding of ERK2 from factor treated cells to Gst-HePTP, similar to what we had observed with pervanadate treated NIH3T3 cells (see Figure 3). Together, these data demonstrate that phosphorylated ERK2 forms a stable complex with catalytically inactive HePTP-C/S and that this association is largely independent of the cell type and the treatment used to activate ERK2.

ERK2 is the predominant HePTP interacting protein from K562 cells

To further address the issue of specificity we wanted to determine if other MAP-kinase family members were potential targets of HePTP. p38 and JNK kinases are structurally related to ERK1 and ERK2, but serve distinct biological functions. In addition they differ in their activation loop sequence from ERK1 and ERK2 and utilize kinases distinct from MEK for their activation (Cobb and Goldsmith, 1995). To test a possible interaction of HePTP with p38 we performed Gst pull down experiments with lysates of sorbitol treated K562 cells. High concentrations of sorbitol induce osmotic stress, leading to the activation of p38 (Raingeaud *et al*, 1995). In previous experiments we were unable to detect any interaction of p38 with HePTP or HePTP-C/S, using our standard lysis buffer (data not shown). We then tested different lysis conditions to see if the presence of glycerol or the salt concentration affects the binding of p38 to HePTP. As shown in Figure 5a, we could detect binding of p38 to Gst-HePTP-C/S only under low salt condition (50 mM NaCl; Figure 5a, lanes 2 and 8). Further, we could only detect binding with lysates from sorbitol treated cells, indicating that activation and phosphorylation of p38 is required for the interaction. As in previous experiments no binding of p38 to HePTP-C/S was detected in the presence of 150 mM NaCl, the physiological salt concentration.

Given these results we also tested the effect of different buffer conditions on the binding of ERK2 to HePTP-C/S. In these experiments we observed constitutive binding of ERK2 to HePTP-C/S in the presence of 50 mM NaCl. At higher salt concentrations (150 mM or 300 mM NaCl) the interaction was strictly dependent on the activation and phosphorylation of ERK2 (data not shown). We also tested a possible interaction of JNK1 and HePTP, but were unable to detect any binding (not shown).

An important question was whether we could detect other HePTP interacting proteins which might play a role in HePTP regulation or in mediating the interaction with MAP-kinase. To this end, we performed binding experiments with lysates from K562 cells that had been metabolically labeled with ^{35}S-methionine. Proteins interacting with Gst-HePTP or Gst-HePTP-C/S were detected by SDS–PAGE and autoradiography (Figure 5b). In this experiment, we detected a single protein from lysate of pervanadate treated K562 cells which specifically bound to Gst-HePTP-C/S, but not to Gst-HePTP or the Gst control. Given its molecular weight of 43 kD, this protein most likely represents activated ERK2.

Binding of activated ERK2 to HePTP-C/S involves the catalytic domain and a region in the N-terminus of the phosphatase

The data presented in Figure 1b suggested that interaction of phosphorylated ERK2 with the active site of the PTP domain may contribute to the overall binding affinity. To further explore this possibility we performed peptide competition experiments. Lysates

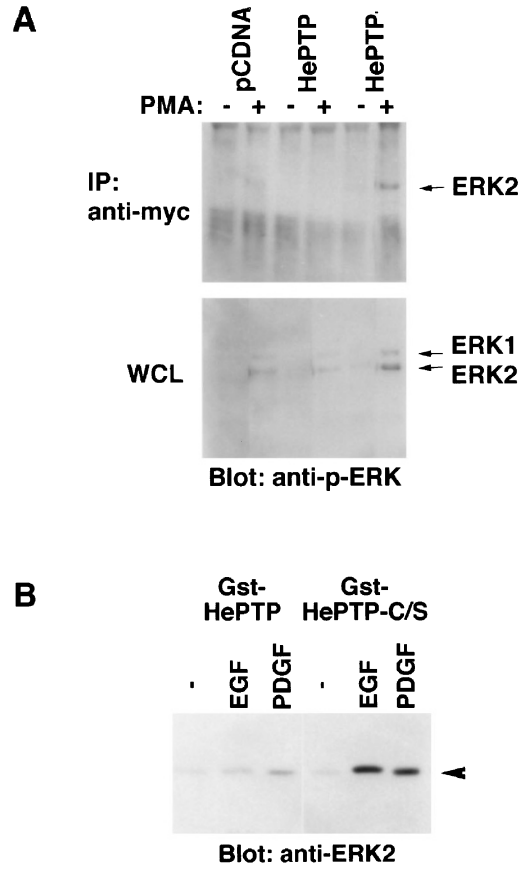

Figure 4 HePTP interacts with ERK2 from PMA and growth factor treated cells. (**a**) Control transfected K562 cells (pCDNA) or cells expressing myc-tagged HePTP or HePTP-C/S were starved overnight and then stimulated with vehicle (−) or PMA (+). The lysates were subjected to immunoprecipitation with mAb 9E10 (anti-myc) and coprecipitation of active ERK2 was detected by immunoblot with a phospho-specific anti-ERK antiserum (anti-p-ERK; upper panel). To control for MAP-kinase activation whole cell lysates (WCL) were analysed using the same antiserum (anti-p-ERK; lower panel). (**b**) U373 cells were starved overnight and then left untreated (−) or stimulated with EGF or PDGF as indicated. The lysate of each dish was split in half and incubated with Gst-HePTP or Gst-HePTP-C/S bound to glutathione agarose beads, followed by SDS–PAGE and anti-ERK2 immunoblot. Activation of MAP-kinase in response to growth factor treatment was verified by immunoblotting whole cell lysates with anti-phospho-p44/p42 (not shown)

from pervanadate treated K562 cells were incubated with Gst-HePTP-C/S bound to glutathione agarose beads, in the absence or presence of phosphopeptides. The peptides used were ERK-pY, ERK-pT, and, as control, a tyrosine phosphorylated peptide corresponding to the regulatory phosphorylation site of the cyclin dependent kinase CDK2 (CDK-pY; see Figure 6a for peptide sequences). As shown in Figure 6a, the peptide ERK-pY competed in a dose dependent manner with the binding of ERK2 to Gst-HePTP-C/S. At a concentration of 10 μM the presence of ERK-pY significantly reduced the binding of ERK2 to HePTP-C/S. In contrast to ERK-pY, ERK-pT and CDK-pY did not affect the interaction of ERK2 with HePTP-C/S. The inability of peptide ERK-pT to compete for ERK2 binding to the phosphatase is in agreement with the results of our phosphatase assays (see Figure 7b) and further indicates that the HePTP catalytic domain does not recognize the ERK activation loop sequence if only phosphorylated on the threonine residue. The results obtained with peptide CDK-pY further suggests that HePTP discriminates between different tyrosine phosphorylated peptide substrates. In contrast, the inability of high concentrations of peptide ERK-pY to completely abolish the interaction of ERK2 with HePTP-C/S suggested that regions outside the catalytic domain may be involved in the interaction. Analysis of the HePTP amino acid sequence revealed a 14 amino acid residue motif within the N-terminus of HePTP, which is also present in the brain specific PTPs, STEP and PTP-SL (LQERRGSNVALMLD; amino acid residues 17–30 in HePTP). Recently, Pulido *et al.* (1998) identified ERK1 and ERK2 as candidate substrates of STEP and PTP-SL. They also demonstrated that both kinases form a stable phosphorylation independent complex with STEP and PTP-SL and that this association requires the conserved N-terminal sequence motif. To determine if this region is also involved in the interaction of HePTP with ERK2 we deleted amino acids 17–30 in HePTP (HePTP-dLD) and HePTP-C/S (HePTP-dLD-C/S). We generated stable K652 cell lines expressing *myc*-tagged versions of HePTP-dLD and HePTP-dLD-C/S, respectively, and tested for coimmunoprecipitation of ERK2 after pervanadate treatment (Figure 6b). As for wild type HePTP, no ERK2 could be detected in anti-*myc* immunoprecipitates from HePTP-dLD expressing cells, while coprecipitation of ERK2 with HePTP-C/S was readily detectable. Deletion of HePTP amino acid residues 17–30 in the HePTP-C/S background resulted in a complete loss of ERK2 binding, suggesting that this region of the phosphatase is required for stable association with its substrate ERK2.

HePTP dephosphorylates ERK1 and ERK2 in vitro *and* in vivo

Our data demonstrate that ERK2 is the predominant HePTP interacting protein from K562 cells. The specific interaction with catalytically inactive HePTP mutants and the requirement of ERK2 phosphorylation for this interaction suggested that ERK2 is a candidate substrate of HePTP. To test this possibility we performed *in vitro* phosphatase assays, using recombinant activated ERK2 and ERK1 as substrates (Figure 7a). Incubation of phosphorylated ERK2 with Gst-HePTP resulted in rapid and almost complete dephosphorylation, as determined by immunoblot with an antibody specific for tyrosine phosphorylated ERK1 and ERK2. As expected, incubation with Gst or Gst-HePTP-C/S did not result in detectable ERK2 dephosphorylation. As compared to ERK2, ERK1 was dephosphorylated less efficiently by Gst-HePTP under identical assay conditions (Figure 7a).

Based on sequence similarities with other protein phosphatases HePTP is a classic PTP and should be specific for tyrosine phosphate (van Huijduijnen, 1998). However, having identified ERK2 as a candidate substrate, we wanted to test if this assumption held true, in that HePTP would only be able to dephosphorylate the phospho-tyrosine residue in the activation loop sequence and not the phospho-threonine residue. Figure 7b shows an *in vitro* phosphatase assay using phosphorylated peptides corresponding to the activation loop sequence of ERK1 and ERK2

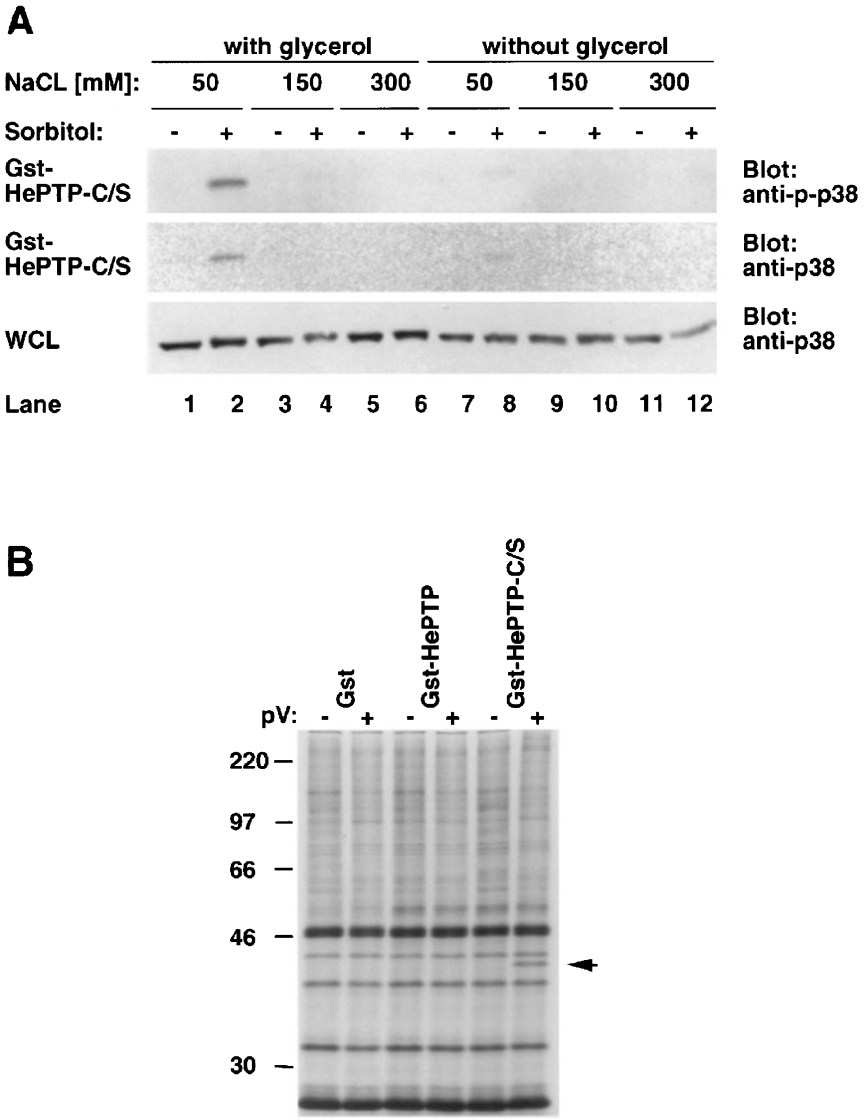

Figure 5 HePTP interacting proteins from K562 cells. (**a**) K562 cells were grown in 10% FBS and treated with 500 mM sorbitol for 15 min as indicated. Cells were lysed under different buffer conditions (with or without 10% glycerol; with 50, 150 or 300 mM NaCl) and the lysates incubated with Gst-HePTP-C/S, bound to glutathione agarose beads. Binding of active p38 was detected by immunoblot with a phosphospecific anti-p38 mAb (anti-p-p38; top panel). The blot was stripped and reprobed with an anti-p38 antiserum (middle panel). The bottom panel shows whole cell lysates (WCL) probed with anti-p38 antiserum. The shift towards higher molecular weight in the sorbitol treated samples demonstrates efficient activation of p38. (**b**) K562 cells were metabolically labeled with ^{35}S-methionine overnight and then stimulated with pervanadate as indicated. Lysates were incubated with Gst-HePTP, Gst-HePTP-C/S, or Gst as a control. Proteins forming a stable complex with fusion proteins were visualized by SDS–PAGE and autoradiography

(DNTGFLTE**Y**VATR) as substrates. In the peptides ERK-pY and ERK-pT the regulatory tyrosine and threonine residues, respectively, are phosphorylated. Incubation of the peptide ERK-pY with Gst-HePTP fusion protein resulted in rapid dephosphorylation, as determined by the release of free phosphate, while no dephosphorylation of peptide ERK-pT could be detected.

We then tested whether expression of HePTP in K562 cells would interfere with PMA induced ERK2 activation. K652 cells stably expressing HePTP or HePTP-C/S or control transfected cells were treated with PMA for the times indicated and the level of ERK2 activation analysed in *in vitro* kinase assays, using MBP as a substrate for phosphorylation (Figure 8a). In control transfected cells PMA treatment resulted in increased ERK2 kinase activity. In cells expressing wild type HePTP, PMA induced ERK2 activation is significantly reduced. Interestingly, cells expressing HePTP-C/S also showed a marked reduction of ERK2 activity. This result may be due to the formation of a stable complex of activated ERK2 with HePTP-C/S, which might interfere with the ability of ERK2 to phosphorylate its substrate. To determine if HePTP could also interfere with MAP-kinase activation in other cell types, we transiently transfected 293T cells with wild type HePTP or empty vector and analysed the effect on EGF induced MAP-kinase activation. In 293T cells, EGF stimulation results in increased ERK1 and ERK2 tyrosine phosphorylation (Figure 8b, top panel). Expression of HePTP in these cells resulted in complete inhibition of phosphorylation of both ERK1 and ERK2.

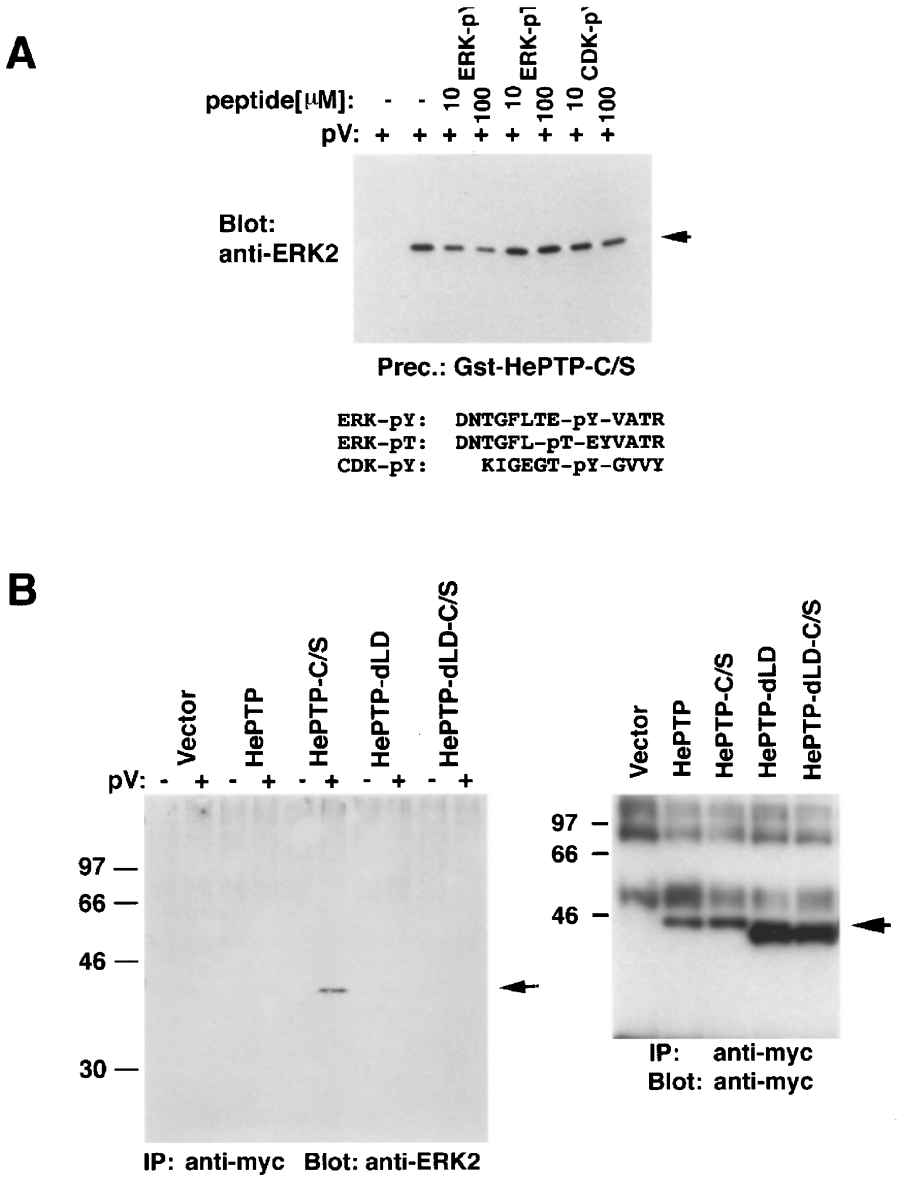

Figure 6 Interaction of HePTP with activated ERK2 requires the catalytic domain and a region within the N-terminus of HePTP. (**a**) A tyrosine phosphorylated peptide corresponding to the ERK activation site inhibits HePTP binding *in vitro*. Lysates from pervanadate treated K562 cells were incubated with Gst-HePTP-C/S in the absence or presence of phosphopeptide. The peptide ERK-pY corresponds to the activation loop sequence of ERK1 and ERK2, with the tyrosine residue phosphorylated; ERK-pT has the same peptide sequence, but with the threonine residue phosphorylated; CDK-pY is a tyrosine phosphorylated peptide corresponding to the regulatory phosphorylation site of cyclin dependent kinase 2 (CDK2). The effect of the phosphopeptides on ERK2 binding was determined by SDS–PAGE and immunoblot with an anti-ERK2 antiserum. (**b**) Deletion of 14 amino acid residues within the HePTP N-terminus abolishes binding to activated ERK2. Vector transfected cells or K562 cells stably expressing wild type HePTP or HePTP mutants were treated with pervanadate prior to lysis as indicated. The lysates were subjected to immunoprecipitation with mAB 9E10 (anti-*myc*) and coprecipitation of ERK2 was analysed by anti-ERK2 immunoblot (left panel). To control for expression of all constructs, cell lysates were analysed by immunoprecipitation and immunoblot with mAb 9E10 (right panel)

Taken together, these results suggest that ERK2 is a HePTP substrate and that direct dephosphorylation of the ERK2 phosphotyrosine residue within the activation loop sequence by HePTP results in inactivation of the kinase. They also indicate that, at least under conditions of overexpression, HePTP can interfere with the activation of ERK1.

Discussion

Aberrant expression of HePTP may be involved in the etiology of various hematopoietic malignancies. The identification of candidate substrates is key to the understanding of HePTP's transforming potential as well as its physiological function. Using the leukemic K562 cell line as a model we have identified the MAP-kinase ERK2 as a specific HePTP substrate. The data show that activated/phosphorylated ERK2 forms a stable complex with catalytically inactive mutants of HePTP, but not with the wild type phosphatase and suggest that ERK2 is a candidate HePTP substrate. The observations that HePTP directly dephosphorylates ERK2 *in vitro* and that HePTP blocks PMA or growth factor induced MAP-kinase activation in transfected cells further support the interpretation that

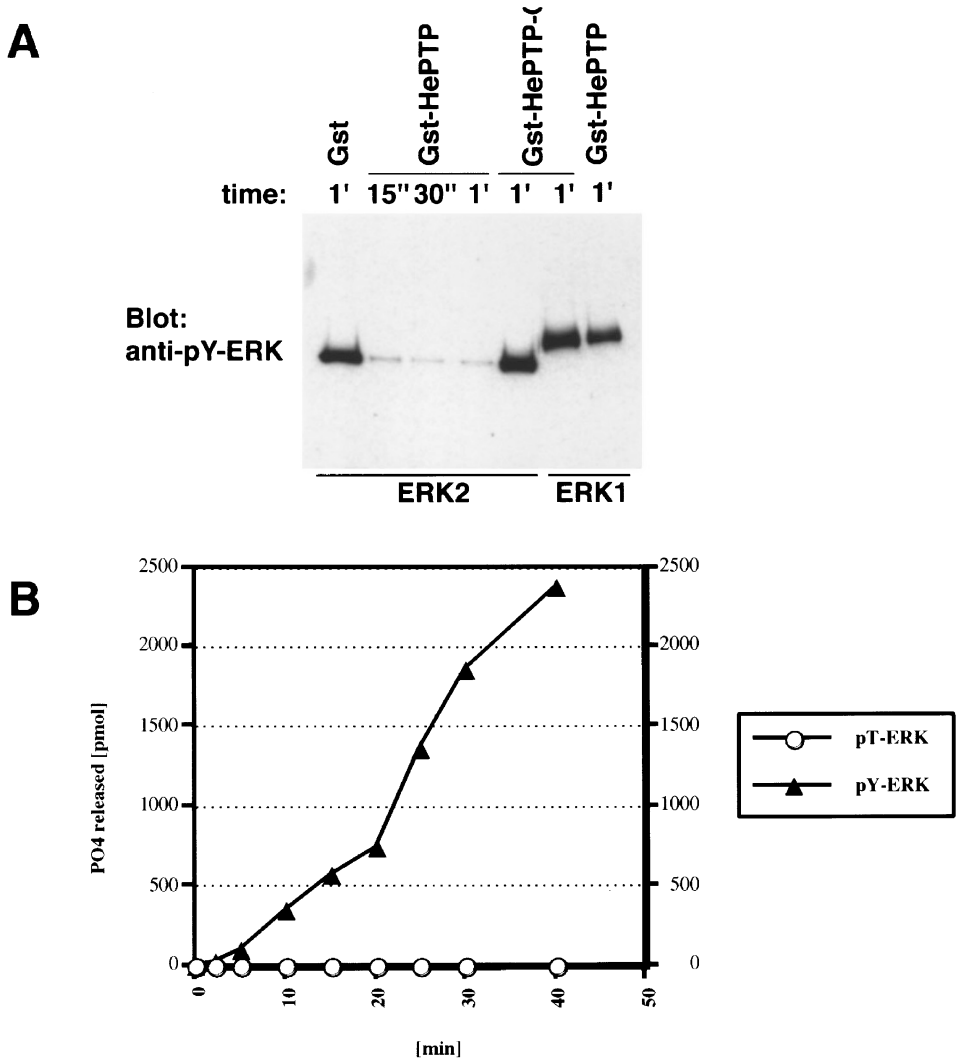

Figure 7 HePTP dephosphorylates MAP-kinase *in vitro*. (**a**) Activated recombinant ERK2 and ERK1 (50 ng per sample), respectively, were incubated with 1 μg Gst, Gst-HePTP or Gst-HePTP-C/S for the indicated time periods. Reactions were stopped by addition of SDS sample buffer and dephosphorylation of ERK2 and ERK1 was monitored by SDS–PAGE and immunoblot with a mAb specifically recognizing tyrosine phosphorylated MAP-kinase (anti-pY-ERK). (**b**) *In vitro* dephosphorylation of MAP-kinase phosphopeptides, corresponding to the activation loop sequence. Peptides ERK-pY and ERK-pT, respectively, were incubated with Gst-HePTP (1 μg per sample) for the indicated time periods and the release of free phosphate determined (see Materials and methods). All measurements were done in duplicate

ERK2 is an *in vivo* substrate of HePTP. These data are in agreement with Saxena *et al*. (1999) who identified ERK2 as a HePTP substrate in T-cells. Our results further demonstrate that the interaction of HePTP with ERK2 is largely cell type independent and can be observed in hematopoietic as well as non-hematopoietic cells. In this regard it should be noted that the expression of HePTP is not restricted to the hematopoietic system. We had previously performed Northern analysis to determine the expression pattern of HePTP in human tissues and observed significant levels of HePTP mRNA in testis, colon, and small intestine (SM Pettiford and R Herbst (1998), unpublished observation). This could indicate that HePTP is also a physiological regulator of ERK2 in non-hematopoietic tissues and cell types.

In T-cells, HePTP negatively regulates the MAP-kinase family members ERK2 and p38 (Saxena *et al*., 1999), while in our study of K562 cells HePTP is selective for ERK2. In *in vitro* binding experiments and by coprecipitation we could only detect a single tyrosine phosphorylated protein of 43 kD, representing ERK2, associated with HePTP. Similarly, the analysis of metabolically labeled cells revealed only binding of a single protein of 43 kD in Gst pull down assays. However, we did detect a weak interaction of activated p38 with HePTP-C/S under less stringent lysis conditions. Cell type specific expression of other proteins interacting with HePTP or MAP-kinase family members may be involved in the regulation of HePTP. Cell type specific substrate selection may be an important aspect of HePTP physiology and will be the subject of future experiments.

A family of dual specificity phosphatases, the MAP-kinase phosphatases or MKPs (reviewed in Lewis *et al*., 1998; Keyse, 1995), is generally involved in the negative regulation of MAP-kinases. MKPs differ with regard to their substrate specificity and tissue distribution. With the exception of MKP-3, MKPs generally localize to the nucleus and of the members known to date, MKP-3 and hVH3 are the only ones specific for ERK1/2. PTPs have also been implicated in the down

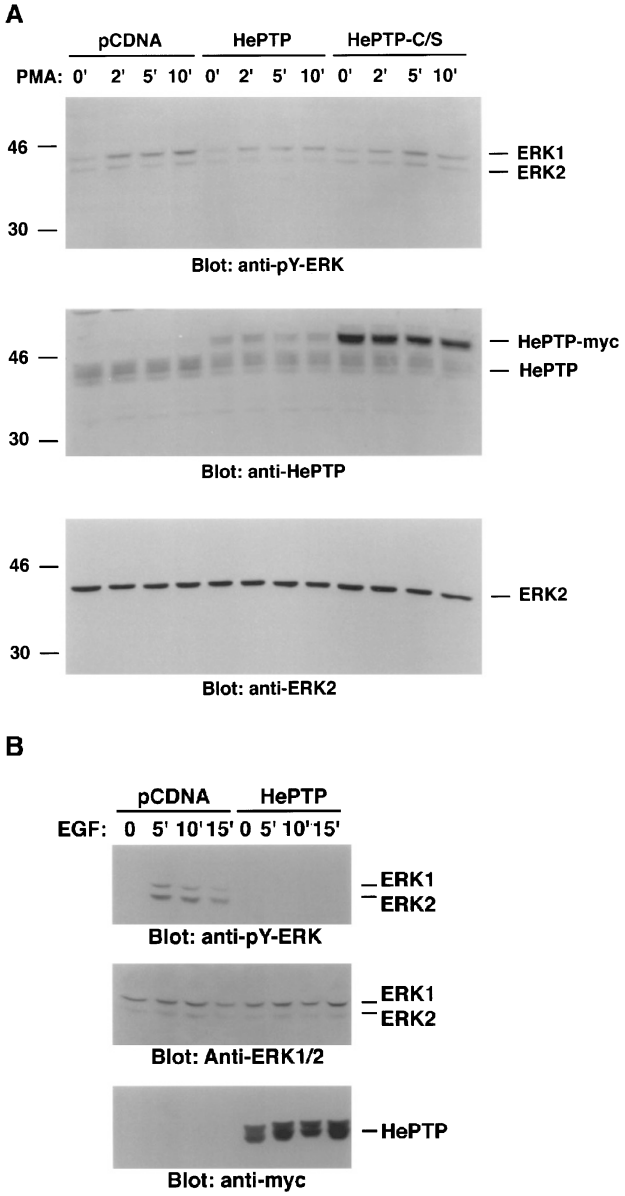

Figure 8 Ectopic expression of HePTP interferes with growth factor induced MAP-kinase activation. (**a**) K562 cells expressing HePTP or HePTP-C/S, respectively, or control transfected cells were stimulated with PMA and lysed. ERK2 was immunoprecipitated with anti-ERK2 agarose beads. Precipitates were washed and phosphorylation of myelin basic protein (MBP) with [^{32}P]γATP was done as described in Materials and methods. Reactions were stopped with SDS sample buffer and applied to SDS–PAGE. Incorporation of ^{32}P into MBP was quantitated using a phosphorimager. (**b**) 293T cells were transfected with HePTP or with empty vector. Cells were starved overnight prior to stimulation with EGF. Whole cell lysates (WCL) were subjected to SDS–PAGE and phosphorylation of ERK1 and ERK2 was determined by immunoblot with anti-pY-ERK (upper panel). Expression of HePTP was detected by anti-*myc* immunoblot (middle panel). Equal loading of samples was verified by probing the blot with an ERK2 specific antiserum (anti-ERK2; bottom panel)

regulation of ERK MAP-kinases (Alessi *et al*., 1995; Gopalbhai and Meloche, 1998). Recently, the PTPs, STEP and PTP-SL have been demonstrated to associate with and dephosphorylate ERK1 and ERK2 (Pulido *et al*., 1998). PTP-SL, STEP, and HePTP share a conserved sequence motif in their otherwise divergent N-termini which is required for their interaction with ERK MAP-kinases. HePTP and STEP/PTP-SL, however differ in two important ways with regard to their substrate interaction. First, HePTP seems to be more selective for ERK2 (see Figure 3), while STEP and PTP-SL bind both ERK1 and ERK2. Second, STEP and PTP-SL associate with ERK1 and ERK2 independent of their activation when overexpressed or in Gst pull down experiments (Pulido *et al*., 1998). Similarly, Saxena *et al*. (1999) observed constitutive association of inactive ERK2 with wild type HePTP in transiently transfected T-cells. In the cell lines we tested we could only observe a weak interaction of inactive ERK2 with wild type HePTP in NIH3T3, RBL-2H3, and U373 cells (Figures 3 and 4b), or when HePTP was transiently overexpressed in 293T cells (not shown). We also detected constitutive binding of ERK2 to HePTP-C/S when the salt concentration in the binding buffer was reduced from 150 mM to 50 mM NaCl, Our data clearly indicate that the HePTP catalytic domain contributes to the overall binding affinity, which may explain the phosphorylation dependent interaction we observed under physiological salt concentrations.

Given their specificity for phosphorylated tyrosine residues together with the identification of ERK MAP-kinases as substrates, HePTP, STEP, and PTP-SL constitute a new family of MAP-kinase phosphatases. While HePTP is predominantly expressed in lymphocytes, the expression of STEP and PTP-Sl is restricted to brain tissue. It is tempting to speculate that novel members of this subfamily of PTPs are expressed in other tissues and might be involved in the regulation of tissue specific growth and/or differentiation signals.

The identification of MAP-kinase family members as HePTP substrates provides possible explanations for a role of this PTP in hematopoietic malignancies. The ERK, p38, and JNK MAP-kinase pathways are important regulators of cell proliferation, survival, and differentiation (reviewed in Seger and Krebs, 1995; Lewis *et al*., 1998). Activation of ERK1/2 has frequently been correlated with increased cell proliferation and/or transformation. The response to ERK activation, however, depends on the cell type as well as the magnitude and duration of ERK kinase activity. For example in rat PC12 cells EGF induces short term ERK activation and proliferation, while NGF treatment results in prolonged ERK activation and neuronal differentiation (Qui and Green, 1992; Traverse *et al*., 1992). Similarly, sustained activation of ERK2 in K562 cells leads to cell cycle arrest and megakaryocytic differentiation (Whalen *et al*., 1997; Racke *et al*., 1997; Herrera *et al*., 1998). Thus, inhibition of ERK activation by HePTP could limit the differentiation of certain cell types and promote a proliferative state. Alternatively, HePTP may be involved in the regulation of cell survival. The MAP-kinase signaling pathways are key regulators of apoptosis induced by stress or physiological stimuli. Activation induced cell death is an important step in the termination of the T-cell immune response. This phenomenon is mediated by the induction of FasL and the nuclear receptor Nur77 (reviewed in Kelly and Siebenlist, 1995; Nagata and Golstein, 1995). van den Brink *et al*. (1999) recently demonstrated that the ERK MAP-kinase pathway is required for FasL and Nur77 expression in activated T-cells and that inhibition of

the pathway with the MEK1 inhibitor PD98059 prevented activation induced cell death. Thus it is possible that HePTP may have a role in regulating T-cell apoptosis.

In conclusion, our data identify the MAP-kinase family member ERK2 as the predominant HePTP substrate and suggest an important function of HePTP in the regulation of the ERK signaling pathway.

Materials and methods

Molecular cloning

The complete human HePTP coding sequence (Zanke *et al*., 1992; GenBank accession #M64322) was amplified by Polymerase Chain Reaction (PCR) from a cDNA library constructed with RNA isolated from an activated human Th1 clonal line, using the oligonucleotide 5′-CCAGCACCC-CACCTCAGC-3′ as forward primer and the oligonucleotide 5′-GTGACTTCCCAGGCTTGAGG-3′ as reverse primer with Pwo DNA polymerase (Boehringer Mannheim). The PCR product was subcloned into pCR®-Blunt (Invitrogen) and the sequence of both strands was determined using an automated DNA sequencer (Applied Biosystems, Perkin-Elmer). The DNA sequence of this clone was identical to the reported sequence for HePTP except for two single base differences that resulted in amino acid changes at position 235 (Glutamine instead of Histidine) and position 236 (Leucine instead of Valine). These amino acids are identical to the amino acids reported by Adachi *et al*. (1992) for LC-PTP at these positions.

Site-directed mutagenesis

Site-directed mutants were made by using Muta-gene® *in vitro* Mutagenesis kit (BioRad) according to manufacturer's instructions. In HePTP-C/S, the active site cystein (C_{269}) residue was mutated to serine (primer 5′-CCTGCACTC-GAGTGGACTACG-3′); in HePTP-D/A, the aspartic acid residue within the catalytic domain (D_{235}) was mutated to alanine, (primer 5′-GGTGTCTGATGGGCCGGCCAGG-CC-3′); in HePTP-dLD amino acids 17–30 were deleted (primer 5′-CCCAGGGACCGAACTCGCACATGCTTCTT-GG-3′).

Cells and transfections

K562 (human chronic myelogenous leukemia), HL-60 (human acute myelogenous leukemia) and Jurkat cells (acute T cell leukemia) were cultured in RPMI 1640 medium with 10% heat inactivated fetal bovine serum (FBS). RBL-2H3 (rat basophilic leukemia) cells were grown in RPMI 1640 medium with 15% FBS. NIH3T3 (mouse embryonic fibroblast), 293T (embryonic kidney fibroblast), and U373 (glioblastoma) cells were grown in D-MEM medium containing 10% FBS. All media contained 100 U/ml penicillin and 100 U/ml streptomycin. Transfectants were obtained by electroporation of 20 μg of DNA with 7.5×10^6 cells in 250 μl 1 × phosphate-buffered saline (PBS) using a BioRad gene pulser equipped with a capacitance extender at 250 microfarads and 300 V (Rossen and O'Brien, 1998). Cells were incubated for 10 min at room temperature before resuspending in 10 ml fresh media and plated in 100 mm dishes. Polyclonal stable lines were established by placing cells under G418 (800 mg/ml) selection 48 h after transfection with continued growth under selection for 5 weeks prior to analysis. 293T cells were transiently transfected using Ca-phosphate (5-prime 3-prime). U373 and transfected 293T cells were starved overnight in media containing 0.5% FBS prior to stimulation with growth factors. U373 and 293T cells were stimulated with epidermal growth factor (EGF; Toyobo) at 100 ng/ml and 50 ng/ml, respectively, and platelet derived growth factor (PDGF; Upstate Biotechnology Inc.) was used at 50 ng/ml. For stimulation with phorbol-12-myristate-13-acetate (PMA; Calbiochem) cells were starved overnight in FBS-free media.

For pervanadate treatment, 1.0×10^7 cells were resuspended in 10 ml fresh media at 1.0×10^6 cells/ml and incubated with or without pervanadate at 1 : 500 for 10 min at room temperature. Cells were pelleted, lysed in 1 ml cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, pH 8.0, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetic acid) and incubated for 20 min on ice. Dithiothreitol (DTT) was added at 10 mM, the lysates were then incubated 10 min at room temperature and clarified by centrifugation at 19 000 r.p.m. for 20 min at 4°C.

GST-precipitations

The complete coding region of wild type HePTP and HePTP mutants were cloned in frame with Gst into pGEX-KG (Guan and Dixon, 1991) for prokaryotic expression. Fusion proteins were expressed in BL21 (DE3) competent cells (Stratagene). Clarified cell lysate was incubated with 5 μg purified Gst or Gst-HePTP fusion protein bound to glutathione agarose beads (Sigma) at 4°C. Precipitates were washed three times with cold lysis buffer containing 0.1% Triton X-100 and resuspended in 1 × SDS sample buffer. At the end of each experiment the filters were stained with amidoblack to control for equal amounts of fusion protein in each sample.

Immunoprecipitations and immunoblotting

Clarified lysates were incubated at 4°C with anti-c-*myc* 9E10 monoclonal antibody (BAbCO) crosslinked to Protein G agarose beads (Sigma). Immune complexes were washed three times in cold lysis buffer containing 0.1% Triton X-100 and resuspended in 1 × SDS sample buffer. Precipitates and total cell lysates were resolved on 10% SDS–PAGE gels and transferred to nitrocellulose. Blots were incubated for 1 h in TBST (50 mM Tris, pH 7.5, 200 mM NaCl, 3 mM KCl, 0.5% Tween 20) containing 5% nonfat milk and incubated overnight with primary antibody in TBST/5% nonfat milk. Blots were washed with TBST and incubated for 1 h with secondary horseradish peroxidase (HRP) conjugated antibody (BioRad) in TBST/5% nonfat milk. After washing with TBST blots were developed using the Enhanced Chemiluminescence Detection System (Amersham). Antibodies used for immunoblotting were anti-c-*myc* 9E10 monoclonal antibody (BAbCO), anti-ERK1/2 polyclonal antibody (Transduction Laboratories), anti-ERK2 polyclonal antibody (Santa Cruz Biotechnology Inc.), anti-phospho-p44/42 MAP kinase monoclonal antibody (New England Biolabs), anti-pERK which specifically detects tyrosine phosphorylated ERK1 and ERK2 (Santa Cruz Biotechnology Inc.), anti-p38 antibodies (New England Biolabs), and for phosphotyrosine blots, PY20 (Transduction Laboratories) was used. Protein concentrations were determined using BCA* Protein assay Reagent (Pierce).

Metabolic labeling

Cells were washed two times with 1 × PBS and then resuspended at 1.0×10^6 cells/ml in 4 ml RPMI 1640 Methionine free media (Gibco-BRL) and plated in 100 mm dishes for 30 min at 37°C, 5% CO_2. After adding dialyzed FBS (5%; v/v) and 100 μCi/ml Pro-mix L-[^{35}S] *in vivo* cell labeling mix (Amersham) to the cells, the cells were incubated overnight. Cells were washed two times with cold 1 × PBS and resuspended in 500 ml lysis buffer. Gst, Gst-HePTP, or

Gst-HePTP-C/S fusion protein bound to glutathione agarose beads was first incubated for 30 min at 4°C with unlabeled cell lysate (10^6 cell equivalents) to reduce the background. The unlabeled lysate was then aspirated, replaced with the radioactive lysate, and the incubation continued overnight. The samples were then processed as described above.

Phosphatase assays

All *in vitro* phosphatase assays were done in a buffer containing 50 mM MES pH 6.5, 150 mM NaCl, 10 mM DTT, and 2 mM EDTA with 1 μg of Gst-fusion protein per sample. Peptide substrates (Research Genetics; purity >80%) were used at a final concentration of 50 mM and phosphorylated ERK2 (New England BioLabs) and ERK1 (Upstate Biotechnology Inc.) were used at 50 ng per reaction. Free phosphate was detected using a malachite green detection system (Promega).

In vitro *MAP-kinase assay*

Anti-ERK2 immunoprecipitates (anti-ERK2 agarose beads; Santa Cruz Biotechnology Inc.) were washed 3× with immunoprecipitation washing buffer containing 1 mM DTT and protease inhibitors and then once in kinase buffer (1 mM DTT, 40 mM MOPS, pH 7.0, 10 mM $MgCl_2$). The precipitates were then resuspended in 30 μl of kinase buffer containing 40 mM ATP 10 μCi/μl [^{32}P]γATP (Amersham) and 30 μg myelin basic protein (MBP; Upstate Biotechnology Inc.). Reactions were incubated at 30°C for 30 min, stopped with SDS sample buffer, and applied to 12% SDS–PAGE. The gel was dried and incorporation of ^{32}P into MBP was quantitated using a Storm 860 phosphorimager (Molecular Dynamics).

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