**Hepatocyte Growth Factor Receptor Tyrosine Kinase Met Is a Substrate of the Receptor Protein-tyrosine Phosphatase DEP-1***

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The receptor protein-tyrosine phosphatase (PTP) DEP-1 (CD148/PTP-ζ) has been implicated in the regulation of cell growth, differentiation, and transformation, and most recently has been identified as a potential tumor suppressor gene mutated in colon, lung, and breast cancers. We have generated constructs comprising the cytoplasmic segment of DEP-1 fused to the maltose-binding protein to identify potential substrates and thereby suggest a physiological function for DEP-1. We have shown that the substrate-trapping mutant form of DEP-1 interacted with a small subset of tyrosine-phosphorylated proteins from lysates of the human breast tumor cell lines MDA-MB-231, T-47D, and T-47D/Met and have identified the hepatocyte growth factor/scatter factor receptor Met, the adapter protein Gab1, and the junctional component p120 catenin as potential substrates. Following ligand stimulation, phosphorylation of specific tyrosyl residues in Met induces mitogenic, motogenic, and morphogenic responses. When co-expressed in 293 cells, the full-length substrate-trapping mutant form of DEP-1 formed a stable complex with the chimeric receptor colony stimulating factor 1 (CSF)-Met and wild type DEP-1 dephosphorylated CSF-Met. Furthermore, we observed that DEP-1 preferentially dephosphorylated a Gab1 binding site (Tyr1349) and a COOH-terminal tyrosine implicated in morphogenesis (Tyr1365), whereas tyrosine residues in the activation loop of Met (Tyr1230, Tyr1234, and Tyr1235) were not preferentially phosphorylated by DEP-1. The ability of substrate-phosphorylated Met to dephosphorylate particular tyrosine residues that are required for Met-induced signaling suggests that DEP-1 may function in controlling the specificity of signals induced by this PTK, rather than as a simple “off-switch” to counteract PTK activity.

A variety of ligands trigger the reversible phosphorylation of tyrosyl residues in cellular proteins, a process that underlies the control of such fundamental cellular functions as growth and proliferation, migration, and morphogenesis. Tyrosine phosphorylation is regulated by the coordinated action of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). Classically it was thought that the PTKs provided the “on-switch” to initiate a physiological response, whereas the PTPs functioned to counteract the PTKs and to return the system to its basal state. However, it was soon shown that PTPs may themselves function positively to promote signaling, for example, by promoting the dephosphorylation and activation of PTKs, thus coordinating with, rather than antagonizing PTK function (reviewed in Ref. 1). A further level of complexity has been introduced with the realization that whether a defined PTP functions positively or negatively may depend upon the signaling context. Thus, SHP-2 is an activator of signaling through the HGF/SF receptor Met (2) and the epidermal growth factor receptor (3), but is an inhibitor of signaling through the platelet-derived growth factor receptor (4). Following ligand binding, a receptor PTK may become phosphorylated on multiple tyrosine residues, which serve as docking sites for distinct signaling proteins. The spectrum of such signaling molecules that associate with the PTK will determine the nature of the response that is initiated following ligand stimulation. The possibility exists, therefore, that a PTP may dephosphorylate a particular site in a receptor PTK and thereby determine the signaling outcome of a particular stimulus. Thus, dephosphorylation of receptor PTKs by members of the PTP family may function as a mechanism for regulating the specificity of a signaling event rather than simply as an “off-switch.”

DEP-1 is a receptor PTP whose expression is enhanced as cells approach confluence (5). Initially cloned from human cDNA libraries (5, 6), DEP-1 homologues were subsequently identified in rat and mouse (7, 8). DEP-1 comprises an extracellular segment of eight fibronectin type III repeats, a transmembrane domain, and a single cytoplasmic PTP domain. Also known as PTP-ζ (6) and CD148 (9, 10), DEP-1 is expressed in a variety of tissues and cell types. There is a growing body of evidence suggesting a role for DEP-1 in the inhibition of cell growth. After vascular injury DEP-1 expression is down-regulated in migrating and proliferating rat endothelial cells (8). Attempts have been made to express DEP-1 constitutively in breast cells and macrophages (11, 12), however, this inhibited development of stable cell lines, further reinforcing a role for DEP-1 in growth inhibition.

In addition to its role in growth inhibition, DEP-1 has also been implicated in differentiation. The levels of DEP-1 mRNA are increased in various cell lines in response to factors that lead to differentiation (8, 11, 13, 14). Interestingly, in rat thyroid cells the expression of DEP-1 (rPTP-ζ) mRNA decreases with increasing levels of transformation (13, 15). Re-introduc-
tation of DEP-1 into the transformed cells leads to reduced growth rates, stabilization of the cyclin-dependent kinase inhibitor p27, and partial re-acquisition of a differentiated phenotype (16). Loss of DEP-1 expression has also been observed in human thyroid tumors (16). Furthermore, the DEP-1 gene Pt-prj was identified as a positional candidate for the mouse colon-cancer susceptibility locus Scet (17). Frequent deletions, loss of heterozygosity, and missense mutations in the human Pt-prj gene have also been identified in colon, lung, and breast cancers (17). Taken together these data indicate that DEP-1 may be a critical factor in controlling cellular growth and transformation.

DEP-1 has recently been shown to localize at cell boundaries in endothelial cells and its staining pattern overlapped with that of the junctional protein VE-cadherin (18). Interestingly, members of the cadherin family of cell-cell adhesion molecules function in the suppression of cell growth and tumor invasion. Junctional components such as β-catenin, however, can also promote cell growth by inducing the transcription of genes involved in proliferation and cancer progression (reviewed in Ref. 19). The growth inhibitory effects of cadherins may involve binding and sequestration of the signaling pool of the catenins (20, 21). Reversible tyrosine phosphorylation is an important aspect of the regulation of junctional integrity and the control of signals emanating from these sites (reviewed in Ref. 22). The identification of the PTKs and PTPs that act upon the components of signals will be important for understanding the regulation of cell morphology and the control of gene expression, events that ultimately influence growth and migration.

Using an in vitro affinity chromatography system we identified a set of proteins from human breast tumor cell lines (MDA-MB-231, T-47D, and T-47D/Met) that interacted specifically with the substrate-trapping mutant form of DEP-1. These proteins included the junctional component p120 catenin (p120(CAM)), the adaptor protein Gab1, and the HGF/SF receptor Met. Met induces mitogenic, motogenic, and morphogenic responses after ligand activation by recruiting a number of signaling and docking molecules and has been implicated in the phosphorylation of cell junction proteins. Disruption of normal signaling through Met has been implicated in certain cancers. Ligand-induced activation of Met by HGF/SF leads to the autophosphorylation of specific tyrosine residues within the PTK. Phosphorylation of Tyr\textsubscript{1234} and Tyr\textsubscript{1235} in the activation loop of Met is required for kinase activity, whereas phosphorylation of COOH-terminal tyrosine residues (Tyr\textsubscript{1349} and Tyr\textsubscript{1356}) is required for the recruitment of signaling and adapter molecules including Gab1 (reviewed in Ref. 23). Additional COOH-terminal tyrosines such as Tyr\textsubscript{1065} appear to be important for mediating a morphogenic signal although the identity of proteins that interact with this site is currently unknown (24). We present evidence that DEP-1 preferentially dephosphorylates specific tyrosine residues in the COOH-terminal domain of Met. By selectively dephosphorylating such sites in the kinase, DEP-1 may attenuate particular signaling events emanating from Met thus, potentially, regulating the outcome of cellular responses induced by HGF/SF stimulation.

EXPERIMENTAL PROCEDURES

Generation of DEP-1 cDNA Constructs—Full-length human DEP-1 cDNA was isolated and subcloned into the mammalian expression vector pMT2 (5). The nucleotide and amino acid numbers listed below correspond to the human DEP-1 sequence reported previously (5) (GenBank\textsuperscript{TM} accession number U10886). DEP-1 point mutants (C1239S and D1205A) were generated by overlap extension using pMT2.DEP-1 as template. The resulting mutant PCR products were exchanged with the wild type sequence in pMT2.DEP-1 and sequenced to confirm the mutations.

DEP-1 cytoplasmic domain constructs were generated using the pMT2.DEP-1 wild type or point mutant (C1239S and D1205A) constructs as template. A 5 primer introduced a BamHI site before the DEP-1 cytoplasmic sequence at nucleotide 3338, whereas a 3 primer added a SalI site after the DEP-1 stop codon. The resulting PCR products (pMT2.DEP-1 nucleotides 3338–4362) were cloned into the BamHI/SalI sites of the pMAL-c2E vector from New England Biolabs (Beverly, MA), generating wild type and point mutant (C1239S and D1205A) pMAL-DEP-1 constructs. The fusion proteins were expressed in Escherichia coli and purified on amylose resin according to the manufacturer’s instructions. The resulting proteins (∼84 kDa) have maltose-binding protein (MBP) fused to the NH\textsubscript{2} terminus of the DEP-1 cytoplasmic domain (amino acids 997–1337). DEP-1 point mutants—DEP-1 monoclonal antibodies A3 and 143-41 used for immunoprecipitations were generous gifts from Dr. Gregorio Aversa and Dr. Antoni Gayà, respectively, and were described previously (9, 10). The DEP-1 polyclonal antibody CS895A was generated against the DEP-1 extracellular domain peptide (DASNTERSRAGSP) corresponding to amino acids 292–307 coupled to KLH (Pierce, Rockford, IL). The Met polyclonal antibody 144 used for immunoprecipitations was generated against a carbamyl-terminal peptide and was described previously (27). The antiphosphotyrosine monoclonal antibodies G98 and G104 were generated in our laboratory and described previously (28). Antiphosphotyrosine-agarose (PT-66) was purchased from Sigma and antiphosphotyrosine (4G10)-agarose conjugate was purchased from Millipore (Lake Forest, IL). The Met-Ab1 C12 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p120(CAM), E-cadherin, Grb2, and phosphotyrosine (PY20) were purchased from BD Transduction Labs (Lexington KY). β-Catenin (6E9) and plakoglobin (15F11) antibodies were purchased from Sigma and the Gab1 COOH-terminal antibody was purchased from Upstate Biotechnology. Anti-α-c-Met (Tyr\textsubscript{1230–1234} Tyr\textsubscript{1235} Tyr\textsubscript{1365}) and Tyr\textsubscript{1356} antibodies were purchased from BioSource International (Camarillo, CA) and phospho-Met (Tyr\textsubscript{1245}) was purchased from Cell Signaling Technology (Beverly, MA).

Substrate Trapping—Prior to lysis, T-47D and T-47D/Met cells were treated with 50 μM pervanadate for 20 min while MDA-MB-231 cells were treated with 100 μM pervanadate for 20 min. Cells were rinsed with phosphate-buffered saline and lysed in 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM benzamidine). For trapping experiments in vitro the lysis buffer also contained 5 mM iodoacetic acid to inhibit cellular PTPs irreversibly. After incubation on ice for 5 min dichloroacetate was added to a final concentration of 10 mM to inactivate any nonreduced iodoacetic acid. Insoluble material was removed by centrifugation. T-47D (1 mg) or MDA-MB-231 lysates (5 mg) were mixed with MBP or the MBP.DEP-1 constructs bound to amylose resin at a 1:1 ratio of 1 for 2 h and washed extensively with 1% Nonidet P-40 buffer. Tyrosine-phosphorylated proteins were immunoprecipitated using 0.1 mg of T-47D cell lysate and a combination of 5 μl each of antiphosphotyrosine antibodies PT-66 and 4G10. Lysates and antibodies were incubated at 4°C for 2 h and washed extensively with 1% Nonidet P-40 buffer. Protein complexes were released by incubation in

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reducing Laemmli sample buffer at 95 °C subjected to SDS-PAGE on 8% gels and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) for immunoblotting.

To determine whether the tyrosine-phosphorylated proteins bound to the substrate-trapping mutants at the PTP active site, we tested the effects of vanadate on complex formation. MBP fusion proteins bound to amylose were preincubated in 1% Nonidet P-40 buffer (without EDTA) with or without 2 mM vanadate. Cells were rinsed with phosphate-buffered saline and lysed in 1% Nonidet P-40 buffer (without EDTA) with or without 2 mM vanadate. For vanadate-competition experiments, the lysis buffer also contained 5 mM iodoacetic acid and after 5 min on ice dithiothreitol was added to a final concentration of 10 mM. Insoluble material was removed by centrifugation and samples were processed as above.

Proteins bound to the DEP-1 substrate-trapping mutant were analyzed by immunoblotting. T-47D and T-47D/Met cells were treated and lysed as above. Lysates (30 mg) were mixed with MBP.DEP-1 or MBP.DEP-1(DA) bound to amylose resin at a ratio of 1 μg of fusion protein to 500 μg of lysate. Lysates and fusion proteins were incubated at 4 °C for 2 h and washed extensively with 1% Nonidet P-40 buffer. Protein complexes were released by incubation in reducing Laemmli sample buffer at 95 °C subjected to SDS-PAGE on 8% gels and transferred onto Immobilon-P membranes for immunoblotting. The samples were divided into 5-mg lysate equivalents per fusion per lane.

**Immunoprecipitations**—Transfected cells were rinsed with phosphate-buffered saline and lysed in 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM benzamidine, 50 mM NaF, 5 mM iodoacetic acid, 1 mM vanadate) and processed as above. For substrate-trapping experiments, DEP-1 was immunoprecipitated from 1 mg of lysate with the DEP-1 antibodies A3 and 143-41 and Met was immunoprecipitated from 1 mg of cell lysate using the Met antibody 144.

For dephosphorylation and recruitment experiments transfected cells were rinsed with phosphate-buffered saline and lysed in 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM benzamidine, 50 mM NaF, 5 mM iodoacetic acid, 1 mM vanadate) and processed as above. Met was immunoprecipitated from 1 mg of lysate using the Met antibody 144. Lysate and antibody were incubated at 4 °C for 1 h. Protein A-Sepharose 4 fast flow (Amersham Biosciences) was added for 45 min at 4 °C. Immune complexes were washed extensively with 1% Nonidet P-40 buffer, released by incubation in reducing Laemmli sample buffer at 95 °C subjected to SDS-PAGE on 8% gels, and transferred onto Immobilon-P membranes for immunoblotting.

**RESULTS**

The DEP-1(DA) Substrate-trapping Mutant Interacted with a Subset of Tyrosine-phosphorylated Proteins from Two Human Breast Tumor Lines—We employed two human breast tumor lines (T-47D and MDA-MB-231), which have been used previously in analysis of Met function, to characterize the broadest spectrum of potential phosphotyrosine-containing substrates for analysis. Further, we determined whether the PTP DEP-1 fused to the NH2 terminus of the DEP-1 cytoplasmic domain (amino acids 997–1337) were generated. Wild type (MBP.DEP-1), catalytically inactive (MBP.DEP-1(CS)), and substrate-trapping (MBP.DEP-1(DA)) mutant forms of DEP-1 were used for purification of potential substrates by affinity chromatography in vitro. DEP-1 fusion proteins were incubated with lysates of pervanadate-treated T-47D cells. Tyrosine-phosphorylated proteins that interacted with the fusion proteins were visualized by immunoblotting with antiphosphotyrosine antibodies. Only the substrate-trapping mutant form of DEP-1 (MBP.DEP-1(DA)) bound tyrosine-phosphorylated proteins (Fig. 1A). In addition, when a comparison was made between the tyrosine-phosphorylated proteins that bound to the DEP-1 substrate-trapping mutant and the proteins immunoprecipitated with antiphosphotyrosine antibodies, we observed that MBP.DEP-1(DA) recognized only a small subset of the tyrosine-phosphorylated proteins from the lysate of pervanadate-treated T-47D cells (Fig. 1A). To determine whether the proteins that interacted with MBP.DEP-1(DA) were potential substrates, the fusion proteins were preincubated with vanadate. Vanadate is a competitive inhibitor that blocks the PTP active site and prevents substrate binding and phosphatase activity (29). The interaction between the tyrosine-phosphorylated proteins and MBP.DEP-1(DA) was inhibited by vanadate, suggesting that they bound to the active site and may represent substrates of DEP-1 (Fig. 1B).

Similarly, DEP-1 fusion proteins were incubated with the lysate of pervanadate-treated MDA-MB-231 cells. As was seen with the T-47D cell lysates, only the substrate-trapping mutant form of DEP-1 (MBP.DEP-1(DA)) interacted with tyrosine-phosphorylated proteins from MDA-MB-231 cell lysates (Fig. 1C) and only a small subset of the pool of available tyrosine-phosphorylated proteins was recognized by the PTP (data not shown). This interaction was also inhibited by vanadate (Fig. 1D). Pervanadate treatment resulted in the accumulation of tyrosine-phosphorylated proteins in both cell lines, however, a lower concentration of pervanadate was needed to induce high levels of tyrosine phosphorylation in T-47D cells compared with MDA-MB-231 cells. Similar results were obtained for both cell lines and we present data obtained from T-47D cells in the subsequent figure.

Identification of Proteins That Interacted with the DEP-1 Substrate-trapping Mutant—Although the tyrosine-phosphorylated proteins that interacted with MBP.DEP-1(DA) were easily detected by immunoblotting with antiphosphotyrosine antibodies, they were difficult to detect on Coomassie-stained gels, suggesting that they were not abundant proteins. After a large scale preparation of DEP-1 substrates from T-47D cells we detected a 100-kDa protein on Coomassie-stained gels (data not shown) that corresponded to a 100-kDa tyrosine-phosphorylated protein detected by immunoblotting (Fig. 1A, arrow). Peptides derived from this protein were sequenced by mass spectrometry. Two individual peptides (NLSYQQVHR, SQSSH-SYDDSTLPLIDR) matched sequences in the Src substrate and adherens junction component p120Ctn (Table I). Both sequences can be found in all the p120Ctn isoforms identified to date (30).

Based on the identification of p120Ctn as a potential substrate of DEP-1, we sought to determine whether the PTP interacted with other junctional components. Immunoblot analysis revealed that the DEP-1 substrate-trapping mutant did not interact with the transmembrane protein E-cadherin from pervanadate-treated T-47D cell lysates (Fig. 2). The cytoplasmic proteins β-catenin and plakoglobin, however, were only found in a complex with MBP.DEP-1(DA). Although p120Ctn interacted with the DEP-1 substrate-trapping mutant, β-catenin and plakoglobin also interacted with the wild type form of the enzyme (MBP.DEP-1) (Fig. 2) suggesting that β-catenin and plakoglobin may interact with DEP-1 constitutively.

The DEP-1 substrate-trapping mutant bound several tyrosine-phosphorylated proteins from both T-47D and MDA-MB-231 cell lines (Fig. 1). Based on the molecular weights of these proteins and our observation that DEP-1 interacted with components of adherens junctions, we probed for signaling molecules known to localize to cell-cell junctions. We observed that MBP.DEP-1(DA) trapped Met, the HGF/SF receptor, from pervanadate-treated MDA-MB-231 cells (data not shown). Because Met is expressed at low levels in T-47D cells we employed a T-47D stable cell line ectopically expressing the PTK (T-47D/Met), which has been used previously in analysis of Met function (26). MBP.DEP-1(DA) also trapped Met from pervanadate-treated T-47D/Met cell lysates and this interaction was not seen with the wild type DEP-1 (MBP.DEP-1) (Fig. 2). This suggests...
a transient interaction between DEP-1 and Met consistent with that of enzyme and substrate.

Met exerts its pleiotropic effects by recruiting a number of docking and signaling molecules (reviewed in Ref. 23). MBP.DEP-1(DA) trapped the docking protein Gab1 from T-47D/Met cell lysates (Fig. 2). Following activation of Met, Gab1 is recruited to the kinase and phosphorylated on tyrosine residues, which allows for the recruitment of other signaling and adapter molecules thereby amplifying downstream signals. Interestingly MBP.DEP-1(DA) also trapped Gab1 from T-47D cells suggesting that the Gab1-DEP-1 interaction is at least partially direct and does not require Met (Fig. 2).

The Full-length DEP-1(DA) Substrate-trapping Mutant Trapped Met from 293 Cells—DEP-1 is a transmembrane PTP, however, in the substrate-trapping experiments described above we utilized only the cytoplasmic domain of the enzyme and observed an interaction with Met. To determine whether the trapping mutant form of full-length DEP-1 also trapped Met, we co-expressed full-length DEP-1 and the mutants DEP-1(CS) and DEP-1(DA) with a chimeric Met construct CSF-Met.

TABLE I

Identification of p120Ctn as a substrate of DEP-1
A potential substrate isolated by affinity chromatography on the substrate-trapping mutant form of DEP-1 was excised from an SDS-PAGE gel. The peptides derived from the protein were identified by mass spectrometry. The table illustrates the peptide sequences and their positions within the various isoforms of p120Ctn.

| p120Ctn isoform | Peptide sequence and matching p120 Ctn amino acids | GenBank™ accession number |
|-----------------|---------------------------------------------------|--------------------------|
| 1ABC            | NLSYQVHR SQSSHYYDDSTLFLIDR                        | AF062321, AF062317       |
| 2ABC            | 585–592                                           | 859–875                  |
| 3AB             | 531–538                                           | 805–821                  |
| 4ABC            | 484–491                                           | 752–768                  |
| 5ABC            | 262–289                                           | 536–552                  |

FIG. 1. Tyrosine-phosphorylated proteins trapped by DEP-1(DA) from pervanadate-treated breast tumor cells. A, immunoblot of tyrosine-phosphorylated proteins trapped by DEP-1(DA) from T-47D cells. T-47D cells were treated with 50 μM pervanadate for 20 min prior to lysis. MBP or MBP.DEP-1 fusion proteins were incubated with cell lysates and protein complexes were analyzed by SDS-PAGE and immunoblotting using antiphosphotyrosine antibodies. An antiphosphotyrosine immunoprecipitation was also performed on pervanadate-treated cell lysates to illustrate the full compliment of tyrosine-phosphorylated proteins (PY IP). B, effects of vanadate on the interaction between tyrosine-phosphorylated proteins with the DEP-1(DA) substrate-trapping mutant. T-47D cells were treated as in A. Cells were lysed in lysis buffer (see “Experimental Procedures”) with (+) or without (−) 2 mM vanadate. MBP and MBP.DEP-1 fusion proteins were preincubated with (+) or without (−) 2 mM vanadate and added to cell lysates. Protein complexes were analyzed by SDS-PAGE and immunoblotting using antiphosphotyrosine antibodies. C, immunoblot of tyrosine-phosphorylated proteins trapped by DEP-1(DA) from MDA-MB-231 cells. MDA-MB-231 cells were treated with 100 μM pervanadate for 20 min prior to lysis. MBP or MBP.DEP-1 fusion proteins were incubated with cell lysates and samples were processed as in A. D, effects of vanadate on the interaction between tyrosine-phosphorylated proteins with the DEP-1(DA) substrate-trapping mutant. MDA-MB-231 cells were treated as in C. Cells were lysed and samples processed as in B, and protein complexes were analyzed by SDS-PAGE and immunoblotting using antiphosphotyrosine antibodies.

T-47D cells suggesting that the Gab1-DEP-1 interaction is at least partially direct and does not require Met (Fig. 2).
This chimeric receptor, which comprises the extracellular domain of human CSF-1R and the transmembrane and cytoplasmic domains of human Met (25), is constitutively active when expressed in 293 cells, bypassing the requirement for ligand stimulation. DEP-1 was immunoprecipitated under conditions that would preserve protein complexes. Immunoblots showed that similar levels of DEP-1, DEP-1(CS), and DEP-1(DA) were immunoprecipitated from 293 cells expressing the Met chimera alone. As with the DEP-1(DA) cytoplasmic domain fusion protein, full-length DEP-1(DA) formed a stable complex with Met (Fig. 3A). Interestingly, the full-length DEP-1(CS) mutant also bound Met, but less efficiently than the DEP-1(DA) mutant. Similar results were observed in the interaction between PTP-PEST and its substrate p130 (28). There appeared to be no stable interaction between wild type DEP-1 and Met when they were co-expressed in 293 cells (Fig. 3A).

**Full-length Wild Type DEP-1 Dephosphorylated Met in 293 Cells**—Because full-length substrate-trapping mutant forms of DEP-1 bound Met when co-expressed in 293 cells (Fig. 3A) we investigated whether full-length wild type DEP-1 could dephosphorylate Met. Full-length DEP-1 and the mutants DEP-1(CS) and DEP-1(DA) were co-expressed with the CSF-Met chimera in 293 cells, as above. The Met chimera was immunoprecipitated from cell lysates with an antibody directed toward the Met portion of the chimera. Immunoblots revealed that similar levels of CSF-Met were immunoprecipitated in each condition (Fig. 3B). We observed that the Met chimera was tyrosine phosphorylated when it was expressed alone in 293 cells, however, we did not detect the presence of tyrosine phosphorylation when it was co-expressed with wild type DEP-1 (Fig. 3B). Although the DEP-1(CS) and DEP-1(DA) mutants interacted with the Met chimera (Fig. 3A), Met was not dephosphorylated in the cells expressing these mutants, suggesting that dephosphorylation required DEP-1 catalytic activity.

**DEP-1 Preferentially Dephosphorylated COOH-terminal Tyrosine Residues in Met**—When equal amounts of DEP-1 and CSF-Met plasmid DNA were transfected into 293 cells, the level of DEP-1 protein expressed was sufficient to dephosphorylate Met (Fig. 3B). We performed a dose-response analysis to determine whether varying the expression level of DEP-1 would affect its ability to dephosphorylate Met. We transfected 293 cells with a constant concentration of CSF-Met DNA (20 μg) and increasing amounts of wild type DEP-1 DNA (0, 1, 2.5, 5, and 10 μg) or 10 μg of the catalytically inactive DEP-1(CS) mutant DNA (Fig. 4A). Immunoblots showed that as the levels of DEP-1 plasmid DNA used for transfection was increased, the level of DEP-1 protein that was expressed also increased.

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**Fig. 2. Identification of tyrosine-phosphorylated proteins that interacted with the DEP-1(DA) substrate-trapping mutant.** Immunoblot analysis of proteins associated with wild type DEP-1 and substrate-trapping mutant DEP-1(DA). T-47D and T-47D/MET cells were treated with 50 μM pervanadate for 20 min prior to lysis. MBP.DEP-1 or MBP.DEP-1(DA) fusion proteins were incubated with cell lysates and protein complexes were analyzed by SDS-PAGE and immunoblotting using antibodies directed toward E-cadherin (E-cad), β-catenin (β-cat), plakoglobin (Pg), p120 Tyr(p120), Met (Met), and Gab1 (Gab1). Cell lysate (50 μg) was loaded to confirm the expression and molecular weight of each of the proteins analyzed by immunoblotting (Lysate).

**Fig. 3. Co-expression of DEP-1 and Met in 293 cells.** A, immuno blot analysis of Met associated with full-length substrate-trapping mutants of DEP-1. 293 cells were transfected with CSF-Met alone or in combination with wild type or mutant forms of DEP-1. Cells were serum starved and DEP-1 was immunoprecipitated from half of the cell lysate using monoclonal antibodies A3 and 143-41. Immunoblots using the polyclonal antibody CS895A revealed the levels of DEP-1 in the immunoprecipitates (DEP-1). Blots were stripped and reprobed for Met (Met). B, immunoblot analysis of the phosphorylation state of Met in the presence of wild type or mutant forms of DEP-1. Met was immunoprecipitated from the other half of the cell lysate from panel A using polyclonal antibody 144. Immunoblots using polyclonal antibody C-12 directed to the intracellular segment of Met revealed the levels of CSF-Met in the immunoprecipitates (Met). Immunoblots were stripped and reprobed with antiphosphotyrosine antibodies (PY).
Dephosphorylation of Met by DEP-1

A

| Met:  | 20 | 20 | 20 | 20 | 20 |
|-------|----|----|----|----|----|
| DEP-1: | 0  | 1  | 2.5 | 5 | 10 |
| DEP-1(CS): | - | - | - | - | - |

B

| Met:  | 20 | 20 | 20 | 20 | 20 |
|-------|----|----|----|----|----|
| DEP-1: | 0  | 1  | 2.5 | 5 | 10 |
| DEP-1(CS): | - | - | - | - | - |

C

| Met:  | 20 | 20 | 20 | 20 | 20 |
|-------|----|----|----|----|----|
| DEP-1: | 0  | 1  | 2.5 | 5 | 10 |
| DEP-1(CS): | - | - | - | - | - |

Fig. 4. The effects of expression of DEP-1 on the phosphorylation of Met and its association with Grb2. A, levels of expression of DEP-1 and Met. 293 cells were transfected with 20 µg of CSF-Met DNA and 0, 1, 2.5, 5, and 10 µg of DEP-1 DNA or 10 µg of DEP-1(CS) DNA. Cell lysates (50 µg) were analyzed for the expression levels of DEP-1 and Met by immunoblots with appropriate antibodies. B, site-specific dephosphorylation of Met by DEP-1. Met was immunoprecipitated from the serum-starved 293 cell lysates shown in panel A using polyclonal antibody 144 and the immunoprecipitates were run in duplicate. Immunoblots using polyclonal antibody C-12 revealed a constant level of Met immunoprecipitated from the cell lysates (Met). This blot was stripped and reprobed with the phospho-specific antibody to Tyr^{1349} in Met (P-Met Y^{1349}). A duplicate blot was probed with antiphosphotyrosine antibodies to illustrate the total phosphotyrosine content (PY), then sequentially stripped and reprobed with phospho-specific antibodies to examine the phosphorylation status of Tyr^{1230}, Tyr^{1234} and Tyr^{1250} (P-Met Y^{1230/1234/1250}), and Tyr^{1365} (P-Met Y^{1365}). C, immunoblot analysis of the association of Grb2 with Met. Blots from panel B were probed with an antibody to Grb2 to reveal the level of Grb2 associated with Met (Met IP/Grb2 IB). Cell lysates (50 µg) were run to determine the level of expression of Grb2 in the transfected cells (Lysate).

whereas the levels of Met protein were similar (Fig. 4A). Although similar amounts of Met were immunoprecipitated from 293 cell lysates, a gradual decrease in the level of phosphorylation of Met was detected with increasing expression of wild type DEP-1 (Fig. 4B). The phosphorylation of Met was similar when Met was expressed either alone or with the catalytically inactive form of DEP-1 (DEP-1(CS)).

Met contains three tyrosines in the activation loop of the catalytic domain (Tyr^{1230}, Tyr^{1234}, and Tyr^{1250}) and phosphorylation of Tyr^{1234} and Tyr^{1250} is required for full activation of the kinase (51). To determine whether DEP-1 acted on these tyrosine residues, phospho-specific antibodies were employed. Fig. 4B shows that similar to the effects on the overall levels of Met phosphorylation, there was a gradual decrease in the level of phosphorylation of the activation loop tyrosine residues with increasing expression of wild type DEP-1 and no effect on phosphorylation with the expression of DEP-1(CS). Phosphorylation of Tyr^{1349} and Tyr^{1356} in the multisubstrate docking site of Met is required for the transduction of downstream signals. Tyr^{1349} is a binding site for the adapter protein Gab1 whereas Tyr^{1356} is primarily responsible for binding Grb2, phosphatidylinositol 3-kinase, phospholipase C (PLC)-γ, and SHP2 (reviewed in Ref. 23). Phospho-specific antibodies toward Tyr^{1349} were used to determine whether DEP-1 dephosphorylated this site. Interestingly, unlike the gradual reduction in phosphorylation seen for the activation loop tyrosine residues, Tyr^{1349} was nearly completely dephosphorylated in the presence of low levels of DEP-1 (Fig. 4B). This dephosphorylation also required DEP-1 catalytic activity because no change in the phosphorylation level of Tyr^{1349} was observed in the presence of DEP-1(CS). The phosphorylation status of the other docking site tyrosine residue (Tyr^{1356}) could not be determined because of the lack of phospho-specific antibodies toward this residue. In addition to the docking site tyrosine residues, other tyrosine residues have been shown to impact Met signaling. For example, Tyr^{1365} is important for mediating a morphogenic signal (24). Interestingly, phospho-specific antibodies directed toward this site revealed that Tyr^{1365} was nearly completely dephosphorylated in the presence of low levels of DEP-1 (Fig. 4B). These observations suggest that DEP-1 may display specificity for certain sites within Met.

Increased Expression of DEP-1 Attenuated the Interaction between Met and Grb2—Ligand-induced activation of Met results in the recruitment of a number of proteins that are important for transmitting downstream signals. The dephosphorylation of a docking site tyrosine residue in Met prompted us to look at the recruitment of Grb2. Met was immunoprecipitated from 293 cells as above co-expressing CSF-Met and varying amounts of DEP-1 and the immunoprecipitates were probed for the presence of the Grb2 adapter protein. Grb2 binds to Met directly via Tyr^{1356} (32, 33). Analysis of the cell lysates revealed that the level of Grb2 was not affected by the expression of DEP-1 and Met in these cells (Fig. 4C). However, we observed that with increasing levels of DEP-1 there was a gradual decrease in the amount of Grb2 that co-immunoprecipitated with Met (Fig. 4C) coincident with the changes in overall tyrosine phosphorylation status of the PTK.

DISCUSSION

Aberrant regulation of tyrosine phosphorylation, for example caused by disruption of the normal balance of activities of PTKs and PTPs, has been implicated in the manifestation of many aspects of the transformed phenotype. An understanding of which PTKs and PTPs contribute to this process will provide important insights into the etiology, and potential avenues for treatment, of cancer. DEP-1 was identified originally as a PTP whose expression is enhanced as cells approach confluence, suggesting a role in contact dependent growth inhibition. DEP-1 has also been implicated in differentiation and loss of DEP-1 expression may contribute to cellular transformation.
Identification of physiological substrates of DEP-1 will be crucial to understanding the role that this enzyme plays in these processes. Using substrate-trapping mutant forms of DEP-1 in vitro we identified several potential substrates including the PTK Met, the adapter protein Gab1, and the junctional component p120™catenin (Fig. 2). In addition to trapping Met in vitro, full-length DEP-1 substrate-trapping mutants trapped a Met chimeric receptor when co-expressed in 293 cells (Fig. 3A). Furthermore, wild type DEP-1 dephosphorylated Met in this cellular context.

Met is the prototypic member of a small subfamily of receptor PTKs that includes Ron and the chicken homologue of Ron, Sea. HGF/SF is the ligand for Met, whereas macrophage-stimulating protein is the ligand for Ron and Sea. Members of this subfamily of PTKs are expressed in a variety of cell types including epithelial, endothelial, and hematopoietic cells. Interestingly, the expression pattern of DEP-1 overlaps with the expression pattern of these receptor PTKs consistent with a possible interaction between these enzymes under physiological conditions.

Following activation by HGF/SF, Met is able to exert a variety of effects by recruiting docking and signaling molecules. Phosphorylation of the tyrosine residues in the activation loop of the PTK domain potentiates the intrinsic kinase activity of Met, whereas phosphorylation of the two docking site tyrosine residues (Tyr1349 and Tyr1356) allows for the recruitment of adaptor molecules including Grb2, SHC, and Gab1 and signaling enzymes including phosphatidylinositol 3-kinase, PLC-γ, the PTK Src, the PTP SHP2, as well as the transcription factor STAT3 (reviewed in Ref. 23). This multisubstrate docking site sequence is primarily responsible for Met-mediated signal transduction and chimeric receptors containing this sequence can induce mitogenic, motogenic, and morphogenic responses similar to Met (25, 34–36). Cells expressing Met with mutations at Tyr1349 and Tyr1356 are unresponsive to HGF/SF stimulation in vitro (33), and transgenic mice with these mutations display a lethal phenotype that resembles the phenotype of mice lacking Met or HGF/SF (37). Modulating the phosphorylation status of the multisubstrate docking site represents an important mechanism for regulating HGF/SF-induced cellular responses. Using phospho-specific antibodies we found that DEP-1 preferentially dephosphorylated the docking site residue Tyr1349 (Fig. 4B). Because of the lack of phospho-specific antibodies we were unable to determine the phosphorylation status of the other docking site residue Tyr1356. In addition, DEP-1 preferentially dephosphorylated Tyr1366, a residue important for mediating a morphogenic signal (24). The tyrosine residues in the activation loop, however, were not preferred sites and were only dephosphorylated when DEP-1 was expressed at higher levels. A recent study on the effect of DEP-1 on platelet-derived growth factor β-receptor phosphorylation has shown that wild type DEP-1 dephosphorylates the platelet-derived growth factor β-receptor in porcine aortic endothelial cells ectopically expressing both proteins (38). Similar to our results with Met, DEP-1 exhibited site selectivity and preferentially dephosphorylated tyrosine residues other than the tyrosine residue in the activation loop of platelet-derived growth factor β-receptor. Taken together these results suggest that DEP-1 may not act to inhibit kinase activity, but rather by dephosphorylating specific docking sites for signaling molecules it may modulate specific signaling pathways emanating from the receptor PTK.

The role of specific adaptor and signaling molecules in transducing Met signals has been studied extensively. The adapter protein Grb2 recruits SOS to activated receptor PTKs to induce Ras-mitogen-activated protein kinase signaling. In Met signaling Ras stimulation is necessary and sufficient to induce proliferation (39). Grb2 binds to Met directly at a binding site that contains phosphorylated Tyr1356 (32, 33, 40). In addition Grb2 can be recruited to Met via the adapter protein SHC (41). We observed a gradual decrease in the recruitment of Grb2 to the Met chimera with increasing expression of DEP-1 (Fig. 4C) coincident with overall dephosphorylation of the PTK (Fig. 4B). These data suggest that unlike Tyr1349 and Tyr1356, Tyr1366 may not be preferentially dephosphorylated by DEP-1 thereby allowing sustained recruitment of Grb2 to Met. In addition, we did not detect a change in mitogen-activated protein kinase activation with increasing expression of DEP-1 (data not shown) suggesting that the level of Grb2 recruited to the Met chimera may be sufficient to activate mitogen-activated protein kinase. As cells reach confluence DEP-1 expression levels increase suggesting a role for this PTP in contact-dependent growth inhibition (5). Interestingly, it was at higher levels of DEP-1 expression that we detected a decrease in the association between Grb2 and Met suggesting that the ability of DEP-1 to affect mitogenic signals may depend upon the level of expression of the PTP. In our system the use of a kinase that was constitutively active bypassed the need for ligand stimulation, however, under conditions in which Met is activated by its physiological ligand the effects of DEP-1 on the phosphorylation of Met at Tyr1356 and the recruitment of Grb2 may be more pronounced.

After Met activation the adapter molecule Gab1 is strongly tyrosine-phosphorylated and recruited to Met directly through Tyr1349 (42) and indirectly via Grb2 bound to Tyr1356 (43–45). Gab1 can amplify and diversify Met signaling by recruiting additional signaling proteins such as phosphatidylinositol 3-kinase, PLC-γ, SHP-2, and the adapter protein Crk. Tyrosine phosphorylation of Gab1 at specific residues is required for the recruitment of the signaling molecules. Transgenic mice lacking Gab1 display a lethal phenotype that resembles the phenotype of mice lacking Met or HGF/SF suggesting that Gab1 is important for Met signaling in vivo (46). Based on the ability of DEP-1 to trap Gab1 (Fig. 2) and specifically dephosphorylate a Gab1 docking site in Met (Fig. 4B) it is tempting to speculate that DEP-1 may act to modulate signals downstream of Gab1. A recent study on the effects of DEP-1 on T-cell receptor signaling has shown that DEP-1 induction in a Jurkat cell line results in reduced tyrosine phosphorylation of the adapter protein LAT and the enzyme PLC-γ1 (47). Interestingly Gab1 and LAT are members of the same family of docking proteins. After receptor engagement LAT is phosphorylated on tyrosine residues and recruits additional molecules including PLC-γ. Although we observed an interaction between DEP-1 and Gab1, Baker et al. (47) did not detect an interaction between a substrate-trapping mutant form of DEP-1 and LAT or PLC-γ1. It will be important to determine whether DEP-1 can preferentially dephosphorylate specific residues in Gab1 thereby influencing specific cellular responses.

The acquisition of a motile phenotype occurs during normal development as well as in tumor progression and requires dissolution of cell-cell junctions. Growth factors such as HGF/SF are able to disrupt cell adhesion and induce phosphorylation of junctional components such as β-catenin and plakoglobin (48). Interestingly, DEP-1 interacted with several known Met substrates including Gab1, β-catenin, and plakoglobin (Fig. 2). In addition DEP-1 trapped the Src substrate and adherens junction component p120 catenin (p120™catenin). The interaction between p120™catenin and DEP-1 was restricted to the substrate-trapping form of DEP-1 while β-catenin and plakoglobin interacted with both the wild type and the mutant form of DEP-1 (Fig. 2). This suggests that p120™catenin may interact with
DEP-1 in a phosphorylation-dependent manner whereas β-catenin and plakoglobin may interact with DEP-1 constitutively. A detailed analysis of the human p120Ctn gene predicts that up to 32 isoforms of the protein may exist based on alternative splicing (30). In addition, most cell types express multiple isoforms of p120Ctn. The sequences we identified by mass spectrometry are present in all the isoforms of p120Ctn identified to date, thus it is unclear whether DEP-1 interacts with specific isoforms. While this article was in preparation, Holsinger et al. (49) also showed that DEP-1 interacts with p120Ctn, β-catenin, and plakoglobin and phosphorylates p120Ctn in vitro (49).

In this study, we have shown for the first time that the PTP DEP-1 recognizes the receptor PTK Met as a substrate. Furthermore, our data suggest that DEP-1 displays selectivity for particular phosphorylation sites within this PTK and therefore, may function in determining which signaling outcomes result from stimulation of Met. Under normal conditions, Met has been implicated in the control of mitogenesis, morphogenesis, and migration. However, aberrant signaling initiated either as a result of mutations in Met, or overexpression of the kinase, have been described in a variety of human cancers (50). Interestingly, frequent deletions, loss of heterozygosity, and missense mutations in the DEP-1 gene have been identified in several human cancers including colon, lung, and breast (17), which have also been associated with aberrant Met signaling. Our demonstration of a functional interaction between DEP-1 and Met as enzyme and substrate raises the intriguing possibility that the up-regulation of Met may be coupled with the down-regulation of DEP-1 in the progression of certain human cancers.

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Hepatocyte Growth Factor Receptor Tyrosine Kinase Met Is a Substrate of the Receptor Protein-tyrosine Phosphatase DEP-1
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