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The EphA8 Receptor Phosphorylates and Activates Low Molecular Weight Phosphotyrosine Protein Phosphatase *in Vitro*

Soochul Park*

Department of Life Science, Sookmyung Women's University, 53-12 Chungpa-Dong 2-Ka, Yongsan-Ku, Seoul 140-742, Korea

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Low molecular weight phosphotyrosine protein phosphatase (LMW-PTP) has been implicated in modulating the EphB1-mediated signaling pathway. In this study, we demonstrated that the EphA8 receptor phosphorylates LMW-PTP *in vitro*. In addition, we discovered that mixing these two proteins leads to EphA8 dephosphorylation in the absence of phosphatase inhibitors. Finally, we demonstrated that LMW-PTP, modified by the EphA8 autokinase activity, possesses enhanced catalytic activity *in vitro*. These results suggest that LMW-PTP may also participate in a feedback-control mechanism of the EphA8 receptor autokinase activity *in vivo*.

Keywords: Eph, EphA8, LMW-PTP, Tyrosine kinase receptor

Introduction

The Eph family is comprised of at least 14 different receptors and nine ligands, all of these are widely expressed in the central and peripheral nervous systems during development and in the adult (Eph Nomenclature Committee, 1997; Flanagan and Vanderhaeghen, 1998; Menzel *et al*., 2001). Individual members of the Eph family have been implicated in axon guidance, cell migration, boundary formation through the restriction of cellular intermingling, and angiogenesis (Mellitzer *et al*., 2000). Numerous reports indicate that members of the Eph family exert their function by a repulsive mechanism. Little is known about the Eph signaling mechanism that mediates repulsion, although the activation of rho and rho kinase was implicated in the collapse of growth cones in retinal ganglion axons (Wahl *et al*., 2000; Shamah *et al*., 2001). More recently, an inhibition of Ras and Raf signaling was shown for the EphB2-mediated signal transduction pathway that leads to axon collapse (Elowe *et al*., 2001). However, in certain cases, such as in the vomeronasal system, it appears that members of the EphA family play an attractive rather than repulsive role (Knoll *et al*., 2001). Identification of the signal transduction pathway that mediates this contradictory effect is likely essential for understanding the mechanistic basis of repulsion versus attraction. The signaling molecules that are involved in Eph receptor-mediated cell adhesion could be important for analyzing these mechanisms, but the interactions among these molecules are apparently quite complex. For example, the EphB1-promoted attachment of cells to fibronectin in a tyrosine kinase-dependent manner is an essential aspect of this signal transduction mechanism. Also, Nck or the low-molecular-weight phosphotyrosine protein phosphatase (LMW-PTP) is also implicated (Stein *et al*., 1998a; Stein *et al*,, 1998b; Huynh-Do *et al*., 1999). EphB2 indirectly controls integrin activity by inducing R-Ras tyrosine phosphorylation, possibly through the intermediary of the Src homology (SH2) domain-containing Eph receptor binding protein 1 (SHEP1) (Dodelet *et al*., 1999; Zou *et al*., 1999). EphA2 also reportedly regulates the integrin function by causing the dephosphorylation of the focal adhesion kinase (FAK) (Miao *et al*., 2000). More recently, EphA8 was shown to enhance integrin activity by a mechanism that requires the p110γ PI-3 kinase, but which is independent of tyrosine kinase activity (Gu and Park, 2001). Other studies have also shown that the binding of EphA receptors to ephrin-A-expressing cells leads to the b1-integrin-dependent upregulation of the adhesiveness of fibroblast cells, and that Fyn or another unidentified protein may play an important role in this process (Davy *et al*., 1999; Huai and Drescher, 2001). However, it was undetermined whether these signaling proteins are expressed, and whether they play a pivotal role in axonal behavior and in the migration of physiologically relevant neurons that express Eph receptors or ephrin ligands.

LMW-PTP is an enzyme that possesses the characteristic PTPase CXXXXXR motif in the active site (Cirri *et al*., 1993). The cysteine residue that is present in this sequence is

*To whom correspondence should be addressed.
Tel: 82-02-710-9330; Fax: 82-02-715-9331.
E-mail: scpark@sookmyung.ac.kr

absolutely necessary for the activity of the enzyme since it forms a phosphointermediate during the reaction mechanism (Chiarugi 1992; Cirri *et al*., 1993). Although the enzyme is localized in the cytosol as a non-receptor PTPase, it is capable of acting upon membrane proteins. For example, LMW-PTP dephosphorylates the EGF receptor *in vitro* (Ramponi *et al*., 1989) and particularly the PDGF receptor *in vivo* (Berti *et al*., 1994). In addition, v-Src phosphorylates and activates LMW-PTP both *in vitro* and *in vivo* (Rigacci *et al*., 1996). Interestingly, the EphB1 receptor complexes recruit LMW-PTP upon treatment with multimeric ligands (Stein *et al*., 1998b). The EphB1-binding site for LMW-PTP was mapped and shown to be required for tetrameric ephrin-B1 to recruit LMW-PTP and promote attachment. These results suggest that LMW-PTP plays an important role in the regulation of Eph-mediated cell-cell interactions, including cell adhesion and migration.

In this study, we demonstrate that LMW-PTP is phosphorylated by EphA8 *in vitro*, and this phosphorylation causes an increase in enzyme activity. Our results suggest that LMW-PTP may also participate in a feedback control mechanism of the EphA8 receptor signaling pathway *in vivo*.

Materials and Methods

Cell culture Two hundred ninety-three cells were routinely cultured in alpha-MEM (Sigma Chemical Co., St. Louis, USA) that contained 10% heat-inactivated fetal bovine serum. For stable transfection with the pcDNA3-derived EphA8 expression plasmid, the calcium phosphate precipitation method was used, as described previously (Graham and van der Eb, 1973). Stable G418-resistant clones were selected by supplementing the culture medium with 250 μg/ml G418. The clones were periodically cultured in the same selection medium to maintain stable expression.

Immunoprecipitation and immunoblotting Confluent 10 cm plates of cells were washed two times with a cold phosphate-buffered saline (PBS) that contained 1 mM sodium orthovanadate, and then lysed in 1 ml of a cold PLC lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 20 μg/ml aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate). Lysates were clarified by centrifugation in a microcentrifuge for 15 min at 4°C, and then incubated with the indicated antibodies for 1 h on ice. Protein A-Sepharose (Pharmacia, Uppsala, Sweden) was then added for 30 min, and the immunoprecipitates were washed three times by pelleting in a cold HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 1 mM sodium orthovanadate).

For Western blot analysis (Yi *et al*., 2001; Kim *et al*., 2002), whole immune complexes were boiled in an SDS-sample buffer, loaded on 7.5% SDS-polyacrylamide gels, and separated by electrophoresis. The proteins that were resolved by SDS-PAGE were electrophoretically transferred to Immobilon-P (Millipore Co., Bedford, USA) membranes. The membranes were blocked in a TN-TX buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.2% Triton X-100) that contained 3% BSA (for anti-phosphotyrosine antibody blots) or skimmed milk, and immunoblotted according to standard protocols. The detection of primary antibodies was performed using horseradish peroxidase-conjugated donkey anti-rabbit IgG or anti-mouse IgG, followed by Enhanced Chemiluminescence (Amersham Pharmacia Biotech., Piscataway, USA).

Expression and purification of recombinant LMW-PTP The LMW-PTP enzyme that was used in the *in vitro* phosphorylation experiments was obtained as a fusion protein with glutathione S-transferase. The fusion protein was cleaved by thrombin, and LMW-PTP was then purified according to the manufacturers protocol (Amersham Pharmacia Biotech., Piscataway, USA).

***In vitro* LMW-PTP phosphorylation and dephosphorylation** To evaluate the extent of LMW-PTP phosphorylation, the kinase reaction mixture included GST or GST-fusion proteins (1 μg), a kinase buffer (20 mM HEPES, pH 7.5, 2.5 mM $MgCl_2$, 4 mM $MnCl_2$, 1 mM sodium orthovanadate), 5 μCi of [γ-^{32}P] ATP (NEN, Boston, USA), and Protein A-Sepharose beads that were bound to the EphA8 receptor, as previously described, in a total reaction volume of 20 μl. The reaction was carried out at 30°C. Unincorporated ATP was removed by washing 2-3 times in a cold HNTG buffer. The whole reaction was loaded on the gel. The labeled proteins were resolved by SDS-PAGE, and the dried gels were autoradiographed for 30 min at room temperature.

To evaluate the extent of the GST-EphA8 JM fusion protein dephosphorylation, the ^{32}P-labeled GST-JM fusion protein was prepared as previously described. The ^{32}P-labeled GST-JM fusion protein was then eluted from the immune complexes by boiling for 5 min, and then further purified by PD-10 column (Amersham Pharmacia Biotech., Piscataway, USA), as described previously (Gu and Park, 2001). The purified ^{32}P-labeled GST-JM fusion protein was incubated with either GST-LMW-PTP or purified LMW-PTP, in the kinase buffer (sodium orthovanadate was removed) that contained 60 mM 2-mercaptoethanol, and 1 μg BSA. The whole reaction was loaded on the gel and analyzed by autoradiography.

To determine the activity of the thio-phosphorylated LMW-PTP, the kinase reaction included active LMW-PTP (1 μg), a kinase buffer (sodium orthovanadate was removed) that contained 60 mM 2-mercaptoethanol, 1 μg BSA, 20 mM γ-thioATP, and Protein A-Sepharose beads that were bound to EphA8 in the same quantities as in the phosphorylation test. The control reaction mixtures did not include γ-thioATP, or LMW-PTP and γ-thioATP. The incubation was carried out at 30°C. Supernatant aliquots were incubated with equal amounts of the ^{32}P-labeled GST-EphA8 JM fusion proteins at two different time intervals for their tyrosine-phosphatase activity. The whole reaction was loaded on the gel and analyzed by autoradiography.

Results

The EphA8 receptor phosphorylates LMW-PTP *in vitro* An earlier work showed that tetrameric ephrin-B1 oligomers

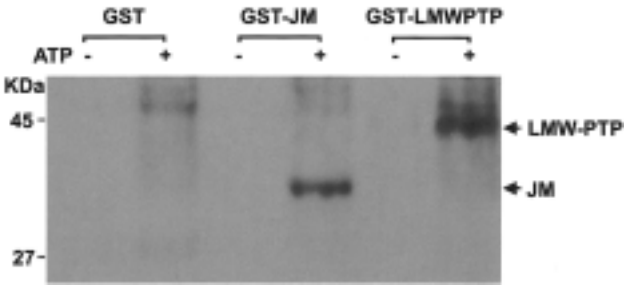

Fig. 1. *In vitro* phosphorylation of GST-fusion proteins by the EphA8 receptor. The purified GST, GST-EphA8 juxtamembrane (JM) region, or GST-LMWPTP was added to a kinase reaction buffer, including [γ-^{32}P] ATP and immunoprecipitated EphA8. Incubation was performed at 30°C for 30 min, and the whole products were boiled in 2 × Laemmli sample buffer, and run on a 12% SDS-PAGE. The gel was dried and exposed to a Kodak X-ray film.

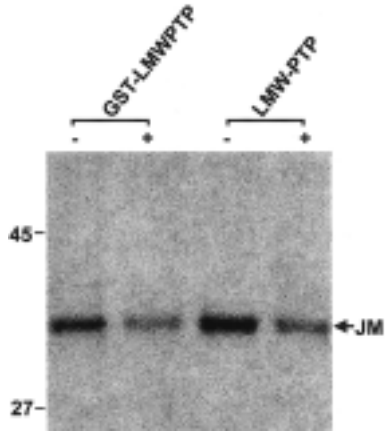

Fig. 2. *In vitro* dephosphorylation of EphA8 juxtamembrane (JM) region by LMW-PTP. The ^{32}P-labeled GST-JM protein was purified as described in Materials and Methods. Equal amounts of the ^{32}P-labeled GST-JM protein was mixed with active GST-LMW-PTP or the purified LMW-PTP enzyme, and allowed to incubate at room temperature for 30 min in the absence of sodium orthovanadate. The reaction was stopped by boiling in 2 × Laemmli sample buffer. After a 12% SDS-PAGE, autoradiography was performed. According to the quantification, phosphorylation of GST-JM decreased approximately 3-fold at lanes 2 and 4, as compared to lanes 1 and 3, respectively.

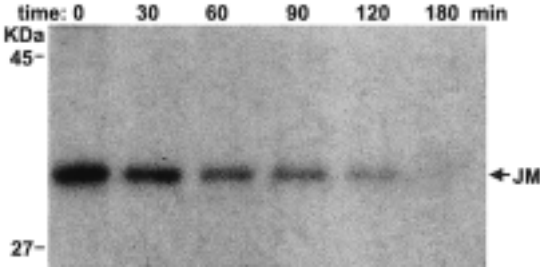

Fig. 3. Time-dependent dephosphorylation of GST-JM by LMW-PTP. Experiments were performed as described in the legend to Fig. 2. At various time points, aliquots were withdrawn and boiled in 2 × Laemmli sample buffer. The quantification indicated that the phosphorylation of GST-JM in each lane decreased at the following ratio; 100% (lane 1), 35% (lane 2), 13% (lane 3), 8% (lane 4), 5% (lane 5), 0% (lane 6).

determine the formation of EphB1-signaling complexes that are marked by the recruitment of LMW-PTP (Stein *et al.*, 1998b). It has been postulated that the recruitment of LMW-PTP may play important roles in the Eph-signaling mechanism that leads to promotion in cell adhesion to fibronectin. To investigate whether LMW-PTP interacts with the EphA8 receptor, we immunoprecipitated the EphA8 protein with an anti-HA antibody from the EphA8-overexpressing HEK 293 cell and used the immobilized protein in a kinase assay, including various GST-fusion proteins and [γ-^{32}P]ATP in the presence of the phosphatase inhibitor (vanadate). For this purpose, we purified GST (GST-JM comprising the entire juxtamembrane region of EphA8 or GST-LMWPTP containing the entire LMW-PTP from bacterial cultures by affinity chromatography). Under these conditions, the GST protein was not phosphorylated by the immobilized EphA8 kinase (Fig. 1, lanes 1 and 2). Consistent with our previous result, the purified GST-JM fusion protein that contained Tyr-615, the major autophosphorylation site of EphA8, was effectively phosphorylated by the EphA8 kinase (Fig. 1, lanes 3 and 4) (Choi and Park, 1999). Interestingly, LMW-PTP was also phosphorylated by EphA8, suggesting the direct involvement of the EphA8 kinase in LMW-PTP phosphorylation (Fig. 1, lanes 5 and 6).

LMW-PTP dephosphorylates the tyrosine residues in EphA8 *in vitro* The discovery that the EphA8 receptor phosphorylates LMW-PTP *in vitro* led us to postulate that the interaction between LMW-PTP and EphA8 may result in the dephosphorylation of EphA8. To verify this hypothesis, we performed an *in vitro* phosphatase assay, including ^{32}P-labeled GST-JM and purified LMW-PTP. In these experiments, the purified GST-JM fusion protein was phosphorylated *in vitro* by incubation with immunoprecipitates that contained wild-type EphA8, and then purified using a gel filtration column. Equal amounts of ^{32}P-labeled GST-JM were incubated with GST, GST-LMWPTP, or LMW-PTP that were obtained from the GST fusion protein in the absence of a phosphatase inhibitor. As shown in Fig. 2, the purified LMW-PTP was able to dephosphorylate the ^{32}P-labeled GST-JM fusion protein *in vitro*, irrespective of its fusion to GST (lanes 2 and 4). To investigate how rapidly LMW-PTP dephosphorylates the ^{32}P-labeled GST-JM fusion protein, the incubation was carried out for various time periods. As shown in Fig. 3, the kinetics showing the dephosphorylation of the ^{32}P-labeled GST-JM fusion protein indicated that LMW-PTP was active in catalyzing the dephosphorylation of ^{32}P-labeled GST-JM fusion protein (up to 3 h in these experiments). In order to

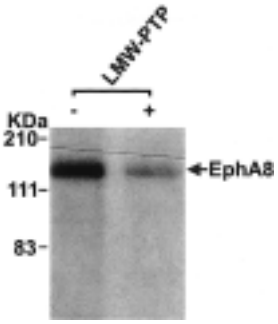

Fig. 4. Dephosphorylation of wild-type EphA8 by LMW-PTP. The tyrosine phosphorylated EphA8 receptor was immunoprecipitated with an anti-HA antibody. The immune complex was mixed with the purified LMW-PTP in a kinase buffer that lacked sodium orthovandate. The reaction was allowed to incubate at room temperature for 30 min, run on 7.5% SDS-PAGE, and immunoblotted with an anti-phosphotyrosine antibody.

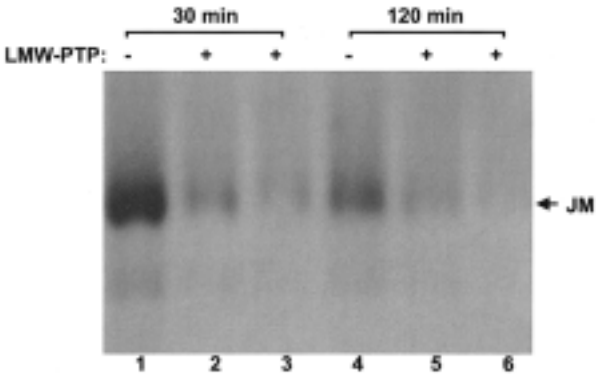

Fig. 5. Activity of enhancement of phosphorylated LMW-PTP. The enzyme was incubated at 30°C with immunoprecipitated EphA8 in the absence (lanes 2 and 5) or presence of γ-thioATP (lanes 3 and 6). The control reaction mixture did not contain both LMW-PTP and γ-thioATP (lanes 1 and 4). At 30 min and 120 min, the aliquots were sampled from the reaction mixtures and assayed for their phosphatase activity using the ^{32}P-labeled GST-JM protein that was prepared as described in the legend to Fig. 2. The quantification indicated that the phosphorylation of GST-JM in each lane decreased at the following ratio; 100% (lane 1), 24% (lane 2), 5% (lane 3), 41% (lane 4), 15% (lane 5), 2% (lane 6).

confirm the hypothesis of a direct LMW-PTP enzymatic action on the Tyr-phosphorylated EphA8 receptor, we performed an *in vitro* assay between the wild-type EphA8 receptor and purified LMW-PTP. For this purpose, we immunoprecipitated the EphA8 protein with an anti-HA antibody from EphA8-overexpressing HEK 293 cell and incubated the immobilized protein with the purified LMW-PTP. The whole complexes were separated by SDS-PAGE, and then assayed by an immunoblot using anti-phosphotyrosine antibodies as a probe. This demonstrates that LMW-PTP effectively dephosphorylated the phosphorylated EphA8 receptor (Fig. 4, lane 2). These results, therefore, suggest that the EphA8 receptor phosphorylates LMW-PTP and, in turn, LMW-PTP dephosphoryates EphA8 as a putative regulator of its autokinase activity.

Tyrosine-phosphorylated LMW-PTP possesses an enhanced enzymatic activity for tyrosine-phosphorylated EphA8-JM domain Our discovery that LMW-PTP is an effective substrate of the EphA8 receptor tyrosine kinase implicates the potential significance of tyrosine-phosphorylated LMW-PTP. To assess whether tyrosine phosphorylation of LMW-PTP could strengthen its activity, we prepared a phosphorylated form of LMW-PTP from a kinase reaction that included the immobilized EphA8 kinase and the purified LMW-PTP in the absence (Fig. 5, lanes 2 and 5) or presence (lanes 3 and 6) of γ-thioATP. The resulting thio-phosphorylated residues in LMW-PTP are resistant to phosphotyrosine phosphatase activity (Rigacci *et al*., 1996). For 30 min or 120 min, the thio-phosphorylated LMW-PTP was incubated with the ^{32}P-labeled GST-JM as a substrate. Interestingly, the enzymatic activity of thio-phosphorylated LMW-PTP increased at least 2-fold higher when compared to unphosphorylated LMW-PTP (Fig. 5, compare lanes 2 and 5 with 3 and 6, respectively). This clearly demonstrates that LMW-PTP that is phosphorylated by the EphA8 kinase *in vitro* causes an increase in the enzymatic activity.

Discussion

The primary discovery of this study is that LMW-PTP can participate in a feedback control mechanism of the EphA8 receptor autokinase activity, although our discovery has not yet been demonstrated in intact cells. Previous studies showed that LMW-PTP interacts with the PDGF receptor and downregulates the mitogenic signals, starting with the PDGF receptor activation (Berti *et al*., 1994). In contrast to the PDGF receptor, the Eph receptor tyrosine kinases do not appear to regulate cell proliferation. This suggests that LMW-PTP plays a role in the Eph signaling pathway via a distinct mechanism rather than its anti-mitogenic role. EphB1 recruits LMW-PTP in response to the tetrameric ephrin-B1 stimulation, and the EphB1-LMW-PTP complexes played an essential role in promoting cell adhesion to fibronectin (Stein *et al*., 1998b). We also found that the EphA8 receptor associates very weakly with LMW-PTP in HEK293 cells, although it was not presented in our current study. For example, when anti-HA immunoprecipitates that contained the EphA8 protein complexes were washed out more than two times, LMW-PTP was barely co-immunoprecipitated with EphA8. However, the complex formation between EphA8 and LMW-PTP was not dependent on the ephrin-A ligand stimulation in HEK293 cells (data not shown). In the HEK293 cells that were deprived of the endogenous ephrin-A ligands

(by treatment of PI-PLC), the EphA8 receptors were still tyrosine-phosphorylated and LMW-PTP was detectable in the EphA8 immune complexes. This suggests that the EphA8 receptors that were overexpressed in the HEK293 cells are multimeric and sufficient for the recruitment of LMW-PTP. For EphB1, the phosphorylation on Tyr-929 was known to be critical in the recruitment of LMW-PTP (Stein *et al*., 1998b). This tyrosine residue is located in the sterile alpha motif (SAM) domain of EphB1, which is well conserved in other members of the Eph receptors and has been postulated as a putative motif that is important for the interaction with LMW-PTP. However, in the case of EphA8, the SAM domain was not required for the complex formation between EphA8 and LMW-PTP (data not shown). This suggests that the EphA8 receptor interacts with LMW-PTP in a distinct mechanism. The molecular basis of the association between EphA8 and LMW-PTP still needs to be determined. In particular, the definition of the EphA8 site of recruitment for LMW-PTP may permit us to determine whether recruitment is functionally significant in mediating the EphA8 signaling process, such as cell adhesion responses.

Our previous study demonstrated that the EphA8 receptor promotes cell adhesion to fibronectin, independent of its tyrosine kinase activity (Gu and Park, 2001). For example, the EphA8-K666M mutant receptor that contains methionine in the place of lysine, ATP binding residue, is defective in its autophosphorylation activity, and yet it is capable of enhancing cell adhesion onto fibronectin. The increased phosphatase activity of tyrosine-phosphorylated LMW-PTP may generate the dephophorylated EphA8, somewhat resembling the kinase-dead EphA8 mutant. Although available data do not paint a clear picture of the functional roles of LMW-PTP, one hypothesis is that LMW-PTP may play a role in enhancing the dephosphorylation of the EphA8 receptor. The dephosphorylated form of the EphA8 receptor is more actively involved in enhancing cell adhesion to fibronectin rather than its phosphorylated form. Further studies to determine the functional role of LMW-PTP in the EphA8-mediated cell adhesion will be an important part of elucidating the exact role for EphA8.

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