Regulation of the EphA2 Kinase by the Low Molecular Weight Tyrosine Phosphatase Induces Transformation*

Keith D. Kikawa†, Derika R. Vidale§, Robert L. Van Etten§, and Michael S. Kinch‡"¶

From the Departments of †Basic Medical Sciences and §Chemistry, Purdue University, West Lafayette, Indiana 47907 and ‡MedImmune, Inc., Gaithersburg, Maryland 20878

Intracellular signaling by protein tyrosine phosphorylation is generally understood to govern many aspects of cellular behavior. The biological consequences of this signaling pathway are important because the levels of protein tyrosine phosphorylation are frequently elevated in cancer cells. In the classic paradigm, tyrosine kinases promote tumor cell growth, survival, and invasiveness, whereas tyrosine phosphatases negatively regulate these same behaviors. Here, we identify one particular tyrosine phosphatase, low molecular weight tyrosine phosphatase (LMW-PTP), which is frequently overexpressed in transformed cells. We also show that overexpression of LMW-PTP is sufficient to confer transformation upon non-transformed epithelial cells. Notably, we show that the EphA2 receptor tyrosine kinase is a prominent substrate for LMW-PTP and that the oncogenic activities of LMW-PTP result from altered EphA2 expression and function. These results suggest a role for LMW-PTP in transformation progression and link its oncogenic potential to EphA2.

Cancer arises when a population of cells gains the ability to inappropriately grow and survive. These biological behaviors often result from genetic and environmental abnormalities that work together to trigger specific signaling pathways that promotes inappropriate cell growth and survival. In particular, increased levels of protein tyrosine phosphorylation are understood to initiate powerful signals that govern many different aspects of cell behavior (1). A popular paradigm suggests that a balance between tyrosine kinase and phosphatase activities determines the cellular levels of protein tyrosine phosphorylation and thereby governs cellular decisions regarding growth, survival, and invasiveness (2). This model predicts that tyrosine kinases are oncogenic, whereas tyrosine phosphatases negatively regulate transformation. Although this paradigm has generally been supported by the identification of oncogenic tyrosine kinases, emerging evidence reveals a more complex interplay between tyrosine kinases and phosphatases. For example, the PTP(CAAX) tyrosine phosphatase has been recently implicated as an oncogene (3). Moreover, the enzymatic activity of Src family kinases is liberated by phosphatase-mediated dephosphorylation of critical tyrosine residues (4, 5). In the latter situation, phosphatases can actually up-regulate protein tyrosine phosphorylation by increasing the enzymatic activity of kinases.

The EphA2 receptor tyrosine kinase is overexpressed in a large number of human cancers. High levels of EphA2 apply to a large number of different cancers, including breast, prostate, colon, and lung carcinomas as well as metastatic melanomas (6–10). The highest levels of EphA2 are consistently found on the most aggressive cell models of human cancer (9–11). Moreover, EphA2 is not simply a marker of transformed disease as ectopic overexpression of EphA2 confers tumorigenic and metastatic potential upon non-transformed epithelial cells (10). In addition to its overexpression, EphA2 is functionally altered in transformed cells as compared with non-transformed epithelia (12). In particular, EphA2 is prominently tyrosine phosphorylated in non-transformed epithelial cells. Yet, despite its overexpression, the EphA2 in transformed cells is not tyrosine phosphorylated (10–12). Recent studies indicate that these differences in EphA2 phosphotyrosine content are important because tyrosine phosphorylation of EphA2 causes it to interact with downstream signaling components that function to negatively regulate cell growth and invasiveness (10, 12–16). In contrast, unphosphorylated EphA2 appears to adopt a different subcellular localization and interacts with different substrates (10–12). Most importantly, recent studies have shown that unphosphorylated EphA2 functions as a powerful oncoprotein, whereas restoration of EphA2 phosphotyrosine content is sufficient to reverse the oncogenic potential of EphA2 (10, 12, 13).

Based on the differential behaviors of EphA2 in normal and transformed cells, our laboratory has been investigating the regulation of EphA2 phosphotyrosine content. Our recent studies have centered upon evidence that cancer cells often demonstrate decreased cell-cell contacts (17–19), which destabilize ligand binding (10, 12). Our present studies investigate an additional possibility, which is that the phosphotyrosine content of EphA2 in transformed cells is negatively regulated by an associated phosphatase. We affirm this hypothesis and identify LMW-PTP1 as a phosphatase that regulates EphA2. Human LMW-PTP has been cloned, sequenced, expressed, and structurally characterized (20–22). We also demonstrate that LMW-PTP is overexpressed in many transformed cell lines and that overexpression of LMW-PTP is sufficient to confer transformation upon non-transformed epithelial cells. Moreover, we demonstrate that the oncogenic activities of LMW-PTP require EphA2.

MATERIALS AND METHODS

Cell Lines and Reagents—Human breast (MCF-10Aneo, MCF-10A neoST), MCF-7, MDA-MB-231, MDA-MB-435, SK-BR-3) epithelial

1 The abbreviation used is: LMW-PTP, low molecular weight tyrosine phosphatase.

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cells were cultured as described previously (23-25). Monoclonal antibodies specific for phosphorysine (PY20) and β-catenin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies specific for phosphorysine (4G10) and EphA2 (clone D7) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibodies against vinculin were purchased from NeoMarkers (Fremont, CA). Purified LMW-PTP was prepared as described (20).

**Cell Lysates**—Cell lysates were harvested and normalized for equal loading as described previously (18). To confirm equal loading, blots were stripped as described previously and reprobed with antibodies specific to β-catenin or vinculin (18).

**Immunoprecipitation and Immunoblot Analyses**—Immunoprecipitation of EphA2 or LMW-PTP were performed using rabbit anti-mouse (Chemicon, Temecula, CA) conjugated protein A-Sepharose (Sigma) as described previously (12). To confirm equal loading, blots were stripped as described previously and reprobed with EphA2- or LMW-PTP-specific antibodies (18). Immunoblot analyses were performed on normalized cell lysates and immunoprecipitations as detailed (12). Antibody binding was detected by enhanced chemiluminescence (ECL, Pierce) and visualized by autoradiography (Kodak X-OMAT; Eastman Kodak Co.).

**EGTA and Pervanadate Treatments**—“Calcium switch” experiments were performed as described previously using MCF-10Aneo cells grown to 70% confluence in medium containing a final concentration of 4 mM EGTA (12). Pervanadate was added to MDA-MB-231 in monolayer culture at a final concentration of 100 μM, and the treatment was allowed to incubate for 10 min at 37 °C, 5% CO2. For the combined EGTA-pervanadate treatment, MDA-MB-231 cells were first treated with 100 μM pervanadate and were then subjected to the EGTA treatment.

**In Vitro Kinase and Phosphatase Assays**—To evaluate LMW-PTP activity against EphA2, EphA2 was immunoprecipitated from MCF-10Aneo cells and incubated with purified LMW-PTP protein at a concentration of 0.45, 7.8, or 62 μg/ml for 0, 5, 15, or 30 min. The assay was terminated through the addition of Laemmli sample buffer. The phosphorysine content of the EphA2 in the treatments was then observed using immunoblot analysis with antibodies specific to phosphorysine. To determine in vitro autophosphorylation activity, immunoprecipitated EphA2 was evaluated using in vitro kinase assays as detailed previously (12).

**Transfection and Selection**—Monolayers of MCF-10Aneo cells were grown to 30-50% confluence and were transfected with pcDNA3.1-LMW-PTP or pcDNA3.1-LMW-PTPΔD129 using LipofectAMINE PLUS (Invitrogen). As a control for the transfection procedure, empty pcDNA3.1 vector was transfected into the same cell line in parallel. Monolayers of MCF-10Aneo human mammary epithelial cells were incubated in the presence or absence (denoted as C for control) of 4 mM EGTA for 20 min before detergent extraction. The samples were resolved by SDS-PAGE and probed with phosphorysine (P-Tyr)-specific antibodies (PY20 and 4G10; top). The membranes were stripped and reprobed with EphA2-specific antibodies to confirm equal sample loading (below). In B, MCF-10Aneo cells were treated with EGTA, as detailed above, in the presence or absence of NaVO₄ to inhibit phosphatase activity. In C, EphA2 was immunoprecipitated from MDA-MB-231 cells that had been incubated in the presence of the indicated concentrations of NaVO₄ for 10 min at 37 °C.

**Antisense Treatment**—Monolayers of MCF-10Aneo cells and MCF-10Aneo cells stably overexpressing LMW-PTP were grown to 30% confluence and were transfected with EphA2 antisense oligonucleotides as detailed (13). Samples that had been transfected with an inverted EphA2 antisense oligonucleotide or with the transfection reagent alone provided negative controls.

**RESULTS**

**EphA2 Is Regulated by an Associated Tyrosine Phosphatase**—Several independent lines of investigation suggested that EphA2 is regulated by an associated tyrosine phosphatase. EphA2 was rapidly dephosphorylated in non-transformed MCF-10Aneo epithelial cells upon disruption of ligand binding. Immunoblot analysis with phosphorysine antibodies (PY20 or 4G10) indicated lower levels of EphA2 phosphorysine content within 5 min following EGTA treatment, which destabilizes EphA2-ligand binding (12) (Fig. 1A). Similarly, tyrosine phosphorylation of EphA2 decreased following incubation of non-transformed epithelial cells with dominant-negative inhibitors of EphA2-ligand binding (e.g. EphA2-Fc, not shown). Similar results were obtained using multiple non-transformed epithelial cell systems, including MCF-12A, MCF10-2, HEK293, Madin-Darby canine kidney, and Madin-Darby bovine kidney cells (not shown). Based on these findings, we asked whether tyrosine phosphatase inhibitors could prevent the loss of EphA2 phosphorysine content in response to EGTA treatment. Indeed, inhibitors such as sodium orthovanadate prevented the decrease in EphA2 phosphorysine following treatment of MCF-10Aneo cells with EGTA (Fig. 1B).

Previous studies by our laboratory have shown that the phosphorysine content of EphA2 is greatly reduced in transformed epithelial cells as compared with non-transformed epithelia (10, 12). Thus, we asked whether tyrosine phosphatase activity could contribute to the reduced phosphorysine content of EphA2 in transformed cells. Although EphA2 was not tyrosine phosphorylated in transformed breast cancer cells (MDA-MB-231, MDA-435, MCFneoST, or PC-3 cells), incubation with increasing concentrations of sodium orthovanadate induced vigorous tyrosine phosphorylation of EphA2 (Fig. 1C). As vanadate treatment of cells can often lead to exaggerated phosphorylation of physiologically irrelevant sites, we performed phosphopeptide mapping studies using EphA2 that had been labeled with 32P[ATP either in vitro or in vivo (not shown). These studies revealed identical patterns of tyrosine phosphorylation in non-transformed MCF-10Aneo cells and vanadate-treated MDA-MB-231 cells (not shown). Although the cytoplasmic domain contains multiple sites that could have been phosphorylated promiscuously, these were not phosphorylated under the conditions utilized here, suggesting that vanadate had not increased the phosphorylation of irrelevant sites. Altogether, these results indicate that EphA2 is regulated by an associated phosphatase that suppresses EphA2 phosphorysine content in transformed cells.

**LMW-PTP Interacts with and Dephosphorylates EphA2**—To identify tyrosine phosphatases that might regulate EphA2 in...
transformed cells, we considered a recent report that LMW-PTP regulates a related molecule, EphB4 (27). Our initial experiments began by assessing the expression and function of LMW-PTP in non-transformed (MCF-10Aneo, denoted as Neo), oncogene-transformed (MCF-10AneoST denoted as NeoST), and tumor-derived (MCF-7, SK-BR-3, MDA-MB-435, MDA-MB-231) mammary epithelial cells. The samples were resolved by SDS-PAGE and subjected to immunoblot analysis using LMW-PTP-specific antibodies (top). Purified LMW-PTP (lane 1) provided a positive control for immunoblot analyses. The membranes were then stripped and reprobed with antibodies specific to vinculin to evaluate sample loading (bottom). Note that LMW-PTP is overexpressed in tumor-derived cells despite the relative overloading of the non-transformed (MCF-10Aneo) samples.

Although in vitro studies indicated that EphA2 could be dephosphorylated by LMW-PTP in vitro, we recognized that in vitro studies are not always representative of the corresponding situation in vivo. Thus, LMW-PTP was ectopically overexpressed in MCF-10A cells. This particular cell system was selected because non-transformed MCF-10A cells have low levels of endogenous LMW-PTP and because the EphA2 in these non-transformed epithelial cells is normally tyrosine phosphorylated. Ectopic overexpression of LMW-PTP was achieved by stable transfection, as determined by immunoblot analyses with specific antibodies (Fig. 5A). Importantly, overexpression of LMW-PTP was sufficient to reduce the phosphotyrosine content of EphA2 as compared with vector-transfected negative controls (Fig. 5B). Identical results were obtained using different experiments with different transfectants and in both stably and transiently transfected samples (not shown), thus eliminating potential concerns about clonal variation. Moreover, the decreased phosphotyrosine content was specific for EphA2 as the overall phosphotyrosine content of LMW-PTP-overexpressing cells was not decreased (Fig. 5C).

LMW-PTP Overexpression Causes Transformation of Epithelial Cells—Tyrosine phosphorylated EphA2 negatively regulates tumor cell growth, whereas unphosphorylated EphA2 acts as a powerful oncogene (10, 12, 13). Thus, we asked whether overexpression of LMW-PTP would be sufficient to induce transformation. To address this question, we utilized the MCF-10A cells, described above, which had been transfected with either wild-type LMW-PTP or a vector control. Our initial studies evaluated the growth rates of control and LMW-PTP-overexpressing cells in monolayer culture. When evaluated using standard, two-dimensional culture conditions, the
samples were probed for EphA2 protein levels (middle). Parallel samples were probed for EphA2 protein levels (middle). The membranes were stripped and reprobed with β-catenin antibodies as a loading control (bottom). In B, equal amounts of EphA2 were isolated by immunoprecipitation, resolved by SDS-PAGE, and subjected to immunoblot analyses with EphA2 (top)- or P-Tyr-specific (bottom) antibodies. Note that this experiment compared the phosphotyrosine content using immunoblot analyses with EphA2 (top)- or P-Tyr-specific (bottom) antibodies. In C, cell lysates were harvested from control or LMW-PTP-transfected cells, and equal amounts of protein were resolved by SDS-PAGE and evaluated by immunoblot analyses using phosphotyrosine-specific antibodies. In D, the protein levels (top) and phosphotyrosine content (bottom) of immunoprecipitated EphA2 were evaluated using material isolated from MDA-MB-231 cells that had been transfected with a dominant-negative phosphatase mutant (LMW-PTPD129A) or a matched vector control.

The Transformed Phenotype of LMW-PTP-Overexpressing Cells Requires EphA2—Tyrosine phosphorylation of EphA2 induces its internalization and degradation (13). Thus, we postulated that overexpression of LMW-PTP might increase the protein levels of EphA2. Indeed, immunoblot analyses of whole cell lysates revealed higher levels of EphA2 in MCF-10A cells that overexpress LMW-PTP as compared with vector-transfected controls (Fig. 5A). Moreover, this EphA2 was not tyrosine phosphorylated (Fig. 5B). However, immunoblot analyses revealed that overexpression of EphA2 as the general levels of phosphotyrosine were not altered in LMW-PTP-transformed cells (Fig. 5C). The finding that overexpression of LMW-PTP increased EphA2 protein levels and decreased its phosphotyrosine content was intriguing since this phenotype was reminiscent of highly aggressive tumor cells (10, 12). Thus, we asked whether selective targeting of LMW-PTP in transformed cells would impact EphA2. To accomplish this, the catalytically inactive (28) mutant LMW-PTP(D129A) was overexpressed in MDA-MB-231 cells, which have high levels of wild-type LMW-PTP (Fig. 2) and which also have elevated levels of EphA2 that is hypophosphorylated. Ectopic overexpression of LMW-PTP(D129A) was found to decrease the levels of EphA2. Moreover, immunoblot analyses of immunoprecipitated material demonstrated that EphA2 was tyrosine phosphorylated (Fig. 5D). These results indicate that overexpression of wild-type LMW-PTP is necessary and sufficient to confer the overexpression and functional alterations of EphA2 that have been observed in tumor-derived cells.

Although the EphA2 in the LMW-PTP-overexpressing MCF-10A cells was not tyrosine phosphorylated, it retained enzymatic activity. In vitro kinase assays verified that the EphA2 from LMW-PTP-transformed MCF-10A cells had levels of enzymatic activity that were comparable with vector-transfected controls (Fig. 7A). To verify equal sample loading, two controls were performed. Equal amounts of input lysate were verified by immunoblot analyses with β-catenin antibodies (not shown). In addition, the immunoprecipitated EphA2 was divided, and half

**Fig. 5.** LMW-PTP dephosphorylates EphA2 in vivo. A–C, MCF-10A cells stably transfected with expression vectors that encode for wild-type LMW-PTP. In A, detergent lysates were resolved by SDS-PAGE and subjected to immunoblot analyses with LMW-PTP antibodies, with purified LMW-PTP providing a positive control (top). Parallel samples were probed for EphA2 protein levels (middle). The membranes were stripped and reprobed with β-catenin antibodies as a loading control (bottom). In B, equal amounts of EphA2 were isolated by immunoprecipitation, resolved by SDS-PAGE, and subjected to immunoblot analyses with EphA2 (top)- or P-Tyr-specific (bottom) antibodies. Note that this experiment compared the phosphotyrosine content using immunoblot analyses with EphA2 (top)- or P-Tyr-specific (bottom) antibodies. In C, cell lysates were harvested from control or LMW-PTP-transfected cells, and equal amounts of protein were resolved by SDS-PAGE and evaluated by immunoblot analyses using phosphotyrosine-specific antibodies. In D, the protein levels (top) and phosphotyrosine content (bottom) of immunoprecipitated EphA2 were evaluated using material isolated from MDA-MB-231 cells that had been transfected with a dominant-negative phosphatase mutant (LMW-PTPD129A) or a matched vector control.

**Fig. 6.** LMW-PTP enhances transformed character. In A, to evaluate anchorage-dependent cell growth, 1 × 10⁶ control or LMW-PTP-transfected MCF-10A cells were seeded into monolayer culture, and cell numbers were evaluated microscopically at the intervals shown. In B, in parallel studies, the control and LMW-PTP-transfected cells were suspended in soft agar. Colony formation (per high powered field (HPF) after 5 days of incubation at 37 °C is shown. These results were representative of at least three separate experiments. *, p < 0.01.
of the material was resolved by SDS-PAGE and analyzed by immunoblot analyses with EphA2 and phosphotyrosine-specific antibodies (Fig. 7, B and C). Thus phosphorylated and unphosphorylated EphA2 were both capable of enzymatic activity.

Having determined that the levels of EphA2 were elevated in LMW-PTP-transformed cells, we asked to what extent the oncogenic activity of EphA2 contributed to this phenotype. To address this, we utilized our experience with antisense strategies to selectively decrease EphA2 expression in LMW-PTP-transformed cells (13, 29). We verified the success of these strategies by immunoblot analyses (Fig. 8A) and then asked whether decreased EphA2 expression would alter soft agar colonization. Indeed, transfection with EphA2 antisense oligonucleotides decreased the soft agar colonization of LMW-PTP-transformed MCF-10A cells by at least 87% (p < 0.01; Fig. 8B).

As a control, transfection of these cells with an inverted antisense or function of LMW-PTP, and future investigation should address this possibility.

The fact that high levels of LMW-PTP were observed in several different cell models of metastatic cancer is notable given that LMW-PTP overexpression is sufficient to confer transformation. LMW-PTP-overexpressing cells gain the ability to colonize soft agar and acquire a transformed phenotype when cultured in three-dimensional basement membranes, such as Matrigel. Notably, however, LMW-PTP-overexpressing MCF-10A epithelial cells displayed reduced rates of cell growth as measured using two-dimensional assays of cell growth. This latter observation is consistent with recent reports that high levels of LMW-PTP similarly decrease the monolayer growth rates of other cell types (30, 31). Although such a finding had been interpreted to suggest that LMW-PTP might negatively regulate transformation, our findings support a very different conclusion. Consistent with this, recent studies by our laboratory and others have shown that transformation of MCF-10A cells is often accompanied by decreased monolayer growth rates and that the most aggressive variants of MCF-10A cells are often accompanied by decreased monolayer growth rates and that the most aggressive variants of MCF-10A in vivo demonstrate the slowest growth in monolayer culture (10, 11, 32). These findings have implications for the design and interpretation of tests of oncogene function when using non-transformed epithelial cell systems.

The biochemical consequences of EphA2 tyrosine phosphorylation remain largely unclear. Unlike other receptor tyrosine kinases, for which autophosphorylation is necessary for enzymatic activity, tyrosine phosphorylation of EphA2 is not required for its enzymatic activity. Consistent with our present results, EphA2 retains comparable levels of enzymatic activity in non-transformed and tumor-derived cells, despite dramatic differences in its phosphotyrosine content (12). Similarly, an-
tibody-mediated stimulation of EphA2 autophosphorylation does not change the levels of EphA2 enzymatic activity (13). Phosphopeptide analyses of the EphA2 cytoplasmic domain provide one potential explanation. Although EphA2 has a predicted activation loop tyrosine at residue 772 (33), neither in vitro nor in vivo phosphopeptide analyses have identified phosphorylation of this site in normal or malignant cells. Thus, the lack of a consensus activation loop tyrosine may account for the retention of EphA2 enzymatic activity in cells where it is not tyrosine phosphorylated.

Tyrosine phosphorylation of EphA2 does not appear to be necessary for its intrinsic enzymatic activity. Instead, ligand-mediated tyrosine phosphorylation regulates EphA2 protein stability (15). Specifically, tyrosine phosphorylation fates EphA2 to interact with the c-Cbl adapter protein and to subsequently be internalized and degraded within proteosomes. Consequently, the phosphatase activity of LMW-PTP would be predicted to increase EphA2 protein stability. Indeed, the highest levels of EphA2 are consistently found in cells with high levels of LMW-PTP. One interesting implication of this finding is that it provides a mechanism, independent of genetic regulation of the EphA2 gene, to explain why high levels of EphA2 are found in many different tumors. An alternative possibility is that LMW-PTP up-regulates EphA2 gene expression, and our present findings do not formally eliminate this possibility. The fact that EphA2 antisense oligonucleotides reversed the transformed character of LMW-PTP-overexpressing cells suggests that the up-regulation of EphA2 is relevant to the cellular behaviors of LMW-PTP-mediated transformation.

In summary, our present studies identify LMW-PTP as a new oncogene that is overexpressed in transformed cells. We also link the biochemical and biological actions of overexpressed LMW-PTP with EphA2. These findings have important implications for understanding the biochemical and biological mechanisms that contribute to the metastatic progression of epithelial cells. Moreover, our present studies identify an important signaling system that could ultimately provide an opportunity to target the large number of cancer cells that overexpress EphA2 or LMW-PTP.

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REFERENCES


2 M. S. Kinch, unpublished information.

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