Loss of protein tyrosine phosphatase N2 potentiates epidermal growth factor suppression of intestinal epithelial chloride secretion

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ABSTRACT

The Crohn’s disease (CD) candidate gene, protein tyrosine phosphatase non-receptor type 2 (PTPN2), has been shown to regulate epidermal growth factor (EGF)-induced phosphatidylinositol 3-kinase (PI3-K) activation in fibroblasts. In intestinal epithelial cells (IECs), EGF-induced EGF receptor (EGFR) activation and recruitment of PI3-K play a key role in regulating many cellular functions including Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion. Moreover, EGFR also serves as a conduit for signalling by other non-growth factor receptor ligands such as the proinflammatory cytokine, IFN\(\gamma\). Here, we investigated a possible role for PTPN2 in the regulation of EGFR signalling and Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion in IECs.

PTPN2 knock-down enhanced EGF-induced EGFR tyrosine phosphorylation in T\(_{84}\) cells. In particular, PTPN2 knock-down promoted EGF-induced phosphorylation of EGFR residues Tyr-992 and Tyr-1068 and led subsequently to increased association of the catalytic PI3-K subunit, p110, with EGFR and elevated phosphorylation of the downstream marker, Akt. As a functional consequence, loss of PTPN2 potentiated EGF-induced inhibition of carbachol stimulated Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion. In contrast, PTPN2 knock-down neither affected IFN\(\gamma\)-induced EGFR transactivation, nor EGF- or IFN\(\gamma\)-induced phosphorylation of ERK1/2.

In summary, our data establish a role for PTPN2 in the regulation of EGFR signalling in IECs in response to EGF but not IFN\(\gamma\). Knock-down of PTPN2 directs EGFR signalling towards increased PI3-K activation and increased suppression of epithelial chloride secretory responses. Moreover, our findings suggest that PTPN2 dysfunction in IECs leads to altered control of intestinal epithelial functions regulated by EGFR.

Keywords: PTPN2, EGFR, PI3-K, IFN\(\gamma\), chloride secretion
INTRODUCTION

Phosphorylation and dephosphorylation of specific amino acid residues, such as tyrosine residues, represents a fundamental mechanism for the activation and inactivation of intracellular signalling molecules. Dephosphorylation is carried out by a large number of different protein phosphatases. One important family of such proteins is the protein tyrosine phosphatases. Members of this family play an essential role in the regulation of critical cell signalling events, i.e. proliferation, differentiation and cell survival (46). The gene locus encoding one member of this protein family, protein tyrosine phosphatase non-receptor type 2 (PTPN2), has recently been associated with Crohn’s disease (CD), ulcerative colitis (UC) and Type I diabetes (17; 43). Nevertheless, a functional role for PTPN2, also known as T-cell protein tyrosine phosphatase (TC-PTP), in the pathophysiology of CD or UC has not been identified. Among PTPN2 substrates are the epidermal growth factor receptor (EGFR) (27; 44; 45), the insulin receptor (18) and the signal transducers and activators of transcription 1 and 3, which are important signalling mediators of IFNγ (42; 52; 57).

EGFR, also known as ErbB1, is a member of the ErbB receptor family and plays a major role in cell growth and wound repair. EGFR can be activated directly by a member of the EGF family of ligands, or indirectly, by transactivation stimulated by non-EGFR ligands such as carbachol (24) or IFNγ (11; 48), which can occur in an EGFR ligand-dependent or -independent manner (12; 16; 48). Upon ligand binding, EGFR undergoes autophosphorylation via its tyrosine kinase activity, and forms catalytically active homo- or heterodimers with other ErbB members such as ErbB2 (56). The recruitment of downstream signalling pathways by EGFR depends on the mode of receptor activation (i.e., direct vs. transactivation), the type of receptor dimer formed (homodimer vs. heterodimers), and the pattern of phosphorylation of specific EGFR tyrosine residues (19; 32). Important downstream signalling pathways,
originating at the EGFR, include mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphatidylinositol 3’-kinase (PI3-K) (5; 32). In addition to the four major autophosphorylation sites of EGFR, Tyr-1068, Tyr-1086, Tyr-1148 and Tyr-1173, the minor autophosphorylation site Tyr-992 serves as a phospholipase C-γ binding site (34; 50). While Tyr-1068 is a binding site for the adaptor protein, Grb2, that is involved in the recruitment of MAPK pathways (4; 26), increased phosphorylation of the residues Tyr-992 and Tyr-1068 has also been associated with elevated activity of PI3-K and Akt (29; 45). Moreover, our laboratory has previously demonstrated that dephosphorylation of these residues by protein tyrosine phosphatase 1B (PTP1B) mediates differential recruitment of PI3-K by EGF but not G protein-coupled receptor-induced transactivation, even though both mechanisms of EGFR activation lead to inhibition of intestinal epithelial calcium-dependent chloride secretion (24; 28; 46; 47).

The intestinal epithelium is responsible for the uptake of nutrients and the absorption and secretion of electrolytes and fluids. The driving force for the intestinal secretion of ions and water is the secretion of chloride. Dysregulation of chloride secretion can result in the hypersecretion of chloride and excessive loss of salt and water into the lumen, as occurs in secretory diarrhea (35), or insufficient chloride secretion as occurs in cystic fibrosis (7) and chronic inflammatory states such as IBD (33; 50). Therefore, precise regulation of the intestinal epithelial chloride secretion is crucial.

As PTPN2 is involved in regulating both EGFR and IFNγ signalling in non-epithelial systems, this led us to the hypothesis that PTPN2 might be involved in modifying the ability of these pathways to regulate intestinal epithelial chloride secretion. Therefore, the aim of this study was to determine whether PTPN2 regulates EGFR-mediated signalling and, subsequently, epithelial chloride secretion in intestinal epithelial cells. We found that PTPN2
promotes EGF, but not IFN\(\gamma\)-induced EGFR activation, and potentiates EGF-induced PI3-K activity and inhibition of intestinal epithelial chloride secretion.

**EXPERIMENTAL PROCEDURES**

**Materials**- Human IFN\(\gamma\) (Roche, Mannheim, Germany), recombinant human EGF (Genzyme, Cambridge, MA), AG1478 (Sigma, St. Louis, MO), LY294002 (Sigma), carbachol (Sigma), mouse anti-PTPN2 antibody that detects both the 45kDa and the 48 kDa splice form of PTPN2 (45) (Calbiochem, San Diego, CA), phosphorylation site-specific rabbit anti-EGFR phosphotyrosine-antibodies (Biosource, Camarillo, CA), mouse anti-phosphotyrosine PY20-antibody (BD Biosciences, Santa Cruz, CA), rabbit anti-Akt-antibody and mouse anti-EGFR-neutralizing-antibody (clone LA1) (Upstate, Lake Placid, NY), rabbit anti-lamin A/C-antibody, rabbit anti-PI3-Kinase p110-antibody and rabbit anti-ERK1/2-antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-EGFR-antibody, rabbit anti-phospho-ERK1/2-antibody and rabbit anti-phospho-Akt (Ser\(^{473}\))-antibody (Cell Signaling Technologies, Danvers, MA) were obtained from the sources noted. All other reagents were of analytical grade and acquired commercially.

**Cell culture**- Human colonic T\(_{84}\) epithelial cells were cultured in a humidified atmosphere with 5% CO\(_2\) as described previously (48) in Dulbecco’s modified Eagle’s/F-12 medium (Mediatech, Inc., Herndon, VA) supplemented with 5% newborn calf serum. Cells were separated by trypsinization and 1x10\(^6\) cells were seeded onto 12 mm Millicell-HA semipermeable filter supports (Millipore, Bedford, MA). T\(_{84}\) cells used for the experiments were between passage 22 and 35. When seeded on filters, T\(_{84}\) cells develop monolayers with
the polarized phenotype of native colonic crypt epithelial cells (25). According to the localization of their receptors, IFN\(\gamma\) (1000 U/ml), EGF (100 ng/ml) and carbachol (100 µM) were added basolaterally. EGFR-inhibitor AG1478 (10 µM) and PI3-K-inhibitor LY294002 (20 µM) were added bilaterally.

**Preparation of cell lysates** - After stimulation, T\(_{84}\) cells were washed three times with ice-cold Ringer’s solution (140 mM Na\(^+\), 5.2 mM K\(^+\), 1.2 mM Ca\(^{2+}\), 0.8 mM Mg\(^{2+}\), 120 mM Cl\(^-\), 25 mM HCO\(_3^-\), 2.4 mM H\(_2\)PO\(_4^-\), 0.4 mM HPO\(_4^{2-}\), 10 mM glucose) and lysed in ice-cold lysis buffer (1% Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml antipain, 100 µg/ml phenylmethylsulfonylfluoride, 1 mM sodium vanadate, 1 mM sodium fluoride, and 1 mM EDTA in PBS) for 45 min. T\(_{84}\) cells were scraped from the filters, transferred to a microcentrifuge tube and centrifuged for 10 min at 13,000 g. Cell lysate supernatants were assayed for protein content using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and adjusted to ensure that each sample contained an equal amount of protein. An aliquot of each lysate was mixed with an equal volume of 2x gel loading buffer (50 mM Tris, pH 6.8, 2% SDS, 200 mM dithiothreitol, 40% glycerol, 0.2% bromophenol blue) and boiled for 4 min.

**Immunoprecipitation** - Each sample was incubated with the immunoprecipitating antibody overnight at 4 °C followed by incubation with protein A agarose beads for 1 h at 4 °C. The beads were centrifuged for 3 min at 13,000 g at 4 °C, washed three times with ice-cold Ringer’s, resuspended in 2x gel loading buffer and boiled for 4 min.

**Western blotting** - Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 1% blocking solution and an appropriate concentration of primary antibody was added in 1% blocking buffer over night. Membranes were washed with
Tris buffered saline containing 1% Tween 20 (1% TBST) for 1h, HRP-labelled secondary anti-mouse- or anti-rabbit-IgG-antibody (BD Biosciences, Santa Cruz, CA) in 1% blocking solution (1:2500) was added for 30 min and membranes were washed for 1h with 1% TBST. Finally, immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, UK). Densitometric analysis of Western blots was performed by NIH Image software.

**Electrophysiological studies**- T₈₄ cell monolayers were mounted in Ussing chambers with a window area of 0.6 cm² and bathed in oxygenated (95% O₂, 5% CO₂) Ringer’s solution at 37°C. Using short-circuit current (Iₛᶜ) the monolayers were continuously voltage-clamped to zero potential difference. A specific finding for T₈₄ cells is that changes in Iₛᶜ (ΔIₛᶜ) under these conditions are exclusively due to changes in electrogenic chloride secretion (13). Transepithelial electrical resistance (TER) across T₈₄ monolayers was assessed by voltohmeter (WPI, Sarasota, FL) and companion electrodes (Millipore, Bedford, MA). Measurements were calculated in Ω · cm².

**Small interfering RNA (siRNA) transfection**- 2x10⁶ T₈₄ cells were seeded 3 days before transfection and grown to 50-70% confluency in T75 flasks. Three different annealed Silencer pre-designed siRNA oligonucleotides targeting PTPN2 were obtained from Applied Biosystems (Foster City, CA). For transfection reactions, a total amount of 100 pmol of the three gene specific siRNA oligonucleotides were transfected into T₈₄ cells using the Amaxa nucleofector system (Amaxa Inc., Gaithersburg, MD) according to manufacturer’s instructions. After transfection, T₈₄ cells were cultured on filter membranes for 48 h before further treatment for Western blotting experiments and for 96 h for electrophysiological studies. A non-specific control siRNA SMARTpool (100 pmol, Upstate Biotechnology/Dharmacon, Chicago, IL) was used as a negative control.
**Statistical analysis**- Data are presented as means +/- S.E.M. for a series of \( n \) experiments. Data are expressed as a percentage of the respective control. Statistical analysis was performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. \( P \) values < 0.05 were considered significant.

**RESULTS**

**Knock-down of PTPN2 enhances EGF-induced EGFR tyrosine phosphorylation.**

EGFR regulates a variety of intracellular signalling pathways. We have previously validated that treatment with EGF at a concentration of 100 ng/ml for 5 min is optimal to cause EGFR tyrosine phosphorylation in \( T_{84} \) cells (24; 27; 29; 45; 47). Using human fibroblasts, it has been elucidated that PTPN2 dephosphorylates, and thereby inactivates, EGFR following EGF treatment (45). Therefore, our first goal was to investigate whether PTPN2 also regulates EGF-induced EGFR tyrosine phosphorylation in human \( T_{84} \) intestinal epithelial cells. To address this issue, we performed PTPN2 knock-down studies and analyzed EGFR tyrosine phosphorylation in EGF-treated \( T_{84} \) cells by Western blotting. \( T_{84} \) cells were transfected with either non-specific control siRNA or specific siRNA targeting PTPN2, and subsequently stimulated with EGF (100 ng/ml) for 5 min. As shown in Figure 1a, PTPN2-specific siRNA caused a dramatic reduction of PTPN2 protein expression. Densitometric analysis revealed a maximal decrease in PTPN2 protein of 93 ± 3 % (Fig. 1a). Equivalent levels of the loading control, lamin A/C, confirmed that the transfection had no non-specific effects on cellular protein expression (Fig. 1a). To study the effect of PTPN2 knock-down on EGF-induced EGFR phosphorylation, whole cell lysates were immunoprecipitated with an anti-EGFR antibody and immunoblotted for phosphotyrosine. Densitometric analysis of
Western blots revealed that EGF treatment of control siRNA transfected T84 cells increased EGFR tyrosine phosphorylation significantly, and this effect was further enhanced in PTPN2-deficient cells (p<0.001; Fig. 1b). In Fig. 1b, we assessed the level of EGF-induced EGFR phosphorylation in relation to the respective untreated control cells. Therefore, the extent of EGFr phosphorylation in untreated cells transfected with control siRNA or PTPN2 siRNA, respectively, was regarded as 100% and the extent of EGFR phosphorylation in EGF-treated cells transfected with control siRNA or PTPN2 siRNA respectively, was calculated as a percentage of each of the respective controls. We then performed a secondary analysis to demonstrate the magnitude of EGFR induction from baseline. As the baseline level, we used the extent of EGFR phosphorylation in untreated cells that were transfected with control siRNA constructs. All of the other EGFR phosphorylation values were then calculated in relation to the extent of EGFR phosphorylation in untreated cells that had been transfected with control siRNA. We found that EGFR phosphorylation in untreated PTPN2-deficient cells differed only to a very limited extent from the level of EGFR phosphorylation in untreated PTPN2-competent cells, especially when this difference was compared to the massive increase in EGFR phosphorylation in response to EGF (Fig. 1c). In contrast and as shown by densitometric analysis, PTPN2 knock-down did not significantly affect the expression of EGFR protein (Fig. 1d). EGF treatment had no effect on PTPN2, lamin A/C or EGFR protein levels (Figs. 1a, d). These data demonstrate that PTPN2 likely dephosphorylates the EGF-activated EGFR in T84 intestinal epithelial cells.

**EGFR phosphotyrosine residues Tyr-992 and Tyr-1068 are targets of PTPN2.**

Having shown that PTPN2 knock-down promotes EGF-induced EGFR tyrosine phosphorylation, we next investigated which specific EGFR tyrosine residues are targeted by the phosphatase. T84 cells were transfected either with control siRNA or PTPN2 siRNA,
treated with EGF (100 ng/ml) for 5 min and analyzed for EGFR phosphorylation patterns using phosphotyrosine-specific antibodies by Western blotting. As shown in Figure 2a, EGF treatment alone significantly increased phosphorylation of Tyr-992, and this effect was further enhanced in PTPN2-deficient cells (144 ± 16 % vs. 183 ± 9 %; p<0.05). Interestingly, loss of PTPN2 led to an increase in baseline EGFR Tyr-992 phosphorylation to a similar extent as EGF treatment of PTPN2-competent cells. However, this effect was statistically not significant (Fig. 2b). While phosphorylation of Tyr-1068 was also induced in response to EGF and further enhanced by PTPN2 knock-down (160 ± 6 % vs. 254 ± 26 %; p<0.001; Fig. 2c), baseline Tyr-1068 phosphorylation levels were not altered in PTPN2-deficient cells (Fig. 2d). In contrast, PTPN2 knock-down did not significantly affect baseline phosphorylation or EGF-induced phosphorylation of Tyr-1148 (Fig. 3a+b) or Tyr-1173 (Fig. 3c+d) in T84 cells as assessed by densitometric analysis of Western blots. Of interest, the phosphorylated form of Tyr-1173 seemed to appear as a double band. However, the lower band was detected at a molecular weight of about 130 kD (EGFR: 180 kD) and is thus unlikely to represent an EGFR doublet, although we cannot exclude that it may represent a truncated form of the EGFR. These data demonstrate that PTPN2 activity selectively modulates the pattern of EGFR tyrosine phosphorylation and may, subsequently, play a role in the regulation of downstream signalling originating at the EGFR.

**PTPN2 regulates EGF-induced PI3-K, but not ERK1/2 activation.**

Having demonstrated that PTPN2 likely dephosphorylates EGFR phospho-residues Tyr-992 and Tyr-1068, we next set out to study the functional consequences of this finding. The selective activation and recruitment of pathways downstream of EGFR depends on the tyrosine phosphorylation pattern of the receptor (32). Two important signalling pathways operating downstream of the EGFR are the PI3-K/Akt and the MAPK pathways (5; 32). Since both Tyr-992 and Tyr-1068 of EGFR have been associated with increased PI3-K and Akt...
activity (29; 45), we first investigated a role for PTPN2 in regulating the activation of PI3-K and its well-described downstream target, Akt (14; 17), in response to EGF. PI3-K consists of a regulatory p85- and a catalytic p110-subunit. To become active, the p85-subunit must bind to specific phosphotyrosine sites of a receptor tyrosine kinase, such as EGFR. This binding allows the recruitment and activation of the p110-subunit (12; 22; 53; 54). Therefore, activation of PI3-K in response to EGF can be assessed as the amount of the p110-subunit in immunoprecipitates of tyrosine-phosphorylated proteins. In this case, we examined levels of EGF-induced p110 association with EGFR by stripping the same membranes that we had previously used for EGFR analysis (c.f. Figs. 1b+d), and re-probed them for p110. As expected, EGF treatment (100 ng/ml, 5 min) increased p110 association with the EGFR in control siRNA transfected cells (Fig. 4a). This effect was significantly enhanced in PTPN2-deficient cells (p<0.001; Fig. 4a). Correspondingly, phosphorylation of Akt, as measured in whole cell lysates, was also elevated in EGF-treated control cells and even further promoted in PTPN2 siRNA-transfected cells (p<0.05; Fig. 4b). These findings indicate that PTPN2, likely through effects on EGFR residues Tyr-992 and Tyr-1068, regulates EGF-induced activation of PI3-K.

EGF activation of the ERK-isoforms (ERK1/2) of the MAPK family is well described (38). To test whether PTPN2 might also be involved in the regulation of EGF-induced activation of MAPK signalling, we examined whether EGF-induced phosphorylation of ERK1/2 was altered in PTPN2 siRNA-transfected T84 cells. Although ERK1/2 phosphorylation was strongly induced in EGF-treated control cells this effect was not altered by PTPN2 knock-down (Fig. 5). These data indicate that PTPN2 is involved in regulating the link of EGFR to the PI3-K/Akt pathway, but not ERK, in response to the EGF.

*Loss of PTPN2 enhances EGF inhibition of Ca^{2+}-dependent chloride secretion.*
Having shown that EGF-induced activation of PI3-K is enhanced in cells that lack PTPN2, we next tried to identify a functional consequence for this observation. Our laboratory has previously demonstrated that PI3-K plays an essential role in mediating EGF-induced inhibition of Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion in intestinal epithelial cells (47; 48). Therefore, we speculated that an increase in PI3-K activity in PTPN2-deficient cells should promote the inhibitory effect of EGF on Cl\(^{-}\) secretion. We performed Ussing chamber studies using control siRNA and PTPN2 siRNA-transfected T\(_{84}\) cells. PTPN2 specific siRNA reduced the PTPN2 protein levels under the cell growth conditions used for Ussing chamber studies clearly. After pretreatment with EGF (100 ng/ml) for 20 min, we assessed the chloride secretory response to carbachol (100µM, basolaterally) (24; 48). As expected, EGF significantly inhibited CCh-stimulated chloride secretion across T\(_{84}\) monolayers transfected with non-specific control siRNA (2.6 ± 0.6 vs. 4.9 ± 1.1 µA/cm\(^2\); Fig. 6a). Corresponding to the elevated PI3-K activity in EGF-treated PTPN2-deficient cells (c.f. Fig. 4a), the inhibitory effect of EGF on Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion was further enhanced in PTPN2-deficient cells (2.0 ± 0.5 vs. 6.2 ± 1.6 µA/cm\(^2\); Fig. 6a). The admittedly rather small I\(_{sc}\) responses could be due to the fact that cells were grown for only 4 days after transfection in order to maintain a PTPN2-deficient phenotype. Therefore, the T\(_{84}\) cells were likely not able to develop as tight a monolayer as “conventionally” grown T\(_{84}\) cells that are usually seeded for 10-14 days before conducting electrophysiologic experiments. This was confirmed by measuring the conductance of these T\(_{84}\) monolayers (Fig. 6b). As expected, the mean conductance values of the transfected T\(_{84}\) monolayers was about 8-fold higher than those across “conventionally” grown monolayers. Since the magnitude of the vectorial ion transport response is related to the tightness of the monolayer, the rather low I\(_{sc}\) response observed in our cells is likely due to the high conductance across the monolayers. Nevertheless, since these cells are obviously able to generate vectorial ion transport responses, albeit with lower magnitudes, our data represent valid physiological findings demonstrating that PTPN2 knock-down promotes EGF-
induced inhibition of CCh-stimulated chloride secretion in intestinal epithelial cells. In subsequent studies, we demonstrated that the PI3-K inhibitor, LY294002, significantly reduced the enhanced inhibitory effect of EGF on CCh-stimulated $I_{sc}$ in PTPN2-deficient cells ($p<0.01; n=5$; Fig. 6c), and this effect was more pronounced in PTPN2-deficient cells than control siRNA transfected cells. This finding supports our biochemical data indicating elevated recruitment of PI3-K signalling following EGF treatment of PTPN2-deficient cells (c.f. Fig. 4). Moreover, we observed more robust ion transport responses to carbachol (ranging from 12-35 $\mu$A/cm$^2$ depending on the treatment conditions) than in the experiment shown in Figure 6a. Although the experiments (Figure 6a vs. 6c) showed quantitative differences in ion transport responsiveness, they were qualitatively similar with respect to the inhibitory effect of EGF on CCh-stimulated $I_{sc}$.

Maintenance of an effective epithelial barrier is also a pre-requisite for regulation of epithelial ion transport. Conflicting roles have been reported for EGFR in the regulation of epithelial barrier function (3; 39). Since we have previously determined that the proinflammatory cytokine, IFN$\gamma$, -induces phosphorylation of the EGFR and suppression of CCh-stimulated Cl$^-\$secretion by a mechanism that is insensitive to AG1478, and thus occurs independently of intrinsic EGFR kinase activity (49), we next wished to identify whether EGFR might be involved in the transduction of IFN$\gamma$-induced effects on epithelial monolayer TER. Although an important pathophysiological feature of IFN$\gamma$ is its capacity to induce a deleterious effect on intestinal epithelial barrier function, the exact signalling mechanisms that mediate the IFN$\gamma$-induced effects on barrier functions are still unclear. Therefore, we treated $T_{84}$ monolayers for 72 h with either IFN$\gamma$ (1000 U/ml), the pharmacologic EGFR inhibitor AG1478 (10 $\mu$M), or both in combination. As shown in Fig. 6d, IFN$\gamma$ treatment significantly decreased the TER across $T_{84}$ monolayers, while EGFR inhibition alone had no effect on TER ($p<0.001; n=4$). Interestingly, co-incubation with AG1478 was not sufficient to prevent the
IFNγ-induced decrease in TER across T84 monolayers. These data demonstrate that increased EGFR activation is likely not involved in the IFNγ-induced barrier defect in intestinal epithelial cells.

**PTPN2 does not modulate IFNγ-induced EGFR transactivation.**

In addition to direct activation by exogenous EGF, EGFR can be transactivated by a number of other ligand-receptor systems, including following activation of the IFNγ receptor by IFNγ (11; 49). We have recently shown that PTPN2 plays a major role in regulating epithelial barrier responses to IFNγ (36). We therefore wished to investigate if PTPN2 modified EGFR transactivation and downstream signalling induced by IFNγ. IFNγ treatment for 24 h exerts an inhibitory effect on Ca2+-dependent Cl− secretion in response to CCh in IECs (9; 49) and increases phosphorylation of ERK1/2 via an EGFR-dependent pathway (11). To test whether PTPN2 regulates EGFR activation in response to IFNγ, we performed PTPN2 knock-down studies and then treated T84 cells with IFNγ for 24 h. As demonstrated previously, PTPN2 siRNA caused a clear reduction of PTPN2 protein in these experiments (36). Using a previously validated concentration of IFNγ (1000 U/ml) (49), IFNγ increased phosphorylation of EGFR in cells transfected with control siRNA (Fig. 7a). However, phospho-EGFR levels of IFNγ-treated PTPN2-deficient cells did not differ significantly from the respective controls or from the level of IFNγ-induced EGFR phosphorylation relative to total EGFR in PTPN2-competent cells (Fig. 7a). In accordance with our previous results (Fig. 1c), baseline EGFR phosphorylation levels did not significantly differ under these conditions (Fig. 7b). Additionally, in an as yet unpublished manuscript, we demonstrate that IFNγ treatment increases EGFR mRNA and protein in T84 monolayers (G. Paul, unpublished observations). These findings are in line with previous data in other epithelial cell systems (6; 21). As expected, and shown in Fig. 7c, IFNγ treatment increased EGFR protein in
immunoprecipitates from cells transfected with control siRNA (p<0.05; n=3). Interestingly, PTPN2 knock-down significantly diminished the cytokine-induced rise in EGFR protein (Fig. 7c; p<0.05; n=3). Additionally, IFNγ did not increase association of EGFR with the p110 subunit of PI3-K in either control siRNA-transfected or in PTPN2 siRNA-transfected cells (Fig. 7d). Similarly, loss of PTPN2 had no effect on IFNγ-induced activation of ERK1/2 by 24 h treatment (Fig. 7e). These data demonstrate that PTPN2 regulates EGFR expression, but not EGFR activation or signalling in response to IFNγ.

DISCUSSION

In this study, we demonstrate that the CD candidate gene, PTPN2, regulates EGFR-mediated downstream signalling in response to EGF. Loss of PTPN2 directs EGF-induced EGFR activity towards the PI3-K pathway and thereby potentiates EGF-induced inhibition of intestinal epithelial Ca2+-dependent Cl⁻ secretion.

Furthermore, PTPN2 knock-down enhanced EGF-induced EGFR tyrosine phosphorylation in IECs. Specifically, PTPN2 knock-down increased the EGF-induced phosphorylation of Tyr-992 and Tyr-1068, and, in agreement with our earlier findings and those in other systems, phosphorylation of these residues likely contributes to increased PI3-K activity and Akt phosphorylation (29; 45). Interestingly, we found that PTPN2 knock-down alone caused a noticeable, but non-significant increase in phosphorylation of Tyr-992, whereas neither the phosphorylation status of the other studied EGFR tyrosine residues, namely Tyr-1068, Tyr-1148 and Tyr-1173, nor the overall phosphorylation status of the EGFR were markedly affected in PTPN2-deficient cells. Therefore, the elevated basal
phosphorylation level of Tyr-992 is likely to be of negligible physiological relevance. We also saw no elevation in phosphorylation of Tyr-1148 or Tyr-1173, or in phosphorylation of ERK1/2 in EGF-treated PTPN2-deficient cells. This indicates that these latter two EGFR tyrosine residues are likely not involved in PTPN2 regulation of the PI3-K/Akt pathway in this system. However, this does not exclude their possible involvement in EGF activation of the MAPK-ERK pathway in T\textsubscript{84} cells. These findings are in accord with our previous work (29), and provide further insight into the mechanisms of EGFR-mediated signal transduction in intestinal epithelial cells. In particular, we have shown that PTPN2 is a key regulator of EGF-induced recruitment of signalling pathways of the EGFR that mediate regulation of ion transport in intestinal epithelial cells.

EGFR plays an important role in the regulation of the intestinal epithelial chloride secretion (8; 23; 24; 28; 47) and inhibits calcium-dependent chloride secretion via two distinct mechanisms. The muscarinic agonist, carbachol, stimulates chloride secretion utilizing intracellular calcium as second messenger. However, carbachol also exerts an inhibitory effect on intestinal chloride secretion via transactivation of the EGFR and the subsequent activation of ERK1/2 (24). On the other hand, direct activation of EGFR by EGF and subsequent recruitment of PI3-K signalling constitutes the second mechanism whereby EGFR inhibits Cl\textsuperscript{-} secretion (48). In this study, we show that knock-down of PTPN2 results in increased activity of the EGFR and subsequently PI3-K in response to EGF treatment. Likewise PTPN2 knock-down potentiates the inhibitory effect of EGF on carbachol-induced chloride secretion in T\textsubscript{84} cells via increased recruitment of PI3-K signaling. These findings not only correlate with previous studies showing a role for the EGFR and PI3-K in the inhibition of intestinal chloride secretion (48), they also imply that PTPN2 critically limits the ability of EGF to regulate ion transport in intestinal epithelial cells. The identification of this functional role for PTPN2 in the regulation of EGFR function in IECs may be important in the setting of chronic
inflammation. This would extend not only to ion transport, but also, perhaps, the involvement of EGFR in epithelial restitution, wound repair and barrier function, all of which are dysregulated in IBD (20; 31; 37; 40; 55). This possibility is of even greater significance following the identification of PTPN2 as an IBD candidate gene (17; 43). While the secretion of ions and water into the intestinal lumen helps to preserve epithelial integrity by flushing the crypts and subsequently removing cell detritus and luminal pathogens, hyposecretory conditions in the intestine promote and sustain intestinal inflammation (1; 2). Therefore, any PTPN2 mutation that results in a loss of protein function could result in increased or sustained EGFR phosphorylation with many consequences for epithelial function. For example, in addition to well-described changes in epithelial transport protein expression due to inflammation (20; 41), prolonged EGFR activation may contribute to a hyposecretory state by further suppressing the function of existing ion transporters, thus exacerbating overall dysregulation of fluid homeostasis in the gut.

The proinflammatory cytokine IFNγ inhibits chloride secretion (9; 41) and also activates EGFR in IECs. However, the inhibitory effect on Cl− secretion could not be reversed by inhibition of EGFR kinase activity (49). Interestingly, and in contrast to EGF-induced EGFR phosphorylation, PTPN2 knock-down did not affect IFNγ-induced phosphorylation of EGFR in our studies. Since IFNγ did not increase p110 association with EGFR, and this was also unaltered in PTPN2-deficient cells, we conclude that the PI3-K/Akt pathway is likely not activated by IFNγ at the time points tested, which is in agreement with observations by other groups (10; 30). In addition, although IFNγ treatment induced phosphorylation of ERK1/2, this was not altered by PTPN2 knock-down. These data suggest that PTPN2 regulates only EGFR-mediated PI3-K, but not ERK1/2 signalling and is likely not recruited to the EGFR in response to IFNγ. However, PTPN2 does appear to regulate the expression of EGFR in
response to the cytokine. Intriguingly, PTPN2 knockdown had the opposite to anticipated effect in that it reduced rather than enhanced the effect of IFNγ on EGFR expression. Although the molecular mechanisms responsible for this have still to be determined, this finding could possibly be of significance for EGFR signalling outcomes in the joint setting of reduced PTPN2 expression or activity, and active inflammation. These questions are being addressed in ongoing investigations by our group. In agreement with our previous work showing EGFR kinase-independent effects of IFNγ on EGFR activation and inhibition of Cl− secretion, we also found no involvement of EGFR kinase activity in the IFNγ-induced decrease of intestinal epithelial barrier function. These data support our hypothesis that PTPN2 plays a more involved role in regulating EGFR signalling, and consequent outcomes, in response to EGF activation rather than IFNγ induced EGFR activation.

In summary, our data establish a role for PTPN2 in the regulation of EGFR signalling in intestinal epithelial cells in response to EGF but not IFNγ. Loss of PTPN2 promotes PI3-K activation and potentiates the suppression of epithelial chloride secretory responses. In addition to changes in ion transporter expression caused by inflammation, our findings conform with hyposecretory states seen in chronic intestinal inflammation. Moreover, given the genetic association of PTPN2 with IBD, it will be important to determine whether PTPN2 plays a greater, or lesser, role in regulating EGF-stimulated EGFR signalling in the setting of inflammation. Thus, our findings highlight the role of PTPN2 in regulating EGFR signalling events in intestinal epithelial cells and suggest that PTPN2 dysfunction could contribute to ion transport suppression in IBD.


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FOOTNOTES

The authors declare that they do not have any competing financial interests.

The abbreviations used are: CCh, carbachol; EGFR, epidermal growth factor (EGF) receptor; ERK1/2, extracellular signal-regulated kinase 1/2; IFNγ, interferon gamma; PI3-K, phosphatidylinositol 3’-kinase; PTPN2, protein tyrosine phosphatase N2.
FIGURE LEGENDS

FIGURE 1. PTPN2-specific siRNA decreases PTPN2 protein expression and promotes EGF-induced EGFR tyrosine phosphorylation. T84 cells were transfected with either non-specific siRNA or PTPN2-specific siRNA and subsequently treated with EGF (100 ng/ml) for 5 min. (a) Representative Western blots and their densitometric analysis below show protein levels of PTPN2 and the nuclear envelope protein lamin A/C, used as a loading control, in whole cell lysates (n=3). (b-d) Whole cell lysates were immunoprecipitated with an anti-EGFR antibody, blotted for phosphotyrosine, stripped and re-blotted for EGFR. (b) Representative Western blot shows tyrosine phosphorylated EGFR. The histogram below demonstrates densitometric analysis of three similar experiments. Data are presented as percentage of the respective controls (EGF treated control siRNA cells as percentage of untreated control siRNA cells and EGF-treated PTPN2 siRNA cells as a percentage of untreated PTPN2 siRNA cells). (c) We performed a secondary densitometric analysis to assess the effect of PTPN2 siRNA on EGFr phosphorylation. This analysis demonstrates the magnitude of EGFR phospho-tyrosine induction above that present in untreated cells that were transfected with control siRNA constructs, described in the y-axis legend as “baseline”. All of the other EGFR phosphorylation values in Fig. 1c were then calculated in relation to the extent of EGFR phosphorylation in these cells. (d) Representative Western blot and the respective densitometric analysis shows EGFR protein level (n=3). Data are presented as a percentage of the respective controls. Asterisks indicate significant difference vs. the respective control, * = p<0.05, *** = p<0.001. ### indicates p<0.001 vs. EGF treated T84 cells transfected with control siRNA.
FIGURE 2. Loss of PTPN2 enhances phosphorylation of the EGFR tyrosine residue Tyr-992 and Tyr-1068 in response to EGF. Either control siRNA or PTPN2 siRNA transfected T84 cells were treated with EGF (100 ng/ml) for 5 min. Analyses were performed using whole cell lysates. (a) Representative Western blots show phosphorylation of the EGFR residue Tyr-992 and expression of total EGFR. Below is the densitometric analysis of three similar experiments. Data are presented as percentage of the respective controls. (b) Secondary densitometric analysis demonstrates the magnitude of Tyr-992 induction from baseline level (analysis was performed as described in Fig. 1c) in percentage of untreated cells transfected with control siRNA. (c) Phosphorylation of the EGFR residue Tyr-1068 in response to EGF and expression of total EGFR is demonstrated by representative Western blots. The densitometric analysis of five similar experiments is shown in the histogram below. Data are presented as percentage of the respective controls (d) Secondary densitometric analysis demonstrates the magnitude of Tyr-1068 induction from baseline level (analysis was performed as described in Fig. 1c) in percentage of untreated cells transfected with control siRNA. Asterisks indicate significant difference vs. the respective control, * = p<0.05, *** = p<0.001. # indicates p<0.05, ### indicates p<0.001 vs. EGF treatment of T84 cells transfected with control siRNA.

FIGURE 3. Phosphorylation of the EGFR tyrosine residue Tyr-1148 and Tyr-1173 in response to EGF is not affected by PTPN2 knock-down. Either control siRNA or PTPN2 siRNA transfected T84 cells were treated with EGF (100 ng/ml) for 5 min. Analyses were performed using whole cell lysates. (a) Representative Western blots show phosphorylation of the EGFR residue Tyr-1148 and expression of total EGFR. Below is the densitometric analysis of three similar experiments. Data are presented as percentage of the respective
controls. (b) Secondary densitometric analysis demonstrates the magnitude of Tyr-1148 induction from baseline level (analysis was performed as described in Fig. 1c) in percentage of untreated cells transfected with control siRNA. (c) Phosphorylation of the EGFR residue Tyr-1173 in response to EGF and expression of total EGFR is demonstrated by representative Western blots. The densitometric analysis of four similar experiments is shown by the histogram below. Data are presented as percentage of the respective controls (d) Secondary densitometric analysis demonstrates the magnitude of Tyr-1173 induction from baseline level (analysis was performed as described in Fig. 1c) in percentage of untreated cells transfected with control siRNA. Asterisks indicate significant difference vs. the respective control, * = p<0.05, ** = p<0.01.

FIGURE 4. PTPN2-deficient T84 cells show enhanced PI3-K activity and phosphorylation of Akt in response to EGF. T84 cells transfected with either control siRNA or PTPN2 siRNA were treated with EGF (100 ng/ml) for 5 min. (a) Whole cell lysates were immunoprecipitated with an anti-EGFR antibody. Membranes as of (1b, d) were stripped and re-probed for p110. Representative Western blot and densitometric analysis show association of the p110 subunit of PI3-K to the EGFR in response to EGF (n=3). (b) Representative Western blots show phosphorylation and expression of the PI3-K downstream marker, Akt, in response to EGF in whole cell lysates. The densitometric analysis of seven similar experiments is shown by the histogram below. Data are presented as percentage of control. Asterisks indicate significant difference vs. the respective control, * = p<0.05, ** = p<0.01, *** = p<0.001. # indicates p<0.05, #### indicates p<0.001 vs. EGF treatment of T84 cells transfected with control siRNA.
FIGURE 5. PTPN2 knock-down does not affect EGF-induced phosphorylation of ERK1/2. T84 cells transfected with either control siRNA or PTPN2 siRNA were treated with EGF (100 ng/ml) for 5 min. Analyses were performed using whole cell lysates. Representative Western blots show phosphorylation and expression of ERK1/2. The densitometric analysis of three similar experiments is shown by the histogram below. Data are shown as a percentage of control. Asterisks indicate significant difference vs. the respective control, ** = p<0.01.

FIGURE 6. PTPN2 knock-down potentiates inhibition of calcium-dependent chloride secretion across T84 monolayers via PI3-K. T84 cells transfected with either control siRNA or PTPN2 siRNA and grown on permeable supports for 4 days, were pretreated with EGF (100 ng/ml, basolaterally) for 20 min and subsequently stimulated with carbachol (100 µM, basolaterally) and/or the PI3-K inhibitor, LY294002 (20 µM, bilaterally). (a) Change in Isc (ΔIsc) in response to carbachol. Data are shown as a percentage of the respective control and represent eight similar experiments. (b) Conductance data for the monolayers used in (a) are expressed in mS/cm². (c) Data are shown as the % inhibitory effect of EGF on the Isc response to carbachol with or without pre-treatment with the PI3-K inhibitor, LY294002 (20 µM, bilaterally). Five similar experiments were performed. (d) TER across T84 monolayers after treatment with IFNγ (100 ng/ml, basolaterally, 72 h), AG1478 (10 µM, bilaterally, 72 h) or a combination of both stimuli is demonstrated in Ohm/cm². Histogram represents data of four similar experiments. Asterisks indicate significant difference vs. the respective control, * = p<0.05; ** = p<0.01. # indicates p<0.05; ## = p<0.01 vs. EGF treatment of T84 cells transfected with control siRNA in (a) or PTPN2/control siRNA in (c).
FIGURE 7. IFNγ-induced phosphorylation of the EGFR and consequent downstream signalling is not affected by PTPN2 knock-down. T₈₄ cells transfected with either control siRNA or PTPN2 siRNA were treated with IFNγ (1000 U/ml, bilaterally) for 24 h. Whole cell lysates were immunoprecipitated with an anti-EGFR antibody, blotted for (a) phosphotyrosine, stripped and re-blotted for (b) EGFR and (c) p110, respectively. (a) Representative Western blot and densitometric analysis represent EGFR tyrosine phosphorylation. Data are expressed as a percentage of the respective controls (n=3). (b) Secondary densitometric analysis demonstrates the magnitude of induction of EGFR tyrosine phosphorylation from baseline level (analysis was performed as described in Fig. 1c) in percentage of untreated cells transfected with control siRNA. (c) Representative Western blot and densitometric analysis show EGFR protein level. Data are expressed as a percentage of the respective controls (n=3). (d) Representative Western blot and densitometric analysis show p110 protein level. (e) Representative Western blots of three similar experiments demonstrate the phosphorylation of ERK1/2 and the expression of total ERK1/2 in response to IFNγ. Asterisks indicate significant difference vs. the respective control, * = p<0.05. # indicates p<0.05 vs. IFNγ treatment of T₈₄ cells transfected with control siRNA.
Figure a: Tyro-992
Figure b: EGFR phosphorylation in percentage of respective controls normalized to total EGFR
Figure c: Tyro-1068
Figure d: EGFR phosphorylation in percentage of baseline level normalized to total EGFR
a: IP: anti-EGFr
   WB: anti-p110

b: pAkt
   75kd ---
   60kd ---
   Total Akt
   60kd ---

P110 association to the EGF
Normalized to total EGF

Basal
Control siRNA
Control EGF
PTPN2 siRNA

Akt phosphorylation
Normalized to total Akt

Control EGF
Control EGF
Control EGF
PTPN2 siRNA