Published in final edited form as:

Gastroenterology. 2009 December ; 137(6): 2030–2040.e5. doi:10.1053/j.gastro.2009.07.078.

Protection of epithelial barrier function by the Crohn's disease associated gene, protein tyrosine phosphatase N2

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Abstract

Background & Aims—Protein tyrosine phosphatase N2 (PTPN2) has been identified as a Crohn's disease (CD) candidate gene. However, a role for PTPN2 in the pathogenesis of CD has not been identified. Increased permeability of the intestinal epithelium is believed to contribute prominently to CD. The aim of this study was to determine a possible role for PTPN2 in CD pathogenesis.

Methods—Intestinal epithelial cell (IEC) lines, T₈₄ and HT29cl.19a, were used in all studies. Protein analysis was performed by Western blotting and protein knock-down was induced by siRNA. Primary samples were from control and CD patients.

Results—Here, we demonstrate increased PTPN2 expression in CD intestinal biopsies and that the pro-inflammatory cytokine, IFN γ , increases PTPN2 expression and activity in IEC. Moreover, IFN γ -induced STAT1 and STAT3 phosphorylation in IEC is enhanced by PTPN2 knock-down. The cellular energy sensor, AMPK, partially regulates the IFN γ -induced effects on PTPN2. Additionally, PTPN2 knock-down potentiates IFN γ -induced increases in epithelial permeability, accompanied by elevated expression of the pore-forming protein, claudin-2.

Conclusions—PTPN2 is activated by IFN γ and limits IFN γ -induced signalling and consequent barrier defects. These data suggest a functional role for PTPN2 in maintaining the intestinal epithelial barrier and in the pathophysiology of CD.

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Author contributions: MS (acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis; obtained funding); GP (acquisition of data; statistical analysis); AW (acquisition of data; analysis and interpretation of data); MH (technical and material support; acquisition of data); BJ, MJD, GR (material support); KEB (critical revision of the manuscript for important intellectual content; obtained funding); DFM (study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; obtained funding; study supervision).

The authors declare that they have no competing financial interests.

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Introduction

The family of protein tyrosine phosphatases (PTPs) plays a critical role in regulating fundamental cellular signalling events, including cell proliferation, differentiation and survival¹. A single nucleotide polymorphism (SNP) in the gene locus encoding one such phosphatase, protein tyrosine phosphatase N2 (PTPN2), has been associated with Crohn's Disease (CD), Type I diabetes, and ulcerative colitis (UC)²⁻⁶. However, a functional role for PTPN2 in CD pathogenesis remains unidentified. PTPN2, also referred to as T-cell protein tyrosine phosphatase (TC-PTP), exists in two different splice forms, a nuclear 45kD and a cytoplasmic 48kD variant bound to the endoplasmic reticulum. Upon activation, the nuclear variant, which seems more important for PTPN2 catalytic activity⁷⁻¹¹, can exit the nucleus¹². In fibroblasts, this is dependent upon activation of the cellular energy sensor, adenosine monophosphate-activated protein kinase (AMPK)⁸. Among the PTPN2 substrates are receptors for epidermal growth factor⁹, 10, and insulin¹³ and the Signal transducers and activators of transcription (STAT) 1+3⁷, 11, 14.

Inflammatory bowel disease (IBD) is comprised of CD and UC. Genetic, immunological and bacterial factors are involved in IBD pathogenesis. Evidence suggests that an epithelial barrier defect, coupled with a dysfunctional immune response to commensal flora, drives the development of chronic intestinal inflammation¹⁵. Both IBD subgroups are characterized by their cytokine profiles, with elevated levels of interferon gamma (IFN γ) predominating in CD¹⁶. The primary mediators of IFN γ -induced signalling are the STATs¹⁷, with upregulation of both STAT1+3 observed in IBD^{18,19}. In addition to a possible role in the onset of CD, increased epithelial barrier permeability may also contribute to the major clinical symptom of CD, diarrhea, due to "leak-flux" of electrolytes and water into the lumen. In cell culture studies, IFN γ reduces intestinal epithelial barrier function. Recent publications demonstrated upregulation of the pore forming claudin-2 in colonic crypt epithelial cells in CD^{20, 21}. Moreover, increased claudin-2 levels correlate with increased permeability^{20, 22-24}.

We found that PTPN2 is upregulated both in IFN γ treated IECs and in CD patient biopsies, and this effect in IECs is partially AMPK-dependent. Moreover, PTPN2 knock-down facilitates elevated claudin-2 expression, correlating with a further increase in IFN γ -induced epithelial permeability. These data establish PTPN2 as a protective factor in a cell model of intestinal inflammation and implicate PTPN2 dysfunction in CD pathogenesis.

Methods

Materials

Human recombinant IFN γ (Roche, Mannheim, Germany), Compound C (CC), AICA-Riboside- 5'-phosphate (AICAR), and monoclonal mouse anti-PTPN2 antibody CF-4, which detects the 45kD and the 48kD isoforms (Calbiochem San Diego, CA), rabbit anti-lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-claudin-2, anti-claudin-4, anti-ZO-1 and rabbit anti-occludin antibodies (Zymed Laboratories, Carlsbad, CA) were obtained from the sources noted. Rabbit anti-phospho-AMPK α (Thr¹⁷²), anti-AMPK α , anti-phospho-STAT1 (Tyr⁷⁰¹), anti-STAT1, anti-phospho-STAT3 (Tyr⁷⁰⁵) and anti-STAT3 antibodies were obtained from Cell Signaling Technologies (Danvers, MA). All other reagents were of analytical grade and acquired commercially.

Cell culture

Human colonic crypt T₈₄ IEC were cultured as described previously²⁵. Human HT29cl.19a IEC were cultured in McCoy's 5A Medium (JRH, Lenexa, Kansas) supplemented with 10%

fetal calf serum. IFN γ was added basolaterally. The AMPK inhibitor, CC (50 μ M), and the AMPK activator, AICAR (1 mM), were added bilaterally.

Patient samples

Tissue specimens were prospectively collected from the terminal ileum, colon or rectum of male and female individuals with active CD (n=9), CD in remission (n=7), or from control subjects (n=9). Experimental details are in the Supplementary Methods while patient characteristics are presented in Supplementary Table 1. Written informed consent was obtained before specimen collection and studies were approved by the Cantonal Ethics Committee of the Canton of Zurich.

RNA isolation and real-time PCR

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and RNA expression was analyzed by real-time PCR. Experimental procedures, real-time PCR conditions and primer sequences (Integrated DNA Technologies, Coralville, IA) are described in the Supplementary Methods.

Preparation of whole cell lysates

T₈₄ and HT29cl.19a lysates were prepared as described previously²⁵. The protein content was adjusted to ensure the same amount of total protein in each sample.

Preparation of cytoplasmic and nuclear lysates

Separate cytoplasmic and nuclear lysates were obtained using a Nuclear Extraction Kit (Imgenex, San Diego, CA). Briefly, T₈₄ cells were scraped from filters and cytoplasmic proteins were collected using a 1x hypotonic buffer and incubated with 10% detergent solution. The nuclear fraction was collected using a Nuclear Lysis Buffer. Protein content was determined and adjusted as described above.

Western blotting

Western analysis of cell lysates was performed as previously²⁵.

Confocal microscopy

5 $\times$ 10⁵ T₈₄ cells were seeded onto 12-mm Millicell-HA filters for 6 days before stimulation. Experimental conditions and staining protocols are described in detail in the Supplementary Methods.

Phosphatase activity assay

Phosphatase activity was assessed using the EnzChek Phosphatase Assay Kit (Molecular Probes) according to manufacturer's instructions and as described in Mattila *et al.*¹⁰. Further details are found in the Supplementary Methods.

Small interfering RNA (siRNA) transfection

T₈₄ (2 $\times$ 10⁶) or HT29cl.19a (1 $\times$ 10⁶) cells were seeded 3 days before transfection. For PTPN2 and AMPK α 1 (PRKAA1) genes, 100 pmol of three different annealed Silencer Pre-designed siRNA oligonucleotides (Applied Biosystems; Supplementary Methods) were transfected into T₈₄ or HT29cl.19a cells using the Amaxa nucleofector system (Amaxa, Gaithersburg, MD). After transfection, IEC were cultured on filter membranes for 48 h before treatment. Control siRNA SMARTpool (Upstate Biotechnology/Dharmacon, Chicago, IL) (100 pmol/transfection) was used as negative control.

Transepithelial permeability studies

Transepithelial permeability was measured as 10 kD fluorescein isothiocyanate-dextran (FITC-Dextran) (Sigma St. Louis, MO) flux across IEC monolayers. Following IFN γ treatment, cells were washed (x3) and incubated for 30 min at 37°C with Ringer's to equilibrate. FITC-Dextran (1 mg/ml) was added to the apical compartment of the monolayer. 1 h later, 100 μ l of the basolateral solution was removed and fluorescence was detected using a microplate reader (Molecular Devices).

Transepithelial electrical resistance (TER)

TER across T₈₄ monolayers was assessed by voltometer (WPI, Sarasota, FL) and companion electrodes (Millipore, Bedford, MA). Measurements were calculated in $\Omega \cdot \text{cm}^2$ and expressed as % control.

Statistical analysis

Data are presented as means $\pm$ S.E.M. for a series of n experiments. Data are expressed as a percentage of the respective controls, arbitrary units (Figure 1d, Supplementary Figure 3) or fluorescence units (Figure 2A). Statistical analysis was performed by Analysis of Variance (ANOVA) and Student-Newman-Keuls post-test or Student's t-test, where appropriate, using Graph Pad InStat 3 software (Graph Pad Software, La Jolla, CA). P values < 0.05 were considered significant.

Results

IFN γ increases PTPN2 expression in IEC

To investigate if IFN γ affects epithelial PTPN2 expression, T₈₄ cells were treated with IFN γ (1000 U/ml) for 24, 48 and 72 h. IFN γ significantly increased PTPN2 mRNA expression (Figure 1a). Western blotting revealed consistently increased levels of cytoplasmic PTPN2 protein (Figure 1b). However, in the nuclear compartment, a significant increase was only observed in 24 h IFN γ -treated cells, with diminishing effects thereafter (Figure 1c). Dose-response studies revealed that 1000 U/ml IFN γ caused a maximal increase in PTPN2 protein by 24 h IFN γ and PTPN2 was not further increased by higher concentrations (Supplementary Figure 1). Previously²⁶, cultured biopsies from patients with active CD have been shown to release IFN γ at levels as high as approximately 1000 U/ml/mg tissue, although the mean level was 75 $\pm$ 215 U/ml/mg tissue. Serum levels of IFN γ in these studies were much lower due to dilution of the high local level of IFN γ originating in the intestinal mucosa. Although much of our data was generated using 1000 U/ml of IFN γ , we do show in Supplementary Figure 1 approximately similar effects of 100 vs. 1000 U/ml of IFN γ on PTPN2 expression in IEC. Therefore, while 1000 U/ml is at the high end of the pathologic range, the experimental use of this concentration is not unreasonable. IFN γ also elevated PTPN2 protein in HT29cl.19a IEC (Supplementary Figure 2a).

To demonstrate the relevance of our studies to human disease, we investigated PTPN2 mRNA levels in human biopsies from macroscopically non-inflamed areas of the terminal ileum, colon or rectum of CD patients in clinical and macroscopic remission, macroscopically inflamed areas of the terminal ileum and colon of CD patients with clinically and macroscopically active disease or healthy control subjects undergoing routine colon cancer screening. We found that PTPN2 mRNA was significantly elevated in active CD patients ($p < 0.05$), but not in CD patients in remission (Figure 1d). Correlating with increased PTPN2 mRNA levels, CD patients with active disease also showed an approximately three-fold increase in IFN γ mRNA levels compared to healthy controls (Supplementary Figure 3). However, the increase did not reach statistical significance due to the marked variability in IFN γ levels among individual samples. In contrast, IFN γ mRNA

levels in CD patients in remission were not different from control subjects (Supplementary Figure 3). Microscopic assessment of H&E-stained biopsy specimens did not show signs of inflammation in either control subjects or patients with CD in remission, while signs of acute inflammation were visible in patients with active CD (Supplementary Figure 4). Overall, our findings of apparently altered PTPN2 expression in active CD are of special interest, since they demonstrate the importance of our study to “*bona fide*” human disease.

PTPN2 activity is elevated by IFN γ

We investigated whether IFN γ alters PTPN2 enzymatic activity in T₈₄ cells and found that IFN γ increases PTPN2 activity maximally within 72 h treatment in PTPN2 immunoprecipitates from whole cell lysates (Figure 2a). Next, we studied the activation status of the PTPN2 target proteins, STAT1 and STAT3 by measuring phosphorylation on tyrosine⁷⁰¹ and tyrosine⁷⁰⁵, respectively. Our hypothesis was that increased PTPN2 activity should correlate with decreased phosphorylation of STAT1+3. Western blot analysis revealed that IFN γ treatment for 24 h not only enhanced cytoplasmic and nuclear STAT1 phosphorylation (Figure 2b,c), but also increased STAT1 expression (Figure 2b,c). In keeping with our hypothesis, however, STAT1 phosphorylation subsequently declined in both compartments in parallel with increased PTPN2 activity, albeit with different kinetics. Nuclear STAT1 phosphorylation was no longer significantly increased at 48 h IFN γ treatment and declined to control levels by 72 h. This suggests that PTPN2 activity was capable of significantly dephosphorylating STAT1 in the nuclear compartment as early as 24 to 48 h after IFN γ treatment ($p < 0.001$). In contrast, cytoplasmic STAT1 phosphorylation, although reduced, was still significantly increased even at 48 h treatment ($p < 0.05$). Since prior observations in fibroblasts point to the 48kD variant of PTPN2 being less active than the nuclear variant, we speculate that this difference in the catalytic activity between the PTPN2 isoforms might also be present in T₈₄ IEC. Therefore, a possible explanation for the observation that cytosolic STAT1 phosphorylation already declines after 48 h IFN γ treatment might be that a small, but sufficient, amount of the nuclear PTPN2 variant is already in the cytosolic compartment and/or has already exited the nucleus. Additionally, since STAT1 is a transcription factor, it translocates after its activation from the cytoplasm into the nucleus, where it becomes dephosphorylated by the nuclear PTPN2 isoform, and is, subsequently, not detectable in the cytosolic protein fraction anymore. Similar data were obtained regarding IFN γ -induced cytoplasmic STAT3 phosphorylation (Figure 2d).

The delay in increased activity of PTPN2 is at odds with increased PTPN2 expression at 24 h. A possible explanation for this dichotomy is that whole cell lysates used for the PTPN2 activity assay contained only a small portion of the nuclear protein fraction (Supplementary Figure 5).

It is possible that only low levels of the nuclear 45kD isoform of PTPN2 were present in the whole cell lysates at the earlier timepoints of 24 and 48 h, whereas by 72 h, significant levels of the 45kD variant had exited the nucleus. Therefore, we speculate that in response to IFN γ treatment, a certain amount of the nuclear 45kD PTPN2 variant shifts from the nucleus into the cytoplasm. Moreover, cytoplasmic accumulation of PTPN2 in T₈₄ cells in response to 72 h IFN γ treatment may contribute to STAT1 dephosphorylation in the cytoplasmic cell compartment, as well as to the significantly increased PTPN2 activity (Figures 2a-c).

Though our data suggest that IFN γ increases PTPN2 activity and this correlates with downregulation of IFN γ -induced STAT signalling in IEC, basal PTPN2 is also likely to be involved in STAT dephosphorylation. Further studies will be needed to investigate the effects of IFN γ on the catalytic activity of the different isoforms of PTPN2 in detail.

Loss of PTPN2 enhances STAT1 and STAT3 phosphorylation

We next investigated whether PTPN2 knock-down conversely elevated STAT1+3 activity. T₈₄ cells transfected with either PTPN2-specific siRNA or non-specific control siRNA sequences, were treated with IFN γ (24 h). PTPN2 siRNA decreased PTPN2 expression with a maximal reduction of $92 \pm 3\%$ (Figure 3a). Non-specific effects on protein levels were not observed, as shown by equivalent levels of the nuclear envelope protein lamin A/C and total STAT1+3 in both subgroups (Figure 3a-c). As expected, IFN γ increased PTPN2 protein (Figure 3a) and STAT1+3 phosphorylation (Figure 3b,c). PTPN2 knock-down amplified STAT1+3 phosphorylation (Figure 3b,c). Similar findings were observed in separately collected cytoplasmic and nuclear protein fractions of 24 h IFN γ -treated T₈₄ monolayers (Figure 3d). siRNA-induced knock-down of PTPN2 in HT29cl.19a cells revealed the same regulatory influence of PTPN2 on STAT1 phosphorylation (Supplementary Figure 2a,b). These data, in addition with those in Figure 2a-d, indicate that PTPN2 is the cellular phosphatase of STAT1+3 in IEC, and that PTPN2 terminates IFN γ -induced STAT signalling.

IFN γ regulates PTPN2 activation via AMPK

The cellular energy sensor, AMPK, has been shown in fibroblasts to participate in regulation of PTPN2 nuclear-cytoplasmic shuttling. Therefore, we investigated if AMPK modified the effects of IFN γ on PTPN2 expression, activity and subcellular distribution in IECs. AMPK activity was assessed by phosphorylation of Thr¹⁷² on the AMPK catalytic α_1 -subunit in T₈₄ cells. Western blot analysis revealed that IFN γ induced AMPK activation in a time-dependent manner, reaching a peak at 6 h (Figure 4a). This effect was sensitive to the AMPK inhibitor, compound C (CC; 50 μ M), which competes for binding of intracellular ATP to AMPK, and inhibits AMPK phosphorylation by upstream kinases. CC co-treatment significantly inhibited IFN γ -induced AMPK phosphorylation (Figure 4b). To determine if AMPK was involved in the regulation of PTPN2 protein, we stimulated T₈₄ monolayers with IFN γ and/or CC for 72 h. IFN γ increased cytoplasmic and nuclear PTPN2 levels, an effect that was partially diminished by CC. Thus, AMPK mediates, at least in part, IFN γ -induced PTPN2 expression although additional pathways may also be involved (Figure 4c,d).

Since we have shown that STAT1 phosphorylation kinetics can be used as a marker of PTPN2 activity, we surmised that STAT1 phosphorylation in cells treated with IFN γ and CC should be higher than in cells treated with IFN γ alone. Indeed, while IFN γ treatment (72 h) increased cytoplasmic and nuclear STAT1 phosphorylation (Figure 4e,f), CC co-treatment potentiated cytoplasmic (Figure 4e), and nuclear (Figure 4f) STAT1 activity above IFN γ alone. Interestingly, CC alone had no effect on STAT1 phosphorylation. In contrast, AMPK activity alone, as induced by the pharmacological activator AICAR (1 mM), neither induced PTPN2 expression nor altered STAT1 phosphorylation (Supplementary Figure 6). These data indicate that AMPK modification of PTPN2 protein levels and STAT1 dephosphorylation is dependent not only on IFN γ -stimulated AMPK activity, but also on other aspects of the signalling milieu existing in IFN γ - vs. AICAR-treated cells. Therefore, further studies will be required to determine additional pathways that are likely involved in the regulation of IFN γ -induced effects on PTPN2 besides AMPK.

AMPK regulates IFN γ -induced PTPN2 cytoplasmic accumulation

Maximal IFN γ -induced PTPN2 activity was detectable by 72 h. However, the peak of IFN γ -induced AMPK phosphorylation was seen by 6 h. We hypothesized that the 45kD PTPN2 isoform, which likely accounts for the majority of PTPN2 activity, shifts from the nucleus to the cytoplasm in response to IFN γ . Therefore, we examined if IFN γ affects the cellular distribution of PTPN2. In untreated T₈₄ cells, PTPN2 was evident in the cytoplasm and the

nucleus (Figure 5a). A similar pattern of PTPN2 distribution was observed in 24 h IFN γ -treated cells (Supplementary Figure 7). This finding supports our Western blot data (Figure 1c) and our hypothesis that nuclear PTPN2 protein is highest from 0-24 h after IFN γ treatment. Therefore, our evidence suggests that 24 h treatment with IFN γ increases PTPN2 protein in both cell compartments independent of alterations in the relative subcellular distribution of PTPN2.

In contrast, after 72 h IFN γ , PTPN2 was more prominent in the cytoplasm than the nucleus compared to untreated control cells (Figure 5a). These findings further support our hypothesis, as well as our previous PTPN2 data (Figures 1b,2a), suggesting that the majority of nuclear PTPN2 shifts from the nucleus to the cytoplasm in response to 72 h IFN γ and accounts for the significant increase in PTPN2 activity at this timepoint. We further investigated whether AMPK inhibition by CC modulates PTPN2 distribution. In T₈₄ monolayers treated with CC alone, PTPN2 was equally distributed in cytoplasmic and nuclear compartments as seen in untreated control cells. However, CC co-treatment clearly attenuated the IFN γ -induced cytoplasmic accumulation of PTPN2. Though PTPN2 was still prominent in the cytoplasm of co-treated cells, PTPN2 was more visible in the nucleus in comparison to IFN γ treatment alone (Figure 5a). Though these data indicate that the extra-nuclear localization of PTPN2 in response to IFN γ is, at least partially AMPK-dependent, additional pathways are likely involved.

AMPK knock-down attenuates IFN γ -induced increases in PTPN2 expression

To confirm the regulatory effect of AMPK on PTPN2 we transfected T₈₄ cells with either control or AMPK- α_1 targeting siRNAs. In cells transfected with AMPK siRNA, AMPK was significantly lowered by $64 \pm 11\%$ (Figure 5b), while lamin A/C and total STAT1 were not affected (Figure 5b-d). IFN γ treatment (24 h) increased PTPN2 expression and STAT1 phosphorylation in control siRNA-transfected cells (Figure 5c,d). However, AMPK-deficient cells showed, in parallel with decreased PTPN2 expression, a potentiation of IFN γ -induced STAT1 phosphorylation (Figure 5c,d). These data confirm that AMPK activity plays a partial but important role in regulating PTPN2 expression and activity in response to IFN γ in IEC.

PTPN2 knock-down increases epithelial permeability

We next determined whether PTPN2 mediates the IFN γ -induced decrease in barrier function. We assessed epithelial permeability by measuring flux of 10kD FITC-Dextran across T₈₄ and HT29cl.19a monolayers, transfected with either control or PTPN2-specific siRNA and subsequently treated with IFN γ (72 h). PTPN2 siRNA significantly decreased PTPN2 expression (Figure 6a; Supplementary Figure 2a). As expected, IFN γ significantly increased permeability in cells transfected with non-specific siRNA. However, PTPN2 knock-down further exacerbated barrier dysfunction following IFN γ treatment in both IEC lines (Figure 6a, Supplementary Figure 2c). Additionally, loss of PTPN2 further aggravated the IFN γ -induced decrease in monolayer TER (Figure 6b). These data suggest that PTPN2 plays an important role in stabilizing epithelial barrier function during exposure to inflammatory cytokines.

The pore-forming protein, claudin-2, plays an important role in regulating epithelial permeability in IBD^{20, 21, 23}. In T₈₄ cells transfected with control siRNA, IFN γ treatment did not affect claudin-2 expression. However, in parallel with the rise in epithelial permeability, IFN γ significantly increased claudin-2 expression in PTPN2-deficient cells (Figure 6c). In contrast, while IFN γ reduced expression of ZO-1 and occludin, this was not further affected by PTPN2 knock-down. Moreover, claudin-4 levels remained stable under all conditions (Figure 6d). Overall, our data indicate that a lack of PTPN2 enables IFN γ -

stimulated expression of claudin-2, which is believed to contribute to increased permeability in IBD. These data thus illustrate a novel role for PTPN2 in protecting epithelial barrier function.

Discussion

PTPN2 plays a critical role in regulating cytokine signalling in immune cells by inactivating STAT1+3^{7, 11, 14}. These findings are supported by observations in PTPN2^{-/-} mice that die within 3-5 weeks from systemic inflammation accompanied by elevated serum levels of IFN γ and TNF α ²⁷. We demonstrated that IFN γ increases PTPN2 expression and activity in IEC. Activated PTPN2, in turn, reduces IFN γ -induced STAT1+3 activation. These data were corroborated by PTPN2 knock-down studies. We can speculate that PTPN2 participates in a negative feedback mechanism activated by IFN γ and thus limits cytokine signalling. STAT1+3 affect different target genes and induce different biological effects. STAT1 plays a decisive role in mediating IFN γ -dependent immunomodulatory, antiproliferative and antiviral effects¹⁷. In contrast, STAT3 has primarily oncogenic and anti-apoptotic effects²⁸, and plays an important role in colon cancer pathogenesis, which has a higher frequency in IBD patients²⁹. Overall, strict regulation of STAT1+3 signalling is crucial for intestinal homeostasis. Our data establish PTPN2 as a key regulator of STAT1+3 phosphorylation and IFN γ -induced signalling in IEC in particular.

We observed that PTPN2 is regulated in T₈₄ cells, at least in part, via IFN γ -induced AMPK activation. In response to reduced cellular energy levels, AMPK limits energy-consuming and activates energy-generating processes³⁰. Energy consuming processes in the intestine include ion transport and epithelial barrier maintenance, both of which are compromised by IFN γ ^{25, 31, 32}, and in IBD^{33, 34}. AMPK is thus a logical mediator of IFN γ signalling. However, AMPK activity alone was unable to affect PTPN2 expression. Therefore, accessory signals activated by IFN γ , in addition to AMPK, may also play an important role in the regulation of PTPN2. Identification of these signals was beyond the scope of this study. Nevertheless, our data indicate that AMPK is necessary, though not sufficient, to mediate important downstream effects of IFN γ relevant to intestinal inflammation.

The ability of IFN γ to increase epithelial permeability and decrease TER was significantly enhanced by PTPN2 knock-down and accompanied by increased claudin-2 expression. The claudins are a family of tight junction proteins that play a crucial role in regulating paracellular permeability^{22,35}. Increased epithelial permeability is believed to contribute substantially to the pathophysiology of CD³⁶. Although the claudins function to seal the tight junction, claudin-2 is unusual as it forms a cation-selective membrane pore for substrates smaller than 4 Angstrom and may therefore directly account for the further decrease in TER across IFN γ -treated PTPN2-deficient T₈₄ monolayers. However, it is not possible that the increased FITC-dextran flux observed in our studies occurs via passage through this specific pore due to the restriction against passage of molecules this size (10 kD). Moreover, increased numbers of claudin-2 molecules do not directly correlate with increased permeability for substrates that exceed its pore size²³. However in CD, claudin-2 is upregulated in intestinal crypt epithelium²⁰, while the number of tight junction strand breaks in the colonic epithelium is increased. These breaks, of ≥ 25 nm, mean that even large macromolecules, such as the 10kD FITC-dextran used here, can pass through²⁰. Moreover, increased claudin-2 correlates with the appearance of discontinuous tight junction strands³⁷. It is plausible that the increased FITC-dextran flux observed reflects such discontinuities. This scenario is compatible with findings showing an essential role for claudin-2 in regulating the structural composition of tight junctions, and consequently in modifying epithelial permeability^{20-24, 38}.

Claudin-2 expression in T₈₄ cells is not increased by IFN γ alone^{21, 38}. However, when PTPN2 expression is repressed, IFN γ increases claudin-2 expression and further elevates epithelial permeability. These findings imply that PTPN2 normally prevents a rise in claudin-2 expression by IFN γ . Moreover, the IFN γ -induced elevation of claudin-2 expression in PTPN2-deficient cells may be mediated by STAT proteins as we have identified a putative STAT1/STAT3 binding sequence in the claudin-2 promoter region^{39, 40}, which is likely needed for STATs to have a regulatory effect on claudin-2 expression. Since, to the best of our knowledge, no STAT binding motif has been described within the claudin-2 promoter, we screened the claudin-2 promoter region³⁹ for such a motif. By blast search, we identified such a palindromic STAT recognition motif, TTCNNNGAA (where N is a variable spacer), 260 to 252bp upstream in the claudin-2 promoter (Supplementary Figure 8). The identified sequence, 5'-TTCCCGGAA-3' matches the criteria for a possible STAT1 binding motif⁴⁰ and matches exactly with the STAT3 binding motif in the gene encoding human p21WAF⁴⁰. Thus, we present a plausible candidate sequence for STAT-dependent regulation of claudin-2 expression. Though the nature of STAT regulation of claudin-2 expression, and the interaction with the putative STAT binding motif are unresolved, these findings support our hypothesis that PTPN2 knock-down leads to increased claudin-2 expression in response to IFN γ , possibly via upregulation of STAT1 and/or STAT3.

Whether PTPN2 restricts the interaction of STAT proteins with various promoters, perhaps by reducing their phosphorylation, remains to be elucidated. Nevertheless, our findings indicate that PTPN2 protects epithelial barrier function during exposure to inflammatory cytokines. Conversely, PTPN2 dysfunction could play an important role in CD, a chronic inflammatory disease featuring elevated levels of IFN γ . A mutation in the PTPN2 gene locus has been associated with CD^{3, 5, 6} and this mutation could possibly lead to a loss of functional PTPN2 in this disease (although this has not been formally demonstrated and, to the best of our knowledge, functional consequences of the specific PTPN2 SNP have not been reported) and to the downstream consequences described herein. Future studies should identify whether loss of functional PTPN2 permits passage of large molecules that might initiate or exacerbate episodes of intestinal inflammation, across the intestinal epithelium. We also observed increased PTPN2 expression in biopsies from CD patients with active inflammation vs. normal subjects, which is in line with our *in vitro* data showing that IFN γ increases PTPN2 expression. These data provide proof of principle of altered PTPN2 expression in CD. However, a more detailed study will be required to determine whether there are differing levels of activity of PTPN2 in active and inactive CD tissues. A similar analysis will need to be performed in tissues from patients harbouring the PTPN2 SNP.

In summary, our data show that loss of PTPN2 leads not only to a prolonged and more severe IFN γ signalling cascade, but critically to a defect in intestinal epithelial barrier function. Taken together, these findings suggest that PTPN2 activity is crucial for regulating inflammatory processes and preserving barrier function in the setting of inflammation. These data indicate a mechanistic role for PTPN2 in restricting the pathogenesis of CD, as well as other inflammatory conditions associated with increased intestinal permeability, and provide a functional basis for the identification of PTPN2 as a CD candidate gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a Crohn's and Colitis Foundation of America Senior Research Award to DFM, a Jon I. Isenberg Award to DFM, grants from the German Research Foundation (DFG) to MS and GP, NIH grant DK28305 to KEB, an unrestricted research gift to KEB from the Shape-Up Settlement fund, and by the University of California, San Diego, Digestive Diseases Research Development Center (DK080506).

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Abbreviations

AICAR	5-aminoimidazole-4-carboxamide-1-beta-d-ribonucleoside
AMPK	adenosine monophosphate-activated protein kinase
CC	Compound C
FITC	fluorescein isothiocyanate
IEC	intestinal epithelial cells
IFN γ	interferon gamma
PTPN2	protein tyrosine phosphatase N2
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
TER	transepithelial electrical resistance

Figure 1.

IFN γ increases PTPN2 levels in T₈₄ cells and human colonic biopsy specimens. (a) IFN γ -stimulated PTPN2 mRNA expression normalized to GAPDH (n=3, performed in triplicate). (b) Cytoplasmic PTPN2 protein in IFN γ -treated T₈₄ cells shown by representative Western blots. Lamin A/C was used throughout as a loading control. The relative protein level was assessed by densitometry (n=4). (c) Nuclear PTPN2 protein in IFN γ -treated T₈₄ cells is demonstrated by representative Western blots and densitometry (n=4). Data are expressed as a percentage of the respective control. (d) PTPN2 mRNA in human terminal ileum and colon biopsies from CD patients with active disease (n=9) and in remission (n=7) as well as from control subjects (n=9). PTPN2 mRNA expression was normalized to the housekeeping gene GAPDH. Measurements were performed in triplicate. Asterisks denote significant differences from the respective control (*=p<0.05, **=p<0.01, ***=p<0.001).

Figure 2. Elevated PTPN2 activity parallels decreased STAT phosphorylation in IFN γ -treated T₈₄ cells. **(a)** PTPN2 was immunoprecipitated from whole cell lysates. Blots were probed for PTPN2 to show equivalent protein loading. The graph demonstrates IFN γ -stimulated PTPN2 activity (n=4). **(b)** Representative Western blots show phosphorylated cytoplasmic (Tyr⁷⁰¹, above) and total STAT1 (below) after IFN γ treatment, followed by densitometric analysis (n=5). **(c)** Western blots and densitometry showing phosphorylated nuclear (above) and total STAT1 (below) in IFN γ -treated cells (n=5). **(d)** Phosphorylated cytoplasmic (Tyr⁷⁰⁵) and total STAT3 in IFN γ -treated T₈₄ cells shown by representative Western blots and densitometry (n=4). Data in (b), (c) and (d) are shown as a percentage of the respective control. Asterisks indicate significant differences compared to the respective control (*=p<0.05, **=p<0.01, ***=p<0.001). #=p<0.05; ###=p<0.001 vs. 24 h IFN γ treatment.

Figure 3.

PTPN2 knock-down enhances IFN γ -stimulated STAT1+3 phosphorylation. T₈₄ cells were transfected with either non-specific or PTPN2 siRNA and treated with IFN γ (24h). **(a)** Representative Western blots for PTPN2 and lamin A/C followed by densitometric analysis (n=3). **(b,c)** Representative Western blots and densitometry showing STAT1+3 phosphorylation and expression (n=3). Figures (a) to (c) represent analyses of whole cell lysates. Asterisks indicate significant differences vs. the respective control (*=p<0.05, **=p<0.01, ***=p<0.001). ###=p<0.001 vs. 24 h IFN γ treatment of control siRNA-transfected cells. **(d)** Representative Western blots showing PTPN2 and lamin A/C, STAT1+3 expression and phosphorylation, in cytoplasmic and nuclear lysates (n=2).

Figure 4.

AMPK activation by IFN γ and regulation of STAT1 phosphorylation in T₈₄ cells. **(a)** Western blots and densitometric analysis of phosphorylated and total AMPK after IFN γ treatment (n=3-7). **(b)** Western blots and densitometric analysis of phosphorylated and total AMPK after IFN γ and/or CC (50 μ M, bilaterally) (n=5). Western blots and densitometric analysis of **(c)** cytoplasmic (n=4), **(d)** nuclear PTPN2 (n=3), **(e)** cytoplasmic (n=4) and **(f)** nuclear (n=4) phosphorylated and total STAT1 in response to IFN γ (72h). Black dashes in (c) to (f) indicate the gel has been cropped at this position. Asterisks indicate significant differences vs. the respective control (*=p<0.05, **=p<0.01, ***=p<0.001). ++=p<0.01 vs. 6h IFN γ treatment and #=p<0.05 vs. 72h IFN γ .

Figure 5.

Effect of AMPK knock-down on PTPN2 distribution. **(a)** Confocal microscopy shows PTPN2 (green) in T₈₄ cells. Nuclear staining is blue. Each panel shows a representative image for one experiment. Three experiments were performed for each condition. PTPN2 was equally distributed between the nucleus (arrow) and cytoplasm in control cells. Cytoplasmic PTPN2 accumulates (arrow) in response to IFN γ treatment (72h). Compound C had no effect on PTPN2 distribution *vs.* control cells. However, CC prevented the IFN γ -stimulated nuclear exit of PTPN2 (arrows). **(b)** Representative Western blots show AMPK α levels in 24 h IFN γ -treated T₈₄ cells transfected with either control or AMPK α 1 siRNA and densitometric analysis (n=4). **(c)** PTPN2 and lamin A/C expression (n=4). **(d)** Representative Western blots and densitometric analysis of phosphorylated and total STAT1 (n=4). Asterisks indicate significant differences *vs.* the respective control (**=p<0.01, ***=p<0.001). #=p<0.05 *vs.* 72h IFN γ treatment of control siRNA cells.

Figure 6.

FITC-dextran flux, TER and claudin-2 protein in PTPN2-deficient T₈₄ cells. **(a,b)** Western blots showing decreased PTPN2 but not lamin A/C protein in PTPN2-deficient T₈₄ cells after IFN γ (72 h) treatment. **(a)** FITC-dextran flux (n=3); **(b)** TER (n=3); **(c)** Western blot and densitometric analysis of claudin-2 expression (n=3); and **(d)** expression of the tight junction proteins claudin-4, occludin and ZO-1 (n=3); in control or PTPN2 siRNA-transfected cells in response to IFN γ . Asterisks indicate significant differences vs. the respective control (*=p<0.05, ***=p<0.001). #=p<0.05, ###=p<0.001 vs. 72h IFN γ treatment of control siRNA cells.