Interferon-γ-induced increases in intestinal epithelial macromolecular permeability requires the Src kinase Fyn

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Interferon-γ (IFNγ) is an important immunoregulatory cytokine that can also decrease intestinal epithelial barrier function. Little is known about the intracellular signalling events immediately subsequent to IFNγ/IFNγ receptor interaction that mediate increases in epithelial permeability; data that could be used to ablate this effect of IFNγ while leaving its immunostimulatory effects intact. This study assessed the potential involvement of Src family kinases in IFNγ-induced increases in epithelial permeability using confluent filter-grown monolayers of the human colon-derived T84 epithelial cell line. Inhibition of Src kinase with the pharmacologic PP1 and use of Fyn kinase-specific siRNA significantly reduced IFNγ-induced increases in epithelial permeability as gauged by translocation of noninvasive *E. coli* (HB101 strain) and flux of horseradish peroxidase (HRP) across monolayers of T84 cells. However, the drop in transepithelial resistance elicited by IFNγ was not affected by either treatment. Immunoblotting revealed that IFNγ activated the transcription factor STAT5 in T84 cells, and immunoprecipitation studies identified an IFNγ-inducible interaction between STAT5b and the PI3K regulatory subunit p85α through formation of a complex requiring the adaptor molecule Gab2. siRNA targeting STAT5b and Gab2 reduced IFNγ-induced increases in epithelial permeability and phosphorylation of PI3K(p85α). PP1 and Fyn siRNA reduced IFNγ-induced PI3K activity (indicated by decreased phospho-Akt) and the formation of the STAT5b/PI3K(p85α) complex. Collectively, the results suggest the formation of a Fyn-dependent STAT5b/Gab2/PI3K complex that links IFNγ to PI3K signalling and the regulation of macromolecular permeability in a model enteric epithelium.

The intestinal epithelium is critical in the maintenance of health. It is responsible for nutrient and water absorption, it acts as an innate immune barrier to microbes entering the mucosa and it is increasingly appreciated as a regulator of mucosal immunity.[1] Controlled and transient increases in epithelial permeability are homeostatic events, yet aberrant and prolonged loss of barrier function could mediate entry of excessive amounts of antigen and microbes into the mucosa to elicit inflammatory disease, as has been proposed for inflammatory bowel disease (IBD).[2,3] Moreover, should bacteria move from the lumen of the gut into the bloodstream, the outcome can be sepsis and multiorgan failure.

Interferon-γ (IFNγ) is an immunoregulatory cytokine whose functions include increasing MHC I and II expression, activation of macrophages and regulation of T helper cell differentiation. Levels of IFNγ are often elevated, locally and systemically, following microbial infection and in chronic inflammatory diseases including IBD.[4] In addition to its pivotal role in immune regulation, numerous *in vitro*, and a lesser number of *in vivo*, studies reveal that IFNγ decreases enteric epithelial barrier function.[5–7] IFNγ-induced disruption of epithelial barrier function is accompanied by reduced expression of the tight junction (TJ) proteins, zonula occludens (ZO)-1 and occludin,[6,7] and this would allow increased permeation of material between adjacent cells via the paracellular pathway.[8] We, and others, have shown that IFNγ can promote the transcytosis of bacteria across epithelial monolayers.[9,10] Consequently, the elucidation of IFNγ-mediated intracellular signalling pathways can be pursued with the ambition of identifying epithelial target molecules, the inhibition of which would ablate IFNγ-induced increases in gut permeability while not interfering with its ability to activate immune cells to eliminate bacteria that gain access to the mucosa.

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Canonical IFNγ signalling involves the phosphorylation, dimerization and nuclear translocation of signal transducer and activator of transcription (STAT)-1.[11] Pharmacological inhibition of STAT1 tyrosine phosphorylation (ie, activation) failed to ameliorate IFNγ-induced reduction in transepithelial electrical resistance (TER, a marker of paracellular permeability), and subsequently a requirement for activation of PI3K during IFNγ-induced increases in epithelial permeability across monolayers of human colon-derived epithelial cell lines was shown.[10,12] However, there is no indication that PI3K can bind the IFNγ receptor,[13] and hence intermediate structural or kinase molecules are needed to convert IFNγ/IFNγ receptor (IFNγR) interaction into PI3K activation.

Src kinases are a family of nonreceptor-associated tyrosine kinases that regulate several cell processes including cell growth and migration.[14] Src has been implicated in STAT5 activation in response to hematopoietic growth factors and malignancy,[15] and may lie upstream of PI3K activation, serving as a component of growth factor signalling.[16,17] Activation of STAT5 in T-cell lines by IFNγ has been shown but similar findings in epithelia have not been reported, whereas Src activity has been implicated in IFNγ regulation of Cl⁻ secretion in the T84 epithelial cell line.[18–20] Thus, we hypothesized that a Src kinase and/or STAT5 were intermediate molecules between the IFNγR and PI3K in the regulation of epithelial barrier function.

The findings presented in this study suggest a novel signalling pathway whereby IFNγ stimulates the activity of the Src kinase Fyn, leading to the formation of a complex containing STAT5b, Gab2 and the p85α regulatory subunit of PI3K. The complex assembly results in PI3K activation and a consequent increase in the macromolecular permeability characteristics of monolayers of the human colon-derived T84 epithelial cell line.

MATERIALS AND METHODS
Reagents
Reagents, including cell culture supplements and pharmacological inhibitors, were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise indicated. Src inhibitor PP1 was purchased from Biomol (Enzo Life Sciences, Plymouth Meeting, PA, USA) and LY294002 and AG490 were purchased from Calbiochem (EMD Chemicals, Gibbstown, NJ, USA). Recombinant human IFNγ was purchased from Ebioscience (San Diego, CA, USA). Rabbit anti-STAT5a/b, anti-phosphotyrosine (694/699) STAT5a/b, anti-Akt, anti-phosphoserine (473) Akt, anti-STAT1a/b, anti-phosphotyrosine (701) STAT1, anti-phosphotyrosine (452) Gab2, anti-phosphotyrosine (416) Src family and mouse anti-c-Src antibodies were purchased from Cell Signaling Technologies (NEB, Oakville, ON, Canada). Mouse anti-STAT5b was from Invitrogen (Burlington, ON, Canada) and mouse anti-Fyn kinase, rabbit anti-interferon response factor-1 (IRF-1) and goat anti-actin antibodies were

purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Mouse anti-PI3K p85α, rabbit anti-Gab2 and mouse anti-phosphotyrosine (4G10) were purchased from Millipore (Billerica, MA, USA). Mouse anti-IFNγR2 was purchased from Abcam (Cambridge, MA, USA). Control mouse IgG and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz. Phosphoinositide 3,4,5, triphosphate (PIP₃) Mass ELISA kits were purchased from Echelon Biosciences (Salt Lake City, UT, USA). Nonpathogenic, noninvasive *E. coli* strain HB101 was a kind gift from Dr PM Sherman (Hospital for Sick Children, University of Toronto, Toronto, ON, Canada).

Cell Culture
The immortalized human colon-derived T84 epithelial cell line (ATCC, Manassas, VA, USA) was cultured at $37\,^{\circ}C/5\%$ CO_2 in 1:1 Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2% (v/v) penicillin–streptomycin, 1.5% HEPES, 5% $NaHCO_3$, 1% L-glutamine, 1% sodium pyruvate (all from Invitrogen) and 10% fetal bovine serum (PAA Laboratories, VWR International, Edmonton, AB, Canada). For assay of epithelial barrier function, 1×10^6 cells (1 ml volume) were seeded onto $12\,mm^2$ semi-permeable filter supports ($3.0\,\mu m$ pore size; Greiner Biosciences (VWR)) and cultured until electrically confluent (see below). For immunoprecipitation (IP) and immunoblotting experiments, 1×10^6 T84 cells/well were seeded in 12-well culture dishes and cultured until confluent as assessed by phase-contrast microscopy. For PIP₃ synthesis assays, 5×10^6 cells were seeded onto 150 mm culture dishes and grown to confluence. In other experiments, cells of the human colon-derived Caco2 cell line were cultured and treated in a manner identical to that used for T84 cells.

In some signalling experiments, THP-1 cells (human monocytic cell line; ATCC) were differentiated by treatment with 20 nM phorbol-12-myristyl-13-acetate (PMA) for 48 h, and cells developed a stellate morphology typical of macrophages (see Figure 3b, inset). Cells were subsequently stimulated with 10 ng/ml IFNγ over the indicated times.

Transient Transfection of T84 Cells with Small Interfering RNA (siRNA)
siRNAs targeting STAT1, STAT5b, Gab2, Fyn and c-Src (and appropriate scrambled oligomer controls) were created using the Stealth siRNA oligomer design platform (Invitrogen); target oligomer sequences used in this study are shown in Table 1. A total of 20 pmol of specific or scrambled control siRNA in Lipofectamine 2000/Opti-MEM (500 μg/ml; Invitrogen) was added to suspension cultures of T84 cells (1×10^6/ml) in antibiotic-free FBS-containing culture medium. The cells were then either seeded onto filter supports for permeability experiments or 12-well culture dishes for signalling experiments. Following an overnight incubation, adherent cells were washed and transferred to antibiotic-containing culture medium until reaching confluence and assays were conducted as described below.

Table 1 Sequences of the siRNA oligomers used in this study

Target	Accession no.	Start site	Sequence
Fyn	NM_002037	5′-1057	5′-GAGCGACAGCTATTGTCCTTTGGAA-3′
		control	5′-GAGACATCGTTACTGTTCGTCGGAA-3′
Gab2	NM_012296	5′-248	5′-CAGGTAGATGCAGGCCTGACCTTTA-3′
		control	5′-CAGTAGAGACGTCCGCCAGTTGTTA-3′
STAT5b	NM_012448	5′-294	5′-CATGGGACTCAGTAGATCTTGATAA-3′
		control	5′-CATAGTCACTGGATATCGTAGGTAA-3′
STAT1	NM_007315	5′-1695	5′-AAACCAGGCTGGCACAATTGGGTTT-3′
		control	5′-AAATAACGTGTGACCGTCCGCCTTT-3′
c-Src	NM_005417	5′-777	5′-CGGCTCCAGATTGTCAACAACACAG-3′
		control	5′-CGGACCTAGTGTAACAACACCTCAG-3′

Sequence selection and accession numbers for gene products selected for siRNA targeting. Shown are the sequences used for targeting of the indicated gene products; multiple siRNAs were designed for each protein target but sequences shown were used for the presented studies. Control oligomeric sequences are listed below each target. RNA oligomers were designed and synthesized using Stealth RNAi (Invitrogen).

Epithelial Barrier Function

For assessment of epithelial barrier function, filter-grown T84 or Caco2 cell monolayers were treated with IFNγ (10 ng/ml) ± pharmacological inhibitors (unless using siRNA transfected cells) added to the basal chamber of the culture well. Treatments were conducted in triplicate.

Transepithelial electrical resistance

TER across filter-grown T84 cells was assessed using a voltmeter and companion electrodes (Millipore). Monolayers in this study were considered electrically confluent when TER values were $\geq 1000 \, Ohms \cdot cm^2$, which typically occurred after 4–7 days of culture.[21] Individual monolayer TER was measured at the start of each experiment (baseline value) and 24 and 48 h thereafter.

HRP fluxes

Immediately after the addition of IFNγ ± pharmacological inhibitors (times and doses denoted on figures) to T84 cell monolayers, HRP (20 ng type VI) was added to the apical chamber (1 ml vol.) of the well, and 48 h later, three 10 μl samples of culture medium were collected from the basolateral chamber, diluted (1:10) in 0.5% HTAB buffer and HRP activity determined by spectrophotometric measurement (OD_{450} nm) of 3,3′,5,5′-tetramethylbenzidine (TMB) oxidation after the reaction was terminated with 2 N H_2SO_4. The amount of HRP was quantified against a standard concentration curve.

Bacterial translocation

Confluent T84 cell monolayers were transferred to antibiotic-free culture medium, noninvasive E. coli (strain HB101, 10^5 CFU) were added to the apical surface of the epithelium, and IFNγ simultaneously added to the basolateral chamber (± inhibitors as indicated). After 24 h, 100 μl aliquots of basolateral media were collected, serial log dilutions made in

PBS and plated on antibiotic-free LB-Agar plates. Cultures were incubated overnight at 37 °C/5% CO_2 and resultant bacterial colonies were counted. Pharmacological inhibitors were tested for bacteriocidal or bacteriostatic activity by conducting E. coli growth assays in the presence or absence of experimental concentrations of the Src inhibitor, PP1 or the PI3K inhibitor, LY294002.

IP and Immunoblotting

Whole cell lysates were prepared by washing with ice-cold PBS, followed by scraping cells into ice-cold Triton X-100 buffer (25 mM Tris-HCl (pH 8), 125 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100) supplemented with protease and phosphatase inhibitors (Complete® protease inhibitor cocktail (Roche/Mannheim), 1 mM sodium orthovanadate, 1 mM sodium fluoride). Lysates were incubated with gentle agitation at 4 °C for 30 min, centrifuged at 10 000 g and supernatants were collected and stored at −80 °C. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Crude membrane extracts were prepared by lysing cells in Triton X-100 buffer as above, followed by dissolution of the pelleted fraction in 1% SDS/PBS at 65 °C for 15 min followed by centrifugation.

For immunoblotting, 20 μg of lysates was added to Laemmli buffer, boiled and resolved on 8% SDS-PAGE. With 1% SDS/PBS (membrane) lysates estimated equivalent quantities (adjusted to Triton X-100 protein lysate concentrations) were used. Separated proteins were blotted to Immobilon nitrocellulose membranes (Millipore), and blots were blocked at room temperature for 1 h in 5% nonfat milk/wash buffer (0.1% Tween-20/Tris-buffered saline (TBS/T)). Primary antibodies (for proteins as indicated in Results) were incubated in 1% bovine serum albumin/TBS/T (for phosphoprotein analysis) or 5% non-fat milk/TBS/T (total proteins) overnight at 4 °C with gentle rocking. Blots were

washed three times in TBS/T and species-appropriate, HRP-conjugated secondary antibodies were applied with gentle rocking for 1 h at room temperature. Blots were washed, subjected to chemiluminescence (Western Lightning® PLUS, PerkinElmer, Waltham, MA, USA) and subsequently exposed to Kodak XB-1 film (Eastman Kodak, Rochester, NY, USA).

For IP, 300 μg of protein from total cell lysates was incubated with gentle rocking overnight at 4 °C with 2 μg/ml of indicated monoclonal antibody for a specific signalling or structural protein or an irrelevant IgG as a control for the IP process. Subsequently, immune complexes were adsorbed with EZ-view protein A-agarose beads (Sigma; 30 μl per sample) by 1.5 h of gentle rocking at 4 °C. Complexes were repeatedly washed with lysis buffer and once with PBS or TBS, and proteins were eluted in Laemmli buffer, boiled and resolved on SDS-PAGE for immunoblotting.

Where indicated, densitometric analyses were conducted on blots using Image J open-access software (NIH, Bethesda, MD, USA).

PIP₃ and IP-10 ELISA

T84 cells (5×10^6) were seeded onto 15 cm culture dishes and cultured to ∼80% confluence and subsequently treated with IFNγ (20 ng/ml) for the indicated times or with human epidermal growth factor (EGF; 20 ng/ml for 30 min; a positive control for PI3K activation). Extraction of phosphoinositide lipids and a competitive ELISA for measurement of PIP₃ was carried out as specified by the manufacturer's protocols.

Plastic-grown T84 epithelial cell monolayers were treated with IFNγ ± LY294002 (pan-PI3K inhibitor), and the amount of IP-10 liberated into the culture medium 24 h (or 48 h) later was determined using Luminex technology.

Statistical analysis

Graphical data are presented as mean ± s.d., with *n* denoting the number of T84 cell preparations from a defined number of experiments. Where appropriate, data were assessed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by *post hoc* pair-wise statistical comparisons with Tukey's or Dunnett's test. A statistical significance was deemed achieved if $P < 0.05$.

RESULTS
Pharmacological Inhibition of Src Kinases Reduces IFNγ-Induced Increases in Epithelial Permeability

A role for Src kinase in IFNγ regulation of Cl⁻ secretion by T84 cells has been shown;[18] therefore, it was of interest to determine if IFNγ-mediated increases in epithelial permeability were influenced by Src kinase activity. As shown in Figure 1a and b, the increased passage of HRP and *E. coli* (strain HB101) across IFNγ-treated (10 ng/ml) T84 cell monolayers was abolished by co-treatment with the Src kinase inhibitor PP1 (125 nM) and the PI3K inhibitor, LY294002 (20 μM) (neither drug had bacteriostatic properties). Using transepithelial flux of HRP as a marker of epithelial barrier function, dose- and

time-response studies revealed that 30 nM PP1 blocked the IFNγ-induced increased transepithelial flux of HRP and the drug was equally effective if added 30 min before or 90 min after IFNγ exposure (Figure 1c and d). IFNγ-induced increased HRP transcytosis across monolayers of Caco2 cells was also significantly reduced by PP1 (Figure 1c and d). In contrast, the drop in TER elicited by IFNγ was not affected by Src kinase inhibition (Figure 1e), whereas consistent with earlier studies,[10,12] inhibition of PI3K activity did reduce, in part, the drop in TER.

To examine Src activation, we assessed IFNγ-stimulated Src phosphorylation at the Y416 residue (an auto-phosphorylation site) 2 h post-cytokine application (Figure 1f). Phosphorylation of two immunoreactive products migrating at ∼60 kDa in size was observed, with marked increase of phosphorylation apparent for the smaller Src form. Phosphorylation of the smaller form was reduced in cells treated with the Src inhibitor, PP1, whereas the larger molecule appeared unaltered.

Downregulation of Fyn Kinase Inhibits IFNγ-Stimulated Increases in Epithelial Permeability

Although PP1 is a general Src kinase inhibitor, at 30 nM only the activity of Src family kinase members Lck, Hck or Fyn are effectively blocked.[22] As Lck and Hck expression is largely restricted to immune cell types,[23] we assessed whether Fyn participated during IFNγ signalling responses in T84 cells. Co-immunoprecipitation studies using Fyn monoclonal antibody showed an increased association of Fyn with the IFNγR2 subunit of the IFNγ receptor within 15 min of IFNγ application to T84 cells cultures (Figure 2a). Subsequently, siRNA targeting Fyn was used to assess a role for this Src kinase in IFNγ regulation of T84 epithelial monolayer permeability. Anti-Fyn siRNA reduced expression of Fyn protein by T84 cells within 96 h of transfection, the time determined to be required for T84 monolayers to reach electrical confluence (Figure 2b). Consistent with studies using PP1, specific Fyn knockdown inhibited IFNγ-stimulated translocation of HRP and *E. coli* fluxes across T84 monolayers (Figure 2c and d), but did not ameliorate the IFNγ-induced drop of TER (Figure 2e). Experiments utilizing c-Src siRNA indicated that knockdown of c-Src did not affect IFNγ-induced increases in translocation of HRP or *E. coli* HB101 to the significant degree achieved by knockdown of Fyn (data not shown).

IFNγ Induces STAT1 and STAT5 Phosphorylation in T84 Cells Independent of Src Activity

IFNγ treatment resulted in STAT1 and STAT5 phosphorylation in both T84 and Caco2 cells (Figure 3a). Analysis of T84 cells revealed that whereas phosphorylation of STAT1 was markedly increased within 30 min of IFNγ exposure, STAT5 phosphorylation was somewhat slower and less robust, peaking at 2 to 3 h post-IFNγ. The protracted expression of phosphorylated STAT1 and STAT5 in IFNγ-treated T84 cells (and STAT5 in Caco2 cells) was not observed in THP-1 cells
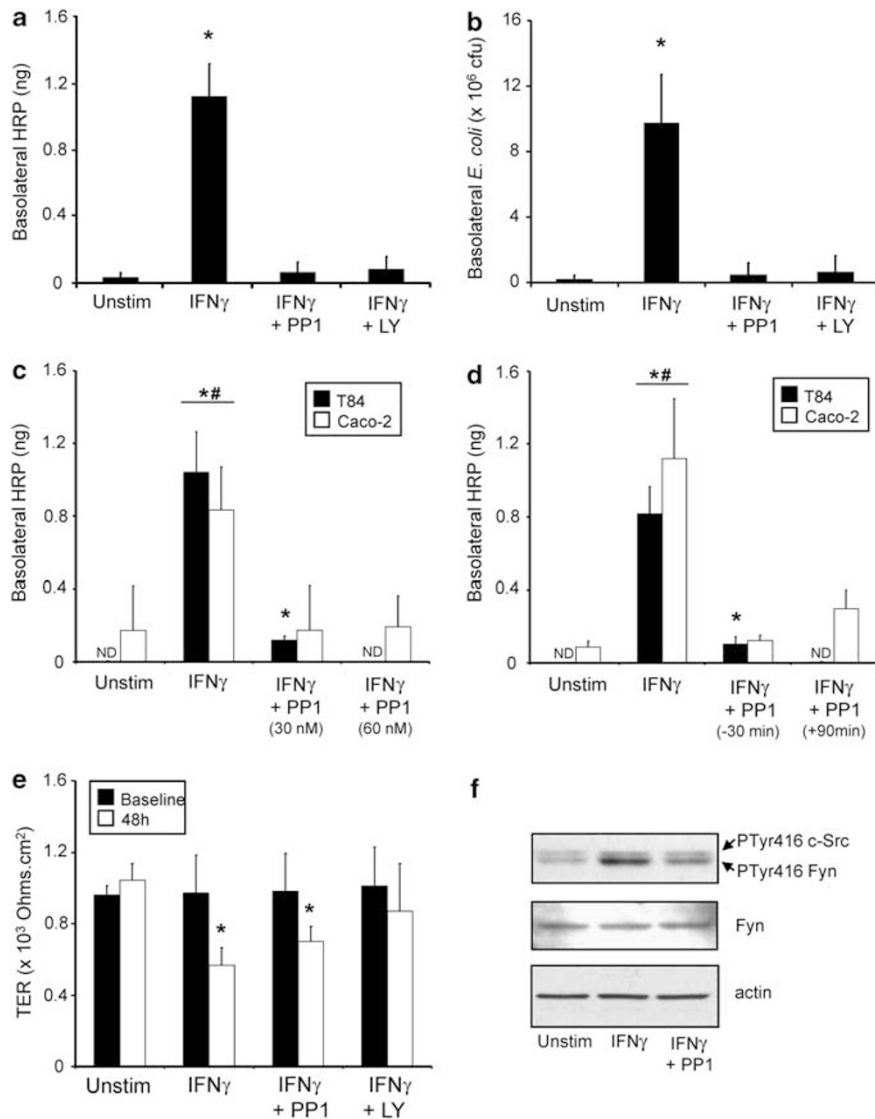
Figure 1 Pharmacological inhibition of Src kinase activity with PP1 (125 nM) administered 90 min following IFNγ stimulation (10 ng/ml; 48 h total exposure time) attenuates increases in T84 epithelial cell monolayer permeability as gauged by transepithelial flux of (**a**) horseradish peroxidase (HRP) and (**b**) *E. coli* (HB101; 10[5] CFU). (**c**) Dose response (90 min post-IFNγ stimulation) and (**d**) time dependence of PP1 (125 nM) inhibition of IFNγ-induced increased flux of HRP across monolayers of the epithelial cell lines T84 (black bars) and Caco2 (white bars). ND, not detected. (**a–e**); Data are presented as mean ± s.d.; $n = 3$ monolayers/treatment/experiment, representative of three experiments; $*P < 0.05$ and $^\#P < 0.05$ compared with control (Unstim) and PP1 treatment, respectively. (**e**) The drop in transepithelial resistance (TER) stimulated by IFNγ is not significantly affected by PP1. (**a, b, e**) Effect of inhibition of PI3K with LY294002 (LY: 20 μM, 90 min post-IFNγ stimulation) is shown for comparison. (**f**) Immunoblots of T84 whole cell extracts showing phosphorylation of Src kinases following 2 h IFNγ stimulation is inhibited by PP1 (30 nM) inhibitor treatment.

differentiated to a macrophage phenotype, where phosphorylation of either molecule was maximal after 15 min and markedly diminished 2 h post-IFNγ treatment (Figure 3b). The increase in STAT1 and STAT5 phosphorylation observed in T84 epithelia at 2 and 6 h after IFNγ treatment was unaffected by Src kinase inhibition with PP1 or Fyn siRNA (Figure 3c and d).

STAT5 Phosphorylation Is Inhibited by Ablation of STAT1 and Antagonism of JAK Kinase Signalling Activity
STAT1 is mobilized in IFNγ-responsive cells, whereas activation of STAT5 following exposure to IFNγ has, to date,

been described in a restricted number of cell types such as hematopoietic cells and mammary epithelia. Earlier pharmacological analyses indicated that STAT1 was not important in IFNγ-induced reduction in TER across T84 cell monolayers;[21] however, involvement of STAT1 or STAT5 in the regulation of epithelial macromolecular permeability has not been assessed. STAT1 siRNA effectively reduced STAT1 expression in T84 cells and also caused a reduction in IFNγ-stimulated expression of IRF-1 (Figure 4a). Knockdown of STAT1 markedly reduced HRP translocation (Figure 4b) but did not affect the IFNγ-induced drop in TER (Figure 4c), findings consistent with earlier pharmacological analyses.
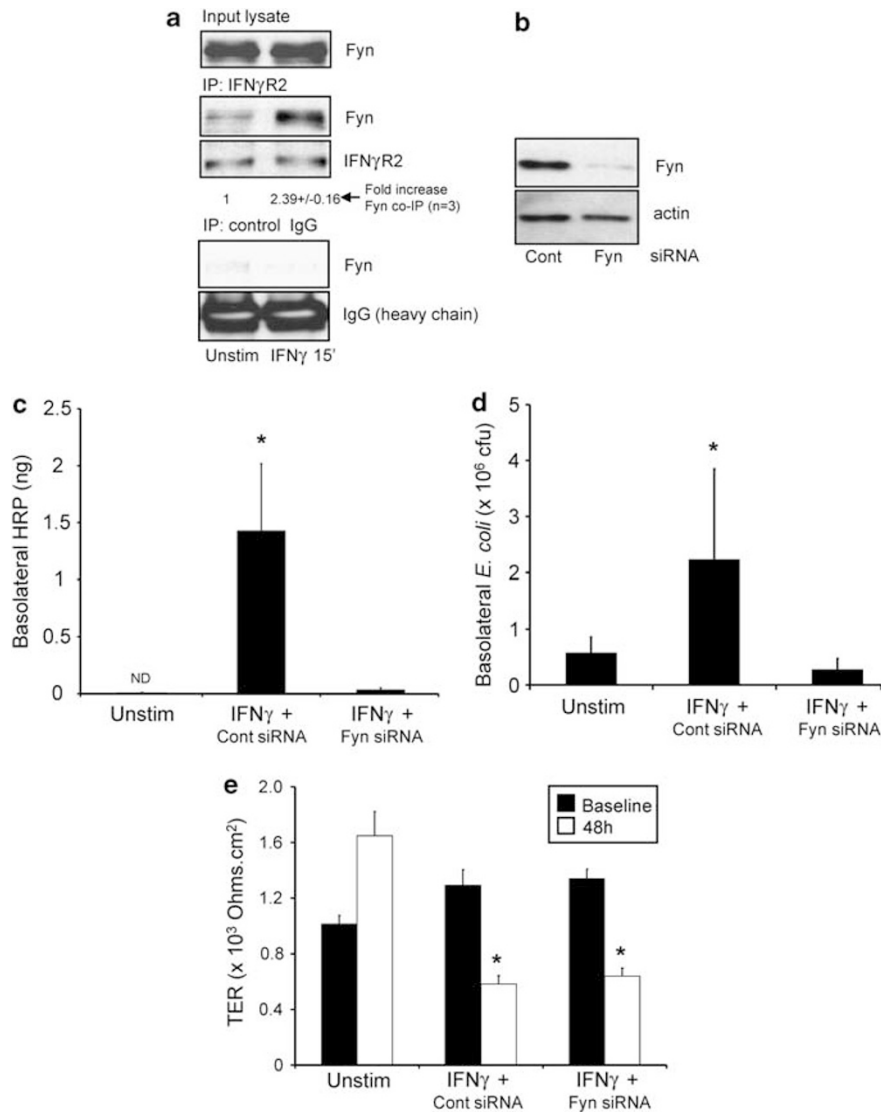
Figure 2 Fyn kinase is a mediator of IFNγ-induced decreases in T84 epithelial monolayer barrier function. (**a**) Increased association of Fyn with immunoprecipitated interferon-γ receptor (IFNγR) 2 subunit 15 min following IFNγ stimulation is shown. Input lysate levels of Fyn protein as well as an absence of immunoprecipitated Fyn from non-immune IgG control incubations are also shown. Densitometric quantification of Fyn immunoprecipitants was performed using Image J. (**b**) Reduction of Fyn protein demonstrated by immunoblotting in T84 cells treated 96 h previous with Fyn siRNA. (**c, d**) T84 cell monolayers exposed to IFNγ (10 ng/ml; 48 h) and transfected with an irrelevant scrambled (Cont) siRNA displayed significant increases in translocation of HRP and *E. coli* (HB101) fluxes that were not apparent in monolayers transfected with Fyn siRNA. In contrast, the drop in transepithelial resistance (TER) that accompanies IFNγ treatment was not inhibited by Fyn knockdown (**e**). (**c–e**) Data represented as mean ± s.d.; *n* = 3 monolayers/treatment/experiment, representative of four experiments; *P < 0.05 compared with control (Unstim). ND, not detected.

Notably, STAT1 siRNA treatment of T84 cells also reduced the IFNγ-evoked phosphorylation of STAT5 (Figure 4d), suggesting a principle requirement of STAT1 during IFNγ-stimulated biological activity including STAT5 activation and barrier dysfunction. Furthermore, inhibition of Janus kinase (JAK) by treatment of T84 cells with 10 μM of the broad-spectrum inhibitor AG490 reduced phosphorylation of STAT1 and STAT5 (Figure 4e) and also prevented translocation of HRP across IFNγ-stimulated monolayer cultures (Figure 4f).

STAT5b and Gab2 Are Required for IFNγ-Stimulated Increases in Epithelial Permeability and to Interact with PI3K in an IFNγ-Inducible Complex

We subsequently focused on STAT5b as a mediator of IFNγ-driven increases of epithelial cell permeability, as it is known to interact with the PI3K pathway in hematopoietic cell lines following growth factor stimulation. Notably, STAT5-dependent, PI3K-activating complexes can be induced via coupling to the signalling adaptor Gab2.[24,25] STAT5b- and Gab2-specific, but not control, siRNAs significantly reduced
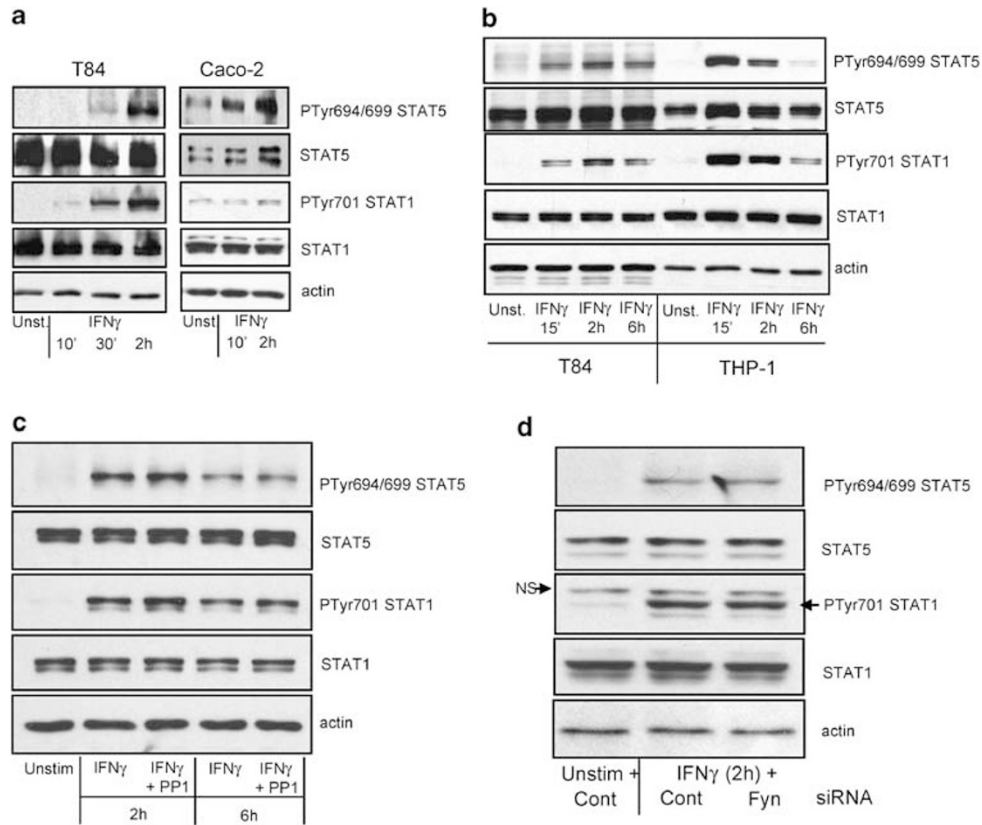
Figure 3 (**a**) Immunoblotting of whole cell protein extracts from T84 (left panels) and Caco2 (right panels) epithelial cell monolayers show phosphorylation of STAT5 and STAT1 after IFNγ (10 ng/ml) exposure. (**b**) Different kinetics of STAT1 and STAT5 phosphorylation were induced by IFNγ in T84 cells and PMA-differentiated THP-1 monocytes. Bright-field micrographs (×20 magnification) below the immunoblots demonstrate the morphology of THP-1 cells before and following 48 h of treatment with 20 nM PMA. Src kinase inhibitor PP1 (125 nM) (**c**) or Fyn siRNA treatment (**d**) of T84 cells did not inhibit IFNγ-induced phosphorylation of either STAT5 or STAT1 (images are representative of three experiments). NS, nonspecific bands.

the IFNγ-induced increase in *E. coli* translocation and transepithelial fluxes of HRP across T84 cell monolayers (Figure 5a and b). Both siRNAs reduced expression of the respective proteins at 96 h after transfection (Figure 5c). Consistent with experiments utilizing the Src inhibitor PP1 or Fyn siRNA, the drop in T84 monolayer TER evoked by IFNγ was affected by knockdown of neither STAT5b nor Gab2 (data not shown). Furthermore, IFNγ activation of Fyn (ie, phosphorylated on Tyr 416) in the cell membrane was markedly reduced by siRNA against both Fyn and STAT5b (Figure 5d).

IFNγ induces PI3K activity as measured by PIP$_3$ formation (Figure 6a) and phosphorylation of the PI3K-regulated serine/threonine kinase Akt (Figure 6b), which was inhibited by LY2940. Immune complexes precipitated with anti-STAT5b show increased association of Gab2 and PI3K p85α 6 h post-IFNγ stimulation (Figure 6c). Reciprocal blots following IP with anti-PI3K p85α antibodies revealed increased association with STAT5b and Gab2 in response to IFNγ (data not shown).

## Fyn Kinase Is Required for Formation of STAT5b/PI3K(P85α) Complexes Stimulated by IFNγ in T84 Cells
PI3K activation in response to select stimuli can be dependent upon the activity of Src kinases including Fyn;[26] thus,

we assessed whether Fyn was required for IFNγ-stimulation of PI3K activity in T84 cells. Fyn knockdown reduced the phosphorylation of Gab2 (Tyr452, contained within a putative YVPM p85α binding site) evoked by IFNγ stimulation (Figure 7a). Akt phosphorylation observed 6 h after IFNγ treatment was also reduced by Fyn siRNA treatment; both findings were corroborated by treatment with PP1, given 90 min after IFNγ treatment (Figure 7b).

Subsequent co-immunoprecipitation studies revealed that association of STAT5b and Gab2 with p85α at 6 h post-IFNγ stimulation was reduced by Fyn-specific siRNA (Figure 7c), findings that were reproduced in PP1-treated T84 monolayers (Figure 7d).

Neither PI3K Inhibition Nor Fyn Knockdown Interfere with STAT1 Signalling in T84 Epithelia
Initial studies showed that Src kinase inhibition with PP1 or Fyn siRNA did not appreciably affect IFNγ-induced STAT1 phosphorylation in T84 cells (Figure 4b and c). Therefore, we assessed the effects of Src inhibition upon expression of a STAT1-dependent gene product, IRF-1. Increased IRF-1 protein expression was observed by 6 h post-IFNγ stimulation and was unaffected by treatment with PP1 (Figure 8a) or
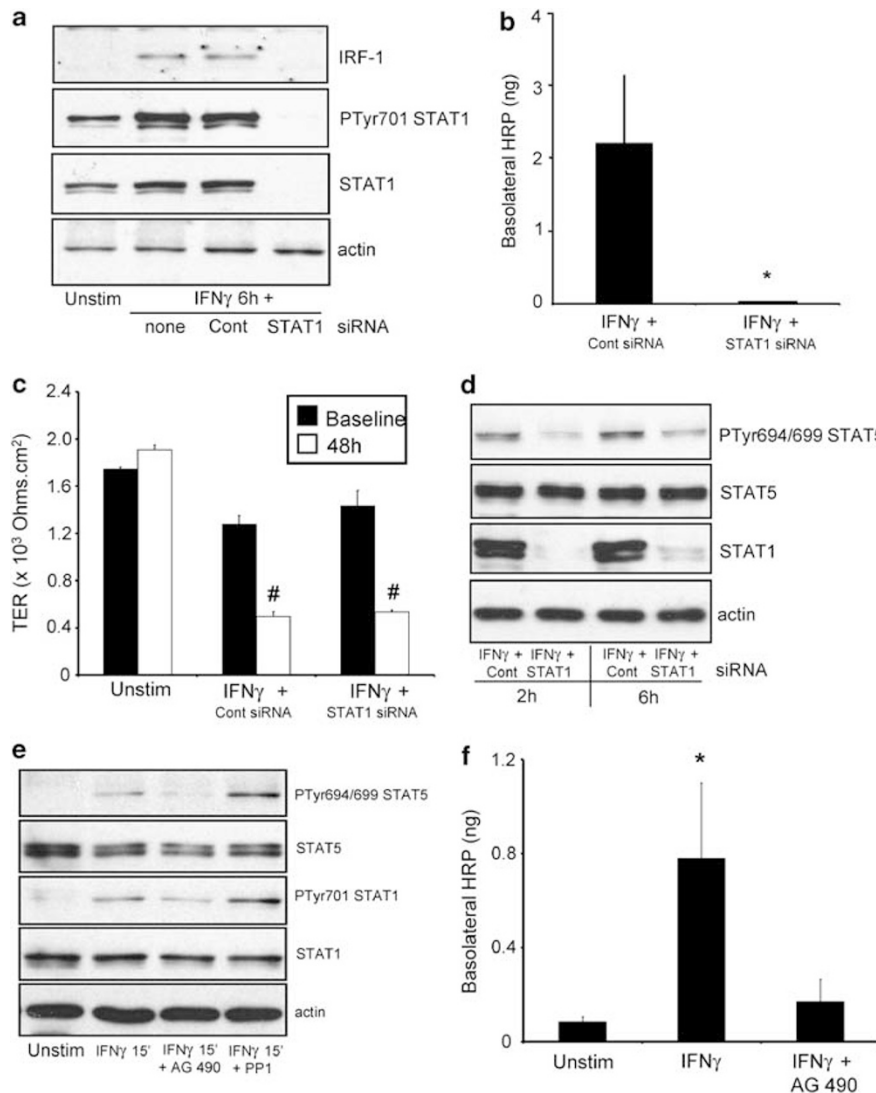
Figure 4 STAT1 is central to IFNγ-stimulated T84 epithelial responses. (**a**) siRNA against STAT1 reduces STAT1 synthesis and consequently IFNγ (10 ng/ml) of IRF-1 protein and also (**b**) blocks IFNγ-induced increases in HRP translocation (measured 48 h post-treatment), but not the drop in TER. (**c**) Data are presented as mean ± s.d.; $n = 3$ monolayers/treatment/experiment, representative of two experiments; *$P < 0.05$ compared with scrambled siRNA control (Cont siRNA) and #$P < 0.05$ compared with control and matched baseline data. (**d**) STAT1 siRNA reduced IFNγ-induced phosphorylation of STAT5 as revealed by immunoblotting (siRNA transfection of T84 was conducted 48 h before stimulation). (**e**, **f**) The general JAK kinase inhibition (10 μM) reduces IFNγ-induced phosphorylation of STAT1 and STAT5 (PP1 does not) and also significantly reduces the increased transepithelial flux of HRP (mean ± s.d.; $n = 3$ monolayers/treatment/experiment, representative of four experiments; *$P < 0.05$ compared with control unstimulated (Unstim) monolayers).

the pan-PI3K inhibitor LY294002 (20 μM) (Figure 8b). Furthermore, the induction and release of the IFNγ STAT1-dependent cytokine, IP-10, was not inhibited in T84 cells co-treated with LY294002 (control = 0.06 ± 0.02, IFNγ treated = 13.7 ± 0.5 and IFNγ + LY294002 treated = 11.9 ± 1.6 ng/ml; $n = 3$).

DISCUSSION

Analyses of human enteric epithelial cell lines and murine models of gut dysfunction reveal that IFNγ can significantly increase epithelial permeability.[27,28] As the intestinal epithelium is the primary interface between the body and the external environment, IFNγ-induced decreases in epithelial barrier function would allow potentially dangerous stimuli (eg, low-grade pathogens, commensal bacteria, unprocessed antigen) into the mucosa that could precipitate or exaggerate existing inflammation. Consequently, knowledge of the epithelial intracellular signalling events engaged by IFNγ that reduce barrier function could allow for the formulation of therapies to limit the increases in gut permeability, with the advantage of leaving the impact of IFNγ on immune cells intact.

The ability of IFNγ to increase epithelial permeability was demonstrated ∼20 years ago.[27] Since then, substantial data have accumulated showing that IFNγ increases epithelial paracellular permeability (evidenced by increased flux of
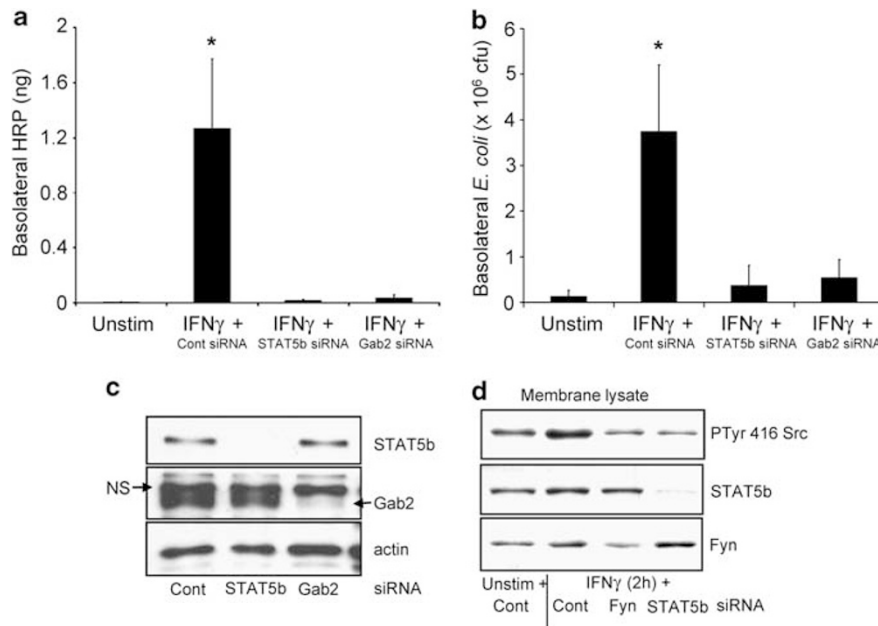
**Figure 5** STAT5b and Gab2 are required to observe IFNγ-induced increases in T84 epithelial monolayer macromolecular permeability. The bar graphs in (**a**, **b**) show that IFNγ (10 ng/ml; 48 h) + scrambled siRNA (Cont) resulted in significant increases in HRP fluxes and *E. coli* (HB101) translocation across confluent T84 monolayers that was blocked by use of STAT5b and Gab2 targeting siRNA (mean ± s.d.; $n = 3$ monolayers/treatment/experiment, representative of four experiments; *$P < 0.05$ compared with control (Unstim); starting TER in these experiments ranged 1200–1600 Ohms/cm^2). (**c**) Reduced expression of STAT5b and Gab2 protein in whole cell extracts of T84 cells treated with specific targeting siRNAs that was not observed in cells treated with an irrelevant scrambled (Cont) siRNA. (**d**) siRNA targeting Fyn or STAT5b reduced IFNγ-stimulated (2 h) membrane-associated activated Fyn in T84 cells. (**c**, **d**) Images are representative of three experiments. NS, nonspecific bands.

small inert molecules and loss or internalization of TJ proteins), that a considerable time lag (24–48 h) exists between IFNγ exposure and reduced epithelial integrity and that apoptosis is not a major cause of the increased epithelial permeability.[6,29] However, there remains a lack of information on the intracellular signalling events proximal to the IFNγR that induce IFNγ-mediated increases of epithelial permeability.

JAK/STAT1 signalling is recognized as an important component of IFNγ-stimulated gene expression.[30] STAT1-independent activities of IFNγ have been described and PI3K has emerged as an important alternative signalling molecule that may also cross-talk with the JAK/STAT1 pathway.[31] Earlier pharmacological analysis,[21] corroborated by current siRNA studies, show that STAT1 does not have a significant role in the IFNγ-induced decreases in TER of monolayers of T84 cells. In contrast, STAT1 knockdown reduced IFNγ-driven increases in the translocation of HRP, indicating a role in the control of the barrier function to macromolecules, and is consistent with the central activity that STAT1 has in IFNγ signal transduction. However, ablation of STAT1 in epithelial cells would have a huge impact on their function, and thus we sought to explore a tangential pathway that would link the IFNγR to the PI3K pathway, which has been shown to mediate IFNγ-induced increases in epithelial permeability.[10,12,32]

Our data presented in this study suggest a novel mechanism for IFNγ-mediated activation of PI3K, involving interactions between STAT5b, Gab2 and the Src kinase Fyn (summarized in Figure 9). Whereas STAT5b can be phosphorylated in monocyte and T-cell lines in response to IFNγ,[33] here we show activation of STAT5, a previously undocumented activity of IFNγ in intestinal epithelial cells. This STAT5 phosphorylation in response to IFNγ was inhibited by use of the broad-spectrum JAK kinase inhibitor, AG490, and was also STAT1 dependent, being significantly reduced by STAT1-specific siRNA. These findings suggest a crucial requirement for STAT1 in transducing cellular responses to IFNγ, corroborating the view presented by others.[34] Moreover, siRNA knockdown of STAT5b (and the use of AG409) resulted in a restoration of T84 cell barrier integrity, preventing IFNγ-stimulated translocation of both macromolecules (HRP) and microbes (*E. coli* HB101), comparable with that achieved by pharmacological inhibition of PI3K activity. Gab2 is a scaffolding adaptor previously shown to mediate STAT5-dependent PI3K activation in hematopoietic cell lines.[25] Aside from a putative STAT5-binding site, Gab2 possesses multiple protein-binding motifs such as Src homology (SH)2 and SH3 domains, proline-rich repeats suitable for interaction with the p85α moiety of PI3K, as well as multiple tyrosine phosphorylation sites that may be targeted by kinases including Src family members.[35,36] Use of
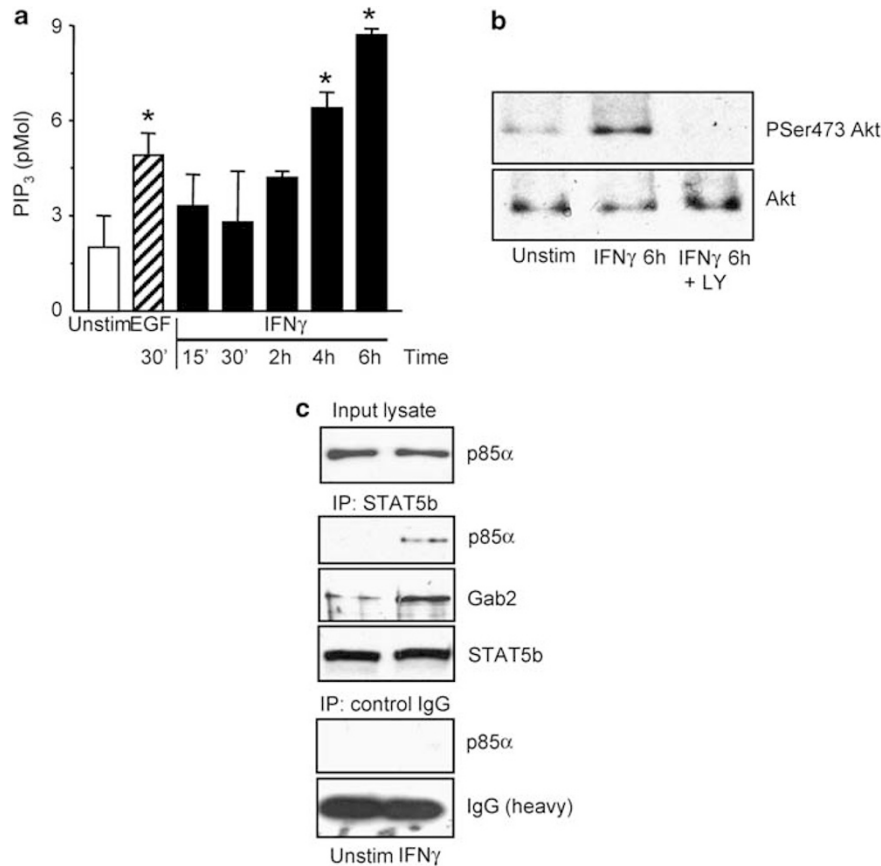
**Figure 6** Treatment with IFNγ evokes formation of a STAT5b/Gab2/PI3K(p85α) complex in T84 epithelia. (**a**) Measurement of PIP$_3$ and (**b**) phosphorylation of AKT following IFNγ (10 ng/ml) indicates activation of PI3K (epidermal growth factor (EGF) at 10 ng/ml; mean ± s.d. of three epithelial preparations/time point; *P<0.05 compared with control (Cont); immunoblotting of phosphor-Ser473 Akt at 6 h post-IFNγ treatment; LY denotes co-treatment of IFNγ-stimulated cultures with 20 μM of the PI3K inhibitor, LY2940). (**c**) Representative immunoprecipitations using STAT5b antibody show increased association of STAT5b/Gab2/PI3K(p85α) at 6 h after treatment with IFNγ. Input and nonimmune controls are shown.

Gab2 siRNA prevented the IFNγ-stimulated increase in T84 monolayer permeability, and knockdown of either Gab2 or STAT5 reduced phosphorylation of the PI3K(p85α), suggesting that interaction of Gab2 and STAT5 is required for activation of PI3K following exposure to IFNγ.

The Src kinases are expressed variably across tissue and cell types, with robust expression of the Fyn, Yes and c-Src isoforms in most cell types including epithelia. Of relevance to our study, Src kinases have been implicated as activators of STAT5 in studies of transformed breast epithelial cell lines.[37–39] Several Src isoforms can interact with adhesion molecules including integrins[40] and E-cadherin,[41] potentially having a role in cell–cell interactions. Src activity has been implicated in the phosphorylation of occludin and its interaction with ZO-1,[42,43] and in IFNγ- and oxidative stress-stimulated reductions in active Cl$^-$ secretion.[18,20] Fyn kinase colocalizes with and phosphorylates both p120-catenin and β-catenin, critical constituents of the cadherin–catenin complex within epithelial cell adherens junctions.[44,45] Furthermore, Fyn has been implicated during enteropathogenic *E. coli* infection, complexing with the *E. coli* receptor in

activation of pedestal formation.[46,47] Thus, a putative role for Fyn as a potential mediator of cytokine-stimulated barrier permeability merited further exploration.

Ablation of Fyn activity by either PP1 (30 nM is highly selective for inhibition of Fyn kinases) or siRNA did not affect the ability of IFNγ to reduce TER, nor did it appreciably affect STAT1 or STAT5 phosphorylation, but it almost completed abolished the concomitant increased transcytosis of HRP and *E. coli*. This suggests recruitment of Fyn after STAT activation and this is substantiated by IP showing Fyn in complex with IFNγR2, the major signalling component of the IFNγR, within 15 min of IFNγ addition to T84 epithelia. Moreover, when applied after IFNγ, PP1 still reduced the barrier defect and this is compatible with the delay in obvious PI3K activation following exposure to IFNγ. Inhibition of Fyn activity reduced phosphorylation of the PI3K-dependent kinase Akt, indicating a functional interaction between Fyn and PI3K. Indeed, blockade of Fyn resulted in a degree of inhibition of IFNγ-induced increases in macromolecular permeability that was not significantly different from that achieved by LY294002 inhibition of PI3K activity. These data
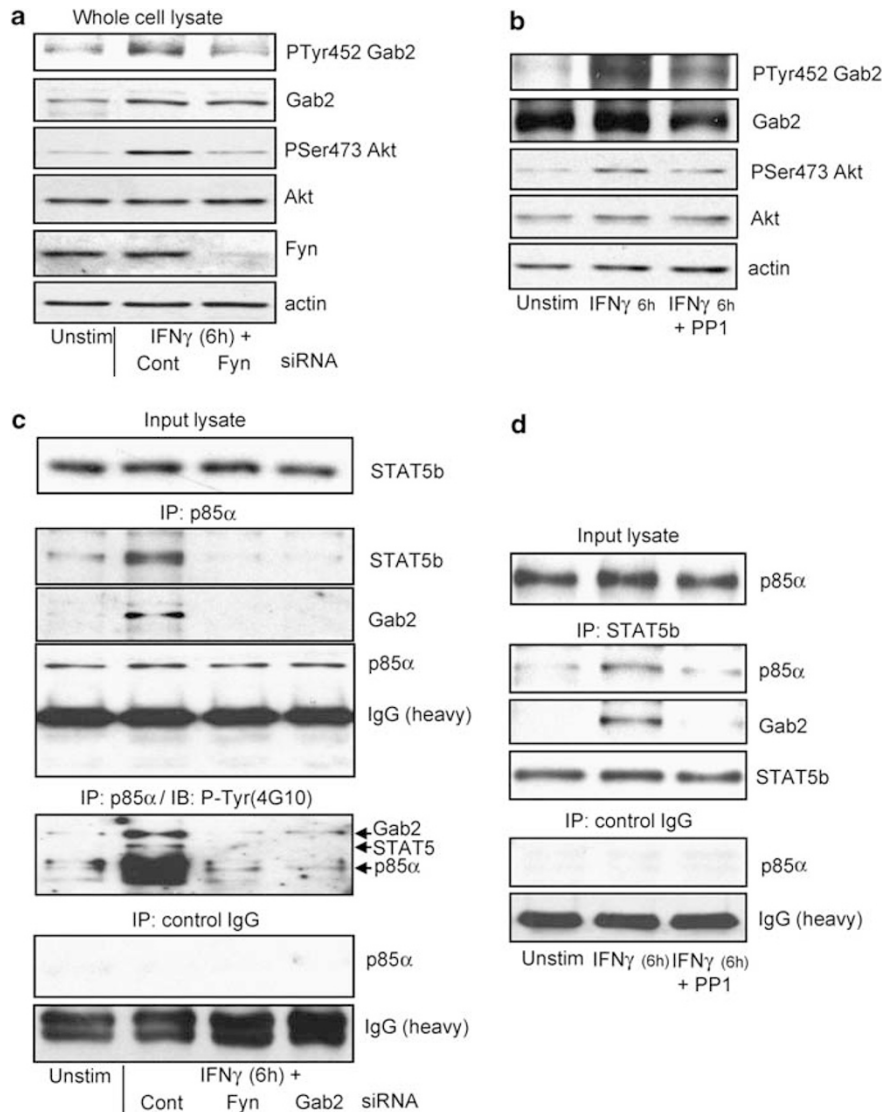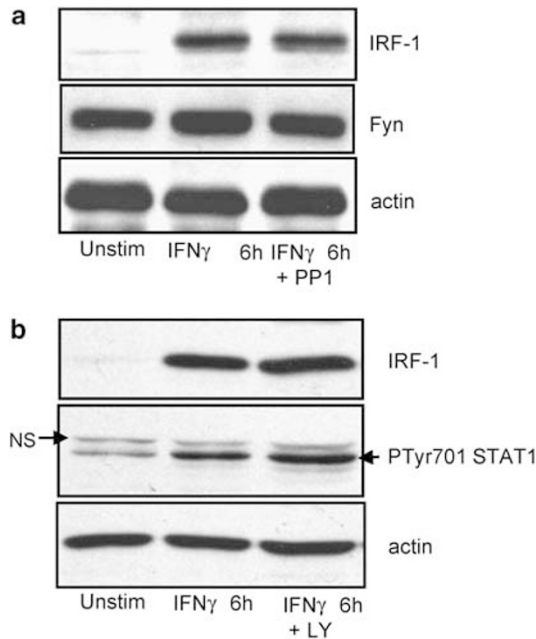
Figure 7 (**a**) Immunoblots of T84 epithelial cell whole cell lysates demonstrate Gab2 was phosphorylated following 6 h of IFNγ stimulation and this was inhibited by transfection of Fyn-targeting siRNA. Levels of IFNγ-stimulated phospho-Akt were also reduced by Fyn siRNA. (**b**) IFNγ-stimulated increases of Gab2 and Akt phosphorylation were reduced by the Src inhibitor PP1 (125 nM, 90 min post-stimulation). (**c**) Treatment of T84 cells with Fyn or Gab2 siRNA reduces PI3K(p85α) and Gab2 interaction with STAT5b at 6 h after treatment with IFNγ (10 ng/ml; images are representative of two experiments). Fyn- and Gab2-siRNA-treated cells demonstrated reduced IFNγ-stimulated tyrosine phosphorylation of products putatively identified as STAT5, Gab2 and p85. (**d**) Reduced association of STAT5b with p85α and Gab2 in STAT5b co-immunoprecipitates following IFNγ stimulation (6 h) in the presence of PP1. Input and nonimmune controls are shown.

indicate that changes in TER are not fully representative of epithelial barrier function and suggest, but do not prove, a Fyn–PI3K axis in the regulation of macromolecule flux across enteric epithelia.

Furthermore, we observed Gab2 phosphorylation and recruitment to STAT5b/PI3K(p85α) complexes in an IFNγ-inducible, Fyn-dependent manner in T84 cells. Co-immunoprecipitations following IFNγ treatment indicated physical association between PI3K(p85α), STAT5 and Gab2 and that this was disrupted by Fyn knockdown. These data are strong evidence of Fyn lying upstream of PI3K during IFNγ signalling responses that regulate epithelial permeability

and suggest a pivotal role for STAT5b, Gab2 and Fyn kinase in the mediation of IFNγ-evoked increases in the transcytosis of macromolecules and microbes across an enteric epithelium. The divergence in the ability of siRNA knockdown of Gab2 and STAT5 and inhibition of Fyn activity in IFNγ control of TER and macromolecule transcytosis is noteworthy and suggest that at least a portion of the increased HRP flux and *E. coli* transcytosis is dependent on a trans-cellular pathway. Others have shown that IFNγ promoted the transcytosis of *E. coli* (strain C25) across Caco2 epithelial monolayers and that this preceded any drop in TER.[9] Having uncovered evidence of Fyn–PI3K interaction, it is interesting

Figure 8 (**a**) Immunoblots of T84 epithelial cell whole cell extracts show that the treatment with Src kinase inhibitor PP1 (125 nM) did not decrease the expression of the IFNγ-responsive gene *IRF-1* following 6 h of treatment with IFNγ (10 ng/ml). (**b**) IRF-1 expression induced by IFNγ was not decreased by treatment with the PI3K inhibitor LY294002 (20 μM). NS, nonspecific band.

that PI3K inhibition blocked the IFNγ-induced drop in TER, whereas Src inhibition with PP1 did not. This may reflect differences in the efficacy of the pharmacologics but could also reflect differences in the signalling pathways that govern paracellular and transcellular permeability. Much remains to be done to precisely elucidate the IFNγ signalling events that control epithelial barrier function, and whereas others have focused on defining TJ protein and cytoskeletal changes,[5–8] the present investigation is in accordance with IFNγ modulation of epithelial endocytosis and transcellular permeability.[10]

The delay in PI3K activity relative to initial STAT phosphorylation is intriguing and suggests mobilization of temporally and spatially distinct signalling pathways, a hypothesis supported by data showing that in T84 cells, neither Src kinase nor PI3K inhibition affected STAT1-dependent immune activities (IRF-1 expression and IP-10 secretion). It is also noteworthy that STAT1 and STAT5 activation is slower and reduced in magnitude in epithelia compared with macrophages (Figure 4b) and this could contribute to a delayed accumulation of other intracellular signals in the enterocyte, and ultimately to the 24–48 h lag period observed between IFNγ treatment and increases in T84 epithelial monolayer permeability. These findings suggest that the immunostimulatory and barrier effects of
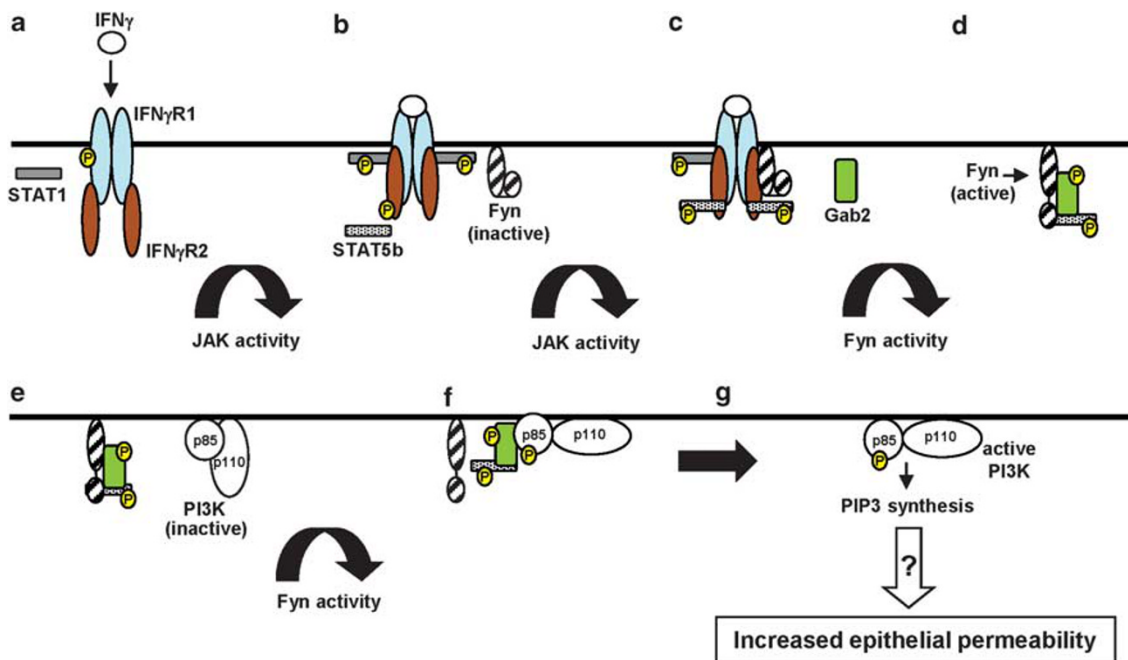
Figure 9 Proposed model of signalling at the IFNγR that control enteric epithelial macromolecular permeability. (**a**) Ligation of the IFNγR stimulates JAK-dependent phosphorylation of IFNγR1 resulting in rapid STAT1 recruitment and phosphorylation (**b**) that allows for a JAK-dependent recruitment and phosphorylation of STAT5b at IFNγR2 along with Fyn Src kinase (**c**). The Gab2 scaffolding protein is recruited to STAT5b and phosphorylated via Fyn, leading to a STAT5b/Gab2 complex that may remain associated with Fyn (**d**). Within 2–6 h, PI3K (p85 recruitment/regulatory domain and p110 catalytic domain) is recruited to Gab2 (**e**), and p85 phosphorylation, possibly via Fyn, relieves inhibition of the catalytic subunit (**f**). The active PI3K is then available to mobilize membrane lipids and a putative host of downstream enzymes that can lead to decreased epithelial barrier function (**g**). P, phosphorylated residue.

IFNγ can be targeted independently. Deficiencies in IFNγR, STAT1 or IRF-1 expression can all result in increased susceptibility to infectious organisms, particularly at mucosal surfaces.[48] Thus, the ability to target the STAT5b/Gab2/Fyn/PI3K pathway in enteric epithelia to preserve the barrier function of the epithelium, while not interfering with the ability of IFNγ to mobilize STAT1-dependent immune events in immune and stromal cells, could be an advantageous therapeutic approach.

In conclusion, using T84 cells as model gut epithelium, data are presented illustrative of a STAT5b/Gab2/Fyn/PI3K interaction that is dependent of STAT1 (perhaps as a prerequisite to allow STAT5/IFNγR interaction) and operates in parallel with STAT1 signal transduction events. When this pathway is inhibited, the ability of IFNγ to decrease the barrier function of epithelial monolayers, as measured by macromolecule and microbe transcytosis, was reduced. Such a specific intervention in the activity of IFNγ at the level of the epithelium in treating gut disease would have the concomitant advantage of preserving immunostimulatory activities of IFNγ on structural and immune cell populations.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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