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Role of ERK/mTOR signaling in TGF β -modulated focal adhesion kinase mRNA stability and protein synthesis in cultured rat IEC-6 intestinal epithelial cells

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Abstract

Increasing evidence is available showing the importance of the FAK (focal adhesion kinase) protein level in the migration and homeostasis of intestinal cells. TGF β (transforming growth factor beta) modulates FAK protein expression in a complex fashion not only by inducing the activation of p38 and Smad signaling resulting in increased *fak* promoter activity and increased FAK protein levels, but also by activating ERK (extracellular signal regulated kinases), p38, and the Smad pathway. We show that the blockade of ERK signaling by a specific MEK (MAPK kinase) inhibitor attenuates TGF β -induced FAK mRNA stability and reduces FAK protein levels in rat IEC-6 intestinal epithelial cells. The mTOR (mammalian target of rapamycin)-specific inhibitor rapamycin and small interfering RNAs for mTOR and p70^{S6} kinase also block TGF β -induced FAK protein synthesis. Furthermore, we have found that a TGF β -induced increase in wound closures in monolayers of these cells is abolished in the presence ERK or mTOR inhibition. Thus, TGF β also modulates FAK protein levels in cultured rat IEC-6 intestinal epithelial cells via ERK activation, acting at the transcriptional level to complement Smad signaling and at on the translational level via the mTOR pathway downstream of ERK, which in turn promotes intestinal epithelial cell migration.

Keywords

Transforming growth factor β (TGF β); Focal adhesion kinase(FAK); Extracellular signal regulated kinases (ERK); Mammalian target of rapamycin (mTOR); mRNA; Rat IEC-6 intestinal epithelial cells

Introduction

The mammalian intestinal epithelium constantly regulates the balance of its cell production and cell loss, with complete mucosal renewal in less than a week (Hall et al. 1994). Such mechanisms are integral not only to normal mucosal biology, but also to pathological processes such as ulcer healing and neoplastic transformation in the gastrointestinal mucosa. The transforming growth factor β (TGF β) pathway plays a major role in the maintenance of epithelial homeostasis (Tanigawa et al. 2005; Sturm and Dignass 2008).

TGF β has been reported to promote the migration and arrest of the growth of proliferating intestinal cells and to induce their terminally differentiated state, acting here as a tumor suppressor (Barnard et al. 1989, 1991; Sancho et al. 2004). This factor may also promote epithelial cell monolayer restitution after injury by stimulating migration (Tanigawa et al. 2005). TGF β classically signals through the canonical Smad signal pathway to elicit transcriptional responses. However, increasing evidence demonstrates the importance of non-Smad TGF β -induced signaling mechanisms that complement the canonical Smad pathway (Moustakas and Heldin 2005; Lamouille and Derynck 2007). Such non-Smad signals include the extracellular signal regulated kinases (ERK) and p38 mitogen-activated protein kinases (MAPK) in intestinal cells (Hartsough and Mulder 1995) with accumulating evidence for cross-talk between them and with the Smad-mediated pathway (Yue et al. 1999; Walsh et al. 2003).

The intracellular tyrosine kinase FAK (focal adhesion kinase) is found prominently within focal adhesions and serves as a convergent integration point in adhesion and cell migration between integrin signaling from the extracellular matrix (ECM) and receptor-mediated signaling activated by growth factors such as TGF β (Parsons et al. 2000; Mitra et al. 2005). Tyrosine phosphorylation of FAK enables FAK binding to other signaling molecules via SH2 and SH3 (Src homology 2 and 3) domains and has been linked to the regulation of diverse cellular events including cell survival, motility, and cell cycle progression (Hanks et al. 2003; Parsons 2003). Integrin-mediated FAK phosphorylation may be necessary for the facilitation of human differentiated intestinal epithelial cell survival by ERK signaling (Bouchard et al. 2007). FAK phosphorylation is also able to suppress apoptosis induced by TNF- α (tumor necrosis factor alpha) and cycloheximide in rat intestinal IEC-6 cells (Zhang et al. 2006). Thus, most studies have focused on understanding the activation of FAK at the posttranslational level. However, increasing evidence demonstrates the importance of small changes in FAK mRNA and protein levels in, for example, migrating intestinal epithelial cells (Yu et al. 2000; Basson et al. 2006), human gastric and colonic ulcers (Walsh et al. 2008b), and human colon cancer cells (Nakagawa et al. 1998; Agochiya et al. 1999).

TGF β increases FAK at the protein level via independent Smad and p38 MAPK signals (Walsh et al. 2008b). Indeed, TGF β seems to induce *fak* transcription effect by inducing *fak* promoter activity through several Smad-binding elements in the *fak* promoter sequence in a Smad-dependent fashion. However, whether ERK signaling by TGF β also influences FAK levels is not known.

The ERK pathway is invoked in various cell types by diverse extracellular stimuli. Stimulation of this pathway, downstream of Ras, activates a series of protein kinases and finally activates ERK1/2, which in turn phosphorylates downstream targets involved in cellular events such as cell proliferation, differentiation, survival, and motility (Kohn and Pouyssegur 2006). TGF β rapidly induces ERK1 activity in intestinal epithelial cells, and this inhibits growth (Hartsough and Mulder 1995). However, the final growth outcome of epithelial cells may be modulated by synergistic pathways (Hartsough and Mulder 1995). Recent studies suggest that ERK modulates translation, in addition to its more classical influence on transcription through the mammalian target of rapamycin (mTOR) pathway (Ma et al. 2005). Targets for mTOR are proteins involved in controlling mRNA translation, including the ribosomal protein S6 kinases (p70^{S6K}) and the initiation factor 4E-binding proteins (4E-BPs; Averous and Proud 2006). mTOR signaling is inhibited by the tumor suppressors TSC1/2, and ERK-dependent phosphorylation of TSC1/2 leads to its dissociation from mTOR enabling translation in cooperation with the PI3K (phosphatidylinositol 3-kinase)/Akt pathway (Ma et al. 2005). Furthermore, recent studies link mTOR signaling with intestinal cell migration by amino acid stimulation in a PI3K-dependent fashion (Rhoads et al. 2006). Interestingly, another recent study has connected the overexpression of the adaptor protein Eps8 to the upregulation of FAK expression via mTOR activation in human colon cancer cell lines (Maa et al. 2007).

In light of previous data showing that TGF β stimulates *fak* promoter activity and FAK protein expression, and against the background of current studies demonstrating mechanistic links between TGF β , ERK, and mTOR in the translational control of the regulation of gene expression, which in turn is crucial for cell growth, survival, and homeostasis (Mamane et al. 2006), we have sought to determine whether ERK and mTOR are involved in the TGF β regulation of FAK expression in cultured rat IEC-6 intestinal epithelial cells. We have observed that TGF β treatment results in the rapid phosphorylation of mTOR and its effectors p70^{S6K} and 4E-BP1 (initiation factor 4E-binding proteins), whereas inhibition of mTOR activation results in a reduced FAK protein level and wound closures. Consistent with this observation, ERK inhibition blocks mTOR activation, which in turn abolishes the stimulation of FAK protein levels and wound closures by TGF β . Therefore, the TGF β -induced FAK protein level modulation via ERK/mTOR promotes rat intestinal epithelial IEC-6 cell migration. Furthermore, we have found that FAK mRNA stability increases with TGF β treatment in an ERK-dependent manner. Taken together with our previous observations (a, Walsh et al. 2008b), these results trace divergent and reconvergent TGF β pathways inducing transcriptional regulation through ERK complementing Smad signaling and translational regulation through ERK/mTOR signaling for the modulation of FAK expression implicated as being important for epithelial intestinal cell homeostasis and migration.

Materials and methods

Cell culture

IEC-6 cells, originally derived from rat jejunum (Quaroni et al. 1979), were purchased from the American Tissue Culture Collection (Rockville, Md.) and maintained by following ATCC recommendations. Cells were cultured in tissue culture flasks and were studied in dishes pre-coated with type I Collagen (Sigma, St. Louis, Mo.) as previously described (Basson et al. 1992). Subconfluent cells were serum-deprived for 6 h before treatment with TGF β 1 (1 ng/ml: R&D Systems, Minneapolis, Minn.; Sigma) in medium containing 0.5% FBS (fetal bovine serum) for a further 24 h before lysis. For pharmacological signal inhibition, cells were pre-treated with PD98059 (10 μ M), actinomycin D (10 μ g/ml), cycloheximide (5 μ g/ml), rapamycin (5–100 ng/ml), or appropriate vehicle controls for 1 h before the addition of TGF β . In all cases, appropriate vehicle controls were used where appropriate. TGF β was dissolved in a stock solution of 4 mM HCl, 0.2% BSA (bovine serum albumin) solution following manufacturer's suggestion and then used at a 1:20,000 dilution in cell culture medium for actual experimentation. This resulted in changing the composition of the cell culture medium by effectively adding 0.08 μ M HCl and 0.00004% BSA. The pH of the HEPES-buffered cell culture medium did not change discernibly after the addition of this vehicle in preliminary studies, and the addition of 0.00004% BSA to the culture medium, which contained 0.5% FBS, was felt not to be biologically significant. Accordingly, a separate vehicle control was not used for the addition of TGF β .

Migration assay

A circular wound model was used for the quantification of epithelial sheet migration. We used a pipette tip to create small circular wounds in confluent IEC-C cell monolayers on collagen I substrates and measured wound closure by quantification of photomicrographs of the original wound and of the same wound after 24 h. Images obtained were analyzed with the Kodak 1D Scientific Imaging computer program.

Western blot analysis

Cells were rinsed twice with ice-cold phosphate-buffered saline and then lysed in buffer containing 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml aprotinin, and 1 mM sodium ortho vanadate (all from Sigma). Equal amounts of protein (10–20 μ g; BCA, Pierce, Rockford, Ill.) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After being blocked, membranes were incubated with the following antibodies overnight: total FAK (clone 4.47, Upstate Biotechnology, Mass.), total p70^{S6K} (Upstate Biotechnology), total mTOR, total p44/42 MAP kinase, phospho-mTOR, phospho- p70^{S6K}, and phospho-p44/42 MAPK (all Cell Signaling Technology, Mass.), with α -tubulin (EMD Chemicals, Gibbstown, N.J.), or D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biodesign, Saco, Me.) as loading controls. After incubation with the appropriate secondary antibody, protein bands were visualized by enhanced chemiluminescence (ECL, GE Health Care, Little Chalfont, Buckinghamshire, England). Imaging and analysis of band density were performed on a Kodak 440CF Image Station. All exposures used for densitometric analysis were within the linear range of the system.

Transfection of small interfering RNA

To inhibit mTOR and p70^{S6K} expression, IEC-6 cells were transfected with small interfering RNA (siRNA). The siRNA duplexes targeted to rat mTOR (simTOR: 5' CCC AGC UUU GUC AUG CCU dT dT 3') and to p70^{S6K} (GGA GAA CUA TTT AUG CAG UUA dT dT 3') and their complementary RNA strands harboring a dT dT tail at the 3' end were synthesized by Dharmacon (Lafayette, Colo.). Non-targeted siRNA duplex (NT1) used as a control was purchased from Dharmacon. One day prior transfection, cells were seeded in type-I-collagen-coated six-well plates (2×10⁵ cells/well). Transfection of siRNAs was performed by using Oligofectamine and Plus Reagent (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, cells were subjected to Western blot analysis.

Dual luciferase reporter assay for FAK promoter activity

To assemble the FAK promoter-luciferase construct, we first obtained a human genomic DNA-BAC clone from BACPAC Resources (Children's Hospital of the Oakland Research Institute, Oakland, Calif.) with an insert containing the human FAK promoter, exon 1, and intron 1 sequences as indicated in the GenBank data base. The BAC plasmid was cut with *SacI* and *StuI* (New England Biolabs, Ipswich, Mass.) releasing a fragment of 1.65 kb that was subcloned into the pBluescript II SK (+) vector (Stratagene, La Jolla, Calif.) for amplification. The pBSK-FAK-promoter clone was subsequently cut with *NarI*, end-filled, and then cut with *KpnI* (New England Biolabs) to produce a 1.3-kb fragment that was subcloned into a pGL2 Basic vector (Promega, Madison, Wis.) to generate the FAK-promoter-luciferase construct used in the Dual Luciferase Reporter Assay (Promega). The 1.3-kb fragment was sequenced by using T3 and T7 primers (New England Biolabs) to verify that it matched the FAK genomic sequence in GenBank (not shown). For transfections, cells were seeded in type-I-collagen-coated six-well plates (2×10⁵ cells/well) one day before transfection with the pGL2-fused FAK promoter construct by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For normalization of luciferase activity, a pRL-TK control vector containing the *Herpes simplex* virus thymidine kinase promoter encoding *Renilla* luciferase was used together with the pGL2 basic and recombinant construct. After 24 h, cells were analyzed by using the dual luciferase reporter assay system kit according to the manufacturer's instructions (Promega). Luminescence was measured with an AutoLumat Plus LB 953 Luminometer (Berthold Technologies, Germany).

Real-time reverse transcription with polymerase chain reaction for FAK mRNA expression

Reverse transcription with polymerase chain reaction (RT-PCR) for FAK was performed as described previously (Basson et al. 2006). Briefly, RNA was isolated from IEC-6 cells, after treatment with PD98059 (10 μM), actinomycin D (10 μg/ml), and respective control agent for 1 h before the addition of TGFβ, by using Trizol reagent (Invitrogen). Total RNA was used for the synthesis of first-strand cDNA by using the Super Script III First Strand Synthesis Super Mix for qRT-PCR (Invitrogen). RT-PCR was performed with rat FAK primer pairs giving a 167-bp product and for ribosomal gene (18S) designed with the help of MIT Prime3 online primer designer protocol. The primers for FAK, viz., sense: 5' ATT

GCT GCC TCG GAA TGT TCT 3' and antisense: 5' GCT GAG GTA AAA CGT CGA AAA 3', and the primers for 18S, viz., sense: 5' CGG CTA CCA CAT CCA AGG AA 3' and antisense: 5' GCT CGA ATT ACC GCG GCT 3' (Integrated DNA Technologies, Coralville, Iowa), were used for the PCR amplification with SYBR Green PCR Master Mix (Applied Biosystems) by using the ABI 7700 Sequence Detection System. Relative FAK mRNA levels were determined by the comparative CT Method (Applied Biosystems User Manual) after ascertaining that the efficiencies of the control 18S and FAK primers were similar. The resulting number of mRNA molecules was calculated by negative log transformation of the differences between CT for 18S and FAK.

Statistical analysis

Results are expressed as mean±SEM. The statistical significance of differences between means of samples in each experiment was assessed by a Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

TGFβ-mediated increase of FAK protein level is abolished by the MEK inhibitor PD98059, but not fak promoter activity in IEC-6 cells

Although TGFβ alters FAK levels in IEC-6 cells via a Smad-dependent and p38-dependent induction of *fak* promoter activity, TGFβ can also signal via ERK (Blanchette et al. 2001; Sanchez-Capelo 2005). We therefore examined the effect of ERK blockade on TGFβ induction of FAK, pretreating subconfluent cells for 1 h with the ERK kinase (MEK) inhibitor PD98059 at various concentrations. As shown in Fig. 1a, FAK protein levels increased similarly after a 24-h treatment with 0.1, 1, or 10 ng/ml TGFβ compared with dimethylsulfoxide (DMSO)-treated controls (143–162±17–23%, **P*=0.03, *n*=3). TGFβ stimulated ERK phosphorylation similarly over the range of 0.1, 1, and 10 ng/ml (Fig. 1b, bars 1–4, **P* 0.01, *n*=3). ERK blockade by the MEK inhibitor PD98059 over the range of 1–20 μM prevented TGFβ-stimulated ERK phosphorylation (Fig. 1b, bars 5–10, *n*=3), suggesting that ERK contributed to the effects of TGFβ on FAK protein levels.

Since the blocking of Smad or p38 signaling prevented the induction of *fak* promoter activity by TGFβ, we further investigated whether ERK signaling was also necessary for FAK induction at the transcriptional level. IEC-6 cells transfected with a pGL2-FAK promoter luciferase construct exhibited an ~1.5-fold increase in luciferase activity after TGFβ treatment, which was in the range (less than twofold) of another published observation of TNF-α-induced FAK promoter activity (Golubovskaya et al. 2004). However, transfected cells pretreated with the MEK inhibitor exhibited a similar ~1.6-fold increase in luciferase activity in response to TGFβ (Fig. 2, *P*=0.007 vs. non-treated respective control, *n*=4 for each). Therefore, the induction of *fak* promoter activity under TGFβ control seemed to be ERK-independent, whereas FAK protein expression required ERK activity.

FAK mRNA stability, but not protein stability, was rescued by TGF β treatment dependent on ERK signaling in IEC-6 cells

TGF β regulates the mRNA stability of genes such as *Furin* (Blanchette et al. 2001), *PTHrP* (Sellers et al. 2002), and *PTEN* (Chow et al. 2007). We therefore investigated whether TGF β affected FAK mRNA stability. FAK mRNA stability in IEC-6 cells was measured after treatment with TGF β , the MEK inhibitor, and actinomycin D, a transcriptional inhibitor. RT-PCR analysis of the samples (Fig. 3a) demonstrated that the addition of TGF β resulted in a twofold (2.3-fold) increase in FAK mRNA stability, increasing the half-life from 22 min to 50 min. The amount of FAK mRNA at 30 min after transcriptional blockade with actinomycin D was significantly higher in DMSO-vehicle-treated cells exposed to TGF β compared with the amount of residual FAK mRNA in control cells treated with the vehicle alone ($n=4$, $*P=0.003$). In contrast, the amount of residual FAK mRNA in cells treated with PD98059 did not differ from vehicle control values regardless of TGF β treatment. Thus, inhibition of MEK signaling negated this TGF β effect on FAK mRNA stability. We next examined whether TGF β also affected FAK protein stability. As shown in Fig. 3b, FAK protein level was highly reduced after a 12-h cycloheximide treatment. Neither TGF β nor PD98059 treatment altered the rate of protein decay after cycloheximide blockade of new protein synthesis. These results suggested that TGF β -mediated ERK signaling contributed to an increase in FAK mRNA stability, but did not prevent FAK protein degradation.

TGF β -mediated increase in FAK protein level is attenuated by inhibition of the mTOR signal pathway in IEC-6 cells

ERK signaling activates the mTOR pathway in some cell systems (Ma et al. 2005; Bermudez et al. 2008). Furthermore, evidence has been provided for a potential translational regulatory effect of mTOR on FAK protein synthesis in the absence of TGF β (Maa et al. 2007). We therefore investigated the effect of mTOR blockade on the TGF β induction of FAK. Although TGF β -treated control cells in this experiment exhibited a $33\pm 2\%$ increase in FAK protein ($n=6$, $P=0.05$), the mTOR-specific blocker rapamycin (5–100 nM) prevented the TGF β stimulation of FAK protein expression (Fig. 4a). We next utilized specific siRNA to reduce mTOR or its common downstream effector p70^{S6K} (Fig. 4b–c). Transfection of IEC-6 cells with 100 nM siRNA for mTOR decreased mTOR protein to $47\pm 4\%$ of that in the non-targeting siRNA (NT1)-treated control cells ($P=0.0004$), whereas transfection with 100 nM siRNA for p70^{S6K} decreased p70^{S6K} to $46\pm 9\%$ of control levels ($P=0.007$). As shown in Fig. 4d–e, TGF β treatment in control transfections (NT1 siRNA) increased FAK protein levels by $60\pm 4\%$ ($n=5$, $*P=0.008$) over the basal level. Reduction of mTOR or its effector p70^{S6K} with 100 nM of specific siRNA completely prevented the increase in FAK protein expression caused by TGF β .

TGF β -stimulated phosphorylation of mTOR and its targets p70^{S6} kinase and 4E-BP1 kinase

Increasing evidence demonstrates that TGF β can increase protein synthesis through the mTOR pathway (Le Pabic et al. 2005; Lamouille and Derynck 2007), and indeed, mTOR signaling in response to other stimuli has been implicated in the control of protein synthesis through the phosphorylation of p70^{S6} kinase and 4E-BP1 kinase (Averous and Proud 2006; Mamane et al. 2006). Because TGF β increases FAK protein synthesis, we explored the

connection of TGF β with mTOR and its effectors p70^{S6} kinase and 4E-BP1 kinase. TGF β induced activation of mTOR, p70^{S6} kinase, and 4E-BP1 (Fig. 5) within 5 min, with maximum phosphorylation (1.19-fold increase) of mTOR (Fig. 5a) within 5 min ($n=14$, $*P<0.05$, $^{\#}P=0.001$). TGF β induced the phosphorylation of 4E-BP1 (Fig. 5b) with a peak (1.19-fold increase) at 15 min ($n=13$, $*P<0.04$), and the phosphorylation of p70^{S6} kinase (Fig. 5c) with a peak (1.18-fold increase) at 30 min ($n=14$, $*P<0.02$), corroborating the downstream signaling effect on translation for mTOR controlled by TGF β .

TGF β -stimulated activation of mTOR signaling is mediated by ERK signaling in IEC-6 cells

To investigate further the involvement of the ERK pathway in TGF β -stimulated mTOR signaling, we added the MEK inhibitor PD98059 to cultured IEC-6 cells treated with TGF β for 5 min. As shown in Fig. 6, the MEK inhibitor significantly blocked the increase of mTOR, p70^{S6} kinase, and 4E-BP1 kinase phosphorylation induced by TGF β treatment. These results suggested that TGF β -mediated ERK-signaling contributed to an increased FAK protein level via de novo protein synthesis by mTOR signaling.

TGF β -stimulated wound closure requires ERK and mTOR signaling in IEC-6 cells

TGF β has been reported to promote epithelial cell monolayer migration (Tanigawa et al. 2005), and mTOR has been suggested as a downstream effector of this effect (Lamouille and Derynck 2007). Therefore, to investigate further the functional impact of the TGF β -stimulated increase of FAK protein levels mediated by ERK/mTOR signaling, we studied the migration of cultured rat IEC-6 cells on collagen I. As shown in Fig. 7, TGF β treatment (1 ng/ml) significantly stimulated wound closure after 24 h by $40\pm 7.6\%$ compared with similarly wounded monolayers not treated with TGF β ($n=24$ pooled from three separate experiments with similar results, $*P=0.05$; Fig. 7b, c, f, g). ERK blockade with PD98059 or blocking the mTOR pathway with rapamycin abolished this stimulation of wound closure by TGF β (Fig. 7d, e, h, i). This suggested that TGF β promoted the migration of cells in monolayers of rat intestinal epithelial cell line IEC-6 via ERK and mTOR signaling.

Discussion

Although TGF β is known to activate pre-existing FAK, our present results suggest that TGF β induces *fak* transcription in a manner not apparently dependent on ERK. In contrast, TGF β seems likely to act via ERK on FAK mRNA stability and FAK translation in rat IEC-6 intestinal epithelial cells, since the blocking of the ERK pathway completely abolishes the ability of TGF β to increase FAK mRNA stability, protein expression, and migration. Furthermore, the rapid activation of the mTOR translational machinery by TGF β -stimulated ERK activation leads to an increase of de novo FAK protein synthesis in cultured rat intestinal cells.

TGF β increases the expression of numerous proteins involved in growth inhibition, transcription, ECM homeostasis, and motility consistent with the distinct biological responses of TGF β signaling in epithelial cells (Hartsough and Mulder 1995). For instance, TGF β increases protein levels of type-1 inhibitor of plasminogen activator, collagen I, TGF β , and the TGF β -converting enzyme furin in various epithelial cell lines via ERK

(Hayashida et al. 1999; Yue et al. 1999; Blanchette et al. 2001; Kutz et al. 2001). FAK is also a critical kinase involved in cell-matrix interactions during adhesion and motility, and a convergent point for trans-activation by many growth factors that regulate proliferation and transcription. Thus, unsurprisingly, TGF β upregulates FAK protein. Even small changes in FAK protein levels may be critical for the amount of FAK available to be activated by other regulatory stimuli such as cytokines or growth factors. For instance, EGF-stimulated cell motility correlates with 45% increased FAK tyrosine phosphorylation in human gastric epithelial monolayers (Szabo et al. 2002).

Although non-transformed rat IEC-6 intestinal epithelial cells are a common model for the study of intestinal epithelial biology (Rhoads et al. 2006; Nicola et al. 2008), manifest differences exist between the behavior of an established cell line in culture and the responses of cells in vivo subjected to a complex array of neurohumoral, matrix-driven, and other stimuli (Basson et al. 1992). However, in vivo observations suggest that efficient ulcer healing requires the interplay of diverse growth factors including TGF β (Tarnawski et al. 2001). Interrupted TGF β -mediated signaling in Smad3 null mice delays ulcer healing (Owen et al. 2008), whereas human ulcers are associated with distinct patterns of TGF β immunoreactivity that colocalize with similar patterns of immunoreactivity for FAK and phosphorylated FAK (Walsh et al. 2008b). Similar changes in TGF β , FAK, and activated FAK immunoreactivity are also found in migrating intestinal epithelial IEC-6 cell monolayers (Walsh et al. 2008b). Although in vitro observations should only be extrapolated to in vivo biology with caution, such findings, together with our present in vitro results, support a role for TGF β in FAK protein induction in vivo and in vitro and the potential significance of such mechanisms in ulcer healing.

TGF β may influence FAK at several levels. For example, TGF β stimulates FAK³⁹⁷ phosphorylation in hepatocarcinoma cells (Cai et al. 2000), keratinocytes (Jeong and Kim 2004), and intestinal epithelial cells (Walsh et al. 2008a, 2008b). However, TGF β also influences FAK expression in intestinal epithelial cells by stimulating *fak* promoter activity via Smad and p38 MAPK (Walsh et al. 2008a, 2008b). In the present study, we have confirmed that TGF β stimulates the *fak* promoter and demonstrate this stimulation to be independent of ERK.

ERK is rapidly activated by TGF β in intestinal epithelial cells (Hartsough and Mulder 1995). However, the TGF β induction of *fak* transcription appears to be independent of ERK, since the MEK inhibitor does not prevent TGF β stimulation of *fak* promoter activity. On the contrary, inhibition of the ERK pathway alone stimulates *fak* promoter activity equivalent to that of TGF β alone. Although these results make it difficult to rule out a small effect of TGF β -mediated ERK activation on FAK promoter activity, we have been unable to demonstrate such an effect, whereas our results clearly demonstrate an effect of TGF β -mediated ERK activation at other regulatory levels. The increase in basal FAK promoter activity observed here with ERK inhibition is consistent with another recent study from our laboratory examining the complex regulation of FAK promoter activity (Walsh et al. 2008a). One explanation may be that ERK activation increases Smad phosphorylation at sites that prevent the nuclear localization of Smad2/3. Inhibition of this cytosolic restraint by ERK

blockade might explain the increase of basal FAK promoter activity independent of TGF β treatment (Kretzschmar et al. 1999; Cushing et al. 2008).

The regulation of mRNA turnover is a crucial mechanism of the post-transcriptional control of gene expression (Mitchell and Tollervy 2000; Newbury 2006). TGF β can modulate mRNA stability in various ways that may involve *cis*-acting or *trans*-acting elements with a range of transcription factors, depending upon the mRNA in question (Beelman and Parker 1995). For instance, TGF β may enhance parathyroid-hormone-related protein mRNA stability in squamous carcinoma cells via the binding of numerous unidentified mRNA-binding proteins to the terminal coding region (Sellers et al. 2002). Our results suggest that TGF β delays FAK mRNA degradation in an ERK-dependent manner in IEC-6 cells. The FAK mRNA half-life is short, suggesting that the strict control of *fak* gene expression is important biologically. TGF β also influences the mRNA stability of urokinase-type plasminogen activator mRNA stability in transformed epidermal keratinocytes by an ERK-dependent mechanism (Santibanez et al. 2000).

Whereas TGF β influences FAK mRNA stability, our study suggests that TGF β also increases FAK protein levels by stimulating de novo protein synthesis. The mTOR pathway controls de novo protein synthesis in response to signals from mitogenic factors, nutrients, cellular energy levels, and stress. TGF β treatment does not significantly inhibit the protein degradation observed in the presence of cycloheximide. Furthermore, the downstream targets of mTOR signaling, viz., p70^{S6} kinase and 4E-BP1 kinase, are phosphorylated in response to TGF β in an ERK-dependent manner. Treating our cells with the mTOR inhibitor rapamycin and specific siRNA for mTOR and p70^{S6K} completely blocks the TGF β stimulation of FAK protein expression. Furthermore, the mTOR inhibitor rapamycin blocks migration. Despite the TGF β modulation of protein synthesis via mTOR not having previously been reported, this model is consistent with a previous report that ERK signaling is implicated in translational control upstream of mTOR via phosphorylation of TSC2 enabling mTOR signaling (Ma et al. 2005), and with the observation that another cytokine, Cyr61, upregulates hypoxia-inducing factor-1 α protein in gastric cancer cells via mTOR-dependent stimulation of de novo protein synthesis (Lin et al. 2008). Although we have not specifically studied the adaptor protein Eps8 here, Eps8 has also been linked to the upregulation of FAK expression via mTOR activation in human colon cancer cell lines (Maa et al. 2007).

Despite other transcription factors possibly playing a role in the TGF β -independent regulation of FAK, we have observed the rapid activation of mTOR, p70^{S6}, and 4E-BP1 kinase in 5–15 min after TGF β treatment. Such rapid activation is not without precedent. TGF β activates ERK itself in 5 min in epithelial cells (Hartsough and Mulder 1995), activates p70^{S6} kinase within 15 min in cultured human hepatic stellate cells (Le Pabic et al. 2005), and activates mTOR, p70^{S6}, and 4E-BP1 kinase within 1 h in murine mammary epithelial cells (Lamouille and Derynck 2007).

Indeed, this rapid activation of mTOR and its downstream effectors may contribute to the TGF β stabilization of the otherwise highly labile FAK mRNA. Such a model would be consistent with the previous demonstration that IGF-1 stimulation increases survivin protein

expression in prostate cancer cells by stabilization and translation of a pool of survivin mRNA through the mTOR pathway (Vaira et al. 2007). mRNA stability is determined by the balance between mRNA translation in polysomes and mRNA degradation in P-bodies. p53 has been associated with mTOR signaling in a human embryonic stem cell line in which rapid inhibition of protein synthesis caused by activated tumor suppressor p53 can be counteracted by mTOR-induced translation of anti-apoptotic proteins (Constantinou and Clemens 2007). However, the nature of a possible interplay between mTOR and p53 signaling in regulating FAK protein levels remains unclear.

Together with our previous observations, our present data delineate distinct and complementary pathways by which TGF β modulates FAK protein. Although TGF β -induced activation of p38 and Smad signaling is required to induce *fak* transcription (a, Walsh et al. 2008b), TGF β activation of ERK might increase the availability of the resulting FAK mRNA, whereas mTOR signaling further potentiates the TGF β response by enhancing FAK protein synthesis. Acute exposure to TGF β also rapidly phosphorylates and activates FAK protein (a, Walsh et al. 2008b), potentially further synergistically activating FAK pathway signaling in response to chronic exposure to TGF β . The complex modulation of FAK transcription, mRNA stability, protein synthesis, and activation might represent an important convergent point for integrin-driven signals and those of growth factors such as TGF β , which might be crucial in promoting motility and maintaining homeostasis in intestinal epithelial cells.

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Fig. 1. MEK signal inhibition abolishes TGF β -mediated increase of immunoreactive FAK protein levels. **a** After a 24-h exposure to TGF β at 0.1 ng/ml, 1 ng/ml, or 10 ng/ml and a 1-h pretreatment with the MEK inhibitor PD98059 at 1 μ M, 10 μ M, or 20 μ M, immunoreactive FAK protein was analyzed in IEC-6 cells (* P 0.03, n =3). A typical Western blot for total FAK and GAPDH (*GDH*) is shown above. **b** TGF β -stimulated ERK phosphorylation (* P 0.03, n =3) is abolished by pretreatment with the MEK inhibitor PD98059 at 1 μ M, 10 μ M, or 20 μ M. A typical Western blot probed for phosphorylated ERK, total ERK, and GAPDH (*GDH*) is shown above. **c** Typical microscopic image of a subconfluent rat intestinal epithelial IEC-6 monolayer used for TGF β treatments. Bar 150 μ m

Fig. 2.

TGFβ-induced *fak* gene transcription and role of ERK signaling. Activity of the *fak* promoter-luciferase construct (*pGL2+FAK*), normalized to *rinella* and expressed as percent of the respective non-treated control, is significantly higher in TGFβ-treated cells ($P=0.007$ vs. non-treated respective controls, $n=4$ for each) and in cells pretreated with the MEK-specific inhibitor PD98059 (*ctr* control)

Fig. 3.

Regulation of FAK mRNA stability and translation by TGF β and the MEK-specific inhibitor PD98059 (*PD*). **a** FAK mRNA stability was increased by TGF β . After treatment with the transcription inhibitor actinomycin D, TGF β was added to cell cultures, and RNA was extracted at the indicated time-points. The amount of transcript was analyzed by RT-PCR in four separate experiments (*ctr* control). The amount of transcript after TGF β -treatment was significantly different from controls at the 30-min time point (* $P=0.003$). Ribosomal gene (18S) transcript was utilized as an internal control for amplification efficiency. **b** Cells were treated with TGF β and PD98059 as indicated in the presence of cycloheximide for 12 h. FAK and α -tubulin were detected by Western blotting. FAK expression was significantly lowered ($n=4$, $P<0.00005$) at 12 h after cycloheximide treatment, but no differences were observed in FAK decay among the cells treated with TGF β and PD98059

Fig. 4.

Inhibition of the mTOR signaling pathway abolishes TGFβ mediated FAK protein expression. **a** Treatment of IEC-6 cells with the mTOR-specific inhibitor rapamycin completely inhibits the TGFβ-induced increase in FAK expression at the indicated concentrations. *Bottom* Densitometric analysis of FAK, expressed as a ratio to α-tubulin and then as a percent of the DMSO control ($n=6$, $*P<0.05$). *Top* Typical Western blots probed for FAK and α-tubulin. **b, c** Typical Western blots probed for FAK, mTOR and p70^{S6K}. Reducing mTOR and p70^{S6K} protein by specific siRNAs (100 nM) attenuates TGFβ-mediated FAK protein expression. Treatment with TGFβ in the control transfection increased immunoreactive FAK protein in IEC-6 cells. mTOR and p70^{S6K} reduction completely inhibited the TGFβ-induced increase in FAK expression (NT non-targeting). **d, e** Densitometric analysis of FAK, expressed as a ratio of α-tubulin and as a percentage of the NT1 control

Fig. 5.

TGFβ stimulates the phosphorylation of mTOR, p70^{S6K}, and 4E-BP1 in cells treated for variable lengths of time before lysis. **a** *Top* Typical Western blot probed for phosphorylated mTOR, stripped, and reprobed for total mTOR and GAPDH. *Bottom* Densitometric results as the ratio of phosphorylated to total mTOR normalized to GAPDH ($n=10$, * $P<0.05$, # $P<0.001$). Control phosphorylation was defined at 1.0. **b** *Top* Typical Western blot probed for phosphorylated 4E-BP1, stripped, and reprobed for total 4E-BP1 and GAPDH. *Bottom* Densitometric results as the ratio of phosphorylated to total 4E-BP1 normalized to GAPDH ($n=10$, * $P<0.04$). Control phosphorylation was defined at 1.0. **c** *Top* Typical

Western blot probed for phosphorylated p70^{S6K}, stripped, and reprobed for total p70^{S6K} and GAPDH. *Bottom* Densitometric results as the ratio of phosphorylated to total p70^{S6K} normalized to GAPDH ($n=10$, * $P<0.02$). Control phosphorylation was defined at 1.0

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Fig. 6. TGFβ stimulated mTOR, p70^{S6K}, and 4E-BP1 phosphorylation in cells treated for 5 min is abolished by pre-treatment with the MEK inhibitor PD98059 (10 μM) for 1 h. **a** Typical Western blot probed for phosphorylated mTOR, phosphorylated p70^{S6K}, phosphorylated 4E-BP1, and GAPDH for the indicated times. **b–d** Densitometric results as the ratio of phosphorylated protein normalized to GAPDH ($n = 4$, * $P < 0.05$). Control phosphorylation (*Ctrl*) at time point 0 was defined at 1.0

Fig. 7.

TGF β (1 ng/ml)-stimulated migration of cultured rat intestinal epithelial IEC-6 cells on collagen I is abolished by 1-h pretreatment with 10 μ M PD98059 or 100 ng/ml rapamycin. **a** TGF β was added before wounding, and migration was quantified after 24 h ($n=3$ for each circular wound, $*P=0.05$). **b–i** Typical microscopic images of cell migration with the original wound (**b–e**) delineated by a *black dashed line* and the final wound at 24 h (**f–i**) by a *white line*. Bars 150 μ m