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TGF- β 1 modulates focal adhesion kinase expression in rat intestinal epithelial IEC-6 cells via stimulatory and inhibitory Smad binding elements

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Abstract

TGF- β and FAK modulate cell migration, differentiation, proliferation and apoptosis, and TGF- β promotes FAK transcription in intestinal epithelial cells via Smad-dependent and independent pathways. We utilized a 1320bp FAK promoter-luciferase construct to characterize basal and TGF- β -mediated FAK gene transcription in IEC-6 cells. Inhibiting JNK or Akt negated TGF- β -stimulated promoter activity; ERK inhibition did not block the TGF- β effect but increased basal activity. Co-transfection with Co-Smad4 enhanced the TGF- β response while the inhibitory Smad7 abolished it. Serial deletions sequentially removing the four Smad binding elements (SBE) in the 5' untranslated region of the promoter revealed that the two most distal SBE's are positive regulators while SBE3 exerts a negative influence. Mutational deletion of two upstream p53 sites enhanced basal but did not affect TGF- β -stimulated increases in promoter activity. TGF- β increased DNA binding of Smad4, phospho-Smad2/3 and Runx1/AML1a to the most distal 435bp containing 3 SBE and 2 AML1a sites by ChIP assay. However, although point mutation of SBE1 ablated the TGF- β -mediated rise in SV40- promoter activity, mutation of AML1a sites did not. TGF- β regulation of FAK transcription reflects a complex interplay between positive and negative non-Smad signals and SBE's, the last independent of p53 or AML1a.

Keywords

FAK; Promoter; TGF- β ; transcription factors; Smad and SBE

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Introduction

The gut epithelium maintains tissue homeostasis despite rapid cellular turnover. This requires an appropriate balance between the proliferation of progenitor cells and the loss of mature cells [1]. Various factors and intracellular signal proteins, such as TGF- β and focal adhesion kinase (FAK), have been reported to influence epithelial restitution and proliferation in intestinal epithelial cells [2–10]. TGF- β contributes to homeostatic control of the intestinal epithelium, acting as a tumor suppressor and growth inhibitor and supporting differentiation [11–13]. FAK, an intracellular tyrosine kinase localized prominently within focal adhesions, participates in a variety of integrin-elicited biological activities, such as migration [14–16]. FAK inhibition in human intestinal epithelial cells produces anoikis while FAK induction in rat intestinal IEC-6 cells suppresses apoptosis [17,18].

FAK has been highly conserved through evolution. Multiple FAK transcripts resulting from alternative splicing and/or promoter usage have been reported in several species [19]. In chicken and mouse, an alternative promoter is transcribed to an inactive FAK analog lacking N-terminal and catalytic domains, termed FAK-related non-kinase (FRNK) [20]. FAK activity is modulated by its tyrosine phosphorylation in response to growth factor receptor activation in adherent cells [14–16,21] and extracellular pressure in suspended cells [22]. Phosphorylated FAK binds to different signaling proteins via Src homology 2 (SH2) and SH3 recognition sites, acting as a scaffold and transmitting signals crucial to cell survival, motility, proliferation and differentiation [15–17,23–25].

FAK activity is influenced not only by integrin signaling but also by many growth factors including transforming growth factor beta1 (TGF- β 1) [26]. Transforming growth factor beta 1 is a 25kDa dimeric polypeptide growth factor/morphogen that functions in a cellular type and context- specific manner to regulate diverse cellular processes including growth, differentiation, migration and apoptosis [27,28]. TGF- β also induces matrix synthesis and remodeling and myofibroblast differentiation [29]. Canonical downstream TGF- β signaling is mediated by the Smad family of transcription factors [30–33], although non-Smad signaling, particularly through MAP Kinase pathways, also occurs [34–36]. Activation of the TGF- β II serine/threonine (Ser/Thr) receptor kinase phosphorylates the type I Ser/Thr receptor, forming an activated receptor complex [37]. The activated type I receptor is then able to phosphorylate intracellular effector substrates that include the highly homologous receptor Smads, Smad2 and Smad3 [33,38,39]. These R-Smads then interact with a common Smad (Co-Smad/Smad4) to form heteromeric complexes which translocate to the nucleus. The nuclear Smad complexes bind to target genes containing Smad binding elements (5'AGAC 3' or 5'GTCT3') (SBE) and modulate transcription in association with other cofactors as well as direct recruitment to specific, promoter-containing sequences [40–43]. Cell-specific positive and negative regulatory cross talk between the Smad and various non-Smad pathways has also been documented [34,44–46].

To date, most attention has focused on the processes that activate FAK after its synthesis, but activated FAK levels are also influenced by alterations in FAK protein and mRNA expression in migrating intestinal epithelial cells [9,47], healing gastric or colonic ulcers, and sarcomas [48,49]. FAK protein is over-expressed in some melanoma cells and in human malignant astrocytoma/glioblastoma [50,51] while increased FAK mRNA levels have been described in adenomatous tissues, and invasive and metastatic tumors [52,53].

TGF- β receptor ligation is known to transactivate FAK [54]. However, recently published studies from our laboratory demonstrated that TGF- β can also enhance FAK protein and mRNA levels and FAK promoter activity in human and rat intestinal epithelial cells [49]. FAK mRNA levels assessed by RT-PCR were elevated within two hours, and luciferase activity of a cloned

FAK promoter, after 24 hours of TGF- β treatment. Chemical and siRNA inhibition of either the Smad or the p38 pathway independently abolished the TGF- β -mediated increases in FAK protein and promoter activity. Finally, TGF- β receptor antagonism had no effect on basal FAK promoter activity [49]. To understand TGF- β -mediated regulation of FAK expression in more detail, we again utilized the cloned 1.32 bp promoter region upstream of exon 4 of the human FAK gene which contains four SBE's and several other transcriptional DNA binding sequences [19,55,56]. We first examined the roles of several non-Smad mediators, ERK, Akt and JNK, in basal expression of the promoter. The promoter construct was then used to investigate the effect of deletions and point mutations of various DNA binding sites on TGF- β -mediated regulation of the FAK promoter.

Materials and methods

Cell culture

All the experiments were performed using IEC-6 cells (passages 15–20) obtained from the American Type Culture Collection (ATCC, Rockville, MD). This cell line, derived from normal rat intestine, was characterized by Quaroni et al [57]. Stock cells were cultured in T-75 flasks in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 10ug insulin/ml and 10ug penicillin/streptomycin (Sigma, St. Louis, MO). Cells were maintained at 37°C in a 5% CO₂ incubator. For the experiments, cells were seeded onto collagen I coated plates (12.5ug/ml in ELISA buffer, 1.5mM Na₂CO₃, 35mM NaHCO₃, pH 9.4) as described by us previously [7]. For *luciferase assays*, subconfluent cells were serum starved for 4–6 hours prior to treatment with TGF- β 1 (1ng/ml; Sigma) in medium containing 0.5% FBS for a further 24 hours. For *signal analysis*, cells were serum-starved as above, pretreated for 1 hour with the following inhibitors, PD98059 (10 μ M), LY294002 (10 μ M), JNK Inhibitor II (or its negative control; 10 μ M) dissolved in 0.1% DMSO (all, EMD Biosciences, Gibbstown, NJ) or the DMSO control, then exposed to 1 ng/ml TGF- β for 1 hour before cell lysis.

Western blot analysis

Treated cells were rinsed twice with ice-cold phosphate buffered saline then lysed in buffer containing 10ug/ml leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF), 1ug/ml aprotinin and 1mM sodium orthovanadate (all, Sigma). Equal amounts of protein (10–20ug; BCA, Pierce, Rockford, IL) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were exposed to the following primary antibodies overnight: total FAK (B/D Transduction, San Jose, CA), phospho-ERK, JNK(Thr183/Tyr185) and Akt(Thr308) (Cell Signaling, Danvers, MA), phospho-Smad2/3, Smad 4, and Runx1 (Santa Cruz, Santa Cruz, CA), and β -tubulin (EMD Chemicals, Gibbstown, NJ) or GAPDH (Biodesign, Saco, ME) as loading controls. Following incubation with the appropriate horseradish peroxidase-conjugated mouse, rabbit or goat secondary antibody, the 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.

Wt.FAK (FAK-A) promoter construct

We obtained a human genomic DNA-BAC clone from BACPAC Resources (Children's Hospital of the Oakland Research Institute, Oakland, CA) 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 Sac I and Stu I (New England Biolabs, Ipswich, MA) releasing a fragment 1.65 kb in size that was subcloned into the pBluescript® II SK (+) vector (Stratagene, La Jolla, CA) for amplification. The pBSK-FAK-promoter clone was subsequently cut with Nar I, end-

filled, and then cut with Kpn I (New England Biolabs) to produce a 1.3 kb fragment that was subcloned into a pGL2 Basic vector (Promega, Madison, WI) to generate the FAK-promoter-luciferase construct used in the Dual Luciferase® Reporter Assay System (Promega). The 1.3 kb fragment was sequenced using T3 and T7 primers (New England Biolabs) to verify that it matched the FAK genomic sequence in GenBank (not shown).

Dual Luciferase Reporter Assay for promoter activity

For transient transfections, 2×10^5 cells were seeded on collagen I coated 6-well plates, cultured overnight and transfected with the pGL2- fused FAK promoter constructs (1 µg/well) 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 (0.1 µg/well) was used together with the pGL2 basic and recombinants plasmids. For some experiments, cells were pre-treated with JNK Inhibitor II, PD98059, LY294002 as above, or the TGF-β receptor (ALK5) inhibitor SB431542 (0.5 µM; Tocris, Ellisville, MO) prior to TGF-β treatment. For other experiments, rat Smad2 SMARTpool® and non-targeting (NT1) duplexes, purchased from Dharmacon (Dallas, TX) were introduced into IEC-6 cells (100 nM) with a mix of Oligofectamine and Plus Reagent in OptiMEM (Invitrogen) 24 hours before luciferase transfection. A time course study indicated that FAK promoter activity was maximal 24 hours after TGF-β treatment (data not shown). Therefore, for all subsequent experiments, cells were lysed with 1× passive lysis buffer and the lysates analyzed using the dual luciferase reporter assay system kit according to the manufacturer's instructions (Promega) 24 hours after treatment. Luminescence was measured on an AutoLumat LB 953 Luminometer (EG and G Berhold).

Transient expression of Smad4 and Smad 7

For transient expression of Smad proteins, the FLAG- Smad4 and Smad7 PcDNA plasmids (kindly provided by Dr. Ana M.Grau, Vanderbilt University) were used. Each well within an experiment was transfected with the same amount of plasmid or the empty parental expression vector with Lipofectamine for 6 hours. After removal of the transfection mixture, cells were incubated in normal growth medium for 24 hours to allow expression of the desired proteins before treatment with TGF-β and luciferase assay as described above.

Subcloning, serial deletion and site-specific mutagenesis of the FAK 5' untranslated region (UTR)

The wt.FAK promoter construct described above, which contains 4 SBE's, was used to generate promoter fragments containing different numbers of SBE's by serial deletion (Fig. 6A). All primers (Integrated DNA Technology, Coralville, IA) were designed to match specific restriction sites within the pGL2 vector in order to clone the fragments directly into that vector without prior subcloning. Thus, for these 5' deletion constructs, the anti-sense primer contained a *Bgl*II cloning site and each sense primer contained either a *Kpn*I or *Mlu*I cloning site. Primer sequences for the FAK-1 (Wt) to FAK-G constructs are listed in Table I. The PCR-amplified fragments were digested with *Kpn*I or *Mlu*I and *Bgl*II, agarose gel purified and sub cloned into the corresponding sites of the pGL2-basic Luciferase vector.

The most distal 130 bp 5' regulatory region of the FAK promoter contains one copy of SBE and two copies of a binding site for the acute myeloid leukemia (AML1a) family of transcription factors, also known as core-binding factor A (CBFA) or polyomavirus enhancer-binding protein 2 alpha (PEBP2α) [58]. This region was cloned using the FAK-pGL2 basic plasmid (wt.FAK/FAK-A) DNA as the template and gene specific primers (sense 5' GGGGTA CC agg cct cgg tgc ctg tc 3' and antisense 5' CCAGATCT ctg gca acc ttg tgg t 3'). The resulting 130 bp PCR fragment was cloned into *Kpn*I and *Bgl*II sites of the pGL2-SV40 promoter luciferase vector. All plasmids were column purified in large scale using Plasmid Midi and

Maxi Kits (Qiagen, Valencia, CA) as per manufacturer's instructions. All positive pGL2-FAK recombinant plasmids were evaluated by PCR, restriction analysis and sequenced in both directions by the WSU core sequencing facility.

Site specific mutation of the SBE, AML1 and p53 sites was accomplished with the Quick Change XL Site-Specific Mutagenesis kit (Stratagene), according to the manufacturer's protocol and using the pGL2-FAK promoter plasmid as DNA template. PCR primers for p53 mutation at the distal and proximal sites (8 deleted bases) and for point mutations at the SEB1 and AML1 sites (mutated bases are underlined and in bold) are shown in Table 1.

ChIP assay

IEC6 cells, grown to 70% confluence and treated with TGF- β (1ng/ml) for 24 hours were cross-linked with 1% formaldehyde in PBS at room temperature for 10 min and then sonicated to shear the chromatin to 500–1000 bp fragments. Samples were pre-cleared with sonicated salmon sperm DNA, 100 $\times$ bovine serum albumin (10 mg/ml BSA New England Biolabs) and protein A/G-Sepharose (50% slurry of 1:1 in dilution buffer; Santa Cruz). Antibodies to phospho-Smad 2/3, Smad4, Runx1 or IgG as control (Santa Cruz) were added to the pre-cleared sample and incubated overnight at 4°C. The beads were harvested by centrifugation and washed serially in low-salt, high salt, LiCl and Tris/EDTA (TE) buffers and the protein-chromatin complexes eluted with 0.1 M NaHCO₃, 1% SDS. Formaldehyde cross-links were reversed by incubation at 65°C with 10 μ g RNase and 5 M NaCl. After addition of proteinase K, chromatin fragments were extracted with phenol: chloroform precipitated in ethanol precipitation and resuspended in TE buffer (pH 8.0). Fragments were then amplified by PCR (30 cycles) using 5 μ l of the immunoprecipitated chromatin as templates and gene-specific primers, 5' tgc ctg tct gtc ctg ctt cct ggc ttg 3' sense and 5' agg gaa cga gcc ctg caa tc3' antisense yielding a 435 bp product that contained both SBE and AML1a sites. The amplified PCR products were visualized on 1.2% agarose-ethidium bromide gels.

Statistical analysis

Data are expressed as Mean $\pm$ SE of at least three experiments. Statistical significance, set a priori at $p \leq 0.05$, was calculated using a one- or two-tailed Student's t-test, paired and non-paired as appropriate.

Results

TGF- β 1 stimulates FAK protein expression and FAK promoter activity in IEC-6 cells

In a recently published study, we demonstrated that a 24 hour exposure to TGF- β 1 significantly enhanced FAK protein levels and FAK promoter activity. Further RT-PCR analysis indicated that FAK mRNA levels were elevated as early as 2 hours after treatment [49]. Similar experiments carried out for the present study showed a similar increase in FAK protein (65% over that of control, non-treated cells) as assessed by Western blot (Fig. 1A). Data are normalized to the α -tubulin control and presented as % of untreated cells. Promoter activity, using the cloned FAK promoter region wt.FAK-luciferase construct (Fig. 1B), was higher than that of the empty pGL2 vector and further increased (by 75%) in TGF- β -treated cells (Fig. 1C), confirming that the increase in FAK protein in response to TGF- β is the result of transcriptional activation.

TGF- β 1 stimulates ERK, Akt and JNK activation/phosphorylation

The actions of TGF- β may be mediated by both Smad and non-Smad signals. The latter include p38, ERK, JNK and Akt [31,54,59–61]. Having previously demonstrated that TGF- β activates p38 [49], we now sought to determine whether TGF- β also specifically activates ERK, JNK

and Akt in IEC-6 cells, and whether this activation plays a role in FAK promoter activity. As shown in Fig. 2, a one hour exposure to 1 ng/ml TGF- β significantly enhanced phosphorylation of ERK (to $184 \pm 20\%$ of control, $p=0.03$; Fig. 2A), Akt at Threonine 308 ($148 \pm 18\%$, $p=0.03$; Fig. 2B), and JNK at Thr183/Tyr185 ($148 \pm 23\%$, $p=0.02$; Fig. 2C). Because some of the signals did not strip completely and reproducibly for re-probing with the appropriate ERK, Akt or JNK total protein antibody, phosphorylation levels were calculated from the ratio of the phosphorylated protein to the GAPDH control and are expressed as percent of the non-treated control. Statistical significance was calculated by paired t-test from the original GAPDH ratios. Each signal was blocked by a one hour pre-incubation with the appropriate inhibitor, PD98059 for ERK (Fig. 2A), LY294002 for Akt (Fig. 2B), or JNK Inhibitor II (Fig. 2C), all at $10 \mu\text{M}$.

Chemical Akt and JNK inhibition ablates TGF- β stimulation of FAK promoter activity while ERK inhibition only affects basal promoter activity

To examine the importance of these signals to both basal and TGF- β -stimulated FAK promoter activity, we pre-treated cells with each of the inhibitor used above for one hour prior to 24 hour exposure to TGF- β and luciferase assay. As an inhibitory control, we used the TGF- β receptor antagonist SB431542 [62], which we have previously shown prevents TGF- β -stimulated FAK promoter activity and protein expression [49]. As expected, SB431542 ($0.5 \mu\text{M}$) inhibited the TGF- β -induced rise in FAK promoter activity. Similar results were obtained with Akt and JNK inhibition with LY294002 and JNK Inhibitor II ($10 \mu\text{M}$ each), respectively. None of these inhibitors significantly altered baseline FAK promoter activity. However, while the PD98059 compound did not inhibit TGF- β enhancement of FAK promoter activity (139 ± 13 of control, $p=0.018$), ERK inhibition significantly increased basal FAK promoter activity (Fig. 3), suggesting that ERK activity exerts a tonic inhibitory effect on the promoter. Although presented as percent of basal DMSO activity, all statistics were calculated from the averages of triplicate luciferase/*renilla* ratios.

TGF- β receptor activation of Smad proteins mediates TGF- β -induced FAK promoter activity

As described above, after TGF- β receptor activation, phosphorylated Smad2/3 complexes with Smad4, entering the nucleus and binding to DNA to activate transcription. In contrast, Smad7 acts outside of the nucleus as a negative regulator of TGF- β signaling by interfering with R-Smad activation at the membrane and/or enhancing degradation of the receptor [63,64]. To examine the molecular interaction between TGF- β -receptor-mediated signaling and the FAK promoter, we co-transfected IEC-6 cells with Smad4 and Smad7 expression vectors along with the FAK promoter. As shown in Fig. 4, basal luciferase activity was nearly equal in cells transiently co-transfected with the luciferase construct and either a wt.FAK-pcDNA or the Smad4 construct. Each responded to TGF- β stimulation as well. However, baseline FAK promoter activity was significantly lower in cells harboring the Smad7 construct. TGF- β responsiveness was abolished in these cells. In addition, when Smad7 was co-transfected with Smad4, it reversed the stimulatory effect of Smad4 on both baseline and TGF- β -stimulated FAK promoter activity. These results suggest that TGF- β -receptor-induced activation of effector Smads is responsible, at least in part, for the TGF- β effect on the FAK promoter.

Lowering Smad2 expression with a specific siRNA does not affect basal FAK promoter activity

We had previously shown that decreasing Smad protein levels with siRNA directed at Smad2 does not alter basal FAK protein expression in IEC-6 cells [49]. To ascertain that this effect occurred at the level of transcription, we treated cells with siSmad2 and a nontargeting control before transfection with the wt.FAK-luciferase construct. Fig. 5 demonstrates that lowering Smad2 protein levels by an average of 68% did not change basal FAK promoter activity,

indicating that this canonical TGF- β -driven pathway is not likely to contribute substantially to basal FAK promoter activity in the absence of exogenous TGF- β .

Different SBE's in the FAK promoter can enhance or inhibit activity

Although the 5' flanking region of the FAK promoter contains a number of putative binding sites for transcriptional factors such as p53, NF κ -B, SP-1, AP-1, and AP-2, among others [55], the data presented above suggests that the four SBE's present in the cloned human FAK promoter must play a role in the regulation of FAK expression. Therefore, we employed a 'serial deletion strategy' and PCR amplification to generate six promoter fragments with sequentially fewer SBE's and lacking both copies of NF κ -B as shown in Fig. 6A. The wt.FAK/FAK-A (-1173 +130) contains 4 SBE's; FAK-B contains 3SBE's; FAK-C contains 2 SBE's and FAK-D contains 1 SBE. Further deletions, eliminated the NF κ -B site (FAK-E) and two other sequences near the putative start site (FAK-F/NoSBE fragment 1 and FAK-G/NoSBE fragment 2). Basal FAK promoter activity of each construct, normalized to *renilla* and presented as fold activity with respect to the intact promoter FAK-A/4 SBE control, is shown in Fig. 6B. Deletion of the two most distal SBE's, FAK-B and FAK-C promoter fragments, decreases basal activity to 11% and 7% of FAK-A, respectively. Further deletion analysis demonstrated that basal promoter activity was regained in FAK-D and greatly increased (3 fold) in the FAK-E construct. The FAK-B and FAK-C constructs also contain known inhibitory p53 binding sites [56] which may account, in part, for the increased basal activity in FAK-D and FAK-E. Normalized luciferase activity was decreased, but still above that of control in the FAK-F construct and again completely lost in the FAK-G construct. TGF- β responsiveness of each construct is depicted in Fig. 6C as fold increase in activity over that of its respective control. Only the FAK-C construct failed to respond to TGF- β . Taken together, these results indicate that, while some SBE's enhance both baseline and TGF- β -stimulated FAK promoter activity, some exhibit a restraining function (FAK-C) which is negated by deletion of the binding site. NF κ -B has been shown to promote basal FAK expression [55]. Our results duplicate these findings but suggest that these binding sites may not be important to TGF- β -mediated FAK regulation as their deletion does not eliminate TGF- β responsiveness.

P53 exerts an inhibitory action on basal FAK promoter activity but does not mediate TGF- β responsiveness

We next sought to analyze the effect of p53 on FAK promoter activity in the presence of TGF- β . A schematic of the mutated distal (upstream) and proximal (downstream) p53 sites is shown in Fig. 7A. As expected from the results presented above and previous reports [56], deletion mutations of either site (mt.p53.d/mt.p53.p) or both sites, significantly enhanced basal FAK promoter luciferase activity, $p=0.02$, $p=0.002$, and $p=0.001$, respectively (Fig. 7B). However, FAK promoter activity of each of the constructs was similar to that of the wt.FAK in response to TGF- β 1 stimulation (Fig. 7C), suggesting that p53 is not involved in TGF- β regulation of the FAK promoter.

The 435 bp upstream region of the FAK promoter binds Smad4, phospho-Smad2/3 and Runx1 proteins

Since the deletion of 5' flanking 130 nucleotides from the FAK promoter significantly ablated FAK promoter activity (Fig. 6), we searched for putative transcription regulating elements in this region by means of TFSEARCH (www.cbrc.jp/htbin/nph-tfsearch.html). The search results indicated that two copies of the AML1a binding site are located within this region. Therefore, we designed a primer pair to amplify a 435 bp region containing those AML1a sites as well as three SBE's to be used in a CHIP assay to examine binding of Smad4 and activated (phospho-) Smad2/3 to the SBE's and Runx1 to the AML1a sites (Fig. 8A). Exposure to TGF- β increased binding of Smad 4 (lane 2), phospho-Smad2/3 (lane 3) and Runx1 (lane 4) to this

fragment of the promoter without affecting any of the controls (Fig. 8B). Thus, TGF- β stimulates binding of these proteins to chromatin, an effect that may enhance promoter activity in response to a TGF- β challenge.

The SBE1 site, but not AML1a binding sites, modulates FAK promoter TGF- β responsiveness

SBE/AML1 interactions in the control of gene expression have been reported by others [65]. To investigate the potential role of SBE/AML1a binding sites in controlling the FAK promoter, we cloned the most distal 130 bp 5' flanking region into a pGL2 vector containing the SV40 promoter and generated point mutated constructs at the SBE (GTCTG to GGCGG) and AML1a (TGTGGT to TATTAT) binding sites (Fig 9A). The wt.SBE1 plasmid increased basal SV40 promoter activity; TGF- β exposure increased this further by 54%, an effect that was completely ablated in cells transfected with the mutated construct (pmt.SBE1; Fig. 9B). On the other hand, mutation of the AML1a binding sites had no effect on TGF- β stimulation of SV40 promoter activity (Fig. 9C), suggesting that there is no co-operation between SBE and AML1a binding sites in TGF- β -mediated regulation of the FAK promoter. The results do confirm the importance of the upstream SBE binding site to the positive regulation of FAK promoter activity by TGF- β .

Discussion

The appropriate regulation of gene expression requires the correct temporal and spatial interaction of a variety of cellular transcriptional factors with their DNA regulatory sequences [66,67]. The mechanisms of TGF- β -mediated transcriptional activation/repression have been extensively investigated [30,39,40,68–70] but little is known about transcriptional regulation of FAK [9,55,56,71]. The FAK 5' untranslated region (UTR) is compatible with the existence of a single promoter region adjacent to exon 4 in both human and rodent transcripts [19]. We used this region, and various serial deletions compatible with previously published constructs [55,56,72], to map TGF- β /SBE FAK promoter responsiveness in rat intestinal epithelial IEC-6 cells. The results of this study, in concert with previous results [49], reveal a complex regulation of FAK promoter activity. In addition to non-Smad regulation by MAP kinases and Akt, both positive and negative SBE regulatory elements in the FAK promoter sequence impact both baseline and TGF- β -stimulated FAK transcriptional activity.

Consistent with reports in other cells [34,36,44], exposure to TGF- β increased phosphorylation/activation of ERK, Akt and JNK in the IEC-6 cells we studied here, but inhibiting these various signals yielded different results with regard to FAK promoter activity. We found that inhibition of either JNK or Akt abrogates TGF- β -stimulated FAK promoter activity. Others have found that, although the PI-3K inhibitor, LY294002 inhibited collagen (COL1A2) gene activation in human glomerular mesangial cells, in the rat osteoblastic cell line, UMR 106-01, JNK inhibition had no effect on collagenase-3 expression [44]. Of interest, are the disparate results obtained with ERK inhibition. We found no decrease in TGF- β -induced FAK promoter activity but others reported that MEK inhibition with PD98059 or U0126 blocks TGF- β induction of AP-1 complex formation in human carcinoma cells [46], aggrecan gene expression in chondrogenic ATDC5 cells [73], and connective tissue growth factor in human fibroblasts [45]. Other investigators, however, suggest that ERK activation may block the activity of Smad transcription factors, thus repressing TGF- β signaling [74,75]. ERK activation was shown to increase Smad2/3 phosphorylation at sites other than those activated by the TGF- β receptor causing cytoplasmic retention and preventing nuclear localization of Smad2/3 [74,75]. Removal of this restraint could explain the increase in basal FAK promoter activity seen with ERK inhibition in our study. We could not document interaction between the Smad pathway and p38 in our previous study [49] but a thorough examination of potential ERK, Akt, JNK and Smad cross talk is beyond the scope of this study.

FAK promoter activity was enhanced by co-transfection with Smad4 and inhibited by co-transfection with Smad7, as reported for various TGF- β target genes [39]. Smad7 also reversed the response to TGF- β , consistent with previous reports. It has been shown that, in response to TGF- β , Smad7 interacts with Smurf1 or Smurf2 ubiquitin ligases leading to TGF- β receptor degradation and the prevention of recruitment and phosphorylation of effector Smads [76–78]. Although ubiquitously involved in Smad-mediated transcription, Smad4 is not essential for TGF- β responses, since some responses have been shown to occur in the absence of Smad4 and some Smad4-deficient cell lines have a limited responsiveness to TGF- β [79]. Our co-transfection results suggest that Smad4 does play a role in transactivation of the FAK promoter. Further, the ChIP assay demonstrated that exposure to TGF- β promoted association of both phospho-Smad2/3 and Smad4 with a 435 bp region of the promoter containing several SBE's, indicating that traditional receptor-activated-Smad-heterodimeric complex binding to SBE DNA sequences [80] mediate some actions of TGF- β on the FAK promoter.

According to a previous report, the region from –774 to the putative initiation site, +47, of the FAK promoter includes several transcriptional regulating elements and binding sites such as SBE, NF-K κ B, SP-1, AP-1, AP-2, GCF-1 TCF-1, PU-1, H4-TF2, and Egr-1 that might be necessary for maximal FAK promoter activity. These investigators also demonstrated that the sequence upstream of the –774 nucleotide contains two p53 binding sites (–965 to –942 and –807 to –790) that inhibit FAK promoter activity [55]. We identified four SBE copies in the intact FAK promoter sequence, one upstream of the two p53 sites. Deletion of the two most distal SBE's (Fig. 6A; FAK-B and FAK-C) significantly decreased basal FAK promoter activity; this was restored by removal of the third SBE (FAK-D), as was TGF- β responsiveness, suggesting that this SBE subserves an inhibitory function. These deletions also resulted in the removal of the two inhibitory p53 binding sites located in this region [55]. Further deletion (FAK-E) led to the greatest increase in basic FAK promoter activity, suggesting that this most proximal SBE may play an inhibitory role as well. These observations of the regulation of basal FAK promoter activity are in accord with those of Golubovskaya et al [55] in that the region of the promoter covered by FAK-D, E, and F, containing the two NF κ -B sites, is responsible for most of the FAK promoter activity.

The significant decrease in FAK promoter activity after deletion of the most distal SBE suggests that this site may play an important role in the constitutive activity of the promoter. Our previous immunohistochemical studies of migrating IEC-6 cells in vitro and human gastric and colonic epithelial cells at in sections of resected tissue taken from human mucosal ulcer margins in vivo show that the intensity and distribution of TGF- β immunoreactivity parallels that of FAK in those cells and tissues. In the human tissues, levels of phosphorylated/activated Smad3 also mimic those of TGF- β , indicating that the TGF- β present can elicit a functional signal. However, the TGF- β receptor antagonist SB431542 did not affect FAK protein expression or basal promoter activity in that study [49]. These promoter results were replicated in this study. In addition, lowering Smad2 expression levels with specific siRNA also had no effect on basal promoter activity. Thus, although autocrine/paracrine relationships cannot be completely ruled out, these results argue against paracrine/autocrine actions of TGF- β as likely mechanisms for this effect.

However, TGF- β -mediated FAK promoter activity was inhibited only in the FAK-C construct, lacking SBE3. TGF- β transcriptional activation of various genes is a highly complex process involving Smad interaction with a number of other binding sites, including AP-1, Sp1, p53, NF κ -B, E2F, and AML1/2, all of which are present in the FAK promoter region under study [65,68,81–85]. In human dermal fibroblasts, p53 was shown to be a potent and selective endogenous repressor of TGF- β -induced collagen expression [86]. Our results do not support such a role in the FAK promoter as TGF- β -stimulated FAK promoter activity was maintained in all but one of the constructs containing the p53 sites. Further, although basal FAK promoter

activity was higher with mutational deletion of p53, singly or in tandem, TGF- β -stimulated promoter activity was not affected.

The ChIP experiments also demonstrated recruitment of Runx1 (AML1a) proteins to FAK chromatin in the 5' UTR. Although AML binding is not required for TGF- β dependent transcription from the FAK promoter, it remains possible that AML proteins cooperate with Smad proteins and enhance the TGF- β effect. AML1/AML2 co expression with Smad3/Smad3/4 results in a strong transcriptional synergy [87]. Indeed, Smad and AML family members, co-operatively activate Ig synthesis [65]. Again, our mutational deletion experiments do not support such co-operation in the FAK promoter.

The most proximal regions of the FAK promoter still responded to TGF- β stimulation. Although we did not investigate this region further, it contains a number of sites, E2F, AP-1 and Sp-1, all of which could be involved [68,81,84]. In addition, NF κ -B appears to have the greatest effect on basal FAK promoter activity in our, and a previous study [55,56]. TGF- β is known to modulate NF κ -B but examination of this effect was beyond the scope of this investigation which focused on the role of the four SBE's present in the FAK promoter region.

Changes in FAK protein levels and promoter activity in response to TGF- β in this and our previous study were modest (50–75%) [49] but basal FAK protein and mRNA levels are generally low in normal tissues and only significantly elevated in pathological states such as malignancy [48,52,56,88]. Thus, small changes in the levels of this tightly regulated gene and protein are likely to have important cellular effects. We have shown that such protein changes occur in two different cell lines, human Caco-2 colon cancer cells and non-transformed rat intestinal epithelial IEC-6, and that changes of this magnitude are associated with changes in function such as motility. Very few studies of the FAK promoter exist. In the largest one, enhancement of luciferase activity varied widely with the genetic make-up of the cell type used, from 1.5 fold (using a promoter construct nearly identical to ours) to ten fold and more using truncated promoter constructs [89]. The changes in TGF- β -stimulated FAK promoter activity were also demonstrated in both cell lines, and were accompanied by elevations in FAK mRNA as assessed by RT-PCR and FAK protein levels as assessed by Western blot [9,49], suggesting that they are relevant. Others have described changes in migration or cell signaling of similar magnitudes in response to a number of stimuli in intestinal epithelial cells [90–92]. The 50–75% increases in FAK protein expression and promoter activity in response to TGF- β seen here may thus be biologically important to intestinal physiology.

Smads can mediate either transcriptional activation or repression depending on their associated partners. For example, Smad-E2F interactions result in myc repression while Smad-Sp1 complexes enhance expression of amyloid-beta precursor protein [68,84]. Our serial deletion experiments indicate that both types of SBE's are contained within the FAK promoter. Our MAP kinase and Akt inhibition experiments also show transcriptional activation in response to TGF- β . TGF- β has been documented to regulate a number of genes encoding members of the cell adhesion apparatus, collagen, fibronectin, integrins, and various pericellular proteases and protease inhibitors [42,68,86,93–97]. Our current results demonstrate that it regulates FAK, an integral member of focal adhesions, expression as well. It has been suggested that the cytostatic/proapoptotic properties of TGF- β may be changed to pro-survival/anti-apoptotic actions via activation of the FAK/Akt pathway [68]. We have demonstrated that the amount of activated FAK can be influenced by the level of FAK protein present [9,71], suggesting that the well-documented effects of TGF- β on intestinal cell restitution and proliferation may be mediated, at least in part, by its induction of FAK expression.

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Figure 1.

TGF- β enhances FAK protein expression and FAK promoter activity. (A) Subconfluent IEC-6 cells were treated with 1ng/ml TGF- β 1 for 24 hours. Cell lysates were analyzed by Western blot for total FAK (tFAK) and tubulin as loading control. Data, normalized to tubulin, is presented as percent of control non-treated cells. Densitometric analysis (Ave $\pm$ SEM) of 4 separate experiments is depicted in the bars; a representative Western blot is shown above. Asterisk denotes significant difference ($p < 0.05$) from control, untreated cells. (B) Agarose gel of the 1.3 kb construct containing the 5' flanking region of the human FAK promoter. The fragment was subcloned into a pGL2 Basic vector to generate a FAK-promoter-luciferase construct. (C) Cells transfected with the control empty vector (pGL2) or the FAK promoter construct (wt.FAK) were exposed to 1ng/ml TGF- β 1 for 24 hours prior to analysis via a Dual Luciferase® Reporter Assay System. Promoter luciferase activity is expressed as the ratio of luciferase to that of a co-transfected pRL-TK control vector encoding *Renilla* luciferase. Asterisk denotes significant fold increase over pGL2 empty vector, pound sign over untreated wt.FAK construct ($p < 0.05$).

Figure 2.

Short-term exposure (1 hr) to TGF- β stimulates phosphorylation/activation of several non-Smad signaling pathways. IEC-6 cells were pretreated for 1 hr with the MEK inhibitor PD98059 (A), the PI-3K (phospho-inositol-3-kinase) inhibitor, LY294002 (B), JNK inhibitor II (JNK Inh II) (C) (each, 10 μ M), or an equivalent concentration of the DMSO vehicle prior to 1 hr stimulation with 1 ng/ml TGF- β (black bars). Cell lysates were analyzed by Western blot with specific antibodies for phospho-ERK (panel A), phospho-Akt (Thr308; panel B) or phospho-JNK (Thr183/Tyr185; panel C) and with antibodies specific to GAPDH as a control. Bar graphs represent densitometric analysis of each specific phosphoprotein signal as a ratio of the GAPDH control; representative Western blots are depicted above the bars. Bars show percent increase over respective control; asterisks indicate statistical significance ($p < 0.03$) from DMSO-treated controls, calculated from the original signal/GAPDH ratios.

Figure 3.

ERK signals exert a significant inhibitory effect on basal FAK promoter activity while PI-3K and JNK signal activation mediates TGF- β stimulation of activity. Cells were co-transfected with the wt.FAK promoter-luciferase and *Renilla*-luciferase constructs 24 hours prior to pre-treatment with the TGF- β receptor antagonist SB431542 (0.5 μ M), the MEK inhibitor PD98059 (10 μ M), the PI-3K inhibitor LY294002 (10 μ M), or JNK Inhibitor II (JNK Inh; 10 μ M) for 1 hr. Control cells were treated with an equivalent concentration of the DMSO vehicle (open bar) or the requisite inhibitor (grey bars), except for JNK Inhibitor II where the inactive (negative) control peptide (Neg Cont; 10 μ M) was added. Cells were then exposed to 1 ng/ml TGF- β (black bars) for 24 hours before lysis and luciferase assay. Results are presented as percent of the DMSO control (Ave $\pm$ SEM of 3–9 experiments); statistical comparisons were calculated from triplicate averages of the original luciferase/*renilla* ratios. Asterisks denote statistical difference in promoter activity over the DMSO control ($p < 0.001$ for TGF- β treatment alone; $p < 0.002$ for PD98059 alone). Pound signs indicate statistical differences of TGF- β -treated cells over their respective controls ($p < 0.05$ for PD98059 and Negative JNK control).

Figure 4.

The activating Co-Smad Smad4 increases FAK promoter activity in response to TGF- β while the inhibitory Smad7 represses it. IEC-6 cells were co-transfected with a vector containing the FAK promoter-luciferase construct (wt.FAK) or FAK-pcDNA3, FLAG- Smad4 and Smad7 PcDNA or both the Smad4 and Smad7 constructs along with the internal *renilla* control pRL-TK vector. Cells were incubated for 24 hours for expression of the desired protein then treated with 1ng/ml TGF- β for a further 24 hours before lysis and analysis of luciferase activity. Results are presented as fold increase of the luciferase/*renilla* ratio over the empty vector controls which were unchanged by transfection with any of the constructs (not shown). Asterisks show significant differences between TGF- β -treated cells (black bars) and their respective controls (open bars; $p < 0.05$ for each).

Figure 5. Decreasing Smad2 expression via specific siRNA has no effect on basal FAK promoter activity. IEC-6 cells were transfected with a specific siRNA pool to Smad2 (siSmad2; black bars) or a non-targeting control sequence (NT1; open bars). After 24 hours, the wtFAK-luciferase and *renilla*/luciferase constructs were introduced into half the cells and all cells incubated a further 24 hours. Corresponding cells were then lysed for either Western blot analysis or luciferase assay. Left panel: Cell lysates were blotted with a specific Smad2 antibody and antibody to GAPDH as control. Bars represent densitometric analysis, expressed as percent of the NT1 control; a representative blot is shown above. A significant difference is denoted by the asterisk ($p < 0.001$ vs NT1). Right panel: Bars depict FAK promoter luciferase activity of the siSmad2-treated cells (black bar) calculated from luciferase/*renilla* ratios and expressed as percent of NT1 control-treated cells (open bar).

Figure 6.

Smad binding elements (SBE) transduce activation and repression, and modulate TGF- β responsiveness of the FAK promoter. (A) Schematic representation of the generation of FAK promoter constructs with consecutive deletions of 4 SBE sites; +47 is the putative transcription start site. Locations of SBE, p53, NF- κ B consensus sequences and forward and reverse primer sites (arrows) are indicated. Each of the seven PCR amplified fragments (FAK-A to FAK-G) were ligated into a promoterless luciferase vector (pGL2) 24 hours before treatment with TGF- β . Cells were lysed for luciferase activity after 24 hours of exposure; activity was normalized to that of the *renilla* internal control. (B) Basal FAK promoter activity in IEC-6 cells transfected with each of the seven deletion mutants (grey bars) is presented as a percent of the activity of the full-length (wt.FAK/FAK-A) construct (open bar). Asterisk denotes a significant increase and pound signs a significant decrease in basal promoter activity over the full-length construct containing all 4 SBE's and both p53 and NF- κ B sites ($p < 0.05$). (C) TGF- β -stimulated FAK promoter activity (black bars) for the full length (FAK-A) and each deletion mutant construct (FAK-B to FAK-G) was calculated as the ratio of luciferase to the internal *renilla* control and expressed as percent of respective control (open bars). Data presented are Ave $\pm$ SEM of three individual experiments. Asterisks indicate significant differences from respective controls; $p < 0.05$ for each.

Figure 7.

p53 DNA binding sites exert an inhibitory action on baseline FAK promoter activity. (A) Sites of single and tandem p53 deletions in the 5' flanking region of the FAK promoter are illustrated. The pGL2 FAK promoter plasmid was used as DNA template for mutation by deletion of 8 bases from p53 sites distal (mt.p53.d) or proximal (mt.p53.p) to the start site (+47), or both (mt.p53.d+p). Cells were treated with 1ng/ml TGF- β for 24 hours before lysis and assessment of promoter activity by luciferase. (B) Basal FAK promoter activity, calculated as the ratio of luciferase/rinella activities for each p53 mutant (grey bars), is shown as a percentage of basal activity of cells transfected with the non-mutated (wt.FAK) construct (open bar). Asterisks denote significant differences from wt.FAK ($p < 0.02$). (C) Normalized luciferase results for wt.FAK and each p53 deletion mutant are presented as fold increase over respective untreated control in IEC-6 cells treated with 1 ng/ml TGF- β for 24 hours (black bars). Bars represent Ave $\pm$ SEM of four experiments. Asterisks indicate statistical difference from untreated control; all statistical comparisons were calculated using the individual, respective controls.

Figure 8.

ChIP assay for Smad4, phospho-Smad2/3 and Runx1/AML1 protein binding to the 5' distal region (−1173 to −738) of the FAK promoter. (A) The primer pair was designed to amplify a 435 bp fragment containing three copies of SBE and two copies of AML1. (B) Chromatin fragments, obtained from cell incubated with and without 1 ng/ml TGF- β for 24 hours, were incubated overnight with A/G Sepharose and antibodies specific for Smad4, phospho-Smad2/3, Runx1 or the IgG control. After elution of the protein-chromatin complexes and phenol:chloroform extraction, the fragments were amplified by PCR using the primer pair. The amplified PCR products for control (top panel) and TGF- β -treated (bottom panel) cells, and control plasmid DNA and antibody lacking (no ab) samples were separated on a 1.2% agarose gel; standard markers are shown in the first and last lane (lanes 1 and 10).

Figure 9.

TGF- β responsiveness of the FAK promoter is abolished by point mutation of SBE1. (A) Schematic illustration of point mutations generated in the 130 bp TGF- β responsive region in the 5' UTR containing one SBE and two AML1a DNA binding sites. The 130 bp (from -1173 to -1043 bases) upstream fragment was PCR amplified and ligated into a pGL2 vector driven by a SV-40 promoter (wt.SBE1). Disabling point mutations were generated in the SBE1 site (pmt.SBE1) or both AML1a sites (pmt.AML1a(d+p)). IEC-6 cells containing each construct were then left untreated (open bars) or treated with 1 ng/ml TGF- β (black bars) for 24 hours. (B) Luciferase activity of the intact SBE1 (wt.SBE1) and point mutated SBE1 (pmt.SBE1) promoters, normalized to the *renilla* control, is shown as fold increase over the empty pGL2-SV40 vector. (C) Normalized luciferase activity of the pGL2-SV40 vector containing either the intact wt.SBE1 or the mutated AML1a (pmt.AML1a(d+p)) inserts is depicted in the bars as fold increase over the empty vector. Asterisks denote statistical significance compared to respective controls in four separate experiments each ($p < 0.05$ for each).

Table 1**FAK 5' UTR Primer and Mutation Sequences**

<i>Serial Deletions</i>	<i>Antisense primer</i>
	5'' ATGGCGCCGGGCCTTTCTTTATGT 3'
<i>Constructs</i>	<i>Sense primers</i>
FAK-B (SBE 2,3,4)	5' <i>GGGGTACC</i> tat cac ttc ctg ctt aaa gcc 3'
FAK-C (SBE3,4)	5' <i>CGACGCGT</i> ttt caa tcc etc caa cct c 3'
FAK-D (SBE 4)	5' <i>GGGGTACC</i> ggc cca gcc caa ctt agc t 3'
FAK-E (NoSBE-NF-KB)	5' <i>GCACGCGT</i> gtg gtg cct gga gag tgc ac 3'
FAK-F (NSBE-1)	5' <i>GGGGTACC</i> gac aaa tta cgc gag cag 3'
FAK-G (NSBE-2)	5' <i>GGG GTA CC</i> acg cag cac ggc etc gag 3'
<i>P53 Deletions</i>	
mt.p53.d (Distal site)	<i>Sense:</i> 5' gaa taa act cca ggc acc t(Δ8) cc acg cag ccg tct gc 3' <i>Anti:</i> 5' gca gac ggc tgc gtg gg(Δ8)a ggt gcc tgg agt tta ttc 3'
mt.p53.p (Proximal site)	<i>Sense:</i> 5' cgg tgt cgg ggc (Δ8) cct cct ttt cct gga gcc cgt etc 3' <i>Anti:</i> 5' gag acg ggc tcc agg aaa agg agg (Δ8) gcc ccg aca ccg 3'
<i>SBE1 Point Mutation</i>	
pmt.SBE1	<i>Sense:</i> 5' agg cct cgg tgc ctg gcg gtc ctg ctt cct 3' <i>Anti:</i> 5' agg aag cag gac cgc cag gca ccg agg cct 3'
<i>AML1 Point Mutations</i>	
Distal site	<i>Sense:</i> 5' gtc ctg ctt cct ggc tat tat tag cat ca3' <i>Anti:</i> 5' tga tgc taa taa tag cca gga agc agg ac 3,
Proximal site	<i>Sense:</i> 5' ttc tgg ttt cat ttc cga ata taa ggt tgc 3' <i>Anti:</i> 5' gca acc taa tat tcg gaa atg aaa cca gaa 3'