TGFβ₁ signaling via αVβ₆ integrin
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Abstract

Background: Transforming growth factor β₁ (TGFβ₁) is a potent inhibitor of epithelial cell growth, thus playing an important role in tissue homeostasis. Most carcinoma cells exhibit a reduced sensitivity for TGFβ₁ mediated growth inhibition, suggesting TGFβ₁ participation in the development of these cancers. The tumor suppressor gene DPC4/SMAD4, which is frequently inactivated in carcinoma cells, has been described as a key player in TGFβ₁ mediated growth inhibition. However, some carcinoma cells lacking functional SMAD4 are sensitive to TGFβ₁ induced growth inhibition, thus requiring a SMAD4 independent TGFβ₁ pathway.

Results: Here we report that mature TGFβ₁ is a ligand for the integrin αVβ₆, independent of the common integrin binding sequence motif RGD. After TGFβ₁ binds to αVβ₆ integrin, different signaling proteins are activated in TGFβ₁-sensitive carcinoma cells, but not in cells that are insensitive to TGFβ₁. Among others, interaction of TGFβ₁ with the αVβ₆ integrin resulted in an upregulation of the cell cycle inhibitors p21/WAF1 and p27 leading to growth inhibition in SMAD4 deleted as well as in SMAD4 wildtype carcinoma cells.

Conclusions: Our data provide support for the existence of an alternate TGFβ₁ signaling pathway that is independent of the known SMAD pathway. This alternate pathway involves αVβ₆ integrin and the Ras/MAP kinase pathway and does not employ an RGD motif in TGFβ₁-sensitive tumor cells. The combined action of these two pathways seems to be necessary to elicit a complete TGFβ₁ signal.

Background

The normal function of transforming growth factor β₁ (TGFβ₁) is essential for the entire organism, representing a multifunctional regulator of cell growth and differentiation [1–5]. TGFβ₁ is a potent inhibitor of epithelial cell proliferation. Upon binding of TGFβ₁, TGFβ₁-receptors phosphorylate SMAD2 or SMAD3 [6–12]. Phosphorylated SMAD2/3 associates with SMAD4 and, as a complex, moves into the nucleus, where it regulates gene expression [13–15].
SMAD4 (DPC4) is essential for this TGFβ signaling and transcriptional activation process [16]. In epithelial cells, TGFβ1 decreases c-myc, cdc2 and cyclin D1 expression, and it increases the expression of c-jun and c-fos [17–23]. Activation of the TGFβ signal pathway in epithelial cells leads to an increased expression of the cell cycle inhibitors p21WAF1 and p15ink4b and to a release of formerly sequestered p27kip [24–26]. It is assumed that the cooperative action of these cell cycle inhibitors results in the growth arrest mentioned above, although p15ink4b does not seem to be necessary in this regard. In addition to mutations in the TGFβ receptors, in a large number of carcinomas disruptions of this signaling pathway by the alteration of a single protein such as p15ink4b, p16, and p21Waf1 are found [2,27–39]. This may result in resistance to the growth-inhibiting action of TGFβ1.

In several cell lines, particularly in pancreatic carcinoma cells, resistance to TGFβ1 could be attributed to a loss of function of the SMAD4 (DPC4) protein [40–43]. However, the pancreatic carcinoma cell line BxPC-3, although homozygously deleted for SMAD4, is growth inhibited by TGFβ1 [30,44]. It is thus speculated that alternative signaling pathways in addition to the SMAD pathway may exist.

After binding to αvβ6 integrin, latent TGFβ1 is activated by processing of latent TGFβ by cleavage of the latency-associated Peptide (LAP) [45–57]. Recently, the interaction of latent TGFβ1 with αvβ6 integrin has been shown [45]. After binding of latent TGFβ1 to αvβ6 integrin, latent TGFβ1 is activated by cleavage of the latency-associated peptide (LAP) [45]. This αvβ6 integrin is also expressed by pancreatic carcinoma cells [58–63]. We hypothesized that there is a SMAD-independent TGFβ signaling pathway in TGFβ1-sensitive carcinoma cells. To address this question, several carcinoma cell lines with different degrees of TGFβ1 sensitivity were chosen as a model system. We investigated the interaction of TGFβ1 with the αvβ6 integrin and its influence on selected target genes known to be involved in cell cycle-regulated growth inhibition. Here, we demonstrate an alternate TGFβ1 signaling pathway via αvβ6 integrin contributing to TGFβ1 growth inhibition in TGFβ1-sensitive carcinoma cells.

Results

Mature TGFβ induces cytoskeletal immobilization of proteins and tyrosine phosphorylation via integrin αvβ6 only in TGFβ sensitive cells

Only integrins that have bound their ligands are anchored to the cytoskeleton [64,65]. In our experiments, mature TGFβ1, αvβ6 integrin, and F-actin colocalize (Figure 1), suggesting association with and activation of this integrin. To further support this finding, we stimulated cells and performed co-immunoprecipitated various integrin subunits of cytoskeletal anchored proteins [66,67] (additional file 1, 2, 3 and 4). Our data strongly suggest that mature TGFβ1 associates with αvβ6 integrin (additional file 1, 2, 3 and 4).

To determine whether binding of mature TGFβ1 leads to integrin-mediated signaling, we looked at the status of integrin-cytoskeleton-associated proteins [66,67] after incubation with mature TGFβ1 in selected carcinoma cell lines with different degrees of sensitivity to TGFβ1 (Table 1). Cytoskeletal anchored proteins were precipitated with anti αv and β6-antibodies. Immobilization of proteins to the cytoskeleton (Triton-X insoluble fraction, Figure 2B) as well as tyrosine phosphorylation of these proteins (Figure 2A) induced through mature TGFβ1 was only seen in the TGFβ1-sensitive carcinoma cell lines (Figure 2 and additional file 5). Notably, tyrosine phosphorylation of cytoskeletonally anchored proteins is further enhanced after combined treatment with mature TGFβ1 and fibronectin in TGFβ1 sensitive cells (Figure 3). In contrast, in the TGFβ1-resistant AsPC-1 and Capan-1 cells, the interaction of mature TGFβ1 with αvβ6 integrin resulted in an immobilization of high molecular weight proteins to the cytoskeleton without tyrosine phosphorylation (Figure 2). Again, stimulation of TGFβ1 sensitive cells BxPC-3, LoVo [68], SW48 [68], Keratinocytes, HeLa and DLD1 [69], results in an enhanced cytoskeletal immobilization and tyrosine phosphorylation of cellular proteins in response to stimulation with mature TGFβ1 (additional file 5). Remarkably, preincubation with the MEK1 inhibitor PD98059 resulted in a reduced cytoskeletal immobilization and tyrosine phosphorylation of cellular proteins in response to stimulation with mature TGFβ1. This finding is in agreement with other observations that MEK1-mediated signal transduction is involved in cytoskeletal remodeling and integrin engagement [70,71].

Activation of p125FAK, a central step in integrin-associated signaling [72,73], was determined to assess integrin-mediated signaling. BxPC-3 cells are sensitive to TGFβ1 but are SMAD4 deleted. We incubated BxPC-3 cells with mature TGFβ1 and observed an association on the cytoskeleton connected with integrin αvβ6 and activation of p125FAK (Figure 4). Indeed, TGFβ1 antibodies, cytochalasin D and BAPTA-AM [66] abolished the association on the cytoskeleton connected with integrin αvβ6 and activation of p125FAK. These data further suggest that TGFβ1 mediated activation of p125FAK depends on free intracellular calcium and an intact actin cytoskeleton.

In order to test whether TGFβ1 signaling via αvβ6 is specific for SMAD4 deleted BxPC-3 cells or if this is a general phenomenon, we investigated signaling in TGFβ1-sensitive carcinoma cell lines HeLa, MCF-7 and MDA-MB-23.1. TGFβ1 induced recruitment of p125FAK, p130Cas and Sos1/2 to the cytoskeleton. Enhanced expression of c-jun, c-fos,
p21WAF1 and p27KIP, while downregulating PCNA, is dependent on ERK1/2 signaling, an intact cytoskeleton and intracellular calcium (Figures 5, 6A, 7, 8 and additional files 6, 7 and 8). We also confirmed the purity of the commercially available mature TGFβ1 used in these experiments by silver stained non-reducing SDS-PAGE, with latent TGFβ1 as control (Figure 6B). We also demonstrated the SMAD4 deficiency of the BxPC-3 cells used (Figure 6C).

**Table 1: SMAD4 status and TGFβ1 response of selected tumor cell lines were: (1) confirmed by PCR sequencing (data not shown) and (2) by [3H] thymidine incorporation assays (data not shown). WT denotes wild type.**

| Cell lines | Smad4 status¹ | Growth inhibition² by TGFβ1 |
|------------|---------------|-----------------------------|
| Panc-1     | + (WT)        | +                           |
| BxPC-3     | - (homozygous deleted) | +                           |
| Capan-1    | - (frame shift mutation) | -                           |
| AsPC-1     | - (amino acid replacement) | -                           |
| HeLa       | + (WT)        | +                           |
| MCF-7      | + (WT)        | +                           |
| MDA-MB-231 | + (WT)        | +                           |

**Figure 1**

*Colocalization of TGFβ1, αVβ6 integrin and the cytoskeleton.* Panc-1 cells were stimulated with mature TGFβ1 and stained using anti TGFβ1 (labeled with goat anti-rabbit IgG conjugate, A-11046), αV/β6 (labeled with goat anti-rabbit IgG conjugate, A-11046) and Actin antibodies. Magnification 1000x.
we assayed for the possible synergistic function of αVβ6 integrin on mature TGFβ1 mediated growth inhibition in Panc-1 cells. As shown in the additional file 10, combined treatment with αV and β6 blocking antibodies almost completely abolished the effect of mature TGFβ1 on the growth of Panc-1 cells. We therefore postulate that the growth inhibition of TGFβ1 is synergistically influenced by αVβ6 integrin.

Discussion

A recent study demonstrated an interaction of latent TGFβ1 with αVβ6 integrin, which led to an activation of latent TGFβ1 [45]. Incubation of different tumor cells with mature TGFβ1 resulted in a direct binding of TGFβ1 to αVβ6 integrin. Certain integrins appear to be preferentially associated with specific growth factor receptors [80]. The interaction of these two receptor classes seems to take place via the actin cytoskeleton. We were able to exclude such signal pathway association, since in our cytoskeletal preparations, no TGFβ1-receptors were detectable, indicating that mature TGFβ1 is a ligand for αVβ6.

It has been reported that activated integrins are associated with the cytoskeleton. Here, we show that binding of mature TGFβ1 to αVβ6 integrin resulted in an association of the cytoskeleton (Figure 10). In a variety of integrin-mediated signaling pathways, tyrosine phosphorylation of proteins immobilized to the cytoskeleton is enhanced [66,67]. The same was true in our experimental settings only for the TGFβ1-sensitive cells. This upregulation of activated SMAD2/3 may translocate to the nucleus and activate gene expression even in the absence of SMAD4. To exclude this possibility, cellular proteins were divided into cytoplasmatic and nuclear fractions after TGFβ1 stimulation, and localization and phosphorylation of SMAD2/3 were investigated. In the SMAD4 deleted BxPC-3 cells, TGFβ1 resulted in phosphorylation of SMAD2/3, but the activated SMAD proteins were retained in the cytoplasmatic fraction (Figure 9). Remarkably, in NP-9 cells [74], SMAD2/3 are translocated into the nucleus upon TGFβ1 stimulation (additional file 9(A)) but we could not observe an enhanced tyrosine phosphorylation of cytoskeletal anchored proteins (additional file 9(B)).

TGFβ1 mediated growth inhibition is dependent on αVβ6 integrin

Influence of TGFβ1 on cell growth is well established, but the mechanisms are not fully understood [75–79]. Here, we observed an enhanced tyrosine phosphorylation of proteins associated with the integrin-cytoskeleton-complex.

Enhanced Tyrosine Phosphorylation of proteins associated with the integrin-cytoskeleton-complex

Cytoskeletally anchored αVβ6 was immunoprecipitated after TGFβ1 and/or fibronectin stimulation (10 nM for 10 minutes) followed by Western analysis with antibodies against tyrosine-phosphorylated proteins (A). Reprobing with αV and β6 antibodies show equal amounts of precipitates used (B).

Figure 2
Phosphorylation and immobilization of proteins associated with the integrin-cytoskeleton-complex.

Cytoplasmatic anchored αVβ6 was immunoprecipitated after TGFβ1 stimulation (10 nM for 10 minutes) followed by Western analysis with antibodies against tyrosine-phosphorylated proteins (A) or Western blotting after biotinylation of all proteins and streptavidin detection (B). Presence of TGFβ1 (C), αV and β6 integrin (D) in the co-precipitates is also demonstrated. TGFβ1-receptor-I and II (TGFβRI and TGFβRII) are expressed at nearly equal levels in all cell lines as demonstrated by western blotting from whole cell extracts (E). In part the cells were preincubated with αV- and β6-antibodies (1:100 each for 30 min) or with a TGFβ antibody (15 µg/ml for 30 min).

Figure 3
Enhanced Tyrosine Phosphorylation of proteins associated with the integrin-cytoskeleton-complex.

Cytoskeletally anchored αVβ6 was immunoprecipitated after TGFβ1 and/or fibronectin stimulation (10 nM for 10 minutes) followed by Western analysis with antibodies against tyrosine-phosphorylated proteins (A). Reprobing with αV and β6 antibodies show equal amounts of precipitates used (B).
tyrosine phosphorylation was inhibited by preincubation with a TGF\(\beta\)_1 neutralizing antibody or by blocking of \(\alpha_\nu\beta_6\) integrin, thus again proving mature TGF\(\beta\)_1 as an initial signaling ligand for \(\alpha_\nu\beta_6\).

Binding of mature TGF\(\beta\)_1 to \(\alpha_\nu\beta_6\) integrin exerts several downstream effects in TGF\(\beta\)_1-sensitive cells (Figure 9). One is a marked phosphorylation of p125FAK. This phosphorylation is dependent on the integrity of the cytoskeleton, as disruption of actin filaments by cytochalasin D completely eliminated this effect, findings which have also been reported for several integrin signaling pathways [66,67]. Moreover, incubation of the TGF\(\beta\)_1 sensitive carcinoma cells with TGF\(\beta\)_1 caused immobilization of the docking protein p130Cas and of the guanine nucleotide exchange factor SOS to the cytoskeleton. Beyond this, a marked induction of the cell cycle inhibitors p21WAF1 and c-fos, and the decrease in p21Ras and MAPK ERK1/2 activation was observable after preincubation of SMAD4 wildtype BxPC-3 cells with a TGF\(\beta\)_1 antibody (15 \(\mu\)g/ml for 30 min), cytochalasin D and BAPTA AM, respectively.

Finally, TGF\(\beta\)_1 caused an activation of p21Ras and the MAP kinases ERK1 and ERK2. This TGF\(\beta\)_1-induced expression profile was not affected by preincubation of SMAD4 deleted BxPC-3 cells with a TGF\(\beta\)_1-RII blocking antibody, which was able to completely block TGF\(\beta\)_1-induced SMAD2/3 phosphorylation, thus demonstrating the independence of the TGF\(\beta\)_1-signaling from the known SMAD pathway in BxPC-3 cells. In contrast, preincubation with \(\alpha_\nu\)- and \(\beta_6\)-blocking antibodies curbed the TGF\(\beta\)_1-induced regulation of these genes as well, indicating the involvement of the MAP kinase pathway in TGF\(\beta\)_1 signaling in BxPC-3 cells. As shown recently, the growth-stimulatory effect of the TGF\(\beta\) superfamily member BMP-2 on CAPAN-1 cells was blocked by this inhibitor as well [81–83], supporting our findings.

Indeed, cytoskeletal immobilization of p130cas and SOS was not prevented by the MEK1 inhibitor PD 98059. Thus, these proteins are good candidates to link the integrin-mediated TGF\(\beta\)_1 signaling to the MAP kinase pathway, as was shown previously for signaling events induced by fluid stress or integrin mediated cell-adhesion in other cell types [71,84–91].

In order to generalize the integrin mediated TGF\(\beta\)_1-pathway identified in the SMAD4 deleted pancreatic tumor cell line BxPC-3, we investigated TGF\(\beta\)_1 signaling in the cervical carcinoma cell line HeLa and the mammary carcinoma cell lines MCF-7 and MDA-MB-231, harboring a wildtype SMAD4-gene. TGF\(\beta\)_1 bound to \(\alpha_\nu\beta_6\)-integrin in these cells as well, and this interaction resulted both in an immobilization of p130Cas and SOS1/2 and in tyrosine phosphorylation of cytoskeleton-associated proteins such as p125FAK. TGF\(\beta\)_1 stimulation of these cells activated p21Ras and MAPK ERK1/2, upregulated c-fos, c-jun/AP1, p21/WAF1 and p27 expression, and resulted a decrease of PCNA, similar to its actions in BxPC-3 cells. Preincubation with a TGF\(\beta\)-RII blocking antibody attenuated the TGF\(\beta\)_1 induced pattern, contrary to SMAD 4 deleted BxPC-3 cells. This preincubation also decreased activation of p21Ras and of MAPK ERK1/2, indicating the participation of the Ras/MAPK-pathway in TGF\(\beta\)_1 induced transcriptional activation.

The same attenuation of TGF\(\beta\)_1 induced gene expression and the decrease in p21Ras and MAPK ERK1/2 activation was observable after preincubation of SMAD4 wildtype cells with \(\alpha_\nu\beta_6\)-blocking antibodies, demonstrating that TGF\(\beta\)_1 signaling via \(\alpha_\nu\beta_6\)-integrin also is linked to the Ras/MAPK-pathway, and that both pathways have synergistic effects in TGF\(\beta\)_1-signaling. Full TGF\(\beta\)_1 induced transcriptional activation is only reached if both pathways are completed. This finding is supported by the observation that activation of p21/Ras and MAPK ERK1/2 induced by TGF\(\beta\)_1 is only reverted to the control level by the combination of the TGF\(\beta\)-RII blocking antibody and the \(\alpha_\nu\beta_6\)-blocking antibodies, or by inhibition of MEK1.

Linking of the TGF\(\beta\)-R pathway to the Ras/MAPK pathway is dependent on a functional SMAD4 gene product, because TGF\(\beta\)_1 induced gene expression and activation of Ras and ERK1/2 is attenuated by the TGF\(\beta\)-RII blocking antibody only in SMAD4 wild type cells, whereas in the...
Cell cycle genes in response to TGFβ1. Western Blot analysis of HeLa cells stimulated with 10 nM of mature TGFβ1 for the time indicated. Cytoskeletally anchored proteins are differentially marked. In part the cells were preincubated with αV and β6-antibodies (1:100 each for 30 min), with a TGFβ-RII antibody (15 µg/ml for 30 min), cytochalasin D, BAPTA AM and MEK1 inhibitor PD98059, respectively.
SMAD4 deleted BxPC-3 cells, no such influence was observable.

Based on our results, we suggest the following model of TGFβ1-signaling, which offers an explanation for the different growth responses to TGFβ1 (Fig. 10). In the TGFβ1-sensitive cell lines with intact SMAD pathway, the TGFβ1 response can be attributed to both the common SMAD signaling pathway and the integrin pathway described above. In the cell line BxPC-3, lacking the SMAD4 gene product, the SMAD4-independent pathway can explain the TGFβ1 sensitivity via αVβ6 integrin, the cytoskeleton and the Ras/MAP kinase pathway, resulting in an upregulation of the cell cycle inhibitors p21/WAF1 and p27, which in turn results in the TGFβ1-induced growth inhibition (additional file 10).

The cell lines Capan-1 and AsPC-1 are not only resistant to TGFβ1 because of their alterations in the SMAD pathway, but also because they cannot complete the alternate pathway, as demonstrated above. Furthermore, this alternate pathway may explain the TGFβ1 resistance of cells with no detectable defect in the SMAD pathway [92–101], as one can imagine that the cooperative action of the...
**Figure 7**

**Cell cycle genes in response to TGFβ1.** Western Blot analysis of MCF-7 and MDA-MB 231 cells as indicated after stimulation with TGFβ1 for the time indicated. Cytoskeletally anchored proteins are differentially marked. In part the cells were preincubated with αVβ6- and β6-antibodies (1:100 each for 30 min), with a TGFβ1-RII antibody (15 μg/ml for 30 min), cytochalasin D, BAPTA AM and MEK1 inhibitor PD98059, respectively.
both pathways is necessary to exert the complete growth inhibitory effect of TGFβ₁. Comparable effects have been described for the synergistic operation of growth factor receptor and anchorage dependent integrin signaling [102–119].

Recombinant mature TGFβ₁ does not contain a RGD motif, and thus binding of TGFβ₁ to the αᵥβ₆ integrin and the subsequent activation of this integrin must rely on a novel motif distinct from RGD. For αᵥβ₆ integrin, a novel non-RGD ligand recognition motif was recently described with the consensus motif DLXXL [120].

This motif has been detected on several proteins, including laminin, collagen and fibrinogen [120]. A BLAST search for this sequence in TGFβ₁ revealed a 70% similar motif in two parts of the molecule; one in the LAP (data not shown) and one in the mature TGFβ₁. In mature TGFβ₁, the DLXXL motif is freely accessible for interactions on the outside of the molecule. Therefore, it may be speculated that TGFβ₁ binding to αᵥβ₆ via this novel ligand recognition motif is facilitating the signaling. Moreover, a non-RGD ligand binding pocket in addition to the usual RGD binding site has been demonstrated for fibrinogen and the α₃β₃ integrin [121], supporting our findings.

**Conclusions**

We demonstrate an alternate TGFβ₁ signaling pathway via αᵥβ₆ integrin, independent of SMAD4. This pathway
seems to be required for full TGFβ1 induced transcriptional activation, which explains the TGFβ1 sensitivity of those cells lacking DPC4/SMAD4 function that still react with growth inhibition.

**Methods**

**Cell Culture and TGFβ1 stimulation**

All cells were obtained from from ATCC and maintained in DMEM supplemented with 17% fetal calf serum. Recombinant human proteins (mature TGFβ1, TNF-α, Fibronectin and Laminin 1) were purchased from R&D Systems. 10^6 cells were grown overnight in 6 cm diameter dishes with DMEM/10 % FCS. After washing twice with PBS (pH 7.4), fresh DMEM without FCS was added to the monolayer. Cells were stimulated with 10 nM of mature TGFβ1 or with fibronectin as described below. In blocking experiments, cells were preincubated with either a TGFβ1-RII-blocking antibody (R&D Systems # AF-241-NA, 15 µg/ml for 30 min), αv and β6-blocking antibodies (Santa Cruz, sc-6617 and sc-6632 respectively, 1:100 each for 30 min), or the MEK1 inhibitor PD98059 (New England Biolabs # 9900S, 7.5 ng/ml for 10 min) before stimulation with mature TGFβ1.

**Indirect immunofluorescence**

For indirect immunofluorescence, 10^4 cells were cultured on glass coverslips, stimulated with 10 nM mature TGFβ1 for 10 minutes, stained as described [66,67] and viewed using a Zeiss LSM-510 confocal microscope. Antibodies used were: actin (sc-8432), TGFβ1 (sc-146), αv (sc-6617) and β6 (sc-6632). The following fluorochrome-labeled antibodies were used (AlexaFluor, Molecular Probes):

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**Figure 9**

**Activation and nuclear translocation of SMAD2/3 in response to TGFβ1 (A).** Nuclear and cytoplasmatic fraction of cellular proteins (BxPC-3) after stimulation with 10 nM of TGFβ1 for 10 minutes and Western blot analysis for SMAD2/3 and phosphorylated SMAD2/3. **Purity of cytoplasmatic and nuclear fraction (B).** Cytoplasmatic and nuclear extracts from K562 cells were probed with p125FAK, PCNA and IκBα antibodies at the same time. As predicted, p125FAK could exclusively be detected in the cytoplasmatic extract, whereas PCNA is found in the nucleus. IκBα served as loading control.
Ras

Immune complexes were washed five times with cold Triton X-100 lysis buffer. For re-precipitation, the pellet was boiled in 10 μl 0.1% SDS for 5 min and diluted 1:20 in the Triton X lysis buffer followed by the precipitation procedure. All samples were boiled in Laemmli denaturing buffer and analyzed by Western blotting. Whole cell lysates serving as positive controls were prepared by incubating monolayers with denaturing Laemmli buffer.

**Treatment with Cytochalasin and Calcium Chelator**

To disrupt the actin filaments of the cytoskeleton, the cell monolayer was treated with 25 nM cytochalasin D for 20 min at 37°C; TGFβ1 was then applied in the presence of 25 nM cytochalasin D. For chelating intracellular calcium, the cells were preincubated with 5 μM of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid, acetoxy methyl ester (BAPTA-AM) for 15 min. TGFβ1 was then applied in the presence of 5 μM of BAPTA.

**[3H]-thymidine incorporation assay**

For the TGFβeta1 growth inhibition assay, cells were seeded in 96-well microtiter plates at 10^4 cells/well in 100 μl of culture medium containing 10% FCS. After 24 h, medium was replaced by culture medium supplemented with 0.5% FCS. After an additional 24 h, cells were treated with 10 nM of mature TGFβ1. After incubation with TGFβ1 for 21 h, cells were pulsed with 200 nCi of [3H]-thymidine (1.74 TBq/mmol; Amersham, UK) for 3 h without changing the medium. Cells were washed once with PBS, incubated with trypsin for 10 min and collected by using a Skatron cell harvester. Radioactivity incorporated was determined by liquid scintillation counting.

**Western Blot**

Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche) as described previously [66]. Blot membranes were blocked for 3 h at 37°C in PBS containing 5 % skim milk and probed with the respective antibodies (16 h at 4°C). The following antibodies were used in a dilution of 1:1,000: TGFβ1 (Santa Cruz [sc], sc-146), p-Tyr (sc-7020), βγ integrin (sc-6632), αβ-integrin (sc-6617), p125FAK (sc-557), TGFβ1-RII (sc-402), TGFβ1-RI (sc-400-G), ERK1/2-P (sc-7383), SMAD2/3 (sc-6033), SOS1/2 (sc-259), p130Cas-P (sc-668), p21Ras (sc-35), PCNA (sc-56), p27KIP (sc-282), p-ERK1/2 (sc-1641), p-ERK1/2 (sc-239), c-jun (sc-44), c-fos (sc-7202), p-ERK1/2 (sc-133), p-ERK1/2 (sc-35) and phospho-threonine antibody (New England Biolabs, # 9381). Detection antibodies (all from Dako; 1:5,000 for 1 h at room temperature) were mouse-anti-goat Ig, mouse-anti-rat Ig, rabbit-anti-mouse Ig, and porcine-anti-rabbit Ig-HRP [66]. To visualize all transferred proteins, we used the ECL protein biotinylation labeling modules (RPN 2202, Amersham) and streptavidin alkaline phosphatase (V020402, Amersham) in accordance with the manufacturer’s instructions.

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**Figure 10** Hypothesis about an alternate TGFβ1 signaling pathway via αvβ6 integrin, independent of RGD. This pathway may be required for full TGFβ1 induced transcriptional activation, which explains the TGFβ1 sensitivity of those cells lacking DPC4/SMAD4 function that still react with growth inhibition.

| Cellular compartment | Proteins Assayed
|----------------------|---------------|
| Cell membrane        | TGFβ1, Ras, βγ integrin, αβ integrin, F-actin, FAK-P |
| Nuclear membrane     | SMAD2/3, SMAD4, p21Ras, p130Cas-P, p27KIP |
| Cytoskeleton         | TGFβ1, Ras, β6 integrin, αvβ6 integrin, F-actin, FAK-P |

**Preparation of cytoplasmatic proteins and of nuclei**

Cellular fractionation was performed as described in earlier reports [122–125]. Cells were scraped into 100 μl of ice-cold buffer A [10 mM Hapes (pH 7.9); 1.5 mM MgCl2; 10 mM KCl; 0.5 mM DTT; 0.05% NP-40]. Nuclei were pelleted in a microcentrifuge for 10 sec. at 4°C and 15,000 G. The supernatant was used to analyze cytoplasmatic proteins. Pre-treatment with trypsin for 10 min and collection by using a Skatron cell harvester. Radioactivity incorporated was determined by liquid scintillation counting.

**Preparation of actin filaments of the cytoskeleton and immunoprecipitation**

The cell monolayer was incubated with cell extraction buffer [0.1% Triton X-100, 80 mM KCl, 20 mM imidazole, 2 mM MgCl2, 2 mM EGTA, pH 7.8] for 5 min at 4°C. The Triton-insoluble fractions were then scraped into cold Triton X-100 lysis buffer [50 mM Tris/HCl (pH 7.4); 100 mM NaCl; 50 mM NaF; 5 mM EDTA; 40 mM glycophosphate; 1 mM sodium orthovanadate; 100 μM PMSF; 1 μM leupeptin; 1 μM pepstatin A; 1% (v/v) Triton X-100] and incubated for 20 min on ice, and clarified by centrifugation at 13000 g for 5 min at 4°C. For immunoprecipitation, the lysates were incubated for 4 h at 4°C with 1 μg of antibody (all from Santa Cruz) pre-adsorbed on Protein A-Sepharose beads (Pharmacia). Immune complexes were washed five times with cold Triton X-100 lysis buffer. For re-precipitation, the pellet was boiled in 10 μl 0.1% SDS for 5 min and diluted 1:20 in the Triton X lysis buffer followed by the precipitation procedure. All samples were boiled in Laemmli denaturing buffer and analyzed by Western blotting. Whole cell lysates serving as positive controls were prepared by incubating monolayers with denaturing Laemmli buffer.
Ras activation assay

Only activated p21<sup>Ras</sup> is able to bind Raf1, leading to a Raf1-translocation to the cell membrane. After stimulation with 10 nM mature TGFβ<sub>1</sub> for 10 minutes, cells were incubated in sterile water until they lysed. The membrane fraction was lysed in Triton X-100 lysis buffer. Precipitation with 10 nM mature TGFβ<sub>1</sub> for 10 minutes was followed by Western analysis with antibodies against tyrosine-phosphorylated proteins (A) or Western blotting after biotinylation of all proteins and streptavidin detection (B). In part the cells were preincubated with α<sub>v</sub>- and β<sub>6</sub>-antibodies (1:100 each for 30 min) or with a TGFβRII antibody (15 µg/ml for 30 min).

Authors’ contributions

CS performed all assays and drafted the manuscript. MPK and GMS provided suggestions and comments for its finalization. All authors read and approved the final manuscript.

Additional material

Additional File 1

Portable Network Graphic (PNG) File showing that mature TGFβ<sub>1</sub> binds to α<sub>v</sub>β<sub>3</sub> integrin. The cells indicated were stimulated for ten minutes with 10 nM of either mature TGFβ<sub>1</sub>, tumor necrosis factor α (TNFα) or fibronectin (FN). Cytoskeletal anchored proteins were extracted, co-immunoprecipitated (IP) and analyzed (Blot) with the antibodies indicated.

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Additional File 2

Portable Network Graphic (PNG) File showing that mature TGFβ<sub>1</sub> binds to α<sub>v</sub>β<sub>3</sub> integrin. The cells indicated were stimulated for ten minutes with 10 nM of either mature TGFβ<sub>1</sub>, tumor necrosis factor α (TNFα), laminin-1 (Lam1) or fibronectin (FN). Cytoskeletal anchored proteins were extracted, co-immunoprecipitated (IP) and analyzed (Blot) with the antibodies indicated.

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Additional File 3

Portable Network Graphic (PNG) File showing that mature TGFβ<sub>1</sub> binds to α<sub>v</sub>β<sub>3</sub> integrin and the specificity of the signals detected as well. The cells indicated were stimulated for ten minutes with 10 µM of either mature TGFβ<sub>1</sub>, tumor necrosis factor α (TNFα), or fibronectin (FN). Cytoskeletal anchored proteins were extracted, and analyzed (Blot) with secondary antibodies (α-mouse HRP plus α-rabbit HRP plus α-goat HRP conjugated antibodies.)

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Additional File 4

Portable Network Graphic (PNG) File showing the specificity of the signals detected. The cells indicated were stimulated for ten minutes with 10 nM of either mature TGFβ<sub>1</sub>, tumor necrosis factor α (TNFα), or fibronectin (FN). Cytoskeletal anchored proteins were extracted, and analyzed (Blot) with secondary antibodies (α-mouse HRP plus α-rabbit HRP plus α-goat HRP conjugated antibodies.)

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Additional File 5

Portable Network Graphic (PNG) File showing enhanced cytoskeletal immobilization and tyrosine phosphorylation of cellular proteins in response to stimulation with mature TGFβ<sub>1</sub>. Cytoskeletal anchored α<sub>v</sub>- and β<sub>6</sub>-antibodies (1:100 each for 30 min) or with a TGFβRII antibody (15 µg/ml for 30 min).

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Additional File 6

Portable Network Graphic (PNG) File showing cell cycle genes in response to TGFβ<sub>1</sub>. Western Blot analysis of HeLa, MCF-7 and Keratinocytes (Keratino) cells as indicated after stimulation with TGFβ<sub>1</sub> for the time indicated. Cytoskeletonally anchored proteins are differentially marked. In part the cells were preincubated with α<sub>v</sub>- and β<sub>6</sub>-antibodies (1:100 each for 30 min), with a TGFβRII antibody (15 µg/ml for 30 min), cytochalasin D, BAPTA AM and MEK1 inhibitor PD98059, respectively.

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Additional File 7

Portable Network Graphic (PNG) File showing that PCNA regulation is dependent on α<sub>v</sub>β<sub>6</sub>-integrins, intact cytoskeleton and free intracellular calcium. BxPC-3 cells were stimulated with 10 nM of mature TGFβ<sub>1</sub> for 6 hours. In part the cells were preincubated with α<sub>v</sub>- and β<sub>6</sub>-antibodies (1:100 each for 30 min), with a TGFβRII antibody (15 µg/ml for 30 min), cytochalasin D and BAPTA AM, respectively. Whole cell extract was probed with PCNA antibodies. Actin served as loading control.

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Additional File 8

Portable Network Graphic (PNG) File showing the regulation of p27, p21, c-fos, and c-jun are dependent on α<sub>v</sub>β<sub>6</sub>-integrins, intact cytoskeleton and free intracellular calcium. BxPC-3 cells were stimulated with 10 nM of mature TGFβ<sub>1</sub> for 6 hours. In part the cells were preincubated with α<sub>v</sub>- and β<sub>6</sub>-antibodies (1:100 each for 30 min), with a TGFβRII antibody (15 µg/ml for 30 min), cytochalasin D and BAPTA AM, respectively. Whole cell extract was probed with PCNA antibodies. Actin served as loading control.

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**Additional File 9**
Portable Network Graphic (PNG) File showing activation and nuclear translocation of SmAD2/3 in response to TGFβ1 (A). Nuclear and cytoplasmic fraction of cellular proteins (NP9) after stimulation with 10 nM of TGFβ1 for 10 minutes and Western blot analysis for SmAD2/3 and phosphorylated SmAD2/3. Cytoselectively anchored α/β1 was immuno-precipitated after TGFβ1 stimulation (10 nM for 10 minutes) followed by Western analysis with antibodies against tyrosine-phosphorylated proteins (C) or Western blotting after biotinylation of all proteins and streptavidin detection (D). In part the cells were preincubated with α- and β-antibodies (1:100 each for 30 min) or with a TGFβ antibody (15 μg/ml for 30 min).

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**Additional File 10**
Microsoft Excel spreadsheet showing TGFβ1 elicited growth inhibition of Panc-1 cells is dependent on α/β1 integrin function. The assay was performed as described in the "Methods" section.

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