TGFβ-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN

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Summary

Transforming growth factor beta (TGFβ) has profound growth-suppressive effects on normal epithelial cells, but supports metastasis formation in many tumour types. In most epithelial tumour cells $TGF\beta_1$ treatment results in epithelial dedifferentiation with reduced cell aggregation and enhanced cellular migration. Here we show that the epithelial dedifferentiation, accompanied by dissociation of the E-cadherin adhesion complex, induced by $TGF\beta_1$ depended on phosphatidylinositol 3-kinase (PI3-kinase) and the phosphatase PTEN as analysed in PANC-1 and Smad4-deficient BxPC-3 pancreatic carcinoma cells. $TGF\beta_1$ treatment enhanced tyrosine phosphorylation of α- and β-catenin, which resulted in dissociation of the E-cadherin/catenin complex from the actin cytoskeleton and reduced cell-cell adhesion. The PI3-kinase and PTEN were found associated with the E-cadherin/catenin complex via β-catenin. $TGF\beta_1$ treatment reduced the amount of PTEN bound to β-catenin and markedly increased the tyrosine phosphorylation of β-catenin. By contrast, forced expression of PTEN clearly reduced the $TGF\beta_1$-induced phosphorylation of β-catenin. The $TGF\beta_1$-induced β-catenin phosphorylation was also dependent on PI3-kinase and Ras activity. The described effects of $TGF\beta_1$ were independent of Smad4, which is homozygous deleted in BxPC-3 cells. Collectively, these data show that the $TGF\beta_1$-induced destabilisation of E-cadherin-mediated cell-cell adhesion involves phosphorylation of β-catenin, which is regulated by E-cadherin adhesion complex-associated PI3-kinase and PTEN.

Key words: Transforming growth factor beta, E-cadherin, Cell-cell adhesion, β-catenin tyrosine phosphorylation, Epithelial cells, Pancreatic cancer, Invasion, Cell migration

Introduction

Proteins of the transforming growth factor beta family (TGFβ) are involved in the regulation of proliferation, differentiation, migration and adhesion of most cell types. $TGF\beta_1$, which represents the best-studied member of this family, inhibits the proliferation and induces differentiation of epithelial cells (Attisano and Wrana, 2002). On the molecular level, binding of TGFβ to type II TGFβ receptors leads to recruitment and transphosphorylation of type I TGFβ receptors and activation of receptor-activated Smad2 and Smad3, which then hetero-oligomerise with the common partner Smad4. This complex regulates the transcription of several target genes [reviewed in (Moustakas and Heldin, 2002)]. Several non-Smad signalling mechanisms have been described in mediating cellular effects of TGFβ. These include the mitogene-activated protein kinases ERK, JNK and p38, the phosphatidylinositol 3-kinase (PI3-kinase) (Bakin et al., 2000) and Ras- and Rho-GTPases (Derynck and Zhang, 2003). Studies have shown that somatic mutations in components of the TGFβ signalling pathway are associated with loss of proliferation control, malignant progression, invasion and metastasis formation both in vitro and in vivo (Akhurst and Derynck, 2001). In this context, $TGF\beta_1$ was described to induce morphological, biochemical and transcriptional changes towards a mesenchymal phenotype designated as epithelial to mesenchymal transition (EMT) (Fensterer et al., 2004; Ellenrieder et al., 2001b; Oft et al., 1998; Boyer et al., 1996). One important feature of EMT is the dissociation of the E-cadherin adhesion complex.

Cadherin molecules represent a family of calcium-dependent transmembrane glycoproteins, which build adherens junctions and contribute to cell-cell adhesion (Takeichi, 1991; Kemler, 1993; Gumbiner, 1996). The cadherin isoforms interact with catenins, which mediate cadherin linkage to the actin cytoskeleton (Kemler, 1993). Either β- or γ-catenin (plakoglobin) binds directly to cadherin and α-catenin, which links this complex directly or indirectly to the actin-based cytoskeleton (Hinck et al., 1994; Herrenknecht et al., 1991). The assembly and maintenance of adherens junctions is under tight control. Tyrosine phosphorylation of β-catenin was shown to be responsible for dissociation of E-cadherin/β-catenin from α-catenin and the actin cytoskeleton. The cellular kinase Src (Behrens et al., 1993; Hamaguchi et al., 1993; Takeda et al., 1995), as well as FER-kinase (Piedra et al., 2003) or receptor kinases such as epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (cMET) are

suggested to contribute to catenin phosphorylation (Hoschuetzky et al., 1994; Birchmeier et al., 1997).

In vivo, protein phosphorylation is a highly reversible and dynamic process, in which the level of phosphorylation reflects the sum of protein kinase and protein phosphatase activity. Several lines of evidence suggest that phosphatases represent important regulators of E-cadherin-mediated cell-cell adhesion. A direct association of different protein tyrosine phosphatases (PTPs), such as PTPμ, PTPκ, LAR or SHP-2 with proteins of the E-cadherin/catenin complex has been described (Brady-Kalnay et al., 1998; Müller et al., 1999). Inhibition of PTPs results in destabilisation of cellular adhesion and enhanced migration (Müller et al., 1999; Volberg et al., 1992) and can be detected in many carcinomas (Streuli, 1996).

In this study, we examine the effect of $TGF\beta_1$ on cell-cell adhesion and cell migration in pancreatic carcinoma cells. Pancreatic adenocarcinomas belong to the most fatal cancers because of their extensive invasion into surrounding tissues and metastases formation (Kern et al., 2001; Poston et al., 1991). We show that PI3-kinase and the phosphatase PTEN are associated with the E-cadherin adhesion complex and play an important role in $TGF\beta_1$-induced phosphorylation of β- and α-catenin, which results in a decrease of cell-cell adhesion with a concomitant increase in cell migration.

Materials and Methods

Antibodies

Monoclonal antibodies against E-cadherin (C20820), α- (C21620) and β-catenin (C19220) were obtained from BD Bioscience. Polyclonal antibodies against α- (C-2081) and β-catenin (C-2206) as well as anti-β-actin (A-4700) antibody were purchased from Sigma-Aldrich. An antiserum against PTEN (210-774-R100) was purchased from Alexis Biochemicals. A HRP-coupled phosphotyrosine-specific antibody was purchased from BD Bioscience (PY20:HRP). Anti-TGFβ receptor type II antibody (AF-241-NA), which inhibits $TGF\beta_1$ binding, was purchased from R&D Systems. Antibody against phospho-PTEN (Ser380) and anti-p110α were obtained from Cell Signaling Technology. Anti-EGFP antibody was purchased from Rockland. Polyclonal antiserum against the p85 subunit of PI3-kinase was kindly provided by M. Thelen (Institute for Research in Biomedicine, Bellinzona, Switzerland).

Plasmids

Human PTEN cDNA was amplified by PCR using cDNA established from mRNA of PANC-1 cells. PCR was performed using specific primers for PTEN according to the published sequence (Li et al., 1997) (sense primer: 3′-ATGACAGCCATCATCAAAGA-5′ and antisense primer: 3′-TTGGATCCTCAGACTTTTGTAATTTG-5′) with Pfu DNA polymerase (Promega). The PCR product was inserted as a *Bam*HI/*Eco*RI-fragment into the pEGFP-C2 vector (BD Clontech). A C-terminally truncated cDNA of PTEN was produced by excision of the *Nhe*I/*Xba*I-fragment from the pEGFP/PTEN-construct. The sequence of individual plasmids was confirmed by DNA sequencing (GATC-Biotech).

Cell culture, transfection and siRNA

PANC-1 cells were obtained from ATCC (CRL-1469) and BxPC-3 cells were available from ECACC (No. 93120816). Cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (Gibco Invitrogen) containing 1% L-glutamine and 1% non-essential amino acids (PAA Laboratories). For transfection, cells were treated with 15 μg DNA per 100 mm dish and DMRIE-C reagent according to the manufacturer's protocol (Gibco Invitrogen). Stably transfected cell clones were selected by addition of 1.5 mg/ml G418 to growth medium (PAA Laboratories). PANC-1 cells stably transfected with an EGFP/H-Ras N17 expression construct were described before (Fensterer et al., 2004). Inhibitory siRNA for silencing PTEN expression was purchased from Cell Signaling Technology (#6250). In 6-well plates PANC-1 cells were transfected with 250 nM siRNA specific for PTEN or unrelated control sequence and DMRIE-C transfection reagent. After 36 hours the medium was changed against DMEM and another 6 hours later $TGF\beta_1$ was added for further 6 hours. Forty eight hours after transfection the cells were lysed in RIPA buffer supplemented with protein and phosphatase inhibitors.

Human recombinant $TGF\beta_1$ was used at a concentration of 10 ng/ml (TEBU). Pharmacological inhibitors were used in the following concentrations: PD98059 (25 μM), LY294002 (25 μM), PP1 (100 μM) and FTI 277 (2 μM). FTI 277 was purchased from Calbiochem, all other inhibitors were from Alexis Biochemical.

Protein analysis

SDS gel electrophoresis (SDS-PAGE) was performed according to standard procedures as described previously (Menke et al., 2001). For total protein analysis, cells were lysed in RIPA-buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). NOP-buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P40, 0.2% Triton X-100) was used for co-immunoprecipitation experiments. Immediately before use, the buffers were supplemented with proteinase inhibitors: 5 μM aprotinin, 1 mM Pefabloc, 5 μM soya trypsine inhibitor (STI; all from Roche Diagnostics). In case of protein phosphorylation analysis, phosphatase inhibitors were added: 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 2 mM sodium orthovanadate (Sigma-Aldrich). Thirty micrograms of lysate were analysed by SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher and Schuell). Immunoreactive proteins were detected with a secondary horseradish peroxidase-coupled antibody (Pierce) and visualised using enhanced chemiluminescence (ECL, Pierce). For co-immunocoprecipitation experiments, 0.3-3.0 mg of NOP-lysates were used for the μMACS protein isolation system from Miltenyi Biotec in accordance to manufactures instruction. The immunoprecipitates were analysed by western blotting as described above.

To obtain Triton X-100-soluble and -insoluble fractions, cells were incubated with Triton-lysis-buffer (1% Triton X-100, 0.3 M sucrose, 25 mM HEPES pH 7.4, 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 5 μM aprotinin, 1 mM Pefabloc, 5 μM STI) for 15 minutes on a rocking platform. After centrifugation, the supernatant (Triton-soluble fraction) was collected. The Triton-insoluble fraction was resuspended in SDS-lysis-buffer (20 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 1% SDS, 0.5 μg/ml DNase I, 1 mM Pefabloc, 5 μM STI, 5 μM aprotinin). Both fractions were reconstituted to equal volumes and 15 μl of each fraction were analysed by western blotting.

Membrane preparations were carried out as described previously (Lutz and Miller, 1993) with few modifications. Cells were scraped into hypotonic buffer (10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 5 μM aprotinin, 1 mM Pefabloc, 0.1 mM STI, 10 μM pepstatin, 10 μM leupeptin) and mechanically broken using a Dounce homogenisator (Braun). Nuclei were removed by centrifugation at 10,000 ***g*** for 30 seconds. Sodium chloride and saccharose were added to a final concentration of 0.2 M and 0.3 M, respectively. Cytosolic and membrane fractions were separated by ultracentrifugation for 1 hour at 4°C and 150,000 ***g*** in a swinging bucket rotor. Membranes were recovered from the interphase by aspiration, diluted with an equal

volume of NOP buffer, and collected by centrifugation at 100,000 ***g*** for 30 minutes at 4°C. The pellets were resuspended in NOP buffer. For immunoprecipitation experiments 0.5 mg of membrane fraction was used.

Protein phosphatase assays

PTEN dephosphorylation of β-catenin was examined using an in vitro phosphatase assay as described (Gu et al., 1999). Briefly, EGFP, WT PTEN or PTENΔC were isolated from 1 mg of pEGFP, pEGFP/PTEN or pEGFP/PTENΔC transfected PANC-1 cells lysed in RIPA buffer with enhanced amounts of detergents to ensure dissociation of associated proteins (50 mM Tris-HCl pH 7.2, 450 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.5% SDS). The proteins were purified by immunoprecipitation with an anti-EGFP antibody as described above and renatured by washing with renaturation buffer (PBS pH 7.0 containing 2 mM $MgCl_2$, 0.5 mM PMSF, 0.005% Tween 20, 10 mM DTT, 1 mM pefabloc, 5 μM STI, 5 μM aprotinin). Purity was confirmed by SDS-PAGE and Coomassie blue staining. Phosphorylated β-catenin was obtained from BxPC-3 cell that had been stimulated with EGF (10 ng/ml for 15 minutes) and 2 mM sodium orthovanadate by immunoprecipitation from 0.5 mg of RIPA lysate. PTEN phosphatase activity against β-catenin was examined by incubation of immunoprecipitated tyrosine phosphorylated β-catenin with immunoprecipitated EGFP, WT PTEN or PTENΔC in 30 μl of 50 mM Tris buffer pH 7.0 containing 50 mM NaCl and 10 mM DTT at 30°C for 30 minutes. Controls were incubated with immunoprecipitates obtained without PTEN antibody. The reaction was terminated by adding reducing SDS sample buffer and heating at 100°C for 5 minutes. The samples were fractionated by SDS-PAGE and immunoblotting.

Immunocytochemical analysis

Cells used for immunostaining were fixed and permeabilised in cold acetone-methanol (1:1) for 15 minutes. Primary antibodies were incubated for 1 hour at 37°C and visualised by incubation with a secondary Cy-3- (Biomol) or Alexa-488-conjugated antibody (Molecular Probes). Staining was examined by fluorescence microscopy (DM RBE, Leica-micosystems) and images were recorded using a CCD camera and analySIS 3.1-software (Soft-Imaging System) or confocal laser microscopy (TCS-4, Leica-microsystems).

Migration and aggregation studies

Migration assays were performed using cell culture inserts with uncoated porous membranes or membranes coated with collagen type I (8.0 μm pore size, BD Bioscience) as described in (Giehl et al., 2000b). To exclude $TGFβ_1$ effects caused by cell proliferation, cell growth was inhibited by mitomycin C-treatment (10 μg/ml for 1 hour). After 48 hours of incubation with 20 ng/ml $TGFβ_1$ or solvent, the number of cells that had migrated through the pores towards $TGFβ_1$ was quantified by counting five independent visual fields in the microscope (Zeiss) using a 20× objective. Three independent assays were performed in triplicates.

To determine the Ca^{2+}-dependent cell-cell adhesion, cell aggregation was determined as described by Ozawa and Kemler (Ozawa and Kemler, 1990). Cells were carefully detached with 0.01% trypsin in HEPES-buffered saline (37 mM NaCl, 5.4 mM KCl, 0.34 mM NaH_2PO_4, 5.6 mM Glucose, 10 mM HEPES) containing 2 mM $CaCl_2$, centrifuged, washed twice with HEPES buffer with 2 mM $CaCl_2$ and resuspended in the same buffer. Cells were singularised using a pasteur pipette until no aggregates were monitored and were allowed to aggregate by incubation with constant rotation of 70 rpm for 30 minutes. Afterwards, the number of aggregates was determined in an invert phase-contrast microscope (objective 10×; Zeiss). The extent of cell aggregation was calculated by the formula $A=(N_o–N_e)/N_o$, with N_o representing the total particle number at the start and N_e the total particle number after incubation for 30 minutes. To examine the calcium dependency, EDTA and EGTA were added to a final concentration of 5 mM each. E-cadherin dependence was proved by addition of neutralising antibody against E-cadherin DECMA-1 (Sigma) to a final concentration of 4 μg/ml assay medium. To remove preservatives of the antisera the solutions were replaced by TBS using centricon centrifugation columns (Filtron, Northborough, MA, USA). Three independent assays were performed in triplicate.

Statistics

The mean values and s.e.m. were calculated from at least three experiments, each performed in triplicate or quadruplicate. For statistical analysis, the Wilcoxon non-parametric test was used and $P<0.02$ was considered significant.

Results

$TGFβ_1$ treatment (10 ng/ml) of the pancreatic carcinoma cell lines PANC-1 and BxPC-3 caused a dramatic change in cell morphology from a more epithelial-like to a spindle-shaped cell morphology typical for mesenchymal cells (Fig. 1A). PANC-1 and BxPC-3 cells scattered with a loss of cell-cell contacts typically observed in mesenchymal cells. Because E-cadherin-mediated cellular adhesion contributes substantially to epithelial cell morphology, we examined the impact of $TGFβ_1$ treatment on the cellular distribution of E-cadherin as demonstrated in Fig. 1B. Control PANC-1 and BxPC-3 cells incubated with the solvent only showed a typical E-cadherin staining at membranes localised at the site of cell-cell contacts. By contrast, $TGFβ_1$ treatment resulted in a loss of E-cadherin in membranes of contacting cells (Fig. 1B). In BxPC-3 cells the E-cadherin immunofluorescence signal was redistributed over the whole cell surface and into the cytoplasm. The observed changes are independent of Smad4, which is homozygous deleted in BxPC-3 cells. In PANC-1 cells, E-cadherin was internalised on $TGFβ_1$ treatment into punctuate structures. Total protein lysates of $TGFβ_1$-treated PANC-1 and BxPC-3 cells were analysed regarding protein levels of E-cadherin, α- and β-catenin. As shown in Fig. 1C, E-cadherin and β-catenin total protein concentrations decreased after $TGFβ_1$-stimulation in PANC-1 cells, whereas BxPC-3 cells exhibited only a slight decrease in E-cadherin, α- and β-catenin protein levels.

$TGFβ_1$ treatment of PANC-1 and BxPC-3 cells reduces cell aggregation and enhances cell migration

To analyse whether reduction of E-cadherin induced by $TGFβ_1$ alters cell-cell adhesion in pancreatic carcinoma cells, we performed aggregation assays in the presence and absence of $TGFβ_1$. As illustrated in Fig. 2A, both analysed cell lines showed cellular aggregation under control conditions. In PANC-1 cells, application of $TGFβ_1$ reduced the diameter of aggregates to 51% (±11%) compared with controls. The additional incubation of $TGFβ_1$-treated cells with a neutralising antibody against E-cadherin does not further reduce cell aggregation (47.8±7.7%), indicating the dependence of cell aggregation on E-cadherin. The diameter of cell aggregates in $TGFβ_1$-treated cells is only slightly more

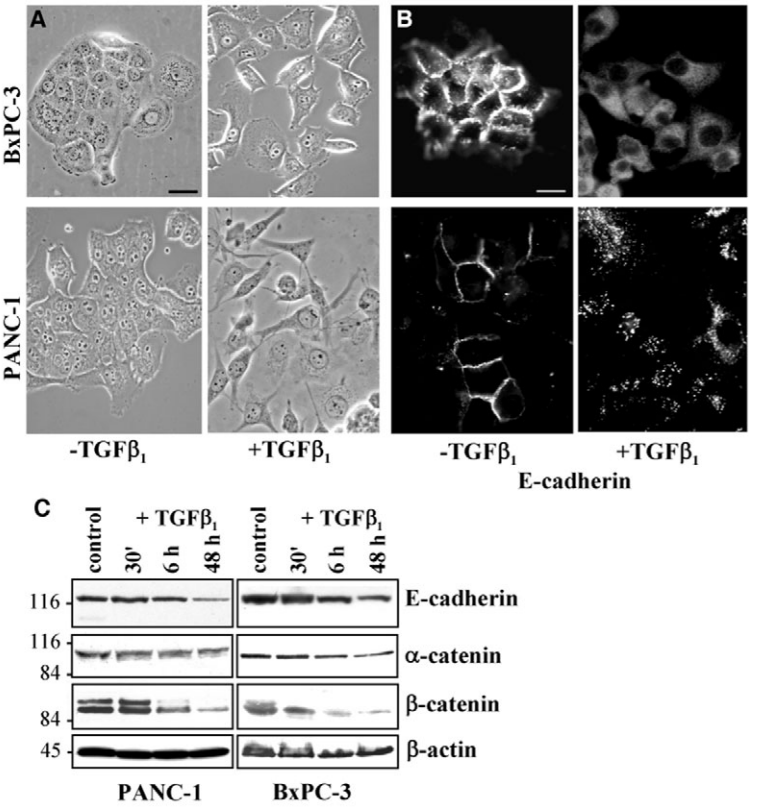

Fig. 1. PANC-1 and BxPC-3 cells were serum-starved for 24 hours and treated with solvent (–TGFβ) or 10 ng/ml $TGF\beta_1$ (+$TGF\beta_1$) for 2 days. (A) The left panel shows phase contrast pictures. (B) The right panel shows immunofluorescence staining of E-cadherin. Bars, 20 μm. (C) Western blot analyses of E-cadherin, α- and β-catenin in total lysates of PANC-1 and BxPC-3 cells treated with 10 ng/ml $TGF\beta_1$ for the indicated periods of time. Equal loading was demonstrated by staining of β-actin. Molecular mass standards are given in $M_r \times 10^3$. Representative blots out of four independent experiments are shown.

then the size of cell clusters after the removal of Ca^{2+} ions using EDTA/EGTA (30±9% compared with untreated control cells), demonstrating that the $TGF\beta_1$ effects most but not all of E-cadherin depend cell aggregation. Similar ratios were observed for BxPC-3 ($TGF\beta_1$: 48±12.2%, $TGF\beta_1$ + neutralising antibody: 42.2±7.2%, EDTA/EGTA: 36.3±10.4%) (Fig. 2A).

In addition to the observed decrease in cell aggregation, we asked whether $TGF\beta_1$ influences the migratory behaviour of pancreatic carcinoma cells. Cell migration was analysed using transwell migration assays with collagen type I-coated or uncoated porous membranes. As shown in Fig. 2B, PANC-1 and BxPC-3 cells showed a threefold increase in cell migration towards the $TGF\beta_1$-containing compartment compared with solvent-containing wells. Interestingly, invasion of BxPC-3 cells was blocked by a collagen type I matrix. In a paper by Ellenrieder et al. it has been shown that TGFβ is able to induce expression of metalloproteinase-2 (MMP-2) and the urokinase plasminogen activator (uPA) system in a Smad4-dependent way (Ellenrieder et al., 2001a). Thus, one might speculate that the failure of Smad4-dependent gene expression in BxPC-3 cells contributes to the reduced invasion through collagen type I in BxPC-3 cells harbouring a homozygous deletion in the Smad4 gene. PANC-1 cells, which have Smad4 protein, were able to invade the collagen type I matrix towards $TGF\beta_1$ (2.5-fold) suggesting that $TGF\beta_1$-induced migration of pancreatic carcinoma cells is independent of Smad4 signalling.

$TGF\beta_1$ induces dissociation of the E-cadherin/catenin adhesion complex

Next, we asked for the effect $TGF\beta_1$ has on E-cadherin-mediated cellular adhesion on a molecular level. As an indicator, how much E-cadherin/catenin is associated with the actin cytoskeleton, we analysed the E-cadherin/catenin distribution in Triton X-100 soluble and insoluble fractions in $TGF\beta_1$-treated and untreated PANC-1 and BxPC-3 cells. As shown in Fig. 3A, the amounts of E-cadherin, α- and β-catenin were reduced in Triton-insoluble fractions already 30 minutes after addition of $TGF\beta_1$ and were further decreasing in the next 48 hours, indicating that the E-cadherin adhesion complex was less associated with the actin cytoskeleton. In the Triton-soluble fractions, the amount of E-cadherin remained constant in PANC-1 cells and was only slightly reduced in BxPC-3 after $TGF\beta_1$ treatment (Fig. 3A). The amounts of α- and β-catenin were also reduced in the Triton-

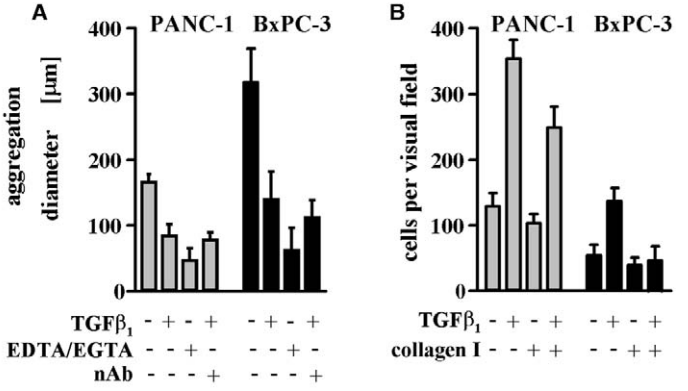

Fig. 2. (A) Cell aggregation assays were performed by incubation of pancreatic carcinoma cells PANC-1 and BxPC-3 under constant agitation in HEPES buffer plus $CaCl_2$ supplemented with solvent, $TGF\beta_1$ (10 ng/ml), EDTA/EGTA (5 mM each) or $TGF\beta_1$ (10 ng/ml) + E-cadherin neutralising antibody (nAB=DECMA1, 4 μg/ml). The aggregation index was determined by $A=(N_o–N_e)/N_o$, N_o represents the total particle number before and N_e the particle number after 30 minutes of incubation with constant rotation at 70 rpm. Mean values ±s.e.m. are shown of three independent experiments. (B) Cell migration of PANC-1 and BxPC-3 was analysed using uncoated or collagen type I-coated transwell cell culture inserts with 8 μm pores. After inhibition of cell proliferation by treatment with 10 μg/ml mitomycin C, 20 ng/ml $TGF\beta_1$ or solvent were added to the lower compartment. After 48 hours of incubation the number of cells, which had migrated through the pores, was estimated by counting 5 independent visual fields. Three independent assays were performed in triplicate. Mean values ±s.e.m. are shown.

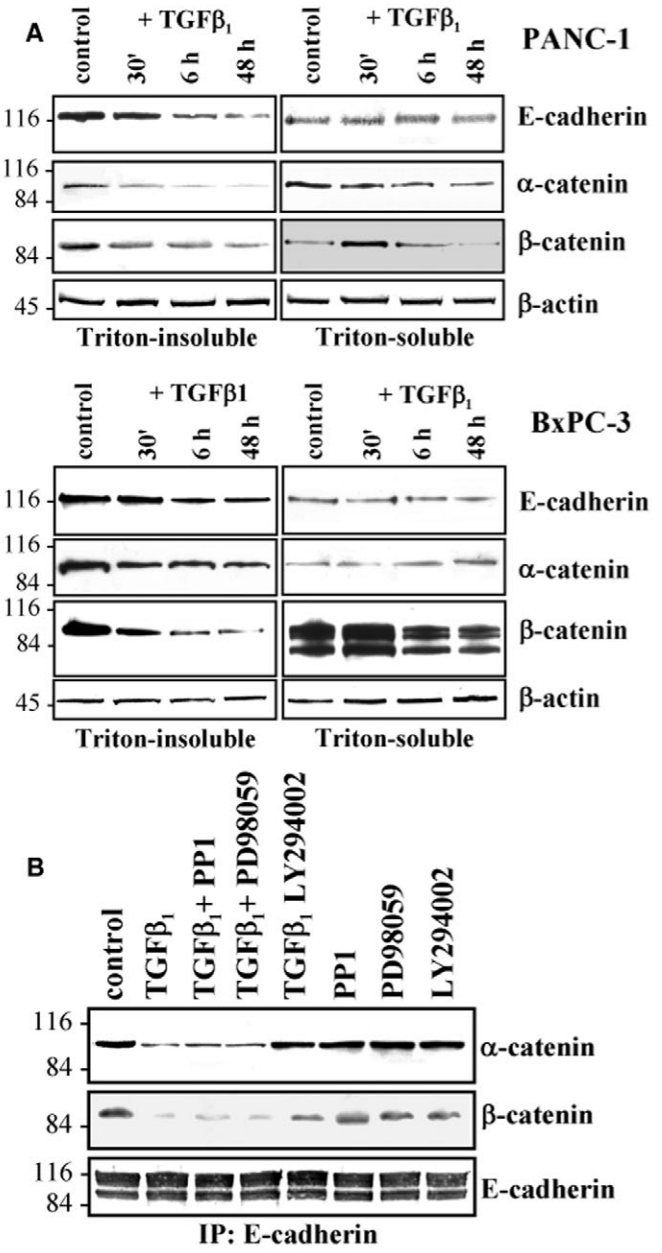

Fig. 3. Triton-soluble and -insoluble protein fractions were analysed regarding their amounts of E-cadherin, α- and β-catenin in PANC-1 and BxPC-3 cells after treatment with TGFβ$_1$ (10 ng/ml) or solvent for the time points indicated (A). Equal amounts of each fraction were separated by SDS-PAGE and blotted onto nitrocellulose. E-cadherin, α- and β-catenin were detected by immunostaining. The β-actin concentration served as control to prove equal loading. Representative blots out of four independent experiments are shown. (B) E-cadherin was precipitated from lysate of TGFβ$_1$-stimulated or unstimulated PANC-1 cells and coprecipitated α- and β-catenin was analysed by western blotting. After serum starvation for 24 hours, cells were incubated for 90 minutes either with 100 μM PP1 to inhibit Src-kinase, 25 μM PD98059 to inhibit MEK-1 or with 25 μM LY294002 to inhibit PI3-kinase and stimulated with 10 ng/ml TGFβ$_1$ or solvent for additional 6 hours. E-cadherin was precipitated from 1 mg of NOP lysate. The amount of co-immunoprecipitated α- and β-catenin was examined by immunoblotting. The blots were restained for E-cadherin to document equal amounts of precipitated protein. Three independent experiments were performed.

soluble fraction of PANC-1 and BxPC-3 cells 30 minutes after addition of TGFβ$_1$ (Fig. 3A).

To further support the observation that TGFβ$_1$ treatment causes a dissociation of the E-cadherin/catenin complex, we immunoprecipitated E-cadherin in TGFβ$_1$-treated and control cells. TGFβ$_1$ treatment of PANC-1 cells for 6 hours resulted in a dissociation of α- and β-catenin from the E-cadherin complex (Fig. 3B).

PI3-kinase mediates TGFβ$_1$-induced disassembly of the E-cadherin/catenin complex

To determine the signalling pathways involved in TGFβ$_1$-induced dissociation of the E-cadherin complex from the actin cytoskeleton, we used pharmacological inhibitors of different signalling molecules, which might be involved in this process. In PANC-1 cells, only the phosphoinositide 3-kinase (PI3-kinase) inhibitor LY294002 (25 μM) caused significant reduction of TGFβ$_1$-induced dissociation of α- and β-catenin from E-cadherin (Fig. 3B). By contrast, inhibition of the cellular kinase Src (100 μM PP1) as well as inhibition of MEK-1 (25 μM PD98059) did not show any effect on TGFβ$_1$-induced disassembly of the E-cadherin/catenin complex. Treatment of cells with inhibitors alone had no effect on the amount of co-immunoprecipitated α- and β-catenin (Fig. 3B).

To analyse whether PI3-kinase activity is necessary for TGFβ$_1$-induced cell migration, we performed transwell migration assays in the presence of the PI3-kinase inhibitor LY294002 using uncoated inserts. As shown in Fig. 4A, 25 μM LY294002 completely inhibited TGFβ$_1$-induced migration of PANC-1 and BxPC-3 cells. Inhibition of MEK-1-activity (25 μM PD98059) reduced TGFβ$_1$-induced cell migration but to a smaller extent than the LY294002 compound. In contrast to the PI3-kinase inhibitor LY294002, the MEK-1 inhibitor PD98059 also reduced the spontaneous migration rate of control cells.

Because TGFβ$_1$-induced cell migration was highly dependent on PI3-kinase activity, we asked if PI3-kinase was associated with the E-cadherin/catenin complex in pancreatic carcinoma cells and if TGFβ$_1$ may modulate this association. By immunofluorescence studies, the p85α subunit of PI3-kinase is localised to the cytoplasm of unstimulated PANC-1 cells (Fig. 4D). TGFβ$_1$ treatment induced an enrichment of PI3-kinase staining in areas of cell-cell contacts at the membrane where the E-cadherin/catenin complex is localised. Co-immunoprecipitation studies using β-catenin antibodies for the precipitation demonstrated that the p85α subunit of PI3-kinase was associated with the E-cadherin/catenin complex in TGFβ$_1$-treated and untreated PANC-1 and BxPC-3 cells (Fig. 4B). As expected from our E-cadherin co-immunoprecipitation data, α-catenin dissociated from β-catenin 6-48 hours after addition of TGFβ$_1$ (Fig. 4B). In reverse, β-catenin protein can be detected when immunoprecipitating with p85α antibodies further strengthening our observation that p85α is in a complex with the E-cadherin/catenin complex (data not shown). In this experiment, we used more stringent conditions by increasing the concentration of detergents in the lysis buffer, which causes a greater dissociation of protein complexes. Under these conditions, neither E-cadherin nor α-catenin can be co-immunoprecipitated with p85α. These data suggest that β-catenin is important for the association of p85α with the E-cadherin/catenin complex. Furthermore, we were able to show

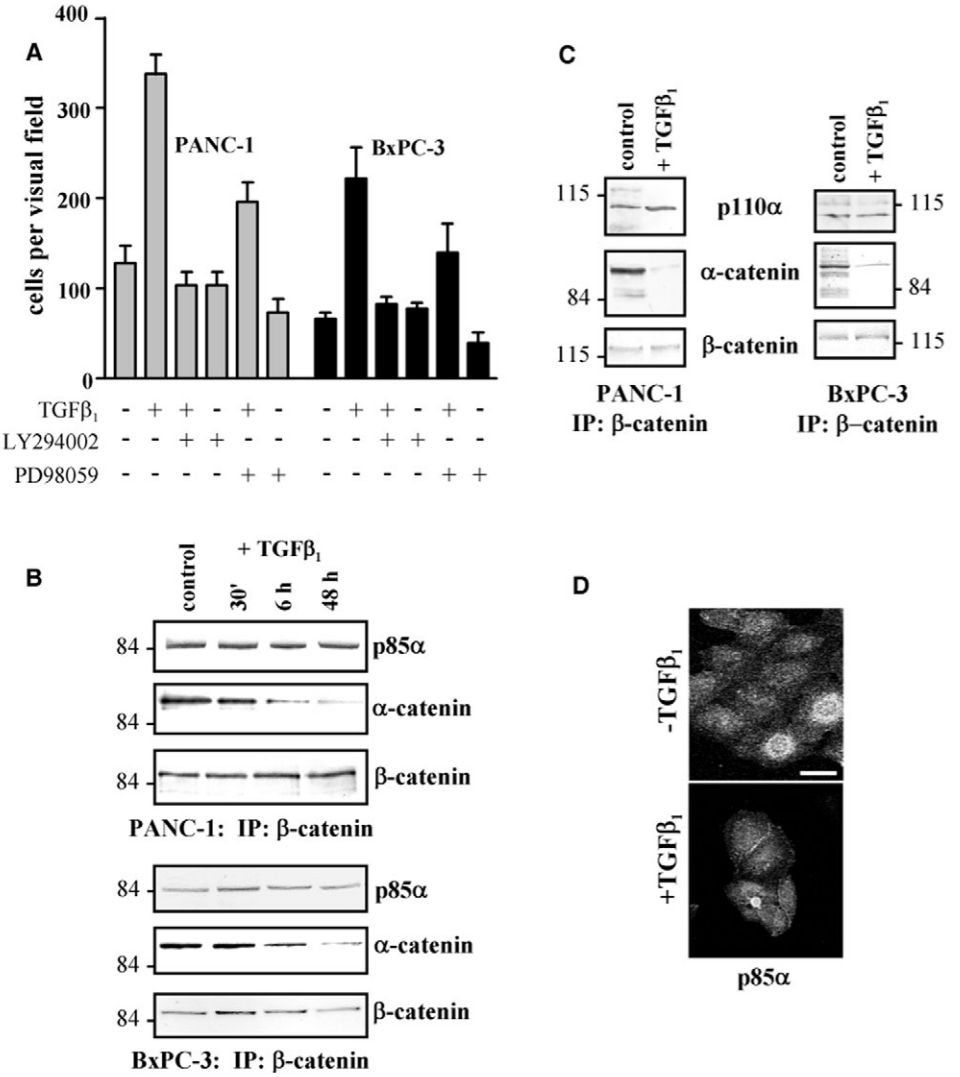

Fig. 4. (A) Transwell migration assays were performed as described before. The inhibitors LY294002 (25 μM) or PD98059 (25 μM) were added to the upper compartment. TGFβ$_1$ (20 ng/ml) or solvent was added to the lower chamber. The number of migrated cells was estimated after 48 hours of incubation. Mean values ±s.d. of one representative assay are shown out of three independent experiments. (B) The amount of PI3-kinase associated with the E-cadherin complex was analysed by co-immunoprecipitation. Beta catenin was precipitated from lysates of PANC-1 or BxPC-3 cells treated for 30 minutes, 6 hours or 48 hours with TGFβ$_1$. The amount of co-precipitated p85α was analysed by western blotting. (C) The amount of p110α co-precipitated with β-catenin was analysed in PANC-1 or BxPC-3 lysates. Beta-catenin was precipitated from 2 mg of PANC-1 or 1 mg of BxPC-3 NOP lysate treated with 10 ng/ml TGFβ$_1$ or solvent. One representative blot out of three is shown. (D) Immunolocalisation of p85α was performed in PANC-1 cells treated with 10 ng/ml TGFβ$_1$ or solvent (–TGFβ$_1$) for 3 hours. P85α localisation was analysed with confocal laser microscopy. Bar, 20 μm.

that the enzymatic subunit of PI3-kinase p110α associated with β-catenin as well (Fig. 4C).

PI3-kinase mediates TGFβ$_1$-induced α- and β-catenin phosphorylation

Tyrosine phosphorylation of catenin proteins is important for regulation of E-cadherin complex assembly and disassembly (Behrens et al., 1993). We analysed tyrosine phosphorylation of α- and β-catenin after addition of TGFβ$_1$ to PANC-1 and BxPC-3 cells. To detect changes of tyrosine phosphorylation levels of functional, membrane-associated catenins, α- and β-catenins were immunoprecipitated from purified membrane fractions of TGFβ$_1$-treated and solvent-treated control cells. Tyrosine phosphorylation was detected using a phosphotyrosine specific antibody. As shown in Fig. 5A, tyrosine phosphorylation of β-catenin increased continuously from 30 minutes up to 6 hours and remained constant for at least 48 hours in both PANC-1 and BxPC-3 cells. The α-catenin phosphorylation increased after 6 h of TGFβ$_1$ application in both cell lines and reached a maximum after 12 hours in PANC-1 cells and 48 hours in BxPC-3 cells, respectively. E-cadherin phosphorylation was hardly detectable in both cell lines after addition of TGFβ$_1$ (data not shown). The kinetics of catenin phosphorylation shown here were in accordance with the observed disassembly of the E-cadherin/catenin complex shown in Fig. 3.

Next we examined the role of PI3-kinase activity in TGFβ$_1$-induced catenin phosphorylation. Immunoprecipitated α- and β-catenins were analysed for their level of tyrosine phosphorylation in the presence or absence of the PI3-kinase inhibitor LY294002 in TGFβ$_1$-treated and control cells. The results presented in Fig. 5B show a marked inhibition of β-catenin phosphorylation in cells treated simultaneously with TGFβ$_1$ and LY294002, in contrast to cells treated with TGFβ$_1$ only or TGFβ$_1$ in combination with the Src inhibitor PP1. TGFβ$_1$-induced phosphorylation of α-catenin was also dependent on PI3-kinase activity as demonstrated in Fig. 5C.

TGFβ-induced catenin phosphorylation requires Ras activity

The TGFβ$_1$-induced phosphorylation of α- and β-catenin in BxPC-3 cells was independent of Smad4, which is

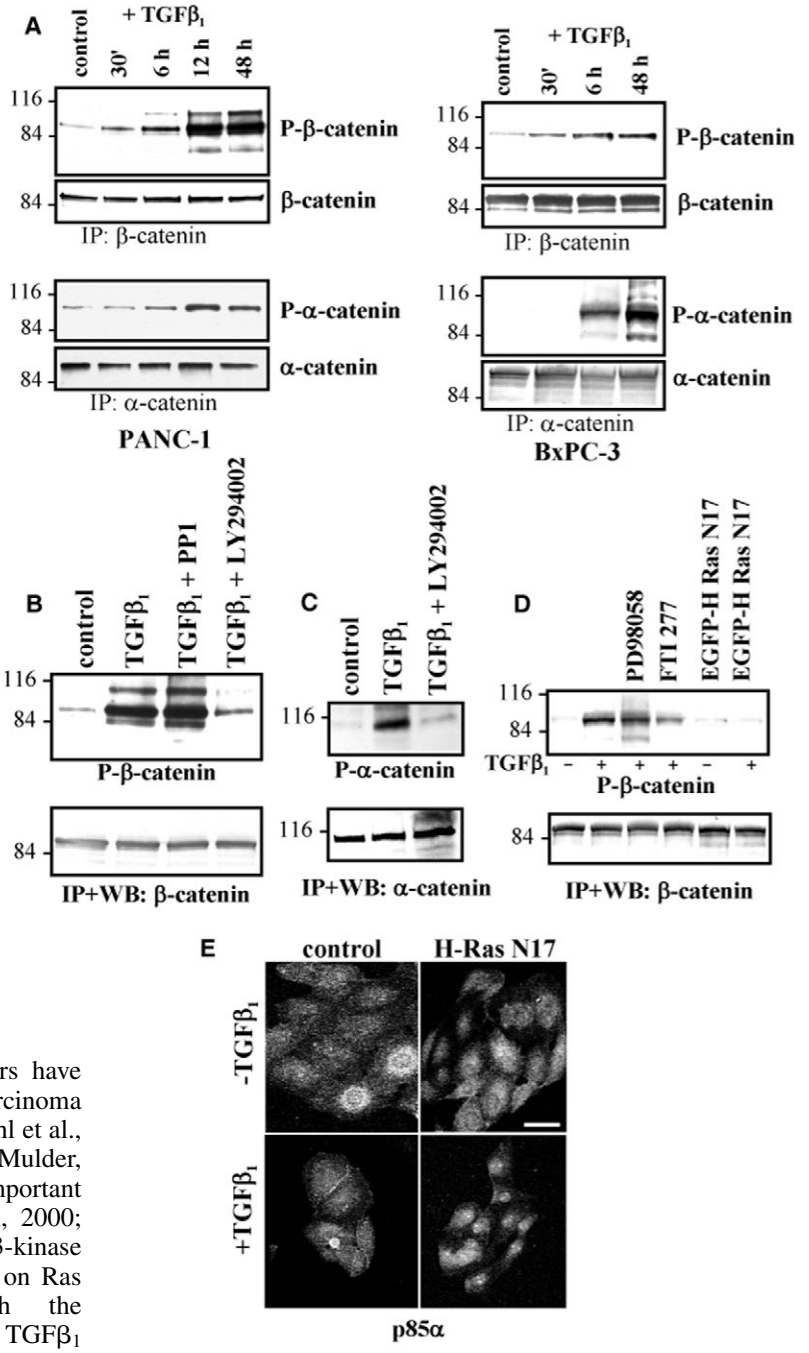

Fig. 5. (A) Phosphorylation of α- and β-catenin after TGFβ$_1$ treatment (10 ng/ml) for 30 minutes, 6 hours and 48 hours for BxPC-3 and additionally after 12 hours for PANC-1 cells was determined by immunoprecipitation of the individual proteins from isolated membrane fractions. Phosphorylated tyrosine was detected using a phosphotyrosine-specific antibody. Blots were restained with antibodies used for immunoprecipitation to document equal amounts of precipitated proteins. Representative blots out of three independent experiments are shown. (B) Phosphorylation of β-catenin or (C) α-catenin was examined after treatment of PANC-1 cells with TGFβ$_1$, the PI3-kinase inhibitor LY294002 or the Src inhibitor PP1 (only for β-catenin phosphorylation). Beta-catenin or α-catenin was precipitated from 1 mg of PANC-1 RIPA-lysates treated with TGFβ$_1$, TGFβ$_1$ plus PP1 or TGFβ$_1$ plus LY294002. Blots were restained for β- or α-catenin to document equal amounts of precipitated protein. (D) TGFβ$_1$-induced β-catenin phosphorylation was analysed in the presence of the farnesyltransferase inhibitor FTI 277 (2 μM) to inhibit Ras activity. In addition PANC-1 cells, which stably expressed EGFP/H-Ras N17, were analysed for β-catenin phosphorylation in response to 10 ng/ml TGFβ$_1$ or solvent. Beta-catenin immunoprecipitated from 1 mg of PANC-1 RIPA lysate was analysed regarding its phosphorylation by western blotting with a phospho-specific antibody. Equal amounts of β-catenin were documented by restaining the blots with β-catenin antibody. Representative blots are shown (n=3). (E) In addition to the pictures shown in Fig. 4D, immunolocalisation of p85α was performed in PANC-1 cells stably expressing EGFP/H-Ras N17 treated with TGFβ$_1$ or solvent (–TGFβ$_1$) for 3 hours and analysed with confocal laser microscopy. Bar, 20 μm.

homozygous deleted in this cell line. We and others have shown before that TGFβ$_1$ stimulation of pancreatic carcinoma cells resulted in the activation of the GTPase Ras (Giehl et al., 2000a; Ellenrieder et al., 2001a; Fensterer et al., 2004; Mulder, 2000). It is well established that Ras-GTPases are important regulators of PI3-kinase activity (Bar-Sagi and Hall, 2000; Giehl, 2005). To test whether the observed effect of PI3-kinase on TGFβ$_1$ induced catenin phosphorylation depends on Ras activity, we incubated PANC-1 cells with the farnesyltransferase inhibitor FTI 277 (2 μM) prior to TGFβ$_1$ treatment to inhibit Ras activity. TGFβ$_1$ induced phosphorylation of β-catenin was clearly reduced by FTI 277 (Fig. 5D). Furthermore, PANC-1 cells stably expressing dominant-negative EGFP/H-Ras N17 did not show enhanced tyrosine phosphorylation of β-catenin after TGFβ$_1$ stimulation (Fig. 5D). These data suggest a role of Ras in TGFβ$_1$-induced activation of PI3-kinase and in the regulation of the E-cadherin/catenin adhesion complex assembly. The importance of Ras in TGFβ$_1$-induced activation of PI3-kinase was confirmed by the localisation of PI3-kinase in TGFβ$_1$-stimulated EGFP/H-Ras N17 expressing PANC-1 cells. Fig. 5E shows that treatment of PANC-1 cells stably expressing EGFP/H-Ras N17 with TGFβ$_1$ did not result in translocation of p85α to sites of cell-cell contacts as demonstrated in control cells (left column, compare also Fig. 4D).

The phosphatase PTEN is associated with β-catenin and dephosphorylates β-catenin

The phosphatase PTEN is a known regulator of PI3-kinase signalling and has been described to be involved in stabilising adherens junctions (Kotelevets et al., 2001). To analyse if PTEN is involved in TGFβ$_1$-regulated E-cadherin/catenin complex disassembly, we immunoprecipitated E-cadherin as well as α- and β-catenin from RIPA cell lysates and examined the amount of PTEN precipitated with E-cadherin, α- and β-catenin. Under the stringent conditions of RIPA cell lysates, indirectly associated proteins dissociate from the E-cadherin/catenin complex, but protein interactions of directly associated proteins are preserved. PTEN was primarily

detected in β-catenin precipitates and to a smaller extent in α-catenin precipitates, but no PTEN was found in association with E-cadherin (Fig. 6A). Treatment of PANC-1 and BxPC-3 cells with TGFβ$_1$ for 6 hours markedly reduced the amount of PTEN bound to β-catenin (Fig. 6B), whereas the small amount of PTEN associated with α-catenin was independent of TGFβ$_1$ treatment (data not shown). The total amount of PTEN protein in lysates of PANC-1 and BxPC-3 cells did not change upon TGFβ$_1$ treatment (Fig. 6B).

To clarify the role of PTEN in TGFβ$_1$-induced E-cadherin/catenin complex disassembly, we transiently transfected PANC-1 cells with expression plasmids for wild type PTEN (PTEN WT) or with a C-terminal deletion mutant of PTEN (PTENΔC), which is assumed to be inactive in its phosphatase function (Tolkacheva and Chan, 2000; Raftopoulou et al., 2004). As documented in Fig. 6C, the TGFβ$_1$-induced decrease of β- and α-catenin proteins co-immunoprecipitating with E-cadherin was inhibited in cells expressing wild type PTEN, whereas the phosphatase inactive form, PTENΔC, had minimal effect on TGFβ$_1$-induced changes on the E-cadherin/catenin protein complex.

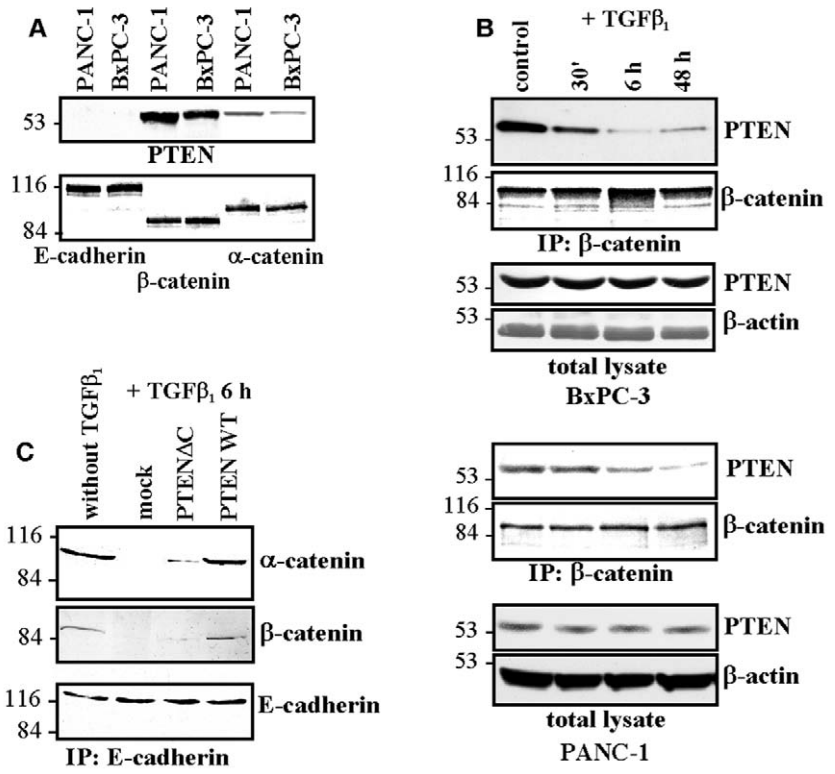

Fig. 6. (A) Immunoprecipitation of E-cadherin, α- or β-catenin was performed from 500 μg of PANC-1 RIPA lysate and PTEN was detected by immunoblotting. Equal amounts of immunoprecipitated proteins were documented by restaining the blots with the appropriated antibody. (B) Beta-catenin was precipitated from 2 mg of BxPC-3 or PANC-1 lysates treated with 10 ng/ml TGFβ$_1$ or solvent. Co-precipitated PTEN was detected by western blotting. The blots were restained with anti-β-catenin antibody to demonstrate equal amounts of protein. (C) E-cadherin was precipitated from 0.5 mg of total lysate from PANC-1 cells transiently transfected with PTEN, PTENΔC or vector alone (mock). Co-precipitated α- and β-catenin was determined by western blotting. Restaining of the blot for E-cadherin confirmed equal amounts of precipitated proteins. In all experiments representative blots out of three independent studies are shown.

TGFβ$_1$-induced dissociation of PTEN from the E-cadherin/catenin complex correlates with enhanced catenin phosphorylation

In addition to the role of PTEN in E-cadherin/catenin complex assembly, we examined whether tyrosine phosphorylation of β-catenin was altered in PTEN-transfected cells. The TGFβ$_1$-induced phosphorylation of β-catenin was diminished after transient transfection of wild type PTEN in PANC-1 cells, whereas only minor changes in β-catenin-phosphorylation were detectable after expression of the phosphatase inactive mutant PTENΔC (Fig. 7A). To determine if PTEN can directly dephosphorylate β-catenin, we established an in vitro phosphatase assay. Incubation of immunoprecipitated EGFP-PTEN and the mutant form EGFP-PTENΔC with tyrosine phosphorylated β-catenin revealed that PTEN, but not PTENΔC, could dephosphorylate β-catenin at tyrosine residues in vitro (Fig. 7B). To confirm the role of PTEN in catenin dephosphorylation, we transfected PANC-1 cells with siRNA against PTEN. The protein level of PTEN was nearly abolished in siRNA treated cells as shown in two independent experiments, which resulted in enhanced β-catenin phosphorylation (Fig. 7C). In addition, PANC-1 cells transfected with PTEN siRNA were stimulated with TGFβ. As demonstrated in Fig. 7C, TGFβ did not increase β-catenin phosphorylation, which was already enhanced by knock down of PTEN.

The regulation of the phosphatase PTEN is mainly unknown, some studies suggest that the activity of PTEN is modulated by regulating total protein levels of PTEN by increased degradation of the unphosphorylated protein (Birle et al., 2002). Using an antibody specific for PTEN phosphorylated at Ser380, we showed that TGFβ$_1$ treatment of PANC-1 cells resulted in a significant reduction of phosphorylated PTEN co-immunoprecipitating with β-catenin (Fig. 7D). The total PTEN phosphorylation was not significantly altered by TGFβ$_1$ (data not shown), probable due to the huge amount of PTEN in PANC-1 cells. The inhibition of TGFβ$_1$-induced Ras activation by the incubation of the cells with the farnesyltransferase inhibitor FTI277 did not suppress the TGFβ$_1$ effect on the phosphorylation of β-catenin associated PTEN (Fig. 7D). Also the PI3-kinase inhibitor LY294002 was not able to inhibit the TGFβ$_1$-induced modification of PTEN phosphorylation in PANC-1 cells (data not shown). Together with our observation that total PTEN protein levels do not change upon TGFβ$_1$ treatment (see Fig. 6B), these data indicate that TGFβ$_1$ indirectly induces PTEN dissociation from the E-cadherin complex which correlates with reduced PTEN phosphorylation and PTEN protein stability.

Discussion

Only little is known how TGFβ$_1$ affects E-cadherin/catenin mediated cell-cell adhesion in tumour cells. Here we demonstrate that PI3-kinase and the phosphatase PTEN mediates the TGFβ$_1$ induced decrease of E-cadherin mediated cell-cell adhesion by phosphorylation of β-catenin in pancreatic carcinoma cells and that this effect is independent of the Smad pathway. PI3-kinase was found in association with the E-cadherin adhesion complex via β-catenin. Whereas TGFβ$_1$ treatment did not alter the association of PI3-kinase with the complex, PI3-kinase activity induced by TGFβ$_1$ and probably mediated by the GTPase Ras was necessary for tyrosine phosphorylation of β-catenin and to a minor extent of α-catenin. We could show for the first time that the phosphatase PTEN is also associated with β-catenin in the E-cadherin adhesion complex and directly dephosphorylates β-catenin. PTEN disassociates from β-catenin upon TGFβ$_1$ treatment, which correlates with an increase in β-catenin phosphorylation. As a consequence of these modifications, the E-cadherin/catenin complex disassembles and detaches from the actin cytoskeleton.

E-cadherin plays an important role in tumourigenesis and metastasis formation (Birchmeier et al., 1996). Expressing wild-type E-cadherin in tumourigenic pancreatic β-cells in a transgenic mouse model, progression from adenoma to carcinoma could be inhibited (Perl et al., 1998). We have shown before that the expression of E-cadherin in E-cadherin-negative pancreatic carcinoma cells restored cellular aggregation and reduced the metastatic potential of these E-cadherin-transfected cells compared with parental cells (Seidel et al., 2004). In our study, TGFβ$_1$ treatment of pancreatic tumour cells did not only disassemble the E-cadherin/catenin complex, but concomitantly decreased cell-cell adhesion and increased cell migration.

Recently Tian and Phillips (Tian and Phillips, 2002) demonstrated that TGFβ$_1$ treatment resulted in a direct association of TGFβ receptor type II with E-cadherin and β-catenin, enhanced β-catenin tyrosine phosphorylation and reduced E-cadherin/catenin complex assembly in a non-tumour renal cell line (Tian and Phillips, 2002). In addition, the authors showed an increase of β-catenin associated with Smad3 and Smad4 in cells treated with TGFβ$_1$. However, the proteins, which phosphorylate β-catenin at tyrosine residues, remained unknown. In our study, we demonstrate for the first time that the TGFβ$_1$-induced tyrosine phosphorylation takes place in Smad4-deficient cells as well (BxPC-3), which shows that the TGFβ$_1$ effects described here are independent of Smad4.

Although important cellular effects of TGFβ$_1$ are transmitted by the Smad pathway, some Smad-independent effects have been described, such as activation of RhoA, Ras and ERK, p38 MAPK or PI3-kinase (Imamichi et al., 2005; Mulder, 2000; Bhowmick et al., 2001; Bakin et al., 2002). Whereas the phosphorylation of β-catenin is Smad4-independent in pancreatic cancer cells, our data show that invasion of a collagen matrix depends on Smad4. The expression of collagen degrading proteases requires Smad4-induced gene transcription (Ellenrieder et al., 2001a), suggesting the cooperation of two separate TGFβ-regulated signalling pathways for cellular invasion through extracellular matrix. However, we cannot exclude that other signalling mechanisms that are potentially defect in BxPC-3 cells may also contribute to the observed difference in cell migration and invasion.

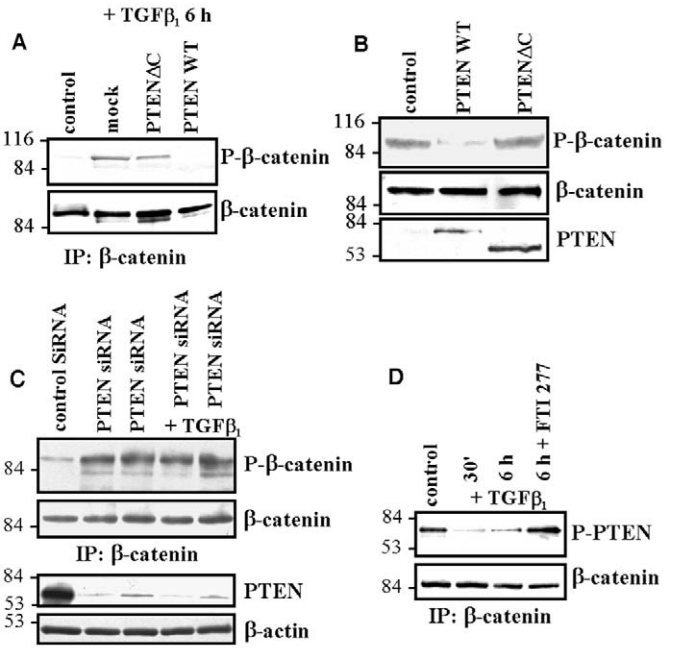

Fig. 7. (A) Beta-catenin was immunoprecipitated from 1 mg of PANC-1 lysate of cells transfected with pEGFP, pEGFP-PTEN or pEGFP-PTENΔC, treated with 10 ng/ml TGFβ$_1$ or solvent for 6 hours and analysed for its tyrosine phosphorylation. The blot was restained for β-catenin to demonstrate equal amounts of protein. (B) For an in vitro phosphatase assay, tyrosine phosphorylated β-catenin was incubated for 30 minutes at 30°C with EGFP-PTEN-constructs, which were immunoprecipitated with anti-EGFP antibody. The amount of phosphorylated β-catenin was analysed by western blotting. Restaining of the blots with anti-β-catenin antibody revealed equal amounts of phosphatase substrate and restaining with anti-PTEN documents the presence of both EGFP-tagged PTEN proteins. Representative assays out of four independent experiments are shown. (C) Beta-catenin was immunoprecipitated from 1 mg of lysate from PANC-1 cells transfected with siRNA for PTEN and treated with TGFβ$_1$ (10 ng/ml) or solvent (two independent experiments each) or an unrelated control siRNA and analysed for its tyrosine phosphorylation. Restaining for β-catenin is shown to document equal loading. PTEN protein expression was analysed by western blots stained for PTEN. Representative assays are shown (n=3). (D) Phosphorylation of Ser380 of PTEN, which was co-immunoprecipitated with β-catenin, was analysed after TGFβ$_1$ treatment. Beta-catenin was immunoprecipitated from 2 mg of PANC-1 lysate treated with TGFβ$_1$ for 30 minutes or 6 hours. In addition PANC-1 cells were examined, which were pretreated with the farnesyltransferase inhibitor FTI 277 (2 μM) for 2 hours prior to addition of TGFβ$_1$ for 6 hours. Co-precipitated PTEN was analysed regarding the phosphorylation at Ser380 with a phospho-specific antibody. Restaining for β-catenin documented equal amounts of precipitated protein. A representative blot out of three independent experiments is shown.

The impact of β-catenin phosphorylation on regulation of the E-cadherin complex assembly is well known and was demonstrated in different studies (Behrens et al., 1993; Hamaguchi et al., 1993; Hoschuetzky et al., 1994). In agreement with Tian and Phillips (Tian and Phillips, 2002), the data shown here demonstrate enhanced β-catenin phosphorylation after TGFβ treatment. In the present study, we provide evidence that PI3-kinase and the phosphatase PTEN

are important for tyrosine phosphorylation of α- and β-catenin after TGFβ$_1$ stimulation. We show that in pancreatic carcinoma cells PI3-kinase is associated with the E-cadherin/catenin complex probably via β-catenin. This is in line with findings reported by Woodfield et al. showing that p85α associates with β-catenin in human keratinocytes (Woodfield et al., 2001) as well as findings by Pece et al., who demonstrated in MDCK cells that p85α can be co-immunoprecipitated with E-cadherin (Pece et al., 1999). Our data show that in epithelial pancreatic carcinoma cells TGFβ$_1$ does not regulate the binding of PI3-kinase protein to β-catenin, but rather regulates its activity necessary for α- and β-catenin tyrosine phosphorylation. Recently, Yi and colleagues demonstrated that the PI3-kinase subunit p85α is associated with the TGFβ receptor type 2 (TβRII) in Cos7 cells. TGFβ$_1$ treatment induced the association of TGFβ receptor type 1 (TβRI) with this TβRII/p85 complex followed by an increase of PI3-kinase activity and Akt phosphorylation (Yi et al., 2005). In agreement with the data from Woodfield et al., we were able to detect TβRII but not TβRI associated with the E-cadherin/catenin complex upon treatment with TGFβ (data not shown). Together, these data strongly support our hypothesis that TGFβ$_1$ mediates the disassembly of the E-cadherin adhesion complex via PI3-kinase in a protein complex consisting of TβRII, PI3-kinase and E-cadherin/catenin. A role for PI3-kinase/Akt in TGFβ$_1$-induced EMT in mammary epithelial cells has been shown also by Bakin et al. (Bakin et al., 2000). The authors demonstrated that TGFβ$_1$ treatment activated PI3-kinase and Akt resulting in a delocalisation of E-cadherin from adherens junctions and ZO-1 from tight junctions as well as altered Smad4-regulated gene expression. In this study the GTPase RhoA was suggested to mediate TGFβ$_1$ activation of Akt-kinase in mouse mammary cells.

In agreement with data published before (Giehl et al., 2000a; Mulder, 2000), the data presented in this study further suggest that Ras activity is important for TGFβ$_1$-induced tyrosine phosphorylation of α- and β-catenin. Interestingly, Yi et al. (Yi et al., 2005) showed in their study that the activation of PI3-kinase via TβRII is indirect. Our data suggest that Ras could be involved in the signalling cascade from TGFβ$_1$ receptors and activation of PI3-kinase upon TGFβ$_1$ treatment. In agreement with data from the literature demonstrating that Ras can activate PI3-kinase (Bar-Sagi and Hall, 2000; Giehl, 2005), we show that the inhibition of Ras activation by pretreatment of cells with a farnesyltransferase inhibitor as well as expression of a dominant-negative Ras mutant abolished the TGFβ$_1$-induced tyrosine phosphorylation of α- and β-catenin. We propose that TGFβ$_1$ activates PI3-kinase mediated by Ras, which leads directly or indirectly to tyrosine phosphorylation of β-catenin and dissociation of the E-cadherin/catenin complex.

In addition to PI3-kinase, we identified the phosphatase PTEN as being involved in TGFβ$_1$-induced phosphorylation of β-catenin. Different phosphatases have been described to interact with the E-cadherin complex or individual components of the complex. Among these are receptor phosphatases such as PTPμ, PTPκ, or PTP LAR, which have been shown to interact directly with the E-cadherin/catenin complex and mediate dephosphorylation of β-catenin as a prerequisite for strong cell-cell adhesion (Aicher et al., 1997; Brady Kalnay et al., 1995; Fuchs et al., 1996). Other studies showed that cytoplasmic tyrosine phosphatases interact directly or indirectly with the E-cadherin complex, such as SHP-1 and SHP-2, PTP1B, PTP and Pez (Balsamo et al., 1998; Taddei et al., 2002; Kotelevets et al., 2001; Müller et al., 1999). In this study, we show for the first time that the phosphatase PTEN is associated with the E-cadherin complex by binding to β-catenin. PTEN dephosphorylates β-catenin at tyrosine residues in pancreatic tumour cells, which was verified in vitro by demonstrating that ectopically expressed PTEN is able to decrease β-catenin phosphorylation.

Indirect evidence that TGFβ affects PTEN in pancreatic cancer derives from a study of Ebert et al. (Ebert et al., 2002), which demonstrated that PTEN expression was dramatically reduced in human pancreatic cancer as well as in the pancreas of TGFβ$_1$-transgenic mice (Ebert et al., 2002). In our study, TGFβ$_1$ treatment results in dissociation of PTEN from β-catenin and the concomitant increase in β-catenin phosphorylation. The molecular mechanism by which PTEN activity is regulated is yet unknown. Reduced phosphorylation of PTEN at the serine residue 380, as shown here upon TGFβ$_1$-treatment, has been suggested to reduce the protein stability of PTEN leading to reduced phosphatase activity (Vazquez et al., 2000). Although the protein phosphatase activity of PTEN is not the main function for which PTEN is known for, which is the conversion of the phospholipid PIP_3 to PIP_2 and termination of PI3-kinase induced PIP_3 production, it was shown that PTEN mediates dephosphorylation of the proteins focal adhesion kinase (FAK) and Shc and concomitantly influences directed cell motility (Tamura et al., 1998; Gu et al., 1999). In the present study tyrosine phosphorylation of β-catenin is directly reduced by PTEN, supporting the hypothesis that PTEN activity is regulating cell-cell adhesion. Interestingly, the lipid but not the protein phosphatase activity of PTEN was necessary to revert Src-induced transformation of MDCK cells (Kotelevets et al., 2001). In this study, overexpression of Src in MDCK cells caused a decrease in cell-cell adhesion, which could be reversed by forced expression of wild type PTEN. However, PTEN expression had no significant effect on β-catenin phosphorylation, which is in contrast with our data. Potentially, this could be due to the ectopic overexpression of Src kinase in the MDCK cells used in their study.

In summary, our data demonstrate that treatment of pancreatic tumour cells with TGFβ$_1$ resulted in reduced cellular adhesion due to a dissociation of E-cadherin/catenin complexes from the actin cytoskeleton. The reduction of E-cadherin/catenin complexes was induced by tyrosine phosphorylation of β-catenin and α-catenin followed by the dissociation of β- and α-catenin from E-cadherin. We have identified PI3-kinase as well as the phosphatase PTEN as important mediators of TGFβ$_1$-induced catenin phosphorylation and cell migration. The direct dephosphorylation of β-catenin by PTEN was reduced after TGFβ$_1$ stimulation. These results suggest that PI3-kinase and PTEN are crucial in controlling the invasive phenotype of pancreatic tumour cells.

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