Adhesion and Rac1-dependent Regulation of Biglycan Gene Expression by Transforming Growth Factor-β

EVIDENCE FOR OXIDATIVE SIGNALING THROUGH NADPH OXIDASE

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Both transforming growth factor-β (TGF-β)-induced expression of biglycan (BGN) and activation of p38 MAPK have been implicated in cellular adhesion and migration. Here, we analyzed the role of adhesive events and the small GTPase Rac1 in TGF-β regulation of BGN. TGF-β1 induction of BGN expression and activation of p38 was abolished or strongly reduced when cells were kept in suspension or exposed to either the actin cytoskeleton-disrupting agent cytochalasin D or a specific chemical Rac1 inhibitor. Ectopic expression of a dominant negative mutant (T17N) of Rac1 abrogated both TGF-β1-induced p38 MAPK activation and BGN up-regulation but did not affect TGF-β1-induced phosphorylation of Smad3 or transcriptional induction of Growth Arrest DNA Damage 45β, previously shown to be crucial for TGF-β regulation of BGN. Overexpression of wild type Rac1 greatly enhanced the TGF-β effect on BGN in adherent cells, whereas ectopic expression of constitutively active Rac1 (Q61L) activated p38 and in the presence of exogenous TGF-β was able to rescue BGN expression in nonadherent cells. Endogenous Rac1 was activated by TGF-β treatment in PANC-1 cells in an adhesion-dependent fashion. Like Rac1-T17N, the NADPH oxidase inhibitor diphenylene iodonium and the tyrosine kinase inhibitor herbimycin A blocked TGF-β-induced p38 activation and BGN expression, suggesting that Rac1 exerts its effect on BGN and p38 through increasing NADPH oxidase activity and subsequent production of reactive oxygen species. These results show that the TGF-β effect on BGN is dependent on cell adhesion and that activated Rac1, presumably acting through NADPH oxidase(s), is necessary but not sufficient for TGF-β-induced BGN expression.

TGF-β2 and its signaling effectors regulate basic cellular functions such as proliferation and apoptosis and act as key determinants of tumor cell behavior (1–3). The cellular activities of TGF-β are mediated by specific receptor complexes that are assembled upon ligand binding and comprise the TGF-β type II receptor and the TGF-β type I receptor/activin receptor-like kinase 5 (4). The activated ligand-receptor complex then activates intracellular signal transducers from the Smad family of proteins, which translocate to the nucleus to modulate the activity of TGF-β target genes (reviewed in Ref. 3). Besides the Smad pathway, other signaling pathways can be activated by TGF-β independently of or downstream of Smads, including p38 mitogen-activated protein kinase (MAPK) (4). Recently, TGF-β-dependent transcriptional induction of MyD118 (encoding growth arrest DNA damage 45 β (GADD45β)) has been shown to be Smad-dependent and to mediate the (delayed) activation of p38 by TGF-β (5). However, the regulation of p38 activation by TGF-β has turned out to be more complex, since it appears to depend also on integrin ligation (6), suggesting that p38 activation represents a point of cross-talk between TGF-β receptor/Smad and adhesion/integrin signaling. The Rho family GTPases Rac and Cdc42 are known to transmit adhesion/integrin-related signals to MAPK activation being able to stimulate the p38 MAPK (and the c-Jun N-terminal kinase) pathway (7). It was thus not surprising that they have been implicated in TGF-β signaling, particularly TGF-β-induced epithelial-to-mesenchymal transdifferentiation (8), which is known to be associated with p38 activation, increased migration, scattering, and mobilization of the actin cytoskeleton (9). TGF-β can also activate p38 through reactive oxygen species (ROS) produced by NAD(P)H oxidase (10), a process that in turn depends on Rac1 as a subunit of this enzyme being absolutely required for its enzymatic activity (for a review, see Ref. 11).

The three best characterized members of the Rho family of small GTPases, Rho, Rac1, and Cdc42, are small (190–250-residue) proteins that consist of a GTPase domain and short N- and C-terminal extensions. They bind to GDP and GTP with high affinity and are thought to cycle between active, GTP-bound and inactive, GDP-bound states (reviewed in Ref. 12). The conformation of the GTP-bound protein results in increased binding affinity for downstream effector proteins (e.g. p21-activated kinase 1) (7). The activities of RhoA, Rac1, and Cdc42 are themselves regulated in the process of cell adhesion, which is initiated by the binding of integrins to their extracellular matrix ligands. These small GTPases in turn regulate cell adhesion and changes in cell morphology by triggering dynamic changes in the actin cytoskeleton (13). Finally, Rac1 (and Rac2) regulate the membrane-associated NADPH oxidase, a multicomponent enzyme that utilizes electrons derived from intracellular NADPH to generate ROS (11, 14–16). Consistent with the essential role of Rac1, the activity of NADPH oxidase is dependent on both cell adhesion (see Ref. 17 and references therein) and tyrosine kinase activity (18, 19) in human neutrophils. Recently, homologs of the phagocyte NADPH oxidase have been found in a variety of tissues. These new nonphagocytic NADPH oxidases/NADPH
oxidase-like enzymes produce low levels of oxidants that appear to be used as signals for a variety of cellular activities, including cell growth and transformation.

Biglycan (BGN) belongs to the family of small leucine-rich proteoglycans (20) and is functionally involved in matrix assembly, cellular migration and adhesion, and the regulation of growth factor (e.g. TGF-β activity) (reviewed in Refs. 21 and 22). BGN is markedly up-regulated in fibrotic diseases, such as diabetic nephropathy (23, 24) and in the stroma of solid tumors, such as pancreatic carcinoma (25), consistent with the central role of TGF-β in the pathogenesis of these diseases (26–28) and as a regulatory factor for BGN. TGF-β signaling to BGN has revealed an unexpected degree of complexity. The TGF-β effect on BGN is strictly dependent on activation of the Smad (29) and the p38 pathway (30) and intermittent transcriptional induction of GADD45β (31). In PANC-1 cells growing on tissue culture plastic, the TGF-β1-induced increase in p38 activation and BGN expression is associated with morphological changes of cellular hypertrophy and well formed actin stress fibers, characteristic of epithelial-to-mesenchymal transdifferentiation (32). Prompted by our original observation that the p38 and BGN responses to TGF-β were lost in cells that were denied cell adhesion, in conjunction with the known role of Rho-like proteins and BGN in cellular adhesion/migration, we hypothesized that the TGF-β control of BGN, too, may involve Rho GTPases. The close functional association between TGF-β regulation of p38 and BGN further opened the possibility that signaling intermediates crucial for TGF-β activation of p38 are likely to be important for TGF-β regulation of BGN too. In the present study, we present evidence that both the activation of p38 and the induction of BGN expression by TGF-β require activation of Rac1. We further show that Rac1 probably promotes the TGF-β effect on p38 activation and BGN expression as part of the NADPH oxidase complex.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**TGF-β1 was purchased from R&D Systems (Wiesbaden, Germany). An antibody to Rac1 was obtained from BD Transduction Laboratories (Heidelberg, Germany); antibody to phospho-p38, p38, phospho-Smad2, and Myc tag (clone 9B11) was from Cell Signaling Technology (Heidelberg, Germany); antibody to phospho-p38, p38, phospho-Smad2, and Myc tag (clone 9B11) was from BD Transduction Laboratories (Heidelberg, Germany); antibody to Rac1 was obtained from Merck Biosciences (Darmstadt, Germany). All other reagents used were from Merck (Darmstadt, Germany). Cytochalasin D was purchased from Sigma (Deisenhofen, Germany). Cytochalasin D was purchased from Sigma, Y-27632 was from Alexis (Grünberg, Germany), and the Rac1 inhibitor, herbimycin A, and dihydropyene iodonium chloride (DPI) were from Merck Biosciences (Darmstadt, Germany). All other reagents used were of analytical grade purity. Pharmacological inhibitors were added to cells 1 h before the addition of TGF-β, which was used at a concentration of 5 ng/ml in all experiments.

**Cell Lines and Cell Culture—**Human pancreatic carcinoma PANC-1 and human osteosarcoma MG-63 cells were maintained as described earlier (31). PANC-1 cells stably transduced with retroviral vectors were cultured in the presence of 2.5 μg/ml puromycin (Sigma), and those transfected with Rac1-Q61L in pcDNA3 or pcDNA3 alone were selected with Geneticin (700 μg/ml, active concentration; Invitrogen, Karlsruhe, Germany). In some experiments, cells were seeded in 3.5-cm well plates precoated with fibronectin and poly-d-lysine (BD Pharmingen, Heidelberg, Germany) or in 10-cm dishes or 3.5-cm well plates with an underlayer of agarose to prevent cells from adhesion (33).

**RNA Isolation and Quantitative RT-PCR Analysis—**Total RNA from PANC-1 cells was isolated with peqGOLD RNAPure (Peqlab, Erlangen, Germany). The general RT-PCR protocol and the PCR primer sequences for BGN and plasminogen activator-inhibitor (PAI-1) were described in detail earlier (29). The mRNA expression was quantified by real-time PCR on an I-Cycler (Bio-Rad, München, Germany) with I-Cycler software (Bio-Rad). SYBR green was used for detection of amplification products. Amplification of β-actin-specific transcripts was achieved as described previously (29). All values for GADD45β, BGN, and PAI-1 mRNA concentrations were normalized to those for β-actin in the same sample to account for small differences in cDNA input.

**Construction of Vectors and Retroviral Infection—**The entire open reading frame of wild type human Rac1 was amplified by RT-PCR with Turbo Pfu polymerase (Strategene, Heidelberg, Germany) and primers Rac1-forward 5′-accagcagccatcaagtgtgg-3′ (start codon underlined) and Rac1-reverse 5′-tacacagcagcatctttcctc-3′ (stop codon underlined) and subcloned in sense orientation into the retroviral vector pBabePuro. cDNA inserts of Myc-tagged versions of dnRac1 (T17N), and caRac1 (Q61L) (kindly provided by G. M. Bokoch) were released from pRK5 and subcloned into pBabePuro and pcDNA3, respectively. Positive clones for Rac1 and Rac1-T17N in pBabePuro (evaluated by PCR, restriction analysis, and sequencing of the plasmid-cDNA junctions) were co-transfected into HEK 293T producer cells along with retroviral packaging vectors as described previously (29). Retroviral particles released by 293T cells were used to infect PANC-1 cells. Pools and individual clones of productively infected cells were obtained after antibiotic selection.

**Transient and Stable Transfections—**For transient transfections followed by immunoprecipitation (IP), cells were seeded at a density of 2 × 10^4 cells/cm^2 on day 1 in 10-cm dishes, and on day 2 they were co-transfected serum-free with Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions with pFLAG-p38 in combination with empty pcDNA3 vector or Myc-tagged versions of dnRac1 and caRac1 as described previously (31) and in the legend to Fig. 4. Following removal of the transfection solution and a recovery period of 24 h in normal growth medium to allow for protein expression from transfected plasmids, cells were starved for 16 h and finally stimulated with TGF-β1 for 1 h. The transfected cells were then lysed in IP buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and processed for anti-FLAG and anti-Myc IP and immunoblotting (see below). Stable transfection of PANC-1 cells with Rac1-Q61L-pcDNA3 was achieved with Lipofectamine Plus.

**Rac1 Activity Assay—**Rac1 activity after TGF-β stimulation was measured using the Rac1 activation kit (Stressgen/Biomol, Hamburg, Germany), which employs the p21-binding domain (PBD) of human p21-activated kinase 1, coupled to GST as a bait. Briefly, 2 × 10^4 cells were seeded in 10-cm dishes on plastic on day 1, treated on the next day with TGF-β1 for various times, and lysed in lysis buffer provided by the supplier. In some assays, cells were kept in suspension for 24 h prior to lysis. The lysate was cleared by centrifugation, and 750 μg of protein were incubated on an immobilized glutathione disc and 20 μg of GST-PAK-PBD for 2 h with gentle agitation. Following washing, bound Rac1 was eluted in boiling Laemmli buffer containing 5% (v/v) 2-mercaptoethanol and subjected to SDS-PAGE and anti-Rac1 immunoblotting. For control reactions, 750 μg of each lysate were incubated with either GSTp6 or GDP for 15 min at 30 °C, after which the reaction was terminated on ice with 60 mM MgCl2 and processed as above.

**Immunoblot Analysis and Immunoprecipitation—**The SDS-PAGE and immunoblotting procedure was performed as described in detail earlier (29). Epitope-tagged proteins were immunoprecipitated from cellular lysates with anti-Myc or anti-FLAG antibodies and Protein A-Sepharose Fast Flow (Amersham Biosciences, Freiburg, Germany) or
FIGURE 1. TGF-β1-induced activation of p38 MAPK and up-regulation of BGN is dependent on cell adhesion.

A, pancreatic PANC-1 and osteoblastic MG-63 cells were seeded in 6-well plates on tissue culture plastics (adherent) or on plastics coated with agarose to prevent cells from attaching (suspended). 24 h later, cells were treated with TGF-β1 (5 ng/ml) in normal growth medium for 24 h, followed by isolation of RNA from the cells and measurement of BGN expression by quantitative RT-PCR. The data shown are from one representative experiment of three experiments performed in total, each with very similar results. Values are given as relative (Rel.) mRNA concentration (Conc.), relative to untreated adherent cells set arbitrarily at 1. Each bar represents the mean ± S.D. calculated from 3-fold determinations. **, p < 0.001; *, p < 0.01. B, PANC-1 cells grown under adherent or nonadherent conditions were treated with TGF-β1 for the indicated times and processed for immunoblotting with antibodies to phospho-Smad2 (p-Smad2) and total Smad2 (t-Smad2) (top), or antibodies to phospho-p38 (p-p38) and total p38 (t-p38) (bottom). C, PANC-1 cells from A, stimulated with TGF-β1 for 24 or 1 h, were subjected to quantitative RT-PCR for PAI-1 (left) and GADD45β (right), respectively. Bars, mean ± S.D. *, p < 0.05. D, PANC-1 cells were seeded in 6-well plates on plastic, plastic coated with fibronectin (FN), or poly-D-lysine. On
Protein G Plus-Sepharose (Santa Cruz Biotechnology), respectively, according to the protocols provided by the suppliers, and subsequently analyzed by immunoblotting.

**Statistical Analysis**—Statistical significance was calculated using the unpaired Student’s t test. Data were considered significant at \( p < 0.05 \).

**RESULTS**

**TGF-β1-induced Activation of p38 MAPK and Up-regulation of BGN but Not Early (Smad-mediated) TGF-β Receptor Signaling Is Dependent on Cell Adhesion**—To examine the role of adhesive events to modulate TGF-β-induced BGN up-regulation, the effect of TGF-β on cells stimulated under nonadherent conditions was investigated. Cells seeded on either plastic or agarose-coated wells were stimulated with TGF-β1 in suspension, and the effect on BGN mRNA expression was assessed at 24 h. TGF-β evoked a 20–30-fold increase in BGN mRNA in cells grown on plastic but was unable to induce BGN expression in suspended cells (Fig. 1A, left). The inability to up-regulate BGN expression in nonadherent cells following TGF-β1 stimulation cannot be explained by loss of cell viability, because these cells are capable of reattaching and growing when replated on plastic; moreover, up-regulation of BGN expression was partially restored when cells treated with TGF-β1 were returned to an adherent state (data not shown). Anchorage-dependent TGF-β regulation was also observed in other BGN-expressing cells (e.g. osteoblastic MG-63 cells) (Fig. 1A, right). This suggested that adhesion-dependent signal(s) are required for the TGF-β effect on BGN. Given the strict dependence on intermittent activation of p38 MAPK, it was of interest to test whether activation of p38 by TGF-β also was adhesion-dependent. To this end, p38 phosphorylation, being indicative of the p38 activation state, was abolished in suspended cells (Fig. 1B, bottom). We have recently shown that BGN induction and p38 activation is induced via GADD45β, a gene transcriptionally activated by TGF-β via Smads (31). To address the question of whether early Smad signaling events require cellular adhesion, TGF-β1-induced phosphorylation of receptor-regulated Smads was examined in adherent and suspended cells. Rapid phosphorylation of Smad2 (Fig. 1B, top) and Smad3 (data not shown) was observed within 15 min of TGF-β1 stimulation of cells under both conditions. In agreement with this finding, PAI-1 expression, which is independent of p38 activation (30), remained unaffected in suspended cells (Fig. 1C, left). Of note, expression of GADD45β was even enhanced \( (p < 0.05) \) (Fig. 1C, right). To determine whether integrin binding is involved in the TGF-β effect on BGN, PANC-1 cells were plated on dishes coated with fibronectin (a ligand for β1 integrins) and on poly-d-lysine, a nonintegrin-binding polypeptide. Whereas fibronectin supported TGF-β induction of BGN to a similar extent as plastic, the TGF-β effect was decreased by 79% in cells grown on poly-d-lysine compared with the respective control cells on plastic (Fig. 1D). Collectively, the results show that loss of cell adhesion prevented activation of p38 and induction of BGN expression by TGF-β but did not interfere with the ability of TGF-β1 to bind/activate its receptor and to mediate early Smad signaling; furthermore, the transcriptional activation of well-characterized Smad-dependent genes (PAI-1, GADD45β) was not reduced under nonadherent conditions. Since the transition from the adherent to the suspended state and *vice versa* is associated with rearrangements in the actin cytoskeleton of the cells, which, in turn, may affect some aspects of TGF-β signaling, we investigated the consequences of disrupting the cytoskeleton for TGF-β regulation of BGN. Disassembly of actin stress fibers by cytochalasin D potently and dose-dependently reduced basal and TGF-β1-stimulated BGN expression in PAN-1 cells (Fig. 1E), whereas both PAI-1 and GADD45β expression were only partially inhibited at the 10 μM concentration (data not shown). This indicates that an intact cytoskeleton is important in TGF-β1 signal transduction leading specifically to BGN expression.

**Dominant Negative Rac1 Blocks TGF-β Induction of BGN Expression by Interfering with Activation of p38 but Not Activation of Smad3 and GADD45β Expression**—Because within the Rho-GTPase family, Rac1 is best known for its role in regulating actin assembly and adhesion/integrin-associated signaling, we determined whether Rac1 is involved in mediating the adhesion-dependent regulation of BGN by TGF-β. For this purpose, we initially employed a newly available specific chemical Rac1 inhibitor. When used at the recommended concentration of 50 μM, this agent significantly reduced the TGF-β effect on BGN but not on PAI-1 (Fig. 2A). Next, PAN-1 cells were infected with a retrovirus encoding dominant negative Rac1 (dnRac1) (Rac1-T17N) followed by puromycin selection of individual clones. Two clones expressing Rac1-T17N (Fig. 2B) were chosen for further analysis and compared with cells expressing empty retrovirus. Strikingly, these clones displayed a drastic decrease in TGF-β induction of BGN, which was most pronounced in clone 21 displaying the highest transgene expression (Fig. 2C, right). To analyze whether Rac1-T17N also inhibited TGF-β-induced activation of p38, we determined the phosphorylation state of p38 in the same clones. As shown in Fig. 2D, dnRac1 suppressed the activation of p38 by TGF-β, and the extent of this inhibition correlated well with that of BGN (compare with Fig. 2C, left). Similar results were obtained in co-transfection experiments of PANC-1 cells with Myc-tagged Rac1-T17N along with FLAG-tagged p38 followed by anti-FLAG IP and anti-p38 immunoblotting (data not shown). We have shown previously that the first step in TGF-β signaling to BGN is activin receptor-like kinase 5-mediated activation of Smad2/3 and Smad-dependent transcriptional induction of GADD45β expression (31), which subsequently activates the p38 MAPK pathway. Since TGF-β-induced p38 activation was suppressed as a consequence of Rac1 inhibition, we tested the possibility that dnRac1 blocked some step upstream of p38 activation. Specifically, we asked whether activated Rac1 is required for Smad2/3 activation and GADD45β expression. Since the GADD45β gene has been shown to be induced by TGF-β primarily by Smad3 rather than Smad2 (34), we determined whether Rac1-T17N also inhibited GADD45β expression and Smad3 activation in TGF-β-stimulated PAN-1 cells stably expressing dnRac1. Notably, TGF-β-induced expression of GADD45β was not reduced and in one clone was even enhanced (Fig. 2C, right panel). The same was true for Smad3 phosphorylation (Fig. 2E). We thus conclude that Rac1-T17N blocked induction of BGN expression by TGF-β at the level of p38 activation without interfering with activation of Smad3, or transcriptional induction of MyD118.

**Ectopic Expression of Wild Type Rac1 Enhances the TGF-β Effect on BGN Expression**—Next, we addressed the question of whether ectopic (over)expression of Rac1 can further amplify TGF-β induction of BGN. Stable transduction of PAN-1 cells with a retrovirus encoding Rac1 increased Rac1 protein levels in these cells (Fig. 3A), and this correlated, in a “dose-dependent” fashion, with enhanced expression of BGN relative to wild type and empty vector expressing control cells following TGF-β treatment (Fig. 3B, upper panel). Ectopically expressed Rac1 did not alter TGF-β-induced PAI-1 mRNA levels (Fig. 3B, lower panel).

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The next day, cells were starved overnight in medium with 0.5% fetal calf serum followed by treatment with TGF-β for 24 h in the same medium. BGN mRNA levels were measured as above and were depicted as -fold induction by TGF-β. Data represent the mean ± S.D. from three independent experiments. **, \( p < 0.001 \).

For the expression of GADD45β, the same media were used for 24 h in the same medium. GADD45β expression was measured as above and were depicted as -fold induction by TGF-β. Data represent the mean ± S.D. from three independent experiments.
again indicating the specificity of the Rac1 effect for BGN. These data show that the endogenous Rac1 levels in these cells are rate-limiting for the TGF-β effect on BGN.

Ectopic Expression of Constitutively Active Rac1 Rescues the TGF-β Effect on BGN in Suspended Cells—Having shown that dnRac1 blocked TGF-β-induced p38 activation and BGN expression, we wondered whether these TGF-β effects can be mimicked by forced expression of Rac1-Q61L, a constitutively active (ca) Rac1 mutant. Again, we performed co-transfection experiments in PANC-1 cells with Myc-tagged Rac1-Q61L along with FLAG-tagged p38 followed by anti-FLAG immunoprecipitation and anti-p38 immunoblotting. As expected, caRac1 mimicked the TGF-β effect on p38 in the absence of TGF-β treatment (Fig. 4A). To analyze the effect on BGN, we utilized PANC-1 cells stably transfected with Rac1-Q61L (in pcDNA3). Notably, caRac1
had no effect on BGN mRNA levels (Fig. 4C, left) despite readily detectable expression (Fig. 4B). The failure of Rac1-Q61L alone to enhance BGN expression suggested that signals delivered through activin receptor-like kinase 5 and the Smad pathway are crucial for the TGF-β effect and synergize with Rac1 in the induction of BGN. We reasoned that if the role of Rac1 in this process is to activate p38, then caRac1 plus TGF-β should be able to rescue TGF-β induction of BGN even in nonadherent cells. To test this prediction, we stimulated caRac1 and vector-transfected control cells for 24 h with TGF-β in suspension culture. Intriguingly, the TGF-β effect on BGN was preserved in the former but not in the latter cells (Fig. 4C, middle). The specificity of this effect for BGN was again confirmed by the demonstration that PAI-1 expression tended to decrease rather than increase, although this difference was not statistically significant (Fig. 4C, right). These data reinforce the concept of a functional segregation of the TGF-β/Smad and the Rac1/p38 signaling pathways, which are both required for BGN expression, and show that caRac1 can substitute for adhesion-induced signals.

**Rac1 Is Activated by TGF-β in Adherent but Not in Suspended PANC-1 Cells**—To address the question of whether Rac1 is activated by TGF-β treatment in adherent cells and whether the inability of this growth factor to up-regulate BGN (and to activate p38) in nonadherent cells reflects a deficit in inducing Rac1 activation (GTP loading), we performed affinity precipitation assays using a GST fusion protein containing amino acids 67–152 of the p21-activated kinase 1 binding domain (GST-PAK-PBD). The PAK family of 62–68-kDa serine/threonine kinases are the best characterized effector proteins of Rac1 and Cdc42 and have been shown to bind preferentially to the GTP-bound forms of these proteins (7, 35). In adherently grown PANC-1 cells, treatment with TGF-β resulted in an increase in Rac1 binding to purified GST-PAK-PBD within 5 min (Fig. 5A, top panel). GST-PAK-PBD effectively interacted with the active GTPγS-bound form of Rac1 but did not bind to the inactive GDP-bound form of Rac1 (Fig. 5A, top panel). In PANC-1 cells that were denied substratum attachment and kept in suspension for 24 h, basal (Fig. 5B, top panel) and TGF-β-stimulated (Fig. 5A, top panel) Rac1 activity was inhibited. The same was true for cells transduced with Rac1-T17N (not shown). Total Rac1 levels were unchanged in TGF-β-stimulated and suspended cells compared with the respective control cells (Fig. 5, A and B, bottom). These results strongly suggest that the failure of TGF-β to trigger GTP loading of Rac1 in the absence of adhesion/integrin signaling underlies its inability to induce BGN expression (and p38 activation) in nonadherent cells.

**DPI, a Pharmacologic Inhibitor of NADPH Oxidase(s), and Herbimycin A Suppress Both TGF-β-mediated BGN Expression and p38 Activation**—Rac1 is one of the subunits of NADPH oxidase and is indispensable for its enzymatic function, namely production of ROS (11), which in turn has been shown to activate p38 in keratinocytes (10). To evaluate the possibility that Rac1 activates p38 and BGN via ROS production through its role as a subunit of NADPH oxidase, we inhibited NADPH oxidase activity using DPI. This agent in a dose-dependent fashion reduced both the induction of BGN by TGF-β (Fig. 6A, left) and the activation of p38 (Fig. 6B). Interestingly, DPI enhanced TGF-β-mediated up-regulation of PAI-1 at the 10 μM concentration (p = 0.045, Fig. 6A, right) and weakly increased phosphorylation of Smad3 (Fig. 6C). Together, these results clearly point to a crucial role of Rac1-containing NADPH oxidase(s) for TGF-β activation of p38 and BGN induction and...
provide a mechanistic explanation for Rac1 function in this process. Finally, NADPH oxidase (of human neutrophils) is not only regulated in an adhesion-dependent manner, requiring signaling via leukocyte integrins (17), but its catalytic activity has also been reported to be sensitive to the tyrosine kinase inhibitor herbimycin A (18). To obtain direct evidence on the involvement of tyrosine kinase activity in TGF-β-induced BGN expression, which would further argue in favor of a role for NADPH oxidase, we measured TGF-β-stimulated BGN expression in the presence of herbimycin A. This drug potently suppressed the TGF-β effect on both BGN expression (Fig. 6D) and p38 phosphorylation (data not shown), with strong inhibition at 10 μM and complete inhibition at 100 μM (36). In contrast, the Rho-associated kinase-specific inhibitor Y-27632 failed to inhibit the action of TGF-β on BGN (Fig. 6D), suggesting that Rho is not involved in this process, at least not through its downstream effector Rho-associated kinase. These data further suggest that Rac1 functions in TGF-β regulation of BGN, at least partially, as part of the NADPH oxidase complex associated with oxidative stress signaling.

**DISCUSSION**

Prompted by our initial observation that the TGF-β effect on BGN was critically dependent on cell adhesion, we studied the role of the small GTPase Rac1 in this process. Using dominant negative inhibition and overexpression of the wild type protein, we present evidence that Rac1 is necessary for TGF-β-induced activation of p38 and BGN expression. Results from ectopic expression of a Rac1 mutant with constitutive activity also indicated that activation of Rac1 signaling alone (without stimulation of the TGF-β/Smad pathway) was not sufficient to up-regulate BGN but that in combination with exogenous TGF-β, expression of this mutant allowed for adhesion-independent TGF-β regulation of this SLRP. This is consistent with the notion that Rac1 acts at the level of p38 without affecting (positively) Smad3 activation and GADD45β induction, two events previously shown to precede the activation of p38 and to be crucial for TGF-β induction of BGN. Finally, using pharmacologic inhibition, we present initial mechanistic insight into Rac1 function by demonstrating that Rac1 may act here as part of the ROS-generating enzyme NADPH oxidase. In Fig. 7, we have inte-
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FIGURE 5. GTP loading of Rac1 is induced by TGF-β in adherent but not in suspended cells. A, PANC-1 cells cultured under adherent (lanes 1–6) or suspended (lanes 7–9) conditions were left untreated or were stimulated with TGF-β for the indicated times followed by lysis in the lysis buffer supplied with the kit. 750 μg of cell lysate was then incubated with 20 μg of GST-PAK-PBD and an immobilized glutathione disc (lanes 2–9) for 2 h at 4 °C. Activated (GTP-loaded) Rac1 was detected by virtue of its binding affinity to GST-PAK-PBD. As controls, cell lysate from adherent PANC-1 cells was electrophoresed without further processing (lane 1), or pretreated in vitro with GTP-y-S (lane 2) or GDP (lane 3) to activate or inactivate Rac1 prior to GST-PAK-PBD binding. Following washing, bound Rac1 was eluted with Laemmli sample buffer. Top, lysate (40 μg, lane 1) and half the volume of eluted samples (lanes 2–9) separated by 4–20% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with Rac1 antibody. Middle, Ponceau staining of GST-PAK-PBD-bound proteins to calibrate GTP loading of GST-PAK-PBD (~35 kDa). Bottom, Rac1 immunoblot analysis of whole cell lysates to demonstrate that TGF-β treatment does not change total Rac1 levels. B, equal amounts (750 μg) of protein from PANC-1 cells cultured in parallel under adherent (adh.) and nonadherent (susp.) conditions were subjected to the Rac1 activation assay as described under A.

An intriguing phenomenon is the functional segregation of the TGF-β/Smad/GADD45β and the Rac1/p38 signaling pathways. Specifically, TGF-β receptor(s)-mediated signaling and Smad3 phosphorylation was preserved upon TGF-β treatment of nonadherently growing PANC-1 cells and their adherent Rac1-T17N-transduced counterparts. Moreover, we assayed two classical TGF-β target genes, which are induced via Smads independently from p38 activation, particularly β1, since these are required and sufficient for integrin-triggered Rac1-GTP loading (37) and TGF-β-induced p38 activation (6).

An intriguing phenomenon is the functional segregation of the TGF-β/Smad/GADD45β and the Rac1/p38 signaling pathways. Specifically, TGF-β receptor(s)-mediated signaling and Smad3 phosphorylation was preserved upon TGF-β treatment of nonadherently growing PANC-1 cells and their adherent Rac1-T17N-transduced counterparts. Moreover, we assayed two classical TGF-β target genes, which are induced via Smads independently from p38 activation, namely PAI-1 and MyD118 and found that their induction by TGF-β was not negatively affected by suspension culture or dnRac1 expression. Rather, GADD45β induction was enhanced under these conditions (see Figs. 1C and 2C). PAI-1 expression, too, displayed a regulation reciprocal to that of BGN, which in DPI-treated cells was statistically significant (see Fig. 6A). Together, this suggests an inhibitory effect of adhesion/Rac1 signaling on Smad signaling. Of note, Smad (2) phosphorylation and PAI-1 promoter activity in response to TGF-β was reported to be higher in suspended versus adherent lung fibroblasts (38). We also studied the effect of a Rac1 mutant with constitutive GTPase activity (Q61L) and found that, although this mutant was able to activate p38 in transient co-transfection assays, it was insufficient to induce BGN by itself (in the absence of TGF-β stimulation) following stable transfection. This is in line with observations that stress stimuli that activate p38 (H2O2, methyl methanesulfonate, sorbitol, UV irradiation) were insufficient to induce BGN.3 Instead, it appears that signaling input from the TGF-β/Smad-pathway is required in order for Rac1 to regulate BGN expression, a notion that was subsequently confirmed by (ectopic) overexpression of wild type Rac1 resulting in considerable enhancement of TGF-β-induced BGN mRNA levels. Final proof of our model then came from the demonstration that expression of caRac1 (which is associated with higher levels of activated p38) was able to rescue the TGF-β effect on BGN in nonadherent cells. Collectively, results from the gene transfer experiments indicate that Rac1 is necessary but not sufficient for TGF-β regulation of BGN expression and that the major, if not the sole, function of Rac1 in this process is to induce activation of p38.

Both BGN and Rac1 have been independently implicated in cellular adhesion and migration. Consistent with hypotheses that predict involvement of BGN in the control of cell migration, BGN RNA expression is up-regulated in migrating endothelial cells after monolayer wounding in vitro, a process that may depend on activation of TGF-β (39). Interestingly, immunocytochemical staining localized BGN to the tips and edges of lamellipodia on migrating cells, indicating that BGN is found at loci at which the formation and dissolution of adhesion plaques occurs (39). Similarly, Rac1 can be activated locally in the context of newly formed adhesions at the leading edge of cells, where also phosphorylated p38 localizes (40). It therefore appears that the close functional connection between Rac1 (and p38) activation and BGN expression, as highlighted here in their common control by TGF-β, is also

3 H. Ungefroren, unpublished data.
reflected spatially in their subcellular distribution. On the other hand, BGN when acting as a ligand can induce morphological and cytoskeletal changes that involve signaling by RhoA and Rac1 and result in lung fibroblast migration (41). Thus, BGN and Rac1 mutually control their regulation and may thus form a feedback loop closely resembling that between BGN and TGF-β.

It has been increasingly realized that many of the downstream signaling effects attributed to Rac1 are executed via the enzyme NADPH oxidase and ROS generation. We therefore investigated whether the function of Rac1 in TGF-β-mediated regulation of BGN too could be related to NADPH oxidase function. This was supported by the observation that the NADPH oxidase inhibitor DPI largely mimicked the effects of dnRac1; DPI dose-dependently suppressed TGF-β-induced BGN expression and p38 activation but did not (negatively) interfere with TGF-β-induced activation of Smad3 and up-regulation of PAI-1 expression. In leukocytes, NADPH oxidase activation is dependent on both cell adhesion (17) and tyrosine phosphorylation (18, 19). Notably, herbimycin A, a potent inhibitor of protein-tyrosine kinases, blunted the TGF-β effect on BGN, providing additional experimental evidence for a role of Rac1 in TGF-β-mediated NADPH oxidase activation. Finally, the involvement of NADPH oxidase(s) is further supported by the known role of ROS in p38 activation (10) and by data from our laboratory that indicate that radical scavengers (vitamin E, N-acetylcysteine) partially inhibit the TGF-β effect on BGN. Based on these data, we speculate that the described mode of TGF-β control of BGN reflects the adhesion dependence of NADPH oxidase function rather than representing an

4 S. Groth, M. Schulze, H. Kalthoff, F. Fandrich, and H. Ungefroren, manuscript in preparation.
adhesion dependence per se. Given the emerging role of ROS and nonphagocytic NADPH oxidase in the etiology of fibrotic lesions, overexpression of Rac1 in the tumor stroma of pancreatic carcinoma (42) provides an appealing pathophysiological explanation for overexpression/misexpression of BGN in this disease.

Taken together, the results of this study establish Rac1 as an important signaling intermediate in TGF-β-regulation of BGN and provide for the first time data on cooperative interactions between adhesion/integrin and TGF-β receptor/Smad-dependent signaling in the regulation of BGN expression.

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