Identification of a novel anoikis signalling pathway using the fungal virulence factor gliotoxin

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Anoikis is a form of apoptosis induced by cell detachment. Integrin inactivation plays a major role in the process but the exact signalling pathway is ill-defined. Here we identify an anoikis pathway using gliotoxin (GT), a virulence factor of the fungus Aspergillus fumigatus, which causes invasive aspergillosis in humans. GT prevents integrin binding to RGD-containing extracellular matrix components by covalently modifying cysteines in the binding pocket. As a consequence, focal adhesion kinase (FAK) is inhibited resulting in dephosphorylation of p190RhoGAP, allowing activation of RhoA. Sequential activation of ROCK, MKK4/MKK7 and JNK then triggers pro-apoptotic phosphorylation of Bim. Cells in suspension or lacking integrin surface expression are insensitive to GT but are sensitised to ROCK-MKK4/MKK7-JNK-dependent anoikis upon attachment to fibronectin or integrin upregulation. The same signalling pathway is triggered by FAK inhibition or inhibiting integrin αV/β3 with Cilengitide. Thus, GT can target integrins to induce anoikis on lung epithelial cells.

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Detachment-induced apoptosis or anoikis is a crucial process to eliminate aberrant cells in the outer layer of epithelia. Lack of anoikis is a hallmark of cancer progression as cells that continue to survive in suspension are prone to metastasis. Originally described by Frisch et al., anoikis is typically induced by detaching cells with trypsin and preventing their re-attachment to polyHEMA-coated plates. However, this system is artificial as trypsin inappropriately modifies adhesion molecules leading to the activation of signalling pathways that may not reflect physiological ways of anoikis. Cells attach to the extracellular matrix via integrins. Integrins consist of transmembrane α and β chains, which form at their extracellular N termini an interaction interface with the Arg-Gly-Asp (RGD) motif of matrix components such as fibronectin or vitronectin. On the intracellular side integrins recruit components that link adhesion signals to cell survival, cell cycle control and cytoskeletal rearrangement. Key players are focal adhesion kinase (FAK), integrin-linked kinase (ILK) and Src tyrosine kinase. FAK is autophosphorylated at Y397 upon integrin activation and subsequently phosphorylates adapter molecules such as paxillin, vinculin and Rho. Rac- and Cdc42 GTPase-modulating proteins to regulate the actin cytoskeleton. FAK and ILK further activate PI3K/AKT, ERK/MAPK and JNK signalling pathways.

Apoptosis can be induced by extrinsic death receptor signalling where activated receptors of the tumour necrosis factor superfamily recruit and activate caspase-8 via FADD leading to the cleavage and activation of effector caspase-3. Alternatively, so-called BH3-only proteins of the Bcl-2 family sense apoptotic signals and convey them to Bax/Bak-mediated mitochondrial outer membrane permeabilization. The subsequent release of cytochrome c induces the formation of the Apaf-1/caspase-9 apoptosome, which results in caspase-3 activation. Although evidence was initially presented that death receptor signalling via FasL and/or FADD plays a role in anoikis induction, this could not be confirmed in other studies. By contrast, several cellular processes seem to block mitochondrial apoptosis signalling in adherent cells. Activation of AKT, elicited by integrin engagement, leads to the phosphorylation of the BH3-only proteins Bad and Bim, which are sequestered and inactivated by 14-3-3 proteins. AKT also inhibits Forkhead transcription factors (FOXOs), which are responsible for the transcriptional upregulation of the BH3-only proteins Bim, Puma and Bmf. Moreover, both ERK and PI3K/AKT-mediated phosphorylation of Bim lead to its proteasomal degradation. However, it has been unclear if activation of these BH3-only proteins during anoikis is indeed linked to AKT and/or ERK inhibition.

The mould Aspergillus fumigatus causes a severe pulmonary disease termed invasive aspergillosis. Under healthy conditions, airborne conidia released by A. fumigatus are successfully eliminated from the pulmonary cavities by alveolar macrophages, neutrophils and leucocytes. In immunosuppressed patients, however, A. fumigatus germinates, invades the lung and causes severe and often lethal systemic infections. The breakage of the epithelial barrier is the most likely cause for the invasive property of A. fumigatus. Accumulating evidence suggests a crucial role of the major virulence factor gliotoxin (GT) in this process because fungi lacking GT production are much less virulent than wild-type (WT) strains. Previously, we showed that GT induces a rapid detachment of human lung epithelial cells and mouse fibroblasts before they undergo caspase-dependent apoptosis. GT-induced apoptosis requires a JNK-mediated triple phosphorylation of Bim at S100/T112/S114, which increases the pro-apoptotic activity of Bim. Moreover, both in vitro and in vivo, GT-induced cytopathy depended on Bak indicating that epithelial barrier breakdown and lung invasion after A. fumigatus infection may be due to GT-mediated anoikis.

Here we use GT to delineate for the first time an anoikis signalling pathway in human lung epithelial cells that leads to the direct activation of the pro-apoptotic family member Bim. GT covalently modifies the RGD-binding domain of integrin α and β chains, leading to rapid cell detachment followed by FAK inactivation and subsequent activation of a RhoA-ROCK-MKK4/MKK7-dependent signalling pathway, which activates JNK- and ERK-mediated apoptosis.

**Results**

**GT employs MKK4 and MKK7 to activate JNK-dependent apoptosis.** We previously reported that JNK is required for GT-induced apoptosis. We therefore sought to identify the kinase(s) responsible for JNK activation. Possible candidates were the mitogen-activated protein kinases MKK4 and MKK7. Indeed, after 4–6 h of GT treatment of human bronchial epithelial cells (BEAS-2B) both MKK4 and MKK7 were phosphorylated in their activation loops (S257/T261 and S271/T275, respectively) as detected by phosphospecific antibodies (Fig. 1a). This coincided with the cleavage of the caspase-3 substrate PARP.

To determine if MKK4 and/or MKK7 were required for GT-induced JNK activation and apoptosis, we analysed WT, MKK4−/−, MKK7−/− and MKK4−/−/MKK7−/− mouse embryonic fibroblasts (MEFs). While WT MEFs exhibited a marked increase in caspase-3/7 activity (Fig. 1c) and cell death (Fig. 1d) after 6 h of GT treatment, this was less the case for MKK4−/− and MKK7−/− cells. MEFs deficient for both MKK4 and MKK7 showed the highest degree of protection against GT-induced caspase-3 activation and cell death (Fig. 1c, d). Western blot analysis confirmed that MKK4 and MKK7 were required for phosphorylation of JNK in its activation loop (Thr183/Tyr185), JNK-mediated triple phosphorylation of Bim (pBim) and caspase-3 processing to the active p17 form (cCasp-3) since all these effects were completely ablated in GT-treated MKK4−/−/MKK7−/− MEFs (Fig. 1b). Thus, both MKK4 and MKK7 link GT to JNK activation along the anoikis signalling pathway (Fig. 1e).

**GT triggers a Rho-dependent phosphorylation cascade.** Since GT causes rapid cell detachment associated with cytoskeletal changes (Supplementary Fig. 1), we looked for an upstream MKK4/MKK7 activator, which is linked to these events. Recent evidence indicated that Rho-related small GTPases such as RhoA, Rac1 and Cdc42 do not only control actin remodelling but also the activity of the JNK cascade. This prompted us to investigate if the Rho-associated protein kinase (ROCK) was involved in GT-induced MKK4/MKK7 activation and detachment-induced cell death.

For that purpose, we treated BEAS-2B cells with two pharmacological ROCK inhibitors, H-1112 and Y-27632, before applying GT for 6 h. Both inhibitors completely abolished GT-induced JNK phosphorylation and caspase-3 and PARP processing (Fig. 2a) as well as Bim phosphorylation at T112/S114 (Fig. 2b). An in vitro JNK activity assay showed that GT-induced c-Jun phosphorylation was abolished after H-1112 treatment (Supplementary Fig. 2E and 2F). Importantly, the general caspase inhibitor QVD did not affect GT-induced JNK phosphorylation but unexpectedly blocked caspase-3 activation (Fig. 2a).

Similar results were obtained in MEFs. H-1112 diminished MKK4 and JNK activation, Bim phosphorylation and caspase-3 processing after GT treatment (Fig. 2c). Consequently, both ROCK inhibitors abrogated caspase-3/7 activity (Fig. 2d) and apoptosis (Fig. 2e) to the same extent as the caspase inhibitor
MKK4 and MKK7 are required for GT-induced anoikis. a Western blot analysis of total extracts of human bronchial epithelial cells (BEAS-2B) showing increased phosphorylation of MKK4 (Ser257/Thr261) (pMKK4) and M KK7 (Ser271/Thr275) (pMKK7) as well as PARP cleavage (PARP/cPARP) after GT treatment for 4 and 6 h. b Western blot analysis showing increased phosphorylation of JNK (T183/Y185) (pJNK) and Bim (T112/S114) (pBim) in total extracts of non-treated (NT) cells or MEFs deficient for either Mkk4 (Mkk4−/−), Mkk7 (Mkk7−/−) or both Mkk4/Mkk7 (Mkk4−/−/Mkk7−/−) exerted reduced caspase-3/7 activity (c) and annexin V-FITC staining (d) as compared to wild-type (WT) cells when treated with GT for 6 h. e Schematic representation of how GT activates MKK4/MKK7 (MKK) and triggers JNK/Bim-mediated anoikis. Tubulin was used as loading control in a and b. Graphs in c and d show the means of at least three independent experiments ± s.e.m.; p-values: *0.05-0.01, **0.01-0.001, ***<0.001; two-way ANOVA, post hoc: Bonferroni compared to WT.

QVD indicating that GT-induced caspase-3 activation was caused by increased ROCK activity.

To ensure that the observed effects of H-1152 and Y-27632 were due to ROCK inhibition, we effectively knocked down ROCK1 by short hairpin (shRNA) in BEAS-2B cells (Supplementary Fig. 2A). Depletion of ROCK1 prevented GT-induced JNK phosphorylation and caspase-3/7 activity (Supplementary Fig. 2D) as well as caspase-3/7 activity (Supplementary Fig. 2B) and apoptosis (Supplementary Fig. 2C). Thus, ROCK links GT to MKK4/MKK7 and JNK activation along the anoikis signalling pathway (Fig. 2f).

We next examined the role of ROCK-activating Rho GTPases in GT-induced anoikis signalling. For this purpose, we took advantage of Rhotekin, a known binding partner and substrate of RhoA and RhoC32. We performed GST-Rhotekin pulldowns of untreated and GT-treated BEAS-2B cell extracts and examined them for the abundance of active RhoA. As a positive control, we used the bacterial toxin CNFy, which inhibits the GTPase activity of Rho proteins, thereby keeping them in a permanent GTP-bound active state (Fig. 3a)33. As shown in Fig. 3a, the amount of active RhoA-GTP in the GST-Rhotekin pulldowns started to increase after 40 min of GT treatment, a time that coincided with GT-induced cell detachment (Supplementary Fig. 1). To confirm that increased Rho activity was crucial for GT-induced anoikis signalling, we treated BEAS-2B cells with the bacterial toxin C3, which inhibits Rho activity by ADP ribosylation34. Rho inhibition by C3 reduced GT-induced caspase-3/7 activity and increased RhoA-GTP in the GST-Rhotekin pulldowns started to increase after 40 min of GT treatment, a time that coincided with GT-induced cell detachment (Supplementary Fig. 1). To confirm that increased Rho activity was crucial for GT-induced anoikis signalling, we treated BEAS-2B cells with the bacterial toxin C3, which inhibits Rho activity by ADP ribosylation34. Rho inhibition by C3 reduced GT-induced phosphorylation of MKK4 and JNK (Fig. 3c) as well as the phosphorylation of Bim at its T112/S114 JNK phosphorylation site when BEAS-2B cells were pretreated with the C3 toxin. Thus, GT triggers Rho activation (particularly RhoA) to stimulate permanent GT-bound active state (Fig. 3a)33. As shown in Fig. 3a, the amount of active RhoA-GTP in the GST-Rhotekin pulldowns started to increase after 40 min of GT treatment, a time that coincided with GT-induced cell detachment (Supplementary Fig. 1). To confirm that increased Rho activity was crucial for GT-induced anoikis signalling, we treated BEAS-2B cells with the bacterial toxin C3, which inhibits Rho activity by ADP ribosylation34. Rho inhibition by C3 reduced GT-induced phosphorylation of MKK4 and JNK (Fig. 3c) as well as the phosphorylation of Bim at its T112/S114 JNK phosphorylation sites (Fig. 3c).

Finally, we wanted to know if the C3 toxin had any inhibitory effect on GT-induced ROCK activation. We therefore monitored the phosphorylation of a major substrate of ROCK, myosin-binding subunit of myosin phosphatase (MYPT1)31 by western blot analysis. As shown in Fig. 3d, e, while the phosphorylation of MYPT1 gradually increased after GT treatment, this was not the case when BEAS-2B cells were pretreated with the C3 toxin. Thus, GT triggers Rho activation (particularly RhoA) to stimulate
ROCK-MKK4/MKK7-JNK-Bim-dependent anoikis signalling (Fig. 3f).

GT disrupts the focal adhesion complex. Adherent cells treated with GT rapidly detach before they die (Supplementary Fig. 1)30. This suggests that disruption of focal adhesions might be an early event of GT action. Paxillin is a scaffold protein at the cytoplasmic side of focal adhesions responsible for recruiting FAK, a crucial mediator of integrin signalling35. We used green fluorescent protein (GFP)-labelled paxillin to monitor its subcellular localisation before and after GT treatment by confocal time-lapse microscopy. While in adherent healthy BEAS-2B cells, GFP-paxillin was present in focal adhesions as well as in the cytosol (Fig. 4a), it mainly localised to vesicular structures after GT treatment before the cell rounded up and died (Fig. 4a and Supplementary Movie 1). Co-transfection with the endosomal marker mRuby-Endo-14 revealed that GFP-paxillin resided on endosomal membranes after GT treatment (Fig. 4b).

Since GT changed the structure/composition of focal adhesions, we sought to study the role of FAK in GT-induced apoptosis. FAK is an interesting downstream target of GT because it is known to regulate Rho GTPases during stress fibre formation and focal adhesion turnover 36. Moreover, it is known to phosphorylate and activate p190RhoGAP, which negatively regulates Rho activity in adherent cells 36. We therefore investigated (i) if FAK activity was regulated by GT and (ii) if this affected the activity state of RhoA and therefore the RhoA-ROCK-MKK4/MKK7-JNK-mediated anoikis signalling.

Within 30 min of GT treatment of BEAS-2B cells, the activating phosphorylation of FAK at Y397 was lost (Fig. 5a). Simultaneously, the phosphorylations of its substrates p190RhoGAP (Fig. 5c) and paxillin (Fig. 5b) diminished and paxillin was degraded (Fig. 5b). Dephosphorylation of p190RhoGAP results in a lower GAP activity towards Rho proteins, therefore favouring their active GTP-bound state36. Indeed, we observed a higher level of active RhoA (Fig. 3a) and phosphorylation of its
downstream target JNK (Fig. 5b) at the time of FAK inactivation (30–60 min). Thus, GT-induced RhoA and JNK activation involves FAK and p190RhoGAP dephosphorylation/inactivation (Fig. 5i).

Inhibition of FAK mimics GT-induced anoikis signalling. If inactivation of FAK by GT triggered apoptosis via Rho-ROCK-MKK4/7-JNK activation, pharmacological inhibition of FAK should activate the same signalling pathway. We therefore used the FAK inhibitor FAK14, which is highly specific for FAK because it blocks recruitment of its downstream target Src at Y39737. As shown for GT, FAK14 induced cell detachment (Fig. 5d) and apoptotic cell death of BEAS-2B cells within 6 h at low (3–10 μM) and high (50 μM) doses (Fig. 5e, f). In addition, both doses of FAK14 caused the rapid dephosphorylation of FAK at Y397, dephosphorylation and hence inactivation of p190RhoGAP, phosphorylation of MKK4 and JNK (Fig. 5g, h) and phosphorylation of Bim at T112/S114 (Fig. 5g). Hence FAK inhibition stimulated the same anoikis signalling pathway as GT (Fig. 5i).

To confirm that inactivation of FAK was crucial for GT-induced JNK activation and caspase-mediated apoptosis, we overexpressed WT FAK or activated mutant forms of FAK (myrFAK, superFAK (FAK K578E/K581E) and myrsuperFAK)38,39 in BEAS-2B cells by lentiviral transduction. All variants of FAK were overexpressed to similar levels, were phosphorylated and inactivated myrFAK, superFAK and myrsuperFAK (Fig. 3A, 3B, 3D and 3E). However, anoikis signalling was not fully blocked most likely because GT could still dephosphorylate and inactivate myrFAK, superFAK and myrsuperFAK (Fig. 3A, 3B, 3D and 3E). As a consequence, GT-induced anoikis signalling, i.e., JNK and Bim phosphorylation and caspase-3 processing and activation were delayed (Supplementary Fig. 3A and 3C). However, anoikis signalling was not fully blocked most likely because GT could still dephosphorylate and inactivate myrFAK, superFAK and myrsuperFAK (Supplementary Fig. 3A and 3C). This finding is consistent with previous observations that the downstream signalling of super-FAK still depends on integrin-mediated adhesion39.

ROCK has been reported to regulate focal adhesions via an inside-out cytoskeletal signalling40. We therefore examined if the ROCK inhibitor H-1152 or the RhoA inhibitor toxin C3 had any effect on FAK phosphorylation and cell detachment.
The inhibitors themselves did not detach BEAS-2B cells (Supplementary Fig. 1) and H-1152 induced a minor FAK dephosphorylation/inactivation without affecting JNK phosphorylation (Supplementary Fig. 2G) as reported before40. In the presence of GT, both H-1152 and C3 slightly delayed cell detachment (Supplementary Fig. 1). This did however not affect focal adhesions since FAK was similarly dephosphorylated by GT irrespective of the presence or absence of H-1152 (Supplementary Fig. 2G). Only JNK and caspase-3 failed to be activated in the presence of the ROCK inhibitor (Supplementary Fig. 2G) confirming that ROCK is a downstream mediator of GT-induced apoptosis and not a major inside-out signalling regulator of cell detachment in this system.

GT can directly target integrins at the RGD binding site. Since GT caused cell detachment and inactivation of the focal adhesion complex, it may directly target integrins. GT contains an intramolecular disulphide bond essential for its cytotoxic activity28. It may therefore covalently modify cysteine residues in integrin α and β chains, which are critical for integrin activation and/or their binding to extracellular matrix components41,42. For that purpose, we incubated recombinant human integrin αVβ3 with GT and determined peptides with possible GT-cysteine adducts by mass spectrometry (MS) analysis. Two cysteines were found to be modified by GT, Cys158 in the seven blade β-propeller domain of αV and Cys258 in the ligand-binding β-1 domain of β3 integrins (Supplementary Fig. 4A). Both cysteines are highly conserved among the α and β chains of various integrins (Supplementary Fig. 4B) and form intracellular disulphide bridges (Cys158 with Cys138 in αV and Cys258 with Cys299 in β3)43,44 that determine efficient binding of integrins to the RGD motif in fibronectin and vitronectin41,42. To provide further evidence that GT modified integrins at cysteines in the RGD binding site, we treated BEAS-2B cells with GT for 30 min and subjected a total cellular extract to anti-GT immunoprecitations (IPs) followed by anti-integrin αVo or β1 western blot analysis. As shown in Supplementary Fig. 5A and 5B, both integrin chains were specifically detected in anti-GT as compared to control IgG1 IPs. This was however not the case when the cells were pretreated with an RGD peptide before GT addition, or the extract was incubated with dithiothreitol (DTT) and iodoacetamide before anti-GT IP (Supplementary Fig. 5A and 5B). Interestingly, GT seemed to also interact with E-cadherin (Supplementary Fig. 5C) but not with the epidermal growth factor receptor (EGFR) (Supplementary Fig. 5D).

To confirm that integrin binding to extracellular matrix components is indeed perturbed by GT, we determined integrin-binding capacity of untreated and GT-treated BEAS-2B cells by fluorescence-activated cell sorting (FACS) analysis using a

![Fig. 4 Paxillin translocates from focal adhesions to early endosomes.](image)
GT-treated cells effectively underwent apoptosis in response to GT, three targets focal adhesions in adherent cells. Indeed, while BEAS-2B cells are used as loading control in a–c, g and h. FAK is active in adherent epithelial cells causing the activation of p190RhoGAP by its phosphorylation at Y1105. This inactivates RhoA. In response to GT, FAK and p190RhoGAP get inactivated, therefore allowing RhoA activation. Graphs in e and f show the means of at least three independent experiments ± s.e.m.; p-values: *0.05–0.01, **0.01–0.001, ***<0.001, one-way ANOVA, post hoc: Bonferroni compared to NT.

**GT-induced anoikis does not occur in suspension cells.** Suspension cells do usually not express active integrins on their surface and hence do not form mature focal adhesions. Therefore, these cells should not be killed by GT if the toxin specifically targets focal adhesions in adherent cells. Indeed, while BEAS-2B cells effectively underwent apoptosis in response to GT, three different human and mouse suspension cell lines, BAF3, Jurkat and FL5.12, were insensitive to GT-induced apoptosis (Fig. 6d). Consistent with this finding, neither MKK4 nor JNK was phosphorylated/activated (Fig. 6e) and caspase-3 was not processed (Supplementary Fig. 8D). All cell lines expressed integrins β1 and β3 on their surface (Fig. 6a–c, Supplementary Fig. 7A and 7B). On treatment FAK gets rapidly dephosphorylated in the focal adhesions at the plasma membrane before it is taken up into the cell together with paxillin.

Integrins are also endocytosed after cell detachment and recycle back to the plasma membrane46. We therefore monitored the fate of integrin β3 surface expression on BEAS-2B cells (Fig. 6b) and integrin β1 surface expression on MEFs (Fig. 6c) after GT treatment by FACS analysis using β chain-specific antibodies. Both integrin chains were removed from the cell surface (Fig. 6b, c). This however occurred only after 2 h of GT treatment suggesting that the primary action of GT was to inactivate RGD binding (Fig. 6a) before integrins were endocytosed.

As shown in Fig. 6a, integrin binding to RGD-FAM rapidly diminished 30 min to 2 h after GT treatment. This kinetic coincided with the time course of FAK and p190RhoGAP inactivation (Fig. 5a, c), paxillin translocation (Fig. 4a) and cell detachment (Supplementary Fig. 1). By confocal immunofluorescence analysis using antibodies against pFAK and the endosomal protein EEA1 as well as GFP-paxillin overexpression we further showed that phosphorylated FAK (pFAK) colocalized with paxillin in focal adhesions in untreated cells (Supplementary Fig. 6B). In response to GT paxillin is endocytosed (Supplementary Fig. 6A). This confirms that after GT treatment FAK gets rapidly dephosphorylated in the focal adhesion plaques at the plasma membrane before it is taken up into the cell together with paxillin.

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GT diminishes RGD binding and surface expression of integrins. a The binding capacity of integrins to extracellular matrix components was measured on untreated (NT) and GT-treated BEAS-2B cells by FACS analysis of RGD-FAM peptide staining. b, c Adherent human lung epithelial cells (BEAS-2B) or MEFs showed reduced surface expression of integrin β3 (b) or integrin β1 (c), respectively, after 2–6 h of GT treatment as evidenced by FACS analysis of anti-integrin-stained cells. Human Jurkat (b) or mouse BAF or FL5.12 suspension cells (c) did not show such a reduction although they expressed integrins on their surface (Supplementary Fig. 7A and 7B), most likely in an inactive form (Supplementary Fig. 7C). d While BEAS-2B cells underwent apoptosis (as determined by annexin V-FITC FACS analysis) after 1 μM GT treatment for 6 h, this was not the case for BAF3, Jurkat or FL5.12 suspension cells. e Western blot analysis of total extracts of BEAS-2B cells (first lane) or BAF3 cells either untreated (NT) or treated with 1 μM GT for 6 h showing that GT did not cause phosphorylation/activation of MKK4 and JNK in BAF3 but does so in BEAS-2B cells. Actin was used as a loading control. Graphs in a–d show the means of at least three independent experiments ± s.e.m.; p-values: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA, post hoc: Bonferroni compared to NT.

BEAS-2B cells, they were in an active state as evidenced by RGD-FAM (Fig. 6a) and anti-active integrin β1 staining (Supplementary Fig. 7C). This was however not the case on Jurkat cells where integrin β1 was not detected with an anti-active integrin antibody (Supplementary Fig. 7C). Moreover, while GT diminished surface staining of integrins β3 (Fig. 6b) and β1 (Fig. 6c) on BEAS-2B and MEF, respectively, none of the suspension cell lines showed such an effect. To further substantiate the integrin-dependence of GT for its pro-apoptotic action, we made use of K562 cells, which also grow in suspension but express very little integrin αV and β3 at all (Fig. 7e). Again, GT could not trigger caspase-3 activity (Fig. 7b) or processing (Fig. 7c) in these cells. However, when the cells were treated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA), they largely upregulated active integrins αV, β1 and β3 on their surface (Fig. 7c–e) and became sensitive to GT-induced cell detachment (Fig. 7a), MKK4, JNK and Bim phosphorylation and caspase-3 processing (Fig. 7e) and activation (Fig. 7b). Similarly, BAF3 suspension cells made adherent by plating them on fibronectin-coated plates overnight displayed activated surface integrins as evidenced by RGD staining (Supplementary Fig. 8B) and acquired sensitivity to GT-induced detachment (Supplementary Fig. 8A), caspase-3 processing (Supplementary Fig. 8D), apoptosis (Supplementary Fig. 8C) and activation of the same MKK4/JNK signalling pathway (Supplementary Fig. 8D) as previously seen in BEAS-2B cells. Hence GT exerts its cytotoxic activity primarily on adherent cells expressing active integrins, which qualifies it as a bona fide anoikis inducer.

Blocking αV/β3 integrins mimics GT-induced anoikis signalling. If activation of the JNK/Bim-dependent anoikis pathway by GT is a consequence of integrin inactivation, the same pathway should be triggered by integrin inhibitory antibodies or drugs. Cilengitide is an RGD-based compound that primarily blocks integrins αvβ3 and αvβ5 at lower and β1 at higher concentrations. We first confirmed that BEAS-2B cells express αv, β3 and β1 integrins (Supplementary Fig. 7D). When these cells were exposed to 25 μg/ml Cilengitide, they exhibited cell detachment (Supplementary Fig. 7E), FAK dephosphorylation and activation of JNK1/2 with subsequent Bim phosphorylation at T112/S114 (Supplementary Fig. 7F and 7G), activation and processing of caspase-3 processing (Supplementary Fig. 7G and 7H) and cell death (Supplementary Fig. 7I) within 6–24 h. However, in contrast to GT, the cells treated with Cilengitide detached in clusters (instead of single cells) (Supplementary Fig. 7E), and FAK dephosphorylation (Supplementary Fig. 7F) and subsequent anoikis signalling were less pronounced and delayed (Supplementary Fig. 7G-I). This was also true when Cilengitide was combined with an anti-integrin α5/β1 inhibitory antibody, which was ineffective alone and did not further enhance Cilengitide-induced anoikis (Supplementary Fig. 7I). This might be due to the...
Discussion

Here we used GT, the major virulence factor of *A. fumigatus*, to identify a novel anoikis signalling pathway. GT qualifies for an anoikis inducer for the following reasons: (i) it induces rapid cell detachment prior to apoptosis induction; (ii) it can directly modify N-terminal cysteines of α and β chains of integrins thereby interfering with their binding to extracellular matrix components; and (iii) it cannot kill suspension cells of the hematopoietic system and does not activate the anoikis signalling pathway in these cells. Only when suspension cells are made adherent, i.e., either plated on fibronectin or induced to express integrins on their surface, they become sensitive to GT-induced anoikis via the same signalling pathway as epithelial cells. Although we provide compelling evidence that GT can directly target integrins by modifying cysteines at the RGD binding interface, we cannot exclude that it also modifies and disrupts binding domains in other adhesion molecules, including inflammatory receptors or cadherins. Indeed, we found that E-cadherin could be co-immunoprecipitated with anti-GT antibodies. This may explain why cells treated with GT detach as single cells while those treated with integrin inhibitory antibodies or compounds detach as cell sheets. Further studies are needed to identify the impact of GT on signalling pathways regulated by cadherins.

Protection from anoikis was suggested to involve activation of ERK/MAPK and PI3K/AKT signalling11-13. These pathways should be turned off upon GT action, resulting in dephosphorylation of Bad and Bim and their release from 14-3-3 proteins21,22, the transcriptional upregulation of Bim, Puma and Bmf by FOXO activation23,24 and the stabilisation of Bim due to lack of proteasomal degradation25. However, as we previously reported, neither Bad, Bmf nor Puma was required for GT-induced apoptosis, and the ERK/MAPK and AKT signalling pathways were still transiently activated after GT treatment30. Moreover, although Bim was essential for GT-induced cytotoxicity it required JNK-mediated phosphorylation at S100/T112/S114 rather than increased protein stability for effective Bax/Bak activation30. This indicated that GT uses a JNK-dependent, but ERK/AKT-independent pathway for anoikis signalling30.
Previously, Stupack et al. reported that unligated integrins or β-integrin tails recruit caspase-8 to the membrane and induce apoptosis distinct from anoikis in death receptor-independent manner. We therefore tested if caspase-8 was activated and required for GT-induced anoikis. However, while recombinant FasL rapidly activated caspase-8 in BEAS-2B cells, we did not detect any caspase-8 activation after GT treatment for up to 6 h (Supplementary Fig. 9A). Moreover, knocking down caspase-8 expression did not affect the kinetics of GT-induced anoikis but blocked FasL-induced apoptosis (Supplementary Fig. 9B). Thus, GT uses a JNK-dependent, but ERK/AKT- and caspase-8-independent pathway for anoikis signalling.

JNK/c-Jun-induced transcription in response to lyso phosphatidic acid was previously shown to be mediated by RhoA-ROCK-MKK4 signalling. The same pathway was involved in arsenic trioxide-induced apoptosis of chronic myelogenous leukaemia cells. However, in both cases it remained elusive how RhoA was activated. Here we show that RhoA is activated by dephosphorylation and inactivation of p190RhoGAP as a result of GT-induced FAK inhibition. This is consistent with a report showing FAK-induced downmodulation of RhoA activity via p190RhoGAP. The role of Rac or Cdc42 GTPases in mediating GT anoikis could be excluded because they do not activate ROCK and have recently been shown to be inactivated by GT.

ROCK1 and 2 play essential roles in regulating cell morphology, motility and cell fate. Whether all these effects are mediated through changes in the actin cytoskeleton is still debated. Whether ethanol, doxorubicin and serum starvation were found to induce caspase-dependent apoptosis via RhoA-ROCK-mediated myosin light chain phosphorylation and subsequent cytoskeletal rearrangements rather than JNK activation although the link to caspase-3 activation was not provided. In other studies ROCK activated JNK but the result was stimulation of cell migration rather than apoptosis. Furthermore, ROCK was shown to affect the membrane blebbing of apoptotic cells. However, in this case ROCK was activated by caspase-3-mediated cleavage, which occurs downstream of cytochrome c release. Although we cannot exclude a cytoskeletal involvement for GT-induced, ROCK-mediated anoikis, our data show that ROCK does not signal back to focal adhesions (FAK) but stimulates the downstream phosphorylation/activation of MKK4 and MKK7 essential for anoikis. The precise mechanism of this activation is not yet understood. It either occurs through intermediate kinases such as MLK or ASK and/or the scaffold proteins hCNK or JIP, which were shown to link ROCK to JNK activation. Once activated MKK4 and MKK7 then phosphorylate different sites in the activation loop of JNK, and we indeed find that both kinases are required for GT-induced JNK activation.

Anoikis has been mainly studied in an artificial system, where cells are detached by trypsinization and prevented from reattaching to polyHEMA-coated plates. This form of anoikis differs from that induced by GT because trypsin at least partially degrades surface integrins. Therefore, the link to caspase-3 activation was not provided. In other studies ROCK activated JNK but the result was stimulation of cell migration rather than apoptosis. Furthermore, ROCK was shown to affect the membrane blebbing of apoptotic cells. However, in this case ROCK was activated by caspase-3-mediated cleavage, which occurs downstream of cytochrome c release. Although we cannot exclude a cytoskeletal involvement for GT-induced, ROCK-mediated anoikis, our data show that ROCK does not signal back to focal adhesions (FAK) but stimulates the downstream phosphorylation/activation of MKK4 and MKK7 essential for anoikis. The precise mechanism of this activation is not yet understood. It either occurs through intermediate kinases such as MLK or ASK and/or the scaffold proteins hCNK or JIP, which were shown to link ROCK to JNK activation. Once activated MKK4 and MKK7 then phosphorylate different sites in the activation loop of JNK, and we indeed find that both kinases are required for GT-induced JNK activation.

The novel anoikis signalling pathway will increase our understanding of diseases caused by excessive cell detachment or the resistance of detached cells to die. Overexpression of FAK has been shown to promote tumour progression and metastasis, which can be counteracted by high levels of RhoA. Moreover, RhoA downregulation is associated with increased breast cancer, cell migration and invasion. Thus, RhoA may act as a tumour suppressor not only by restricting tumour cell motility but also by inducing anoikis. Moreover, FAK is an attractive target for anticancer therapy as it has recently been proposed for pancreatic cancer.

On the other hand, activation of FAK or integrins may render lung epithelial cells less sensitive to GT-induced anoikis and hence diminish increased epithelial cell permeability and subsequent lung invasion during A. fumigatus infections. An even better strategy is to develop GT inhibitors, which would prevent integrin inactivation and thereby completely block lung epithelial cell anoikis.

**Methods**

**Reagents and inhibitors.** The ROCK inhibitors Y-27632 (Rho Kinase inhibitor) and H-1152 were obtained from EMD Millipore (Billerica, MA, USA); Q-VD-OPh from MP Biomedicals (Eschwege, Germany); and GT from AppliChem (Darmstadt, Germany). The CNF toxin (150 ng/ml) from Yersinia.
pseudotuberculosis and the C2I/C3 fusion toxin (Clostridium botulinum and ClostridiumLimousinum, respectively) combined with the C2I toxin from Clostridium tetani. Anti-
C2II (200 ng/ml) and C2I/C3 (100 ng/ml) mixed in cell culture media. The FAK inhibitor 14 (FAK14)
was purchased from Tocris (Bristol, UK), and Cilengitide, a cyclic pentapeptide RGD compound (cyclor-RGDIN(Nal)y)6-35, the RGD peptide, DTT and
iodocetamide were from Sigma (Taufkirchen, Germany).

Plasmids
shRNA against ROCK1 (SHCLNG-NM_005406.1, shROCK1: GCGCGGCAAGATGTACCCGCCGATTTACTCGAGTAAATCGGGTACAACT
CCGGGCACCAGTTG TACCCGATTTACTCGAGTAAATCGGGTACAACT
CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTT
CCGGACATGAACCTGCTGGATATTTCTCGAGAAATATCCAGCAGGTTCA
and only used until passage number 20. Typically, 1 × 106 cells were seeded in

*Human bronchial epithelial cells (BEAS-2B) were obtained from American Type
Culture Collection (ATCC® CRL-16411®). Cells were cultured in Dulbecco’s
modified Eagle’s medium or RPMI supplemented with 10% foetal calf serum (FCS)
and 1% penicillin/streptomycin, respectively. BEAS-2B cells were kept subconfluent
and only used until passage number 20. Typically, 1 × 106 cells were seeded in
10 cm plates to reach a confluency of approximately 60–70% on the day of the
experiment. Murine suspension cells BAF3 (ACC300, DSZM Germany), FLS12
(Thermo Fisher, Darmstadt, Germany) and human Jurkat T cells (Clone E61,
ATCC® TIB152®) were maintained at cell densities lower than 500 000 cells/ml.
The former two cell lines were daily supplemented with 1 ng/ml interleukin-3
(Peprotech, Rocky Hill, NJ, USA). Human leukaemia K562 suspension cells
(ATCC® CCL44®) were kindly provided by Ilmin Brummer (University of
Freiburg, Germany). In all, 3 × 105 cells/well were plated in six-well plates for
differentiation into megakaryocytes by the addition of 10 ng/ml PMA (Sigma)
for 9 h. The medium was replaced daily with fresh medium containing PMA.
Anokias was induced by treating BEAS-2B cells with 1 μM for 6 h or 10–50 μM FAK14
for 6–12 h. BEAS-2B cells were also treated with Fas-L (20 ng/ml, Adipogen,
Epalinges, Switzerland) for up to 4 h to induce extrinsic apoptosis signalling.
All cell lines were regularly tested for mycoplasma contamination using a PCR
based mycoplasma detection kit (mycoMyCMycoplasm PCR Detection Kit, INTRON
Biotechnology Inc., Seongnam, South Korea).

Attachment of suspension cells
Human fibroblasts (Advanced BioMatrix, Carlsbad, CA, USA) was used for the attachment of BAF3 suspension cells. Twelve-
well plates were coated with 50 μg/ml fibronectin in phosphate-buffered saline
(PBS) overnight. On the next day, 100 000 cells were seeded per well and grown
without attachment. Non-attached cells were removed by washing with PBS before
GT treatment or further analysis.

Immunoblotting and antibodies
Cells were lysed on ice for 15 min in 50–100 μl whole-cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM
EDTA (pH 8.0), 5 mM Na2-phyrophosphate, 1 mM Nα-sulfo-L-nitrocellulose membranes by wet blotting (GE Healthcare Europe,
Epalinges, Switzerland) for up to 4 h to induce extrinsic apoptosis signalling. All
Cell pellets were lysed on ice for 15 min in

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|                        | 11                                 |                                          |
| Anti-GT immunoprecipitations. A volume of 500 μl (1 mg) of a TX-solubilized
 whole-cell extract from BEAS-2B cells treated with GT for 30 min was pre-
 cleared with 100 μl of a 50% slurry of Protein G Sepharose® 4 Fast Flow recombinant
 protein G beads (GE Healthcare Life Sciences) on a turning wheel at 4 °C for 1 h. After
 incubating the supernatant with 100 μl of Protein A-Sepharose (GE Healthcare Life
 Sciences) beads and the mixture rotated at 4 °C for 2 h. All beads were
 centrifuged at 8200 × g, 4 °C for 3 min, washed three times with lysis buffer and
 incubation complexes eluted by boiling in Lämmli buffer. The eluted samples were
 run on SDS-PAGE and subjected to Western blotting. Immunoreactive bands were cut
 out and subjected to in-gel digestion (Promega, Madison, WI, USA). Mass spectrometry
 analysis was performed with the LTQ Orbitrap Velos mass spectrometer (Thermofisher
 Scientific) using a LTQ ion trap as the mass analyser. The LC-MS/MS data were imported
 into Progenesis software and the results were validated with Mascot (version 2.3.08, Matrix
 Science, London, UK). A false discovery rate of 5% was set as cut-off for peptide
 identification.

Cell death assays. Cells were plated in 96-well plates, treated on the next day as indicated and incubated
with XTT solution for 4 h at 37 °C before analysis in the Tecan instrument or
identified with annexin V-FITC and incubated for at least 15 min in the dark. The percentage of
apoptotic cells was analysed on a Calibur or LSRII equipment (BD Biosciences). Video
analysis was performed with the CellQuest Pro software (BD Biosciences) for
quantification and analysis of the data. Cell death was calculated as the percentage
of cells showing up to 30% annexin V and 10% PI staining.
reader (Männedorf, Switzerland). Increasing fluorescence due to substrate cleavage was monitored in intervals of 2 min for 30 min. The slope of the linear regression was used to determine the relative caspase activity.

**Rhotekin pulldown.** GST-Rhotekin-RBD beads (Cytoskeleton Inc., Denver, CO, USA) were used to pull down active Rho-GTP from whole cell lysates. Samples were adjusted to the same cell numbers and lysed in GST-Fish buffer (10% (v/v) glycerol, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% (v/v) NP-40, 2 mM MgCl2 and 1% (v/v) Polybrene (Fluoride)). Lysates were incubated with GST-rhotekin beads for 45 min at 4 °C on an orbital shaker. After washing, the samples were boiled in SDS sample buffer and RhoA was detected on western blots.

**Kinase assays.** The KinaseSTAR JNK Activity Assay Kit (BioVision, Milpitas, CA, USA) was used to measure JNK activity after GT treatment. The kit was used as stated in the manufacturer’s protocol. In brief, JNK was pulled down from cell lysates and incubated with recombinant c-Jun. Phosphorylation of c-Jun was quantitated by phosphorimaging of its substrate MYPT1 after anti-phospho MYPT1 immunoblotting.

**Site-directed mutagenesis of FAK.** To generate superFAK and myr-superal, which are activated forms of FAk mutated in their kinase activation loop (K578E/K581E)[12], we performed site-directed mutagenesis on the lentiviral WT FAK and myrFAK plasmids obtained from Andrew Gilmore[14] using the QuikChange II XL site-directed mutagenesis kit (Agilent, Santa Clara, USA). The following primers were designed using the Agilent QuickChange Primer Design Tool: FAK K578/581E_fwd: CCATCCATTAAATAGTTATTTTCCATCGAGACCTCATACAGTAACGTGCTTCCATATATGGGAATA; FAK K578/581E_rev: TTAATCTCTGATATGAAAGCAGACTATGACTGTACGTTCCGGAGGAAAATTACCTTATTAAATGATGG.

**Lentiviral and retroviral transductions.** In all, 2 × 10^6 HEK 293T cells were transfected with 3 μg of the plasmid of interest, 3 μg envelope vector pMD2G and 3 μg packaging vector pSPAX using Attractene Transfection Reagent (Qiagen, Hilden, Germany). After 12 h protein biosynthesis was augmented using 5 mM fluoride (Koegel, Hilden, Germany). For live cell imaging, 6 × 10^4 BEAS-2B cells were seeded in glass-bottom dishes (Greiner Bio One, Frick, Switzerland) and transfected with 5 μg GFP-paxillin and mRuby-Endo-14 (Bio-Rad AbD Serotec). BEAS-2B cells were seeded in glass-bottom dishes (Greiner Bio One, Frick, Switzerland) and transfected with 5 μg GFP-paxillin and mRuby-Endo-14 (Bio-Rad AbD Serotec). After 12 h post transfection, lentiviruses were harvested on the next morning and supplemented with 100 μg/ml of the inhibitory anti-integrin antibodies (1:100 each): anti CD62-FITC (integrin αvβ1, clone P4C10, MAB1987Z, Merck Millipore) and 1 μM Cyclo-RGD-5-FAM (AnaSpec, MoBiTech, Göttingen, Germany) was used to pull down active (αvβ3)-integrins by GT. Human recombinant αvβ3 integrin (5 μg) was mixed with 5 μg bovine serum albumin as an internal control in 50 nM HEPS, pH 7.5, incubated with 100 μM to 1 mM GT at room temperature for up to 6 h and then denatured in 0.1% RapiGest (Waters, Eschborn, Germany) for 45 min at 50 °C. Peptides were generated by digestion with 0.2 μg trypsin overnight at 37 °C and then purified using C18 STAGE solid phase extraction columns (Varian, Palo Alto, CA, USA). A Q-Exactive Plus Hybrid Quadrupole-Orbitrap system (Thermo Scientific, Darmstadt, Germany) was used for mass spectrometry and operated in the data-dependent mode[26]. Peptide sequences were identified using Mascot (Version 2.4.0.2) in conjunction with PeptideProphet using the reviewed canonical human (20272 protein sequences) and bovine (5994 proteins) combined sequence database, downloaded from Uniprot on 26.11.2013 and 15.07.2015 respectively, together with an equal number of randomised decoy sequences, generated by DBooRkit. The PeptideProphet minimum probability threshold was set to 0.05. The examination of peptides showed that this could be just an adduct with a variable modification with a mass difference of 326.0395 Da. Only peptides with a probability score > 0.99 were taken into consideration. Identified peptides were mapped on the protein sequence using the software Proteorat.

**Integrin Affymetrix microarray and RNAseq analyses.** To quantify the expression of integrin genes, raw data of the transcriptome of untreated Beas-2B cells from three high-throughput experiments, one microarray and two RNAseq assays data were downloaded from Gene Expression Omnibus and Array Express with respective series/sample IDs GSE24025/GSM591439 as well as E-MTAB-4729/ERR1406031 and ERR1406032. Affymetrix Human Gene 1.0 ST microarray was normalised using Single-Channel Array[10,41] and exon expression was summarised to the gene level using the R/Bioconductor package pd.hugene.1.0st.v1 (version 3.14.1). Transcripts were considered to be present in the cell if they have an absolute expression value > 0. RNAseq data were pseudolabeled to the cDNA transcript sequences Ensembl version 87 and quantified using kallisto (Version 0.43.1)[46]. Transcripts abundance was summarised to the gene level via the R library txipnorn[40]. Genes were considered to be expressed, if they have a minimal transcript per million value > 1.

**Data availability**

All data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request.

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Author contributions

C.B., U.M., O.S. and K.A. conceived the project. F.H., S.N., L.P., K.W., C.S., K.Ø., M.M.K., Ma.B., J.H. and M.K. carried out the experiments. H.B. and Me.B. performed data analysis. R.J.D. contributed the Mkk4+/−, Mkk7+/− and Mkk4+/−/Mkk7+/− MEFS. F.H. prepared the figures, and F.H. and C.B. wrote the manuscript.

Additional information

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