Oncogene (2005) 24, 8240–8251
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Commutators of PAR-1 signaling in cancer cell invasion reveal an essential role of the Rho–Rho kinase axis and tumor microenvironment

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We recently reported that proteinase-activated receptors type I (PAR-1) are coupled to both negative and positive invasion pathways in colonic and kidney cancer cells cultured on collagen type I gels. Here, we found that treatments with the cell-permeant analog 8-Br-cGMP and the soluble guanylate cyclase activator BAY41-2272, and Rho kinase (ROK) inhibition by Y27632 or a dominant negative form of ROK lead to PAR-1-mediated invasion through differential Rac1 and Cdc42 signaling. Hypoxia or the counteradhesive matricellular protein SPARC/BM-40 (SPARC: secreted protein acidic rich in cysteine) overexpressed during cancer progression also commutated PAR-1 to cellular invasion through the cGMP/protein kinase G (PKG) cascade, RhoA inactivation, and Rac1-dependent or -independent signaling. Cultured primary cancer cells isolated from peritoneal and pleural effusions from patients with colon cancer or other malignant tumors harbored PAR-1, as shown by RT–PCR and FACS analyses. These malignant effusions also contained high levels of activated thrombin and fibrin, and induced a proinvasive response in HCT8/S11 human colorectal cancer cells. Our data underline the essential role of the tumor microenvironment and of several commutators targeting cGMP/PKG signaling and the RhoA–ROK axis in the control of PAR-1 proinvasive activity and metastatic potential of cancer cells in distant organs and peritoneal or pleural cavities. We also add new insights into the mechanisms linking the coagulation mediators thrombin and PAR-1 in the context of blood coagulation disorders and venous thrombosis often observed in cancer patients, as described in 1865 by Armand Trousseau.
Oncogene (2005) **24,** 8240–8251. doi:10.1038/sj.onc.1208990; published online 8 August 2005

Keywords: PKG; hypoxia; SPARC/BM-40; tenascin-C; peritoneal and pleural effusions; metastasis and thrombosis

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Received 13 April 2005; revised 15 June 2005; accepted 1 July 2005; published online 8 August 2005

Introduction

The multifunctional serine protease thrombin exerts direct actions on a wide variety of cell types, including epithelial cells, endothelial cells, vascular smooth muscle cells, leukocytes, neurons, and glial cells (Coughlin, 2000). Thrombin is a potent mitogen for fibroblasts and epithelial cells, and potentiates the proliferative responses of tumor cells to classical growth factors, such as epidermal growth factor and insulin (Van Obberghen-Schilling *et al*., 1995). Both thrombin and proteinase-activated receptors type I (PAR-1) are expressed in invasive cancer cell lines and breast carcinoma biopsy specimens (Wojtukiewicz *et al*., 1995; Even-Ram *et al*., 1998, 2001; Nguyen *et al*., 2002; Darmoul *et al*., 2003). However, the signaling pathways triggered by thrombin and PAR-1 signaling during tumor progression and invasion remain poorly documented.

Thrombin interacts with the G-protein-coupled protease-activated receptors PAR-1, -3, and -4. Activated PAR-1 are coupled via several members of the heterotrimeric G-proteins Gαo/i, Gα12/13, and Gαq to transduce a substantial network of signaling pathways (Coughlin, 2000). We have recently demonstrated that PAR-1 are functionally connected to both negative and positive invasion pathways in colon and kidney cancer cells (Faivre *et al*., 2001; Nguyen *et al*., 2002). In the collagen type I substratum, PAR-1 and the pertussis toxin (PTx)-sensitive Gαo/i subunits were shown to exert a dominant invasion suppressor role toward several proinvasive pathways controlled by oncogenes and tumor-secreted growth factors (Faivre *et al*., 2001). Similar findings were reported by Kamath *et al*. (2001) for the inhibition of invasion and migration in the highly invasive MDA-MB231 breast cancer cell line, through PAR-1- and Gαi-dependent pathways. Conversely, we have shown that neutralization of Gαo/i signaling by PTx led to the proinvasive activity of thrombin and PAR-1 through the Gα12/13/RhoA cascade, myosin light chain (MLC) phosphorylation, and activation of the actomyosin system (Kureishi *et al*., 1997; Nguyen *et al*., 2002). We presented evidence that the proinvasive potential of PAR-1 in collagen type I is also revealed by

inhibition of RhoA GTPase by RhoD, C3 exoenzyme, the dominant negative N19-RhoA, and cGMP-elevating agents sodium nitroprusside and guanylin, acting through soluble and membrane-bound guanylate cyclases, respectively (Nguyen *et al*., 2002). In this case, PAR-1 is connected with the Gαq/phospholipase Cβ–Ca^{2+}/calmodulin-MLC kinase (CaM-MLCK) cascades that bypass RhoA blockade (Nguyen *et al*., 2002). Thus, we defined a new function for the small GTPases RhoA and RhoD acting as a molecular switch controlling the negative and positive invasive pathways triggered by PAR-1 through PTx-independent heterotrimeric G-proteins. Our data were reminiscent of the demonstration that PAR-1 induce invasion of Matrigel by breast cancer cells through requirement of αvβ5 integrins (Even-Ram *et al*., 1998, 2001), suggesting that the PAR-1 invasive potential is controlled by the matricellular context (Nguyen *et al*., 2002).

Important features of invasive cancers include dramatic changes in the tumor microenvironment and infiltration by immune and mesenchymal cells and myofibroblasts, and remodeling of several extracellular matrix (ECM) components such as tenascin-C (TN-C) and SPARC/BM-40 (SPARC: secreted protein acidic rich in cysteine; Porte *et al*., 1995; Ledda *et al*., 1997; Thomas *et al*., 2000). In addition, hypoxia and increased production of the free radical nitric oxide (NO) play a central role in invasive tumor growth, angiogenesis, and metastasis. Inducible isoforms of nitric oxide synthase (iNOS) are overexpressed in human colonic tumors, while genetic ablation or inhibition of iNOS reduces aberrant crypt foci, colon tumor formation, and lung tumorigenesis (Ahn and Ohshima, 2001 ; Kisley *et al*., 2002). Here, we examined the impact of the RhoA effector Rho kinase (ROK), the soluble guanylate cyclase (sGC) activator BAY41-2272 (Stasch *et al*., 2001), as well as hypoxia and matricellular proteins SPARC and TN-C, on the commutation of negative and positive signaling pathways controlled by PAR-1 in cancer cell invasion. We demonstrate that these molecular and pharmacological interventions targeting the cGMP and RhoA–ROK signaling pathways, as well as other key parameters linked to the tumor microenvironment and metastatic process, exert a permissive role on PAR-1 proinvasive activity.

Results

Promotion of PAR-1 proinvasive activity by inhibition of the RhoA–ROK axis

The Rho-GTPase subfamily members are involved in the regulation of cell shape, cytoskeletal reorganization, and motility through activation of the actin microfilament network and establishment of intercellular and adhesive contacts with ECM components. RhoA regulates the formation of stress fibers, focal adhesions, and cell contraction, Rac1 induces the formation of lamellipodia and membrane ruffling, whereas Cdc42 is involved in filopodia formation (Ridley, 2001). We have shown that small GTPases RhoA and RhoD act as molecular switches at the negative and positive invasion pathways controlled by PAR-1 in kidney and colon cancer cells (Nguyen *et al*., 2002). Moreover, the RhoA antagonist RhoD and the RhoGTPase inhibitor C3 exoenzyme promoted the PAR-1 proinvasive activity through a signaling cascade using Gαq, phospholipase-Cβ, and CaM-MLCK. In this signaling network, cGMP-elevating agents (sodium nitroprussiate, guanylin) and protein kinase G (PKG) act as RhoA inhibitors, as previously reported (Sauzeau *et al*., 2000; Sawada *et al*., 2001).

In order to determine the molecular specificity of the PAR-1 commutation by the Rho-GTPase family members, we have established a series of human HCT8/S11 colon cancer cell lines expressing dominant negative mutants of RhoA, Rac1, and Cdc42, as well as the constitutively activated form of RhoA. As previously demonstrated in kidney cancer cells, parental and stably transfected HCT8/S11 cells by DN-Rho GTPase and DC-RhoA vectors express PAR-1 receptors as a 90 kDa band downregulated by specific PAR-1 antisense (top of Figure 1a; Nguyen *et al*., 2002). Similarly, invasion of type I collagen gels was induced by the PAR-1 agonist TRAP in HCT8/S11 cells stably transfected with the interfering mutant DN-RhoA (T19N), as shown in Figure 1, and previously in kidney epithelial cells MDCK-T23 (Nguyen *et al*., 2002). Such a commutation cannot be accomplished by the constitutively active DC-RhoA (G14V) or dominant interfering mutants DN-Rac1 (T17N) and DN-Cdc42 (T17N) in colorectal cancer cells HCT8/S11 (Figure 1a), as well as in kidney epithelial cells MDCK-T23 expressing interfering mutants DN-RhoA and DN-Rac1 (Jou and Nelson, 1998) under the tetracycline-repressible transactivator (not shown).

Since RhoA and its direct effector ROK regulate the dynamic reorganization of the cytoskeletal actin network leading to activation of the actomyosin system, stress fibers assembly, and cell motility (Amano *et al*., 1997), we decided to examine the role of the RhoA–ROK axis in the regulation of the thrombin PAR-1 receptor proinvasive behavior. Here, we found that the ROK inhibitor Y27632 reveals the proinvasive activity of PAR-1 through Rac1-dependent and Cdc42-independent signaling pathways (Figure 1b and c). In order to verify this observation, we next established stably transfected cell lines HCT8/S11-DN-ROK and -DC-ROK expressing dominant negative and constitutively activated forms of ROK, respectively (Amano *et al*., 1997). As shown in Figure 1d, both parental and DN-ROK-transfected HCT8/S11 cells were noninvasive under control conditions. Interestingly, the dominant negative form of ROK established the proinvasive activity of PAR-1, but failed to produce the same response following activation of the G-protein-coupled PAF-R. As expected (Rodrigues *et al*., 2001), DN-ROK abrogated the proinvasive activities of pS2 and leptin (Figure 1d), whereas DC-ROK was ineffective. We confirmed that this dominant active mutant was functionally competent in our system since DC-ROK

abrogated insulin-induced invasion. Indeed, activation of the RhoA–ROK axis was shown to negate insulin signaling via association of insulin receptor substrate IRS-1 with ROK (Begum *et al*., 2002). The half-maximal effect of insulin on collagen invasion was observed at the potency EC_{50} of 0.6 nM consistent with activation of insulin receptors in intestinal epithelial cells. Alternatively, insulin may act through additional targets by modulating positively cGMP generation and PI3-kinase pathways (Emami and Perry, 1984; Begum *et al*., 2002) involved in cancer cell invasion (Kotelevets *et al*., 1998). Interestingly, PAR-1 commutation induced by DN-RhoA, ROK inhibition, and DN-ROK (Figure 1a, b, and d) is not associated with significant changes in PAR-1 expression in HCT8/S11 cells incubated with TRAP and under control conditions (Figure 1a and b, top panels).

Thrombin is an endogenous PAR-1, PAR-3, and PAR-4 agonist. PAR-2 is activated by trypsin, tryptase, and coagulation factors VIIa and Xa, but not by thrombin. There is evidence that PAR-2 is involved in colon cancer cell growth through transactivation of EGF-R receptors (Darmoul *et al*., 2003). As previously observed in kidney cancer cells (Nguyen *et al*., 2002), PAR-2 and PAR-4 agonists are ineffective in inducing collagen type I invasion in HCT8/S11 cells incubated with the Rho-GTPase inhibitor C3T and under control conditions (not shown). In contrast, the PAR-1 commutators C3T and Y27632 were effective in determining the invasive phenotype in human colorectal cancer cells HCT8/S11, HT29, and HCT116 (not shown). Moreover, both PAR-2 and -4 activating peptides failed to downregulate invasiveness determined by the trefoil peptide pS2 (not shown). Taken together, our data confirm that invasive growth of human colon cancer cells in collagen type I is not controlled by PAR-2 and -4 agonists (Nguyen *et al*., 2002).

Promotion of PAR-1 proinvasive activity by direct activation of soluble guanylate cyclase

The importance of the cGMP pathways in RhoA inactivation is well established (Sauzeau *et al*., 2000; Sawada *et al*., 2001), while its significance in the neoplastic progression and cancer cell invasion is still obscure. We have shown that PAR-1-mediated invasive potential of human colorectal cancer cells in collagen type I gels can be achieved with the cGMP-elevating agents sodium nitroprusside and guanylin acting as PAR-1 commutators through soluble and membrane-bound guanylate cyclases, respectively (Nguyen *et al*.,

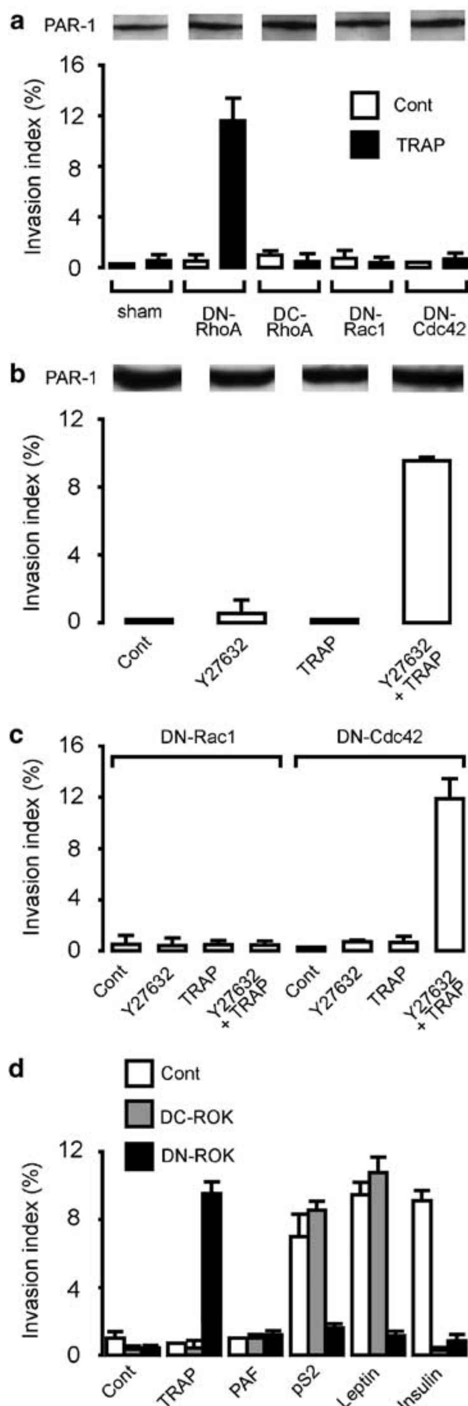

Figure 1 Induction of PAR-1 proinvasive activity by inhibition of the RhoA–ROK axis. (**a**) Invasion of collagen type I gels by HCT8/S11 human colorectal cancer cells stably transfected by expression vectors encoding dominant negative forms of small GTPases (DN-RhoA, DN-Rac1, and DN-Cdc42), a constitutively activated form of RhoA (DC-RhoA), or the control pcDNA3 vector (control sham-transfected cells). Cells were incubated at 37°C for 24 h in the presence or absence of 10 μM PAR-1 agonist TRAP. (**b**) HCT8/S11 cells were treated with 10 μM TRAP and ROK inhibitor Y27632 (10 μM), either alone or combined. Western blot analysis (**a** and **b**, top panels) showed the expression of PAR-1 (90 kDa) in HCT8/S11 cells submitted to the same experimental conditions. (**c**) HCT8S/11 cells expressing the DN-Rac1 and DN-Cdc42 mutants were treated with TRAP and Y27632 (each at 10 μM), either alone or combined. (**d**) Parental HCT8/S11 cells (Cont) and their counterparts stably transfected with either DN-ROK (lacking the RB/PH domain) or DC-ROK (containing exclusively the catalytic CAT domain) were treated with the proinvasive agents TRAP (10 μM), pS2 (100 nM), leptin (100 ng/ml), and insulin (100 nM). PAR-1 and PAF-R responses were compared using 0.1 μM PAF

2002). Here, we report that the direct activator of sGC BAY41 promotes a permissive signal for the proinvasive activity of PAR-1 (Figure 2a). The sGC activator BAY41 at 10 nM–1 μM induced the invasive capacity of HCT8/S11 cells exposed to a fixed concentration of TRAP (10 μM). Similarly, the PAR-1 agonist TRAP dose-dependently (10 nM–10 μM) stimulated collagen type I invasion by HCT8/S11 cells treated with a fixed concentration of BAY41 (1 μM), but was ineffective when tested alone at the same concentrations (not shown). The invasive response induced by TRAP in the presence of BAY41 was blocked by PLCβ and CaM-MLCK inhibitors (U73122 and KT5926), but was insensitive to the ROK inhibitor Y27632, as expected (Figure 2b). Thus, the two PAR-1 commutators BAY-41 and ROK inhibitor Y27632 are not mutually exclusive and instead produce, as expected, additive responses to PAR-1 activation. In the presence of BAY41, PAR-1 operate through PLCβ/CaM-MLCK-dependent proinvasive pathways. As control, we checked that BAY41 dose-dependently (0.1 and 1 μM) abrogated cellular invasion induced by leptin and the trefoil factors pS2 and ITF (not shown), both acting through Rho-dependent signaling pathways (Rodrigues *et al.*, 2001). As shown in Figure 2c, the proinvasive activity determined by PAR-1 and the sGC activator BAY41 was blocked by the DN-Rac1 interfering mutant, but was insensitive to DN-Cdc42.

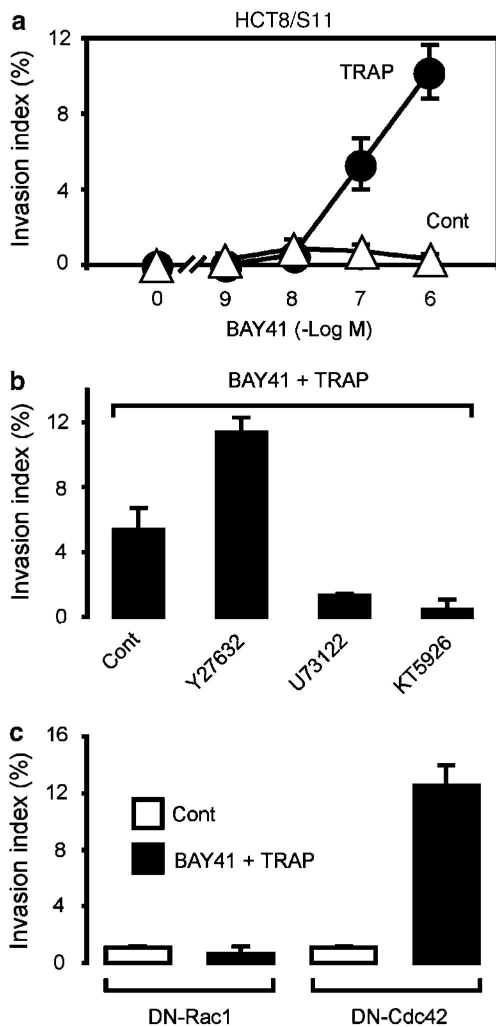

Figure 2 Induction of PAR-1 invasive activity by the sGC activator BAY41. (**a**) Induction of cellular invasion by TRAP (10 μM, filled circles) in HCT8/S11 cells exposed for 24 h to various concentrations of BAY41 (1 nM–1 μM, open triangles). (**b**) Effects of inhibitors targeting ROK (Y27632, 10 μM), PLCβ (U73122, 1 μM), and Ca-MLCK (KT5926, 20 nM) on invasion induced by 10 μM TRAP combined with 1 μM BAY41 (Control: Cont). (**c**) HCT8/S11 cells stably expressing the DN-Rac1 and DN-Cdc42 were treated with TRAP (10 μM) combined with BAY41 (1 μM)

Promotion of PAR-1 proinvasive activity by the matricellular proteins SPARC and TN-C

Interactions between cancer cells, tumor stromal cells, and matricellular molecules participate in the mechanisms of invasive growth in malignant tumors. Invasive cell migration is controlled by a vast array of extracellular factors, which require the coordinated activity of cell adhesion molecules facing ECM components. Adhesion molecules and cell–matrix communications control immediate cellular responses, such as activation of the cytoskeleton, cell shape, and motility, as well as delayed responses, including gene transcription. SPARC or BM-40, a secreted protein, acidic and rich in cysteine, also known as osteonectin, is a 43-kDa ECM glycoprotein involved in cell–ECM interactions during wound healing, tissue remodeling, and cancer progression (Bradshaw and Sage, 2001). Both SPARC and TN-C are ECM components highly expressed in cancers of the gastrointestinal tract and a wide range of human malignant neoplasms. Recently, TN-C was shown to downregulate activated forms of GTP-bound RhoA in fibroblasts and HCT8/S11 epithelial cells (De Wever *et al.*, 2004), suggesting that this matricellular component can function as a PAR-1 signaling commutator (Nguyen *et al.*, 2002). We have therefore investigated the proinvasive potential of PAR-1 in HCT8/S11 cells cultured on collagen type I in association with these ECM components. As expected, both SPARC and TN-C-containing collagen type I matrices promoted the PAR-1 invasive potential (Figure 3a), as demonstrated for TN-C and HGF, another proinvasive agent acting through the Met oncogene (De Wever *et al.*, 2004). Preincubation of HCT8/S11 cells plated on SPARC for 15 min with the PKG inhibitor KT5823 (2 and 20 μM) abrogated invasion promoted by activated PAR-1 ($n = 3$ experiments, data not shown). The matricellular protein SPARC induced a dramatic neutralization of RhoA activity in HCT8/S11 cells plated on top of SPARC-containing matrix, as compared to collagen type I (Figure 3b). Conversely, HCT8/S11 cells cultured on SPARC exhibit a twofold increase in Rac1 activity (from 1 to 2.05 relative intensity), which was not further elevated in the presence of the PAR-1 agonist TRAP (1.96 relative intensity). As shown in Figure 4a, this was associated with decreased cellular adhesion and multiple cellular extensions in HCT8S11 cells cultured on

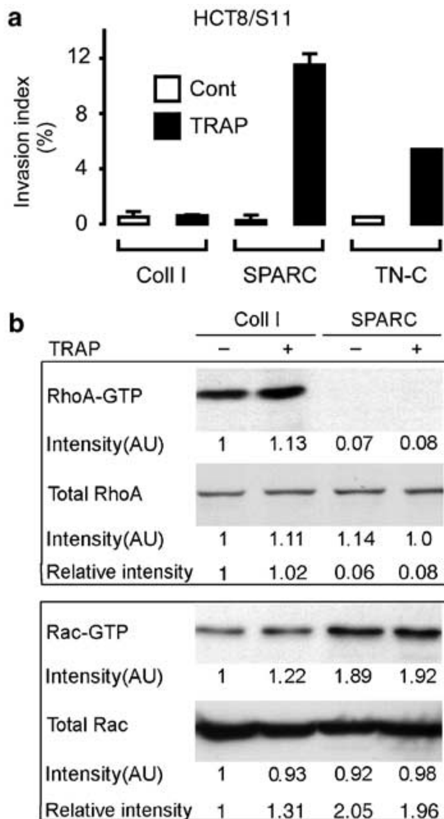

Figure 3 Induction of PAR-1 proinvasive activity by the matricellular proteins SPARC and TN-C. (**a**) Invasive potential of HCT8/S11 cells cultured on collagen type I alone (Coll I), or a mixture of collagen type I containing either SPARC (10 nM) or TN-C (50 pg/ml). (**b**) RhoA and Rac1 activity in cell lysates prepared from HCT8/S11 cells grown in serum-free conditions and seeded on substrates coated with collagen type I (Coll I) or SPARC (10 nM), in the presence or absence of TRAP (10 μM). Activated GTP-bound RhoA and Rac1 were assayed using agarose beads linked to the Rho-binding domain of Rhotekin or the Rac effector p21-activated kinase PAK-1. Relative intensity of the RhoA-GTP and Rac1-GTP immunoreactive bands was quantified, according to total RhoA and Rac1 levels detected by direct Western blots (arbitrary units, AU), using the ImageQuant Software (Amersham Biosciences). Data are representative of 1–4 other experiments

SPARC, as compared to control collagen type I (white arrowheads), and 82% reduction of focal adhesions ($P<0.05$), as monitored by vinculin staining (white arrows in control cells). These results are consistent with previous data showing that SPARC exerts counter-adhesive effects through the disassembly of focal adhesion plaques in endothelial cells (Murphy-Ullrich *et al*., 1995). Most interestingly, we observe in Figure 4b that TRAP-stimulated HCT8/S11 cells entering SPARC-containing collagen type I gels are not blocked by dominant interfering Rac1 and Cdc42 mutants. In this situation, we hypothesize that PAR-1 proinvasive activity is mainly driven by the RhoA status since RhoA inactivation can be achieved through serine phosphorylation and translocation from the plasma membrane to the cytosol. Such an event is induced by the cGMP-dependent protein kinase PKG (Sauzeau *et al*., 2000; Sawada *et al*., 2001).

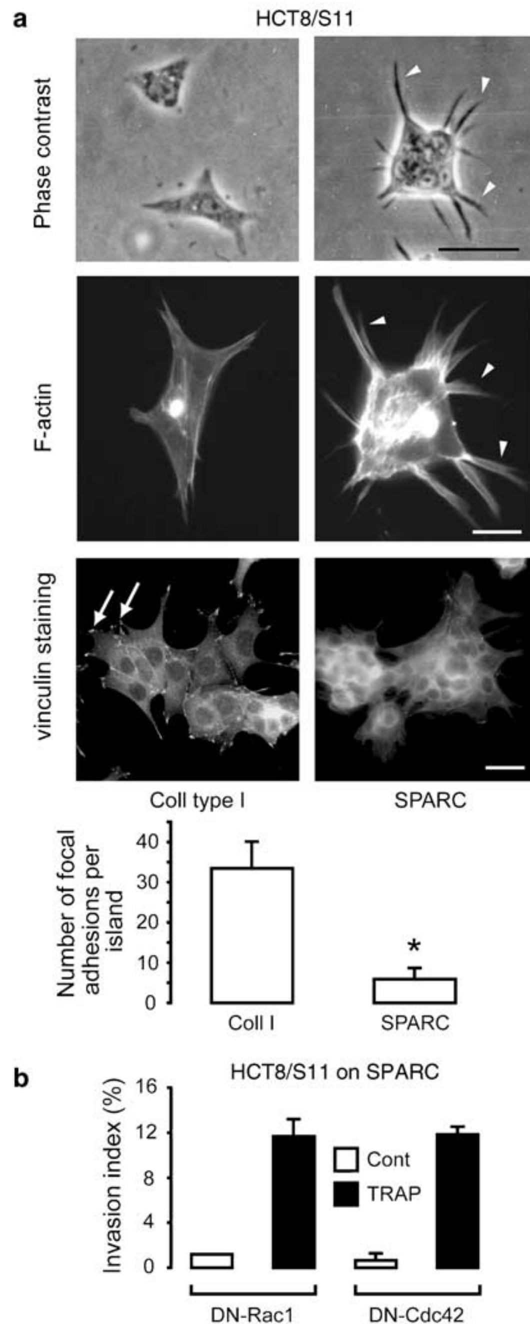

Figure 4 Induction of a RhoA neutralization phenotype, activation of the actin cytoskeleton, and focal adhesion disassembly by the matricellular protein SPARC. (**a**) HCT8/S11 cells were seeded on collagen type I (10 μg/ml) or SPARC (10 nM). After 24 h, adhesive cells were examined by phase-contrast microscopy (bar = 40 μm). Cells adherent to collagen type I showed typical cellular spreading with formation of focal adhesions and stress fibers. SPARC remarkably induced actin belts and formation of cellular extensions (white arrowheads), as evidenced by F-actin staining (white arrowheads) and reduced focal adhesions by 82% ($P<0.05$*) assayed by vinculin staining (white arrows, bar = 20 μm). The number of focal adhesions was measured by a direct counting on the computer screen. Data are means ± s.e.m. from at least 10 different fields. (**b**) HCT8/S11 cells stably expressing DN-Rac1 and DN-Cdc42 were cultured on top of collagen type I gels containing 10 nM SPARC and treated by TRAP (10 μM)

Promotion of PAR-1 proinvasive activity by hypoxia

Low oxygen availability during intratumoral hypoxia is a general feature of growing tumors. Hypoxic environment has been shown to play a major role in tumor angiogenesis, cancer growth, and metastasis. The hypoxia-dependent angiogenic switch in human solid tumors is an early event linked to the secretion of high levels of VEGF and several angiogenic factors by cancer cells, as well as by endothelial cells or other cell types in the tumor stroma (Andre *et al.*, 2000). The NO/cGMP/PKG and calcium-dependent signaling pathways are also involved in the regulation of tumor cell invasiveness and angiogenesis linked to endothelial cell proliferation, morphogenesis, and tumor invasion (Kawasaki *et al.*, 2003). These observations led to the hypothesis that hypoxic conditions could exert a direct or a permissive role on the PAR-1 invasive potential. As shown in Figure 5a, low oxygen levels were inefficient in inducing a spontaneous invasive phenotype. Under hypoxia, the PAR-1 agonist dose-dependently promoted this response, according to the potency $EC_{50} = 0.1\,\mu M$ TRAP. Hypoxic conditions were associated with significant 2.2- and 2.5-fold increase in cGMP levels in HCT8/S11 cells cultured under control conditions (10.6 *versus* 23.6 pmol/10^6 cells, $P<0.05$) or in the presence of the phosphodiesterase inhibitor IBMX (14 *versus* 35.6 pmol/10^6 cells, $P<0.05$), as shown in Figure 5b. This hypoxia-induced cGMP generation was not further elevated in the presence of TRAP (not shown). Hypoxia-mediated PAR-1 activity was abrogated by inhibitors of the Gαq–PLCβ–CaM-MLCK cascade (U73122 and KT5926), but was unaffected by inhibitors of the RhoA–ROK axis, as expected (Figure 6a). Hypoxia induced a remarkable downregulation of RhoA activity in the presence or absence of TRAP, a response associated with increased Rac1 activation (1.6-fold) in the presence of the PAR-1 agonist (Figure 6b). In agreement, invasiveness induced by PAR-1 activation in hypoxic HCT8/S11 cells was not reversed by DN-RhoA and DN-Cdc42, but was impaired by the interfering form of Rac1 (DN-Rac1). Our data are reminiscent of the observation that hypoxia sensitizes cancer cells to HGF proinvasive activity (Pennacchietti *et al.*, 2003).

Since both hypoxia and TN-C/SPARC expression correlate with tumor progression, we next examined the impact of oxygen deficiency on the expression of these two matricellular proteins. Our data in Figure 7a and b provide the first evidence that the SPARC and TN-C genes are expressed in human colorectal cancer cells at both transcriptional and protein levels. While both TN-C and SPARC transcripts were significantly elevated under hypoxia (5.8- and 6.2-fold induction, respectively, $P<0.05$), their protein levels were found downregulated 10-fold ($P<0.05$) in cellular extracts prepared from hypoxic HCT8/S11 cells. Since both TN-C and SPARC proteins were undetectable in the conditioned media and extracellular matrices prepared from normoxic and hypoxic cells (not shown), one can postulate that the permissive action of hypoxia on the PAR-1 proinvasive action is mediated through cGMP-dependent and TN-C/SPARC-independent mechanisms. In line with this, recent studies indicate that both Ras activation and TGFβ are implicated in TN-C secretion and matrix deposition (Maschler *et al.*, 2004). As control, we confirmed that hypoxic conditions were associated with a remarkable 7.8-fold induction of the hypoxia-inducible factor 1α (HIF-1α) subunit (Figure 7b). To our knowledge, there is no information on TN-C and SPARC degradation by the proteasome. Interestingly, we found that the proteasome inhibitor MG132 (100 nM, 2 h preincubation of HCT8/S11 cells before the 24 h invasion assay in the presence of MG132) selectively obliterates cellular invasion determined by leptin or PAR-1 activation in the presence of BAY41 (not shown).

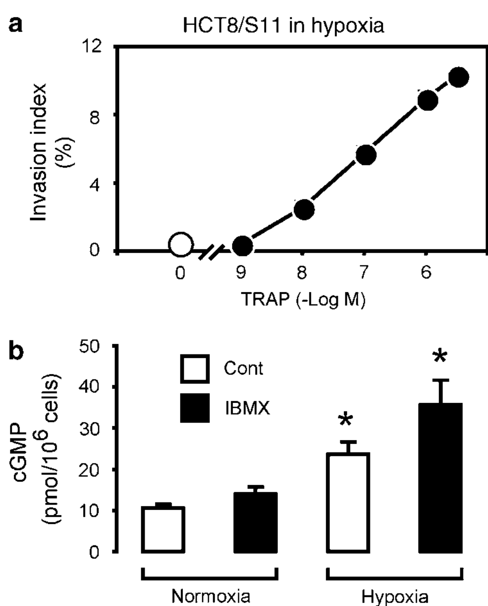

Figure 5 Induction of PAR-1 invasive activity and cGMP generation by hypoxia. (**a**) HCT8/S11 cells were cultured in hypoxic conditions for 24 h in the presence or absence of increasing concentrations of the PAR-1 agonist TRAP (1 nM–30 μM). Invasion assays were performed on type I collagen gels. (**b**) HCT8/S11 cells were cultured in normoxia or hypoxia on collagen type I gels for 24 h, in the presence or absence of the phosphodiesterase inhibitor IBMX (1 mM). cGMP generation was quantified by radioimmunoassay

Implication of the cGMP–PKG pathways in PAR-1 proinvasive activity

Since hypoxic conditions promote the proinvasive activity of PAR-1 and significant elevation of cGMP levels in HCT8/S11 cells (Figure 5), we next examined the possible implication of PKG in this commutation. As observed above for the PAR-1 commutator SPARC, 15 min preincubation of HCT8/S11 cells with the PKG inhibitor KT5823 (2 and 20 μM) abrogated the PAR-1 proinvasive activity determined by subsequent treatment with TRAP in hypoxia (Figure 8a). This blockade was not observed when KT5823 and TRAP were added simultaneously in hypoxic conditions (not shown).

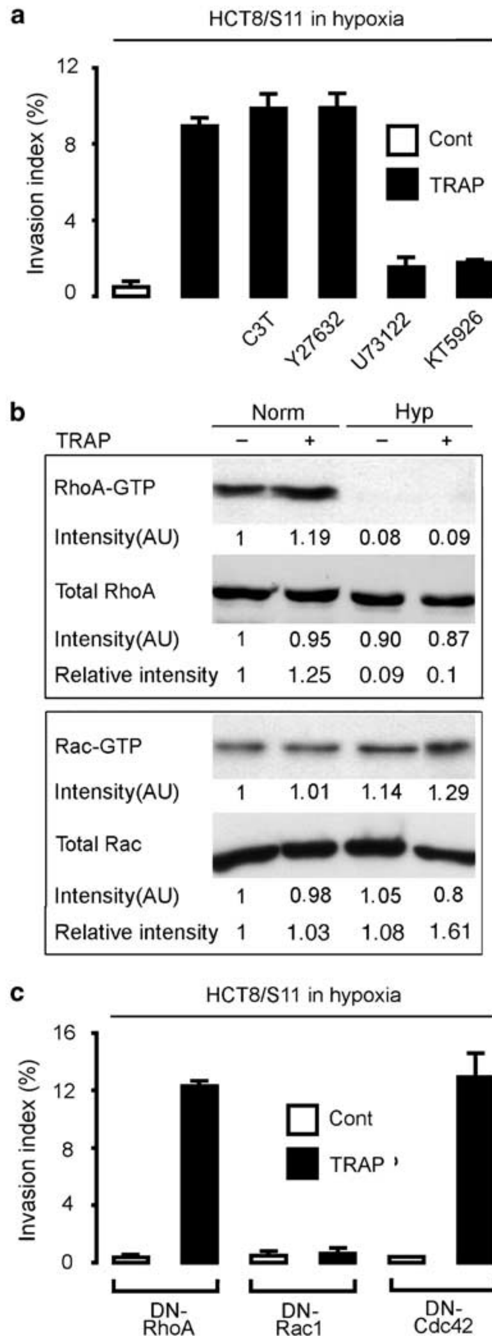

Figure 6 Induction of PAR-1 proinvasive activity by hypoxia is mediated by the $G\alpha q$/PLCβ/Ca-MLCK cascade and small GTPase Rac1. (**a**) The invasive phenotype of HCT8/S11 cells in collagen gels was induced by hypoxic conditions. Cells were incubated for 24 h in control conditions (Cont: no addition) or in the presence of TRAP (10 μM), either alone or combined with selective inhibitors of the RhoA/ROK cascade (C3T, 5 μg/ml; Y27632, 10 μM), PLCβ (U73122, 1 μM), and Ca-MLCK (KT5926, 20 nM). (**b**) Activity of RhoA and Rac1 in HCT8/S11 cells cultured under normoxic and hypoxic conditions in the presence or absence of TRAP. Data are representative of another experiment. (**c**) HCT8/S11 cells expressing DN-RhoA, DN-Rac1, and DN-Cdc42 were assayed for their invasive capacity in collagen type I under hypoxia, in the presence or absence (Cont: no addition) of TRAP (10 μM)

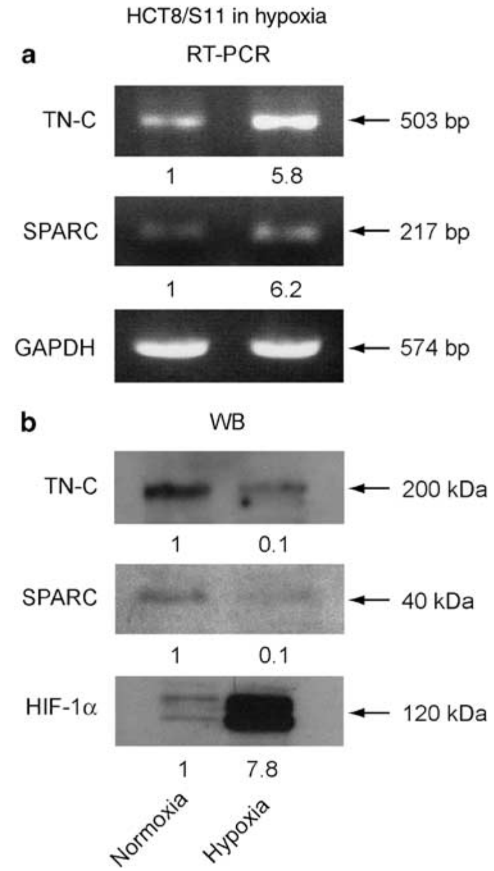

Figure 7 Impact of hypoxic conditions on TN-C and SPARC expression. Identification of the TN-C and SPARC gene transcripts (**a**) and proteins (**b**) in HCT8/S11 cultured for 24 h at 37°C on top of collagen type I gels under normoxic or hypoxic conditions. RT–PCR reactions amplified the expected 503 and 217 bp products corresponding to the TN-C and SPARC amplimers, respectively. GAPDH cDNA was amplified as a loading control (574 bp). Western blot analysis of TN-C and SPARC in HCT8/S11 cells using 100 μg protein in each lane and 12% SDS–PAGE. Immunodetection was performed using the anti-TN-C mAb BC8 (v/v dilution of the hybridoma supernatant) and the anti-SPARC pAb (1 : 1000). Membranes were stripped and reprobed with the anti-HIF-1α mAb (1 : 250) as control for hypoxia. Two major bands of HIF-1α were detected in normoxic conditions and were remarkably induced by 24 h hypoxia. These slower and faster migrated bands are phosphorylated and dephosphorylated forms of HIF-1α, respectively. Data are representative of two other separate experiments

Notably, cellular invasion induced by PAR-1 activation in the presence of the sGC/cGMP activator BAY41 in normoxia was blocked by simultaneous addition of KT5823 (2 and 20 μM), as shown in Figure 8b. To further demonstrate the direct implication of the cGMP/PKG cascade in the PAR-1 proinvasive activity, we confirmed that 8-Br-cGMP (5 μM) behaved as a selective PAR-1 commutator (Figure 8c): (1) this cell-permeant cGMP analog was ineffective in promoting the proinvasive activity of PAF and Met receptors (Kotelevets *et al*., 1998; De Wever *et al*., 2004) and (2) the invasive response determined by 8-Br-cGMP and PAR-1 activation was blocked by 15 min preincubation of HCT8/S11 cells with the PKG inhibitor (data not shown, $n = 3$ experiments).

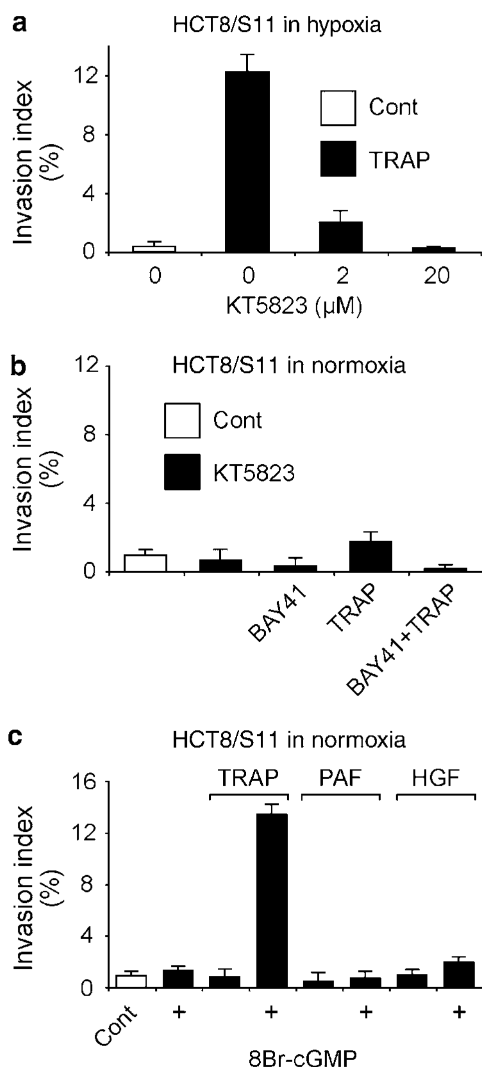

Figure 8 Impact of cGMP and PKG inhibition on PAR-1 proinvasive activity in hypoxia and normoxia. **(a)** HCT8/S11 cells in normoxia were preincubated for 15 min in the presence or absence of the PKG inhibitor KT5823 (2 and 20 μM), and then exposed to hypoxia and TRAP (10 μM), or control vehicle. **(b)** HCT8/S11 cells in normoxia were treated for 24 h with the cGC activator BAY41 (1 μM) and TRAP (10 μM), either alone or in combination. **(c)** HCT8/S11 cells in normoxia were treated for 24 h with either TRAP (10 μM), PAF (0.1 μM), or the Met activator HGF (10 U/ml), alone or combined with 8Br-cGMP (5 μM). Collagen type I gel invasion was then measured using our standard assay in these respective conditions, in comparison with control experiments performed without additions in normoxic and hypoxic conditions (Control: Cont)

We found that the PAR-1 proinvasive activity promoted by the sGC activator BAY41 is Rac dependent and Cdc42 independent (Figure 2c), whereas an inverse situation was observed for the cGMP analog 8Br-cGMP (Rac independent/Cdc42 dependent, not shown). This difference can be attributed to the possibility that sGC and PKG are not the only BAY41 and cGMP signaling targets. Alternatively, cGMP levels and PKG enzymes type I (a/b isoforms) and type II might be differentially induced, activated, and phosphorylated by these two cGMP-inducing agents in terms of duration, extent, and cellular localization, as suggested by the differential impact of the PKG inhibitor KT5823 in preincubation and simultaneous addition experiments (Figure 8a–c).

Thrombin activation and PAR-1 expression in peritoneal and pleural effusions and their derived cancer cells in culture

Activation of the coagulation system is an invariable problem for patients with colorectal carcinoma and other solid tumors, as previously described by Pr. Armand Trousseau. As fibrinolysis is mediated by plasmin, which degrades fibrin clots into D-dimers and fibrin degradation products, the detection of D-dimers is frequently used to verify the activation of fibrinolysis. Since thrombin cannot be measured directly, prothrombin fragment 1 + 2, which is released from prothrombin during thrombin activation, can be used as an indirect marker for thrombin activation. We observed that thrombin is already activated in the serum of the majority of cancer patients, as shown by the detection of higher prothrombin 1 + 2 levels (1.70 ± 1.01 nmol/l), as compared to 0.4 ± 1.1 nmol/l in serum from normal volunteers. Interestingly, prothrombin 1 + 2 levels were remarkably increased to 9.80 ± 5.2 nmol/l ($P < 0.0001$) in malignant effusions of the same patients. Interestingly, malignant effusions from gastric, colorectal, and esophageal cancer patients (respectively, samples 523–539–591; dilution range of the initial fluid, 1:9–1:15) induced the invasive response in HCT8/S11 cells (invasion index range: 12–20%, $n = 3$ experiments).

As shown in Table 1, PAR-1 expression was positive by RT–PCR and FACS analysis in cultured cancer cells isolated from 15 out of 22 malignant effusions (68%) from patients with gastrointestinal or lung cancers, melanoma, or lymphoma. In four peritoneal effusions from colorectal cancer patients, cultured cancer cells were 100% positive by RT–PCR analysis and 75% positive by FACS analysis.

Discussion

It is now well recognized that cancer cells and the tumor stroma form a 'new organ' that promotes invasive tumor growth and metastasis through complex interactions between cancer cells with cellular and matricellular components of the tumor, as well as several other effectors linked to immune and blood cells, hypoxia, and the angiogenic switch (Porte *et al.*, 1995; Bissell and Radisky, 2001; Liotta and Kohn, 2001). The matricellular proteins TN-C and SPARC/BM-40/osteonectin are induced during wound healing, angiogenesis, and inflammatory and neoplastic diseases. Tumor matricellular components such as TN-C (De Wever *et al.*, 2004) and SPARC in the present study suppress RhoA activity, cellular spreading, and focal adhesions, and induce PAR-1-mediated invasive migration of cancer cells. Consistently, high levels of TN-C and SPARC in

Table 1 Expression of PAR-1 by RT–PCR and FACS analyses in cultured cancer cells isolated from peritoneal, pleural, and pericardial effusions from patients bearing colorectal tumors or other malignancies

Diagnosis	*Effusion sites*	*PAR-1 expression*		*Patient no.*
		RT–PCR	*FACS*	
Gastrointestinal cancers				
Colorectal carcinoma	Peritoneum	+	+	539
Colorectal carcinoma	Peritoneum	+	–	546
Colorectal carcinoma	Peritoneum	+	+	534
Colorectal carcinoma	Peritoneum	+	+	568
Gastric carcinoma	Peritoneum	+	+	523
Pancreatic carcinoma	Peritoneum	+	+	496
Pancreatic carcinoma	Peritoneum	+	+	467
Lung and pleural cancers				
Lung cancer (NSCLC)	Pleura	–	–	550
Lung cancer (NSCLC)	Pleura	–	+	499
Lung cancer (NSCLC)	Pleura	+	+	524
Lung cancer (NSCLC)	Pleura	+	+	459
Lung cancer (NSCLC)	Pleura	+	+	535
Lung cancer (NSCLC)	Pleura	+	+	552
Lung cancer (SCLC)	Pleura	+	–	551
Lung cancer (SCLC)	Pleura	+	+	506
Lung cancer (SCLC)	Pericardium	–	+	556
Pleural mesothelioma	Pleura	+	–	548
Pleural mesothelioma	Pleura	+	+	527
Pleural mesothelioma	Pleura	+	+	558
Miscellaneous cancers				
Mammary carcinoma	Peritoneum	+	+	545
Melanoma	Pleura	–	–	536
Non-Hodgkin's lymphoma	Pleura	+	+	533

PAR-1 mRNA and cell surface expression was determined by RT–PCR and FACS analysis. PAR-1 RT–PCR has been considered as positive when amplicons are clearly detected after 30 amplification cycles. At least 10 000 cells were examined by FACS analysis using anti-PAR-1 mouse mAb (clone WEDE15, Immunotec/Coulter, Marseille, France). + and – indicate positive and negative expression signals

many solid tumors correlate with the higher incidence of metastases and a poor prognosis (Porte *et al*., 1995; Ledda *et al*., 1997; Thomas *et al*., 2000; Maschler *et al*., 2004). We also identified a complex signaling interplay between the cGMP/PKG cascade, the RhoA–ROK axis, and hypoxia in the determination of the proinvasive activity of PAR-1 through heterotrimeric G-protein signaling. In support of this model, recent studies demonstrated that NO and cGMP pathways inactivate the RhoA–ROK axis (Sandu *et al*., 2001) involved in Rac1 inhibition, suggesting that upstream elements using this cascade function as potential Rac1 activators. The proinvasive small GTPase Rac is activated by PKG (Hou *et al*., 2004) and G-protein coupled receptors (GPCR) using Gαq subunits, such as PAR-1 (Booden *et al*., 2002). Small GTPase RhoA is involved in cell migration via its direct effector ROK, which promotes MLC phosphorylation, through MLC phosphatase inhibition and direct MLC phosphorylation (Kaibuchi *et al*., 1999). MLC phosphorylation is also induced by PAR-1, and the MAPK- and calcium-activated MLCK cascade (Hansen *et al*., 2000; Nguyen *et al*., 2002). Reducing RhoA activity has two opposing effects: it promotes migration by lowering adhesion and decreases cell migration by inhibiting cell body contraction. Consistently, the DN-ROK interfering mutant in the present study abrogated several forms of invasion induced by pS2, leptin, and insulin. This picture further illustrates our model of the reciprocal RhoA–Rac1 crosstalk and antagonism as a molecular sensor for PAR-1 commutation in cancer cell invasion. Integration of these counteractive mechanisms can originate from actin-associated molecular scaffolds containing guanine nucleotide-exchange factors (GEF), as shown for Trio and juxtaposition of GEF for RhoA/Rac1 small GTPases at cell migration (Bellanger *et al*., 2000).

We have observed that calcium translocations induced by TRAP are preserved in HCT8/S11 cells exposed to the Rho–ROK inhibitors C3T, BAY41, and Y27632 (not shown), suggesting that these PAR-1 commutators are acting downstream the formation and release of functional heterotrimeric G-proteins complex at activated PAR-1 and Gα subunits. In this area, Rho-GTPases play pleiotropic roles in cell movements, polarity, vesicle trafficking, and EGF-R processing and degradation, as recently demonstrated for Cdc42 (Cerione, 2004). GPCR controlled by thrombin, bombesin, neurotensin, endothelin, and LPA are also implicated in metalloprotease-mediated EGF-R transactivation (Darmoul *et al*., 2004; Schafer *et al*., 2004; Zhao *et al*., 2004), a major signaling platform in invasive growth (Rodrigues *et al*., 2003). More recently, the fibroblast-derived matrix metalloprotease MMP-1 in the stromal-tumor microenvironment has been designed as a new PAR-1 activator that promotes invasion and tumorigenesis of breast cancer cells (Boire *et al*., 2005). Moreover, MMP-1 is a new breast cancer predictive marker to identify patients with lesions that may develop into cancer (Poola *et al*., 2005). There are some early clues suggesting that the RhoA–ROK axis is also connected with JNK- and c-Jun/AP-1-dependent transcription (Marinissen *et al*., 2004) involved in several proinvasive pathways, including src and Wnt (Rivat *et al*., 2003; Le Floch *et al*., 2005). In the present study, we have clearly shown that several PAR-1 commutators are acting as invasion promoters through the cGMP/PKG cascade and inhibition of the RhoA/ROK axis. This finding is reminiscent of the ability of macrocyclic alkaloid analogs to exert tumor invasion inhibition through persistent Rho activation (McHardy *et al*., 2004). Further studies on PAR-1 signaling targets are, therefore, needed to delineate the relative contribution of these integrative levels on invasion and metastasis by real-time kinetics of the activation status of signaling elements involved in cellular invasion and survival, including proteomic screens and gene expression profiling.

Understanding the pathogenesis of cancer invasion and metastasis at cellular and molecular levels constitutes a major challenge for the development of efficient therapeutic strategies against neoplastic progression, recurrence, and death. We have observed that PAR-1 are expressed in metastatic cancer cells isolated from

peritoneal and pleural effusions in patients with colon cancer and other carcinomas. In the majority of cancer patients, we found that coagulation and thrombin activation markers such as d-dimers or prothrombin fragment 1+2 are remarkably elevated in serum. Moreover, up to 30% of patients developed manifest deep vein thrombosis during the natural course of their disease, according to the interconnection between cancer and coagulation disorders (Xie *et al*., 2005) referred to as the Trousseau disorder in 1865. Accordingly, we found high levels of activated thrombin as well as fibrin, a product of fibrinogen degradation by thrombin, in pleural and peritoneal effusions from colorectal cancer patients and other malignancies. Overall, we propose that thrombin and PAR-1 might participate, at least in part, in metastasis of human solid tumors in peritoneal and pleural cavities. In agreement, HCT8/S11 cell invasiveness was induced by pleural and peritoneal effusions from cancer patients with digestive tumors. Thus, the thrombin/PAR-1 connection in the present study may play additional roles with the HGF/Met connection to cyclooxygenase and prostanoid formation (Boccaccio *et al*., 2005) in cancer-associated metastasis and thrombosis. It is anticipated that the design of new therapeutic strategies targeting invasive tumor growth should be defined in view of the diversity and redundancy of the proinvasive networks connected with PAR-1 signaling in the tumor stroma and regarding the matricellular and oncogenic context of a given tumor in cancer patients.

Materials and methods

Cell culture, expression vectors, and stable transfections

The human colon cancer cells HCT8/S11 were maintained in RPMI 1640 (Gibco BRL, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FBS, PAA Laboratories GmbH, Pasching, Austria) plus L-glutamine and antibiotics (Gibco BRL). Human colon cancer cell lines HT29 and HCT116 were cultured in DMEM (Life Technologies Inc., Cergy Pontoise, France) supplemented with 10% FBS and antibiotics. The cDNAs encoding the myc-tagged dominant active (CAT, i.e. DC-ROK) or dominant negative ROK (DN-ROK) lacking the PH and Rho-binding domains (ΔRB/PH) were previously described (Kaibuchi *et al*., 1999). The vectors pcDNA3 encoding the HA-tagged dominant negative RhoA (T19N, DN-RhoA), dominant negative Rac1 (T17N, DN-Rac1), dominant negative Cdc42 (T17N, DN-Cdc42), and constitutive active RhoA (G14V, DC-RhoA) were from The Guthrie cDNA Resource Center (Sayre, PA 18840, USA). HCT8/S11 cells were stably transfected according to described methods (Nguyen *et al*., 2002). MDCKT23 cells expressing mutant small GTPases RhoAV14, RhoAN19, or Rac1V12 were a generous gift of Dr WJ Nelson (Jou and Nelson, 1998).

Thrombin activation in peritoneal and pleural effusions from colon cancer patients and their derived cancer cells in culture

From November 2002 to November 2004, we collected 58 samples of effusions from patients admitted to the Oncology Center (Kiel University Hospital, Germany). Cancer cells were harvested from eight peritoneal, 13 pleural, and a pericardial effusion sites. The mean age was 63±9.1 years (range: 53–87 years). Cancer cells derived from these malignant effusions were established in long-term culture (6–26 months), using RPMI 1640 medium containing 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% cell-free original effusion fluid. Repetitive cytopathological examinations included cell morphology by phase-contrast microscopy, determination of mitotic rates and epithelial markers (e.g. human epithelial EpCAM-antigen BerEP4), absence of contaminating lymphocytes and mesothelial cells by immunocytochemistry (e.g. leukocyte common antigen LCA and thrombomodulin, respectively), using specific antibodies from Dako (Hamburg, Germany). Quantitative determination of prothrombin fragment 1+2 in the serum and malignant effusions was performed by ELISA (Behring GmbH, Germany).

Collagen type I invasion assays and hypoxia

Collagen type I invasion by cancer cells was monitored as previously described (Bracke *et al*., 2000). The invasion index is the percentage of cells invading the collagen gel over the total number of cells. None of the compounds tested in the 24 h assay interfered with cell growth and viability. Where indicated, HCT8/S11 cell cultures were placed for 24 h at 37°C in a sealed incubator chamber device (OXOID Ltd, Basingtoke, Hampshire, England) containing an AnaeroGen bag lowering oxygen levels below 1% within 30 min.

Western blot and immunofluorescence analyses

For immunoblotting, cultured cells were homogenized at 4°C in lysis buffer containing phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), aprotinin, pepstatin A, and leupeptin as protease inhibitors, as previously described (Nguyen *et al*., 2002). The blots were probed with one of the following antibodies: anti-HA monoclonal (mAb) clone 12CA5 (1 : 100; Roche Laboratories, Meylan, France), anti-myc mAb 9E10 (1 : 500; Santa Cruz Biotechnology, CA, USA), mAb anti-thrombin receptor (1 : 4000; clone IIaR-A) labeling the 90 kDa PAR-1 receptor (Biodesign International, Kennebunk, ME, USA), anti-TN-C supernatant clone BC8 (v/v) kindly provided by Dr L Zardi (Laboratory of Cell Biology, Genoa, Italy), anti-SPARC goat antibody (1 : 1000; R&D Systems Europe Ltd, Lille, France), and anti-HIF-1α mAb (1 : 250; Becton Dickinson France SA, Le Pont de Claix, France). Membranes were probed for 1 h with peroxidase-linked goat anti-mouse IgGs (1 : 2000; Santa Cruz Biotechnology, CA, USA) and revealed by enhanced chemiluminescence (ECL plus, Amersham Pharmacia Biotech). For F-actin and vinculin staining, HCT8/S11 cells were seeded under serum-free conditions on coverslips coated with collagen type I (10 μg/ml) or SPARC (10^{-8} M). Cells were then incubated for 1 h with phalloidin-FITC or the anti-vinculin mAb clone hVIN-1 (1 : 400; Sigma), and incubated for 1 h with a secondary antibody (Alexa Fluor 488 anti-mouse, 1 : 400; Molecular Probes, Eugene, USA). Samples were analysed with a Leica DMR fluorescence microscope (Leica Microsystèmes SA, Reuil-Malmaison, France) and a Nikon Eclipse TE300 inverted phase-contrast microscope (Micromécanique, Evry, France). For the detection of cell surface PAR-1 in malignant effusions-derived cancer cells, a FACS scan analysis was performed. About 5×10^5 cells were washed twice in ice-cold PBS containing 0.05% sodium azide in PBS, and incubated for 1 h at 37°C with anti-PAR-1 mouse mAb, clone WEDE15 (2 μg, i.e. 1 : 100; Immunotec Coulter, Marseille, France). Cells were incubated for 1 h at 37°C with anti-mouse

FITC-conjugated secondary antibody (1 : 1000, Dako), and resuspended in 1 ml PBS for FACS analysis. Cells incubated without primary antibody followed by labeling with secondary antibody were used as controls. Flow cytometric analysis was carried out using a 'Galaxy Argon Plus' and the results were analysed with 'Flomax Software' (Dako). At least 10 000 cells were examined for each determination, and expression $<10\%$ has been considered as negative.

GTP-bound RhoA and Rac1 pull-down assays

HCT8/S11 cells were washed twice with ice-cold PBS and lysed in Mg^{2+} lysis/wash buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, and 10 μg/ml leupeptin or aprotinin). Cell lysates were cleared by centrifugation (14 000 *g* , 5 min, 4°C), and equal volumes of lysates were incubated for 45 min at 4°C with either Rhotekin RBD-agarose beads (Upstate Biotechnology, Campro Scientific) or PAK-1-agarose beads (25 μg). The beads were washed four times with Mg^{2+} lysis/wash buffer. GTP-bound and total levels of RhoA and Rac1 proteins were detected by immunoblotting (15% SDS–PAGE), using a mAb against RhoA (Santa Cruz Biotechnology) or Rac1 (Transduction Laboratories, Lexington, KY, USA). The relative intensity was determined with the ImageQuant software (Amersham Biosciences).

cGMP radioimmunoassay and intracellular free Ca^{2+} concentration

HCT8/S11 cells were seeded in 35 mm Petri dishes (200 000 cells per dish) and incubated overnight with the indicated effectors. cGMP production was measured by radioimmunoassay (Amersham Biosciences). Changes in HCT8/S11 intracellular Ca^{2+} concentrations in response to TRAP were monitored using the QuantiCell 700 dynamic imaging microscopy system (Visitech International Ltd, UK), as described (Ricort *et al*., 2002). The cells were cultured overnight on glass coverslips in a six-well plate (200 000 cells per well), in the presence or absence of C3T, BAY41, or Y27632. Then, control and treated cells were washed twice and incubated for 2 h at 37°C in PBS-calcium-free HEPES medium containing 5 μM FURA2/AM (Molecular Probes Inc., Eugene, USA). After background recording for 40 s (20 images), the experiment was initiated by adding TRAP (20 μM).

RNA isolation and RT–PCR amplification

Total RNA was extracted using the Trizol reagent (Invitrogen) or the 'Qiagen RNeasy Mini Kit' (Qiagen). RT–PCR analyses were performed with the SuperScript One-Step RT–PCR kit (Invitrogen). Fragments of the human TN-C and SPARC cDNAs were amplified using the following primers: TN-C: 5′-CCC TGC AGT GAG GAG CAC GGC ACA-3′ and 5′-TGC CCA TTG ACA CAG CGG CCA TGG-3′; SPARC: 5′-AAG ATC CAT GAG AAT GAG AAG-3′ and 5′-AAA AGC GGG TGG TGC AAT-3′. The amplified amplicons (503 and 217 bp, respectively) were resolved in 1.5% agarose gel stained with ethidium bromide. For PAR-1 gene expression, the following PCR primers and conditions were used: F, 5′-GTGC TGTTTGTGTCTGTGCT-3′; R, 5′-CCTCTGTGGTGGAA GTGTGA-3′ (30 cycles, annealing temperature 55°C, 598 bp product); nested F, 5′-GGGCTTCCTTCACTTGTCT-3′; R, 5′-ACTTCTTGCTGCGGTTGG-3′ (30 cycles, 54°C, 273 bp); and β-actin F, 5′-ATCTGGCACCACACCTTCTACAAT GAGCTGCG3′, R, 5′CGTCATACTCCTGCTTGCTGATC CACATCTGC-3′ (16 cycles, 58°C, 838 bp product). Amplifications were performed using a Perkin Elmer Cycler (Applied Biosystem 2400) and 30 μl reaction mixtures containing 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.5 μM each primer, and 1.5 U of *Taq* DNA polymerase (Invitrogen). After an initial denaturation step (5 min at 94°C), thermal cycling was performed for 30 cycles (1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min synthesis at 72°C), followed by a 7 min final extension step. A 1 μl portion of the first-round PCR product was reamplified in the nested PCR at the following profiles: 94°C for 30 s, 54°C for 30 s, 72°C for 1 min and 30 cycles. PCR products were electrophoresed on agarose gels, stained with 5 μg/ml ethidium bromide, and visualized under UV light.

Peptides and chemicals

The thrombin PAR-1 receptor activating peptide TRAP (hexapeptide SFLLRN), PAR-2 and PAR-4 agonists (SLIGRL and GYPGQV, respectively), platelet activating factor (PAF), and insulin were purchased from Bachem Biochimie (Voisins-le-Bretonneux, France), Neosystem (Strasbourg, France), and Sigma-Aldrich (Saint-Quentin Fallavier, France), respectively. The direct activator of sGC BAY41-2272 (abbreviated as BAY41 in the present study), the ROK inhibitor Y27632, and the *Clostridium botulinum* exoenzyme C3 transferase (abbreviated as C3T), which ADP-ribosylates and inactivates the small GTPases RhoA, B, and C, were generous gifts from Dr J-P Stasch (Wuppertal, Germany), Yoshitomi Pharmaceutical Industries Ltd (Osaka, Japan), and Dr Gilles Flatau (INSERM U627, Nice, France), respectively. TN-C and SPARC were from Chemicon International Inc. (Temecula, CA, USA) and Calbiochem, respectively. Inhibitors targeting CaM-MLCK, PLCβ, and proteasome (KT5926, U73122, and MG132) were from Calbiochem (Meudon, France); 8-bromo-cGMP and the PKG inhibitor KT5823 were from BIOMOL Res. Labs. (Tebu- Bio, France); leptin was from R&D Systems Europe Ltd; and recombinant hpS2 (TFF1) produced in *Escherichia coli* was generously provided by Dr B Westley (University of Newcastle upon Tyne, UK).

Statistical analyses

Significance between experimental values was assessed by the unpaired Student's *t*-test at *P*-values <0.05. Data are means $\pm$ s.e.m. from three to four experiments.

Abbreviations
ECM, extracellular matrix; HIF-1α, hypoxia-inducible factor 1α; PAR-1, proteinase-activated receptors type I; PKG, protein kinase G; ROK, Rho-associated coiled-coil-containing protein kinase; TN-C, tenascin-C; SPARC, secreted protein acidic rich in cysteine.

Acknowledgements
This work was aided by INSERM, IPSEN (PhD grant to Q-DN), and the FORTIS Verzekeringen (Brussels, Belgium). We thank Dr Rudolph for the characterization of malignant effusions-derived cancer cells, Miss C Boissard and Mr M Clark for valuable technical assistance, and Dr K Kaibuchi, Dr L Zardi, Dr G Flatau, Dr WJ Nelson, Dr J-P Stasch, Dr B Westley, and Yoshitomi Pharmaceutical Industries Ltd (Osaka, Japan) for sharing materials.

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