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# Proteinase-Activated Receptors 1 and 2 Regulate Invasive Behavior of Human Melanoma Cells via Activation of Protein Kinase D1

**Cordula Kempkes**[1,2,8], **Anke Rattenholl**[1,3,8], **Jörg Buddenkotte**[1], **Elwira Strozyk**[4,5], **Jürgen Eberle**[6], **Angelika Hausser**[7], **Ferda Cevikbas**[2], **Stefan W. Schneider**[4], and **Martin Steinhoff**[2]

[1]Department of Dermatology and IZKF Münster, University of Münster, Münster, Germany [2]Departments of Dermatology and Surgery, University of California, San Francisco, San Francisco, California, USA [3]Department of Product Engineering, Applied Biotechnology Division, University of Applied Sciences Bielefeld, Bielefeld, Germany [4]Department of Dermatology, Venereology and Allergology, Institute of Experimental Dermatology, Medical Center Mannheim, University of Heidelberg, Mannheim, Germany [5]Department of Cancer Immunology, University of Medical Sciences at Great Poland Cancer Center, Poznan, Poland [6]Department of Dermatology and Allergy Skin Cancer Center, Charité Berlin, Berlin, Germany [7]Department of Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany

## Abstract

Recent studies have indicated an important role of proteinases and proteinase-activated receptors (PARs) in tumorigenesis. Although a role for PARs has been described in various skin tumors including melanoma, the underlying cellular mechanisms have not been understood. Recent studies have suggested $PAR_1$ as a regulator of melanoma cell growth and metastasis by affecting angiogenic and invasive factors. Moreover, changes in the expression patterns of $PAR_1$ and $PAR_2$ correlate with skin cancer progression, and $PAR_1$ is overexpressed in melanoma. Therefore, we sought to elucidate the putative role of $PAR_1$- and $PAR_2$ -mediated signal transduction pathways during melanoma progression. Activation of both $PAR_1$ and $PAR_2$ led to rapid phosphorylation of protein kinase D1 (PKD1) in cultured WM9 melanoma cells. PKD1 is known to be involved in cell migration, integrin regulation, and intracellular vesicle transport. Downregulation of PKD1 by siRNA resulted in diminished proliferation, decreased αvβ3 integrin regulation, and secretion of pro-angiogenic chemokine IL-8 in WM9 cells. In conclusion, our results show that $PAR_1$ and $PAR_2$ are involved in WM9 cell proliferation and secretion of IL-8 by activation of PKD1. Inactivation of the PKD1 pathway may be beneficial for the inhibition of PAR-induced melanoma proliferation and for maintenance of the inflammatory tumor environment.

## INTRODUCTION

Proteinase-activated receptors (PARs) are G-protein-coupled receptors with seven transmembrane domains that are stimulated by a unique activation mechanism. The extracellular N-terminus is specifically cleaved by certain serine proteinases (e.g., thrombin,



Correspondence: Martin Steinhoff, Departments of Dermatology and Surgery, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, California 94143, USA. SteinhoffM@derm.ucsf.edu.
[8]These authors contributed equally to this work.

**CONFLICT OF INTEREST**
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trypsin, tryptase, cathepsin G), exposing a formerly cryptic peptide sequence of six amino acids. Subsequently, this “tethered ligand” can bind to the second extracellular loop of PARs, leading to the induction of specific intracellular cell signaling events, which can also be mimicked by stimulating PARs with synthetic peptides of the tethered ligand sequence. PARs are expressed in numerous tissues that exert several biological effects, including cell proliferation, inflammation, and blood coagulation. In addition, both $PAR_1$ and $PAR_2$ expressions are abundantly upregulated in tumors of various tissues (Tellez and Bar-Eli, 2003; Steinhoff *et al.*, 2005; Melnikova *et al.*, 2008). Semiquantitative RT-PCR revealed that $PAR_1$, but not $PAR_2$, is expressed by primary melanocytes of both newborn and adult origin. Nevertheless, $PAR_2$ on keratinocytes is essential for melanosome transfer between melanocytes and keratinocytes (Seiberg *et al.*, 2000).

The incidence of malignant melanoma is still increasing and causes most deaths related to skin cancer (Bowden, 2004; Larson *et al.*, 2009). $PAR_1$ was reported to be significantly overexpressed in malignant melanomas (Tellez *et al.*, 2003; Melnikova *et al.*, 2008; Tellez and Bar-Eli, 2003; Villares *et al.*, 2009) and $PAR_2$ immunoreactivity was found to be upregulated both in nevi and malignant melanomas (Massi *et al.*, 2005). Thrombin-induced activation of $PAR_1$ on murine melanoma cells led to increased tumor growth and enhanced pulmonary metastasis *in vivo* (Nierodzik *et al.*, 1992; Nierodzik *et al.*, 1998). *In vitro* overexpression of $PAR_1$ in a nonmetastatic melanoma cell line (SB2) led to increased adhesion to extracellular matrix molecules and also modulated cytoskeletal reorganization (Nierodzik *et al.*, 1998). Furthermore, activation of $PAR_1$ induced the recruitment of $\alpha v\beta 5$ integrin to focal contact sites (Hazarika *et al.*, 2004).

$PAR_1$ expression is controlled by the transcription factor activator protein-2α, and loss of activator protein-2α causes overexpression of $PAR_1$, which correlates with increased metastatic potential (Tellez *et al.*, 2003). Accordingly, thrombin-induced activation of $PAR_1$ on M24met melanoma cells enhanced their migration and metastasis. Interestingly, thrombin coactivated $PAR_2$ in these cells by an indirect cleavage-independent mechanism, indicating that $PAR_2$ may have an additional, possibly collaborative role in melanoma metastasis. In fact, treatment of melanoma cells with a selective $PAR_2$ agonist also enhanced experimentally induced metastasis (Even-Ram *et al.*, 2001). Finally, increased expression of $PAR_1$ in malignant melanomas was recently correlated with increased expression of IL-8 and integrins (Shi *et al.*, 2004).

Activated PARs induce production of diacylglycerol, an important second messenger in cells of which the primary cellular targets are protein kinase C (PKC) kinases, leading to their translocation to the plasma membrane and subsequent phosphorylation of various molecules. Thus, the family of protein kinase D (PKD) kinases is a direct substrate of PKCs. The PKD family of serine/threonine PKs comprises three members: PKD1 (PKCμ), PKD2, and PKD3 (PKCν). PKD1 has important roles in cell migration and invasion. This kinase reportedly arranges cell motility by aiding the transport of $\alpha v\beta 3$ integrin to emerging focal adhesions (Tellez *et al.*, 2007). Moreover, PKD1 was found to be directly involved in tumor cell invasion and metastasis, and it promotes proproliferative and anti-apoptotic effects (Tellez *et al.*, 2007). Here, we provide early evidence that both $PAR_1$ and $PAR_2$ can activate PKD1 in malignant melanoma cells, thereby enhancing cell proliferation and motility.

## RESULTS

### Stimulation of $PAR_1$/$PAR_2$ on WM9 cells leads to phosphorylation of PKD1

WM9 melanoma cells respond with a fast $[Ca^{2+}]_i$ signal to synthetic PAR agonists (Figure 1a). As both $PAR_1$ and $PAR_2$ are known to be involved in melanoma invasion and metastasis, we searched for possible signal transduction molecules after PAR stimulation in

melanoma cells. Both $PAR_1$ and $PAR_2$ agonists led to a rapid phosphorylation of PKD1 within 5 minutes after stimulation (Figure 1b). Immunoblot analysis using a specific antibody against phospho-Ser916-PKD1 gave an immunoreactive band of about 110 kDa, which corresponds to the molecular mass of PKD1. Activation started to fade 30 minutes after stimulating peptides were added. However, PKD1 remained partly phosphorylated even after 4 hours. We confirmed this observation by immunofluorescence: in unstimulated WM9 cells no phospho-PKD1 was detectable, whereas in PAR-agonist-stimulated WM9 cells, phospho-PKD1 was detected in intracellular compartments, which appeared to be partly aligned along cytoskeletal fiber structures (Figure 1c). Unstimulated WM9 cells did not show a positive staining for phospho-Ser916-PKD1, unless the cells were in a mitotic state (Figure 1d).

## Knockdown of PKD1 in WM9 melanoma cells

We investigated the role of PKD1 in invasiveness and metastasis of melanoma cells by using RNA interference for PKD1-specific knockdown. For high RNA interference efficiency, WM9 cells were transfected with pSuppressor construct with or without a short interfering RNA specific for PKD1, and cells were selected with the antibiotic G418 for 14 days. Stable cell clones were screened for their ability to silence PKD1 expression by real-time PCR analysis (Figure 2a). In all stable clones, the PKD1 mRNA level was reduced by at least 80% as compared with untransfected WM9 cells, which was confirmed by immunoblot analysis (Figure 2b). The slightly decreased expression of PKD1 in mock-transfected WM9 cells was not significant (Figure 2b). The maintenance of PKD1 suppression was monitored for each experiment (Livak and Schmittgen, 2001).

## Stable PKD1 antisense clones exhibit diminished proliferation

Recently, PKD1 has been reported to be involved in the proliferation of a pancreatic carcinoma cell line (Guha *et al.*, 2002). We elucidated the impact of PKD1 on the proliferation of WM9 cells. First, we compared the capability of cell proliferation of normal (untransfected) WM9 cells and PKD1 antisense-expressing cells by means of a $^3$[H]-thymidin incorporation assay. Normal WM9 cells, as well as mock-transfected WM9 cells, revealed high $^3$[H]-thymidin incorporation levels, whereas WM9 cells with low PKD1 expression showed significantly diminished proliferation with a lower $^3$[H]-thymidin incorporation level (Figure 3a).

Activation of PARs on melanoma cells was recently reported to increase proliferation (Even-Ram *et al.*, 2001). Therefore, we analyzed the proliferation capacities of untransfected WM9 cells after stimulation with $PAR_1$- or $PAR_2$-activating peptides. Interestingly, we found that stimulation of $PAR_1$ or $PAR_2$ on WM9 cells had no obvious effect on cell proliferation (Figure 3b). This could be explained by the possibility that proliferation of WM9 cells was already at a maximum. Kinase protein analyses showed a constitutive activation of ERK1/2 kinases in unstimulated WM9 cells (data not shown). The constitutive activation of ERK1/2 could account for a sustained cell proliferation, which was affected by neither $PAR_1$ nor $PAR_2$ activation in these cells (Satyamoorthy *et al.*, 2003). WM9 cells are highly proliferative but do not display an invasive phenotype, because of which we failed to establish migration assays using Boyden Chamber, wound closure experiments, or time-lapse microscopy.

## Knockdown of PKD1 in WM9 cells affects $PAR_1$-mediated secretion of IL-8

Both $PAR_1$ and $PAR_2$ are known to stimulate the secretion of IL-8 in endothelial and epithelial cells, as well as in fibroblasts (Amadesi *et al.*, 2009). In addition, IL-8 is known to promote progression of prostate cancer and melanoma (Huang *et al.*, 2002; Araki *et al.*, 2007). Thus, we analyzed the role of $PAR_1$ and $PAR_2$, as well as that of PKD1, for IL-8

secretion of WM9 cells. Only $PAR_1$ significantly increased the moderate baseline of IL-8 secretion (216±34% compared with 100±20% of unstimulated cells), whereas $PAR_2$ did not significantly affect IL-8 secretion (135±29%; Figure 4a). Conversely, IL-8 secretion was markedly inhibited in PKD1-knockdown cells down to 3±1.5% (Figure 4b), and stimulation of $PAR_1$ did not increase IL-8 secretion in these cells (Figure 4c). We cannot exclude the possibility that the transfection and selection conditions influenced the secretion of IL-8, because the mock-transfected WM9 cells presented a nonsignificant reduced secretion of IL-8 (63±29%). Together, these results suggest that $PAR_1$-mediated melanoma progression involves the induced secretion of IL-8 by $PAR_1$. This mechanism is partly mediated by PKD1, because the loss of PKD1 expression results in a significant decrease in IL-8 secretion.

### Knockdown of PKD1 in WM9 cells leads to changes in cell shape and morphology

Immunofluorescence analysis revealed that unstimulated WM9 cells contained a large amount of β-actin at the cell rims. $PAR_1$ and $PAR_2$ activation provoked rapid formations of β-actin-positive stress fibers (Figure 5). These fibers were still present after 2 hours. Unstimulated PKD1-knockdown melanoma cells also showed predominant immunoreactivity for β-actin along the cell rim with some cytoskeletal fibers that contained β-actin. However, these cells revealed much less β-actin-containing filopodia. After stimulation of either $PAR_1$ or $PAR_2$, some stress fibers had formed in the cell cytosol, and filopodia formation was sparse. Nevertheless, no major changes in β-actin redistribution were observed.

### Knockdown of PKD1 in WM9 cells affects integrin αvβ3 distribution

For normal cell migration, integrins (heterodimeric cell adhesion receptors consisting of alpha and beta subunits) are indispensable. Proper function involves a controlled cycling of the integrin receptors between the cell surface and cytosol. Thus, it is not surprising that several integrins are known to have a direct role in tumor progression. For example, integrin αvβ3 participates in tumor-induced angiogenesis and metastasis (Kumar *et al.*, 2001; Nemeth *et al.*, 2007; Neto *et al.*, 2007). Integrin αvβ3 binds to vitronectin, fibronectin, laminins, matrix-metalloproteinase-2, fibrinogen, prothrombin, thrombospondin, and von-Willebrand factor. Therefore, we analyzed the distribution of αvβ3 after $PAR_1$ and $PAR_2$ activation in wild type and siPKD1-transfected WM9 cells. In unstimulated cells, αvβ3 was mainly found at the cell surface. Fifteen minutes after PAR activation, αvβ3 was widely distributed in the cell; after 60 minutes, αvβ3 was back at the cell membrane (Figure 6a). The PKD1 knockdown in WM9 cells led to a reduced staining of αvβ3 integrin on the cell membrane (Figure 6b). However, PAR activation did not lead to a visible change in integrin distribution in siPKD1-transfected WM9 cells.

## DISCUSSION

This study shows that the activation of both $PAR_1$ and $PAR_2$ in the melanoma cell line WM9 leads to fast activation of PKD1, a previously unreported member of the PKC family of kinases involved in tumorigenesis. PKD1 is apparently an important intracellular kinase, as its presence and its $PAR_1$- and $PAR_2$-mediated activation are important for cell proliferation, αvβ3 integrin distribution, and secretion of inflammatory cytokines such as IL-8.

PKD1 is important for cancer cell migration in myeloma and breast cancer (Bowden *et al.*, 1999; Qiang *et al.*, 2004), and activation of PKD1 prevents apoptosis in pancreatic tumor cells (Trauzold *et al.*, 2003). Pancreatic tumor cells present an increased transcription of neurotensin receptor type-1 during tumor progression (Wang *et al.*, 2000). Neurotensin

receptor type-1 is a member of the heptahelical G-protein-coupled receptor family, and activation of neurotensin receptor type-1 resulted in rapid intracellular calcium mobilization and PKC phosphorylation (Ryder *et al.*, 2001), properties that are also well known for both $PAR_1$ and $PAR_2$ (reviewed in Amadesi *et al.*, 2009). Neurotensin receptor type-1-mediated PKC activation further led to phosphorylation of PKD1 in the pancreatic tumor cell line PANC-1 (Huang *et al.*, 2002). Recent data showed the direct phosphorylation of PKD isoforms by agonist-activated $PAR_2$ in dorsal root ganglia, indicating PKD1 to depict an important component of a signal transduction pathway for proteinase-induced activation of nociceptive neurons and inflammatory responses (Amadesi *et al.*, 2009). Therefore, we investigated whether PKD1 contributes to $PAR_1$- and/or $PAR_2$-induced modification of melanoma cell behavior.

$PAR_1$ is known to be overexpressed in malignant melanoma, and the expression of $PAR_1$ is known to contribute to melanoma cell invasion (Bromberg *et al.*, 2001; Tellez *et al.*, 2003; Shi *et al.*, 2004; Boire *et al.*, 2005; Goerge *et al.*, 2006). Until now, the underlying mechanisms of $PAR_1$ activation and melanoma progression have barely been understood. $PAR_1$ is normally expressed by thrombocytes, neutrophils, mast cells, granulocytes, endothelial cells, fibroblasts, myocytes, neurons, and astrocytes. $PAR_1$ is upregulated during inflammation and triggers degranulation of mast cells. It can be activated by various serine proteinases such as thrombin, kallikreins, plasmin, and cathepsin G, as well as by metalloproteinase-1, all of which are involved in inflammation and tumorigenesis (Hollenberg *et al.*, 2008; Stefansson *et al.*, 2008; Villares *et al.*, 2009; Wilson et al., 2009). Tumors are often called wounds that do not heal because noticeable similarities exist, similar to neovascularization and extracellular matrix deposition (Dvorak, 1986; Chantrain *et al.*, 2006). Therefore, we investigated the activation and role of both $PAR_1$ and $PAR_2$ in WM9 melanoma cells. In normal skin, $PAR_2$ is expressed by keratinocytes, endothelial cells, fibroblasts, sensory nerve fibers, and cells of the immune system (e.g., mast cells and neutrophils). Our own data revealed upregulation of $PAR_2$ in these cells during inflammation (Shpacovitch *et al.*, 2007). Activation of $PAR_2$ has an important role in diseases that involve acute or chronic inflammation of the skin (e.g., contact dermatitis and atopic dermatitis; Buddenkotte *et al.*, 2005; Grant *et al.*, 2007). The expression of $PAR_2$ in keratinocytes is also important for melanosome transfer and skin tanning. Normal melanocytes do not express $PAR_2$, whereas nevi and malignant melanomas express PAR2 but present no significant differences between benign and malignant melanocytic lesions (Massi *et al.*, 2005).

Others have shown that both $PAR_1$ and $PAR_2$ contribute to melanoma cell metastasis. Interestingly, $PAR_2$ can promote metastasis by means of a thrombin-dependent mechanism. However, the details of this mechanism remain elusive (Even-Ram *et al.*, 2001). We therefore analyzed possible signal transduction pathways triggered in WM9 cells after $PAR_1$ or $PAR_2$ activation. We found that either $PAR_1$- or $PAR_2$-activating peptides temporarily induced the phosphorylation of PKD1 at Ser916. We found phosphorylated PKD1 close to α-tubulin fibers during mitosis of WM9 cells. PKD1 is known to have a role in the cell cycle (Rykx *et al.*, 2003). Indeed, we found that cell proliferation was significantly downregulated in PKD1-silenced melanoma cells.

The immunofluorescence staining of activated PKD1 in WM9 cells revealed that after $PAR_1$ and $PAR_2$ stimulation, PKD1 was mainly found in structures that appeared to be vesicle-like. It is appreciated that PKD1 is important for vesicle transport from the trans-golgi network to the cell surface and for recycling of internalized αvβ3 integrin vesicles back to the cell membrane (Baron and Malhotra, 2002; Jaggi *et al.*, 2007). Therefore, activation of $PAR_1$ and $PAR_2$ could lead to a PKD1-dependent vesicle transport to the cell membrane.

However, we could not detect any colocalization of PKD1-positive vesicles with either β-actin or α-tubulin fibers.

Here, we also demonstrate that melanoma cells can secrete the chemoattractive and proangiogenic molecule IL-8 after stimulation of $PAR_1$. IL-8 expression by human melanoma cells is known to correlate with their metastatic capability *in vivo* (reviewed in Tellez *et al.*, 2007). The secretion of IL-8 is influenced by PKD1, as knockdown of PKD1 led to a decrease in the baseline secretion of IL-8. A reduced release of IL-8 could lead to a diminished invasion and metastasis of melanoma (Bar-Eli, 1999). However, $PAR_1$ stimulation in siPKD1-WM9 cells did not increase IL8-secretion. Therefore, secretion of IL-8 in WM9 cells depends only on PKD1 and could be independent from other signaling pathways. $PAR_2$ expression had no significant impact on IL-8 secretion, which could be explained by the lower $PAR_2$ expression compared with $PAR_1$ expression in these cells. We hypothesize that PKD1 is involved in the secretion of pro-inflammatory cytokines, which will be exocytosed from tumor cells to establish a pro-inflammatory environment and induce neovascularization.

Invasive tumor cells form membrane protrusions to come in contact with extracellular matrix proteins. At these invadopodia, metalloproteinases are secreted, which degrade the extracellular matrix to enable tumor cell invasion (Mauch *et al.*, 1994; Schnaeker *et al.*, 2004; Villares *et al.*, 2008). PKD1 is also enriched at invadopodias (White *et al.*, 2007). Thus, we asked whether filopodia formation might be impaired in PKD1-silenced WM9 cells. In addition, non-transfected cells contained numerous filopodia, whereas WM9 cells expressing PKD1-antisense displayed a rounded appearance with only sparse filopodia formation. The cytoskeletal protein β-actin is abundant in filaments along the cell rims, giving WM9 cells a wispy appearance. When either $PAR_1$ or $PAR_2$ was activated, stress fiber formation was pronounced in WM9 cells. In contrast, WM9 cells expressing PKD1 antisense showed almost no filopodia positive for β-actin. Moreover, PAR activation in these cells induced only minor formation of actin stress fibers. These observations are in accordance with a recent report demonstrating that active PKD1 interacts with F-actin at the leading edge of migrating cells, strongly suggesting a direct role of PKD1 in actin remodeling (Eiseler *et al.*, 2007).

PKD1 was shown to be indispensable for the fast recycling of αvβ3 by antagonizing α5β1 recycling in NIH3T3 fibroblasts, thus enabling persistent migration of cells (Woods *et al.*, 2004; White *et al.*, 2007). Integrin αvβ3 is highly expressed in metastatic melanoma cells, and it contributes to the malignant phenotype (Nemeth *et al.*, 2007). Integrin recycling to the cell surface is an important mechanism for cell migration and invasion. Because we saw that PAR agonists can induce a temporary phosphorylation of PKD1 at Ser916 in WM9 cells, we wondered whether PAR activation or silencing of PKD1 in WM9 cells would induce changes in αvβ3 integrin distribution. Activation of either $PAR_1$ or $PAR_2$ induced a fast internalization of αvβ3 integrin from the cell membrane and recycling of the integrin within 60 minutes. Therefore, activation of $PAR_1$ or $PAR_2$ in melanoma cells could influence cell migration. However, we failed to present this possible phenotype for WM9 cells in the Boyden Chamber assay, wound closure experiments, and time-lapse video microscopy because of a low migration phenotype of the cell line. Knockdown of PKD1 in WM9 cells results in lower cell membrane expression of αvβ3 integrin, and PAR stimulation induced no obvious changes in αvβ3 distribution. Our results maintain the important role of PKD1 for αvβ3 integrin recycling in tumor cells and hint that PAR-induced activity of PKD1 could be important for αvβ3-dependent migration of tumor cells. However, future studies with highly invasive and migrating melanoma cell lines must be conducted to improve this hypothesis.

In conclusion, we present early evidence that activation of both $PAR_1$ and $PAR_2$ induces rapid and transient phosphorylation of PKD1 in WM9 cells, serving as an *in vitro* model system for studying the mechanisms of melanoma progression, remodeling, and metastasis. Further elucidation of the signal transduction pathways involved in PAR-mediated PKD1 activation may identify new targets for therapeutic intervention in malignant melanoma.

## MATERIALS AND METHODS

### Materials

Reagents were purchased from the indicated providers: rabbit polyclonal anti-PKD1 and anti-phospho-Ser916-PKD1 from Cell Signalling (Boston, MA), mouse anti-αvβ3-antibody (clone LM609) from Millipore (Billerica, MA), mouse anti-tubulin antibody (clone DM1A) from Calbiochem (Darmstadt, Germany), Fura2AM and FITC-conjugated anti-β-actin and secondary antibodies from Sigma-Aldrich (Taufkirchen, Germany), [$^3$H]-thymidine from GE Healthcare (Braunschweig, Germany), and activating peptides for $PAR_1$ and $PAR_2$ from Bachem (Weil am Rhein, Germany). The PKD1-gene silencing construct and pSuppressor plasmid were described previously (Eiseler *et al.*, 2007).

### Cell lines

The human melanoma cell line WM9 was maintained in a standard culture medium containing enriched Earle's salts, nonessential amino acids, glutamic acid, and 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany). Culture medium for stably transfected PKD1-knockdown WM9 cells and mock-transfected WM9 cells was supplemented with 150 $\mu g ml^{-1}$ G418 (Sigma-Aldrich, Taufkirchen, Germany). Cells were changed to serum-free medium 24 hours before the experiments (except for proliferation assay).

### Generation of transfected cells

WM9 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsberg, Canada) with pSuppressor-PKD1 or control vectors. After 2 days the cells were cultured in standard culture medium supplemented with 400 $\mu g ml^{-1}$ G418 sulfate. The expression of PKD1 was assessed by real-time PCR (forward: 5′-TGCTGTGGGGGCTGGTA CGT-3′ and reverse: 5′-GTGCGGATGGTGCTGACCCC-3′; Livak and Schmittgen 2001).

### Calcium mobilization assay

The calcium mobilization assay was performed as described elsewhere (Bocheva *et al.*, 2009) with minor changes. WM9 cells were incubated with 2.5 μM Fura-2AM for 60 minutes in HEPES-buffered Ringer solution and then washed and stimulated with $PAR_1$ and $PAR_2$ agonists ($10^{-4}$ M).

### Immunoblot analysis

WM9 cells were harvested after treating several times with hot lysis buffer (100mM Tris (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 5% β-mercaptoethanol). Equal volume samples (20 μl) were separated by denaturing SDS-PAGE and were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk powder/1× phosphate-buffered saline+Tween-20 and incubated with specific antibodies against phosphorylated and unphosphorylated PKD1 and peroxidase-conjugated secondary antibodies. The membranes were developed using the ECL Plus Western blotting detection system (GE Healthcare).

### Proliferation assay

For the proliferation assay, 150,000 cells per ml were cultured in 96-well flat-bottom plates in a final volume of 200 μl. Simultaneously, 1 μCi per well [$^3$H]-thymidine was added for 48 hours and thymidine incorporation was measured by liquid scintillation counting.

### Immunofluorescence microscopy

Transfected and nontransfected WM9 cells were washed with cold phosphate-buffered saline and then fixed and permeabilized for 30 minutes in 1× phosphate-buffered saline/1% fetal calf serum/ 0.05% saponin at 4 °C. Primary antibodies were detected with FITC-conjugated antimouse IgG (1:250, 1 hour, room temperature). Cells were embedded in Vectashield mounting medium (Vector, Burlingame, CA). Specimens were observed using a Bio-Rad MRC 1000 confocal microscope (Dreieich, Germany). Images were collected at 0.68 μm intervals using a Zeiss 100 Plan Apo 1.4 NA objective and a zoom of × 1.5–2 (Zeiss, Jena, Germany).

### Statistical analysis

Microsoft Excel software was used to manage data and create graphs. The mean data of the experiments are given in percentage ± SD unless otherwise indicated. Statistical significance was tested with unpaired Student's *t*-test; significant differences of compared values are indicated ($P \leq 0.05$).

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## Abbreviations

| | |
|---|---|
| **PAR** | proteinase-activated receptor |
| **PKC** | protein kinase C |
| **PKD** | protein kinase D |

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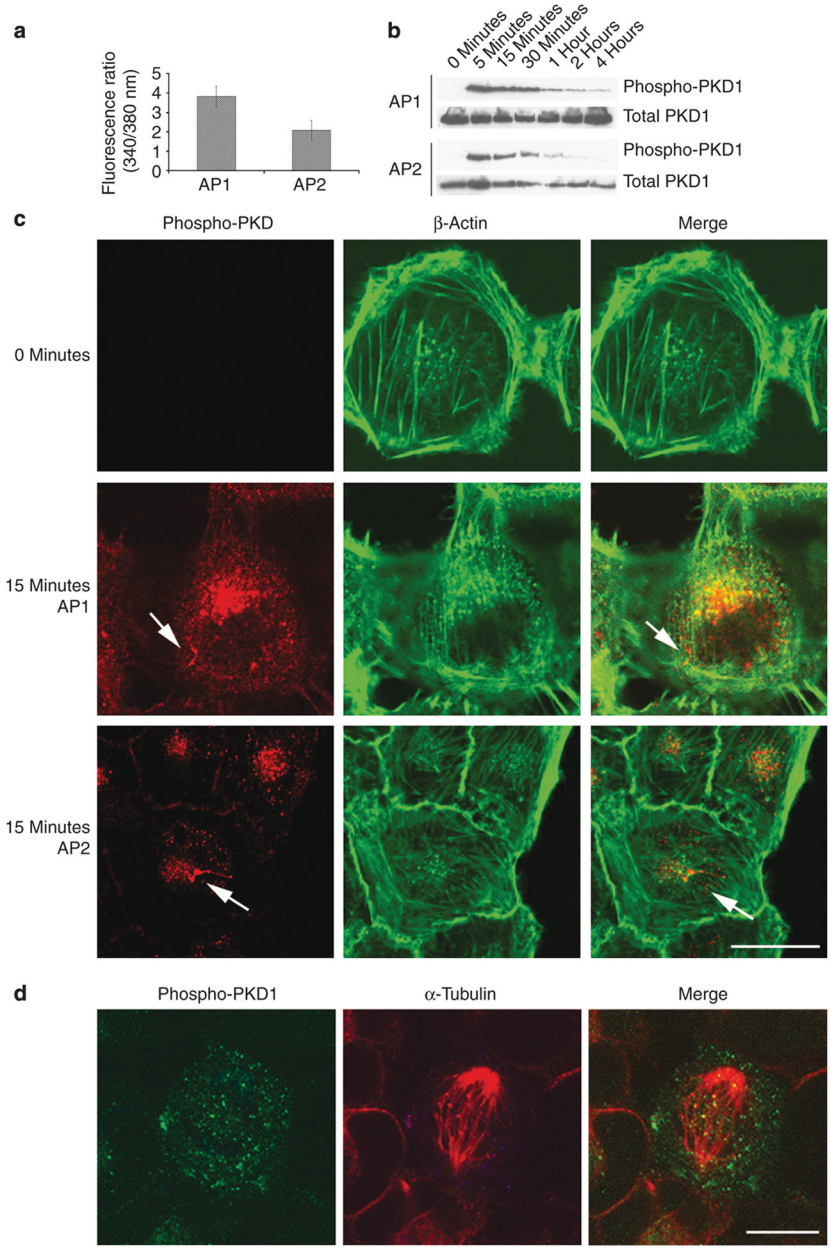


**Figure 1. Stimulation of proteinase-activated receptor $(PAR)_1$ and $PAR_2$ induces phosphorylation of protein kinase D (PKD)1**

Incubation with $PAR_1$- and $PAR_2$-activating peptides (activating peptide for $PAR_1$ (AP1), activating peptide for $PAR_2$ (AP2); 0.1mM) induces fast activation of PKD1. (**a**) Stimulation with AP1 and AP2 induces increase of $[Ca^{2+}]_i$ in WM9 cells. (**b**) AP1- or AP2-induced phosphorylation of PKD1 (110 kDa) peaked after 5 minutes, decreased rapidly within 15 minutes, but lasted up to 4 hours in WM9 cells. (**c**) Immunoreactivities of phospho-Ser916-PKD1 and β-actin were localized in WM9 cells after 15 minutes of stimulation with AP1 or AP2 (0.1mM). Only agonist-induced phosphorylation of PKD1 was detectable, which was localized in vesicular compartments in the cells. However, there was no costaining with β-actin fibers. (**d**) Unstimulated cells only showed positive staining for phospho-Ser916-PKD1 during mitosis (bar=10 μm).

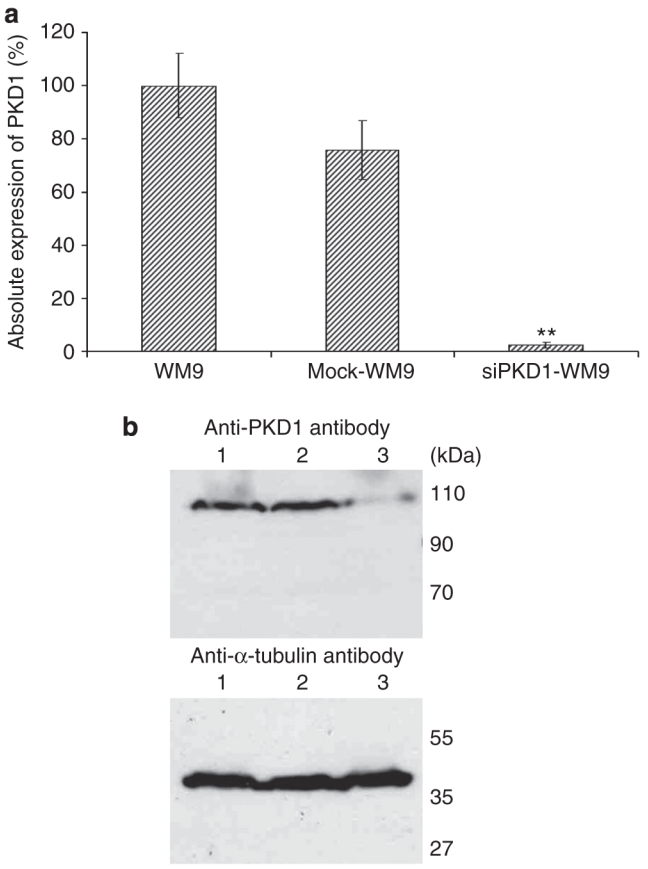


**Figure 2. Protein kinase D (PKD)1 downregulation in WM9 cells by selective knockdown**

PKD1 expression analysis of either normal or PKD1-knockdown WM9 cells. (**a**) PKD1-knockdown cells present a significant decrease of PKD1 expression in real-time PCR. However, control-transfected cells present a nonsignificant decreased expression of PKD1. Triplicate observation in $n=3$ experiments; data are presented as means±SEM, and differences between data were tested by Student's $t$-test for unpaired data. $^{**}P<0.005$. (**b**) Short interfering RNA to PKD1 led to a significant reduction of protein translation in PKD1-knockdown cells (lane 3). Although transcription of PKD1 was decreased in mock cells (lane 2), translation of the PKD1 protein is comparable to that of untransfected WM9 (lane 1) cells.

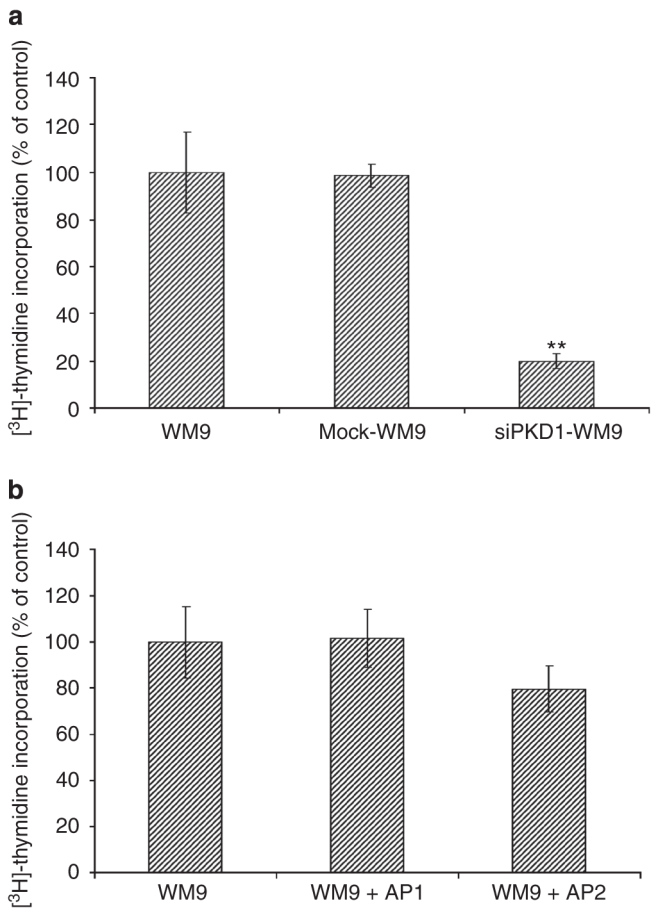


**Figure 3. Proliferation is compromised in protein kinase D (PKD)1-knockdown melanoma cells**

(**a**) Proliferation of normal, mock-transfected, and PKD1-knockdown WM9 cells was determined by [$^3$H]-thymidine incorporation. Stably transfected PKD1-knockdown WM9 cells display an 80% inhibition of cell proliferation as compared with nontransfected and mock-transfected WM9 cells. Data are presented as means±SEM. Differences between data were tested by Student's *t*-tests for unpaired data. $^{**}P<0.005$. (**b**) Stimulation of either proteinase-activated receptor $(PAR)_1$ or $PAR_2$ (0.1mM agonists) had no effect on WM9 cell proliferation. The data shown are $n=3$ and presented as means±SEM.

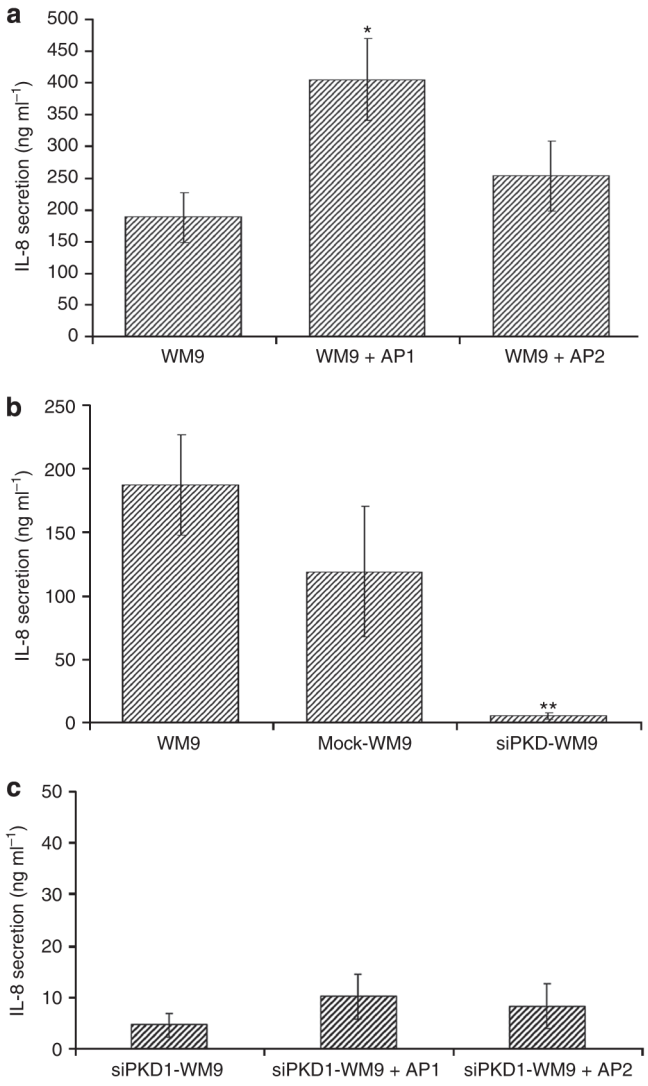


**Figure 4. IL-8 secretion is enhanced after proteinase-activated receptor (PAR)$_1$ stimulation and decreased after protein kinase D (PKD)1 knockdown in cultured WM9 melanoma cells**

(**a**) WM9 cells were stimulated with either activating peptide for $PAR_1$ (AP1) or activating peptide for $PAR_2$ (AP2) (0.1mM), and the secretion of IL-8 was determined by ELISA. Activation of $PAR_1$ induced enhanced IL-8 release in WM9 cells, whereas $PAR_2$ did not exert this effect. (**b**) Secretion of IL-8 is inhibited after PKD1 silencing in WM9 cells (3±1.5%). Triplicate observation in $n$=4 experiments. (**c**) Neither stimulation with AP1 nor stimulation with AP2 increased IL-8 secretion of PKD1-knockdown WM9 cells significantly. Data are presented as means±SEM. Differences between data were tested by Student's $t$-tests for unpaired data. *$P$<0.05; **$P$<0.005.

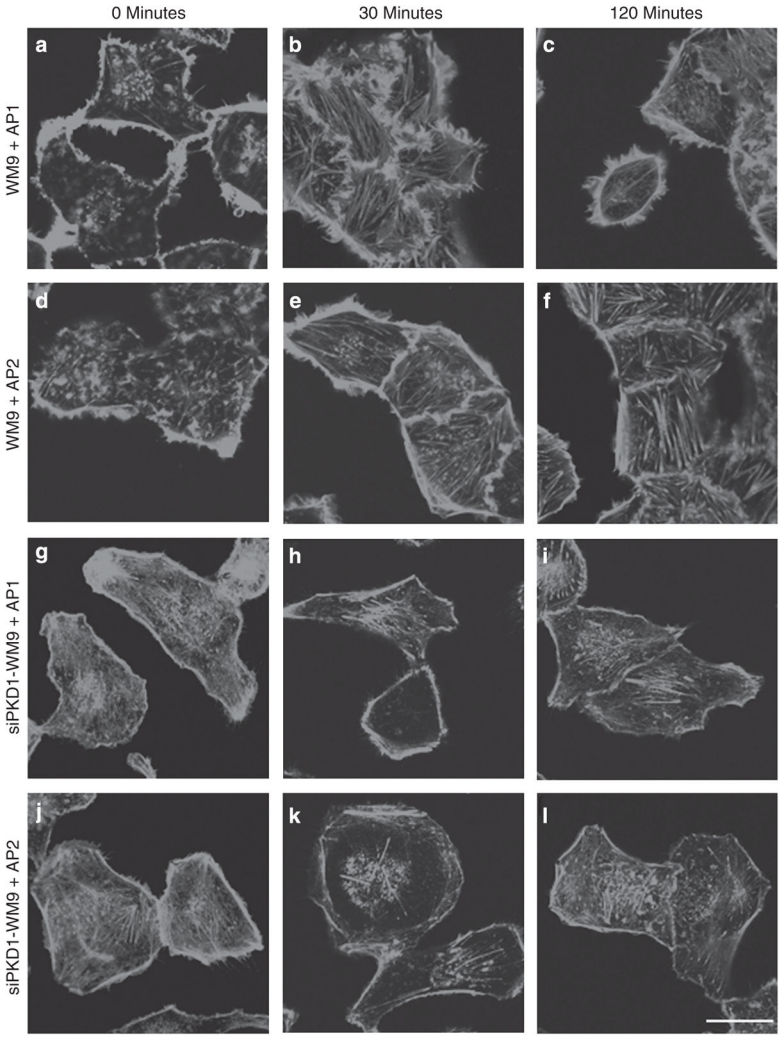


**Figure 5. Knockdown of protein kinase D (PKD)1 in WM9 cells leads to changes in cell shape**

Untransfected and PKD1-knockdown WM9 cells were stimulated with activating peptide for proteinase-activated receptor $(PAR)_1$ (AP1) or activating peptide for $PAR_2$ (AP2) (0.1mM) for 120 minutes and fixed, and fiber formation was monitored with β-actin-FITC antibody. In normal, unstimulated WM9 cells, stress fibers did not form (**a, d**). When $PAR_1$ (**b, c**) and $PAR_2$ (**e, f**) were activated, formation of stress fibers was significantly increased in WM9 cells. PKD1 knockdown induced marked changes in the actin cytoskeleton structure (**g–l**). Unstimulated PKD1-knockdown cells display actin-positive fibers (**g, j**). Here, the activation of $PAR_1$ and $PAR_2$ in PKD1-knockdown cells did not induce formation of stress fibers, and no changes to unstimulated cells were observed (**h, i, k, l**) (bar=10 μm).

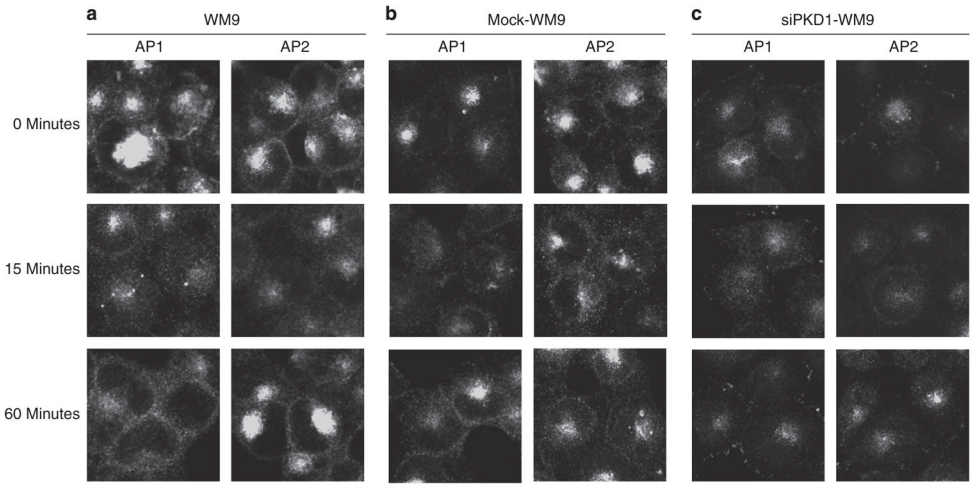


**Figure 6. Protein kinase D (PKD)1 knockdown affects αvβ3-integrin distribution in WM9 cells**

(**a**, **b**) Indirect immunofluorescence revealed fast internalization of αvβ3-integrin after activating peptide for proteinase-activated receptor $(PAR)_1$ (AP1) and activating peptide for $PAR_2$ (AP2) stimulation in either WM9 or mock-transfected WM9 cells (0.1mM, 15 minutes) and recycling of the integrin back to the cell membrane after 60 minutes. (**c**) Staining for αvβ3-integrin on the cell membrane in siPKD1-WM9 cells appears to be reduced, and the integrin is only found in clusters in filopodia-like structures. Stimulation with AP1 and AP2 induced internalization, and recycling of αvβ3-integrin is reduced (bar=10 μm).