Keratin 8 phosphorylation regulates keratin reorganization and migration of epithelial tumor cells

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Summary
Cell migration and invasion are largely dependent on the complex organization of the various cytoskeletal components. Whereas the role of actin filaments and microtubules in cell motility is well established, the role of intermediate filaments in this process is incompletely understood. Organization and structure of the keratin cytoskeleton, which consists of heteropolymers of at least one type 1 and one type 2 intermediate filament, are in part regulated by post-translational modifications. In particular, phosphorylation events influence the properties of the keratin network. Sphingosylphosphorylcholine (SPC) is a bioactive lipid with the exceptional ability to change the organization of the keratin cytoskeleton, leading to reorganization of keratin filaments, increased elasticity, and subsequently increased migration of epithelial tumor cells. Here we investigate the signaling pathways that mediate SPC-induced keratin reorganization and the role of keratin phosphorylation in this process. We establish that the MEK–ERK signaling cascade regulates both SPC-induced keratin phosphorylation and reorganization in human pancreatic and gastric cancer cells and identify Ser431 in keratin 8 as the crucial residue whose phosphorylation is required and sufficient to induce keratin reorganization and consequently enhanced migration of human epithelial tumor cells.

Key words: Intermediate filaments, Mitogen-activated protein kinases, Sphingosylphosphorylcholine, Gastric cancer cells, Pancreatic cancer cells

Introduction
Cell migration and invasion are markedly dependent on the complex organization of the cytoskeleton (Ballestrem et al., 2000). The cytoskeleton of epithelial cells is a network of three major classes of filamentous biopolymers: microfilaments, microtubules and intermediate filaments. Intermediate filaments are composed of a large family of cell-specific proteins that organize to form 10 nm filaments sharing sequence homology and structural features. Among the cytoplasmic intermediate filament proteins, keratins are expressed preferentially in epithelial cells (Fuchs and Weber, 1994; Coulombe and Omary, 2002) and constitute nearly 5% of the total protein in these cells (Omary et al., 1998). Keratin filaments are obligate heteropolymers of at least one type I (relatively acidic keratins K9–K28, K31–K40) and one type II keratin (relatively basic keratins K1–K8, K71–K86) (Schweizer et al., 2006). These filaments are usually organized into bundles, the so called tonofibrils, which form cage-like structures around the nucleus and extend from the perinuclear region to the cell periphery (Hatzfeld and Franke, 1985).

K8 and K18 are the major components of intermediate filaments of simple epithelia as found in intestine, liver and exocrine pancreas (Fuchs and Weber, 1994; Coulombe and Omary, 2002). The expression pattern of these proteins is generally persistent in carcinomas arising from tissues that normally express K8 and K18 (Oshima et al., 1996). Keratins play a crucial role in maintaining the structural integrity and the mechanical properties of cells and thereby protect cells from a variety of environmental insults (Yamada et al., 2002). Furthermore, they are major determinants for the mechanical features of the cytoplasm and the nucleus (Maniotis et al., 1997; Fuchs and Cleveland, 1998).

The structure and function of keratins are probably regulated through posttranslational modifications, particularly phosphorylation on serine residues within the so-called ‘head’ (N-terminal) and/or ‘tail’ (C-terminal) non-α-helical end domains (Fuchs and Cleveland, 1998; Omary et al., 2006). Ser52 is the major phosphorylation site of human K18 in vivo. This site has been implicated in increased keratin solubility and altered polymerization (Ku and Omary, 1994; Liao et al., 1995a), keratin reorganization (Ku et al., 1999), apoptosis (Caulin et al., 1997) and cellular stress (Omary et al., 1998). Increased phosphorylation of K18 has also been implicated in the reorganization of keratin filaments in hepatocytes treated with protein phosphatase inhibitors (Toivola et al., 1998).

Ser431 is a major in vivo phosphorylation site in human K8. Ser431 is phosphorylated by mitogen-activated protein kinases (MAPKs) in response to activation of the EGFR (Omary et al., 1998). Phosphorylation at this site has also been described during hyperosmotic stress, whereas hypo-osmotic stress leads to
dephosphorylation at Ser431 of K8 (Tao et al., 2006), and also occurs in human and mouse liver upon injury resulting in Mallory–Denk body formation (Stumptner et al., 2000) or during mouse liver and gallbladder injury induced by a high-fat diet (Tao et al., 2003).

Sphingosylphosphorylcholine (SPC) is a naturally occurring bioactive lipid that acts as an intracellular and extracellular signaling molecule in numerous biological processes including proliferation (Seufferlein and Rozengurt, 1995), cell migration (Boguslawski et al., 2000), wound healing (Wakita et al., 1998) and differentiation (Kleger et al., 2007). Similar to other bioactive lipids such as lysophosphatidic acid (LPA) or sphingosine-1-phosphate, many of its actions are mediated by the activation of a subfamily of low- and high-affinity G-protein-coupled receptors (An et al., 1995; Meyer zu Heringdorf et al., 2002).

Previously, we have shown that SPC is one of the few naturally occurring compounds that can induce a perinuclear reorganization of the keratin cytoskeleton in human pancreatic cancer cells. This reorganization is accompanied by keratin phosphorylation, including phosphorylation at K18(S52) and K8(S431), and an increase in cellular elasticity and enhanced migration of cancer cells through size-limited pores (Beil et al., 2003). However, the precise downstream signaling mechanisms by which SPC induces keratin reorganization and the role of keratin phosphorylation in this process are as yet unknown.

Here we show that the MEK–ERK signaling cascade regulates both SPC-induced K8 phosphorylation at Ser431 and keratin reorganization in human pancreatic and gastric cancer cells. We identify Ser431 in K8 as the crucial residue whose phosphorylation is required and sufficient to induce keratin reorganization and consequently enhanced migration of human epithelial tumor cells.

Results
Role of the ERK cascade for SPC-induced keratin reorganization

Previously we have demonstrated that SPC reorganizes the keratin cytoskeleton in Panc-1 and AGS human cancer cells from a branched phenotype into a perinuclear, ring-like formation and increases migration of epithelial tumor cells through size-limited pores (Beil et al., 2003). These cell lines express K8 and K18 as their major keratins, as shown using a pan anti-keratin antibody and individual K8 and K18 antibodies (supplementary material Fig. S1A). This effect of SPC is probably mediated by a G-protein-coupled receptor. SPC interacts with S1P receptors 1–5, GPR4 and OGR1 with different affinities (Meyer zu Heringdorf et al., 2002). Both pancreatic and gastric cancer cell lines express S1P1–S1P5, GPR4 and OGR1, as determined by RT-PCR (supplementary material Fig. S1B).

Activation of the ERK signaling cascade has been implicated in cell migration (Huang et al., 2004; Rajalingam et al., 2005; Bove et al., 2008). We have previously shown that SPC potently induces activation of ERKs in fibroblasts (Seufferlein and Rozengurt, 1994). SPC was also able to stimulate ERK activation in human pancreatic and gastric cancer cells, reaching

![Fig. 1. SPC-induced activation of p42 and p44 (MAPK1 and MAPK3) phosphorylation.](image-url)

(A) Panc-1 and AGS were incubated with 12.5 μM SPC for the indicated time points. (B) Cells were treated with various concentrations of SPC as indicated for 15 minutes (Panc-1) or 30 minutes (AGS). (C) Panc-1 and AGS were preincubated with PD98059 using the concentrations indicated and subsequently treated with 12.5 μM SPC for 15 minutes (Panc-1) or 30 minutes (AGS). (D) Panc-1 and AGS cells were preincubated with the U0126 as indicated and subsequently treated with 12.5 μM SPC for 15 minutes (Panc-1) or 30 minutes (AGS). Immunoblotting was performed using antibodies against p44 and p42 (p44/42) and phosphorylated p44 and p42 (Ph-p44/42).
a maximum after 15 and 30 minutes of incubation in Panc-1 and AGS cells, respectively. ERK activation in Panc-1 and AGS cells in response to SPC was prevented by incubation of cells with the selective MEK inhibitors PD98059 or U0126 (Fig. 1). Interestingly, SPC failed to activate other signaling pathways in pancreatic cancer cells including the PI3K–AKT, the PKC–PKD and the PKM2 signaling pathways (supplementary material Fig. S2).

ERKs also regulate keratin phosphorylation (Ku and Omary, 1997; Huang et al., 2004; Omary et al., 2006). Therefore, we determined whether ERK-mediated phosphorylation of keratins could play a role in SPC-induced keratin reorganization and migration. SPC induced perinuclear reorganization of both endogenous keratin and overexpressed K8 or K18 (Fig. 2A). Keratin perinuclear reorganization was confirmed by quantifying the fluorescence intensity distribution on the y-axis (Fig. 2B, see results below). SPC-induced keratin reorganization was completely prevented upon incubation of Panc-1 or AGS cells with the MEK inhibitors PD90859 or U0126, respectively (Fig. 3). Thus, activation of the MEK–ERK cascade appears to be crucial for perinuclear keratin reorganization in response to SPC.

ERKs mediate SPC-induced phosphorylation of K8(Ser431)

Next we wanted to identify the site(s) in keratins that become phosphorylated in response to SPC. SPC stimulated phosphorylation of K8 at Ser431 in both epithelial cancer cell lines – a site that is also phosphorylated by active ERK (Omary et al., 1998; Ku et al., 2005). K8(S431) phosphorylation in response to SPC was prevented when cells were incubated with either MEK inhibitor (Fig. 4A). To determine the effect of ERK-induced K8 phosphorylation on the organization of the keratin cytoskeleton we performed immunocytochemistry using a phosphospecific antibody against phosphorylated K8(S431).

Fig. 2. Keratin reorganization in epithelial tumor cells after SPC treatment. (A) Panc-1 and AGS cells were transfected with eCFP-tagged K8, eYFP-tagged K18 or left untransfected and were subsequently incubated with 15 μM SPC for 45 minutes. Endogenous keratins were stained with pan-CK antibody, followed by Alexa Fluor 488 staining. Images were taken using a confocal microscope and keratin was detected in the 488 channel (green emission). Representative images show reorganization of endogenous or ectopic keratin filaments to a perinuclear, ring-like structure upon SPC incubation. Scale bars: 10 μm. (B) Quantification of cytokeratin organization. Cytokeratin organization was quantified in cells treated with or without 12.5 μM SPC for 1 hour, as described in the Materials and Methods. Images represent ortho-max projections of confocal image sections with three linear ROIs in the perinuclear and the cytoplasmic region, respectively. Left graph shows intensity ratio of perinuclear to cytoplasmic ROIs. Cytokeratin organization was quantified in cells treated with or without 12.5 μM SPC for 1 hour, as described in the Materials and Methods. Images represent ortho-max projections of confocal image sections with three linear ROIs in the perinuclear and the cytoplasmic region, respectively. Left graph shows intensity ratio of perinuclear to cytoplasmic ROIs. Cells treated with SPC exhibit an increase in fluorescence intensity in the perinuclear area compared with untreated cells, demonstrating a marked difference in cytokeratin redistribution upon phosphorylation that can be quantified. Right graph shows height of cells (Z-volume) calculated from confocal image stacks. **p<0.01.
Using this antibody we detected intense pK8(S431) immunoreactivity exclusively upon incubation of cells with SPC, but not in unstimulated, control cells. pK8(S431) immunoreactivity was predominantly detectable in reorganized, perinuclear keratin filaments, indicating that K8 phosphorylation strictly correlates with keratin reorganization (Fig. 4C). In the presence of U0126 or PD98059, the SPC-stimulated increase in Ser431 immunoreactivity was virtually abolished (Fig. 4D). Similarly, the SPC-stimulated increase in K8(S431) immunoreactivity was completely prevented when p42 and p44 (MAPK1 and MAPK3, respectively) were depleted by specific siRNAs (Fig. 4E). Thus, SPC-induced K8(S431) phosphorylation requires ERK activity.

SPC also induces keratin phosphorylation at other sites. Incubation of cells with SPC increased the phosphorylation of K18(S52) in both pancreatic and gastric cancer cells. However, K18(S52) phosphorylation in response to SPC was not prevented by inhibition of the MEK–ERK signaling cascade with PD90859 or U0126, respectively (Fig. 4B). Collectively, these findings indicate that: (1) SPC stimulates ERK activity; (2) SPC-induced keratin reorganization requires ERK activity; (3) SPC-induced phosphorylation of K8(S431) is also dependent on ERK activity; and (4) K8(S431) phosphorylation and keratin reorganization by SPC go hand in hand. These data suggest a relationship between K8(S431) phosphorylation and keratin reorganization in epithelial tumor cells.

Role of phosphorylation at K8(Ser431) and K18(Ser52) in SPC-induced keratin reorganization in human cancer cells

To examine whether keratin phosphorylation at K8(S431) or K18(S52) was required and/or sufficient for SPC-induced keratin reorganization in Panc-1 and AGS cells, we generated eCFP-tagged mutants of K8 and eYFP-tagged mutants of K18 that mimic phosphorylation at K8(S431) and K18(S52), respectively (S$\rightarrow$E), or exhibit a non-phosphorylatable site (S$\rightarrow$A). It has been shown previously that keratin phosphorylation affects its solubility. Indeed, there was more K8(S431E) detectable in the cytosolic/soluble fraction compared with K8(S431A) and K8 WT (supplementary material Fig. S2A).

Upon incubation of cells with SPC, endogenous as well as exogenously expressed K8 and K18 exhibited the typical pattern of ‘reorganized’ keratin, with a predominant perinuclear keratin organization in Panc-1 and AGS cells that was prevented in the presence of U0126 (Fig. 5A; supplementary material Fig. S3A). When cells were transfected with an eCFP–K8(S431A),K18(S52A) double mutant there was no detectable keratin reorganization in response to SPC (Fig. 5B; supplementary material Fig. S3B). In marked contrast, transfection of cells with the
Fig. 4. See next page for legend.
K8(S431E), K18(S52E) mutants resulted in a strictly perinuclear redistribution of these keratin mutants that was not further increased in presence of SPC. Incubation of K8(S431E), K18(S52E) transfected cells with U0126 did not prevent the perinuclear organization of the transfected keratins (Fig. 5C; supplementary material Fig. S3C). Thus, phosphorylation of K8 and/or K18 is sufficient to trigger keratin reorganization.

To determine whether and which of these phosphorylation sites were required and/or even sufficient to trigger perinuclear keratin reorganization, we expressed K8 and K18 expression plasmids that contained only one keratin modification either in K8 or K18. Organization of K8(WT), K18(S52E) transfected Panc-1 or AGS cells was comparable with that of wild-type keratins. Perinuclear organization of K8(WT), K18(S52E) was only detectable in the presence, but not in the absence of SPC (Fig. 5D; supplementary material Fig. S3D). This SPC-induced perinuclear reorganization of K8(WT), K18(S52E) could be prevented by treatment of cells with U0126. Organization of the K8(WT), K18(S52A) mutant was comparable to that of wild-type K8 or K18 in the presence or absence of SPC (Fig. 3; Fig. 5E; supplementary material Fig. S3E). Thus, K18(S52) phosphorylation was neither sufficient nor required for SPC-induced perinuclear keratin reorganization in epithelial cancer cells.

Transfection of Panc-1 or AGS cells with K8(S431E), K18(WT) resulted in marked perinuclear reorganization of keratin in the absence of SPC. Incubation of cells with SPC did not further enhance perinuclear organization of K8(S431E), K18(WT).

Furthermore, incubation of cells with U0126 did not prevent the perinuclear organization of K8(S431E), K18(WT) (Fig. 6A; supplementary material Fig. S4A). In turn, K8(S431A), K18(WT) did not exhibit perinuclear keratin organization either in the absence or in the presence of SPC. The selective MEK inhibitor U0126 had no effect on the subcellular organization of K8(S431A), K18(WT) (Fig. 6B; supplementary material Fig. S4B).

Furthermore, there was no difference in the perinuclear organization of K8(S431E), K18(WT), K8(S431E), K18(S52E) or K8(S431E), K18(S52A), indicating that phosphorylation of K18 at Ser52 is indeed not required for perinuclear keratin organization in human epithelial tumor cells (Fig. 6C; supplementary material Fig. S4C). Interestingly, transfection of K8(S431E) alone was also sufficient to induce perinuclear organization of endogenous K8 and K18 (Fig. 6D). Thus phosphorylation of K8 at Ser431 is required and sufficient for perinuclear keratin reorganization in human pancreatic and gastric cancer cells.

Keratins regulate the motility of epithelial tumor cells

Previously, we have shown that SPC reorganizes the keratin cytoskeleton and facilitates migration of tumor cells through size-limited pores, suggesting a link between keratin organization and tumor cell migration (Beil et al., 2003). To further determine the precise role of keratin in tumor cell migration, we targeted K8 using small interfering (si)RNA oligonucleotides. Depletion of K8 by siRNA was confirmed by qRT-PCR, immunocytochemistry and western blot analysis. K8 mRNA transcripts were reduced by 80% 24 hours after transfection (Fig. 7A; supplementary material Fig. S5). A maximum effect on K8 protein expression was observed after 72 hours of incubation with the specific siRNA (Fig. 7B). Depletion of K8 or K18 resulted in a significantly enhanced random migration of tumor cells (siK8 = 0.31 μm/minute vs siControl = 0.18 μm/minute; Fig. 7C,D). Thus, under basal conditions, the established keratin cytoskeleton obviously restricts tumor cell motility.

SPC stimulates random motility of Panc-1 cells (Fig. 7C; supplementary material Fig. S6A). This increase in motility corresponds to the changes in keratin organization in response to SPC (supplementary material Fig. S6B) and it was prevented when cells were incubated with the MEK inhibitor U0126 (Fig. 7E). This was not only the case for random motility, but also for tumor cell migration through size-limited pores in Boyden chamber assays (Fig. 7F). Interestingly, SPC stimulated random tumor cell migration to a similar degree as keratin depletion and was not able to further enhance the random motility of K8-depleted cells (Fig. 7C). Thus, SPC-induced cell migration requires ERK activity and SPC affects tumor cell migration by a mechanism that requires MEK-mediated phosphorylation.

Phosphorylation of K8 at Ser431 leads to enhanced tumor cell migration

Because perinuclear keratin reorganization correlates with increased tumor cell motility, we next examined the effect of K8(S431E) phosphorylation on random migration of tumor cells. Pancreatic cancer cells expressing wild-type K8 and K18 move with a speed of about 0.19 μm/minute, which corresponds to the speed of untransfected control cells (0.21 μm/minute). K8(S431E), K18(WT)-expressing cells moved twice as fast as wild-type cells (0.39 μm/minute) and as fast as cells expressing...
wild-type K8 and K18 in the presence of SPC (0.36 μm/minute; Fig. 7G). In addition, the migration speed of K8(S431E),K18(WT)-expressing cells was comparable to that of K8-depleted pancreatic cancer cells using specific siRNA (0.31 μm/minute). Pancreatic cancer cells expressing the K8(S431A),K18(WT) mutant exhibited the same migratory behavior as cells expressing wild-type K8 and K18 (0.21 μm/minute). SPC failed to enhance the migration velocity of cells expressing K8(S431A),K18(WT) (0.24 μm/minute; Fig. 7G). This indicates that phosphorylation of K8 at Ser431 plays a crucial role for both SPC-induced keratin organization and SPC-induced release of the keratin-mediated inhibition of human tumor cell migration. It has been reported that K8 and K18 modulate the distribution of focal adhesions in simple epithelial cells (Bordeleau et al., 2010). Our data (supplementary material Fig. S7) show that upon expression of K8(S431E), there was a significant increase in the number but not the size of focal adhesions. The focal adhesions aligned at the margin of the cells. Thus, keratin phosphorylation affects focal adhesion formation in pancreatic cancer cells and could thereby contribute to the pro-migratory effect observed. These data provide a direct link between keratin organization, ERK-mediated keratin phosphorylation and tumor cell migration. They further demonstrate that K8(S431) phosphorylation resembles the effects of knockdown of keratins by releasing the restrictive function of keratins on tumor cell migration.

**Discussion**

Sphingosylphosphorylcholine (SPC) is a naturally occurring, bioactive lipid that acts as an intracellular and extracellular signaling molecule in numerous biological processes including proliferation (Seufferlein and Rozengurt, 1995), cell migration (Boguslawski et al., 2000), wound healing (Wakita et al., 1998).
and differentiation (Kleger et al., 2007). Previously, we established that SPC is one of the few physiological compounds that is able to reorganize the keratin cytoskeleton. SPC induced reorganization of keratin from a widespread, ramified network to a strictly perinuclear, ring-like structure in human epithelial tumor cells (Beil et al., 2003). This reorganization led to an increase in the elasticity of the tumor cells, facilitated migration of cells through size-limited pores and was accompanied by keratin phosphorylation at K8(S431) and K18(S52).
Fig. 7. Effect of keratin depletion on basal and SPC-induced random migration of pancreatic cancer cells. (A) K8 and/or K18 were depleted in Panc-1 cells using specific siRNAs (siK8, siK18). Relative K8 mRNA (upper panel) and K18 mRNA levels (lower panel) were determined in Panc-1 cells by qRT-PCR using the ICyclerIQ system from Bio-Rad. Graphs depict percentage mRNA expression as compared with control. Figures shows representative data obtained in triplets of at least three independent experiments. (B) Top panel shows Panc-1 cells transfected with specific K8 siRNA (siK8) and/or K18 siRNA (siK18). After 72 hours, keratins were extracted and expression analyzed by western blotting using a pan-CK antibody. Bottom panel is densitometric evaluation of keratin protein levels. Data represent the means ± s.e.m. of three independent experiments. (C) Panc-1 cells were transfected with scrambled siRNA (siCon) or siK8. After 48 hours, cells were incubated with 10 μM SPC (+) or solvent (–) and subjected to a random migration that was analyzed by time-lapse video microscopy. Cells were tracked and the velocity was calculated using the ImageJ program. Data represent the fold increase in migration above control and are the means ± s.e.m. of three independent experiments. (D) Panc-1 cells were transfected with scrambled siRNA (siCon) or siK18. 24 hours after knockdown, cells were incubated with solvent or 10 μM SPC and subjected to random migration. Migration was analyzed as described above. Data represent the fold increase in migration above control and are the means ± s.e.m. of three independent experiments. (E) Panc-1 cells were incubated with 10 μM U0126, 10 μM SPC and 5 ng/ml TGF-β as indicated. Migration through size-limited pores was determined using a Boyden chamber assay as described previously (Beil et al., 2003). Data represent the fold increase in migration above control and the means ± s.e.m. of three independent experiments. (F) Panc-1 cells were incubated with scrambled siRNA (siCon) or siK8, 24 hours after knockdown, cells were incubated with solvent or 10 μM SPC and subjected to random migration. Migration was analyzed as described above. Data represent the fold increase in migration above control and are the means ± s.e.m. of three independent experiments. (G) Panc-1 cells were transfected with K8(WT), K18(WT), K8(SE), K18(WT) or K8(SA), K18(WT) and subsequently incubated with 10 μM SPC (+). Random migration was determined as above. Data represent the fold increase in migration above control and are the means ± s.e.m. of four independent experiments. In all experiments described in 7C-G significant differences were tested using the Student’s t-test. *P<0.05; **P<0.01; ***P<0.001.
Our previous data suggested that SPC-induced keratin reorganization and cell migration are linked (Beil et al., 2003). In addition, enhanced migration often corresponds to increased activity of the ERK signaling cascade (Huang et al., 2004). Thus, we investigated the role of SPC-induced ERK-dependent K8 phosphorylation and reorganization in cell migration, both in the absence and presence of SPC. Inhibition of MEK blocked K8(S431) phosphorylation, keratin reorganization and tumor cell migration. Interestingly, depletion of K8 in epithelial tumor cells also resulted in a marked increase in tumor cell motility compared with that in cells transfected with a scrambled siRNA construct. These data are in line with observations in wound healing, where knockdown of K8 with siRNA resulted in accelerated wound closure in vimentin-positive HeLa and Panc-1 cells (Long et al., 2006). Notably, compared with control cells, scratch-wound edges were irregular after K8 depletion and frequently contained cells that were migrating individually in both cell lines (Long et al., 2006). This behavior of K8-knockdown cells is comparable with our single-cell-based experiments, and indicates that the basal organization of the keratin cytoskeleton in simple epithelial tumor cells serves to restrict cellular migration. Interestingly, SPC failed to further increase migration velocity of tumor cells depleted of K8. This shows that keratin organization is crucial for the effect of SPC on tumor cell migration. In addition, our data clearly show that ERK-induced phosphorylation of K8 at Ser431 is sufficient to stimulate tumor cell migration to a similar degree as SPC treatment. Accordingly, phosphorylation at K8(S431) was required for SPC-induced tumor cell migration. The migratory behavior of tumor cells depleted of K8 was similar to that of cells expressing the phosphomimetic K8(S431E) mutant. This shows that ERK-mediated K8(S431) phosphorylation functionally mimics K8 depletion of tumor cells and abolishes the otherwise restrictive function of the keratin cytoskeleton on tumor cell migration. The fact that this effect can be so clearly demonstrated using SPC, but has so far not been described for other activators of the ERK cascade such as EGF–EGFR might lie in the fact that SPC, by acting through a G-protein-coupled receptor (GPCR), induces only few signaling pathways in human epithelial tumor cells, in particular an activation of the ERK cascade (our own unpublished observations). Upon induction of multiple pathways there are likely to be other modifications of keratins that attenuate or modulate the effect of ERK activation with respect to keratin reorganization.

Our data have implications for tumor biology. The ability of tumor cells to migrate is pivotal for tumor progression and metastasis (Chambers et al., 2002). Our data show that SPC-dependent phosphorylation of K8(S431) leads to increased tumor cell migration as a result of reorganization of the keratin cytoskeleton and this is likely to contribute to the metastatic properties of these cells. Thus, we provide a mechanism to explain how ERKs can regulate tumor cell migration and identify keratin phosphorylation as an interesting novel therapeutic target to prevent invasion, and potentially, metastasis.

### Materials and Methods

#### Materials

The MEK inhibitor U0126 was obtained from Promega (Fitchburg, WI). PD98059 and SPC were from Calbiochem (Merck Chemicals, Nottingham, UK), TGF-β from BD Biosciences (San Jose, CA). Antibodies directed against Akt, phospho-Akt (Ser 473), phospho-PKD (Ser744/748) and phospho-PKM2 (Ser440/441) were from Cell Signaling Detection (Danvers, MA), antibodies detecting Pan-Cytokeratin (Pan-CK, clone KL1) are from Immunotech (Praha, Czech Republic).

The phosphospecific antibodies detecting K18(S52) (3055) and K8(S431) (S53) have been described previously (Liao et al., 1995a; Ku et al., 1997). Antibodies directed against Akt, phopho-Akt (Ser 473), phospho-PDK (Ser744/748) and phospho-PKM2 (Tyr105) were obtained from Cell Signaling. The mouse monoclonal antibody against PKM2 was purchased from Abcam (Cambridge, UK). The antibodies detecting PKD2 were purchased from Calbiochem (Merck Chemicals). Phorbol-12-myristate-13-acetate (PMA) was obtained from Calbiochem (Merck Chemicals). IgF1 was from Peprotech (Hamburg, Germany).

#### Cell culture and reagents

Panc-1 human pancreatic cancer cells were purchased from American Type Culture Collection (Manassas, VA). AGS cells were a gift from Michael Hoecker (Charite, Berlin, Germany). This is a human gastric cancer cell line that also exclusively expresses keratins, but no vimentin. Cells were maintained in DMEM (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (PAA, Pasching, Austria) in a humidified atmosphere and 5% CO₂, 95% air at 37°C and passaged every 2–3 days. Cells were incubated in serum-free DMEM (Gibco) for 18 hours according to the experiment.

#### Cell transfection

For immunofluorescence microscopy and live-time imaging, cells were either transfected with Fugene (Roche, Basel, Switzerland) or Metafectene (Biontex, Martinsried, Germany). For migration assays, transfection was performed with Nucleofector (Amaxa, Cologne, Germany) using Kit R and program X-005, siRNA was transfected with RNAiFacT (Qagen).
mRNA was prepared from either Panc-1 or AGS cells and semi-quantitative RT-PCR analysis was conducted with specific primers for SPC receptors as described previously (Kleger et al., 2007).

Random migration
Serum-starved Panc-1 cells were subjected to a random migration on fibronectin and imaged by the time-lapse video microscopy. Glass bottom culture dishes (MaTek Corporation) were coated with 50 μg/ml of fibronectin (Roche). Cells were allowed to spread for 3 hours on the fibronectin-coated dishes in DMEM with 1% FCS. Imaging was performed using a BZ-8000 Keyence microscope. During imaging cells were kept at 37 ºC in an atmosphere containing 5% CO₂. A motion picture (AVI format) was created from time-lapse images using the BZ-Analyzer software (Keyence Corporation). Cell movement was analyzed using tracking routines implemented in ImageJ software. Three independent experiments were done for each condition.

Isolation and analysis of keratin fractions
Keratins were isolated from three cellular fractions. The cytosolic fraction was obtained after disrupting cells by centrifugation at 100,000 g for 90 minutes in Buffer A [PBS with 10 mM EDTA and protease and phosphatase inhibitor cocktail (Roche)]. The pellet was then resuspended using 1% NP-40 in buffer A (30 minutes at 4 ºC) followed by centrifugation (16,000 g, 15 minutes, 4 ºC) and collecting the NP-40 fraction. The remaining cytoskeletal fraction was solubilized in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA with 9.5 M urea. GelTol Aqueous Mounting Medium (Immunotech). Imaging was performed with confocal laser-scanning microscope LSM510 Meta (Carl Zeiss, Jena, Germany) equipped with a 63 x 1.4 NA objective using the indicated filters or a Keyence BZ-8000 fluorescence microscope. Images show representative cells from at least three independent experiments.

Site-directed mutagenesis
To obtain amino acid exchange of K8(Ser431) and K18(Ser52), site-directed mutagenesis with pk8-ΔCFP and pk18-ΔYFP (Rudolf Leube, Universitätssklinikum Aachen, Germany) (Wöll et al., 2005) as matrices were performed using QuikChange XL-smd-Kit (Stratagene, La Jolla, CA) according to the instruction manual. To replace K8(Ser431) and K8(Ser52) with Alanine (A) or Glutamic acid (E), primers as follows were used: K8(Ser431): 5′-GGAGAGAGAGAGAGAAAGAAGGAGGAGGTTTGG-3′; K8(Ser52): 5′-GAGGAGAGAGAGAGAAAGAAGGAGGCTCAGTT-3′; K18(Ser52): 5′-GGAGAGAGAGAGAAAGAAGGAGGAGGTTTGG-3′; K18(Ser52): 5′-GGAGAGAGAGAGAAAGAAGGAGGCTCAGTT-3′. pk8-Alexa-488 was used. As a control, an AllStar siRNA labeled with Alexa Fluor 546 (Qiagen, Hilden, Germany) was used. To deplete K18, we used a mixture of two different siRNA constructs: Stealth RNAi K8-1HSS142770 (cat. no. 5194538, Invitrogen) and Hs-KRT18-3 CK (5′-ccgccccagttgacgatc-3′, -gagaagagagagagagaaaaa-3′) (Qiagen). AllStar siRNA labeled with Alexa Fluor 546 (Qiagen) was used as a control. For knockdown of p44 and p42 MAPKs, ERK1/2 siRNA from Cell Signaling was used. Panc1 cells were transfected using Hyperfect (Qiagen) according to manufacturer’s instructions.

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References


A. cytosolic/soluble  membrane  cytoskeletal

WB:pan-K

- CFP-K8
- endo-K8
- endo-K18

WB:K8

- endo-K8

WB:K18

- endo-K18

WB:Actin

B. AGS  Panc1  H2O  pos

S1P1

S1P2

S1P3

S1P4

S1P5

GPR4

OGR1
A. 

<table>
<thead>
<tr>
<th></th>
<th>cytosolic/soluble</th>
<th>membrane</th>
<th>cytoskeletal</th>
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<tbody>
<tr>
<td>K8wt</td>
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<td></td>
<td></td>
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<tr>
<td>K8SA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K8SE</td>
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</table>

WB: GFP

WB: Actin

B. 

12.5μMSPC

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<tr>
<th>Time (min)</th>
<th>M[kDa]</th>
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</tbody>
</table>

Ph-K8-S431

Pan-K

Ph-p44/42

P44/42

Ph-PKM2

PKM2

Ph-Akt

Ph-PKD2

PKD2

Ph-Akt

Akt
Fig. S5

K8-mRNA expression [% of maximum]

- siCon
- siK8

Timepoints:
- 24 h
- 48 h
- 72 h
A. Random Migration

![Graph showing cell velocity over time with and without SPC treatment.]

B. Images showing cellular morphology with and without SPC treatment over different time points.

C. Bar graph showing the percentage of perinuclear and ramified cells with SPC treatment over durations of 45' to 16h.

D. Bar graph showing the percentage of perinuclear and ramified cells without SPC treatment over durations of 45' to 16h.
A. CFP-K8wt
   Actin
   Paxillin

CFP-K8SE
   Actin
   Paxillin

B. Average FA size per cell [%]

   K8wt | K8SE
   ----|----
   70  | 90

   Number of FA per cell [%]

   K8wt | K8SE
   ----|----
   50  | 70

n.s. **