Prolactin upregulates sphingosine kinase-1 expression and activity in the human breast cancer cell line MCF7 and triggers enhanced proliferation and migration

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Abstract

Sphingosine kinases (SK) catalyze the formation of sphingosine-1-phosphate (S1P) which plays a crucial role in cell growth and survival. Here, we show that prolactin (PRL) biphasically activates the SK-1, but not the SK-2 subtype, in the breast adenocarcinoma cell-line MCF7. A first peak occurs after minutes of stimulation and is followed by a second delayed activation after hours of stimulation. A similar biphasic effect on SK-1 activity is seen for 17 β -estradiol (E₂). The delayed activation of SK-1 derives from an upregulated mRNA and protein expression and is due to increased SK-1 promoter activity and mechanistically involves STAT5 activation as well as protein kinase C and the classical mitogen-activated protein kinases. Furthermore, glucocorticoids also block both hormone-induced SK-1 expression and activity. Functionally, long-term stimulation of MCF7 cells with PRL or E₂ is well known to trigger increased cell proliferation and migration. Both hormone-induced cell responses critically involve SK-1 activation since the depletion of SK-1, but not SK-2, by siRNA transfection abolishes the hormone-induced cell proliferation and migration. In summary, our data show that PRL and E₂ cause a pronounced delayed SK-1 activation which is due to increased gene transcription, and critically determines the capability of cells to grow and move. Thus, the SK-1 may represent a novel attractive target for anti-tumor therapy.

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Introduction

Sphingosine-1-phosphate (S1P) has turned out to be a bioactive lipid critically involved in various cell responses such as cell proliferation and differentiation, survival, and migration (Huwiler *et al.* 2000, Spiegel & Milstien 2003, Hait *et al.* 2006). Although S1P may exert its effects via both intracellular and extracellular actions and targets, most of the so-far-described cellular responses triggered by S1P are mediated through specific cell surface S1P receptors, the S1P_{1–5} receptors, which belong to the class of G-protein-coupled receptors (Hla 2003).

S1P is produced by sphingosine kinases (SKs) which can be activated by a variety of stimuli that couple either to receptor tyrosine kinases like the platelet-derived and

epidermal growth factors (EGFs) or to various G-protein-coupled receptors (Alemany *et al.* 2000, Huwiler *et al.* 2006). To date, two SK subtypes have been cloned and partially characterized, SK-1 and SK-2 (Kohama *et al.* 1998, Liu *et al.* 2000). SK-1 was suggested to be involved in certain forms of cancer (Milstien & Spiegel 2006), whereas SK-2 is speculated to contribute to immunological responses (Baumruker *et al.* 2005). Thus, overexpression of SK-1 leads to a transformed phenotype of NIH 3T3 fibroblasts causing foci formation, colony growth in soft agar, and tumor formation in SCID mice (Xia *et al.* 2000). In addition, overexpression of SK-1 in MCF7 breast cancer cells causes enhanced proliferation, decreased apoptosis, and leads to larger tumors in nude mice in an estrogen-dependent manner (Nava *et al.* 2002,

Sukocheva *et al.* 2003). Moreover, several tumor types showed an overexpression of SK-1 when compared with normal tissue (French *et al.* 2003). All these data strongly suggest a critical contribution of SK-1 in tumor development and growth.

The involvement of the mammogenic hormones prolactin (PRL) and 17 β -estradiol (E₂) in normal mammary growth and development is well documented (Couse & Korach 1999, Ormandy *et al.* 2003) and it has been suggested that these hormones may also contribute to the development and progression of breast cancer. E₂ has been clearly defined as a mammary mitogen, and anti-estrogen therapy is the most successful treatment for patients with estrogen receptor α (ER α) positive breast tumors (Jordan 2004). But this therapy is restricted mainly because of a wide range of side effects of E₂ on bone, brain, cardiovascular, and other targeted tissues. Thus, understanding the signaling pathways that couple mammogenic hormones like PRL and E₂ to a specific cellular function has become a primary focus of inquiry.

In this study, we investigated the contribution of SKs in PRL- and E₂-mediated cell responses in the breast cancer cell line MCF7. We show for the first time that PRL is able to induce a prolonged SK-1, but not SK-2, enzymatic activity which is due to a stimulation of the SK-1 promoter activity causing increased mRNA and protein expression.

Furthermore, cell proliferation and migration, which are both triggered by PRL and E₂ and are critical for tumor development, strongly depend on SK-1 activity since cellular depletion of SK-1 by siRNA transfection abolishes both cell responses.

Materials and methods

Chemicals

[³²P]ATP (specific activity, >5000 Ci/mmol) was from GE Health Care; dexamethasone, PRL, E₂, and 4,6-diamino-2-phenylindole (DAPI) were from Sigma-Aldrich Fine Chemicals; staurosporine and Ro 31-8220 were from Biomol, Hamburg, Germany; U0126 was from Merck Bioscience; monoclonal STAT5a/b antibody was from BD Biosciences, Heidelberg, Germany; siRNA of human STAT5 (sc-29495) was from Santa Cruz, Heidelberg, Germany; OligofectAMINE and all cell culture nutrients were from Invitrogen.

Cell culturing and siRNA transfections

MCF7 cells were cultured as previously reported (Döll *et al.* 2005), and gene silencing of SKs was conducted exactly as described (Huwiler *et al.* 2006).

Western blot analysis

Confluent MCF7 were stimulated for the indicated time periods in phenol red-free Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg/ml fatty acid-free BSA. Protein extracts were prepared exactly as previously described (Döll *et al.* 2005). About 30 μ g protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western blot analysis was performed as previously described using a polyclonal anti-peptide antibody against human SK-1 at a dilution of 1:2000 (Döll *et al.* 2005).

Quantitative real-time PCR (TAQMAN)

About 1.0 μ g total RNA isolated with TRIZOL reagent was used for reverse transcriptase-PCR (First strand synthesis kit, MBI) utilizing an oligo(dT)₁₈ primer for amplification. The real-time PCR was carried out in ABgene plates. Probes and primers for human SK-1, SK-2, and GAPDH were obtained from Applied Biosystems, Darmstadt Germany. The reporter dyes chosen were 6-FAM/Tamra and VIC/Tamra. The PCR buffer used in the experiments was from ABgene. After cDNA synthesis, 100 ng was used for further analysis. The run was performed on the Applied Biosystems 7700 HT sequence detection system. The cycling conditions were as following: 95 °C for 15 min (1 cycle), 95 °C for 15 s, and 60 °C for 1 min (40 cycles). SDS version 1.9.1 software (ABI Prism, Applied Biosystems) was used to analyze real-time and endpoint fluorescence.

Promoter studies

A 2217 bp fragment of the human SK-1 promoter was cloned by RT-PCR using the following primers: forward with a BglIII site: 5'-gaagatctcaccaagtcctcgctggag-3'; reverse with a HindIII site: 5'-gggaagcttttctggagag-gatccctg-3'. A 1053 bp SK-1 promoter fragment was cloned by RT-PCR using the same reverse primer and the forward primer: 5'-gaagatctgcttggttccaatcccgac-3'. A 619 bp SK-1 promoter fragment was cloned by RT-PCR using the same reverse primer and the forward primer: 5'-gaagatctgccttctagccagacgctag-3'.

The promoter fragments were fused into the pGL3 basic luciferase reporter gene-containing vector (Promega). To measure SK-1 promoter activity, a luciferase reporter gene assay was performed exactly as previously described (Huwiler *et al.* 2006) using a Lumat LB9507 luminometer (Berthold Detection Systems, Pforzheim, Germany).

SK activity assay

SK-1 activity was measured exactly as previously described (Döll *et al.* 2005, Huwiler *et al.* 2006). In brief, 30 µg protein extracts and 10 µl of 1 mM sphingosine (dissolved in 1 mg/ml BSA) were mixed with SK-1 buffer (20 mM Tris, pH 7.4, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride) in a total volume of 190 µl. For SK-1 activity, 0.5% Triton X 100 was included to block SK-2 activity (Liu *et al.* 2000). For SK-2 activity, 1 M KCl was included to block SK-1 activity (Liu *et al.* 2000). Reactions were started by addition of 10 µl [γ - 32 P]ATP (10 µCi, 20 mM) in 200 mM MgCl₂ and incubated for 15 min at 37 °C. Reactions were terminated by addition of 20 µl 1 N HCl followed by 800 µl chloroform/methanol/HCl (100/200/1, v/v), 240 µl chloroform and 240 µl of 2 M KCl followed by vigorous vortexing. After phase separation, 50 µl of the lipids in the organic lower phase were resolved by thin layer chromatography with 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v). Spots corresponding to S1P were analyzed and quantified using an

Imaging System (Fujifilm Europe, Düsseldorf, Germany).

Migration assay

Chemotaxis was measured in Nunc cell culture inserts (polycarbonate membrane, 10 mm, 8.0 µm pore size) exactly as described (Döll *et al.* 2005).

Proliferation assay

Transfected cells were starved for 24 h with serum-free DMEM. Proliferation of cells was assessed using 1 µCi/ml [3 H]methyl-thymidine in the absence of serum for the last 4 h of stimulation. After 24 h of stimulation, cells were washed twice with PBS and incubated in 5% trichloroacetic acid at 4 °C for 30 min, and the DNA was solubilized in 0.5 M NaOH for 30 min at 37 °C. Finally, [3 H]thymidine incorporation was determined in a β-counter.

Statistical analysis

Statistical analysis was performed by one-way ANOVA. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

Figure 1 Time-dependent effects of prolactin and 17β-estradiol on SK-1 activity in MCF7 cells. Confluent MCF7 cells were treated with 5 µg/ml prolactin (A and B) or 10 nM 17β-estradiol (C and D) for short-term time periods (0–60 min; A and C) or long-term time periods (1–48 h; B and D). Thereafter, cell lysates were taken for a SK-1 activity assay as described in the Materials and methods section. Data are expressed as percentage of control values and are means ± s.d. ($n=3$), * $P<0.05$, ** $P<0.01$, *** $P<0.001$ when compared with control values.

Results

Stimulation of MCF7 cells with PRL leads to a biphasic increase in SK activity. A first peak of activation occurs after 15 min of stimulation (Fig. 1A) which declines again after 30 min. A second peak of activity is observed after 1 h of stimulation reaching a plateau after 24 h and stays elevated for at least 48 h the latest time point measured (Fig. 1B). The delayed activation of SK-1 by PRL after 16 h is only slightly seen at 50 ng/ml of concentration but is more pronounced at 100 ng/ml and reaches maximal values at 1 µg/ml PRL (Fig. 2A). Another mammogenic hormone E₂ is also able to induce a similar biphasic activation of SK-1 in MCF7 cells (Fig. 1C and D) which confirms previous data of Sukocheva et al. (2003, 2006). Dose-dependency experiments reveal a maximal effect on SK-1 activity after 16 h already at

Figure 2 Dose-dependent effects of prolactin and 17β-estradiol on SK-1 and SK-2 activities in MCF7 cells. Confluent MCF7 cells were stimulated with the indicated concentrations of prolactin (A) or 17β-estradiol (B) for 16 h. Thereafter, cell lysates were taken for an *in vitro* kinase assay for SK-1 activity (closed symbol) or SK-2 activity (open symbol) as described in the Materials and methods section. Results are expressed as percentage of control values and are means ± s.d. ($n=3-6$), * $P<0.05$ when compared with control values. SK-1 activity in control samples was at 13.9 ± 1.9 c.p.m./min per mg protein; SK-2 activity of control samples was at 2.15 ± 0.44 c.p.m./min per mg.

the low concentration of 10 nM E₂ (Fig. 2B). In contrast, SK-2 activity, which was assayed under conditions where SK-1 is inhibited, i.e. by including 1 M KCl (Liu et al. 2000), is not activated by PRL or E₂ (Fig. 2A and B, open symbols).

To see whether the second delayed phase of SK activation depends on increased SK-1 protein expression, Western blot analyses were performed, using a specific antibody against SK-1 which was previously characterized (Döll et al. 2005, Huwiler et al. 2006).

As seen in Fig. 3A, PRL stimulation of MCF7 cells leads to a dose-dependent increased expression of the 44 and 51 kDa form of SK-1 (Fig. 3A). Similarly, E₂ also upregulates SK-1 protein expression (Fig. 3B). To further characterize whether the enhanced SK1 activity and protein expression is preceded by an enhanced mRNA expression, quantitative real-time PCR was performed. As shown in Fig. 3C, SK-1 mRNA expression is dose-dependently enhanced by PRL and also by E₂. In contrast, SK-2 mRNA expression is rather downregulated by hormone treatment.

The increased mRNA expression of SK-1 is owing to increased gene transcription as shown by SK-1 promoter studies. To this end, a long (2217 bp) and two shorter (1053 and 691 bp) fragments of the human SK-1 promoter were cloned according to Nakade et al. (2003) and fused to a luciferase-containing vector. Transfection of MCF7 cells with these fragments followed by PRL or E₂ stimulation reveals that only the long 2217 bp promoter fragment is able to increase luciferase activity, whereas the two shorter fragments are ineffective (Fig. 4).

To further document that the delayed activation of SK-1 by PRL and E₂ is due to increased *de novo* protein synthesis, the general translation inhibitor cycloheximide (CHX) was applied. As seen in Fig. 5A, CHX completely abolishes the delayed activation of SK-1 by both PRL and E₂. In contrast, CHX has no reducing effect of the short-term PRL- or E₂-stimulated SK-1 activity (Fig. 5B), whereas the MEK inhibitor U0126 completely abolishes the early SK-1 activation (Fig. 5B), confirming previous reports that the rapid activation of SK-1 requires the classical mitogen-activated protein kinase (MAPK) cascade.

The mechanisms of delayed SK-1 activation were further characterized. The MEK inhibitor U0126 not only suppresses the early effect of PRL on SK-1 activity (Fig. 5B), but also blocks the delayed effect (Fig. 6A). Similarly, the potent but unspecific protein kinase C (PKC) inhibitor staurosporine and the highly specific PKC inhibitor Ro 31-8220 completely abolish the effect of PRL on SK-1 activity (Fig. 6A). All these inhibitors that reduced SK-1 activity also

Figure 3 Effect of prolactin and 17 β -estradiol on SK-1 protein and mRNA expression in MCF7 cells. MCF7 cells were stimulated for 16 h with either vehicle (Co) or the indicated concentrations of prolactin (A) or 17 β -estradiol (B). Thereafter, cell extracts were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and subjected to western blot analysis using a specific anti-SK-1 antibody at a dilution of 1:2000. Bands were visualized by using the ECL detection system. Data are representative of three independent experiments giving similar results. (C) Stimulated MCF7 cells were taken for RNA extraction and subjected to quantitative real-time PCR of hSK-1 (closed columns) or hSK-2 (open columns) as described in the Materials and methods section. Results are expressed as percentage of control values and are means $\pm$ S.E.M. ($n=3$), *** $P<0.001$ when compared with the control hSK-1 values; ## $P<0.01$, ### $P<0.001$ when compared with the control hSK-2 values.

downregulated SK-1 mRNA expression (data not shown). Similarly, E₂-stimulated delayed SK-1 activity also involves PKC and the classical MAPKs (Fig. 6B).

Furthermore, since PRL has previously been reported to activate the Janus kinase (JAK)–STAT pathway especially involving STAT5 (Liu *et al.* 1995, Hynes *et al.* 1997), the involvement of STAT5 in SK-1 activation was investigated by depleting STAT5 by siRNA transfection. As seen in Fig. 7A inset, the STAT5 siRNA completely blocks STAT5 protein expression. Under this STAT5-depleted condition, PRL-triggered delayed SK-1 activity is abolished, suggesting that STAT5 is a critical transcription factor involved in PRL-stimulated delayed SK-1 upregulation. In contrast, depletion of STAT5 has no effect on the acute activation of SK-1 (Fig. 7B).

Interestingly, the synthetic glucocorticoid dexamethasone also reduces both PRL- and E₂-induced delayed SK-1 activity (Fig. 8) and mRNA expression (data not shown).

Increased proliferation and cell migration is not only characteristic for cancer cells but also normal breast epithelial cells are characterized by their ability to migrate and proliferate during the lactation cycle. To see whether SK-1 is involved in these cell responses, we depleted SK-1 from MCF7 cells by using the technique of siRNA transfection. As previously shown, transfection of MCF7 cells with siRNA targeted to SK-1 specifically reduced the expression of SK-1 without affecting SK-2 (Döll *et al.* 2005). For MCF7 cells, both PRL and E₂ are well-established growth factors causing

an increased [³H]thymidine incorporation into newly synthesized DNA (Fig. 9). However, when SK-1 was depleted from the cells, neither PRL nor E₂ triggered cell proliferation anymore (Fig. 9), whereas neither a scrambled oligonucleotide nor siRNA of SK-2 affected the PRL- and E₂-stimulated proliferation (Fig. 9).

Finally, we measured the migratory capacity of MCF7 cells upon PRL and E₂ stimulation. As seen in Fig. 10,

Figure 4 Effect of prolactin and E₂ on SK-1 promoter activity in MCF7 cells. Subconfluent MCF7 cells were co-transfected with the wild-type 2217 bp SK-1 promoter DNA, a 1053 bp fragment, or a 691 bp fragment plus the plasmid coding for Renilla luciferase (PRL-CMV). After 24 h of transfection, cells were rendered serum-free and stimulated for 16 h with either vehicle (Co), prolactin (PRL, 5 μ g/ml), or 17 β -estradiol (E₂, 1 μ M). The ratio between beetle and Renilla luciferase activities was calculated and is depicted as relative luciferase activity (RLU). Data are means $\pm$ S.E.M. ($n=3$), *** $P<0.001$ when compared with the vehicle-stimulated control value.

Figure 5 Effect of cycloheximide on prolactin- and 17 β -estradiol-induced SK-1 activity in MCF7 cells. (A) Cells were stimulated for 24 h with either vehicle (Co), 5 μ g/ml prolactin (PRL), or 10 nM 17 β -estradiol (E₂) in the absence (–, closed columns) or presence (+, open columns) of 100 ng/ml cycloheximide (CHX). (B) Cells were pretreated for 20 min with either vehicle, U0126 (20 μ M), or cycloheximide (CHX, 100 ng/ml) prior to stimulation for 15 min with 5 μ g/ml prolactin (PRL) or 10 nM 17 β -estradiol (E₂). Thereafter, cell lysates were taken for an SK-1 activity assay as described in the Materials and methods section. Results are expressed as percentage of control values and are means $\pm$ s.d. ($n=3$), * $P<0.05$, ** $P<0.01$, *** $P<0.001$ considered statistically significant when compared with control values; ## $P<0.01$, ### $P<0.001$, when compared with the stimulated values in the absence of CHX.

PRL and E₂ triggered a pronounced increase of cell migration. This effect is significantly reduced when depleting cells of SK-1, but not when using scrambled siRNA oligonucleotides or siRNA of SK-2. Additionally, CHX treatment abolishes both the PRL and E₂ effect on cell migration (Fig. 11A), whereas the depletion of STAT5 from cells only abrogates the PRL effect, but not the E₂ effect on cell migration (Fig. 11B).

Discussion

In this study, we show for the first time that the mammogenic and lactogenic hormone PRL activates SK-1 in MCF7 breast cancer cells. PRL triggers a biphasic activation pattern of SK-1, i.e. a rapid and

Figure 6 Effect of various inhibitors on prolactin- and estradiol-induced SK-1 activity in MCF7 cells. (A) MCF7 cells were pretreated for 20 min with either vehicle (–), Ro 31-8220 (Ro, 10 nM), staurosporine (stau, 10 nM), or U0126 (U, 10 μ M) prior to stimulation for 16 h with either vehicle (–), prolactin (PRL, 5 μ g/ml; A), or 17 β -estradiol (E₂, 10 nM; B). Cell extracts were taken for an SK-1 activity assay as described in the Materials and methods section. Results are expressed as percentage of control values and are means $\pm$ s.d. ($n=3$), ** $P<0.01$ when compared with unstimulated control values; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ when compared with the PRL- or E₂-stimulated values respectively.

transient first peak and a second more delayed peak of activity occurring after 20 h and lasting up to 48 h.

PRL is one important hormone required for mammary gland differentiation promoting morphological development and milk protein production in the lactating gland. Recent evidence has risen that PRL also plays a role in breast cancer development and growth (Tworoger & Hankinson 2006). In rodents, PRL shows a clear tumor-promoting efficiency as transgenic female mice overexpressing the PRL gene spontaneously develop mammary carcinomas (Rose-Hellekant et al. 2003), whereas in humans, the role of PRL in tumor formation and progression is less clear due to the failure of clinical trials evaluating the potential of functional PRL antagonists. Thus, although dopamine agonists (*in vivo* dopamine is the physiological negative regulator of PRL release from the anterior pituitary gland) drastically reduce circulating

Figure 7 Effect of STAT5 depletion by siRNA transfection on prolactin-induced SK-1 activity in MCF7 cells. MCF7 cells were transfected with either vehicle (control) or siRNA of human STAT5 as described in the Materials and methods section. Thereafter, cells were rendered serum-free and stimulated for 24 h with either vehicle (–) or 5 µg/ml prolactin (PRL, +) (A), or (B) for 15 min with vehicle (Co), 5 µg/ml prolactin (PRL) or 10 nM 17β-estradiol (E₂). Cell extracts were taken either for SDS-PAGE, protein transfer, and Western blot analysis of STAT5 (at a dilution of 1:250; inset) or for an SK-1 activity assay as described in the Materials and methods section. Results are expressed as percentage of control values and are means ± s.d. (*n* = 3), ****P* < 0.001 when compared with unstimulated control values; ###*P* < 0.001 when compared with the PRL-stimulated values.

PRL levels, no therapeutic benefit in breast cancer patients is seen by dopamine agonists (Bonneterre *et al.* 1988).

Besides PRL, the SK-1 and its product S1P have also been proposed to play a critical role in tumor development and growth. Thus, S1P is a potent mitogen for many cell types (Huwiler *et al.* 2000, Spiegel & Milstien 2003). Furthermore, overexpression of SK-1 leads to a transformed phenotype of NIH 3T3 fibroblasts causing tumor formation in SCID mice (Xia *et al.* 2000). In addition, overexpression of SK-1 in MCF7 breast cancer cells causes enhanced proliferation, decreased apoptosis, and leads to larger tumors in nude mice in an estrogen-dependent manner (Sukocheva *et al.* 2003).

All these data made it very tempting to speculate on a relation between PRL and SK-1. The upregulation of

Figure 8 Effect of dexamethasone on prolactin- and 17β-estradiol-induced SK-1 activity in MCF7 cells. Confluent MCF7 cells were stimulated for 24 h with either vehicle (Co), 5 µg/ml prolactin (PRL), or 10 nM 17β-estradiol (E₂) in the absence (–, closed columns) or presence (+, open columns) of dexamethasone (Dexa, 100 nM). Cell extracts were taken for an *in vitro* SK-1 activity assay. Results are expressed as percentage of control values and are means ± s.d. (*n* = 3), ***P* < 0.01 when compared with unstimulated control values; #*P* < 0.05 when compared with the PRL- or E₂-stimulated values respectively.

SK-1 by PRL reported in this study was shown to mechanistically involve an activation of the *SK-1* gene promoter (Fig. 4). The human SK-1 promoter was first cloned by Nakade *et al.* (2003) and it was shown to be highly stimulated by the direct PKC activator TPA. The involvement of PKC in PRL-induced SK-1 upregulation is confirmed by the blocking effect of the selective PKC inhibitor Ro 318220 (Fig. 6A). In agreement with these findings, Marte *et al.* (1994) reported that PRL signaling involves PKC-α activation

Figure 9 Effect of SK-1 and SK-2 depletion by siRNA transfections on prolactin and 17β-estradiol-induced proliferation of MCF7 cells. MCF7 cells were either left untransfected (–) or transfected with 200 nM of a scrambled siRNA (Scr-siRNA), siRNA of human SK-1 (SK1-siRNA), or siRNA of human SK-2 (SK2-siRNA) as described in the Materials and methods section. Thereafter, cells were rendered serum-free for 48 h followed by a 24-h stimulation period with either vehicle (Co, open columns), prolactin (PRL; 5 µg/ml, hatched columns), or 17β-estradiol (E₂; 10 nM; closed columns). Cell proliferation was determined as described in the Materials and methods section. Data are means ± s.d. (*n* = 3), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 when compared with the respective unstimulated control values.

Figure 10 Effect of SK-1 and SK-2 depletion by siRNA transfection on prolactin- and 17 β -estradiol-induced migration of MCF7. MCF7 cells were either left untransfected (open columns) or transfected with 200 nM siRNA of human SK-1 (closed columns), human SK-2 (hatched columns), or a scrambled siRNA (gray columns) as described in the Materials and methods section. Thereafter, cells were rendered serum-free for 24 h and stimulated with either vehicle (Co), 5 μ g/ml PRL, or 10 nM E₂ and allowed to migrate through 8 μ M membrane inserts for 24 h. Data are means $\pm$ s.d. ($n=3$), *** $P<0.001$ when compared with the unstimulated control values; # $P<0.05$ when compared with the untransfected PRL-stimulated values; \$\$\$ $P<0.001$ when compared with the untransfected E₂-stimulated values; ns, not significant when compared with the untransfected PRL or E₂ values.

in the mouse mammary epithelial cells HC11. Similarly, Franklin *et al.* (2000) reported that PKC- α mediates PRL-stimulated aspartate aminotransferase expression in rat lateral prostate epithelial cells. On the other hand, Peters *et al.* (1999) reported that in a luteinized granulosa cell model, PRL receptor activation rather promotes activation of the PKC- δ isoenzyme. Which PKC isoenzyme is finally involved in PRL-triggered SK-1 upregulation in MCF7 cells cannot be concluded unequivocally from our data. However, the fact that staurosporine, which shows a high selectivity for the Ca²⁺-dependent PKC isoenzymes when compared with the Ca²⁺-independent PKC isoenzymes (Meyer *et al.* 1989), blocks PRL-induced SK-1 activity in the low nanomolar range (Fig. 6) rather favors the involvement of a Ca²⁺-dependent PKC isoenzyme, i.e. PKC- α which is the only Ca²⁺-dependent PKC isoenzyme expressed in MCF7 cells (Marino *et al.* 2001). An involvement of PKC- α in SK-1 mRNA upregulation and activation has also been shown for histamine-stimulated endothelial cells (Huwiler *et al.* 2006); thus, a general regulatory function of this PKC subtype in SK-1 expression may be envisioned.

Recently, Perks *et al.* (2004) reported that in MCF7 cells, PRL acts as a survival factor and protects cells from ceramide-induced apoptosis. However, they could not find a proliferative effect of PRL in MCF7

Figure 11 Effect of cycloheximide and STAT5 depletion on prolactin- and 17 β -estradiol-induced migration of MCF7. (A) MCF7 cells were stimulated for 24 h with either vehicle (DMEM), 5 μ g/ml prolactin (PRL), or 10 nM E₂ in the absence (open columns) or presence (closed columns) of 100 ng/ml cycloheximide. (B) MCF7 cells were transfected with either vehicle (open columns) or siRNA of human STAT5 (closed columns) prior to stimulated for 24 h with either vehicle (DMEM), 5 μ g/ml prolactin (PRL), or 10 nM E₂. Cells were allowed to migrate through 8 μ M membrane inserts for 24 h. Data are means $\pm$ s.d. ($n=3$), ** $P<0.01$, *** $P<0.001$ when compared with the unstimulated control values; ### $P<0.001$ when compared with the PRL-stimulated values; \$\$\$ $P<0.001$ when compared with the E₂-stimulated values; ns, not significant when compared with the E₂-stimulated values.

cells which contrasts to other reports in the same cell system (Bernard *et al.* 1991, Acosta *et al.* 2003; and our finding (Fig. 9)) and *in vivo* (Chen *et al.* 2002). Although the mechanism of this PRL-mediated cell protection was not further addressed, it is tempting to speculate that PRL stimulated S1P generation, which is a well-known anti-apoptotic factor and counterregulator of ceramide action (Hait *et al.* 2006).

Similar to PRL, two other mammogenic factors also activate SK-1 in the same biphasic manner in MCF7 cells, i.e. E₂ (Sukocheva *et al.* 2003, 2006; and this study) and EGF (Döll *et al.* 2005). Interestingly, all three agonists act via different receptor classes. Whereas the PRL receptor (PRLR) belongs to the

class of cytokine receptor superfamily and classically, but not exclusively couples to JAK/STAT, the EGF receptor belongs to the class of receptor tyrosine kinases and couples to various signaling devices including phospholipase C- γ and PI3K. E₂ primarily acts through specific high-affinity ERs including α - and β -isoforms located within the cell nucleus. Although still controversial, many studies have shown an association of the PRLR with the ER receptor expression. Thus, PRLR and ER α are co-expressed in many breast tumors and PRL and E₂ can cross-regulate their receptors in breast cancer cells (Shafie & Brooks 1977, Ormandy *et al.* 1997, Gutzman *et al.* 2004a). Furthermore, it was reported that E₂ contributes to tumor development by trans-activating the EGF receptor via S1P generation and S1P₃ receptor activation (Sukocheva *et al.* 2006).

All the three mammogenic agonists cause not only SK-1 activation but also increased proliferation and migration of MCF7 cells and these cell responses depend critically on SK-1, but not on SK-2. This is based on the findings that selective depletion of SK-1, but not SK-2, by siRNA transfections reduces MCF7 proliferation and migration in response to all three agonists (Figs 9 and 10 of this study; Döll *et al.* 2005). Since all three agonists activate the *SK-1* gene promoter, the question arises whether the same transcription factors are involved. SK-1 promoter analysis reveals the existence of several potential transcription factor-binding sites such as for Sp1, AP-1, AP-2, and AP-4. Recently, it was shown that PRL can activate AP-1 in breast cancer cells (Olazabal *et al.* 2000, Gutzman *et al.* 2004b), and also EGF is a well-known activator of c-Jun and c-Fos which are the relevant constituents forming AP-1 (Manfroid *et al.* 2005). Moreover, E₂ can stimulate the recruitment of ER α and its cofactor p300 to the AP-1 site (Jeffy *et al.* 2005). In addition, an interaction of PRL and E₂ was reported to cause an enhanced AP-1 activity by increasing MAPK/ERK and c-Fos phosphorylation, and c-Fos promoter activity (Gutzman *et al.* 2004b, Gutzman 2005).

Furthermore, a critical involvement of the STAT5 transcription factor in PRL-induced SK-1 upregulation and activation became evident from this study (Fig. 7A). In this context, it has been extensively shown that PRL is an activator of the STAT5 transcription factor (Liu *et al.* 1995, Hynes *et al.* 1997) and that various PRL-regulated genes contain a STAT-binding site in their promoter sequences (Groner & Gouilleux 1995). Sequence analysis of the SK-1 promoter indeed reveals such a putative STAT-binding site and future experiments will be needed to show whether this binding element is functional and able to bind STAT5.

Our data further suggest that especially the delayed second phase of SK-1 activation may be involved in

the process of migration. This is stressed by the finding that either STAT5 depletion (Fig. 7) or incubation of cells in the presence of CHX (Fig. 5A) abolishes the delayed SK-1 activation as well as the migratory capacity of the cells (Fig. 11), whereas the acute PRL-triggered SK-1 activation still occurs.

Interestingly, PRL- and also E₂-stimulated SK-1 upregulation is prevented by the glucocorticoid dexamethasone. The mechanism of this suppressive effect is presently unclear. However, various studies have provided evidence that glucocorticoids can interact with transcription factors such as NF κ B and thus prevent its nuclear translocation. Furthermore, glucocorticoids can induce the NF κ B-inhibitory protein- α (I κ B- α) and thereby prevent NF κ B activity (Auphan *et al.* 1995). Indeed, a potential NF κ B DNA-binding element is found in the 5'-flanking region of the human *SK-1* gene although the functionality of this element still needs to be proven. *In vivo* glucocorticoids not only act as immunosuppressive and anti-inflammatory agents but are also effective in the treatment of leukemia as they act as inducers of apoptosis (Distelhorst 2002). It is tempting to speculate that glucocorticoids promote apoptosis by suppressing SK-1 expression and activity and thereby reduce cellular S1P, which is considered not only a mitogenic factor but also an anti-apoptotic factor.

In summary, our data have shown that mammogenic hormones including PRL, estrogen, and EGF all induce SK-1 mRNA and protein expression via promoter activation which essentially contributes to increased cell proliferation and migration of breast cancer cells. Thus, the SK-1 may represent a novel and attractive pharmacological target to interfere with cancer cell growth and migration.

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