The secretory leukocyte protease inhibitor gene is a target of epidermal growth factor receptor action in endometrial epithelial cells

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Abstract

The over-expression of epidermal growth factor receptor (EGFR) and its ligands, epidermal growth factor (EGF) and transforming growth factor- α , is a common feature of epithelial carcinomas and correlates with neoplastic progression. Secretory leukocyte protease inhibitor (SLPI), a member of the Kazal superfamily of serine anti-proteases, induces proliferation and promotes malignancy of epithelial cells and is expressed at high levels in multiple tumor types. In the present study, we have demonstrated that EGF increases SLPI expression in the human endometrial epithelial cell line Ishikawa in a dose- and time-dependent manner. We have shown that this effect of EGF occurs, in part, at the level of the SLPI promoter

and involves the MAP kinase signaling pathway. We have further shown that EGF promotion of cell proliferation, but not induction of cyclin D1 gene expression, involves SLPI. Our results suggest that the regulation of SLPI expression by EGFR ligand(s) may represent a 'feed-forward' mechanism by which the enhanced proliferative and migratory properties of EGFR over-expressing cancer cells are sustained. Increased SLPI expression is likely an important component of altered EGFR signaling in human tumors and may have significant therapeutic implications in cancer progression.

Journal of Endocrinology (2005) **184**, 141–151

Introduction

Secretory leukocyte protease inhibitor (SLPI), a 12 kDa protein secreted by epithelial cells at mucosal surfaces and a member of the Kazal superfamily of serine protease inhibitors, inhibits the activities of elastase, cathepsin G, trypsin, and chymotrypsin via the carboxy-terminal portion of its two homologous cysteine-rich domains (Thompson & Ohlsson 1986, Abe *et al.* 1991, Potempa *et al.* 1994). SLPI plays important and diverse roles in cellular immunity, inflammatory response, normal development, reproduction, and tissue growth (Hiemstra *et al.* 1996, Zhang *et al.* 1997, Denison *et al.* 1999, Song *et al.* 1999, Ashcroft *et al.* 2000, Wang *et al.* 2003). While the specific mechanisms underlying the multiple actions of SLPI remain largely unknown, increasing data have emerged to support the growth regulatory functions of this protease inhibitor. Over-expression of SLPI has been noted in human tumors and human carcinoma cell lines of ovarian, endometrial, respiratory tract, and neural origins (Koshikawa *et al.* 1996, Ameshima *et al.* 2000, Hough *et al.* 2001, Shigemasa *et al.* 2001, Westin *et al.* 2002). In endometrial clonal cell lines engineered to under-express

SLPI by forced expression of antisense SLPI mRNA, a direct linkage between cellular SLPI production and proliferation has been observed (Zhang *et al.* 2002a). In these cell lines, expression of SLPI was positively correlated with increased expression of the cell cycle progression factors cyclin D1 and proliferating nuclear antigen, and was inversely correlated with expression levels of the epithelial growth inhibitors, transforming growth factor- β (TGF- β) and insulin-like growth factor-binding protein (IGFBP)-3, and of the tumor suppressor lysyl oxidase (Zhang *et al.* 2002a). A causal role for SLPI in the promotion of malignant behavior was also recently demonstrated in lung carcinoma cells stably transfected with mouse or human SLPI expression constructs (Devoogdt *et al.* 2003). This function of SLPI was attributed largely to its anti-protease activity, and was hypothesized to occur via SLPI inhibition of the elastase-mediated processing of the angiogenesis inhibitor endostatin (O'Reilly *et al.* 1997).

The epidermal growth factor (EGF) family of polypeptide hormones, which includes EGF, TGF- α , amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin, regulates cell proliferation,

motility, differentiation, and survival by modulating the expression of many cell growth-related genes that control cell cycle progression (Yarden 2001). This action is mediated by four members of the EGF receptor (EGFR) family of transmembrane receptor tyrosine kinases: ErbB-1, also called EGFR, ErbB-2 (also called HER2 or neu), ErbB3, and ErbB4. Auto-phosphorylation of these proteins upon binding of their ligands triggers the activation of multiple signaling cascades resulting in altered gene transcription (Alroy & Yarden 1997, Hackel *et al.* 1999). The activation of particular signaling pathways (ras/raf/MAP kinase (MAPK), Akt/protein kinase B, and STAT) by activated EGFR is dependent on cell context (Zhong *et al.* 1994, Li *et al.* 2002, Schafer *et al.* 2004). The EGFR has been localized to the nucleus in many tissues and cell lines (Carpenter 2003), where it was demonstrated to transactivate cyclin D1 promoter (Lin *et al.* 2001). Although the significance of nuclear EGFR in the global expression of growth-regulatory genes is not well understood, the findings that EGFR and its ligands are commonly amplified and/or over-expressed in cancer cells (Jasonni *et al.* 1995, Niikura *et al.* 1996, Pfeiffer *et al.* 1997, Yarden 2001) support their patho-physiological roles in tumor initiation and progression.

In light of the coincident expression of SLPI, EGFR, and EGFR ligands in many tumors and carcinoma cell lines, we hypothesized that the induction of SLPI gene expression may represent one mechanism by which EGFR signaling enhances cell proliferation. In the present study we have shown that EGF increases SLPI gene expression in a dose- and time-dependent manner, that this occurs, in part, at the level of SLPI transcription, and that MAPK signaling, while involved in this inductive process, does not directly modulate SLPI promoter activity. We have further shown that EGF induction of Ishikawa cell proliferation, but not of cyclin D1 gene expression, is mediated by SLPI. Increased levels of SLPI may thus facilitate cell cycle progression by an as yet unknown mechanism, leading to the enhanced proliferative and migratory properties of EGFR over-expressing cancer cells.

Materials and Methods

Materials

Reagents, enzymes, and laboratory supplies were purchased from the following vendors: ULTRAhyb reagent from Ambion (Austin, TX, USA); restriction enzymes from Roche Molecular Biochemicals (Indianapolis, IN, USA); nick-translation kit from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, USA); [α - 32 P]dCTP (3000 Ci/mmol) and Biotrans nylon membranes (0.2 μ m) from ICN Radiochemicals (Irvine, CA, USA); MAPK inhibitor (MKI) PD98059 from Cell Signaling Technology Inc. (Beverly, MA, USA); CellTiter 96 AQueous non-

radioactive cell proliferation assay kit from Promega Corp. (Madison, WI, USA); cell culture media and supplies and TriZol reagent from Invitrogen (Carlsbad, CA, USA); antibiotic/antimycotic (ABAM) solution and glutamine from GIBCO (Carlsbad, CA, USA); and oligonucleotides from Integrated DNA Technologies, Inc. (Coralville, IA, USA). All other molecular reagents and solvents, when not listed, were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Cell culture

The human endometrial epithelial carcinoma cell line Ishikawa (courtesy of Dr Bruce Lessey, Greenville, SC, USA) was routinely grown at 37 °C in an atmosphere of 5% CO₂/95% air in minimal essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% ABAM. Medium was replaced every 2–3 days, and cells were split after reaching confluence. For growth factor addition studies, cells were seeded in six-well plates at a density of 2×10^5 cells per well. Sub-confluent cells (80%) were serum-starved for 24 h prior to treatment with vehicle (phosphate-buffered saline; PBS), recombinant human EGF (rhEGF) (R&D Systems Inc., Minneapolis, MN, USA), recombinant human TGF- α (rhTGF- α) (R&D Systems) or recombinant human insulin-like growth factor-I (rhIGF-I; Upstate Biotechnology, Lake Placid, NY, USA) at the concentrations indicated (10–100 ng/ml). All treatments were performed under low serum-containing medium (0.5% FBS), unless otherwise indicated. For antibody treatments, goat anti-recombinant human SLPI IgG (R&D Systems) and rabbit anti-recombinant rat basic transcription element binding protein-1 (BTEB1) IgG (Zhang *et al.* 2002b) were added to cells at a dose of 2.5 μ g/well 30 min prior to the addition of vehicle or rhEGF (50 ng/ml). For gene expression and protein studies, cells were collected 4, 6, or 24 h after treatment. For proliferation assays, cell density was evaluated 24 h after treatment. Images of cell densities were captured at 200 $\times$ magnification using the Spot program, Version 4.0 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Pictures were adjusted to 800 $\times$ 600 pixels using Adobe Photoshop, and the area occupied by cells (in mm²) was measured using the Scion Image program, Beta 4.0.2 (Scion Corporation, Frederick, MD, USA). All experiments were repeated at least three times, with each experiment performed in triplicate.

RNA isolation, Northern blot analysis and quantitative RT-PCR

Total cellular RNA was prepared from Ishikawa cells using the TriZol reagent following the manufacturer's protocol. Total RNA (30 μ g/sample) was electrophoresed in 1% agarose/formaldehyde gels in 1 $\times$ 3-(N-morpholino) propanesulphonic acid (MOPS) buffer and blotted onto BioTrans membranes using the TurboBlotting

system (Schleicher & Schuell, Keene, NH, USA). RNA was immobilized to the membranes by u.v. cross-linking for 90s followed by baking at 80 °C for 25 min. The pre-hybridization and hybridization of membranes with ³²P-labeled human SLPI cDNA fragment (399 bp) using ULTRAhyb followed previously described protocols (Zhang *et al.* 2002a). Membranes were washed twice in 2 × SSC, 0.1% SDS and in 0.1 × SSC, 0.1% SDS at 42 °C for 15 min each time to remove non-specifically bound probe. Resultant hybridization signals on X-ray films were quantified using the Alpha Imager 2000 documentation and analysis system (Alpha Innotech Co., San Leandro, CA, USA). To adjust for loading differences among samples, the membranes were stripped with 1% SDS at 90 °C, and re-probed with ³²P-labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment (971 bp).

mRNA levels were also determined by real-time quantitative RT-PCR (qPCR). Total RNA was quantified and analyzed for integrity using the Agilent 2100 bioanalyzer and RNA 6000 NanoLabChip kit (Agilent Biotechnologies, Palo Alto, CA, USA). RNA samples were reverse-transcribed using random primers and a cDNA synthesis kit following the manufacturer's protocols (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Each primer set was designed to flank an intron to prevent the amplification of genomic DNA, using PrimerExpress (Perkin Elmer Applied Biosystems). The forward and reverse primers respectively and resultant PCR product sizes (in parentheses) were: (a) human SLPI: 5'-GCTGTGGAAGGCTCTGGAAA-3' and 5'-TGCCCATGCAACACTTCAAG-3' (297 bp); (b) human cyclin D1: 5'-AATGACCCCGCACGATTT-3' and 5'-ATGGAGGGCGGATTGGAA-3' (144 bp); (c) human p21^{WAF1}: 5'-CAGCGACCTTCCTCATCCAC-3' and 5'-GAGAAACGGAACAGGACA-3' (155 bp); and (d) human 18S: 5'-TCTTAGCTGAGTGTCGCG-3' and 5'-ATCATGGCCTCAGTTCCGAA-3' (151 bp). The latter was used to evaluate the efficiency and variability of the reverse transcription step. cDNA samples (1/25 of reverse transcription reaction) were amplified using the SYBR Green PCR Master Mix under conditions recommended by the manufacturer (Perkin Elmer Applied Biosystems): (a) preincubation at 50 °C for 2 min; (b) DNA polymerase activation at 95 °C for 10 min; and (c) 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were assayed in duplicate using the ABI Prism 7000 detection system (Perkin Elmer Applied Biosystems). For each primer set, a standard curve was generated by serial dilution of pooled cDNAs. The threshold cycle, which represents the fractional cycle number where the fluorescent signal exceeds background was obtained for each reaction and used to calculate the mean RNA quantity. The melting points of all samples were routinely determined to confirm that the expected products were generated with minimal primer-dimer formation.

Western blot analysis

Cells were harvested and lysed by sonication in ice-cold lysis buffer (10 mM Hepes, pH 7.9; 150 mM NaCl; 1 mM EDTA; 0.6% IGEPAL CA-630 (Sigma Chemical Co.); 0.5 mM phenylmethylsulfonyl fluoride) (Liu *et al.* 1997). Proteins were fractionated on SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane. Immunoblotting was performed with rabbit polyclonal anti-human SLPI antibody (1 µg/ml) that was generously provided by Dr Pieter S Hiemstra (Leiden University, Leiden, The Netherlands). Horseradish peroxidase-conjugated goat anti-rabbit antibody (BioRad; diluted 1:2000 as per the manufacturer's instructions) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) were used to detect immunoreactive bands followed by exposure to X-ray film.

Transient transfection and luciferase assays

Ishikawa cells were seeded at a density of 5×10^4 cells/well in six-well dishes in serum-containing medium and incubated for 24 h to allow cells to adhere. Cells (70% confluent) were transfected using lipofectAMINE reagent (Invitrogen) in OPTI-MEM I reduced serum medium (Invitrogen) for 6 h with SLPI reporter-promoter plasmids (containing 1385, 552, and 248 bp respectively of the 5' regulatory region of the porcine SLPI gene) linked to the reporter plasmid luciferase E (pGL2-E; Promega) or empty vector plasmid (10 µg DNA/well) (Reed *et al.* 1996). After transfection, cells were incubated in serum-containing medium for an additional 18 h, and then transferred to low serum-containing (0.5% FBS) medium with or without added rhEGF (50 ng/ml). The MKI PD98059 (2 µM) was added 30 min prior to rhEGF treatment. In a number of experiments, β-galactosidase expression plasmid pSV-GAL (5 µg/well; Promega) was co-transfected with SLPI-LucE-reporter constructs to evaluate transfection efficiency. Luciferase activity (measured as relative light units; RLU) was measured in cell lysates using the Promega luciferase assay system and an MLX microtiter plate luminometer (Dynex Technologies, Inc., Chantilly, VA, USA). Two independent transfection experiments were performed, with each experiment carried out in triplicate ($n=6$ samples/treatment group). Protein concentration of extracts was determined by the Lowry method using bovine serum albumin as a reference standard. Results were normalized to the protein content of each sample and are presented as least square means ± S.E.M.

MTS cell proliferation assay

Ishikawa cells were seeded at a density of 1.2×10^4 cells/well in 96-well culture plates in MEM containing 10% FBS, and 1 h later, were transferred to low serum

(0.5%)–containing MEM. After overnight incubation at 37 °C in a humidified, 5% CO₂ atmosphere, cells were treated with rhEGF (50 ng/ml) in the presence or absence of anti-recombinant human SLPI IgG (2.5 µg/well) for another 24 h. *In vitro* proliferation assay was performed using the MTS assay kit following the manufacturer's instructions (Promega). The reduction of MTS solution into formazan, which measures the numbers of metabolically active cells, was quantified by obtaining the absorbance at 570 nm.

Statistical analysis

Data obtained from transfection, Northern, qPCR, and cell density assays were analyzed by one-way or two-way ANOVA, followed by inspection of all differences between pairs of means by the Tukey test. Differences were considered significant at $P \leq 0.05$.

Results

EGF and TGF- α induce SLPI mRNA expression in Ishikawa cells

To determine if events downstream of EGFR signaling pathways involve SLPI, the induction of SLPI mRNA expression by the EGFR ligands EGF and TGF- α was examined in Ishikawa cells. SLPI mRNA levels, normalized to those of the housekeeping gene GAPDH, were increased by both growth factors, with EGF showing a greater inductive effect than TGF- α when analyzed by two-way ANOVA ($P < 0.001$) (Fig. 1). At the range of concentrations tested (10, 50, and 100 ng/ml), the effect of EGF was dose-dependent ($P < 0.05$), in contrast to that of TGF- α ($P = 0.118$). rhIGF-I at 100 ng/ml concentration did not affect SLPI mRNA levels in these cells (data not shown).

We next determined whether SLPI mRNA expression is decreased in cells in which EGFR signaling was inhibited. In this experiment, rhEGF was used because of its greater inductive effect on SLPI gene expression than rhTGF- α (Fig. 1). Since the ras/raf/MAPK pathway is a major signaling cascade involved in the proliferative activity of EGFR (Olayioye *et al.* 2000), Ishikawa cells were incubated with rhEGF (50 ng/ml) in the presence or absence of the MKI PD98059 (2 µM). Cells were harvested 24 h after treatment, and analyzed for SLPI mRNA and protein. As shown in Fig. 2 (A and B), EGF increased SLPI mRNA abundance. Treatment of Ishikawa cells with EGF in the presence of MKI reduced the amounts of SLPI mRNA. The inhibitor alone had no effect on basal SLPI mRNA expression (Fig. 2A). The amounts of SLPI protein, which were increased by EGF, were also decreased in cells treated with EGF/MKI, consistent with the effect observed for mRNA (Fig. 2C). MKI alone had

no effect on basal SLPI protein levels. The identity of the higher M_r immunoreactive band is unclear at present, although we have previously reported the presence of a comparably sized protein (~16 kDa) with the same amino-terminal amino acid sequences as authentic SLPI (14 kDa) from the pig uterus (Badinga *et al.* 1999).

EGF increases SLPI promoter activity

To examine if EGF induction of SLPI gene expression occurred at the transcriptional level, luciferase reporter constructs containing 1385, 552, and 248 bp respectively of the 5'-flanking and regulatory region of the porcine SLPI gene (Reed *et al.* 1996) were transiently transfected into Ishikawa cells, which were then treated with rhEGF (50 ng/ml) for 24 h. Previous studies have shown the porcine SLPI gene promoter to exhibit highest homology to human and mouse SLPI gene promoter sequences within the 248 bp region most proximal to the translation initiation site, and less so in more upstream regions (Stetler *et al.* 1986, Reed *et al.* 1996, Kikuchi *et al.* 1998). The longest SLPI-reporter construct (–1385 SLPI-LucE) had robust promoter activity in Ishikawa cells, and this was further increased (by at least 2.5-fold) with EGF ($P < 0.05$) (Fig. 3A). Reporter constructs containing shorter regions of the SLPI promoter (–552 SLPI-LucE; –248 SLPI-LucE) showed basal activities that were comparable with those of the longest construct and were also inducible by EGF. Interestingly, the induction by EGF was greater with the –248 SLPI-LucE construct than with –552 SLPI-LucE ($P < 0.01$) and –1385 SLPI-LucE ($P < 0.001$) constructs respectively. Sequence analysis of the entire 1385 bp promoter region using the Transfac6.0-PublicDatabase (www.generegulation.com/pub/databases.html#transfac) showed the presence of several regulatory motifs that can mediate the transcriptional activation of the SLPI promoter by EGF. In particular, we identified potential activated protein-1 (AP-1), serum-responsive element (SRE) and STAT-inducible element (SIE) motifs that were shown previously to mediate the regulation by EGF of various other gene promoters (Shi & Teng 1996, Kloth *et al.* 2002, Li *et al.* 2003) in the SLPI promoter region (Fig. 3B).

To determine if the enhancement of SLPI promoter activity by EGF is mediated by MAPK signaling, the MKI PD98059 (2 µM) was added to cells previously transfected with the –1385 SLPI-LucE construct 30 min prior to incubation in medium with or without added rhEGF for 24 h. MKI did not affect the basal or the EGF-induced SLPI promoter activities (Fig. 3C).

EGF-induced proliferation of Ishikawa cells involves SLPI

In previous studies (data not shown), we found that rhEGF added at a range of concentrations (10–100 ng/ml) increased DNA synthesis in Ishikawa cells over that of

Figure 1 Regulation of SLPI mRNA levels by EGF and TGF- α . Ishikawa cells were incubated in medium containing 10% FBS (S) or in low serum (0.5% FBS)-containing medium (LS) with or without added rhTGF- α (10–100 ng/ml) or rhEGF (10–100 ng/ml) for 24 h. (A) Total RNA was isolated and analyzed by Northern blot (30 μ g RNA/sample) using 32 P-labeled SLPI or GAPDH cDNA fragments as probe. (B) The bar graphs (least square means $\pm$ S.E.M.) represent the hybridization intensities of the SLPI transcript after normalizing to that of GAPDH. Differences between S and LS were evaluated by *t*-test. Two-way ANOVA (Tukey) was performed to evaluate the effects of treatment, dose, and dose $\times$ treatment interaction for EGF and TGF- α . Asterisks indicate differences at $P < 0.05$ (*) and $P < 0.001$ (**) from LS.

untreated cells, as quantified by labeled thymidine incorporation. To determine if the proliferative activity of EGF is mediated, in part, by its induction of SLPI synthesis, we determined if the mitogenic signal of EGF was inhibited by the addition of anti-SLPI antibody. Ishikawa cells (seeded at 5×10^4 cells/well) in serum-containing MEM for 24 h were transferred to serum-free MEM to synchronize cell cycle stage. After 24 h, cells were incubated in fresh low serum (0.5%)-containing MEM supplemented with vehicle (PBS), rhEGF (50 ng/ml), anti-SLPI IgG (2.5 μ g/well), or a combination of EGF and anti-SLPI IgG. Cells were examined under a light microscope 24 h later, and average cell density was measured for each treatment group by tracing the cell monolayer density in four randomly chosen fields (200 $\times$ magnification)/

culture plate. Results of three independent experiments are summarized in Fig. 4 (A and B). EGF increased cell density over those of control (untreated) cells. Addition of anti-SLPI antibody did not alter cell density, relative to controls. However, the overall cell density in cultures treated with EGF+anti-SLPI IgG was visibly diminished relative to those treated with EGF alone, and was comparable with control cells. An antibody against basic transcription element binding protein-1 (BTEB1), a protein unrelated to SLPI, did not affect basal cell density, when added alone or in combination with rhEGF (data not shown).

To validate the above results, Ishikawa cells were subjected to the same treatments and cell proliferation was measured 24 h later by the MTS assay. In preliminary

Figure 2 The MKI PD98059 inhibits EGF-induced SLPI expression. Ishikawa cells were incubated in low serum-containing medium (0.5% FBS) alone or in the same medium containing rhEGF (50 ng/ml) with (EGF+MKI) or without (EGF) added MKI PD98059 (2 μ M) for 24 h. (A) Total RNA was isolated and analyzed for SLPI mRNA levels (30 μ g RNA/sample) by Northern blot analysis. (B) The bar graphs (least square means $\pm$ S.E.M.) represent the hybridization intensities of the SLPI transcript after normalizing to that of GAPDH, in control cells and cells treated with EGF with and without added MKI. Results presented are from five independent experiments. Values with different subscripts are significantly different ($P < 0.05$). (C) Cells were incubated in low serum-containing medium alone (Control) or in the presence of added rhEGF, rhEGF+MKI, and MKI alone. After 24 h, cells were lysed (Materials and Methods) and equal amounts of protein (100 μ g/sample) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Levels of SLPI protein were determined using a polyclonal antibody against anti-human SLPI. A representative Western blot is shown.

studies (data not shown), we confirmed that the spectrophotometer absorbance at 570 nm was directly proportional to the number of cells in the culture wells. Results from two independent experiments (where each experiment had $n=6$ culture wells/treatment) indicated that while EGF-treated cells had increased absorbance values over those of control cells (0.756 ± 0.009 vs 0.602 ± 0.003 ; $P=0.002$) cells with added anti-SLPI IgG had absorbance units comparable with control cells (0.655 ± 0.021 vs 0.602 ± 0.003 ; $P=0.55$). Cells treated

with EGF+anti-SLPI IgG had absorbance values (0.695 ± 0.008) that tended to be lower than those treated with EGF (0.756 ± 0.009 ; $P=0.07$).

Induction of cyclin D1 by EGF is independent of SLPI

To determine if SLPI mediates EGF induction of cell cycle transit through an effect on cyclin D1 gene expression, we evaluated the temporal correspondence in the induction of cyclin D1 and of SLPI mRNA by EGF at 4 and 24 h after growth factor addition. EGF significantly increased ($P < 0.05$) cyclin D1 mRNA levels over those for control (untreated) cells at 4 h post-treatment (Fig. 5, top panel). Cyclin D1 mRNA levels in cells treated with EGF for 24 h tended to remain higher ($P=0.10$) than in untreated cells, although these levels were lower ($P < 0.05$) than those of 4-h EGF-treated cells. The inductive effect of EGF on SLPI mRNA levels was observed at 24 h and not at 4 h (Fig. 5, middle panel). The abundance of mRNA for p21^{WAF1}, a cell cycle inhibitor, was also evaluated since a previous study has shown increased levels of p21^{WAF1} mRNA in EGFR null epidermal keratinocytes, suggesting inhibition by EGFR ligand of p21^{WAF1} gene expression (Woodworth *et al.* 2000). In Ishikawa cells, p21 mRNA levels did not change from those of untreated cells after incubation with EGF for 4 and 24 h (Fig. 5, bottom panel).

To further evaluate the relevance of SLPI on EGF-induced cyclin D1 gene expression, we next examined the temporal induction by EGF of cyclin D1 gene expression in the presence or absence of anti-SLPI antibody. Cells treated with EGF (50 ng/ml) for 4, 6, and 24 h had higher cyclin D1 mRNA levels than those of corresponding untreated cells or of cells incubated with anti-SLPI IgG (added at 2.5 μ g/well) alone (Fig. 6). Anti-SLPI IgG did not affect basal or EGF-enhanced cyclin D1 gene expression at all incubation times.

Discussion

Results from this study support a model in which the transmission of the EGF/EGFR mitogenic signal in human Ishikawa endometrial epithelial cells involves the up-regulation of SLPI synthesis. Our data showed that: (a) EGFR signaling induced SLPI gene transcription, leading to increased levels of SLPI mRNA and protein; (b) this effect of EGF was mediated, in part, through the MAPK signaling pathway; and (c) EGF-stimulated increase in cell proliferation was mediated, in part, by SLPI. Although a stimulatory role for SLPI in cell proliferation and promotion of tumor progression has been reported previously (Zhang *et al.* 2002a, Devoogdt *et al.* 2003), the present study constitutes the first to link the mitogenic activities of SLPI and EGF in the uterine endometrium. Our findings are consistent with the reported high basal expression of SLPI in carcinoma and transformed cells and tissues

(Shigemasa *et al.* 2001, Westin *et al.* 2002, Schlingemann *et al.* 2003) where over-expression of EGFR and/or its ligands has been similarly observed (Jasonni *et al.* 1995, Niikura *et al.* 1996, Pfeiffer *et al.* 1997).

The induction of SLPI gene expression and proliferation of human epithelial Ishikawa cells by EGF appears to be mediated, in part, by the MAPK signaling cascade. This is suggested by the findings that addition of the MKI PD98059 abrogated EGF-induced SLPI mRNA abundance and that the EGF-mediated increase in cell proliferation (measured by cell density and MTS assay) was diminished by anti-SLPI antibody. Our data are in agreement with a previous report that responsiveness of Ishikawa cells to EGF growth stimulation was lost with the mutation of the K-ras oncogene (Kato *et al.* 1998), a

component of the ras/raf/MAPK signaling cascade. Although we cannot eliminate the involvement of the PI3K pathway, which signals through the activation of the downstream serine/threonine kinase Akt (Mendelsohn & Baselga 2003), in the induction by EGF of SLPI gene expression reported here, the utilization of either pathway has been reported to be highly dependent on cell context (Martin *et al.* 2000, Kruger & Reddy 2003, Shin *et al.* 2003). Indeed, the activation of Akt is largely linked to the anti-apoptotic function of EGFR (Grant *et al.* 2002). Interestingly, PD98059 failed to block EGF induction of SLPI promoter activity, suggesting that other ERK-dependent mechanisms such as an increase in SLPI mRNA stability may also contribute to enhanced SLPI expression. SLPI mRNA transcripts have been reported to be relatively stable, consistent with the potential regulation of SLPI expression at the post-transcriptional level (Abe *et al.* 1991). Alternatively, the promoter constructs used here may not contain all the requisite regions for manifestation of an MKI response. The finding that the promoter activity of the shortest construct (– 248 SLPI-LucE) exhibited the most responsiveness to EGF induction, despite the presence of multiple consensus EGF-responsive motifs in the two longer constructs, suggests several possibilities including: (a) the EGF effect is mediated by non-consensus EGF-response element(s); (b) negative regulatory element(s) that mask the positive effects of EGF are present in the longer constructs; and (c) the upstream regulatory sequences of the porcine SLPI gene, which are distinct from those of the human (Stetler *et al.* 1986), do not contain the functional EGF response elements. However, the latter possibility is not supported by previous findings that TGF- α and, to a lesser extent, EGF stimulated SLPI gene expression in porcine uterine endometrium *in vitro* (Reed *et al.* 1998).

Figure 3 EGF increases SLPI promoter activity. (A) Ishikawa cells were transfected with luciferase promoter-reporter DNA constructs containing 1385, 552, and 248 bp of the porcine SLPI gene promoter with or without added rhEGF (50 ng/ml), as described under Materials and Methods. Reporter activity was normalized to protein content, and results are expressed as least square means $\pm$ S.E.M. from two individual experiments, with each experiment carried out in triplicate. The asterisk (*) indicates significant difference ($P < 0.05$) of EGF-treated from corresponding untreated (– EGF) cells. Significant differences between treatment groups (indicated by brackets) were identified by two-way ANOVA. (B) The sequence of the 1385 bp region of the porcine SLPI promoter was evaluated using the Transfac6.0-Public Database. The locations of putative motifs for AP-1, SIE, and SRE are indicated. (+1) represents translation initiation site. (C) Cells transfected with the – 1385 SLPI-LucE reporter construct were incubated in serum-free medium in the presence or absence of rhEGF (50 ng/ml) with or without added PD98059 (2 μ M). Results (least square means $\pm$ S.E.M.) are from two individual experiments, with each experiment carried out in triplicate. The asterisk (*) indicates significant difference ($P < 0.05$) from corresponding untreated cells.

Figure 4 Effect of anti-SLPI antibody on Ishikawa cell proliferation. Ishikawa cells seeded at equal densities (1×10^5 cells) in 60 mm culture dishes were incubated in low serum (0.5% FBS)-containing medium or in medium containing rhEGF (50 ng/ml), anti-SLPI antibody (Ab) (IgG fraction; 2.5 µg/well), or a combination of rhEGF and anti-SLPI antibody. Twenty-four hours later, cells were visualized under a light microscope. (A) A representative picture of resultant cell densities after the indicated treatments is shown. (B) Cell density for each treatment group was quantified as described under Materials and Methods, and cell surface areas (in mm²) are reported as least square means $\pm$ S.E.M. Results are from three independent experiments. Means with different superscripts differ ($P < 0.05$).

Our findings that anti-SLPI antibody can attenuate EGF induction of cellular proliferation provide strong support for the involvement of SLPI in growth control. On the other hand, the distinct temporal induction by EGF of cyclin D1 and SLPI gene expression, which occurred maximally at 4 h and 24 h respectively, and the inability of anti-SLPI antibody to inhibit the EGF-mediated increase in cyclin D1 gene expression are consistent with the notion that EGF induction of SLPI expression is not required to initiate cell proliferation. Since Ishikawa cells endogenously express SLPI, and addition of anti-SLPI antibody alone had no demonstrable effect on basal cyclin D1 gene expression or on basal cell proliferation status, the collective data suggest that SLPI may function in a supporting role, albeit a biologically important one, under conditions of persistent growth stimulus, whereby activation of additional growth signaling pathways may be initiated. Such is likely the case in epithelial carcinomas, which are characterized by over-expression of EGFR and/or corresponding ligands (Jasonni *et al.* 1995, Niikura *et al.* 1996, Pfeiffer *et al.* 1997) and increased levels of SLPI protein (Koshikawa *et al.* 1996, Ameshima *et al.* 2000, Hough *et al.* 2001, Shigemasa *et al.* 2001).

Studies by our group have demonstrated that SLPI can inhibit the expression of a number of anti-proliferative factors including IGFBP-3 and the ras inhibitor lysyl oxidase (Zhang *et al.* 2002a). SLPI has also been reported to prevent proteolysis of progranulin, a growth factor highly expressed in aggressive cancer cell lines and which mediates cell cycle progression via its induction of cyclins D and B (He & Bateman 2003). Moreover, SLPI has been shown to increase the expression of hepatocyte growth factor in human lung fibroblasts, albeit the mechanism for this up-regulation remains unclear (Kikuchi *et al.* 2000). Taken together, these data suggest that cell proliferation

mediated by EGF through induction of SLPI gene expression can result in the activation of distinct downstream pathways and demonstrate a versatile role for SLPI in growth-associated events.

Although our results suggest a linear pathway between EGF and cell proliferation via EGF induction of synthesized and secreted SLPI, the precise cellular signaling underlying SLPI modulation of cell proliferation remains unknown. A membrane receptor for SLPI has not been equivocally established (McNeely *et al.* 1997, Tseng & Tseng 2000). Moreover, SLPI has been shown to bind to extracellular matrix components, including elastin, fibronectin, heparin, and the high molecular weight glycoprotein mucins (Kramps *et al.* 1981, Van-Seuning *et al.* 1992, Llewellyn-Jones *et al.* 1994, Walter *et al.* 1996). Further, the induction by SLPI of cyclin D1 promoter activity (Zhang *et al.* 2002a) and its inhibition of the nuclear accumulation of nuclear factor- κ B via maintenance of inhibitor of κ B β levels (Lentsch *et al.* 1999) imply intracellular functions of the protein as well. Future studies to delineate the intra- and extra-cellular modes of SLPI signaling are warranted. Nonetheless, it is tempting to speculate, based on the results presented here, that SLPI may function as a key component of a positive 'feed-forward loop' mechanism that confers a growth advantage to tumor cells.

Finally, it is of interest to note that, aside from their involvement in tumorigenesis, EGF and SLPI may also be linked to wound healing, a complex process requiring cell proliferation, migration, and differentiation. EGF has been reported to promote wound closure and epidermal regeneration (Pilcher *et al.* 1999, Gibbs *et al.* 2000). Similarly, mice null for the SLPI gene show impaired cutaneous wound healing (Ashcroft *et al.* 2000), that was corrected by administration of proepithelin, an epithelial growth factor

Figure 5 Temporal induction by EGF of cyclin D1, SLPI, and p21^{WAF1} gene expression. Ishikawa cells were incubated in low serum (0.5% FBS)-containing medium with or without added rhEGF (50 ng/ml) for 4 or 24 h, after which cells were harvested and analyzed for levels of cyclin D1 (top), SLPI (middle), or p21^{WAF1} (bottom) transcripts by quantitative RT-PCR, as described under Materials and Methods. Results (least square means $\pm$ S.E.M.) were normalized to the transcript levels of 18S rRNA and are from three independent experiments. Significant differences between treatment groups (indicated by brackets) as a function of treatment duration were identified by two-way ANOVA. The asterisk (*) indicates significant difference ($P < 0.05$) from corresponding control (untreated) cells. The symbol (^) indicates tendency for significant difference ($P = 0.10$) from corresponding control (untreated) cells.

Figure 6 EGF induction of cyclin D1 expression is independent of SLPI. Ishikawa cells were treated with rhEGF or buffer (PBS) alone, as described in Fig. 1, with or without anti-SLPI antibody (Ab) (IgG fraction, 2.5 μ g/well). After 4, 6, and 24 h, cells were harvested and analyzed for cyclin D1 transcript levels by quantitative RT-PCR, as described under Materials and Methods. Results from three independent experiments are shown and are expressed relative to transcript levels of control (untreated cells) after normalization of each transcript level to that of 18S. Means with different superscripts differ at $P < 0.05$ (for a and b) and at $P = 0.07$ for ^ (relative to a).

whose activity is regulated by SLPI (Zhu *et al.* 2002). The up-regulation by EGF of SLPI expression in keratinocytes (Lai *et al.* 2004) was recently reported, albeit this study did not evaluate the level of EGF regulation.

In summary, the present results identify SLPI as an EGFR target gene. Given the recently described roles for SLPI in cell proliferation, tumor progression, and metastasis (Zhang *et al.* 2002a, Devoogdt *et al.* 2003), these findings suggest a novel pathway by which over-expression of EGFR and its ligands can lead to tumor progression and carcinoma. We propose that future studies of SLPI and possibly other serpin family members (Potempa *et al.* 1994) could provide significance to their potential pathophysiological actions in cancer progression and may lead to novel strategies for cancer treatment downstream of or in concert with those involving EGFR.

Acknowledgements

The authors thank Frank J Michel for expert technical assistance, Jason Blum and Lindsey Smith for help with cell culture, and Dr Pieter S Hiemstra for the gift of anti-human SLPI antiserum.

Funding

This study was supported by a grant from the National Institutes of Health (HD21961). There is no conflict of interest.

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Received 3 September 2004

Accepted 20 September 2004

Made available online as an

Accepted Preprint 1 October 2004