Proepithelin Promotes Migration and Invasion of 5637 Bladder Cancer Cells through the Activation of ERK1/2 and the Formation of a Paxillin/FAK/ERK Complex

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Abstract

The growth factor proepithelin (also known as progranulin, acrogranin, PC-derived growth factor, or granulin-epithelin precursor) is a secreted glycoprotein that functions as an important regulator of cell growth, migration, and transformation. Proepithelin is overexpressed in a great variety of cancer cell lines and clinical specimens of breast, ovarian, and renal cancer as well as glioblastomas. In this study, we have investigated the effects of proepithelin on bladder cancer cells using human recombinant proepithelin purified to homogeneity from 293-EBNA cells. Although proepithelin did not appreciably affect cell growth, it did promote migration of 5637 bladder cancer cells and stimulate invasion in vitro wound closure and invasion. These effects required the activation of the mitogen-activated protein kinase pathway and paxillin, which upon proepithelin stimulation formed a complex with focal adhesion kinase and active extracellular signal-regulated kinase. Our results provide the first evidence for a role of proepithelin in stimulating migration and invasion of bladder cancer cells, and support the hypothesis that this growth factor may play a critical role in the establishment of the invasive phenotype. (Cancer Res 2006; 66(14): 7103-10)

Introduction

Proepithelin, also known as granulin-epithelin precursor, progranulin, PC cell-derived growth factor, or acrogranin, is a growth factor that plays a critical role in development, cell cycle progression, cell motility, and tumorigenesis (1, 2).

Proepithelin, originally isolated from different sources by several independent laboratories (3–6), is a 593-amino-acid secreted protein with a predicted Mr of ~68 kDa, but due to its high degree of glycosylation, it migrates on SDS-PAGE as a ~80 kDa protein (5, 7). The precursor protein has a secretory signal peptide and seven and one-half cysteine-rich 6 kDa tandem repeats (8). Notably, proepithelin undergoes proteolytic processing with the liberation of small, ~6 kDa peptides, which retain biological activity (9); peptides are active in cell growth assays (3) and may be proinflammatory (10). Zhu et al. have recently shown that the secretory leukocyte protease inhibitor (SLPI) interacts with proepithelin. Elastase digests proepithelin exclusively in the interpeptidic linkers with the generation of epithelin peptides, suggesting that this protease may be an important component of a proepithelin convertase. SLPI blocks this proteolysis by either direct binding to elastase or by sequestering epithelin peptides from the enzyme (10).

The role of proepithelin in the regulation of cellular proliferation has been well characterized using mouse embryo fibroblasts derived from mice with a targeted deletion of the insulin-like growth factor receptor (IGF-IR) gene (R– cells). These cells are unable to proliferate in response to IGF-I and other growth factors (epidermal growth factor and platelet-derived growth factor) necessary to fully progress through the cell cycle (11). In contrast, proepithelin is the only known growth factor able to bypass the requirement for the IGF-IR, thus promoting growth of R– cells (4, 12). Proepithelin promotes the sustained expression of cyclin B, enabling R– cells to traverse mitosis (10). Proliferation of R– cells correlates with the ability of proepithelin to promote the activation of both the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (12). Although proepithelin is able to replace the function of the IGF-IR in cell growth, it does not allow R– cells to grow in anchorage independence and does not protects R– cells from anchorage-independent apoptosis (anokias; ref. 12). Conversely, in SW13 carcinoma cells, proepithelin-dependent activation of the PI3K and MAPK pathways protects cells from anokias, confers anchorage-independent growth, and promotes tumor formation in nude mice (13). In several breast cancer cell lines, proepithelin expression correlates with an aggressive phenotype (14, 15), and immunoneutralization of proepithelin inhibits estrogen-mediated proliferation of MCF-7 cells (16). Block of proepithelin expression by antisense strategy inhibits tumorigenicity of the human breast carcinoma cell line MDA-MB-468 (15). Moreover, high proepithelin expression plays also a significant role in adipocytic teratoma, glioblastomas, multiple myeloma, renal cell, gastric, and ovarian carcinoma (1, 2, 17–19). By immunohistochemistry, stromal proepithelin was mainly identified in microvascular structures (2, 7).

Taking into account the biological properties of proepithelin, it has been hypothesized that proepithelin could act through a “classic” membrane receptor(s), as for the other known growth factors. Thus far, a functional receptor has not been identified, although proepithelin (20) and epithelins (21) bind specifically to membrane proteins. Data from competitive binding experiments indicate that a long list of known growth factors and cytokines are unable to displace radiolabeled epithelin binding to its putative receptor (21), suggesting that the receptor for proepithelin is not a known tyrosine kinase receptor.

Although bladder cancer is the 5th most common cancer in the United States (22), very little is still known about the molecular
mechanisms that determine tumor formation in the bladder urothelium. Most bladder cancers arise in the bladder epithelium, and regardless of treatment with surgery, chemotherapy, or immunotherapy, they often recur and/or metastasize. Using 5637 transitional cell carcinoma-derived cells, we provide the first evidence for a role of proepithelin in promoting migration and invasion of bladder cancer cells, supporting the hypothesis that this growth factor may play a significant role in the establishment of the transformed phenotype in bladder cancer. Furthermore, proepithelin may prove a useful clinical marker for diagnosis and prognosis in bladder tumors.

Materials and Methods

Cell lines. Transitional cell carcinoma-derived human 5637 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI supplemented with 10% fetal bovine serum. Serum-free medium (SFM) is DMEM supplemented with 0.1% bovine serum albumin and 50 μg/mL of transferrin (Sigma-Aldrich, St. Louis, MO).

Purification of human recombinant proepithelin. Human proepithelin was purified from conditioned medium of 293-EBNA/proepithelin cells. This cell line expresses a 6His-tagged human proepithelin (7). Serum-free conditioned medium was concentrated with polyethylene glycol, dialyzed, and purified on Ni-NTA resin eluted with 250 mmol/L imidazole, as previously described (25).

Migration assay. Cells were serum starved for 24 hours. Cells (2 × 10^4 in 200 μL) were then seeded in Boyden chambers (upper chamber). Lower chambers contained 500 μL of SFM or purified recombinant proepithelin (0.5, 1, or 2 μg/mL). After 24 hours, the cells in the upper chamber were removed, whereas the cells that migrated to the lower chamber were counted after fixation and staining in Coomassie blue solution for 5 minutes as described (26). Cells migrated to the lower chamber were counted under the microscope.

Wound-healing migration assay. 5637 cells were seeded onto 35-mm plates in serum-containing medium until subconfluence and then transferred to SFM. After 24 hours, the plates were scratched with a thin disposable tip to generate a wound (500 μm/0.3) in the cell monolayer (12, 24, 25). The cells were incubated for additional 24 hours in SFM without or with purified proepithelin (1 or 2 μg/mL). Cells were analyzed and photographed with a Zeiss Axiovert 200 M cell live microscope using the Metamorph Image Acquisition and Analysis software (Universal Imaging, Downingtown, PA) at the Kimmel Cancer Center Confocal Microscopy Core Facility.

Invasion assay. Cell invasion through a three-dimensional extracellular matrix was assessed by a Matrigel invasion assay using BD Matrigel Invasion Chambers (BD Biocoat, Bedford, MA) with 8.0 mm filter membranes. Cells (2.5 × 10^5) resuspended in 200 μL of SFM or SFM supplemented with 2 μg/mL of purified proepithelin were plated onto each filter, and 750 μL of serum-free medium supplemented placed in the lower chamber. After 24 hours, filters were washed, fixed, and stained with Coomassie brilliant blue. Cells on the upper surface of the filters were removed with cotton swabs. Cells that had invaded to the lower surface of the filter were counted under the microscope.

Immunoblot detection of activated signaling molecules. 5637 cells were serum starved for 24 hours and then stimulated for 10, 30, and 120 minutes with purified proepithelin (2 μg/mL). Lysates (20 μg) were run on a 12% gel SDS-PAGE. The activation of p90RSK, Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), and 56 ribosomal protein was analyzed by Western immunoblot using the PathScan Multiplex Western Cocktail I (Cell Signaling, Beverly, MA), which provides a mix of phospho-specific antibodies for different activated protein. EIF4E protein is the control to monitor the loading of the samples. The anti-α-actin polyclonal antibodies are from Sigma (St. Louis, MO).

Activation of paxillin was determined in 5637 cells unstimulated or stimulated with 2 μg/mL of proepithelin for 10 minutes. Proteins (800 μg) were immunoprecipitated with anti-paxillin monoclonal antibodies (BD PharMingen, San Diego, CA). Tyrosine-phosphorylated paxillin and focal adhesion kinase (FAK) were detected by Western immunoblot using anti-phospho-tyrosine/horseradish peroxidase–conjugated monoclonal antibodies (BD PharMingen), whereas total paxillin and FAK were detected using anti-paxillin monoclonal antibodies (BD PharMingen) and anti-FAK polyclonal antibodies (BD PharMingen). Active MAPK in the paxillin immunoprecipitates was detected using an anti-phospho-MAPK/2/4 polyclonal antibody (Thr186/Tyr195) from Cell Signaling.

To detect serine phosphorylation of paxillin, 5637 cells were unstimulated or stimulated with 2 μg/mL of proepithelin for 10 and 30 minutes with or without 20 μg/mL U0126. Proteins (1 mg) were immunoprecipitated with anti-paxillin monoclonal antibodies (BD PharMingen). Serine-phosphorylated paxillin was detected by immunoblot using anti-phospho-serine polyclonal antibodies (BD PharMingen).

Gene silencing. Gene silencing of human ERK1/2 and paxillin was obtained by RNA interference using small interfering RNA (siRNA). 5637 cells were transfected with vehicle (DEPC-treated water), control siRNA (scrambled), or siRNA directed against ERK2 or paxillin (75 pmol) using either TransIT-sQUEST or TransIT-sTKO reagents (Mirus Corp., Madison, WI) according to the manufacturer’s instructions. Scramble and anti-ERK2 (NM 002745, exon 3, sequences not disclosed by the vendor) and paxillin (NM002859, 5’–GCAAAUAUGUUCUAGCUTT–3’) and scramble control (four mismatches) Silencer siRNA oligos were from Ambion (Austin, TX). Twenty-four hours after transfection, 5637 cells were starved in SFM for 24 hours and then stimulated with 2 μg/mL of purified proepithelin. After 24 hours, cells were processed and analyzed for migration and invasion as described above. The expression of ERK1/2 and paxillin proteins was detected by immunoblot using anti-ERK1/2 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-paxillin monoclonal antibodies (BD PharMingen).

Statistics. Experiments were carried out in triplicate and repeated at least thrice. Results are expressed as mean ± SD.

Immunofluorescence microscopy. 5637 cells were plated onto coverslips and serum starved for 24 hours and then stimulated for 10 and 30 minutes with 2 μg/mL of purified proepithelin. After two washes in PBS, cells were fixed in 3.7% paraformaldehyde for 10 minutes. After blocking for 30 minutes at 37°C in PBS/1% bovine serum albumin, 0.5% Triton X-100 (blocking buffer), coverslips were incubated with anti-paxillin monoclonal antibody (BD PharMingen) and anti-active MAPK p2/4 polyclonal antibody (Cell Signaling: 1:100) in blocking buffer for 30 minutes at 37°C. After washing five times with the same solution and blocked again for 30 minutes at 37°C, samples were incubated for 30 minutes at 37°C with secondary antibodies Alexa Fluor 647 (blue) and Alexa Fluor 488 (green) from (Molecular Probes, Invitrogen, Carlsbad, CA) used at 1:100 dilution in blocking solution. The third additional staining implies a repetition of the protocol after the double staining. FAK was stained using anti-FAK polyclonal antibodies (BD PharMingen). Secondary antibodies were Alexa Fluor 594 (red: Molecular Probes). The coverslips were washed twice with PBS and mounted using Slow-Fade mounting medium (Molecular Probes). Coverslips were analyzed at the Kimmel Cancer Center Confocal Microscopy Core Facility with a Zeiss Axiosvert 200 M cell live microscope using the Metamorph Image Acquisition and Analysis software (Universal Imaging) using a ×63 objective. The images were merged using Photoshop.

Results

Proepithelin promotes migration of 5637 bladder cancer cells. The growth factor proepithelin has been recently shown to promote wound healing (1) and migration of breast cancer cells (26).

To investigate whether proepithelin plays any role in migration of bladder cancer cells, we purified human proepithelin from conditioned medium of 293-EBNA/proepithelin cells, which stably express a HIS6-tagged human proepithelin (7). Purified proepithelin was visualized by Coomassie blue–stained SDS-PAGE and Western immunoblotting with anti-proepithelin polyclonal antibodies (Fig. 1A). The purified proepithelin migrated as ~85 kDa broad band, in addition to a minor component migrating at ~65 kDa,
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Signaling pathways stimulated by proepithelin in 5637 bladder cancer cells. Previous studies have shown that proepithelin promotes the activation of the MAP kinase pathway in MCF-7 breast cancer cells (16) and the activation of both the PI3K and MAPK pathways in mouse embryo fibroblasts, adrenal carcinoma, and multiple myeloma cells (12, 13, 18). Thus, we examined whether proepithelin would also mediate the activation of Akt and MAPK pathways in 5637 cells. We employed the PathScan Multiplex Western Cocktail I (Cell Signaling), which allows the testing of multiple pathways in one single blot. Serum-starved 5637 cells were stimulated with 2 μg/mL of proepithelin for 10, 30, and 120 minutes. Proepithelin induced a rapid (within 10 minutes) and transient activation of ERK1/2, with a concurrent, albeit less marked, and transient activation of p90RSK, one of ERK1/2 downstream effectors (refs. 28, 29; Fig. 3A). In contrast, Akt was not activated by proepithelin in 5637 cells (Fig. 3A) compared with unstimulated cells but was activated by IGF-I that we used as a control for Akt activation (data not shown).

Next, we confirmed that proepithelin-mediated MAPK activation was required for promoting migration and invasion of 5637 cells by performing the same experiments as above but in the presence of U0126, a specific inhibitor of the MAPK pathway (30). Both migration (Fig. 3B) and invasion (Fig. 3C) were markedly reduced

which we consider the less glycosylated species. As previously shown (7), human proepithelin purified using this methodology is biologically active, as determined by proliferation assays on SW13 adrenal carcinoma cells. We then used purified proepithelin for cell migration assays on 5637 transitional cell carcinoma–derived human cells.

Increasing concentration of purified proepithelin from 0.5 to 2 μg/mL ( ~ 60, 120, and 240 nmol/L) induced cell migration of 5637 bladder cancer cells in a dose-dependent manner (Fig. 1B), suggesting that proepithelin may play a critical role in migration of bladder cancer cells.

We further determined the ability of proepithelin to induce migration of 5637 bladder cancer cells using an in vitro “wound-healing” motility assay (12, 25). The 5637 cells were plated at high density in serum-containing medium (Fig. 2A, time 0). After 24 hours of starvation in SFM, confluent 5637 cells were wounded (Fig. 2A, wound) and incubated for additional 24 hours in SFM with or without proepithelin (1 μg/mL). In contrast to control, proepithelin evoked a substantial migration of the cells into the denuded area (Fig. 2A).

Although proepithelin can effectively promote migration of 5637 cells at nanomolar concentrations, it does not induce an appreciable mitogenic response at equimolar concentrations (data not shown).

Proepithelin enhances invasiveness of bladder cancer cells. The acquisition by cancer cells of an invasive phenotype is a critical step for tumor progression (27). Matrigel-coated filters are widely used to examine invasive migration through a three-dimensional extracellular matrix (8, 26). The 5637 cells stimulated for 24 hours with 2 μg/mL of recombinant proepithelin, which was added either to the upper or lower chamber (Fig. 2B and C, respectively), showed a marked increase in the ability to traverse Matrigel-coated filters compared with controls SFM-stimulated cells.

Collectively, our results suggest that proepithelin not only stimulates the migratory ability of urothelial cancer cells but also the cells’ ability to migrate and invade a complex three-dimensional matrix, such as Matrigel.

Figure 1. Purified proepithelin induces cell migration of 5637 cancer urothelial cells. A, 600 ng of purified proepithelin were visualized by Coomassie blue–stained SDS-PAGE (lane 2) and immunoblot (WB) with anti-proepithelin polyclonal antibodies (lane 3; Oncogene Sciences, Calbiochem, San Diego, CA). Lane 1, molecular weight markers. B, 5637 cells were serum starved for 24 hours. Migration experiments on 5637 bladder cancer cells were done using SFM or SFM supplemented with 0.5, 1, and 2 μg/mL of purified proepithelin. % Increase over control (SFM). Control value is 30 ± SD. Columns, average of four (B) independent experiments run in triplicate; bars, SD.

Figure 2. Proepithelin promotes in vitro closure of a wound and invasion of 5637 cells. A, in vitro wound healing motility assay in 5637 cells in SFM without or with purified proepithelin (1 μg/mL) was done as described in Materials and Methods. Cells were analyzed with a cell live microscope using the Metamorph Image Acquisition and Analysis software (Universal Imaging; ×63). Ten fields per plate were examined. Cell invasion was assessed in 5637 cells as described in Materials and Methods. C, proepithelin (2 μg/mL) in lower chamber only. B, proepithelin in lower and upper chamber. Cells that had invaded to the lower surface of the filter were counted under the microscope. A minimum of 100 cells was counted per sample. Time 0 value is 25 ± SD. Columns, average of four independent experiments run in duplicates; bars, SD.
in the presence of 10 μmol/L U0126. The same concentration of U0126 completely abolished proepithelin-induced ERK1/2 and p90RSK activation (Fig. 3D). In agreement with these findings, inhibition of the MAPK pathway by U0126 also blocked the ability of proepithelin to promote migration of 5637 cells and fill a wound in in vitro “wound-healing” assays (Fig. 4).

To further determine the role of MAPK activation in proepithelin-induced migration and invasion, we used ERK-specific siRNA. Our approach yielded a ~80% suppression of endogenous ERK protein expression compared with vehicle-treated or control-treated cells (Fig. 5A) and a concurrent suppression of proepithelin-mediated migration (Fig. 5B) and invasion (Fig. 5C) through Matrigel-coated filters.

Collectively, our findings reveal an essential role for MAPK signaling in the proepithelin functional regulation of tumor cell motility and invasion, two key properties of aggressive cancer phenotype.

**Proepithelin promotes paxillin activation and the formation of a paxillin/FAK/ERK complex.** Oncogene- or growth factor–mediated activation of the scaffolding protein paxillin is important for cell attachment, spreading, and morphogenesis (31–35). We therefore investigated whether proepithelin could promote paxillin phosphorylation in 5637 cells. Indeed, proepithelin stimulation of 5637 cells led to a marked enhancement of paxillin tyrosine phosphorylation (Fig. 6A), indicating that proepithelin mediates in vivo the formation of a complex that contains active paxillin, FAK, and active ERK.

We further confirmed that paxillin upon proepithelin stimulation complexes with active MAPK and FAK by determining whether paxillin colocalizes with phospho-MAPK and FAK using immunofluorescence microscopy. In unstimulated 5637 cells, activated ERK was below detectable levels, whereas FAK and paxillin showed a diffused stain in the cytoplasm (Fig. 6B, top). Upon proepithelin stimulation activated ERK colocalized with both FAK and paxillin (Fig. 6B, middle) and the association increased upon prolonged proepithelin stimulation (Fig. 6B, bottom). These results confirmed the immunoprecipitation experiments and indicate that proepithelin stimulation of 5637 cells promotes tyrosine phosphorylation of paxillin and the formation of a ternary complex among paxillin, FAK, and activated ERK.

**Proepithelin induces MAPK-dependent serine phosphorylation of paxillin.** The ability of proepithelin to promote tyrosine phosphorylation of paxillin, activation of ERK, and the association of paxillin with active ERK suggest that paxillin in 5637 cells may be a direct substrate of ERK. To test this possibility, we immunoprecipitated paxillin, and we tested whether proepithelin promoted serine phosphorylation of paxillin.

In unstimulated 5637 cells, serine phosphorylation of paxillin was barely detectable (Fig. 6C). In contrast, proepithelin-stimulated 5637 cells showed a time-dependent increase in serine phosphorylation of paxillin (Fig. 6C) consistent with the time-dependent

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**Figure 3.** Proepithelin-mediated activation of the MAPK pathway promotes migration and invasion in 5637 cells. A, 5637 cells were serum starved for 24 hours and stimulated with proepithelin (2 μg/mL) for 10, 30, and 120 minutes. The activation of (top to bottom) p90RSK, Akt, ERK1/2, and S6 ribosomal protein was analyzed by Western immunoblot using phospho-specific antibodies as described in Materials and Methods. Representative of four independent experiments. Migration (B) and invasion (C) experiments were carried out on 5637 cells as described in Materials and Methods. U0126 (Calbiochem) was used at 10 μmol/L concentration. % Increase over SFM (30 ± SD). D, 5636 cells were serum starved for 24 hours and then stimulated for 10 minutes with purified proepithelin (2 μg/mL). The activation of p90RSK and ERK1/2 in the presence of 10 μmol/L U0126 was detected by immunoblot with anti-phospho-specific p90RSK and ERK1/2 (Cell Signaling). Samples were normalized using anti-α-actin polyclonal antibodies (Sigma).

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**Figure 4.** Proepithelin-mediated activation of the MAPK pathway promotes migration and invasion in 5637 cells. A, 5637 cells were serum starved for 24 hours and stimulated with proepithelin (2 μg/mL) for 10, 30, and 120 minutes. The activation of (top to bottom) p90RSK, Akt, ERK1/2, and S6 ribosomal protein was analyzed by Western immunoblot using phospho-specific antibodies as described in Materials and Methods. Representative of four independent experiments. Migration (B) and invasion (C) experiments were carried out on 5637 cells as described in Materials and Methods. U0126 (Calbiochem) was used at 10 μmol/L concentration. % Increase over SFM (30 ± SD). D, 5636 cells were serum starved for 24 hours and then stimulated for 10 minutes with purified proepithelin (2 μg/mL). The activation of p90RSK and ERK1/2 in the presence of 10 μmol/L U0126 was detected by immunoblot with anti-phospho-specific p90RSK and ERK1/2 (Cell Signaling). Samples were normalized using anti-α-actin polyclonal antibodies (Sigma).
increase in paxillin/phospho-ERK association (Fig. 6B). Notably, serine phosphorylation of paxillin detectable after 30 minutes of proepithelin stimulation was decreased about 50% in the presence of 10 μmol/L U0126 (Fig. 6C) as determined by densitometric analysis. Taken together, these results suggest that paxillin upon proepithelin stimulation associates with ERK and may be a direct substrate of ERK-mediated phosphorylation.

**Depletion of endogenous paxillin inhibits proepithelin-mediated migration and wound healing of 5637 cells.** The finding that proepithelin promotes tyrosine phosphorylation of paxillin combined with the association of paxillin with active ERK2 and subsequent MAPK-dependent serine phosphorylation of paxillin suggest that paxillin may play a critical role in proepithelin-mediated migration of 5637 cells. To assess this possibility, we used siRNA to target endogenous paxillin protein. Our approach yielded a ~100% depletion of endogenous paxillin protein compared with vehicle-treated or control-treated cells (Fig. 7A) and a robust inhibition of proepithelin-mediated migration (Fig. 7B) and wound healing (Fig. 7C) in 5637 cells. Collectively, our findings reveal an essential role for paxillin in the proepithelin functional regulation of tumor cell motility, a key property of the aggressive cancer phenotype.

**Discussion**

Although bladder cancer is one of the most common malignancies, very little is known about the molecular mechanisms that determine malignant transformation in the urothelial lining the bladder wall. Most bladder cancers are characterized by frequent recurrences and often progress into an invasive phenotype, regardless of treatment with surgery, chemotherapy, or immunotherapy. The present study provides the first evidence that the growth factor proepithelin might play a significant role in bladder tumor formation and progression by promoting migration and invasion of bladder cancer cells. Proepithelin is expressed in normal urothelium and bladder cancer cells (data not shown), suggesting that endogenous proepithelin might also contribute in an autocrine fashion to these processes.

In this study, we have shown that human recombinant proepithelin induces migration and invasion of 5637 bladder cancer cells. Moreover, nanomolar concentrations of exogenous proepithelin stimulate in vitro closure of a wound. We further show that proepithelin-mediated migration and invasion of 5637 bladder cancer cells requires the activation of the MAPK pathway and paxillin, as determined by using pharmacologic and genetic inhibitors.

Proepithelin has an interesting molecular architecture insofar as it harbors seven and one-half protein repeats (P-G-F-B-A-C-D, where P is the half motif) called epithelins or granulins (36). Although the proepithelin precursor is clearly mitogenic, the 6 kDa epithelins can instead promote opposite effects (8). For example, epithelin A induces mitogenesis of keratinocytes and promotes anchorage-independent growth of rat kidney cells but inhibits proliferation of A431 squamous carcinoma cells (3, 37). Elastase digests proepithelin in the linker region between the epithelin domains, with the generation of epithelin peptides, whereas SLPI blocks this proteolysis by either directly binding to elastase or by sequestering proepithelin from the enzyme (10). In leukocytes, the protection of proteolysis by SLPI controls a switching mechanism between intact proepithelin, which is anti-inflammatory through the inhibition of tumor necrosis factor–mediated biological effects, and its proteolytic peptides, which are instead proinflammatory by stimulating the production of interleukin-8 (10).

**Figure 4.** U0126 inhibits in vitro closure of a wound of 5637 cells. For in vitro wound-healing motility assay, 5637 cells were incubated in SFM and SFM with purified proepithelin (2 μg/mL) supplemented or not with 10 μmol/L U0126. Cells were analyzed as described in Fig. 2 legend.

**Figure 5.** Depletion of endogenous ERK1/2 proteins effectively reduces proepithelin-mediated migration and invasion of 5637 cells. Gene knockdown for ERK1/2 was achieved by siRNA. Twenty-four hours after transfection, 5637 cells were serum starved for 24 hours and then stimulated with 2 μg/mL of purified proepithelin. After 24 hours, cells were processed and analyzed for migration (B) and invasion (C). % Increase over SFM (30 ± SD). The expression of ERK1/2 proteins (A) was detected by immunoblot using anti-ERK1/2 polyclonal antibodies (Santa Cruz Biotechnology). Protein loading was normalized using anti-α-tubulin polyclonal antibodies (Sigma).
Proepithelin is also detectable intracellularly, where it interacts specifically with cyclin T1 (38), and this interaction plays a regulatory function on transcription elongation (38, 39). Colocalization experiments have shown that proepithelin colocalizes with cyclin T1 predominantly in the cytoplasm, whereas a protein encompassing epithelins CDE mostly translocates to the nucleus, where it interacts with cyclin T1 (38). These data strongly suggest that intracellular proepithelin may play a biological role not only in the cytoplasm but also in the nucleus, where it might affect the transcriptional machinery (38, 39).

We have examined the signaling pathways stimulated by proepithelin in transitional cell carcinoma–derived cell lines and have shown that the activation of the MAPK pathway is required for proepithelin-induced migration and invasion of 5637 human bladder cancer cells. Our results are in agreement with previous data, which have shown that proepithelin promotes the activation of the MAPK pathways in MCF-7 breast cancer cells (16), whereas proepithelin promotes the activation of both the PI3K and MAPK pathways in mouse embryo fibroblasts, adrenal carcinoma, and multiple myeloma cells (12, 13, 18). We note that in 5637 cells, proepithelin does not promote the activation of Akt, suggesting that the PI3K pathway does not play a role in migration and invasion in these cells.

Our study identified for the first time paxillin as a proepithelin-activated protein and showed that paxillin upon proepithelin stimulation associates with FAK and active ERK. Our results suggested that paxillin may be also directly phosphorylated by ERK in a proepithelin-dependent fashion. Paxillin knockdown in 5637 cells completely abolished proepithelin-induced cell migration and wound healing, contributing the first demonstration that paxillin is a major regulator of proepithelin signaling in these cells. Our data provide therefore a step further in the understanding the mechanism(s) by which MAPKs contribute to regulate proepithelin-induced cell migration.

5637 cells respond in migration and invasion to the purified proepithelin precursor. Furthermore, it should be pointed out that we were able to detect only the 88 kDa precursor by immunoblot on concentrated medium of 5637 cancer cells (data not shown). These results would indicate that these cells secrete proepithelin, which is likely the bioactive form on bladder cancer cells. However,
we cannot totally rule out the possibility that some intracellular processing into epithelin peptides may occur and contribute to the biological response. Although proepithelin is effectively promoting migration and invasion of 5637 cells at nanomolar amounts, it is not strongly mitogenic for these cells at the same concentration (data not shown), suggesting that proepithelin in some cell systems may play a more critical role in promoting a stimulus for migration and invasion than for proliferation (13, 40).

The ability of cancer cells to migrate and invade through the extracellular matrix is a critical step for tumor metastasis to occur (27). Our results suggest that proepithelin is not only important for migration of bladder cancer cells but may also play a physiologic role in regulating cell migration of normal urothelial cells.

In SW13 adrenal carcinoma cells, proepithelin activates FAK (13): FAK activation links growth factors and integrin signaling pathways and has an important function in promoting growth factor- and integrin-mediated signaling (31, 41). Furthermore, integrins through FAK activate Ras signaling to MAPK (27, 32), which in turn regulate cell migration by phosphorylating cytoskeletal proteins and by inducing gene expression. Both FAK and ERK interact with and phosphorylate the focal adhesion scaffolding protein paxillin (33, 42, 43), which is required for epithelial morphogenesis and motility (33, 42–44). Thus, a plausible mechanism by which proepithelin promotes migration of bladder cancer cells would be by activating the FAK/integrins complex and the signaling cascade leading to MAPK activation and MAPK-dependent paxillin phosphorylation.

As we mentioned in the Introduction, the proepithelin membrane receptor has not yet been identified precluding at the moment the possibility of clearly define the early stages of proepithelin-mediated signaling from the plasma membrane.

In conclusion, the identification of the growth factor proepithelin as a novel regulator of tumor cell motility and invasion could represent an additional molecular target for bladder cancer and could provide new leads for developing novel tumor markers and for improved therapeutic approaches against bladder cancer.

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