CKAP4/p63 Is a Receptor for the Frizzled-8 Protein-related Antiproliferative Factor from Interstitial Cystitis Patients*

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Antiproliferative factor (APF) is a low molecular weight sialoglycopeptide that is secreted by bladder cells from interstitial cystitis patients and is a potent inhibitor of both normal bladder epithelial and bladder carcinoma cell proliferation. We hypothesized that APF may produce its antiproliferative effects by binding to a transmembrane receptor. This study demonstrates that cytoskeleton-associated protein 4/p63 (CKAP4/p63), a type II transmembrane receptor, binds with high affinity to APF. The antiproliferative activity of APF is effectively inhibited by precubation with anti-CKAP4/p63-specific antibodies, as well as by short interfering RNA knockdown of CKAP4/p63. Immunofluorescent confocal microscopy showed co-localization of anti-CKAP4/p63 and rhodamine-labeled synthetic APF binding in both cell membrane and perinuclear areas. APF also inhibits the proliferation of HeLa cervical carcinoma cells that are known to express CKAP4/p63. These data indicate that CKAP4/p63 is an important epithelial cell receptor for APF.

Antiproliferative factor (APF)2 is a sialoglycopeptide inhibitor of bladder epithelial cell proliferation that is secreted specifically by bladder epithelial cells from patients with interstitial cystitis (IC) (1, 2), a disorder commonly associated with denudation or thinning of the bladder epithelium (3–5). APF was discovered to be the active factor in urine from IC patients that reversibly inhibited the growth of bladder epithelial cells in vitro (2, 6). The specificity of APF for urine from IC patients (versus normal controls or patients with a variety of other urogenital disorders (7)) indicates that it may be useful as a diagnostic marker for IC and that it may play an important role in the pathogenesis of this disorder.

APF is the first naturally occurring, low molecular weight sialoglycopeptide negative growth regulator to have been completely characterized. The peptide sequence of APF is identical to residues 541–549 of the 6th transmembrane domain of Frizzled 8, a Wnt ligand receptor. The glycosyl moiety of APF consists of sialic acid α-2,3 linked to galactose β1–3-N-acetylgalactosamine, which is α-O-linked to the N-terminal threonine residue of the nonapeptide (1).

APF has been shown to profoundly inhibit the proliferation of both normal bladder epithelial cells and bladder carcinoma cells in vitro (1, 2, 6). Furthermore, APF can induce multiple changes in the pattern of cellular gene expression, including decreased production of heparin-binding epidermal growth factor–like growth factor and increased production of E-cadherin, resulting in a more differentiated bladder epithelial cell phenotype (2, 8). APF was also recently determined to decrease tight junction protein (zonula occludens-1 and occludin) production and increase paracellular permeability of normal bladder epithelial cell monolayers similar to changes seen in cells from patients with IC in vitro (9). Because decreased urine levels of heparin-binding epidermal growth factor–like growth factor (10), bladder epithelial thinning or ulceration (3, 4), abnormal expression of some of the same proteins (11), and bladder epithelial leakiness (12) have all been described in IC patients in vivo, APF appears to play a pivotal role in the pathogenesis of IC. Identification of a receptor for APF on human bladder epithelial cells will therefore advance understanding of the mechanism of pathogenesis for IC, as well as aid in the development of specific therapies for this disorder.

The potency of APF (IC50 in the high picomolar range), its varied effects on bladder epithelial cell protein expression and proliferation, and the requirement for a hexosamine-galactose disaccharide linked in a specific α configuration to the backbone peptide for activity (1, 2, 8, 9), all suggest that the effects of APF may be mediated by binding to a transmembrane receptor. Microarray analysis suggested a potential role for specific transcription factors such as AP-1, SP-1, and TCF/LEF-1 in abnormal gene expression in cells explanted from IC patients or fol-
lowing APF treatment of normal cells, providing additional evidence for involvement of a receptor (8). Identification of a receptor for APF is important for understanding its mechanism of action and could lead to development of therapeutic agents for IC that specifically bind to APF or its receptor to block its effects on the bladder epithelium.

To isolate potential receptor proteins, solubilized microsomal preparations from APF-sensitive bladder epithelial cells were passed over an avidin column to which biotinylated, synthetic APF was attached. After a series of high stringency washing steps, two proteins were observed in the eluate and identified by mass spectrometry as cytoskeletal associated protein 4 (CKAP4/p63) and vimentin. Demonstration that CKAP4/p63 is a functional receptor for APF was accomplished through the use of anti-CKAP4/p63 antibodies that effectively blocked APF activity. Additional evidence was provided by decreased normal bladder epithelial cell sensitivity to APF following siRNA knockdown of CKAP4/p63, as well as by APF sensitivity of another cell line (HeLa) known to express CKAP4/p63. Co-localization of CKAP4/p63 and APF binding in bladder epithelial cells was confirmed by confocal immunofluorescence microscopy.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Explanted bladder epithelial cells were propagated from biopsies of four patients who had undergone cystoscopy and fulfilled the NIDDK/National Institutes of Health diagnostic criteria for interstitial cystitis (13) and their age-, race-, and gender-matched controls. (Three of the four normal control explants were obtained from biopsies of patients undergoing cystoscopy following living kidney donation or bladder resuspension surgery who also consented to undergo bladder biopsy at that time for research purposes. The fourth bladder resuspension surgery who also consented to undergo cystoscopy following living kidney donation or bladder resuspension surgery who also consented to undergo bladder biopsy at that time for research purposes. The fourth normal control explant was grown from tissue obtained at autopsy from a patient who had no history of bladder disorder.) These cells were grown from the tissue specimens using Dulbecco’s modified Eagle’s medium/F-12 (Mediatech, Herndon, VA) with 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution, 2 mM L-glutamine, 0.25 units/ml insulin (all from Sigma), and 5 ng/ml recombinant human epidermal growth factor (R & D Systems, Minneapolis, MN) at 37 °C in a 5% CO2 atmosphere and were characterized by binding of AE-1/AE-3 pan-cytokeratin antibodies (Signet, Dedham, MA), as described previously (1, 2). All patients were at least 18 years old and enrolled in accordance with guidelines of the Institutional Review Board of the University of Maryland School of Medicine.

HeLa cells were obtained from the American Type Culture Collection (CCL-2) and grown in MEM containing 1 mM sodium pyruvate and 2 mM L-glutamine (Invitrogen), plus 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (Sigma).

**Biotinylated Synthetic APF**—APF biotinylated on the C-terminal lysine residue was synthesized up to the N-terminal valine by solid phase methods on a Nautilus 2400 synthesizer (Argonaut Technologies, Foster City, CA) utilizing standard Fmoc chemistry on N-α-Fmoc-N-ε-biotinyl-L-lysyl 2-chlorotrityl resin. Fmoc-protected L-amino acids (Novabiochem) were coupled using O-(7-azbenzotriazol-1-yl)-N,N,N′,N′′-tetramethyluronium hexafluorophosphate (Sigma) and 1-hydroxy-7-azabenzotriazole (Anaspec) reagents. The Fmoc-protected Galβ1-3GalNACα-O-threonine was then coupled to the remaining peptide backbone, as described previously (1). After purification by HPLC on a C8 column, the identity of the biotinylated synthetic APF was confirmed by mass spectrometry, and its antiproliferative activity was confirmed in primary normal bladder epithelial cells (this congener has ~80% activity as compared with the parent synthetic GalNACβ1–3Gal-APF congener).

**Receptor Purification**—Explanted bladder epithelial cells were grown in Dulbecco’s modified Eagle’s medium/F-12 with supplements as described above until confluent and then cultured in serum-free MEM (containing only 1% L-glutamine and 1% antibiotic/antimycotic solution) for 48 h at 37 °C, rinsed with cold phosphate-buffered saline (PBS) in the culture flasks, scraped with sterile cell scrapers into ice-cold PBS, pelleted by low speed centrifugation at 4 °C, and then immediately frozen at −80 °C. The frozen pellet (containing 3 × 107 cells) was then washed three times with 0.5 ml of ice-cold wash buffer (phosphate-buffered saline (PBS), pH 7.2, 1 mM sodium vanadate (NaVO3), 10 mM sodium fluoride (NaF), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and repelleted by centrifugation for 15 min at 5000 × g. Cells were then further resuspended in 0.5 ml of ice-cold wash buffer and sonicated with a microtip sonifier (Branson Digital Sonifier, model 250, Branson Ultrasonics, Corp., Danbury, CT) for 30 s at 20% power on ice. Sonication was performed three times allowing for a 5-min cool down on ice between sonic bursts. The cell lysate was centrifuged at 15,000 × g for 30 min at 4 °C to pellet the microsomal fraction. The pellet was then washed three times by resuspending in 0.5 ml of wash buffer followed by centrifugation at 15,000 × g for 30 min. The microsomal fraction was finally solubilized in PBS, pH 7.2, containing 1 mM NaVO3, 10 mM NaF, 1 mM EDTA, 0.1 mM PMSF, and 1% Triton X-100. This solution was allowed to incubate on ice for 30 min and diluted to 0.5% Triton X-100 with ice-cold wash buffer.

The solubilized protein fraction was then loaded onto an APF-bound affinity spin column generated by immobilizing synthetic biotinylated APF to a streptavidin-agarose bead stationary phase support (400-ml bed volume; Pierce). The column was washed six times with 500 ml of PBS, pH 7.2, containing 1 mM NaVO3, 10 mM NaF, 1 mM EDTA, 0.1 mM PMSF, and 0.2% Triton X-100, where each wash contained an increasing concentration of NaCl as follows: 10, 100, 250, and 500 mM and 1 mM NaCl. The remaining bound proteins were eluted with 1% formic acid containing 0.2% Triton X-100. Each of the eluates was lyophilized to dryness, resuspended in Laemmli buffer, boiled for 5 min, and resolved by one-dimensional SDS-PAGE followed by visualization by silver stain. Sections were cut from the one-dimensional SDS-polyacrylamide gel containing the protein bands uniquely isolated by high affinity binding to the immobilized APF (as compared with the control isolations), and proteins in these slices were digested according to Wilm et al. (14). The extracted tryptic peptides were then analyzed by nanoflow reverse phase liquid chro-
matography (nano-RPLC) coupled on line with tandem mass spectrometry (MS/MS).

**Nanoflow Reverse Phase Liquid Chromatography-Tandem Mass Spectrometry**—Chromatographic separations of the tryptic peptides were conducted using a microcapillary column with an integrated electrospray ionization (ESI) emitter constructed by flame-pulling a fine tip (∼5–7-mm orifice) on a 75-mm inner diameter × 360-mm outer diameter × 10-cm long segment of fused silica (Polymerico Technologies Inc., Phoenix, AZ). This integrated ESI column was slurry-packed in-house with 5-mm, 300-Å pore size C-18 stationary phase (Jupiter, Phenomenex, Torrance, CA). The integrated ESI column was connected via a stainless steel zero dead volume union to an Agilent 1100 nanoflow LC system (Agilent Technologies, Palo Alto, CA), coupled on line to a linear ion-trap mass spectrometer (LTQ, ThermoElectron, Inc., San Jose, CA). After sample injection, a 20-min wash with 98% mobile phase A (0.1% HCOOH in water) was applied, and peptides were eluted using a linear gradient of 2% mobile phase B (0.1% HCOOH in CH₃CN) to 42% mobile phase B over 40 min with a constant flow rate of 200 nl/min. The column was washed for 15 min with 98% mobile phase B and re-equilibrated with 98% mobile phase A prior to subsequent sample loading.

The integrated nano-RPLC ESI column was coupled on line to a linear ion-trap-MS using the manufacturer’s nanoelectrospray source with an applied electrospray potential of 1.5 kV and capillary temperature of 160 °C. The linear ion-trap-MS was operated in a data-dependent mode where each full MS scan was followed by five MS/MS scans, in which the five most abundant peptide molecular ions detected from the MS scan were dynamically selected for five subsequent MS/MS scans using a collision-induced dissociation energy of 35%. Dynamic exclusion was utilized to minimize redundant MS/MS acquisition.

The collision-induced dissociation spectra were analyzed using SEQUEST operating on a 40-node Beowulf parallel virtual machine cluster computer (ThermoElectron) and the Homo sapiens proteome data base. Only peptides with conventional tryptic termini (allowing for up to two internal missed cleavages) possessing δ scores (DC₅) >0.08 and charge state-dependent cross-correlation (Xcorr) criteria were considered as legitimate identifications as follows: >1.9 for [M + H]⁺, >2.2 for [M + 2H]²⁺, and >3.1 for [M + 3H]³⁺ peptide molecular ions.

**Western Blots**—Solubilized high affinity APF-binding bladder epithelial cell proteins were prepared as described above. Solubilized bladder epithelial cell membrane proteins were prepared by rinsing cells in the culture flasks with ice-cold PBS and then scraping the cells into ice-cold extraction buffer (20 mM Tris/HCl, pH 7.4, containing 50 μg/ml PMSF, 2 μg/ml aprotinin, 100 μM tosyl-lysine chloromethyl ketone, 1 μM pepstatin A, and 1 μg/ml diisopropyl fluorophosphate). The cell slurry was then vortexed until homogeneous, and centrifuged at 4 °C for 10 min at 14,000 rpm. The supernatant was removed, and the pelleted membrane fraction was washed once with ice-cold extraction buffer and repelleted, and membrane proteins were solubilized using ice-cold extraction buffer containing 1% Triton X-100. Solubilized total cell proteins were extracted into ice-cold RIPA buffer containing 1.0% Nonidet P-40, as described previously (9).

Protein concentration of solubilized protein samples was measured using a Folin reagent-based protein assay kit (Bio-Rad). The solubilized proteins were then incubated for 10 min at 70 °C in sample reducing buffer; each lane was loaded with 20 μg of protein, and proteins were separated by electrophoresis using 4–12% NuPAGE NOVEX BisTris polyacrylamide gels (Invitrogen) in MOPS/SDS running buffer (Invitrogen), according to the manufacturer’s instructions, and transferred to nitrocellulose membranes (Invitrogen) according to the NuPAGE gel manufacturer’s protocol for Western transfer (30 V constant voltage for 1 h). Following protein transfer, the nitrocellulose membranes were blocked with 5% nonfat dry milk in TBS-T buffer (Tris-buffered saline, pH 7.4, with 0.1% Tween 20) and incubated overnight at 4 °C in TBS-T buffer containing mouse monoclonal anti-CKAP4/p63 antibodies (“anti-CLIMP-63,” clone G1/296) (Alexis Biochemicals), mouse monoclonal anti-vimentin antibodies (BD Biosciences), or mouse monoclonal anti-β-actin antibodies (Sigma); when more than one antibody was used for binding to proteins on a single membrane, the membrane was stripped between antibody incubations using Re-Blot Plus Mild solution (Chemicon) according to the manufacturer’s instructions. The membranes were subsequently washed three times with TBS-T, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature, and developed with ECL chemiluminescence Reagent (Amersham Biosciences).

**[³H]Thymidine Incorporation**—Cell proliferation was measured by [³H]thyidine incorporation into explanted normal human bladder epithelial cells, as described previously (1, 6), or into HeLa cells. Briefly, purified lyophilized synthetic APF was resuspended in acetonitrile/distilled water (1:1), diluted in serum-free MEM (containing only glutamine and antibiotics/antimycotics), and applied to normal bladder epithelial or HeLa cells; cell controls received acetonitrile/distilled water diluted in serum-free MEM alone. Cells were then incubated at 37°C in a 5% CO₂ atmosphere for 48 h. The cell contents were harvested and methanol-fixed onto glass fiber filter paper, and the amount of radioactivity incorporated was determined. Significant inhibition of [³H]thyidine incorporation was defined as a mean decrease in counts/min of greater than 2 S.D. from the mean of control cells for each plate.

**Rhodamine-labeled Synthetic APF**—Rhodamine-APF was synthesized in a similar manner to the bionitylated APF using solid phase methods, starting with N̅-α-Fmoc-N-ε(iV-Dde)-L-lysyl-2-chlorotriyl resin. After coupling of the sugar-threonine residue to the peptide, the glycopeptide was treated with hydrazine hydrate (1:11 in MeOH) to remove the sugar-protecting groups and also the lysine ivDde-protecting group. The glycopeptide was cleaved from the resin and purified by HPLC on a C₈ column. It was then dissolved in N,N-dimethylformamide, and 5(6)-rhodamine B isothiocyanate (Sigma) (1 eq) and triethylamine (3 eq) were added. The solution was left stirring in the dark for 1.5 h. After removal of the solvent, the residue was dried under vacuum overnight and purified by HPLC. The
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**RESULTS**

Solubilized bladder epithelial cell membrane proteins that bound with high affinity to biotinylated APF were eluted with 0.1% formic acid, 20% acetonitrile. The eluate was resolved by denaturing SDS-PAGE, and proteins were visualized by silver staining. Two protein bands were evident with molecular masses of ~63 and 54 kDa (Fig. 1A). These bands were excised and in-gel digested with trypsin, and the resulting tryptic fragments were analyzed by nano-RPLC coupled with a linear ion-trap mass spectrometer operating in a data-dependent MS/MS mode. This analysis resulted in the unequivocal identification of the 63-kDa band as CKAP4/p63 (16 unique peptides, 30% sequence coverage) and the 54-kDa band as vimentin (13 peptides, 28% sequence coverage) (Fig. 1B).

The identities of the 63- and 54-kDa proteins were confirmed by Western blot using monoclonal antibodies specific for CKAP4/p63 and vimentin. As shown in Fig. 2, these antibodies...
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FIGURE 3. Western blot identification of CKAP4/p63 in membrane proteins extracted from bladder epithelial explants. Epithelial cell explants were grown from bladder tissue of four IC patients and their four age-, race-, and gender-matched normal controls, and solubilized cell membrane preparations were generated from each using ice-cold extraction buffer containing 1% Triton X-100 and protease inhibitors. These proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes under reducing conditions; the membranes were further blocked, incubated with antibodies against CKAP4/p63 or β-actin (the same membrane was used for both antibodies and stripped between runs), followed by secondary horseradish peroxidase-labeled secondary antibodies, and developed using ECL chemiluminescence reagent. NBC, normal bladder cells; IC, cells from IC patients.

bound specifically to the 63- and 54-kDa proteins, respectively, that had been isolated by high affinity binding to biotinylated APF.

To establish that CKAP4/p63 may indeed be a cell membrane receptor for APF required evidence that this protein is present on the cell membrane of bladder epithelial cells from both normal controls and IC patients. Cell membrane preparations from four IC patients and their age-, race-, and gender-matched controls all expressed CKAP4/p63 protein on the cell membrane as determined by Western blot analysis (Fig. 3). Although the amount of CKAP4/p63 expression relative to β-actin expression varied between individuals, there was not a notable difference in expression between cells explanted from IC patients versus controls.

APF was discovered as a cell growth inhibitor from the urine of patients with IC. To establish whether CKAP4/p63 is a true receptor for APF, we determined whether antibodies that bind to CKAP4/p63 prevent the antiproliferative effects of APF on bladder epithelial cells. Normal human bladder epithelial cells were preincubated with monoclonal anti-CKAP4/p63 antibodies for 1.5 h at 37 °C prior to APF exposure. As shown in Fig. 4, preincubation with anti-CKAP4/p63 antibodies decreased the sensitivity of normal bladder epithelial cells to APF in a dose-dependent manner. In comparison, preincubation of cells with the same concentrations of anti-vimentin antibodies or an isotype control antibody directed against an unrelated cell protein did not inhibit APF activity (data not shown), and incubation of the cells with anti-CKAP4/p63 antibodies alone neither stimulated nor inhibited thymidine incorporation (Fig. 4).

Additional evidence that CKAP4/p63 is a functional cell membrane receptor for APF was provided by transient transfection of cells with siRNA for CKAP4/p63 prior to APF exposure. Electroporation of normal bladder epithelial cells with double-stranded siRNA for CKAP4/p63 resulted in decreased protein expression of CKAP4/p63 relative to β-actin (Fig. 5A) as well as approximately 1000-fold decreased sensitivity of the cells to APF (Fig. 5B). In comparison, control cells electroporated without siRNA or with a scrambled siRNA had little or no change in CKAP4/p63 protein expression relative to β-actin,

FIGURE 4. Decreased sensitivity of normal bladder cells to APF following preincubation with anti-CKAP4/p63 antibodies. Normal bladder epithelial cells were grown to confluence and then preincubated with varying concentrations of anti-CKAP4/p63 antibodies prior to incubation with synthetic APF and performance of the [3H]thymidine incorporation assay for cell proliferation. Each data point was performed in triplicate; data are presented as percent inhibition of thymidine incorporation compared with untreated cell controls.

FIGURE 5. siRNA knockdown of CKAP4/p63 in normal bladder epithelial cells. Normal bladder epithelial cells were electroporated with or without CKAP4/p63 double-stranded siRNA and cultured for an additional 48 h. Cell expression of CKAP4/p63 protein was assessed at that time by Western blot of solubilized cell proteins, and duplicate cell culture sensitivity to APF was assessed by inhibition of [3H]thymidine incorporation. A, Western blot of CKAP4/p63 protein expression was normalized to β-actin expression in normal bladder epithelial cells following electroporation procedure alone (without siRNA) (lane a); following electroporation with double-stranded siRNA to CKAP4/p63 (lane b); receiving neither electroporation nor siRNA (lane c); or following electroporation with a scrambled double-stranded siRNA (lane d). These experiments were repeated three times with similar results each time. B, decreased sensitivity to APF following electroporation with double-stranded siRNA to CKAP4/p63 (○); first negative control (electroporation without siRNA) (○); second negative control (no electroporation or siRNA) (○); third negative control (electroporation with scrambled siRNA) (○). These experiments were performed in triplicate on three separate occasions; data are expressed as the mean percent change in [3H]thymidine incorporation following APF treatment as compared with untreated cell controls, and vertical lines indicate standard error of the mean for all data points. (Data indicating the effect of each treatment alone with no added APF on thymidine incorporation are indicated by the appropriate disconnected symbol and error bars close to the y axis.)
HeLa cervical carcinoma cells that are known to express endogenous CKAP4/p63 might also be sensitive to the antiproliferative effects of APF, the urinary bladder that expressed endogenous CKAP4/p63 resulted in a dose-dependent decrease in APF binding. Preincubation of cells with varying concentrations of anti-CKAP4/p63 followed by FITC-labeled secondary antibodies, and subsequently 0.7 μg/well HPLC-purified rhodamine-labeled synthetic APF. The distribution of anti-CKAP4/p63 binding (Fig. 6A) overlapped with rhodamine-labeled APF binding (Fig. 6B), with both entities present on the cell membrane as well as concentrated in the perinuclear cytoplasm (Fig. 6C). Controls with secondary FITC-labeled anti-mouse antibody alone were negative for fluorescent signal (data not shown). Co-localization of CKAP4/p63 and APF was further confirmed by incubating cells with varying concentrations of anti-CKAP4/p63 followed by a constant amount of rhodamine-APF. As shown in Fig. 7, preincubation of cells with increasing concentrations of anti-CKAP4/p63 resulted in a dose-dependent decrease in APF binding.

To determine whether epithelial cells from tissue other than the urinary bladder that expressed endogenous CKAP4/p63 might also be sensitive to the antiproliferative effects of APF, HeLa cervical carcinoma cells that are known to express endogenous CKAP4/p63 (15) were incubated with varying concentrations of purified synthetic APF. APF inhibited HeLa cell proliferation at an IC50 (~1 nM) value that is similar to its IC50 value in primary bladder epithelial cells (~0.4 nM) (1) (Fig. 8); the same concentrations of a negative control nonglycosylated APF peptide had no effect on HeLa cell proliferation (data not shown).

**DISCUSSION**

This study presents evidence that CKAP4/p63 is a functional bladder epithelial cell receptor for APF, an inhibitor of cell proliferation secreted from bladder epithelial cells in patients suffering from the chronic painful bladder disorder called interstitial cystitis. In addition, the inhibition of HeLa cell proliferation by APF suggests that nonbladder cells expressing CKAP4/p63 may also be sensitive to its antiproliferative effects.

CKAP4/p63 was isolated by affinity binding to APF, and its identity was determined by mass spectrometry and confirmed by Western blot analysis. The role of CKAP4/p63 in mediating the activity of APF was determined by showing that both anti-CKAP4 antibodies and siRNA knockdown of CKAP4/p63 expression block the effects of APF on normal bladder epithelial cell proliferation.

CKAP4/p63 was first described as a reversibly palmitoylated type II transmembrane receptor (also called CLIMP-63) and was originally localized to the rough endoplasmic reticulum of fibroblast-like cells, epithelial cells, and plasma cells (16, 17). It has been shown recently to be a functional vascular smooth muscle cell membrane receptor for tissue plasminogen activator (18) and is one of several endoplasmic reticulum transmembrane proteins known to also localize at the cell membrane (19, 20). CKAP4/p63 has been shown to be present also in several types of epithelial cells (including COS and HeLa cells) where it helps to anchor the rough endoplasmic reticulum to microtubules (21), a function that requires direct interaction of the cytoplasmic tail of the receptor to microtubules (21) and is regulated by CKAP4/p63 phosphorylation (15).
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The mechanism by which CKAP4/p63 binding may mediate APF activity is unknown. No data yet exist to determine whether this transmembrane protein functions in signaling activity. However, because of its association with cytoskeletal proteins, it is possible that CKAP4/p63 functions as a chaperone for internalization (and perhaps also secretion) of APF. This role may also help to explain the intranuclear fluorescent signal following exposure of cells to rhodamine-labeled APF (see Figs. 6 and 7). (Following the original submission of our study, a similar role for CKAP4/p63 was also hypothesized for surfactant protein A in rat type II pneumocytes, in which it was noted that its possible role as a protein transporter is indeed compatible with CKAP4/p63 being a reversibly palmitoylated, cytoskeleton-binding protein (22).)

Although the association between CKAP4/p63 and the cytoskeleton has been demonstrated (18, 21), a specific association with vimentin has not been shown previously. The significance of CKAP4/p63 isolating with vimentin, which is expressed in high concentrations in basilar but not terminally differentiated bladder epithelial cells (23), is unknown. It is interesting to note, however, that vimentin was one of only nine bladder epithelial cell proteins shown to be significantly down-regulated in IC (versus normal) or APF-treated (versus mock APF-treated) bladder epithelial cells in our previous microarray studies of the mRNA expression of ~4000 genes and Western blot analysis of specific cytoskeletal and tight junction protein expression (8, 9). Although the Western blot data in this study indicate no significant difference in the amount of CKAP4/p63 total protein made by IC and normal bladder cells, whether APF binding affects the binding of CKAP4/p63 to the cytoskeleton or its distribution in bladder epithelial cells, remains to be determined.

The discovery of APF and its association with the CKAP4/p63 receptor provide several lessons in biomarker discovery. APF activity was first noted to be present in urine from IC patients (6); the subsequent determination that this factor was probably made or activated in the distal urinary tract of these patients (24) led to the discovery that APF appears to be made exclusively by explanted bladder cells from IC patients but not from controls (2). Much of the current focus in the discovery of novel biomarkers leverages technologies that acquire large amounts of data (e.g. mass spectrometry and mRNA arrays) and typically search for changes in the abundance of wild-type proteins between disease-affected persons compared with normal controls (25). Although mass spectrometry was instrumental in determining the structure of purified APF (1), we think that the approach of using biological activity to identify substances that are pathogenically related to disease may be more fruitful, particularly for abnormal, disease-specific proteins. The identification of such proteins using proteomics alone can be difficult because disease-specific biomarkers are often present at much lower (nanomolar to picomolar) concentrations than other proteins (26), and the structure and presence of APF in any cell or body fluid could not have been predicted or identified based on presently annotated genomic and proteomic data bases. However, based on the high activity of APF and its specificity for urine samples and cells from IC patients, it may represent a novel class of small, modified bioactive peptides that can also function as disease-specific biomarkers.

The unique nature of APF as the first small sialoglycopeptide growth inhibitor to be completely characterized, and the first secreted frizzled-related protein to be identified that contains a transmembrane region of a frizzled protein exclusively, suggests the intriguing possibility that other such negative growth regulators may exist, either in association with normal embryonic development or with other disease states in which tissue development is impaired. Two other natural sialoglycopeptide growth inhibitors have been identified but have yet to be completely characterized (27, 28), one of which is known to cause reversible growth inhibition like APF (28, 29). However, the relationship of either of these negative growth factors to APF or frizzled proteins is unknown at this time.

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