Quantitative Proteomics Identifies a β-Catenin Network as an Element of the Signaling Response to Frizzled-8 Protein-Related Antiproliferative Factor*§

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Antiproliferative factor (APF), a Frizzled-8 protein-related sialoglycopeptide involved in the pathogenesis of interstitial cystitis, potently inhibits proliferation of normal urothelial cells as well as certain cancer cells. To elucidate the molecular mechanisms of the growth-inhibitory effect of APF, we performed stable isotope labeling by amino acids in cell culture analysis of T24 bladder cancer cells treated with and without APF. Among over 2000 proteins identified, 54 were significantly up-regulated and 48 were down-regulated by APF treatment. Bioinformatic analysis revealed that a protein network involved in cell adhesion was substantially altered by APF and that β-catenin was a prominent node in this network. Functional assays demonstrated that APF down-regulated β-catenin, at least in part, via proteasomal and lysosomal degradation. Moreover, silencing of β-catenin mimicked the antiproliferative effect of APF whereas ectopic expression of nondegradable β-catenin rescued growth inhibition in response to APF, confirming that β-catenin is a key mediator of APF signaling. Notably, the key role of β-catenin in APF signaling is not restricted to T24 cells, but was also observed in an hTERT-immortalized human bladder epithelial cell line, TRT-HU1. In addition, the network model suggested that β-catenin is linked to cyclooxygenase-2 (COX-2), implying a potential connection between APF and inflammation. Functional assays verified that APF increased the production of prostaglandin E₂ and that down-modulation of β-catenin elevated COX-2 expression, whereas forced expression of nondegradable β-catenin inhibited APF-induced up-regulation of COX-2. Furthermore, we confirmed that β-catenin was down-regulated whereas COX-2 was up-regulated in epithelial cells explanted from IC bladder biopsies compared with control tissues. In summary, our quantitative proteomics study describes the first provisional APF-regulated protein network, within which β-catenin is a key node, and provides new insight that targeting the β-catenin signaling pathway may be a rational approach toward treating interstitial cystitis. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.007492, 1–11, 2011.

Antiproliferative factor (APF), a nine-residue sialoglycopeptide whose peptide chain is 100% homologous to the putative sixth transmembrane domain of Frizzled-8 (1), is secreted by bladder epithelial cells from patients with interstitial cystitis (IC) (2, 3), a prevalent and debilitating pelvic disorder (4, 5). Studies suggest that APF is a potent negative growth factor, which markedly inhibits the proliferation of not only normal bladder epithelial cells but also T24 bladder carcinoma cells and HeLa cervical carcinoma cells (1, 6, 7). Studies have been conducted to investigate the molecular mechanisms underlying the antiproliferative effect of APF using the hypothesis-driven approach; these led to the discoveries that (a) cytoskeleton-associated protein 4 (CKAP4), also known as CLIMP63, is a high-affinity receptor for APF (6); (b) palmitoylation of CKAP4 by the palmitoyl acyltransferase...
APF regulates a β-catenin network

DHHC2 plays a critical role in regulating APF-mediated signaling (7); (c) APF specifically inhibits the production of the urothelial cell mitogen, heparin-binding epidermal growth factor-like growth factor (HB-EGF) (8); (d) HB-EGF functionally antagonizes APF activity (8) via parallel mitogen-activated protein kinase signaling pathways (9); and (e) the transcription factor p53 is an important mediator of APF-induced growth inhibition (10).

To provide a broader view of the role of APF in abnormal bladder mucosal features seen in IC, DNA microarray analysis identified several genes differentially regulated by APF treatment of urothelial cells (11). Though DNA microarray technology is powerful, RNA expression does not necessarily reflect the number of functional protein molecules present, or their quantitative differences (12). Another approach to expression profiling is to quantitatively assess protein levels directly using MS. In the past decade, several quantitative proteomics techniques have been developed to accurately measure protein level changes. Among these methods, stable isotope labeling by amino acids in cell culture (SILAC) (13), when combined with high-resolution mass spectrometry, is generally thought to give the lowest technical variation because minimal manipulations are required before the differentially labeled proteins are combined and processed as a single sample (14, 15).

In the present study, we employed a SILAC-based quantitative proteomics approach to identify proteins significantly regulated by exposure of human bladder cells to APF. Bioinformatic analyses uncovered a potentially critical molecular network, within which β-catenin was identified as a prominent, functionally relevant node. Functional assays validated that β-catenin is likely to be a key mediator of APF’s anti-proliferative and inflammatory effects on human bladder epithelial cells.

EXPERIMENTAL PROCEDURES

Reagents—APF peptides were purified from the supernatant of bladder epithelial cells explanted from IC patients using molecular weight fractionation, ion exchange chromatography, hydrophobic interaction chromatography, and reversed-phase high-performance liquid chromatography (1). Mock APF peptides were purified from the supernatant of bladder epithelial cells from age-, race- and gender-matched controls who were asymptomatic for urinary tract disease by ultrafiltration (1). Mock APF peptides were purified from the supernatant of bladder epithelial cells cultured in the “light” medium that was serum starved for 16 h and maintained in serum-free “light” medium containing 10 ng/ml APF or mock APF peptide for an additional 3 days. Cells grown in serum-containing “heavy” medium, which would be used as an internal standard, were serum starved and maintained in serum-free “heavy” medium containing 10 ng/ml mock APF peptide in parallel with the cells cultured in the “light” medium.

Protein Preparation, Separation, and Tryptic Digestion for Mass Spectrometric Analysis—Whole-cell lysates of APF- and mock APF-treated T24 cells were prepared with lysis buffer (1% Nonidet P-40, 50 mM Tris pH 7.4, 10 mM NaCl, 1 mM NaF, 5 mM MgCl2, 0.1 mM EDTA, 1 mM PMSF, and protease inhibitor mixture) and centrifuged at 12,000 × g for 15 min. Protein concentrations were determined by Micro BCA assay according to the manufacturer’s protocol. Proteins extracted from “heavy” cells treated with mock APF peptide were mixed at equal amounts with proteins extracted from “light” cells treated with APF or mock APF peptide. The “heavy” and “light” protein mixtures were resolved on a 12.5% SDS-PAGE gel, and visualized with Coomassie Blue R-250 staining solution. Each gel lane was excised into seven slices of similar size and cut into ~1 mm3 particles prior to in-gel reduction, alkylation, and trypptic digestion as described (17, 18). Tryptic peptides were extracted, dried down in a SpeedVac (Thermo Savant, Holbrook, NY), and stored at ~80 °C until mass spectrometric analysis.

Mass Spectrometric Analysis—Peptides were redisolved with 10 μl 1.5% acetic acid and 7.5% acetonitrile solution. 5 μl samples were analyzed by online C18 nanoflow reversed-phase HPLC (Eksigent nanoLC-2D™) connected to an LTQ Orbitrap mass spectrometer (Thermo Scientific) as described previously (19, 20). Briefly, samples were separated at about 200 nL/min with 80 min linear gradients from 5 to 31.5% acetonitrile in 0.4% formic acid. The survey spectra were acquired in the Orbitrap with the resolution set to a value of 30,000. Up to five of the most intense ions per cycle were fragmented and analyzed in the linear ion trap.

Database Searching—Peak lists of the 200 most abundant fragment ions from each product ion spectrum were extracted out of Thermo .raw files and converted into .mgf files using in-house software (21). No further data processing such as smoothing, de-isotoping, and/or filtering were carried out. All MS data sets were searched against a concatenated International Protein Index human protein database (v3.52; 148,380 sequences) using the Mascot search engine (Matrix Science, v2.2). Protein modifications were set as Carbamidomethyl (C) (fixed) and Arginine (R-full), Lysine (K-full), N-Acetyl (Protein), and Oxidation (M) (variable). Up to one missed tryptic cleav-
APF Regulates a β-Catenin Network

Molecular & Cellular Proteomics 10.6

10.1074/mcp.M110.007492–3

age was allowed. The MS tolerance was set as ±10 ppm and tandem MS ±0.6 Da. All peptides were identified with ion scores no less than 32 (p < 0.05). The proteins identified with at least two unique peptides were classified as a high-confidence data-set, for which the false discovery rate was assessed using a target-decoy search strategy (22). For all the quantified proteins that were identified with only one peptide, the tandem MS spectra were manually checked to ensure the accuracy of protein identification. Raw MS data files can be accessed at Tranche (https://proteomecommons.org/data_set.jsp?ix=74516).

Protein Quantification—Identified proteins were quantitated using the MSQuant open-source software program (v1.4.3, msquant.sourceforge.net) (23). All SILAC pairs were manually inspected to minimize potentially incorrect quantifications. Each quantitative data set was then normalized using a multiple-point strategy to minimize systematic errors introduced by the Micro BCA assay and sample mixing as described (24). To minimize false discoveries arising from differential SILAC labeling and arginine-to-proline conversion, the Mock/APF (H/L) ratios were normalized against Mock/Mock (L/L) ratios to generate APF/Mock (L/L) ratios. The proteins with more than 1.4-fold change (i.e., >1.400 or <0.714) were accepted as significantly (p < 0.05) regulated by APF treatment.

Network Modeling—Differentially expressed proteins (DEPs) were analyzed for functional enrichment using GeneGo's Metacore program. Subsequently, the DEPs were subjected to network modeling essentially as described (25, 26). We first generated an initial network using the first neighbors of the DEPs from protein-protein and protein-DNA interactions gathered from the Kyoto Encyclopedia of Genes and Genomes and National Center for Biotechnology Information databases. The initial network was then reduced to generate a minimal subnetwork optimized to maximally include active nodes (DEPs) by removing the first neighbors of the DEPs that do not contribute to connecting the active nodes. Subsequently, PubMed was explored to obtain more comprehensive and updated information about the functions of the significantly regulated proteins. Kyoto Encyclopedia of Genes and Genomes pathways were used to organize the active nodes and to identify the biological processes they mediate.

Immunofluorescence Assay—Cells were fixed with 4% paraformaldehyde and incubated with 1:1000 anti-β-catenin primary antibody, prior to incubation with 1:250 Cy3.5-conjugated secondary antibody. Anti-Rpt1 antibody and LysoTracker were used to show proteasomes and lysosomes, respectively. Slides were mounted in Vectashield with DAPI and observed under a Zeiss microscope (Burlingame, CA).

Transient Cell Transfection—T24 bladder cells were grown to ~80% confluence in 100-mm dishes, at which time they were transiently transfected with small interfering RNAs (siRNAs) against β-catenin or OFF-TARGET controls using Lipofectamine 2000 according to the manufacturer's instructions. To ectopically express nondegradable β-catenin, a plasmid construct kindly provided by Dr. Sean Li at Children's Hospital Boston was transiently transfected into T24 or TRT-HU1 cells.

Cell Proliferation Assay—Cell proliferation rate was determined by crystal violet staining. Briefly, cells were stained with crystal violet solution and quantified by dissolving stained cells in 10% acetic acid solution. Absorbance was measured at 570 nm.

Measurement of PGE2 Production—T24 cells were plated in 6 well plates at a density of 2 × 10³ cells per well. After APF treatment for the indicated time, the medium was collected and PGE2 was analyzed using a Parameter™ PGE2 Assay kit according to the manufacturer's instructions.

descriptive and Kidney Diseases diagnostic criteria for IC excluding bladder capacity measurements. Age- and gender-matched controls were asymptomatic for urinary tract disease. All participants were at least 18 years old and were enrolled in accordance with guidelines of the Institutional Review Board of the University of Maryland School of Medicine.

Culture of Patient-derived Bladder Epithelial Cells—Transitional epithelium with submucosal bladder tissue were obtained from six IC patients and six age- and gender-matched controls to propagate primary bladder epithelial cells as previously described (11). Briefly, epithelial cells were seeded into T75 culture flasks at a density of 1 × 10⁴ cells per ml. When the cells reached confluence, they were subcultured in serum-free Eagle's minimal essential medium containing 1% antibiotic/antimycotic solution and 1% glutamine at 37 °C in a 5% CO₂ atmosphere for 72 h. Cells were rinsed with cold phosphate buffer saline prior to the extraction of proteins.

Western Blot Analysis—Whole-cell lysates were extracted from T24 or TRT-HU1 cells using 1% Nonidet P-40 containing lysis buffer, or from explanted patient cells using ice-cold RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, and centrifuged at 12,000 × g for 15 min. Nuclei or membrane fractions were prepared (20) and lysed with 1% SDS-containing lysis buffer. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes for immunoblotting. To standardize protein levels, the blots were reprobed with antibodies against β-actin, lamin A/C, or caveolin-1.

Routine Statistics—All functional validation experiments were repeated at least three times. Data were compared using a paired Student's t test. p < 0.05 was considered to be statistically significant.

RESULTS

To comprehensively characterize proteins and pathways downstream from APF, we attempted to identify signaling network perturbations in T24 bladder cancer cells after treatment with physiologic concentrations of purified APF or mock APF peptides. For quantitative proteomics analysis we used the SILAC method, which is widely considered one of the most reliable procedures for relative protein level quantitation by MS (14, 15).

SILAC Identifies Proteins Perturbed by APF—APF-responsive T24 cells (1) were labeled with “heavy” and “light” Arg-and Lys-containing medium, and SILAC analysis was performed to identify proteins perturbed by APF treatment (Fig. 1A). Although it is generally believed that SILAC labeling will not alter protein levels, quantitative analyses of proteins extracted from control cells cultured in parallel in “light” and “heavy” media have shown that levels of a minor fraction of proteins can be significantly changed by differential labeling (24, 27). In addition, conversion of arginine to proline, which results in a reduction in intensity of “heavy” peptides (28), has been observed in certain cell lines. To minimize false discoveries resulting from differential SILAC labeling and/or arginine-to-proline conversion, we used mock APF-treated “heavy” cells as an internal standard so that the levels of proteins from APF- or mock APF-treated “light” cells could be compared (Fig. 1A).

Proteins extracted from mock APF-treated “heavy” cells were mixed in the same amounts with proteins isolated from
APF- or mock APF-treated “light” cells. The two samples were separated by SDS-PAGE and the results are shown in Fig. 1B. No remarkable differences in electrophoretic patterns were observed, suggesting that the great majority of abundant proteins were not significantly changed by APF treatment. MS analysis identified a total of 9571 unique peptides with MOWSE score no less than 32 ($p$-value $H110210.05), corresponding to 2098 different proteins, of which 1359 were identified with at least two unique peptides (supplemental Table S1). Fig. 1C shows a representative tandem mass spectrum, derived from the analysis of a doubly charged peptide ($m/z$ 930.96) from pyruvate kinase isozymes M1/M2 (PKM2). A target-decoy search (22) showed that the false discovery rate was less than 0.6% for all peptides and less than 0.1% for all proteins identified with a minimum of two unique peptides.

Unsupervised MSQuant analysis, followed by manual inspection of all the SILAC pairs, resulted in the quantitation of 1780 and 1394 different proteins from the APF-mock and mock-mock mixtures, respectively, with 1170 different proteins in common (supplemental Table S2). Fig. 1D shows a representative SILAC pair derived from prostaglandin G/H synthase 2 (PTGS2), also called cyclooxygenase-2 (COX-2), from PKM2. A, Diagram describing our approach toward minimizing false discoveries caused by differential SILAC labeling and/or arginine-to-proline conversion. APF and mock APF (abbreviated as Mock) were purified from the supernatant of bladder epithelial cells explanted from IC patients and healthy controls, respectively, as described (1). T24 cells cultured in “heavy” (“H”) medium were treated with mock APF peptide and used as an internal standard so that the levels of proteins extracted from APF- or mock APF-treated cells, both cultured in “light” (“L”) medium, can be quantitatively compared. B, SDS-PAGE gel image of two samples of “heavy” and “light” protein mixture. Each gel lane was cut into 7 slices with approximately the same length. C, A representative tandem mass spectrum derived from the analysis of a doubly charged peptide ($m/z$ 930.96) from PKM2. D, Representative SILAC pairs showing that COX-2 (i.e. PTGS2) was up-regulated by APF treatment and unchanged by differential SILAC labeling and/or arginine-to-proline conversion.

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which was up-regulated 1.8-fold by APF treatment. All protein ratios in each data set, except certain extreme ratios, were logarithmically plotted and normalized against the mean ratios so that the Log$_2$(ratios) were centered around 0, the theoretical mean log-ratio for light and heavy samples mixed at 1:1 ratio (supplemental Fig. S1) (24). Subsequently, the mock/APF (H/L) ratios were normalized against the corresponding mock/mock (H/L) ratios to generate APF/mock (L/L) ratios with the purpose of minimizing false discoveries arising from differential SILAC labeling and/or arginine-to-proline conversion (24). Statistical analysis of the Log$_2$-transformed APF/mock (L/L) ratios showed that the standard deviation is 0.216. Thus, 1.4-fold was set as a significant cutoff ($p < 0.05$), given that Log$_2$(1.4) (i.e. 0.485) is greater than two standard deviations (supplemental Fig. S1). Using these criteria, we identified 54 and 48 different proteins that were significantly up- or down-regulated by APF treatment, respectively (supplemental Table S2). For those quantitated proteins identified with only one peptide, their tandem MS spectra were manually checked to ensure the accuracy of protein identification (supplemental Fig. S2).

Bioinformatic Analysis Identifies a Network Perturbed by APF Treatment—As an initial assessment of the overall effect of APF on the signal transduction network, the proteins whose levels were significantly changed as determined by SILAC (supplemental Table S3) were analyzed for functional enrichment using GeneGo’s MetaCore, a bioinformatics program that interrogates a curated database (http://www.genego.com/metacore.php). Pathway analysis using this tool suggested that mediators of cell adhesion and cell structure (cytoskeleton) are over-represented among the differentially expressed proteins (DEPs) in the APF treatment condition in comparison to the mock APF peptide. The top five enriched pathways ($p < 0.0007$) were cell adhesion-related (Table I), suggesting that cell adhesion pathways are strongly affected by APF.

Further bioinformatic analysis of the DEPs allowed us to construct a hypothetical signaling network downstream from APF. We generated an initial network comprised of network modules made up of groups of proteins that functionally interact with each other, using the nearest neighbors of the DEPs from protein-protein and protein-DNA interactions gathered from Kyoto Encyclopedia of Genes and Genomes and National Center for Biotechnology Information databases. The initial network was then reduced to generate a subnetwork to maximally include DEPs with minimal connections by removing the first neighbors of the DEPs that do not contribute to connecting the network modules (Fig. 2A).

Consistent with the pathway findings obtained from MetaCore (Table I), this network analysis suggested that several modules are significantly perturbed by APF. Modules relevant to cell adhesion, β-catenin, epidermal growth factor receptor (EGFR), mitogen-activated protein kinase, cell cycle, and GTPases were significantly down-regulated, whereas those relevant to fibronectin, integrin, actin polymerization, p53, and cytochrome c oxygenase were significantly up-regulated (Fig. 2A).

Differential expression was verified by Western blot analysis of nine proteins, including seven in the network model, for which specific antibodies are available. As shown in Fig. 2B, β-catenin (CTNNB1), γ-catenin (junction plakoglobin, JUP), EGFR, calpain-2 (CAPN2), and signal transducer and activator of transcription 3 (STAT3) were down-regulated, whereas integrin β-1 (ITGB1), COX-2, tissue factor (F3), and N-myc downstream-regulated gene 1 (NDRG1) were up-regulated in response to APF, consistent with the SILAC quantitation results.

Validation of β-Catenin as an Important Node in the APF Network—The SILAC and bioinformatics analyses described above identified β-catenin as one potentially informative node within the APF network. We decided to focus on β-catenin for validation experiments because of the previously established links between this protein and both cell adhesion and cell proliferation mechanisms (29). Another rationale was that a role for β-catenin in IC pathogenesis was not identified prior to this study. We reasoned that, if the involvement of β-catenin in the APF network could be confirmed, the pre-existing literature on the protein’s adhesion and transcription functions might facilitate a rapid expansion of our knowledge of the APF network using the β-catenin node as a point of departure. β-catenin protein levels, as assessed by Western blotting, were decreased in whole-cell lysates and isolated nuclear and membrane fractions in response to APF (Fig. 3A), verifying the SILAC finding that β-catenin is significantly down-regulated by APF treatment. Immunofluorescence cell staining showed that the protein levels of plasma membrane-associated β-catenin and nuclear β-catenin were significantly reduced 3 days after treatment with APF, compared with mock APF (Fig. 3B).

Previous reports have shown that when β-catenin is not assembled into adherens junctional complexes along with

### Table I

| Map | Cell process | $p$ value |
|-----|--------------|-----------|
| 1   | Cell adhesion: Integrin-mediated cell adhesion and migration | Cell adhesion | 1.98E-06 |
| 2   | Cell adhesion: Endothelial cell contracts by nonjunctional mechanisms | Cell adhesion | 1.10E-05 |
| 3   | Cytoskeleton remodeling: Integrin outside-in signaling | Cell adhesion | 1.47E-04 |
| 4   | Cell adhesion: Role of tetraspanins in the integrin-mediated cell adhesion | Cell adhesion | 2.26E-04 |
| 5   | Cell adhesion: Integrin inside-out signaling | Cell adhesion | 6.88E-04 |
cadherins, the protein can be phosphorylated by glycogen synthase kinase 3 (GSK-3) prior to ubiquitin-dependent degradation by proteasomes (29). β-catenin can also be degraded via lysosomal trafficking, resulting in the inhibition of β-catenin’s transcriptional function (30). We observed that APF treatment induced β-catenin accumulation as discrete puncta in the cytoplasm within 6 h (Fig. 4A), implying that β-catenin was being internalized and routed toward a proteolytic degradation pathway. Consistent with this interpretation, APF treatment led to the colocalization of β-catenin with proteasome subunit Rpt1 and with Lysotracker, a fluorescent acidotropic probe that decorates acidic organelles, predominantly lysosomes (Fig. 4B). Treating the cells with specific inhibitors of proteasomal or lysosomal degradation (MG132 or leupeptin) suppressed β-catenin degradation, resulting in higher levels of β-catenin (Fig. 4C). These data indicate that APF downregulates β-catenin, at least in part, by proteasomal and lysosomal protein degradation.

To assess the biological consequences of β-catenin downregulation, we performed loss- and gain-of-function analyses. We first measured the proliferation rate of T24 cells after depletion of β-catenin by RNA interference. Silencing of
β-catenin using siRNA oligonucleotides resulted in a substantial reduction of cell proliferation in the absence of APF (Fig. 5A). Importantly, inhibitory effects of APF and the siRNAs were not additive, consistent with the hypothesis that β-catenin is a key mediator of APF’s antiproliferative activity. Moreover, enforced expression of a nondegradable β-catenin mutant, which lacks all serine/threonine phosphorylation target residues (31), led to robust rescue of growth inhibition in response to APF (Fig. 5B).

To rule out the possibility that the key role of β-catenin in APF-induced cell proliferation inhibition is restricted to T24 bladder cancer cells, we used an hTERT-immortalized human
APF Regulates a \( \beta \)-Catenin Network

**Fig. 6.** **APF inhibits the proliferation rate of immortalized human bladder epithelial cells (TRT-HU1) via a \( \beta \)-catenin-dependent pathway.** A, APF inhibits the proliferation of TRT-HU1 cells. Cells were incubated with APF or mock APF peptides for 3 days. Cell proliferation rate was determined using crystal violet assay. \( ** \) indicates \( p < 0.01 \). B, APF inhibits the expression of \( \beta \)-catenin, p53, and NDRG1 in TRT-HU1 cells. The protein levels of \( \beta \)-catenin, p53, and NDRG1 were determined by Western blot analysis. \( \beta \)-actin was used as a loading control and blots are representative of at least three independent experiments. C, Ectopic expression of nondegradable \( \beta \)-catenin rescues cell proliferation inhibition caused by APF. A nondegradable \( \beta \)-catenin construct or vector only was transiently transfected into TRT-HU1 cells. Cell proliferation was evaluated using crystal violet 3 days after APF or mock APF treatment. In this figure, “Mock” stands for mock APF peptide and “ND \( \beta \)-catenin” represents nondegradable \( \beta \)-catenin.

bladder epithelial cell line (TRT-HU1) that we recently generated and characterized (16). Fig. 6A shows that TRT-HU1 cell proliferation can be substantially inhibited by APF treatment. Fig. 6B shows that TRT-HU1 cells responded to APF with decreased expression of \( \beta \)-catenin and increased expression of p53 and a p53 target protein, NDRG1, consistent with the findings in T24 cells (Fig. 2B and ref(10)). As in T24 cells, ectopic expression of nondegradable \( \beta \)-catenin in TRT-HU1 cells resulted in significant rescue of APF-induced inhibition of cell growth (Fig. 6C). These findings suggest that \( \beta \)-catenin is a key mediator of APF’s antiproliferative effect in human bladder cells generally.

**APF Evokes an Inflammatory Response by Down-modulating \( \beta \)-Catenin—Our network model (Fig. 2A) suggested that prostaglandin G/H synthase 2 (PTGS2), more commonly known as cyclooxygenase-2 (COX-2), lies downstream of the \( \beta \)-catenin module and is up-regulated by APF treatment. Given that COX-2 is an inducible enzyme critical for the biosynthesis of proinflammatory prostaglandin E\(_2\) (PGE\(_2\)) (32, 33) and that IC is characterized by chronic inflammation of the bladder wall, we hypothesized that APF evokes an inflammatory response by down-modulating \( \beta \)-catenin and consequently up-regulating COX-2 and PGE\(_2\). Indeed, consistent with the increase in COX-2 levels, PGE\(_2\) production was remarkably increased in the presence of APF (Fig. 7A). In addition, siRNA knockdown of \( \beta \)-catenin increased COX-2 expression in the absence of APF stimulation, which was not further enhanced in the presence of APF (Fig. 7B). Moreover, forced expression of nondegradable \( \beta \)-catenin significantly inhibited APF-induced up-regulation of COX-2 (Fig. 7B). Collectively, the results confirmed that APF evokes an inflammatory response by down-modulating \( \beta \)-catenin, which in turn increases COX-2 expression and PGE\(_2\) production.

**\( \beta \)-Catenin Expression is Suppressed Whereas COX-2 Expression is Increased in IC—The biological activity of APF is detectable in urine specimens from over 94% of IC patients (34). To determine whether bladder epithelial cell explants from IC patients have reduced protein levels of \( \beta \)-catenin and increased levels of COX-2 similar to those observed in APF-treated T24 cells, we performed Western blot analysis of protein lysates from bladder epithelial cells explanted from IC patients and age- and gender-matched healthy controls. As shown in Fig. 8, the protein level of \( \beta \)-catenin was significantly lower whereas that of COX-2 was significantly higher in blad-
APF Regulates a β-Catenin Network

Using an unbiased approach on a proteome scale, we have described a provisional molecular network downstream from APF, a urine biomarker associated with IC (34). Comprehensive proteomic analysis using SILAC led to the identification of more than 2000 proteins, including over 1300 proteins with high confidence, representing one of the largest protein databases of human bladder cells ever assembled. To the best of our knowledge, this is the first quantitative proteomics study aiming to elucidate the molecular mechanisms underlying IC pathogenesis.

SILAC analysis followed by network modeling and functional assays suggested that β-catenin, a cell adhesion protein and a transcription factor, is a pivotal component in the APF-regulated protein network. However, it remains unclear how APF suppresses the expression of β-catenin in human bladder cells. Though the peptide portion of APF is 100% homologous to the putative sixth transmembrane domain of Frizzled-8, a Wnt receptor (1), it is unknown whether APF arises from specific proteolytic cleavage of Frizzled-8. Moreover, unlike Wnt-antagonizing secreted Frizzled-related proteins, which are ~300 amino acids in length (35, 36), APF is only a nine-residue sialoglycopeptide and its peptide portion is very hydrophobic. Thus, it is unlikely that APF can interact with Wnt and consequently lead to the down-regulation of β-catenin. Instead, APF might down-modulate β-catenin by inhibiting the production of HB-EGF (8) and/or antagonizing the binding of tissue plasminogen activator (tPA) with CKAP4, a high-affinity receptor for APF (6, 7). This speculation is based on previous findings that HB-EGF may activate the phosphatidylinositol 3-kinase/Akt pathway and in turn inhibit GSK3β and stabilize β-catenin (37), and that CKAP4 is also a receptor for tPA (38), which may activate β-catenin signaling by transactivating EGFR (39). In addition, the down-modulation of EGFR by APF (see Fig. 2) may play a role in APF-induced β-catenin down-regulation.

Interestingly, our findings suggest that inflammation associated with IC may be caused, at least in part, by APF. FDA-approved COX-2 inhibitors, nonsteroidal anti-inflammatory drugs (e.g. celecoxib and refecoxib) or their chemical derivatives, which to our knowledge have not been applied to treat IC as monotherapy, may be beneficial to IC patients by suppressing inflammation and inhibiting certain APF-regulated pathways. However, we found that NS-398, a potent COX-2-specific nonsteroidal anti-inflammatory drugs (40), did not change β-catenin level and significantly inhibited rather than increased T24 cell proliferation (supplemental Fig. S3). This finding is suggestive of the possibility that, although nonsteroidal anti-inflammatory drugs may reduce inflammation, they may promote rather than reduce the destruction of bladder mucosal cells caused by APF. This possibility waits to be tested in animal models.

In summary, a quantitative proteomics analysis revealed, for the first time, a signaling network at the protein level downstream from APF. We verified that β-catenin is a pivotal component within this network and that COX-2 is regulated by APF, at least in part, through a β-catenin-dependent pathway. These findings reinforce published data that proteins participating in intercellular junctions are affected by APF (41), and provide new insight that targeting the β-catenin signaling pathway in addition to APF itself may be a rational approach toward treating IC with novel therapeutics.

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**Fig. 8. β-catenin expression is suppressed whereas COX-2 expression is increased in epithelial cells explanted from bladder tissue from IC patients compared with healthy controls. A, Human bladder epithelium was harvested from six IC patients and six age- and gender-matched healthy controls. Protein extracts from primary cell culture of those explants were subjected to Western blot analysis to detect β-catenin and COX-2 protein expression. Blots are representative of at least three independent experiments. NB, normal bladder. B, Densitometry analysis of the Western blots. Western blots were quantified using ImageJ software. The intensities of β-catenin or COX-2 bands were normalized against corresponding β-actin blot bands. The normalized intensities were then used to compute the relative protein expression of β-catenin or COX-2 in epithelial cells derived from IC bladder and paired normal bladder (abbreviated as NB).**

**Table 1.**

| Case # | NB | IC |
|-------|----|----|
| 1     | NB | IC |
| 2     | NB | IC |
| 3     | NB | IC |
| 4     | NB | IC |
| 5     | NB | IC |
| 6     | NB | IC |

**DISCUSSION**

Using an unbiased approach on a proteome scale, we have described a provisional molecular network downstream from APF, a urine biomarker associated with IC (34). Comprehensive proteomic analysis using SILAC led to the identification of more than 2000 proteins, including over 1300 proteins with high confidence, representing one of the largest protein databases of human bladder cells ever assembled. To the best of our knowledge, this is the first quantitative proteomics study aiming to elucidate the molecular mechanisms underlying IC pathogenesis.

SILAC analysis followed by network modeling and functional assays suggested that β-catenin, a cell adhesion protein and a transcription factor, is a pivotal component in the APF-regulated protein network. However, it remains unclear how APF suppresses the expression of β-catenin in human bladder cells. Though the peptide portion of APF is 100% homologous to the putative sixth transmembrane domain of Frizzled-8, a Wnt receptor (1), it is unknown whether APF arises from specific proteolytic cleavage of Frizzled-8. Moreover, unlike Wnt-antagonizing secreted Frizzled-related proteins, which are ~300 amino acids in length (35, 36), APF is only a nine-residue sialoglycopeptide and its peptide portion is very hydrophobic. Thus, it is unlikely that APF can interact with Wnt and consequently lead to the down-regulation of β-catenin. Instead, APF might down-modulate β-catenin by inhibiting the production of HB-EGF (8) and/or antagonizing the binding of tissue plasminogen activator (tPA) with CKAP4, a high-affinity receptor for APF (6, 7). This speculation is based on previous findings that HB-EGF may activate the phosphatidylinositol 3-kinase/Akt pathway and in turn inhibit GSK3β and stabilize β-catenin (37), and that CKAP4 is also a receptor for tPA (38), which may activate β-catenin signaling by transactivating EGFR (39). In addition, the down-modulation of EGFR by APF (see Fig. 2) may play a role in APF-induced β-catenin down-regulation.

Interestingly, our findings suggest that inflammation associated with IC may be caused, at least in part, by APF. FDA-approved COX-2 inhibitors, nonsteroidal anti-inflammatory drugs (e.g. celecoxib and refecoxib) or their chemical derivatives, which to our knowledge have not been applied to treat IC as monotherapy, may be beneficial to IC patients by suppressing inflammation and inhibiting certain APF-regulated pathways. However, we found that NS-398, a potent COX-2-specific nonsteroidal anti-inflammatory drugs (40), did not change β-catenin level and significantly inhibited rather than increased T24 cell proliferation (supplemental Fig. S3). This finding is suggestive of the possibility that, although nonsteroidal anti-inflammatory drugs may reduce inflammation, they may promote rather than reduce the destruction of bladder mucosal cells caused by APF. This possibility waits to be tested in animal models.

In summary, a quantitative proteomics analysis revealed, for the first time, a signaling network at the protein level downstream from APF. We verified that β-catenin is a pivotal component within this network and that COX-2 is regulated by APF, at least in part, through a β-catenin-dependent pathway. These findings reinforce published data that proteins participating in intercellular junctions are affected by APF (41), and provide new insight that targeting the β-catenin signaling pathway in addition to APF itself may be a rational approach toward treating IC with novel therapeutics.

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APF Regulates a \(\beta\)-Catenin Network

This article contains supplemental Figs. S1 to S3 and Tables S1 to S3.

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