Expression of the Calcium-binding Protein S100A4 Is Markedly Up-regulated by Osmotic Stress and Is Involved in the Renal Osmoadaptive Response*

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Proteomic analysis of Inner Medullary Collecting Duct (IMCD3) cells adapted to increasing levels of tonicity (300, 600, and 900 mosmol/kg H₂O) by two-dimensional difference gel electrophoresis and mass spectrometry revealed several proteins as yet unknown to be up-regulated in response to hypertonic stress. Of these proteins, one of the most robustly up-regulated (22-fold) was S100A4. The identity of the protein was verified by high pressure liquid chromatography-mass spectrometry. Western blot analysis confirmed increased expression with increased tonicity, both acute and chronic. S100A4 protein expression was further confirmed by immunocytochemical analysis. Cells grown in isotonic conditions showed complete absence of immunostaining, whereas chronically adapted IMCD3 cells had uniform cytoplasmic localization. The protein is also regulated in vivo as in mouse kidney tissues S100A4 expression was many -fold greater in the papilla as compared with the cortex and increased further in the papilla upon 36 h of thirsting. Increased expression of S100A4 was also observed in the medulla and papilla, but not the cortex of a human kidney. Data from Affymetrix gene chip analysis and quantitative PCR also revealed increased S100A4 message in IMCD3 cells adapted to hypertonicity. The initial expression of message increased at 8–10 h following exposure to acute sublethal hypertonic stress (550 mosmol/kg H₂O). Protein and message half-life in IMCD3 cells were 85.5 and 6.8 h, respectively. Increasing medium tonicity with NaCl, sucrose, mannitol, and choline chloride stimulated S100A4 expression, whereas urea did not. Silencing of S100A4 expression using a stable siRNA vector (pSM2; Open Biosystems) resulted in a 48-h delay in adaptation of IMCD3 cells under sublethal osmotic stress, suggesting S100A4 is involved in the osmoadaptive response. In summary, we describe the heretofore unrecognized up-regulation of a small calcium-binding protein, both in vitro and in vivo, whose absence profoundly delays osmoadaptation and slows cellular growth under hypertonic conditions.

The cells that inhabit the hypertonic environment of the inner medulla possess a number of adaptive mechanisms that allow them to survive this harsh environment. This survival is mediated initially by the activation of ion transport systems (1) and thereafter by the cellular accumulation of a number of organic osmolytes (2). It has become increasingly evident that in addition to the proteins required for the cellular uptake and/or synthesis of these osmolytes (transporters and enzymes), hypertonic stress brings about a coordinated response involving the up- and down-regulation of hundreds of genes (3, 4), many of which may be critical to cell viability and adaptation.

The use of genomic and proteomic tools, including gene arrays and two-dimensional difference gel electrophoresis (DIGE) analysis allows for the discovery of proteins that heretofore were not attributed to the osmotic stress response in kidney tissues. A useful tool in this discovery process is the development of stable cell lines that are chronically adapted to increasing levels of hypertonicity. One such cell line, the Inner Medullary Collecting Duct (IMCD3) cells originally developed by Rauchman et al. (5), has been adapted to 600 and 900 mosmol/kg H₂O and can be directly compared with cells grown at isotonic conditions and thereby provides an excellent model for genomic and proteomic changes in response to osmotic stress. Identification of perspective proteins that are involved in the osmotic response has been underway in our laboratory and has given rise to a large number of candidate proteins to study. One such heretofore unrecognized osmoregulated protein described in this report is S100A4, a 101-amino acid, calcium-binding protein with a molecular mass of ~11.5 kDa, also known as metastasin or metastatic cell protein 1 (mts1), pEL-98, 18A2, p9Ka, CAPL, calvasculin, and fibroblast-specific protein 1 (Fsp1), a member of the S100 family of calcium-binding proteins (6).

In general, S100 proteins are small, acidic proteins of 9–14 kDa and contain two distinct EF-hands (7, 8). Members of this family of proteins have been related to the intracellular- and extracellular Ca²⁺-dependent regulation of multiple activities such as calcium homeostasis, the dynamics of cytoskeleton components and myofilaments (F-actin, muscle and non-mus-
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Although S100A4 has been shown to be present in kidney tissues and in chronic renal allograft dysfunction (8, 14, 27, 28), its regulation by hypertonicity has not been described. The present work was undertaken to confirm the observations made by proteomic analysis and to further characterize the osmotic regulation of the protein as to timing, half-life, cellular distribution, in vivo effects, and possible physiologic role in osmoadaptation.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium, serum, and antibiotics were obtained from Invitrogen. Antibodies to S100A4 were from Abcam (ab27957) and Covance (Berkeley, CA) (PRB-497P). Both manufacturers assert that this antibody is specific for the S100A4 protein and does not recognize other members of the S100 family. We also undertook studies to determine whether the antibody recognizes both the calcium-bound and free forms of S100A4 and found that it recognizes both equally. All other chemicals were UHP grade from Sigma.

Cell Culture—The established murine inner medullary collecting duct cell line IMCD3 was previously provided by Dr. Steve Gullans (Boston, MA). Cell stocks were frozen in liquid N₂ and propagated in 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen). IMCD3 cultures chronically adapted to increasing levels of tonicity (6, 9, 10). S100A4 has been implicated as a metastasis-associated protein (6, 11, 12). As a mediator of metastasis, S100A4 has been implicated in the epithelial to mesenchymal transition (13, 14). Because S100A4 does not have known enzymatic activity, interaction with other proteins, both intracellular and extracellular, is functionally critical (6). S100A4 is expressed in normal tissues such as smooth muscle, liver, bone marrow, smooth muscle cell of arteries, kidney, and osteoblastic cells in rats and humans. However, the physiological functions of S100A4 in normal tissues have not been clarified yet (15, 16). A specific functional activity of S100A4 has been reported in its colocalization with cytoskeletal proteins F-actin and non-muscle myosin (17–24). S100A4 has also been reported to prevent the phosphorylation of non-muscle myosin by protein kinase C (19). Therefore, it is possible that S100A4 can regulate the function of cytoskeletal proteins or impinge on signal transduction pathways ultimately controlling cell movement or adhesion (26). In this regard, Bustamante et al. (25) working with rat kidney MTAL tubules described that the cellular regulatory volume increase is associated with a p38 kinase-dependent actin cytoskeleton remodeling. Upon exposure to hypertonic stress with NaCl or sucrose, but not urea, the actin cytoskeleton of MTAL cells is reorganized.

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**Confocal Microscopy**—Confluent culture cells were grown on cover glasses and fixed for 15 min with 4% paraformaldehyde in phosphate-buffered saline, pH 7.3. They were then rinsed, permeabilized with 0.2% saponin in 0.5% bovine serum albumin in phosphate-buffered saline, and incubated in polyclonal rabbit anti-S100A4 antibodies (Abcam) that were detected with goat anti-rabbit antibodies labeled with Alexa 488 and counterstained with To-Pro nuclear stain. Cultures were observed with a Leica TCS SL confocal laser scanning microscope (Mannheim, Germany).

**Mass Spectrometry/Proteomics**—Cultures of IMCD3 cells were cultivated from independent inocula in medium supplemented with NaCl at total osmotic concentrations of 300, 600, and 900 mosmol/kg H2O (n = 6 at each osmotic pressure). Each replicate was washed with isosmotic buffer, lysed with 50 μl of 50 mM Tris, pH 7.5, 150 mM NaCl, 0.3% SDS with a protease and phosphatase inhibitor as described previously (30). Protein extracts were prepared from the lysates and subjected to DIGE as described previously (30). Analysis was performed with DeCyder software (version 6.5; GE Healthcare) with false discovery rate correction enabled and individual p values generated from the multiple comparisons test of analysis of variance as implemented in the Extended Data Analysis module of that software. Initial identification of spots by mass spectrometry with a Voyager DE-STR (Applied Biosystems, Foster City, CA) with an Agilent 3.5-m pore size reversed phase Zorbax brand C18-alto, CA) with an Agilent 3.5-μm pore size reversed phase Zorbax brand C18-μm ID at 300 nl/min.

**RNA Preparation**—Confluent culture plates were harvested by trypsinizing the cells followed by centrifugation to a cell pellet. RNA was extracted using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer’s protocol. The RNA content was determined by spectrophotometry at 260 nm. The integrity of the RNA preparation was determined using an Agilent Bioanalyzer (model 2100). Reverse transcription of RNA to cDNA was prepared using the Omniscript kit from Qiagen according to the manufacturer’s protocol.

**Gene Arrays**—The mouse gene array 430-2.0, which contains 43,000 transcripts (Affymetrix Inc., Santa Clara, CA), was employed. RNA transcription to cDNA biotinylated to cRNA, fragmentation, hybridization to the chip, and chip analysis using a HP Gene Array Scanner were according to the manufacturer’s recommendations and performed by the University of Colorado Gene Array Core. Gene array data were analyzed using both the Affymetrix Microarray Suite 5.0 and GeneSpring GX 7.3 Expression Analysis (Agilent Technologies) software analysis programs.

**Quantitative PCR (QPCR) Analysis**—QPCR was performed using a MyCycler (Bio-Rad) with the Quantitech SYBR Green kit (Qiagen). Primer sets for specific genes were determined using Beacon Designer software (version 4.0; Premier Biosoft International, Palo Alto, CA). Primers for QPCR for S100A4 were (forward) 5’-GCTGCCCAGATGAGGAAACC-3’, (reverse) 5’-GCTGGAGAAGCCAGAGTAAGG-3’ producing a product of 124 bp. QPCR cDNA samples were normalized to 18 S rRNA content using the following primers (forward) 5’-GCTGCTCCTCTCCTACCTG-3’ and (reverse) 5’-GCCTGCTGCCTCCTTG-3’, producing a product of 355 bp. QPCR runs were analyzed by agarose gel electrophoresis and melt curve to verify that the correct amplification was produced.

**Western Blot Analysis**—Cell lysates were prepared from confluent cell cultures in 100 × 20-mm tissue culture dishes as previously described (29, 31). Sample protein content was determined by the BCA protein assay (Pierce). Depending on the experiment, from 25 to 150 μg of protein was loaded per lane for PAGE analysis. The primary antibody against S100A4 (mouse) was from Abcam. Gels were visualized using an alkaline phosphatase secondary antibody and Lumi-Phos reagent (Pierce) as described by the manufacturer. Chemiluminescence was recorded with an Image Station 440CF and results analyzed with the 1D Image Software (Kodak Digital Science, Rochester, NY).

**Statistical Analysis**—All data are presented as the means ± S.E. of the mean (S.E.). Data graphics and statistical analysis were performed using Instat (version 3.0) and Prism 4 (GraphPad Software, San Diego, CA). Independent replicates for each data point (n) are identified in the figure legends. p values <0.05 were recognized as statistically significant.

**RESULTS**

**Proteomic Analysis of IMCD3 Cells Adapted to Hypertonicity**—Analysis of the proteomic data revealed that in addition to aldose reductase (27-fold increase), the most up-regulated protein in cells adapted to hypertonicity was spot 3022, depicted in Fig. 1A (22-fold increase). This difference was highly significant (p = 8 × 10^{-11}) for comparison of cells adapted to 900 mosmol/kg H2O and was 8.6-fold increased (p = 7 × 10^{-11}) in cells adapted to 600 mosmol/kg H2O as compared with isotonic controls (Fig. 1B). Peptide mass fingerprinting identified the protein in this spot as S100A4 calcium-binding protein, and this was further confirmed by liquid chromatography/mass spectrometry (Fig. 1C). Peptide mass fingerprinting data indicated a significant score (66) for S100A4 with 5 peptides matching out of 22 submitted, representing 30% sequence coverage (root mean square error was 3 ppm). Image analysis of the gel indicated a protein mass of 10.3 kDa and pl of 5.0, in good agreement with published values for S100A4 protein of 11.7 kDa and a pI of 5.2.

**Genomic Analysis of S100A4 Expression in IMCD3 Cells Adapted to Hypertonicity**—To complement the observation made in the proteomic analysis, we studied the expression of S100A4 message both by gene chip analysis (Affymetrix) and by QPCR. Data shown in Fig. 2 indicate very low levels of message in cells grown under isotonic conditions, with a substantial increase in message with adaptation of cells to increasing levels of tonicity. This pattern observed by gene chip was confirmed by QPCR. Although a further increment in message was not appreciated in the gene chip data when comparing expression in cells adapted to 600 and 900 mosmol/kg H2O, a marked increase was evident by QPCR.
Western Blot Analysis of S100A4 in Cells Adapted to Hypertonicity and Acute Increments in Tonicity—Because antibodies to S100A4 are available, to confirm the observations obtained in the proteomic analysis we examined the expression of the protein in the adapted cells and extended it to study the response to acute increments in tonicity. As depicted in Fig. 3, although undetectable at isotonic conditions the protein is robustly expressed in adapted cells, further confirming the proteomic data. Of added interest is the time-dependent expression of the protein with acute sublethal osmotic stress at 24 h, further increased at 48 h.

Expression of S100A4 in the Renal Cortex and Medulla from Rodents and Human Kidneys—To assess whether the changes seen in cultured cells are also observed in renal tissues, protein expression was examined in kidney tissues when mice were subjected to ad libitum water following thirsting for 36 h (urine osmolality increased from 1424 ± 211 to 3105 ± 524 mosmol/kg H2O, n = 6). Western blot data shown in Fig. 4A indicate minimal S100A4 protein expression in the cortex and substantial protein in the papilla. Furthermore, S100A4 protein expression increased 31.0 ± 4.2% (p < 0.01) in the papilla tissues upon thirsting animals for 36 h, with no substantial change in the cortex. Analysis of S100A4 protein expression in normal human kidney is shown in Fig. 4B for comparison and demonstrates the greatest expression in the hypertonic medulla followed by papilla tissues and essentially no protein expression in the isotonic cortex tissues.

Immunocytochemical Localization of S100A4 in Cells Adapted to Hypertonicity—Immunocytochemical staining was undertaken to assess the localization of S100A4 protein in IMCD3 employing confocal microscopy. The upper panel of Fig. 5 demonstrates a complete lack of staining for cells...
grown under isotonic conditions, whereas a consistent high level of staining occurs in cells adapted to 900 mosmol/kg H2O (lower panel). Cells adapted to 600 mosmol/kg H2O demonstrate a somewhat intermediate level of staining. In all cases immunocytochemical staining for S100A4 occurred uniformly within the cytoplasm and without significant structural details.

Kinetics of S100A4 Message and Protein Expression following Exposure to Hypertonicity—The onset of S100A4 message in IMCD3 cells subjected to acute sublethal osmotic stress was evaluated to determine whether the response is rapid or delayed. To this end we undertook QPCR measurements for message and Western blot analysis for protein at several time points following exposure to sublethal hypertonicity. Data shown in Fig. 6 demonstrate a significant increase in message only after 8–10 h of hypertonic stress (550 mosmol/kg H2O) with a maximum level of message after 16 h. S100A4 protein expression following acute sublethal osmotic stress is depicted in Fig. 7 with increasing protein levels determined over the 32-h analysis period. Analysis of half-life for S100A4 message and protein in IMCD3 cells is shown in Fig. 8. Data were curve fit for decay and the half-life calculated to be 6.8 and 85.5 h for S100A4 message and protein, respectively.

Studies on the Osmotic and Ionic Mediators of the Up-regulation of S100A4 Expression—To assess whether the effects observed with NaCl stress were unique to this solute, S100A4 expression was measured after exposure to other solutes as well. As depicted in Fig. 9, other osmotically active solutes, including sucrose and mannitol, also caused a marked increase in protein expression, but not so with a more permeable solute such as urea. Replacement of sodium with choline did not affect the response.

Effects of Silencing S100A4 Expression in IMCD3 Cells on Survival during Acute Hypertonic Stress—To assess the physiologic significance of the osmotic up-regulation of S100A4, we under-
took to silence the expression. A plasmid-based system generating fold back stem-loop structures that are processed into the specific small interference RNA was used (pSM2-S100A4; Open Biosystems). This vector was lipid-transfected into IMCD3 cells and stable clones selected by growth in puromycin-containing medium. Expression of S100A4 protein in three

FIGURE 5. Comparison of cellular expression of S100A4 protein in IMCD3 cells chronically adapted to increasing hypertonicity with control cells at isotonic conditions. Coverglass-grown cells were imaged when nearly confluent with the polyclonal S100A4 antibody (Abcam) and Alexa 488-conjugated secondary antibody. Cell nuclei were stained with To-Pro and imaged with a Leica TCS SL confocal laser scanning microscope. Cells grown at 300 mosmol/kg H2O (top panel) demonstrate little or no S100A4 protein as compared with cells adapted to 600 (middle panel) or 900 mosmol/kg H2O (bottom panel). Size bars, 20 μm.

FIGURE 6. Effects of acute sublethal osmotic stress (550 mosmol/kg H2O) on the expression of S100A4 message in IMCD3 cells. Significant increases in message occurs following 10 h of acute osmotic stress and appears to be maximal at 16 h. Data represent the mean ± S.E. of three independent experiments performed in triplicate (n = 9).

FIGURE 7. Effect of acute sublethal osmotic stress (550 mosmol/kg H2O) on S100A4 protein expression in IMCD3 cells. Cell lysates (0–32 h) were analyzed by Western blot. Data depict the mean ± S.E. from three Western blots (50 μg of total protein/lane, n = 6). A representative Western blot is shown.

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representative clones and the empty vector control following incubation under acute osmotic stress for 48 h are shown in Fig. 10. All three clones were totally silenced for expression of S100A4 protein.

A comparison of the effect of silencing S100A4 protein expression on the survival of IMCD3 cells during acute sublethal hypertonic stress is shown in Fig. 11A. Cell number data indicate that silencing of S100A4 results in a 48-h delay in the onset of adaptation of IMCD3 cells to the hypertonic stress. We then studied the impact of silencing the protein on cell growth. As depicted in Fig. 11B, silencing had no effect on growth rates under isotonic conditions but significantly (p < 0.001) decreased cell growth in chronically adapted cells (40.6 ± 4.9% reduction in cellular growth, slope of the linear regression curve fit).

**DISCUSSION**

The IMCD3 cell line from mouse provides a unique tool to study the adaptive changes in the collecting duct of the kidney. Unlike mouse papilla, IMCD3 cells may be examined under isotonic growth conditions as well as chronically adapted to the hypertonic setting. In this work we have applied the DIGE approach to study the changes in the cellular proteome in response to chronic adaptation to hypertonic stress in IMCD3 cells. As with any current proteomic technique, DIGE allows for the study of changes to only a fractional subset of the proteome (32, 33). In our studies, the small, calcium-binding protein S100A4 was determined to be highly up-regulated by DIGE analysis, Western blot, gene chip analysis, and quantitative PCR. The expression of S100A4 was also found in the hypertonic papilla tissues of mice with little or no expression in the cortex of the kidney. This expression of S100A4 protein in the papilla was further increased with thirsting of animals and concentration of urine for a 36-h period. Comparison of S100A4 expression in human kidney tissues followed a similar pattern with essentially no expression in the cortex and substantial expression in the hypertonic medulla and papilla.

The onset of expression of message for S100A4 with acute hypertonic stress was evaluated in comparison to other important osmotic stress response proteins. As an example, TonEBP-binding Protein (TonEBP) is a transcriptional activator involved in the cellular response to increased tonicity (34). Osmoprotective genes regulated by TonEBP include aldose reductase (AR) for sorbitol production and the betaine transporter (BGT1) among others. Following exposure of IMCD3 cells to acute hypertonic stress, a variety of osmoprotective genes are rapidly up-regulated under the control of the TonEBP promoter. We have previously determined that the up-regulation of message for several of these TonEBP-controlled genes, including AR and BGT1, occurs within 6 h in IMCD3 cells exposed to acute osmotic stress (35). In contrast, the onset of expression of message for S100A4 with acute hypertonic stress (10 h) suggests that it is a delayed response gene and
possibly more involved in adaptation than initial osmotic stress response.

Expression of S100A4 protein was absent in IMCD3 cells under isotonic conditions. In chronically adapted cells, however, localization of the S100A4 protein appears in the cytoplasm with increasing intensity in cells grown at higher tonicity. Whereas cells at 900 mosmol/kg H₂O demonstrated uniform staining, a more variable staining of S100A4 protein in IMCD3 cells adapted to an intermediate level of hypertonicity (Fig. 5, middle panel) may be associated with the phase of the cell cycle. Additional efforts are underway to better describe any potential connections between cell cycle and S100A4 protein expression.

The extremely long half-life of S100A4 protein in IMCD3 cells may indicate a role in structural related proteins. In fact, S100A4 has been identified to interact with F-actin and non-muscle myosin-IIA (6, 11, 17–24). Previous studies demonstrated that S100A4 acts to disorganize filaments due to a cross-linking activity (23). Our laboratory has evaluated changes in F-actin structure in IMCD3 cells with chronic adaptation to hypertonic stress and found a substantial reduction in the peripheral ring. This reduction in F-actin may be involved in adaptation and growth rate for S100A4-silenced cells under hypertonic stress. We envision that the hypertonic expression of S100A4 in adapted cells provides a “preconditioning” by reducing the amount of F-actin in the peripheral ring. This action enhances the normal cellular regulatory volume changes (i.e. cell shrinkage) that is important during additional acute changes in the hypertonic conditions (36). Conversely, silencing S100A4 expression may reduce the dissociation of F-actin filaments resulting in a more substantial peripheral ring and reduce the efficacy of regulatory volume changes. Changes in F-actin may also alter the activity of ion transporters, leading to intracellular ion accumulation and an increased demand for ion transport with a resulting lower cellular energy level. Lower cellular energy levels may hamper the adaptive response and delay active cellular functions, including resumption of cell growth.

In conclusion, expression of the small, calcium-binding protein S100A4 is induced by hypertonic stress in both IMCD3 cells and the papilla of mouse kidneys. Silencing the normal up-regulation of S100A4 during exposure to acute hypertonic stress profoundly delays the osmoadaptive response and slows further growth under hypertonic conditions. The mechanism whereby S100A4 impacts the osmoadaptive response and cell growth in hypertonic states is the subject of ongoing studies in our laboratory.

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REFERENCES

1. Chamberlin, M. E., and Strange, K. (1989) Am. J. Physiol. 257, C159–C173
2. Garcia-Perez, A., and Burg, M. B. (1991) Physiol. Rev. 71, 1081–1115
3. Rivard, C. J., Capasso, J. M., Heasley, L. E., and Berl, T. (2003) J. Am. Soc. Neph. 14, 56A
4. Tian, W., and Cohen, D. M. (2002) Am. J. Physiol. 283, F388–F398
5. Rauchman, M. I., Nigam, S. K., Delpierre, E., and Gullans, S. R. (1993) Am. J. Physiol. 265, F416–F424
6. Garrett, S. C., Varney, K. M., Weber, D. I., and Bresnick, A. R. (2006) J. Biol. Chem. 281, 677–680
7. Marenholz, I., Heizmann, C. W., and Fritz, G. (2004) Biochem. Biophys.

T. Berl, unpublished study.

FIGURE 11. Effect of silencing S100A4 expression on IMCD3 cells under hypertonic conditions. A, comparison of cell viability of IMCD3 clones expressing the empty vector or silencer for S100A4 with incubation time in the setting of acute sublethal osmotic stress (550 mosmol/kg H₂O) after cells reached confluence at isotonic conditions. Cell counts were performed as detailed under “Experimental Procedures” over a 5-day period. Data represent the mean ± S.E. for three independent experiments performed in triplicate (n = 9) and demonstrate a 48-h delay in adaptation and resumption of cell growth. B, comparison of cell growth rates for empty vector and S100A4-silenced cells under isotonic conditions and chronically adapted to 550 mosmol/kg H₂O. No significant difference was determined for cells grown in the isotonic setting. However, a 40.6 ± 4.9% reduction in the growth rate for S100A4-silenced cells as compared with the empty vector controls was determined in cells chronically adapted to 550 mosmol/kg H₂O (n = 6, p < 0.0001).
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Res. Commun. 322, 1111–1122

8. Ravasi, T., Hsu, K., Goyette, J., Schroder, K., Yang, Z., Rahimi, F., Miranda, L. P., Alewood, P. F., Hume, D. A., and Geczy, C. (2004) Genomics 84, 10–22

9. Donato, R. (1999) Biochim Biophys Acta 1450, 191–231

10. Davies, M., Harris, S., Rudland, P., and Barraclough, R. (1995) DNA Cell Biol. 14, 825–832

11. Gibbs, F. E., Barraclough, R., Platt-Higgins, A., Rudland, P. S., Wilkinson, M. C., and Parry, E. W. (1995) J. Histochem. Cytochem. 43, 169–180

12. Barraclough, R. (1998) Biochim. Biophys. Acta 1448, 190–199

13. Kalluri, R., and Neilson, E. G. (2003) J. Clin. Investig. 112, 1776–1784

14. Robertson, H., Ali, S., McDonnell, B. I., Burt, A. D., and Kirby, J. A. (2004) J. Am. Soc. Nephrol. 15, 390–397

15. Kato, C., Kojima, T., Komaki, M., Mimori, K., Duarte, W. R., Takenaga, K., and Ishikawa, I. (2005) Biochem. Biophys. Res. Commun. 326, 147–153

16. Levett, D., Flecknell, P. A., Rudland, P. S., Barraclough, R., Neal, D. E., Mellon, J. K., and Davies, B. R. (2002) Am. J. Pathol. 160, 693–700

17. Davies, B. R., Davies, M. P., Gibbs, F. E., Barraclough, R., and Rudland, P. S. (1993) Oncogene 8, 999–1008

18. Gibbs, F. E., Wilkinson, M. C., Rudland, P. S., and Barraclough, R. (1994) J. Biol. Chem. 269, 18992–18999

19. Kriaievska, M., Tarabykina, S., Bronstein, I., Maitland, N., Lomonosov, M., Hansen, K., Georgiev, G., and Lukardin, E. (1998) J. Biol. Chem. 273, 9852–9856

20. Kriaievska, M. V., Cardenas, M. N., Grigorian, M. S., Ambartsumian, N. S., Georgiev, G. P., and Lukardin, E. M. (1994) J. Biol. Chem. 269, 19679–19682

21. Mandinova, A., Atar, D., Schafer, B. W., Spiess, M., Aebi, U., and Heizmann, C. W. (1998) J. Cell Sci. 111, Pt. 14, 2043–2054

22. Mazzucchelli, L. (2002) Am. J. Pathol. 160, 7–13

23. Watanabe, Y., Usada, N., Minami, H., Morita, T., Tsugane, S., Ishikawa, R., Kohama, K., Tomida, Y., and Hidaka, H. (1993) FEBS Lett. 324, 51–55

24. Li, Z. H., Spektor, A., Varlamova, O., and Bresnick, A. R. (2003) Biochemistry 42, 14258–14266

25. Bustamante, M., Roger, F., Bochaton-Piallat, M. L., Gabbiani, G., Martin, P. Y., and Feraillé, E. (2003) Am. J. Physiol. 285, F336–F347

26. Li, Z. H., and Bresnick, A. R. (2006) Cancer Res. 66, 5173–5180

27. Psitkun, T., Bieniek, J., Tchapyjnikov, D., Wang, G., Wu, W. W., Shen, R. F., and Knepper, M. A. (2006) Physiol. Genomics 25, 263–276

28. Le Hir, M., Hegyi, I., Cueni-Loffing, D., Loffing, J., and Kaissling, B. (2005) Histochem. Cell Biol. 123, 335–346

29. Capasso, J. M., Rivard, C. J., and Berl, T. (2001) Am. J. Physiol. 280, F768–F776

30. Blake, C. A., Brown, L. M., Duncan, M. W., Hunsucker, S. W., and Helmke, S. M. (2005) Exp. Biol. Med. (Maywood) 230, 800–807

31. Capasso, J. M., Rivard, C., and Berl, T. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13414–13419

32. Anderson, N. L., Polanski, M., Pieper, R., Gatlin, T., Tirumalai, R. S., Conrads, T. P., Veenstra, T. D., Adkins, J. N., Pounds, J. G., Fagan, R., and Lobley, A. (2004) Mol. Cell. Proteomics 3, 311–326

33. Li, X., Gong, Y., Wang, Y., Wu, S., Cai, Y., He, P., Lu, Z., Ying, W., Zhang, Y., Jiao, L., He, H., Zhang, Z., He, F., Zhao, X., and Qian, X. (2005) Proteomics 5, 3423–3441

34. Woo, S. K., Lee, S. D., and Kwon, H. M. (2002) Pflugers Arch. Eur. J. Physiol. 444, 579–585

35. Klawitter, J., Rivard, C. J., Capasso, J. M., Almeida, N. E., Christians, U., Leibfritz, D., Berl, T., and Chan, L. K. (2005) J. Am. Soc. Nephrol. 16, 571A

36. Ebner, H. L., Cordas, A., Patundo, D. E., Schwarzbaun, P. J., Pelster, B., and Krumanschnabel, G. (2005) Am. J. Physiol. 289, R877–R890