Proteomic Analysis of Cellular Response to Osmotic Stress in Thick Ascending Limb of Henle’s Loop (TALH) Cells*

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Epithelial cells of the thick ascending limb of Henle’s loop (TALH cells) play a major role in the urinary concentrating mechanism. They are normally exposed to variable and often very high osmotic stress, which is particularly due to high sodium and chloride reabsorption and very low water permeability of the luminal membrane. It is already established that elevation of the activity of aldose reductase and hence an increase in intracellular sorbitol are indispensable for the osmotic adaptation and stability of the TALH cells. To identify new molecular factors potentially associated with the osmotic stress-resistant phenotype in kidney cells, TALH cells exhibiting low or high levels of resistance to osmotic stress were characterized using proteomic tools. Two-dimensional gel analysis showed a total number of 40 proteins that were differentially expressed in TALH cells under osmotic stress. Twenty-five proteins were overexpressed, whereas 15 proteins showed a down-regulation. Besides the sorbitol pathway, enzymes aldose reductase, whose expression was 15 times increased, many other metabolic enzymes like glutathione S-transferase, malate dehydrogenase, lactate dehydrogenase, α-enolase, glyceraldehyde-3-phosphate dehydrogenase, and triose-phosphate isomerase were up-regulated. Among the cytoskeleton proteins and cytoskeleton-associated proteins vimentin, cytokeratin, tropomyosin 4, and annexins I, II, and V were up-regulated, whereas tubulin and tropomyosins 1, 2, and 3 were down-regulated. The heat shock proteins α-crystallin chain B, HSP70, and HSP90 were found to be overexpressed. In contrast to the results in oxidative stress the endoplasmic reticulum stress proteins like glucose-regulated proteins (GRP78, GRP94, and GRP96), calreticulin, and protein disulfide isomerase were down-regulated under hypertonic stress. Molecular & Cellular Proteomics 4: 1445–1458, 2005.

Elevation of extracellular osmolality induces a decrease in the cell volume in most cells. Mammalian kidneys are regularly exposed to steep osmotic gradients because of the urine concentration mechanism. Kidney cells, especially the epithelial cells, are protected from the osmotic effect of concentrated sodium ions, chloride ions, and urea in the interstitium by accumulating organic osmolytes such as sorbitol, betaine, inositol, glycerophosphocholine, and taurine (1). These organic osmolytes are involved in maintaining cell volume and electrolyte contents without perturbing the protein structure and function over a wide concentration range (2).

The cells of the renal medulla are exposed under normal physiological conditions to widely fluctuating extracellular solute concentrations (3). Outer medullary cells of the thick ascending limb of Henle’s loop (TALH)1 participate to a considerable degree in the regulation of the osmotic gradient due to their strong NaCl transport activity and low water conductivity. Thus, they have to possess specific osmoregulatory mechanisms to tolerate and counteract changes of extracellular osmolality (4).

Sorbitol is one of the principal organic osmolytes in the renal medulla. It is already established that a high extracellular concentration of NaCl or glucose induces sorbitol accumulation in the cells of the inner renal medulla and TALH cells. This increase of intracellular sorbitol content is accompanied and augmented by an induction of aldose reductase (AR), which reduces α-glucose in the polyl pathway (5, 6). Aldose reductase is present in a variety of tissues including kidney (7), liver (8), ocular lens, and retina (9) and in erythrocytes (10). In renal epithelial cells a 4-fold increase in AR mRNA expression was observed after 24 h of osmotic stress (11).

In addition, recent investigations on renal cells have disclosed that osmotic stress is also a trigger for enhanced expression of heat shock proteins (HSPs). So far five target HSPs for this kind of stress have been identified: two members of the HSP70 family, two small HSPs, HSP25/27, α-crystallin B chain, and a new member of the HSP110 subfamily, the so-called osmotic stress protein OSP94, which is highly inducible by hyperosmolality (12). Modulation of intracellular

1 The abbreviations used are: TALH, thick ascending limb of Henle’s loop; 2D, two-dimensional; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; GRP, glucose-regulated protein; AR, aldose reductase; BiP, binding protein; TM, tropomyosin; ER, endoplasmic reticulum.

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contents of organic osmolytes, elevated activity of AR, and the expression of specific HSPs are aspects of the complex response of medullary renal cells, including TALH cells, to widely fluctuating extracellular solute concentration (12).

Proteomics uniquely allows delineation of global changes in protein expression resulting from transcriptional and post-transcriptional control, post-translational modification, and shifts in protein between different cellular compartments and under different conditions. The purpose of the present study was to investigate the protein expression changes in the epithelial cells of the thick ascending limb of Henle’s loop under different conditions of osmotic stress using proteomic tools.

**EXPERIMENTAL PROCEDURES**

**Materials**— Dulbecco’s modified Eagle’s medium was from Invitrogen. L-Glutamine, urea, DTT, and monoclonal anti-β-actin antibody were from Sigma. Culture flasks were from Falcon. CHAPS was from Merck. Precision Plus protein marker and Bio-Lyte® were from Bio-Rad. Bovine serum albumin was from Roche Applied Science. Protease Inhibitor Mix 100 was from Amersham Biosciences. The Lambda2 UV/visible spectrophotometer was from PerkinElmer Life Sciences. The Sequazym™ peptide mass standard kit was from Applied Biosystems. Colloidal Coomassie Blue stain (Roti-Blue) was from Carl-Roth. Monoclonal mouse anti-vimentin antibody and monoclonal mouse anti-human cytokeratin antibody were from Dako. Rabbit anti-tropomyosin 4 polyclonal antibody was from Chemicon International. Mouse anti-HSP70 monoclonal antibody, mouse anti-Hsp90 monoclonal antibody, and rabbit anti-ERP72 (protein-disulfide isomerase) polyclonal antibody were from Stressgen. Mouse anti-β-actin monoclonal antibody and rabbit anti-GRP78/BIP polyclonal antibody were from Sigma. Mouse anti-calcitrocin monoclonal antibody was from BD Biosciences.

**Cell Line and Culture Procedure**—The epithelial cell line used in these experiments was derived from a rabbit kidney’s outer medulla. Cultured cells were immortalized by SV40 early region DNA. They show a high degree of differentiation and specialization and provide a suitable model to study TALH cell function in vitro.

The TALH cell line was maintained as a monolayer culture in Dulbecco’s modified Eagle’s medium including 5.5 mmol/l d-glucose supplemented with 10% fetal calf serum, 1% minimum Eagle’s medium with nonessential amino acids, 1% l-glutamine, and 1% penicillin/streptomycin. Cells were routinely cultured in 75-cm² tissue culture flasks at 37 °C in a humidified 5% CO₂, 95% air atmosphere.

**Osmotic Stress Experiments**—After reaching 70% confluence, TALH cells cultivated in 300 mosmol/kg medium (TALH-STD) were stressed with 600 mosmol/kg medium. TALH cell lines exhibiting a high resistance to osmolarity (600 mosmol/kg) (TALH-NaCl) were established. The osmolarity was controlled routinely. All osmotic stress experiments were repeated three times.

**Sample Solubilization and Protein Determination**—Confluent cultures were scraped and washed three times with PBS of different osmolarity (300 or 600 mosmol/kg). The cells were harvested by centrifugation at 1800 rpm for 10 min, the pellet was treated with 0.3–0.5 ml of lysis buffer containing 9.5 mm urea, 2% (w/v) CHAPS, 2% (w/v) amphoteries, 1% DTT, and 10 mM PMSE. Aliquots of 500 μl of urea buffer were frozen till use; Ampholine, DTT, pepstatin (to a final concentration of 1.4 μM), and Complete™ from Roche Diagnostic (according to the manufacturer’s protocol) were freshly added. After adding the lysis buffer the samples were incubated for 30 min at 4 °C. For removing the cell debris sample centrifugation was carried out at 15,000 rpm and 4 °C for 45 min. Supernatant was recentrifuged at 15,000 rpm and 4 °C for an additional 45 min to get maximal purity. The resulting samples were used immediately or stored at –80 °C until used. Protein concentration was measured according to Bradford (13) using bovine serum albumin as a standard.

**Two-dimensional Electrophoresis**—Each sample was diluted in rehydration buffer (8 M urea, 1% (w/v) CHAPS, 0.2% amphoteries pH 3–10, 15 mM DTT, and a trace of bromphenol blue) to a final volume of 350 μl. The mixture containing 400 μg of proteins from cell lysate was used for the hydration of IPG strips. The strips (pH 3–10, 17 cm) were allowed to rehydrate for 1 h before adding mineral oil. The passive hydration of the gels was carried out overnight for at least 12 h at room temperature in a focusing chamber. Isoelectric focusing with a Protean IEF cell was performed at 20 °C using the following multistep protocol: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 4 h. After the first dimension, the individual strips were equilibrated in 6 M urea, 30% (w/v) glycerol, 2% SDS (w/v), 0.05 M Tris-HCl, pH 8.8, and 15 mM DTT for 20 min. An additional incubation in the same buffer supplemented with iodoacetamide (40 mg/ml) was carried out for another 20 min. The second dimension was performed overnight at 120 V using a homogenous acrylamide gel (12% T, 200 × 230 × 1.5 mm) applying a continuous Laemmli buffer system.

**Protein Visualization and Image Analysis**—Gels were stained with colloidal Coomassie Brilliant Blue G-250 as described by previously Neuhoff et al. (14). Image analysis was performed using the PDQuest system according to the protocols provided by the manufacturer. To account for experimental variation three gels were prepared for each experiment. The gel spot pattern of each gel was summarized in a standard after spot matching. Thus, we obtained one standard gel for each experiment. These standards were then matched to yield information about new spots related to the stress resistance in TALH cells (up- or down-regulation of spots).

**In-gel Digestion and MALDI-TOF MS Analysis of Protein Spots**—Coomassie Brilliant Blue-stained spots were manually excised from the gels and then washed with distilled water for 15 min. The destaining procedure was carried out by washing the spots alternately with 50% ACN and 100 mM ammonium bicarbonate three times for 5 min. After dehydrating the spots with ACN for 15 min, they were dried in a vacuum centrifuge for 15 min. Thereafter the gel spots were rehydrated for digestion with 40 μl of trypsin (10 ng/μl in 100 mM ammonium bicarbonate) and incubated at 37 °C overnight. The peptide samples were extracted with different concentrations of ACN and TFA. The peptide samples were then co-crystallized with matrix (α-cyano-4-hydroxycinnamic acid) on a stainless steel target using 1 l of matrix and 1 μl of sample. An Applied Biosystems Voyager-DE STR time-of-flight mass spectrometer, operating in delayed reflector mode with an accelerated voltage of 20 kV, was used to generate peptide mass maps. Mass spectra were obtained by averaging 50 individual laser shots. All samples were externally calibrated with a peptide mixture of des-Arg-bradykinin ([M + H]⁺ 904.46), angiotensin I ([M + H]⁺ 1296.68), Glu1-fibrinopeptide B ([M + H]⁺ 1570.67), ACTH-(1–17) ([M + H]⁺ 2093.08), and ACTH-(18–39) ([M + H]⁺ 2465.19), and the resulting mass spectra were internally calibrated with trypsin autolysis products (m/z 842.50 and m/z 2211.10). Monoisotopic peptide masses were assigned and then used in Mascot database searches.

**Database Search**—A database search with the peptide masses was performed against the Mass Spectrometry Protein Sequence Database (MSDB) or the National Center for Biotechnology non-redundant (NCBI) database using the Mascot peptide mass fingerprint software provided by Matrix Science (Oxford, UK, www.matrixscience.com/search_form_select.html) (15). Carboxyamidomethylation and methionine oxidation were considered as variable modifications. A database search was performed so that after identification each hit was inspected visually to match as much spectral information as
possible. The quality criteria encompassed optimized mass accuracy (~50 ppm), minimal mass deviation (in the mDa range), maximized sequence coverage, and highest possible probability score had to be assigned to the identified protein.

Peptide Sequence Analysis—To confirm and accomplish the data obtained from mass finger print analysis, all samples were subjected to peptide sequence analysis. After in-gel digestion the extracted peptides were dissolved in 0.1% formic acid. One microliter of sample was introduced using a CapLC autosampler (Waters) onto a μ-pre-column™ cartridge, a C18 pepMap (300 μm × 5 mm, 5-μm particle size), and further separated through a C18 pepMap100 nano-Series™ (75 μm × 15 cm, 3-μm particle size) analytical column (LC Packings).
## Table I

List of all identified proteins (pH 3–10) in TALH cell lines using MALDI-TOF, microsequencing, and database comparisons

| Spot | Protein name | Molecular mass (Daltons) | pI | Score MS/MS | Score fingerprint |
|------|--------------|--------------------------|----|-------------|-------------------|
| 1    | 14-3-3 protein $\beta$ | 28,054 | 4.81 | 483 | 95 |
| 2    | 14-3-3 protein $\epsilon$ renal | 29,155 | 4.64 | 342 | 102 |
| 3    | 14-3-3 protein $\zeta$ | 27,728 | 4.73 | 187 | 119 |
| 4    | 3-Hydroxy-CoA dehydrogenase | 27,140 | 8.45 | 197 | 85 |
| 5    | 3-Ketoacyl-CoA thiolase | 41,844 | 175 | 96 |
| 6    | 5-Aminomidazole-4-carboxamide ribonucleotide formyltransferase | 64,117 | 6.65 | 221 | 68 |
| 7    | Aconitase 2 | 85,410 | 8.08 | 125 | 90 |
| 8    | Aconitase hydratase | 82,473 | 7.12 | 156 | 86 |
| 9    | Actin $\beta$ | 41,579 | 5.25 | 419 | 163 |
| 10   | Actin $\beta$ | 41,579 | 5.25 | 325 | 145 |
| 11   | Actin $\beta$ | 41,579 | 5.25 | 156 | 125 |
| 12   | Actin $\gamma$ | 41,579 | 5.25 | 212 | 158 |
| 13   | Adenylate kinase 3 | 25,595 | 8.69 | 228 |
| 14   | Alcohol dehydrogenase class III | 39,439 | 7.57 | 79 |
| 15   | Aldose reductase | 35,763 | 6.48 | 296 | 204 |
| 16   | Aldose reductase | 35,763 | 6.48 | 165 | 151 |
| 17   | Aldose reductase | 35,763 | 6.48 | 98 |
| 18   | Aldose reductase | 35,763 | 6.48 | 74 | 115 |
| 19   | Aldose reductase | 35,609 | 6.40 | 221 |
| 20   | Aldose reductase | 35,609 | 6.48 | 290 |
| 21   | Aldose reductase | 35,609 | 6.48 | 112 |
| 22   | $\alpha$ enolase | 47,172 | 6.16 | 616 | 122 |
| 23   | $\alpha$ enolase | 47,040 | 5.84 | 810 | 71 |
| 24   | $\alpha$ enolase | 47,008 | 6.99 | 187 | 110 |
| 25   | $\alpha$ enolase | 47,008 | 6.99 | 221 |
| 26   | $\alpha$-Crystallin chain B | 20,024 | 6.76 | 205 | 110 |
| 27   | $\alpha$-Crystallin chain B | 20,024 | 6.76 | 103 | 98 |
| 28   | Annexin I | 38,711 | 6.28 | 336 | 111 |
| 29   | Annexin II (lipocortin II, calpactin I) | 38,448 | 8.36 | 360 | 100 |
| 30   | Annexin II (lipocortin II, calpactin I) | 38,448 | 8.36 | 194 | 99 |
| 31   | Annexin V | 35,914 | 4.94 | 49 | 98 |
| 32   | Annexin VIII | 35,914 | 4.94 | 89 | 120 |
| 33   | ATP synthase $\beta$ chain | 36,657 | 5.53 | 128 |
| 34   | A-X-actin ($\gamma$ actin-like protein) | 41,667 | 5.21 | 236 | 85 |
| 35   | A-X-actin ($\gamma$ actin-like protein) | 41,667 | 5.21 | 95 | 96 |
| 36   | Bat 1 | 48,944 | 5.43 | 49 | 98 |
| 37   | Bicaudal D protein | 93,334 | 5.26 | 119 |
| 38   | BiP protein dnak-type molecular chaperone | 72,302 | 5.07 | 408 | 80 |
| 39   | Calmodulin | 16,884 | 4.11 | 255 | 114 |
| 40   | Calreticulin | 67,277 | 4.30 | 321 | 102 |
| 41   | Calreticulin | 67,277 | 4.30 | 254 | 89 |
| 42   | Chaperonin groEL | 60,903 | 5.67 | 270 | 162 |
| 43   | Creatine kinase B | 42,648 | 5.34 | 193 | 119 |
| 44   | Cytokeratin 19 | 44,609 | 5.21 | 668 | 101 |
| 45   | Cytokeratin 8 | 54,018 | 5.80 | 320 |
| 46   | Dnak-type molecular chaperone grp75 | 73,744 | 5.87 | 562 | 227 |
| 47   | Dnak-type molecular chaperone hsc73 | 73,808 | 6.04 | 699 | 132 |
| 48   | Desmin | 34,726 | 4.90 | 120 |
| 49   | Dihydrolipoamide dehydrogenase | 54,143 | 7.59 | 110 |
| 50   | Dnak-type molecular chaperone HSPA5 | 72,423 | 5.01 | 329 |
| 51   | Elongation factor 1 | 95,335 | 6.31 | 165 | 102 |
| 52   | Elongation factor 2 | 95,146 | 6.41 | 308 | 109 |
| 53   | EndoA' cytoxin | 53,210 | 5.42 | 173 | 116 |
| 54   | ERP57 protein | 56,761 | 5.98 | 215 |
| 55   | Ezrin (p81, cytovillin) | 69,046 | 6.09 | 182 |
| 56   | GRP78 | 73,744 | 5.78 | 133 |
| 57   | GRP94 | 82,557 | 4.90 | 246 | 120 |
| Spot | Protein name | Molecular mass | pl  | Score MS/MS | Score fingerprint (p < 0.05) |
|------|--------------|----------------|-----|-------------|-----------------------------|
| 58   | Glutathione S-transferase | 26,617 | 6.82 | 175 | 152 |
| 59   | Glutathione S-transferase | 25,678 | 6.45 | 272 | 102 |
| 60   | GAPDH        | 35,827 | 8.51 | 512 | 120 |
| 61   | G-protein β subunit-like protein | 34,504 | 7.72 | 141 | |
| 62   | GTP-binding protein Ran | 22,339 | 9.16 | 111 | |
| 63   | Guanosine diphosphate dissociation inhibitor 2 | 50,236 | 6.30 | 122 | |
| 64   | H+ -transporting two-sector ATPase | 50,770 | 4.90 | | 123 |
| 65   | Heat shock cognate protein 70 kDa | 42,081 | 6.62 | 326 | |
| 66   | HSPA6        | 70,828 | 5.28 | 345 | 98  |
| 67   | Heat shock cognate protein 70 kDa | 42,081 | 6.62 | 579 | |
| 68   | Heat shock protein 60 kDa | 60,950 | 5.84 | 102 | |
| 69   | Heat shock protein 8 | 70,828 | 5.28 | 129 | 120 |
| 70   | Heat shock protein 8 | 70,828 | 5.28 | 129 | 120 |
| 71   | HSP84        | 83,229 | 4.97 | 159 | 110 |
| 72   | Heat shock protein 90-α | 84,480 | 4.96 | 143 | 95  |
| 73   | Heat shock protein 90-α | 84,480 | 4.96 | 216 | 125 |
| 74   | Heat shock 20-KDa-like protein p20 | 20,024 | 7.67 | 115 | |
| 75   | Heme-binding protein 1 | 21,039 | 5.18 | | 110 |
| 76   | Heterogeneous nuclear ribonucleoprotein G | 42,208 | 9.96 | 125 | |
| 77   | HSP 70-kDa protein 5 | 70,038 | 5.48 | 155 | |
| 78   | Hspa1 protein | 70,050 | 5.53 | 302 | |
| 79   | Hspa1a protein | 70,050 | 5.53 | 239 | |
| 80   | Hypothetical protein leucine-rich repeat signature | 34,223 | | 165 | |
| 81   | Isocitrate dehydrogenase | 50,877 | 518 | 80 | |
| 82   | Lactate dehydrogenase | 36,564 | 8.17 | 303 | 132 |
| 83   | Lamin A | 74,279 | 6.53 | 251 | 121 |
| 84   | Lamin C | 65,096 | 6.54 | 210 | 152 |
| 85   | Long-chain-acyl-CoA dehydrogenase | 47,842 | 7.63 | 210 | 89  |
| 86   | Malate dehydrogenase | 35,589 | 8.93 | 467 | 82  |
| 87   | Methyltransferase | 55,992 | 8.76 | 166 | |
| 88   | Modulator recognition factor 1 | 65,948 | 9.40 | 135 | |
| 89   | Moesin | 67,584 | 6.22 | 156 | |
| 90   | Moesin | 67,584 | 6.22 | 141 | |
| 91   | Mortalin-2 (HSP70 9B) | 70,827 | 5.37 | 163 | |
| 92   | Myosin phosphatase target subunit 1 | 108,719 | 5.30 | | 110 |
| 93   | Nebulin-related anchoring protein isoform S | 195,791 | 9.24 | 123 | |
| 94   | Nucleophosmin | 32,540 | 4.62 | 161 | |
| 95   | p60 protein (Hsp70/Hsp90 organizing protein) | 62,611 | 6.40 | 102 | |
| 96   | p66 mot1 (hsp70 family) | 73,528 | 5.91 | 165 | 91  |
| 97   | Phosphoglycerate kinase | 44,377 | 7.54 | 136 | 191 |
| 98   | Phosphoglycerate mutase | 28,655 | 6.67 | 163 | |
| 99   | Phosphatidylethanolamine-binding protein | 20,913 | 7.42 | 115 | |
| 100  | Peptidyl-prolyl cis-trans isomerase A | 17,166 | 8.44 | | 120 |
| 101  | Protein-disulfide isomerase | 56,761 | 4.77 | 838 | 218 |
| 102  | Protein-disulfide isomerase | 57,203 | 4.80 | 568 | 147 |
| 103  | Protein-disulfide isomerase A4 (ERp72) | 72,932 | 4.96 | 692 | 139 |
| 104  | Protein phosphatase 1 regulatory subunit 12A | 109,654 | 5.35 | 213 | |
| 105  | Pyrophosphatase-5-peptidase I | 22,439 | 5.21 | 321 | |
| 106  | Pyruvate kinase | 57,737 | 7.96 | 745 | |
| 107  | S-Adenosylhomocysteine hydrolase | 47,663 | 5.88 | 101 | |
| 108  | Stress-induced phosphoprotein 1 | 62,528 | 6.40 | 125 | 98  |
| 109  | Stathmin (phosphoprotein p19) | 17,166 | 5.76 | 252 | 132 |
| 110  | Superoxide dismutase | 23,221 | 8.18 | 283 | 70  |
| 111  | Thioredoxin peroxidase | 22,096 | 8.27 | 261 | |
| 112  | Transforming protein VAV | 98,031 | 6.31 | | 98  |
| 113  | Transketolase | 60,545 | 6.54 | 107 | |
| 114  | Translation initiation factor eIF-4A.I | 45,262 | 5.20 | 228 | |
| 115  | Triose-phosphate isomerase | 26,609 | 7.10 | 242 | 100 |
| 116  | Tropomyosin 1 | 32,708 | 4.71 | 1065 | 122 |
The mobile phase consisted of solution A (5% ACN in 0.1% formic acid) and solution B (95% ACN in 0.1% formic acid). The total sample run time was 60 min. The first step consisted of injecting onto a precolumn and washing for 5 min with 0.1% formic acid. The washing step was followed by an elution step with an exponential flow rate down-regulated by a flow splitter from 5 μl/min to 0.25 μl/min. The precolumn was reequilibrated with 0.1% formic acid (30 μl/min) for 5 min. The nanospray needle was held at 2 kV, and the source temperature was held at 40 °C. After chromatographic separation, peptide sequencing was performed on a Q-TOF Ultima Global (Micromass, Manchester, UK) mass spectrometer equipped with a nanoflow ESI Z-spray source in positive ion mode. Multiple charged peptide parent ions were automatically marked and selected in the quadrupole and fragmented in the hexapole collision cell, and their fragment patterns were analyzed by time-of-flight. The data acquisition was performed using MassLynx (Version 4.0) software on a Windows NT PC, while data were further processed on Protein-Lynx-Global-Server (Version 2.1), (Micromass). The raw data files were deconvoluted and deisotoped using the Max Ent™ lite algorithm.

processed data were searched against MSDB and Swiss-Prot database searches 132 proteins were identified (Table I). For Western blot analysis after SDS-PAGE, a standard protocol of Towbin et al. (16) was used.

### RESULTS

**Morphology Changes of TALH Cells under Osmotic Stress**—To investigate the effect of elevated osmolarity on the cell morphology, TALH cells were exposed to physiological osmolarity of 300 mosmol/kg or an increased extracellular toxicity of 600 mosmol/kg. The osmotic stress resulted in shrinkage of the cell as a consequence of osmotically induced water loss (Fig. 1, A–C). Morphological changes in TALH-STD cells were already observed after 8 h of exposition, and significant distinctive changes were observed after 35 h. We also investigated whether TALH cells exhibiting resistance to 600 mosmol/kg were able to adapt to higher osmolarity or not. For this purpose, the cells were exposed to an external osmolarity of 900 mosmol/kg (Fig. 1, D and E). Under these conditions morphological changes occurred later (first after 12 h) than these changes of TALH cells shifted to 600 mosmol/kg, and furthermore they were less intense. After 35 h of incubating the cells in 900 mosmol/kg, these cells also showed a marked change in forms.

**Conventional 2D Electrophoresis**—The morphological changes of the TALH cells under osmotic stress are very intense. In our study we investigated whether these modifications were accompanied by changes in the cell proteome. For this purpose cell extracts were prepared from TALH-STD and -NaCl cells and separated by 2D gel electrophoresis. Comparison of protein expression in 2D gel images was carried out using the PDQuest software. An analysis of TALH imaged gels derived from conventional 2D gel electrophoresis in the pH range 3–10 identified more than 2000 (2552 for TALH-NaCl and 2654 for TALH-STD) protein spots in both stressed and non-stressed cells when 20,000 pixels were used as the filter limit. The pixel volume of each spot was calculated based on spot intensity and spot area and was followed by the normalization with the total pixel volume of all the spots in the gel image. The pixel volume of each spot provides the basis for comparison of protein expression between NaCl-stressed and non-stressed cells. A representative electropherogram from TALH-NaCl is shown in Fig. 2; all proteins that were found to be differentially expressed in these cells are numbered. Using mass spectrometry and database searches 132 proteins were identified (Table I).

Further examination of the gels showed significant differences between the protein compositions from stressed and non-stressed TALH cells. In the high molecular mass region, prominent differences in protein expression (Fig. 3A) were
recorded for protein spots 40, 57, and 101 that were only present in non-stressed cells as strongly expressed proteins. In stressed cells, these spots were either completely absent or much less intensely expressed. By contrast, in the middle molecular mass regions, new prominent protein spots (Fig. 3D, spots 15, 29, 61, and 82) were exclusively observed in gels derived from stressed cells. The same results were also observed in the low molecular mass regions for spots 15 and 26 (Fig. 3E).

Changes of Protein Expression Pattern between Stressed and Non-stressed TALH Cells—Software analysis of the gel spot images showed that 40 proteins were differently expressed in the stressed TALH cells. Twenty-five proteins from three different protein classes, namely HSPs, metabolism proteins, and structure proteins, were differentially overexpressed (Table II). Fifteen proteins belonging to endoplasmic reticulum stress proteins, structure proteins (tubulin), and other proteins were differentially down-regulated (Table III). Fig. 3 (A–G) shows gel sections of each cell line in comparison. The differentially expressed proteins are numbered corresponding to Tables I–III. The expression quantification is presented as a grouped bar chart with error bars. Each bar represents the intensity means ± S.D. of gels from three independent experiments. As a representative example of our MS/MS analysis Fig. 4A shows a representative MS/MS spectrum from the peptide fragment 345–356 of α enolase (P25704), while Fig. 4B shows a MS/MS spectrum from the peptide fragment 158–171 of creatine kinase (KIRBCB).

Western Blot Analysis of Up- or Down-regulated Proteins—To highlight the protein expression changes after osmotic stress and to confirm the results obtained in 2D gels especially in the case of endoplasmic reticulum and cytoskeleton proteins, Western blot analysis was carried out for prominent proteins (Fig. 5). The Western blot data correlate with the 2D gel results for the analyzed proteins.

DISCUSSION

Renal TALH cells are exposed to a highly variable extracellular environment in which NaCl and urea concentrations regularly reach high levels. The cells adjust osmotically by accumulating large amounts of sorbitol (17, 18). Accumulation of sorbitol protects the cells, and its level varies with extracellular osmolyte concentrations (19). In the present study we used proteomic tools to investigate the impact of the osmolarity change on the proteome pattern in TALH cells. Forty proteins were found to be differentially expressed in TALH cells under osmotic stress. Fifteen were down-regulated, and 25 up-regulated. Only some of these proteins have been linked previously to renal osmotic stress resistance (20–23). This highlights the importance of proteomic approaches, which allow new protein targets for osmotic stress resistance to be defined.

Impact of Osmotic Stress on Metabolic Protein Expression—To avoid excessive alterations in volume, cells have developed regulatory mechanisms including ion transport across the membrane and changes in metabolism. The ability of cells to resist osmotic shrinkage by cell volume regulation parallels their resistance to apoptosis after osmotic shock (24). Cells adapt to hyperosmotic stress by a variety of mechanisms that restore cell volume by restoring intracellular salt and osmolyte concentrations (19). The most evident change observed in our results was represented by overexpression of AR. However, the extent in the increase in protein expression (more than 15-fold) was surprising when compared with the increase in mRNA expression in inner medulla cells (more than 4-fold) reported previously (11). High extracellular osmolarity induces renal sorbitol synthesis and accumulation in inner medullary collecting duct cells by increasing the AR activity and decreasing membrane permeability (25, 26). The expression of the AR gene in the mammalian renal medulla has been shown to be osmotically regulated. In this tissue, AR mRNA and sorbitol content increase during dehydration or antidiuresis and decrease during diuresis (27). Moreover experiments on PAP-H25 cells derived from rabbit renal papillae demonstrated that external hyperosmolarity enhances transcription of the AR gene (28) resulting in a rapid increase in AR mRNA (29) followed by an increase in AR activity and sorbitol content (30).

α enolase was described as a heat shock protein in yeast because it shares some degree of homology with DNAK (31). Interestingly it has already been demonstrated to be an early target of oxidative damage by carbonylation in different cell systems, ranging from yeast (32) to humans (33). An involvement of enolase in oxidative stress in lens cells has been suggested previously by Paron et al. (34). The role of the overexpression of α enolase under osmotic stress remained unclear. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a central glycolytic protein with a pivotal role in energy production. However, studies have demonstrated that GAPDH or some of its isoforms display a number of activities that are unrelated to its glycolytic function (35–39). Its importance in oxidative stress and apoptosis has already been demonstrated (40). In the case of osmotic stress the pivotal role in energy production may be the main function of GAPDH. Lactate dehydrogenase and malate dehydrogenase are enzymes involved in carbohydrate metabolism. One of the reasons for the overexpression of these proteins is the activation of the gluconeogenesis pathway to provide enough glucose for the synthesis of sorbitol in the polyol pathway.

Among the overexpressed proteins adenylate kinase and creatine kinase are known to maintain the ATP level in tissues with high energy demands like brain and muscle (41). The overexpression of these proteins in TALH-NaCl highlights the higher energy needed in stressed TALH cells and the role of the energy balance under hyperosmotic stress conditions.

Changes in Cytoskeleton and Cytoskeleton-associated Protein Expression—Based on its properties and subcellular organization, vimentin is considered to be the major contributor
FIG. 3. Close-up of the region of the gels showing differential expression of proteins in non-stressed TALH-STD and their stressed counterparts, TALH-NaCl, in the range of pH 3–10. A–G, close-ups of different regions of the gels showing up- or down-regulated proteins. The protein spots are numbered corresponding to Tables I–III. The names of the proteins differentially expressed are shown in Tables II and III. The degree of differential expression in the TALH cell lines is shown in the histogram on the right side of the corresponding gel part. The expression quantification is presented as a grouped bar chart with error bars. Each bar represents the intensity means ± S.D. of gels from three independent experiments.
Proteomics, Renal Epithelial Cells, Osmotic Stress

D

TALH-STD

TALH-NaCl

E

TALH-STD

TALH-NaCl

F

TALH-STD

TALH-NaCl

G

TALH-STD

TALH-NaCl

Fig. 3—continued
to the mechanical integrity of cells and tissue. The unique viscoelastic properties of vimentin render it more resistant than either microtubules or microfilaments to deformation and other external physical stress (42). The increase in vimentin expression in stressed TALH cells seems to play an important role by stabilization and reinforcement of the cells exposed to high salt concentration. Further studies are needed to investigate the role of vimentin in osmotic stress resistance.

Tropomyosins encompass a large family of actin-regulatory proteins that are expressed by muscle and non-muscle cells. The transition of lens epithelial cells from the undifferentiated to the differentiated state is characterized by a shift in tropo-
**FIG. 4.** Representative example of MS/MS analysis. A, a representative MS/MS spectrum from the peptide fragment 345–356 of α enolase (P25704), B, a MS/MS spectrum from the peptide fragment 158–171 of creatine kinase (KIRBCB).
myosin isoform expression from high molecular weight (TM1, TM2, TM3, and TM4) to low molecular weight (TM5) and by a resulting reorganization of actin (43–45). The down-regulation of TM1–3 and up-regulation of TM4 seem to be an indication of the necessity of reorganization of actin in TALH cells under osmotic stress condition.

Fig. 5. Western blot analysis of proteins found to be up- or down-regulated. TALH cells cultivated in 300 mosmol (TALH-STD) or 600 mosmol (TALH-NaCl) medium were tested for protein expression changes with antibodies against the appropriate proteins. A, blots probed with antibodies against the appropriate protein. B, blots quantification. The degree of differential protein expression in the TALH cell lines is shown in the histogram. The expression quantification is presented as a grouped bar chart with error bars. Each bar represents the intensity means ± S.D. of blots from three independent experiments. PDI, protein-disulfide isomerase.
Molecular Chaperones—One of the major protein groups found to be influenced by osmotic stress in TALH cells is the group of chaperones, including HSPs. HSPs are a group of highly conserved proteins, some of which are expressed constitutively and/or induced by stress. Constitutively expressed HSPs participate in protein folding and assembly, elimination of misfolded proteins, and stabilization of newly synthesized proteins in various intracellular compartments. Exposure of cells to diverse physical or chemical stressors such as heat, heavy metals, hypoxia, arsenite, and amino acid analogues evokes string induction of HSP synthesis. Our results obtained from TALH-STD and its counterpart TALH-NaCl show differential expression in several major HSPs. In TALH-NaCl cells there was an increased expression of the small stress protein α-crystallin chain B and an up-regulation of members of the HSP70 family (HSC73 and HSP8a). Furthermore proteome analysis revealed up-regulation of HSP90-α in TALH-NaCl cells. HSPs enhance survival of the stressed cells by acting as molecular chaperones when normal cellular protein synthesis is inhibited through denaturation. Recent investigations on both renal and non-renal cells have reported similar effects of osmotic stress concerning the expression of α crystallin B chain and HSP70 under stress situation (48–50).

Down-regulated Proteins—In addition to up-regulated proteins, osmotic stress resulted in down-regulation of 15 proteins. Nucleophosmin that serves as a shuttle protein for the nuclear transport of ribosomal components and presumably collaborates with other nucleolar proteins in ribosome assembly (51) is known to be up-regulated under oxidative stress in lens cells (35) or as a response to genotoxic stress (52). The role of expression changes of nucleophosmin in the context of osmotic stress is still unclear.

Another interesting finding is the involvement of the endoplasmic reticulum stress proteins in osmotic stress resistance. The glucose-regulated proteins (GRPs) are a family of endoplasmic reticulum (ER) molecular chaperones and Ca\(^{2+}\)-binding stress proteins. These proteins are also induced under ER stress (53–55). Induction of GRPs by ER stress protects cells against a variety of toxic insults including Ca\(^{2+}\) ionophores, oxidative stress, toposomerase inhibitors, and cytotoxic T-cells (56–59). In our case the GRP78, GRP94, GRP96, protein-disulfide isomerase, and calreticulin were found to be down-regulated. The role of the down-regulation of ER stress proteins in the context of hyperosmolarity is still unclear. One possible aspect is that the down-regulation of these proteins leads to an increase of free calcium and so prevents disturbance of intracellular Ca\(^{2+}\) homeostasis under osmotic stress.

The use of proteomic tools to study global changes of protein expression under osmotic stress provides a more complete picture of protein targets of hypertonic stress resistance in TALH cells. This information is very important and will lead to better understanding of the urinary concentrating mechanism in renal epithelial cells. Our results provide a stage from which mechanistic and biological aspects of proteins involved in hyperosmotic stress resistance can be examined.

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