Dynamics of the G Protein-coupled Vasopressin V2 Receptor Signaling Network Revealed by Quantitative Phosphoproteomics*†

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G protein-coupled receptors (GPCRs) regulate diverse physiological processes, and many human diseases are due to defects in GPCR signaling. To identify the dynamic response of a signaling network downstream from a prototypical Gs-coupled GPCR, the vasopressin V2 receptor, we have carried out multireplicate, quantitative phosphoproteomics with iTRAQ labeling at four time points following vasopressin exposure at a physiological concentration in cells isolated from rat kidney. A total of 12,167 phosphopeptides were identified from 2,783 proteins, with 273 changing significantly in abundance with vasopressin. Two-dimensional clustering of phosphopeptide time courses and Gene Ontology terms revealed that ligand binding to the V2 receptor affects more than simply the canonical cyclic adenosine monophosphate-protein kinase A and arrestin pathways under physiological conditions. The regulated proteins included key components of actin cytoskeleton remodeling, cell-cell adhesion, mitogen-activated protein kinase signaling, Wnt/β-catenin signaling, and apoptosis pathways. These data suggest that vasopressin can regulate an array of cellular functions well beyond its classical role in regulating water and solute transport. These results greatly expand the current view of GPCR signaling in a physiological context and shed new light on potential roles for this signaling network in disorders such as polycystic kidney disease. Finally, we provide an online resource of physiologically regulated phosphorylation sites with dynamic quantitative data (http://helixweb.nih.gov/ESBL/Database/TiPD/index.html).

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lates the water channel aquaporin-2 (Aqp2) (reviewed in Ref. 5) and urea channel Slc14a2 (6) through the heterotrimeric G protein G_{12} and subsequent activation of adenylyl cyclases that mediate a rise in intracellular cAMP. Vasopressin also increases intracellular calcium through V2R and cAMP (7).

Despite a number of recent studies that have explored the steady state response of cells to vasopressin through phospho-

proteinomic methodologies (8–11), a comprehensive, dynamic profile of vasopressin signaling has yet to emerge. To carry out a dynamic phosphoproteomic analysis, we utilized a multi-

plexed labeling strategy (iTRAQ) allowing analysis of four dis-

tinct time points following the addition of vasopressin. Other studies have utilized various phosphoproteomic strategies to quantify biological responses (12–18). To our knowledge, the present study is the first temporal quantitative phosphoprote-

omic analysis of a physiological response using a native mammalian model. It also represents the largest collection of quan-

titative phosphorylation data on vasopressin signaling to date. These new data implicate a number of previously unidentified pathways that are regulated downstream of V2R, including the Wnt/β-catenin signaling and apoptosis pathways, which are relevant to the pathogenesis of polycystic kidney disease, as well as a variety of water balance disorders.

**EXPERIMENTAL PROCEDURES**

Brief descriptions of key experimental procedures are provided below. For complete details, see supplemental materials online.

**Sample Preparation**—IMCD suspensions were prepared from rat kidney inner medullas (150–250 μg of protein/inner medulla) using the method of Stokes et al. (19) with modifications (20). After isolation, IMCD suspensions were incubated for 0.5, 2, 5, and 15 min at 37 °C in the presence or absence of 1 nM [deamino-Cys1, D-Arg8]-vasopressin (dDAVP), a V2 receptor-specific analog of vasopressin, followed by centrifugation at >10,000 × g for 10 s. Pelleted IMCD tubules were lysed in 150 μl of lysis buffer containing 8 M urea, 50 mM Tris-HCl, 75 mM NaCl with 1 × HALT protease and phosphatase inhibitor (Pierce). The four time points are labeled based on the time of incubation with dDAVP and do not include the 30 s of additional preparation time for centrifugation and lysis.) Protein samples were sonicated for 1 min with 0.5-s pulses on ice. The samples were spun for 10,000 × g for 10 min to pellet the debris, and the supernatant was saved for further analysis. Five microliters of each sample was saved for analysis of protein concentration by the BCA method (Pierce). Another 15 μl was removed for protein immunoblotting. The remain-

der of each sample (500 μg of protein) was reduced with 10 mM DTT for 1 h at 37 °C, alkylated with 40 mM iodoacetamide for 1 h at room temperature in the dark, and then quenched with 40 mM DTT for 15 min. The samples were diluted to <1 μl urea with 50 mM ammonium bicarbonate buffer and then digested with trypsin overnight at 37 °C using an enzyme-to-protein ratio of 1:25 (w:w). After acidiﬁcation with 0.5% formic acid, the samples were desalted on a 1-cc Oasis HLB cartridge (Waters, Milford, MA) prior to iTRAQ labeling. This entire sample preparation process was repeated two additional times on separate days to produce a total of three biological replicates.

**iTRAQ Labeling**—iTRAQ labeling was performed according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Briefly, the peptide samples were resuspended in 150 μl of iTRAQ dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5). Each peptide sample was labeled with 5 units of iTRAQ 8-plex reagent for 2 h at room temperature according to the experimental design shown in Fig. 1. The reaction was quenched by adding 0.5% formic acid. All eight iTRAQ-labeled samples were combined into a single sample, then desalted (HLB cartridges; Waters), and dried in vacuo.

**Fractionation and Phosphopeptide Enrichment**—iTRAQ-labeled peptide samples were fractionated by strong cation exchange chro-

matography, and phosphopeptides were enriched by Ga-13 IMAC.

**LC-MS/MS Analysis**—The samples were analyzed on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). The average precursor isolation window was 3 m/z. MS data files are available via the Proteomic Commons Tranche Repository (https://proteomecommons.org/tranche/). (See the supplemental data online.)

**Database Searching**—MS2 spectra were searched with Proteome Discoverer software (version 1.1.0.263; Thermo Scientiﬁc) running the Sequest algorithm on a concatenated database containing both the forward and reversed complement of the Rat RefSeq Database (Na-

tional Center for Biotechnology Information, March 3, 2010, 30,734 entries), which included a list of common contaminating proteins from other species. Precursor ion tolerance was 25 ppm, whereas frag-

ment ion tolerance was 0.05 Da. Three missed tryptic cleavage sites were allowed. Static modifications included carbamidomethylation of cysteine (+57.021 Da) and iTRAQ 8-plex modification of lysine and peptide N termini (+304.205 Da). Variable modifications included oxidation of methionine (+15.995 Da); phosphorylation of serine, threonine, and tyrosine (+79.966 Da); and iTRAQ 8-plex modification of tyrosine (+304.205 Da). Known contaminant ions were excluded. The data sets were ﬁltered to include <1% false positive hits (esti-

mated based on target decoy analysis (21)) based on the following Xcor threshold values for each charge state for the each replicate: +2 (2.14, 1,965, 2,355); +3 (2.38, 2,315, 2,81); +4 (2.66, 2.4, 3.06); and +5 (2.7, 2.405, 3.065). Phosphorylation site assignment was performed using a dynamic programming algorithm (false localization rate < 1%) that is currently under review. PubMed (http://www.ncbi. nlm.nih.gov/pubmed/), PhosphoSitePlus (http://www.phosphosite.org), and HPRD (http://www.hprd.org) (22) were used to search for known phosphorylation sites. Phosphopeptides matching to multiple protein isoforms were identiﬁed using ProMatch software (23).

**iTRAQ Quantification**—Reporter ion intensities for redundant pep-

tides were summed for each iTRAQ channel. (We deﬁned peptides as redundant if the charge states, site(s) of modiﬁcation, and amino acid sequences were identical.) Abundance ratios (dDAVP/control) for all four time points were calculated, and each was normalized using a cor-

crection factor that was based on the ratio of the summed reporter ion intensities for the corresponding dDAVP and vehicle control channels. The log2 of the normalized ratio was used as the basis for calculation of the mean and standard deviation for each peptide across all three biological replicates. Unpaired t tests determined whether changes in phosphopeptide abundance were signiﬁcant. Background variability across the three replicate time courses was assessed for all phosphopeptides (mean log2 (0.5 min control/5 min control) = −0.02 ± 0.22 (S.E.) (Fig. 1C). In addition, average non-

phosphorylated peptide abundance ratios for each protein were ob-

tained by analyzing the IMAC flow-through fractions (included in supplemental Table S1). The majority of proteins did not change abundance with vasopressin (average log2(dDAVP/control) = −0.025 ± 0.14 (S.E.), indicating that changes in phosphopeptide abundance are not likely due to changes in total protein abundance. Average precursor ion isolation purity (i.e. the percentage of target ion intensity compared with the complete ion intensity in the precursor isolation window) for the three replicate data sets was 83%. Because precursor isolation purity is not always a reliable indicator of accurate iTRAQ quantitation (24), data sets were not ﬁltered for a particular isolation purity threshold. The isolation purities for individual phos-

phopeptides are included in supplemental Table S1.
Cluster Analysis—Cluster analysis of phosphopeptides with similar temporal profiles was performed using a temporal pattern mining (TPM) algorithm (http://helixweb.nih.gov/TPM) (25). To be included for cluster analysis, a phosphopeptide had to be present in at least two of the three time courses, and all eight iTRAQ reporter ions needed to be present in each spectrum to obtain quantifiable iTRAQ ratios at each of the four time points. This analysis produced 30 distinct temporal clusters. Phosphorylation motif analysis was then performed on each of these clusters by using the motif-x algorithm (26) (supplemental Table S5). Some of the clusters contained too few phosphopeptides to produce a motif. In house software (ABE; see “Bioinformatics”) was then used to extract Gene Ontology (GO) terms for each phosphopeptide in these clusters, as well as for all identified phosphopeptides (i.e. background). The proportion of each GO biological process term in each cluster was then calculated, and this value divided by the proportion of that particular GO Biological Process term in the background data (supplemental Table S6). The values for the 100 most abundant GO biological process terms for all clusters were then hierarchically clustered using Gene Cluster software (27) to detect patterns in GO term enrichment between the various temporal phosphopeptide clusters.

Bioinformatics—Automated Bioinformatics Extractor, ABE (http://helixweb.nih.gov/ESBL/ABE/), was used to extract GO terms and conserved protein domains through NCBI Entrez Programming Utilities (http://eutils.ncbi.nlm.nih.gov). The DAVID bioinformatic tool (Database for Annotation, Visualization and Integrated Discovery, NIAID, http://david.abcc.ncifcrf.gov/) (28) was used to extract the list of PANTHER pathway terms (http://www.pantherdb.org) (29) associated with each phosphopeptide.

RESULTS

Summary of Phosphoproteomic Profiling—Three replicate 8-plex iTRAQ time course experiments were performed as indicated in Fig. 1A to quantify the effects of vasopressin at four separate time points (0.5, 2, 5, and 15 min), each with its own time point control. Immunoblotting for Aqp2 phosphorylated at Ser-256 confirmed the response of the IMCD cells to the vasopressin analog dDAVP (supplemental Fig. S1). Aqp2 phosphorylated at Ser-256 was significantly increased during all four time points, whereas the total protein abundance of Aqp2 did not change with vasopressin.

A combined total of 12,167 phosphopeptides corresponding to 2,783 proteins were identified, although not all of the phosphopeptides were present in all three replicate time courses (Fig. 1B and supplemental Table S1; nonphosphorylated peptide data in supplemental Table S2). A database of all phosphopeptide identifications including dynamic, quantitative data for both phosphorypeptide and corresponding protein level abundances is available online (http://helixweb.nih.gov/ESBL/Database/TiPD/index.html). Background variability across the three replicate time courses was assessed for all phosphopeptides (mean log2 (0.5 min control/5 min control) = −0.02 ± 0.22 (S.E.) (Fig. 1C). The majority of phosphopeptides (90% of the total) were singly phosphorylated, and serine residues were the most commonly modified amino acid (83% of the total). 4,202 nonredundant phosphorylation sites (50% of the total) were previously unidentified based on information from various online phosphorylation site databases (see “Experimental Procedures”). Approximately 28% of phosphorylation sites were present in known “regions of interest” (based on the current NCBI Reference Sequence record), which include binding sites, enzyme active sites, regions of local secondary structure, and conserved protein domains. (Full results are provided in supplemental Table S3.)

Of the phosphopeptides that were present in all three replicate time courses, 273 changed significantly during at least one time point, and 40% of these changes occurred within 2 min of exposure to vasopressin. Analysis of PANTHER Pathway terms for these 273 phosphopeptides revealed that a number of signaling pathways are regulated by vasopressin (Fig. 1D). These included pathways previously implicated in vasopressin signaling (the phosphatidylinositol 3-kinase pathway, the protein kinase B/Akt pathway, various MAP kinase pathways (30), and signaling through Rho kinases (31, 32)), as well as pathways not known to be regulated downstream from the V2 receptor (angiogenesis, Wnt signaling pathway, and heterotrimeric G protein Gαi- and Gαs-mediated pathway).

Many of the phosphoproteins that changed with vasopressin were shared among multiple signaling pathways, suggesting that there may be considerable interpathway connectivity. The full results of the PANTHER Pathway analysis are available in supplemental Table S4.

Forty-six phosphopeptides changed significantly (p < 0.05) in abundance during at least one time point by at least 40% (−0.5 ≤ log2(dDAVP/control) ≥ 0.5) (value is based on 2× S.E. of control:control quantitation; see “Experimental Procedures”). Phosphopeptides that increased or decreased in the presence of dDAVP based on these criteria are presented in Table I. This table includes numerous membrane channels (Aqp2 and Slc14a2), trafficking proteins (Lrba, Sec22b, Agfg1, and Sept9), and protein kinases (Camk2, Prkar1a, Ptk2, Map3k7, Map4k6, and Pak2). There were also a number of phosphoproteins involved in actin binding and cytoskeletal reorganization (Eps8l1, Lcp1, Ctnna1, and Kif13b), a result consistent with prior studies showing that vasopressin regulates cytoskeletal dynamics (33) and that depolymerization of the cortical actin network promotes Aqp2 trafficking (31, 34). Many of the proteins listed in Table I have not been implicated previously in vasopressin signaling.

Phosphopeptide Time Course Clustering—Phosphopeptides were further analyzed by cluster analysis using a TPM algorithm (http://helixweb.nih.gov/TPM) (25) to detect groups of peptides that changed in abundance with similar temporal profiles. A total of 30 distinct phosphopeptide time course clusters were identified from 3,198 phosphopeptides (Fig. 2), indicating the wide variety of phosphorylation dynamics triggered by exposure to vasopressin. An interactive version of this figure that lists the phosphopeptide sequences and associated data contained within each cluster is available online at http://helixweb.nih.gov/ESBL/Database/TiPD/cluster.html. Clusters showing increases in phosphopeptide abundance with dDAVP at all time points consistently contained more phosphopeptides than clusters showing decreases, indicat-
ing that vasopressin may trigger an increase in net phosphorylation at the cellular level. It also suggests that kinase activation and/or phosphatase inactivation dominates the physiological response to vasopressin.

A cluster that increased at all time points is shown in Fig. 3A. Based on linear motif analysis of the residues surrounding the site of phosphorylation (motif-x (26)), this cluster was enriched for “basophilic” motifs consistent with activation of AGC family kinases (e.g. PKA) and/or Ca$^{2+}$-calmodulin-dependent kinases (35) (Fig. 3A, inset). A phosphopeptide cluster that decreased at all time points was enriched in “proline-directed” phosphorylation motifs, suggesting a general inactivation of MAP and/or cyclin-dependent kinases (Fig. 3B). Similar up- and down-regulated kinase motifs have been

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Fig. 1. Overview of phosphoproteomic profiling. A, experimental workflow for iTRAQ-based quantitative phosphoproteomics of native rat IMCD. SCX, strong cation exchange chromatography; IMAC, immobilized metal affinity chromatography. B, the distribution of phosphopeptides based on the number of phosphorylation sites identified for each peptide. C, the histogram shows the background variability for all phosphopeptides across the three biological replicates (mean log$_2$(0.5 min control/5 min control) = −0.02 ± 0.22 (S.E.). D, an overview of signaling pathways regulated by vasopressin. PANTHER Pathway terms were extracted for each phosphopeptide that changed significantly ($p < 0.05$) with vasopressin during at least one time point and compared with pathway terms for all identified peptides (i.e. background). The gene names for the corresponding phosphoproteins that changed with vasopressin are listed for each pathway. Only the pathways that contained more than one identified protein are included.

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| Protein name | RefSeq | Gene symbol | Peptide sequence | Phosphosite | Average log₂(dDAVP/Control) |
|--------------|--------|-------------|-----------------|-------------|--------------------------|
|              |        |             |                 | 0.5 min     | 2 min                    | 5 min | 15 min |
| 1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase β3 | NP_203501 | Pib3 | NNS#1SEAK | Ser-1107 | 0.41 | 0.68<sup>b</sup> | 0.65<sup>b</sup> | 0.67 |
| 2-Oxoisovalerate dehydrogenase subunit α, mitochondrial | NP_039614 | Bokda | IQH5FSSTEDISSR | Ser-383<sup>a</sup> | -0.16 | -0.26 | -0.07 | 1.00<sup>d</sup> |
| 6-Phosphoheptadecanoylphosphatidylethanolamine | NP_476476 | Ph6b3 | NSTTIFASPEKP | Ser-496 | -0.26 | 0.76<sup>b</sup> | -0.08 | 0.40 |
| α-Synuclein | NP_00109371 | Snta1 | NYASQGWY#PFAFPQ | Ser-183<sup>b</sup> | -0.09 | 0.56 | -0.33 | 1.15 |
| Arl5 repeat and SOCS box protein 4 | NP_001019489 | Asb4 | SKPLNLX | Ser-408 | 1.40<sup>b</sup> | 1.96<sup>b</sup> | 1.20 | 1.26<sup>d</sup> |
| Aquaporin-2 | NP_037041 | Aqp2 | RFS#VIELSF#PSLFR | Ser-256, Ser-261 | 0.72 | 0.66<sup>b</sup> | 0.83 | -0.15 |
| Aquaporin-2 | NP_037041 | Aqp2 | RFS#VIELSF#PSLFR | Ser-256 | 0.89 | 0.83<sup>b</sup> | 1.09 | 1.13 |
| Bcl-1-related ovarian killer protein | NP_059008 | Bok | RRS#PIRAKNEIFAP | Ser-8 | 1.99 | 1.21 | 2.92<sup>b</sup> | 2.28<sup>b</sup> |
| Calcium/calmodulin-dependent protein kinase 2 | NP_11268 | Camk2 | SPF#PDEDFSLRL | Ser-494 | 0.26 | 0.22<sup>b</sup> | 0.46<sup>b</sup> | 0.76<sup>b</sup> |
| cAMP-dependent protein kinase type I-α regulatory subunit | NP_037313 | Pka1α | TDS#KEELSPFPPEPK | Ser-77<sup>c</sup> | 0.65<sup>b</sup> | -0.16 | 0.16 | 0.32 |
| Cytochrome c oxidase complex IV subunit 2 | NP_064440 | Cc2 | GS#FPFLFX | Ser-278 | -0.1 | 0.41 | 0.26 | 0.59<sup>b</sup> |
| Focal adhesion kinase 1 | NP_037213 | Fak | LQPS#FPY#PVTLR | Ser-913 | 0.17 | 0.01 | 0.51<sup>b</sup> | 0.20 |
| Hypothetical protein | NP_001079852 | Fam83e | EPT#DFALPSFELR | Ser-363 | 1.19<sup>b</sup> | 0.16 | 1.70 | 0.47 |
| Keratin, type I cytoskeletal 19 | NP_955792 | Krt19 | GGS#FSALSTVTDLLGNEK | Ser-67 | 0.08 | -0.11 | 0.19<sup>b</sup> | 0.62<sup>d</sup> |
| Kinesin-like protein KIF13B | NP_980791 | Kif1b3 | SSMQLPQGPEARP | Ser-1719<sup>b</sup> | 1.09<sup>b</sup> | 0.99<sup>b</sup> | 0.83<sup>d</sup> | 1.07<sup>d</sup> |
| Leucine-rich repeat proteoglycan-interacting protein 1 | NP_001014291 | Lrnp1 | RG#GTDQGNIWEYAI | Ser-88 | 0.64 | 1.23<sup>b</sup> | 0.67 | 0.86<sup>b</sup> |
| Leucine-rich repeat proteoglycan-interacting protein 2 | NP_001019302 | Lrnp2 | NSAT#PFTLSONSS#F | Ser-129<sup>c</sup> | 0.27<sup>b</sup> | 0.70 | 0.53<sup>d</sup> | 0.67<sup>d</sup> |
| Lipoplysaccharide-responsive and beige-like anchor protein | NP_001102025 | Lrb | QTAPVDF#PDQQR | Thr-1231 | 0.42<sup>b</sup> | 0.33 | 0.52<sup>b</sup> | 0.51<sup>b</sup> |
| Mitogen-activated protein kinase kinase 7 | NP_001101390 | Map3k7 | S#IQDLTVTQGREQSSLR | Ser-439 | 0.02<sup>c</sup> | 0.55 | 0.64 | 0.60<sup>c</sup> |
| Nuclear receptor coactivator 3 | NP_00107069 | Ncoa3 | ALS#LDFVGVSPRP | Ser-848 | 0.53<sup>c</sup> | - | - | - |
| OCA1 domain-containing, member 5 | NP_001015896 | Oca1d | SKTVPFR | Ser-198 | 0.54 | 0.58 | 0.70 | 0.67<sup>d</sup> |
| Phosphatidylinositol glycan anchor biosynthesis, class B | NP_001101636 | Pgbp | STQVQVAEQE | Ser-29 | 1.00 | 1.23<sup>b</sup> | 0.84 | 0.83 |
| Rap1 GTPase-activating protein | NP_001070178 | Rap1gap | SQ#EMRAMLKSLR | Ser-472 | 1.19 | 0.78<sup>b</sup> | 0.65<sup>b</sup> | 1.08 |
| Signal transducer and activator of transcription 5A | NP_058370 | Stat5a | LSW#FAPL#SAR | Ser-779 | 0.56 | 0.38 | 0.50<sup>b</sup> | 0.50<sup>b</sup> |
| Similar to Map66-pending protein | NP_00272458 | Loc303259 | S#NGS#M#TVTLG | Ser-714 | 0.82<sup>b</sup> | 0.75<sup>b</sup> | 0.83<sup>d</sup> | 0.70<sup>d</sup> |
| Splicing factor, proline- and glutamine-rich | NP_001020442 | Styf | NOS#D#TVQDRO | Thr-679 | -0.37 | 0.34 | 0.07 | 0.87<sup>b</sup> |
| TBC1 domain family, member 1 | NP_001071842 | Tbc1d1 | SKTLESLSK | Ser-497 | 0.56<sup>c</sup> | 0.91<sup>b</sup> | 0.86<sup>d</sup> | 1.08<sup>d</sup> |
| Tyrosine-protein phosphatase nonreceptor type 12 | NP_674666 | Ptpn12 | DAVDZZ#FFFPFLER | Ser-667 | -0.20 | 1.35 | -0.13 | 0.72<sup>d</sup> |
| Urea transporter 2 isoform 1 | NP_0622596 | Slc14a2 | Slc14a2 | Ser-486 | 1.63 | 2.03<sup>b</sup> | 1.95 | 1.92<sup>b</sup> |
| Urea transporter 2 isoform 2 | NP_808877 | Slc14a2 | RER#EFLR | Ser-84 | 1.61 | 1.81<sup>b</sup> | 1.74<sup>b</sup> | 1.85<sup>b</sup> |
| Vesicle-traffic protein 4 | NP_001020597 | Sec22b | NIOS#TEVLQP#QR | Ser-137 | 3.02 | 0.68 | 2.90 | 1.75<sup>b</sup> |

**Average log₂(dDAVP/Control) < 0.5**

**AHNAK nuclear protein isoform 1**

**α-Endosulfine isoform 1**

**Ahn**

**A1a**

**Aquaporin-2**

**Art-GAP domain and FG-repeats-containing protein 1**

**Catennin-α1**

**CLIP-associated protein 2**

**Erd2 growth factor receptor kinase substrate 8-like protein 1**

**Ernhytrocyte protein band 4.1-like 4a**

**Hypothetical protein LOC500552**

**Plastin-2**

**Sparc isoform 2**

**Serine/threonine-protein kinase PAK 2**

**Tumor protein p53 binding protein 2, isoform 1**

<sup>a</sup> Ambiguous phosphorylation site.

<sup>b</sup> p < 0.05.

<sup>c</sup> —, no quantifiable iTRAQ ratio.
demonstrated both in a mouse cortical collecting duct cell line (8) and in rat kidney thick ascending limb (11) in the presence of dDAVP. These general motifs are apparent in many of the peptide sequences presented in Table I. It is important to realize that these motifs are predictive only of general kinase classes and that other contextual information is often required to identify kinase-substrate relationships (36). The full results of TPM clustering and kinase motif analysis are provided in supplemental Table S5.

**Clustering of Gene Ontology Terms**—To determine which particular classes of phosphoproteins were regulated by vasopressin, we performed hierarchical clustering of GO biolog-

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**Fig. 2.** **TPM-based clustering of phosphopeptides.** Phosphopeptides that were quantified in at least two of the three time courses were clustered based on temporal patterns across four different time points (0.5, 2, 5, and 15 min). The color of each node (circles) in the tree indicates whether the cluster increased (red, positive slope) or decreased (green, negative slope) at that particular time point. The zero time point is represented by a theoretical node (yellow) that is used as a baseline reference. iTRAQ ratios are represented as the average log$_2$(dDAVP/control) for each time point. The numbers in parentheses indicate the four-digit binary label for each cluster based on the slope of the line between each time point ($0 = \text{negative slope}; 1 = \text{positive slope}$). $n$ indicates the number of phosphopeptides in each cluster.
Phosphoproteomics of G Protein-coupled Receptor Signaling

Fig. 3. Kinase motif analysis of temporal clusters. A, an example of a phosphopeptide cluster that decreased in abundance with dDAVP at all time points and the most predominant phosphorylation motif for this phosphopeptide cluster (inset). B, an example of a phosphopeptide cluster that increased in abundance with dDAVP at all time points and the most predominant phosphorylation motif for this phosphopeptide cluster (inset). Phosphopeptides from the following proteins showed a significant (p value < 0.05) change in abundance: Ahnak (point a), Tpcn1 (point b), Slc14a2 (points c and d), Bok (point e), and Tbc1d1 (point f).

We found that apoptosis was one of the processes most broadly regulated by vasopressin, with the GO biological process terms “apoptosis” (GO cluster B) or “anti-apoptosis” (GO cluster A) included in 23 of 30 phosphopeptide time course clusters. Phosphoproteins associated with apoptosis points for the glycolytic and hexosamine pathways, respectively (39). Regulated phosphorylation of the Zn^{2+} carrier Mt2a and Zn^{2+} transporter Slc30a1 within the same cluster suggests that dDAVP may also affect the cellular distribution of Zn^{2+}, a cation that has been shown to inactivate Pfk1 (40). Increased phosphorylation of Gfpt1 at Ser-243 identified in our study has been shown to activate Gfpt1 (41), which leads to an elevation in intracellular levels of O-GlcNAc.

GO cluster E included Aqp2 and Slc14a2 (UT-A1), the major channels responsible for collecting duct water and urea transport, respectively, as well as a number of other proteins that may regulate channel trafficking. The heavy chain of non-muscle myosin IIa (Myh9) was present in this cluster and has been implicated in Aqp2 trafficking via vasopressin-mediated activation of the Ca^{2+}-calmodulin pathway (42). Also included in this cluster were three regulators of Rab GTPase activity: Ais2, Rabgap1, and AS160 (Tbc1d4). The Rab family of proteins has been implicated in the regulation of Aqp2 localization and trafficking (43, 44).

We found that apoptosis was one of the processes most broadly regulated by vasopressin, with the GO biological process terms “apoptosis” (GO cluster B) or “anti-apoptosis” (GO cluster A) included in 23 of 30 phosphopeptide time course clusters. Phosphoproteins associated with apoptosis
Fig. 4. Hierarchical clustering of GO biological process terms and phosphopeptide clusters. Shown is a heat map visualizing the proportion of the 100 most abundant GO biological process terms (displayed vertically) enriched in all 30 temporal phosphopeptide clusters (displayed horizontally) relative to the background proportion of each GO term for all identified phosphopeptides. GO terms that displayed similar enrichment patterns have been colored accordingly. Squares above the heat map indicate whether a phosphopeptide increased (red squares) or decreased (green squares) in abundance with dDAVP relative to the previously measured time point based on TPM clustering. The symbols directly above these squares indicate whether the temporal profile was located entirely above the x axis (+), was located entirely below the x axis (−), or crossed the x axis (0). Numerical designations for each temporal phosphopeptide cluster are located above these values.
included Bok, Bad, Bcl2l14 (Bcl-G), Ptk2, Tp53bp2, Ctnna1, Map3k7 (Tak1), Stat5a, Krt8, and Krt18. Three of these proteins (Bok, Bad, and Bcl2l14) contain Bcl-2 homology 3 domains and are pro-apoptotic members of the Bcl-2 family. Bad phosphorylation increased with vasopressin at Ser-137 and Ser-156 in our study, as well as in mpkCCD cells (8). Phosphorylation of Bad at these sites by PKA has been shown to block dimerization with the anti-apoptotic protein Bcl-xL, thus promoting cell survival (45, 46). Similar to Bad, which is a pro-apoptotic ligand, Bok is a pro-apoptotic channel that contains additional Bcl-2 homology 1 and 2 domains that are important for channel formation and cytochrome c release from mitochondria (47). Bok phosphorylation significantly increased at Ser-8 (Table I) in response to dDAVP nearly 8-fold (log2(dDAVP/control) = 2.92), the largest significant increase in phosphorylation of any protein identified in this study. Ser-8 is contained within a potential PKA consensus motif (…VLRRSS*VF…). To address whether this sequence can be phosphorylated by PKA, we performed an in vitro kinase assay on a synthetic Bok peptide containing Ser-8 and quantified the signal for both the unphosphorylated and phosphorylated peptides by LC-MS/MS. The addition of PKA resulted in a prominent peak for the phosphorylated peptide that was not present without kinase (Fig. 5A), as well as a 74% reduction in the MS1 peak intensity for the unphosphorylated peptide (Fig. 5B). This result demonstrates that Bok can be phosphorylated by PKA in vitro. Although very little is known regarding the regulation of Bok, this result raises the possibility that the pro-apoptotic function of Bok may be inhibited through phosphorylation by PKA in a fashion similar to Bad. We also found increased phosphorylation of Stat5a, an anti-apoptotic transcription factor responsible for transcription of a variety of genes including Bcl-xl and Bcl-2 (48). Phosphorylation of Stat5a at this site (Ser-779) promotes its transcriptional activity (49). Vasopressin regulated the phosphorylation of the anti-apoptotic kinase Fak (Ptk2), as well as the pro-apoptotic kinase Pak2 (Table I). We also found reduced phosphorylation of cytokeratins Krt8 and Krt18 in the

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**Fig. 5.** Vasopressin regulates a variety of apoptotic proteins in the collecting duct. A kinase assay was performed to determine whether residue Ser-8 of the pro-apoptotic factor Bok can be phosphorylated by PKA (A and B). A synthetic Bok peptide containing Ser-8 was incubated in the absence (no kinase) or presence (PKA) of the catalytic subunit of PKA. LC-MS/MS quantitation of the relative abundances of the phosphorylated peptide signal (A) and the unphosphorylated peptide signal (B). The asterisk indicates the site of phosphorylation. C, immunoblots of the cleaved forms of caspase-3 and caspase-7 in the presence (+) or absence (−) of dDAVP. D, band densities from replicate immunoblots (n = 5) are plotted as the mean log2(dDAVP/control). The error bars indicate S.E. *, p < 0.05.
presence of vasopressin. Hyperphosphorylation of these particular isoforms of cytokeratin has been associated with increased apoptosis (50). Taken together, the phosphorylation data from this large array of proteins involved in apoptosis suggest that vasopressin may inhibit this process and promote IMCD cell survival.

The anti-apoptotic effects of vasopressin mediated via the V1a receptor have been documented in neurons (51, 52) and glomerular mesangial cells (53). To further address whether vasopressin, via the V2 receptor, may have similar effects in kidney collecting duct, we incubated IMCD tubule suspensions in the presence and absence of dDAVP for 1 h and then quantified the levels of caspase-3 and caspase-7 cleavage products, which are hallmarks of apoptosis (54–57). The amounts of the cleaved forms of both caspases were significantly reduced in the absence of dDAVP (Fig. 5, C and D), consistent with the conclusion that vasopressin has a net anti-apoptotic effect in the renal collecting duct. Inhibition of apoptosis may provide a protective effect in the kidney inner medulla, which is often subjected to high osmotic stress during the urine concentration process (58). Inhibition of the apoptosis pathway has also been implicated in the pathogenesis of polycystic kidney disease (4), but a role for vasopressin in inhibition of apoptosis was not previously recognized.

Another pathway with many phosphoproteins regulated by vasopressin was the Wnt receptor signaling pathway (Figs. 1D and 4, GO cluster “C”). The Wnt signaling pathway plays a critical role in the development of the renal collecting duct system. Wnt family members are ligands for the Frizzled family of G protein-coupled receptors (59). Transcripts for both Wnt (Wnt4 and Wnt5a) and Frizzled (Fzd1 and Fzd4) gene family members are highly enriched in rat inner medullary collecting duct (http://dir.nhlbi.nih.gov/papers/lkem/imcdtr) (60). We identified a total of 15 Wnt pathway phosphoproteins based on GO term analysis, including β-catenin, an integral downstream component of this pathway that has been implicated in the pathogenesis of polycystic kidney disease (61, 62). Akt and PKA, which are both activated by vasopressin (30), phosphorylate β-catenin at Ser-552, which induces β-catenin accumulation in the nucleus and increases its ability to regulate gene transcription (63–65). Prior studies have demonstrated that phosphorylation of β-catenin at Ser-552 increases with vasopressin (9) (8, 11). We confirmed this increase in phosphorylation by LC-MS/MS and demonstrated through quantitative immunoblotting that this increase occurs within 0.5 min of vasopressin exposure (Fig. 6, A and B). A number of additional proteins in this GO cluster (Apc, Lrrfip1, Lrrfip2, Tnik, and Csnk1e) have been shown to regulate the function of β-catenin. Phosphorylation of Apc (adenomatosis polyposis coli) was decreased by vasopressin. Apc promotes the degradation of β-catenin, and its activity directly correlates with its phosphorylation state (66). Phosphorylation of Lrrfip1 at Ser-88 and Lrrfip2 at Ser-133, both part of putative PKA consensus sites, were increased by vasopressin. Lrrfip isoforms positively regulate β-catenin-dependent gene transcription (67–69). These data are consistent with the hypothesis that vasopressin may act as a potentiator of Wnt pathway signaling.

Global Analysis of Protein Domains—We next extracted conserved protein domains for all vasopressin-regulated phosphoproteins. The most prevalent domain was the catalytic domain of serine/threonine protein kinases (smart00220: S_TKc) (12 hits) (supplemental Table S7). We identified a total of 17 serine/threonine protein kinases, including kinase regulatory subunits, that changed phosphorylation status with vasopressin. Among these were nine members of MAP kinase signaling pathways. Vasopressin has been shown to decrease
signaling through MAP kinases (8, 30, 70), and the results of the current study confirm and expand on this hypothesis. We were able to detect significant changes in phosphorylation of A-raf and Raf1 (upstream kinases in the ERK pathway), which were consistent with down-regulation of the ERK pathway. In addition, Raf1 signaling can be negatively regulated by interaction with the small GTPase Rap1 (71). In the current study, vasopressin increased phosphorylation of Rap1gap (an inhibitor of Rap1) at Ser-472 (Table I) and Ser-556 (Fig. 6, C and D), two sites that are known to inhibit GAP activity when phosphorylated by PKA (72). Theoretically, this event could lead to an increase in Rap1 activity and subsequent inhibition of the Raf-MEK (MAP kinase/ERK kinase)-ERK pathway. This mechanism could contribute to the vasopressin-mediated decrease in ERK MAP kinase activity found in previous studies (8, 30).

We also found reduced phosphorylation of Jnk2 (Mapk9) and p38α (Mapk14), as well as the upstream activating kinases Map2k3/6. In contrast, phosphorylation of Map3k7 (Tak1) at Ser-439, a known target of PKA (73), was significantly increased by vasopressin as detected by LC-MS/MS, as well as immunoblotting (Fig. 6, E and F). Reduced phosphorylation of Map2k5 at Ser-311 (a known activating site (74)) along with increased phosphorylation of its corresponding regulatory kinase Map3k2 at Ser-331 (a known inhibitory site (75)) provides initial evidence that vasopressin can also down-regulate members of the ERK5 pathway. Thus, the majority of phosphorylation changes in MAP kinase family members detected in the present study are consistent with vasopressin triggering an overall reduction in MAP kinase signaling under normal physiologic conditions.

**DISCUSSION**

We have applied a temporal quantitative phosphoproteomic strategy to probe the dynamics of signaling through a prototypical GPCR, V2R, in native kidney cells. This study has greatly expanded knowledge about vasopressin signaling, producing a network model that serves as a basis for further study of GPCR signaling (Fig. 7). The study provides a temporal ordering of phosphorylation changes following vasopressin binding. These temporal data extend our knowledge about vasopressin signaling well beyond the existing evidence obtained in experiments in which measurements were made at a single time point (8–11). Although causality cannot be established using this approach, these time course data can provide the basis for hypotheses about causality. Clustering of the time courses revealed that the phosphorylation dynamics triggered by the activated V2 receptor are complex, with many different response patterns. The initial response was very rapid, with a large number of phosphorylation changes occurring within 30 s of exposure to ligand. These phosphorylation changes coincide temporally with increases in water and solute permeability in isolated, perfused renal collecting ducts following vasopressin exposure (76).

This study has demonstrated that binding of the V2R by vasopressin affects more than simply the canonical cAMP-PKA and β-arrestin pathways in the kidney collecting duct. Previous studies have demonstrated that arrestin binding to liganded GPCRs causes activation of MAP kinases (77–79), whereas our current results, as well as prior studies of the general response to vasopressin (8, 30, 70), show an inactivation of multiple MAP kinase pathways. Thus, it appears that the effect of vasopressin on MAP kinase signaling cannot be attributed to β-arrestin binding and MAP kinase scaffolding, but rather to additional effects, possibly related to PKA-mediated phosphorylation of Rap1gap (72). Additional evidence for the varying effects of GPCR signaling to MAP kinases comes from a recent phosphoproteomic study of lysophosphatidic acid (LPA) signaling (18). In that study, Schreiber et al. report that LPA (signaling through a GPCR) and EGF (signaling through the EGF receptor) synergistically activate the ERK MAP kinase pathway, which potentiates downstream mitogenic effects in a renal carcinoma cell line. This interplay between GPCRs and receptor tyrosine kinases has not been implicated in vasopressin signaling. In fact, perfusion of isolated collecting ducts with EGF actually inhibits vasopressin-induced osmotic water permeability (supplemental Fig. S2). These apparent differences in LPA and vasopressin signaling may be due to differences in the specific G protein isoforms activated by these receptors; AVP triggers Gs-mediated signaling, whereas many LPA receptors signal through Gαi, Gq, and G12/13 (18).

From this study we have demonstrated that vasopressin affects many other pathways including Wnt/β-catenin, Ca2+, calmodulin, and apoptosis pathways. An anti-apoptotic effect of vasopressin was confirmed through demonstration of a decrease in the proteolytic fragments of caspase-3 and -7 following vasopressin exposure in independent experiments. Our data also indicate that vasopressin regulates the phosphorylation of many proteins associated with structural components of the collecting duct cell including the actin cytoskeleton, microtubules, and various tight junction and adherens junction proteins. Although the signaling network that we have described shows a high degree of connectivity, the basis of the connectivity is not yet clear. It is important to note that many of the phosphorylation sites that were regulated by vasopressin in this study were not previously known to be regulated by any physiological stimulus. In addition, many of the phosphorylated proteins within a given pathway demonstrated coordinate regulation. An important question for future studies is whether the findings of this work can be generalized to other Gαi-coupled receptors.

The resource produced by this study is an online database for temporal regulation of protein phosphorylation in a physiological setting. Along with the quantitative phosphoproteomic data, this study provides a large amount of relevant metadata including gene ontology-based classification, identification of conserved protein domains, identification of ki-
Fig. 7. An updated view of the vasopressin signaling network. Select phosphoproteins identified in this study that increased (red nodes) or decreased (green nodes) with dDAVP at one or more time points (small squares) have been integrated with the established vasopressin signaling network (gray nodes). Orange nodes indicate proteins that contain multiple dDAVP-regulated phosphorylation sites; some sites increased, whereas others decreased with dDAVP. A small letter a indicates a known activating phosphorylation site, whereas a small letter i indicates a known inhibitory phosphorylation site. The network was formatted using Ingenuity IPA software (www.ingenuity.com).
nase phosphorylation motifs, and pathway analyses. These new data provide a systems view of GPCR signaling that can lead to a deeper understanding of disorders associated with perturbed vasopressin signaling, including the water retention seen in congestive heart failure, water wasting in nephrogenic diabetes insipidus, hyponatremia seen in many forms of cancer caused by the syndrome of inappropriate anti-diuretic hormone hypersecretion, and autosomal dominant polycystic kidney disease.

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Molecular & Cellular Proteomics 11.2

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