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The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases

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SUPPLEMENTARY MATERIALS AND METHODS

Materials

Sequencing-grade trypsin, DNase1 and protease-inhibitor cocktail tablets Complete were from Roche; dialysed fetal-bovine serum and other tissue-culture reagents were from Life Technologies; [γ-^{32}P]ATP, Protein G−Sepharose, glutathione−Sepharose 4B and ECL® (enhanced chemiluminescence) reagent were from Amersham Biosciences; myelin basic Protein (MBP), the precast 10% and 4−12% Bis-Tris/SDS/polyacrylamide gels and 3−8% polyacrylamide/Tris/Acetate gels were from Invitrogen. Tween-20, 4-vinylpyridine, and dimethyl pimelimidate were from Sigma. Lysozyme from hen's- egg white and Nonidet P40 were from Fluka; GST-PreScission™ Protease was expressed and purified from *Escherichia coli* using plasmids kindly provided by Professor John Heath (School of Biosciences, University of Birmingham, Edgbaston, Birmingham, U.K.) and Professor David Barford (Institute of Cancer Research, Chester Beatty Laboratories, Fulham, London, U.K.). Human H2A expressed and purified from *E. coli* was kindly provided by Dr Nicola Wiechens and Dr Tom Owen-Hughes (School of Life Science, University of Dundee, Dundee, Scotland, U.K.). All peptides were synthesised by Dr Graham Bloomberg (Molecular Recognition Centre, University of Bristol School of Medical Sciences, Bristol, U.K.).

Antibodies

The WNK1-(total) antibody was raised in sheep against WNK1 protein encompassing residues 61--661 expressed in *E. coli*, the WNK1-(C-terminus) was raised in sheep against residues 2360--2382 of human WNK1, QNFNISNLQKSISNPPGSNLRTT, and affinity purified on the peptide antigen affinity column [1]. Total ERK1/2 (#9102) antibody was purchased from Cell Signalling Technology (ERK is extracellular-signal-regulated kinase). Mouse monoclonal antibodies anti-FLAG M2 (#F3165) and mouse monoclonal antibodies recognizing glutathione S-transferase were purchased from Sigma (#G1160). Secondary antibodies coupled to horseradish peroxidase were from Pierce.

General methods and buffers

Tissue culture, transfection, immunoblotting, restriction-enzyme digests, DNA ligations and other recombinant DNA procedures were performed using standard protocols. All

mutagenesis were carried out using the QuikChange Site-Directed Mutagenesis system (Stratagene). DNA constructs used for transfection were purified from *E. coli* DH5α using Qiagen plasmid Mega or Maxi kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland, U.K., using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. Rat tissues were rapidly excised and frozen in liquid N_2, lysed and stored at –80°C, as described previously [2]. Lysis Buffer was 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (w/w) Nonidet P40, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM DTT (dithiothreitol) and Complete protease inhibitor cocktail (one tablet per 50 ml). Buffer A was 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 1 mM DTT. Sample Buffer was 2% (w/w) lithium dodecyl sulphate, 10% (v/v) glycerol, 0.2 M Tris, pH 8.5, 25 mM DTT and 0.5 mM EDTA.

DNA constructs

The cloning of human WNK1 [1], STRADα and STRADβ [3] were described previously. A full-length human WNK4 (NCBIAAK91995) cDNA was obtained by combing EST (expressed sequence tag) NCBI CA388932 (EST1, kindly provided by Dr G. Wistow, National Eye Institute, National Institutes of Health, Bethesda, MD, U.S.A.) and IMAGE clone 2583218 (EST2, NCBI AW082836, ordered from the IMAGE Consortium). Residues 1--680 with an N-terminal BamH1-site and FLAG-tag were amplified by PCR from EST1, whereas residues 681--1243 was amplified from EST2. The full-length cDNA clone was assembled in the pEBG2T vector, using BamH1--Spe1--Spe1--Not1 three-way ligation. Full-length WNK4 was subcloned into the pCMV5 vector as a BamH1--Not1 fragment, and residues encompassing 1--593 were amplified by PCR and subcloned into pEBG2T in order to express an active fragment of WNK4 in mammalian cells. The coding region of human SPAK (NCBI AF099989) was amplified from IMAGE clone 4825063 (NCBI BG724360) with an N-terminal HA (haemagglutinin) tag and subcloned into pEBG6P and pGEX6P-1 (Amersham) expression vectors as a BamH1--BamH1 fragment. Human OSR1 (NCBI NP_005100) was cloned from the IMAGE clone 5271312 (NCBI BI464286). This EST possessed an intronic insertion of 100 nucleotides in the coding region, which was eliminated by PCR. Full-length OSR1 with an N-terminal HA tag was amplified and subcloned as a BamH1--Not1 fragment into pEBG6P and pGEX6P-1 expression vectors. Human Claudin-4 (NCBI NP_001296) with an N-terminal HA tag was amplified from EST IMAGE clone 3349211 (NCBI AAS07556) and subcloned as a BamH1--Not1 fragment into pEBG2T and pGEX-6P-1 expression vectors. A cDNA coding for the shark NKCC1 co-transporter (NCBI P55013) was kindly provided by Dr Florian Lang (Department of Physiology, University of Tübingen, Tübingen, Germany). Amino acid residues 1--260 of NKCC1 were amplified by PCR and subcloned as a BamH1--Not1 fragment into pGEX6P-1 expression vector.

Immunoblotting

Samples were heated in SDS sample buffer, subjected to SDS/PAGE and transferred to nitrocellulose. Membranes were blocked for 5 min in TBST [50 mM Tris/HCl (pH 7.5)/0.15 M NaCl/0.2% Tween-20], containing 10 % (w/v) skimmed milk. The membranes were then incubated for 16 h at 4°C with 1 μg/ml of the sheep antibodies or 1000-fold dilution for commercial antibodies in TBST containing 10 % (w/v) skimmed milk for the sheep, mouse antibodies and 5 % (w/v) BSA for commercial anti-ERK1/2 antibody. Detection of proteins was performed using horseradish peroxidase-conjugated secondary antibodies and the ECL® reagent.

Multiple sequence alignment

The protein sequence alignments of human SPAK, OSR1, STRADα and STRADβ were performed using the program T-Coffee [4]. The alignment was graphically represented with BOXSHADE version 3.21 at http://www.ch.embnet.org/software/BOX_form.html using standard parameters. The pairwise alignments and the calculation of the percentage of sequence identity were performed with EMBOSS (parameters: Matrix Blosum50; open gap penalty 10; gap extension penalty 0.5) [5].

SUPPLEMENTARY REFERENCES

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