

Regulation of sodium channels by the ubiquitin-protein ligases Nedd4 and Nedd4-2

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Enrolled through the Department of Medicine, Faculty of Health Sciences, University of Adelaide.

Research conducted at the Institute of Medical and Veterinary Science, Division of Haematology, Hanson Institute, Adelaide.

A thesis submitted for the degree of Doctor of Philosophy, in the Faculty of Health Sciences, University of Adelaide.

November 2004

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Abstract

Protein modification by ubiquitination regulates protein abundance, function and localisation. Specificity of ubiquitination is largely determined by ubiquitin-protein ligases (E3s). The Nedd4-family proteins are a group of E3s containing a conserved domain structure of a C2 domain, multiple WW domains and a carboxyl terminal HECT domain, which is responsible for E3 activity. The prototypical member of this family, Nedd4, is known to down-regulate the epithelial Na⁺ channel (ENaC) by ubiquitination. This process requires interactions between ENaC and specific WW domains of Nedd4. Mutation or deletion of WW domain binding sites in ENaC leads to Liddle's syndrome, an autosomal dominant form of hypertension. At the beginning of this study there was evidence to suggest that Nedd4-2, a Nedd4-family protein closely related to Nedd4, could also regulate ENaC. The focus of this study was to characterise the ability of Nedd4-2 to regulate ENaC and other potential substrates.

Two major splice variants of Nedd4-2 were identified, which were both found to down-regulate ENaC in *Xenopus* oocytes. *In vitro* binding studies and whole cell functional analysis showed that interactions between ENaC and Nedd4-2 occur via two of the four Nedd4-2 WW domains. The E3 activity of Nedd4-2 was further examined, revealing that it can use the same ubiquitin-conjugating enzymes as Nedd4 and exhibits strongest activity in the presence of UbcH5b. An *in vitro* ubiquitination assay and whole cell functional analysis provided evidence that Nedd4-2 down-regulates ENaC via ubiquitination.

The possibility that Nedd4 and Nedd4-2 could down-regulate a number of voltage-gated Na⁺ channels (Na_vs) by a similar mechanism to regulation of ENaC was investigated. Not only were Nedd4 and Nedd4-2 found to interact with seven

Na_vs, but these channels and ENaC have conserved WW domain binding specificity. Ubiquitination studies indicated that these channels can be ubiquitinated by Nedd4 and Nedd4-2. Co-expression of Nedd4 or Nedd4-2 with neuronal Na_vs in *Xenopus* oocytes reduced channel activity to varying degrees. These data indicate that Nedd4 and Nedd4-2 are likely to be key regulators of neuronal Na_v channels *in vivo*.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Acknowledgments

I thank Professor Sharad Kumar for giving me the opportunity to conduct research in his laboratory and for helping me to develop my scientific skills. I thank the University of Adelaide for awarding me an APA scholarship for the first three and a half years of my PhD, and Sharad for supporting me for the final months of my PhD. I give thanks to Sharad, Linda and Loretta for their help in the editing process of this thesis.

A special thanks goes to our collaborators who have provided us with valuable assistance in physiological experiments: Jan-Peter Koch and Christoph Korbmayer (University of Oxford/Universität Erlangen-Nürnberg) for analysis of ENaC activity in *Xenopus* oocytes, Anuwat Dinudom and David Cook (University of Sydney) for whole-cell patch-clamp analysis of ENaC activity in mouse cells, and Jenny Ekberg and Philip Poronnik (University of Queensland) for analysis of Na_v activity in *Xenopus* oocytes. I would also like to thank Keith Shearwin (University of Adelaide) for his valuable assistance in the SPR binding experiments.

I thank everyone in the Molecular Regulation Laboratory for their support, friendship, and for smiling politely at my jokes. I would especially like to thank Jim and Tasman for the intense toilet-humour sessions! I give thanks to my family and friends for their support and encouragement. I would finally like to thank my wife, Marijke, for her love, support, humour, and for helping me keep things in perspective. I could not have done it without you Marijke.

Publications

The following publications have resulted from the work performed by the candidate during the period of this candidature.

Harvey, K. F., Shearwin-Whyatt, L. M., **Fotia, A.**, Parton, R. G., and Kumar, S. (2002). N4WBP5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel golgi-associated protein. *J Biol Chem* 277, 9307-17.

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Abbreviations

The following abbreviations appear throughout the text of this thesis.

Ac	acetate
bp	base pair
BSA	bovine serum albumin
CNS	central nervous system
cRNA	capped RNA
DEPC	diethyl pyrocarbonate
dNTP	deoxynucleotide-triphosphate
DRG	dorsal root ganglion
DTT	dithiothreitol
DUB	de-ubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
ENaC	epithelial Na ⁺ channel
EST	expressed sequence tag
GEFS+	generalised epilepsy with febrile seizures plus
GST	glutathione S-transferase
h	hour(s)
HBS	HEPES-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HYPP	hyperkalaemic periodic paralysis
IVT	<i>in vitro</i> translation/translated
kb	kilobase
kDa	kilo Dalton
LB	Luria-Bertani
LQT3	long QT syndrome type 3
min	minute(s)
MOPS	3-(N-morpholino) propanesulphonic acid

Na _v	voltage-gated Na ⁺ channel
NMDG	<i>N</i> -methyl-D-glucamine
PAGE	polyacrylamide gel electrophoresis
PAM	potassium-aggravated myotonia
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA1	pseudohypoaldosteronism type 1
PKA	protein kinase A
PKC	protein kinase C
PMC	paramyotonia congenita
PNS	peripheral nervous system
PVDF	polyvinylidene difluoride
PY motif	PPxY or LPxY sequence
RT	room temperature
RTK	receptor protein tyrosine kinase
RT-PCR	reverse transcriptase-PCR
RU	resonance units
SCF	Skp1 Cullin F-box
SDS	sodium dodecyl sulphate
SE	standard error
sec	second(s)
Sgk	serum and glucocorticoid-regulated kinase
siRNA	small interfering RNA
SMEI	severe myoclonic epilepsy of infancy
SPR	surface plasmon resonance
TAE	Tris-Ac-EDTA
TEMED	<i>N, N, N', N'</i> -tetramethylethylene diamine
TGFβ	transforming growth factor-β
Ub	ubiquitin
UBP	ubiquitin-specific processing enzyme
UCH	ubiquitin carboxyl terminal hydrolase
UEV	ubiquitin E2 variant protein
WW1, 2, 3 or 4	WW domain 1, 2, 3 or 4