ORIGINAL ARTICLE

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Common variant of human NEDD4L activates a cryptic splice site to form a frameshifted transcript

Received: September 9, 2002 / Accepted: October 2, 2002

Abstract The ubiquitin ligase *NEDD4L* is a candidate gene for essential hypertension on both functional and genetic grounds. By targeting the epithelial sodium channel (ENaC) for degradation, *NEDD4L* is a significant determinant of sodium reabsorption in the distal nephron. Genetic linkage has been reported to a region of chromosome 18q harboring the gene, with phenotypes that include a rare orthostatic hypotension disorder, essential hypertension, and postural change in systolic blood pressure. A systematic search for genetic polymorphisms by resequencing exons and intron boundaries in 48 Caucasians yielded 38 variants. Among these, variant 13 is common, with either G (70%) or A (30%) as the last nucleotide of a putative exon 1. This mutation could affect the generation of a previously unrecognized splice isoform. In subsequent experiments, (1) we confirmed the presence of this putative isoform in both kidney and adrenals; (2) we established that variant 13-A leads to the systematic use of an alternative splice site, generating a transcript encoding a nonfunctional protein; and (3) we demonstrated differences in tissue-specific expression of the novel isoform relative to its previously

reported counterpart. Variant 13-A precludes the formation of a transcript encoding a full-length Ca^{2+} -dependent lipid-binding (C2) domain with very high evolutionary conservation among *NEDD4L* orthologs. A similar C2 domain in the paralogous *NEDD4* gene plays a significant role in the transfer of its product to the apical membrane of epithelial cells. Differential function of *NEDD4L* isoforms could prove significant in blood pressure regulation through an effect on ENaC-dependent sodium reabsorption.

Key words *NEDD4L* · Hypertension · Common variants · Aberrant splicing · Kidney

Introduction

Genetic determinants of essential hypertension have proven elusive, as no single common variant is likely to exert a major effect on blood pressure (Corvol et al. 1999; Luft 2000). In rare Mendelian syndromes of hypertension, in contrast, gain- or loss-of-function mutations account for the physiology and the molecular basis of disorders. Such remarkable advances have pinpointed critical elements of regulatory pathways involved in sodium homeostasis and blood pressure control (Lifton et al. 2001). Thus, in Liddle's syndrome (Liddle et al. 1963), mutations affecting PPxY (PY) domains of the epithelial sodium channel (ENaC) lead to increased sodium reabsorption as a result of increased ENaC activity (Schild et al. 1995; Shimkets et al. 1994). ENaC mediates sodium reabsorption in the cortical collecting tubules of the kidney, accounting for the fine regulation of sodium balance. The cell surface expression of ENaC is regulated by hormones such as aldosterone and vasopressin and by intracellular signaling, including ubiquitination and phosphorylation (Debonneville et al. 2001a; Snyder et al. 2002).

The members of the *Nedd4/Rsp5* protein family have a unique modular structure consisting of an N-terminal Ca^{2+} -dependent lipid-binding (C2) domain, multiple WW protein–protein interaction domains, and an E3 ubiquitin ligase

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A 5' exon containing the initiation codon for the *NEDD4L* isoform II (AB071179) is present in the human genome assembly Build 30 from the National Center for Biotechnology Information (NCBI; UCSC version hg12). This assembly was produced at NCBI based on sequence information submitted into GenBank as of June 28, 2002. This 5' exon is located 121 kb upstream of exon 2. SNP data has been deposited in dbSNP at NCBI (ss ASSAY ID: ss 5604308 to ss 5604345).

HECT domain. The WW domains of the NEDD4 family of ubiquitin ligases specifically interact with the PY domains in ENaC subunits, leading to monoubiquitination of ENaC and down-regulation via endocytosis from the plasmid membrane. The vesicles carrying ENaC enter the vacuolar protein sorting pathway leading to recycling to the plasma membrane or targeting to lysosome for degradation (Lemmon and Traub 2000). There are two Nedd4 paralogs in human: *NEDD4* (chromosome 15q, GenBank LocusID 4734) and *NEDD4L* (chromosome 18q21, GenBank LocusID 23327). Although NEDD4 orthologs were originally identified as ENaC binding partners using yeast two-hybrid experiments (Staub et al. 1996), it has recently been established that NEDD4L orthologs (also termed human, mouse Nedd4-2 proteins) are more potent regulators of ENaC than are NEDD4 orthologs (Harvey et al. 2001b; Kamynina et al. 2001a, 2001b).

Suggestive linkage in a region of human chromosome 18q has been reported for essential hypertension (Atwood et al. 2001) and postural change in systolic blood pressure (Pankow et al. 2000). Significant linkage has also been published for essential hypertension (Kristjansson et al. 2002) and orthostatic hypotensive disorder (DeStefano et al. 1998) in the vicinity of the peaks of these other reports. This general region includes the ubiquitin ligase NEDD4L (GenBank LocusID 23327). It follows that *NEDD4L* is a candidate gene for susceptibility to hypertensive and hypotensive disorders on both functional and genetic grounds. Because the gene may span more than 300kb, our systematic search for polymorphisms by resequencing was limited to exons and their splice junctions. Sequence alignments based on the comparative analysis of the human genomic *NEDD4L* sequence with cDNA sequences from several organisms suggested the existence of putative exons upstream of the previously reported mammalian NEDD4L transcripts that, in apparent contrast with NEDD4, lack an intact amino-terminal C2 domain (Harvey et al. 2001a; Kamynina et al. 2001a, 2001b). A resequencing survey including these putative exons revealed 38 polymorphisms. Among these, a common variant (allelic frequency of 30% in Caucasians) at the splice junction of a putative exon 1 disrupts the formation of a transcript capable of producing a NEDD4L protein containing a conserved C2 domain. Characterization of the 5' end of NEDD4L transcripts reveals multiple transcriptional isoforms that contain or lack this C2 domain. These isoforms exhibit significant differences in their tissue-specific distribution.

These novel observations may be of major significance for the function of NEDD4L. The presence of a Ca^{2+} -dependent C2 domain in previously unreported isoforms of the mammalian gene may be of critical relevance for its regulated targeting in microdomains of the cell membrane, where it may interact with ENaC to affect its residence time at the cell surface (Plant et al. 2000), or, alternatively, may play a role in the budding of enveloped viruses (Strack et al. 2000) or in viral latency (Winberg et al. 2000). The common variant observed, markedly biasing the representation and subcellular targeting of NEDD4L isoforms, would have a major impact on these cellular functions.

Materials and methods

Sequence analysis

Genomic, cDNA, and expressed sequence tag (EST) databases included GenBank nr, Human and Mouse EST entries, the human genome draft assembly hg8 06 Aug 2001 freeze (<http://genome.ucsc.edu>), the mouse phusion and arachne draft assembly (<http://mouse.ensembl.org>), and the *Fugu rubripes* draft genome assembly 1 (<http://www.jgi.doe.gov/fugu/index.html>). Results from BLAST and cross_match analysis were parsed with Perl scripts that iterated the sequence search to form a consistent assembly of EST, cDNA, and genomic sequence. Inclusion of EST and cDNA sequence into the assembly required at least one confirmed splice junction on genomic DNA. All genomic coordinates are referenced to the chromosome 18 draft assembly hg8 06 Aug 2001 freeze. The coordinates are reported locally, indexed to base 65000000 in that assembly.

Genomic DNA used for polymorphism analysis

The HyperGEN network recruited hypertensive sibpairs and normotensive controls for the investigation of genetic linkage to a wide variety of phenotypes (Williams et al. 2000). DNA samples were selected from 48 normotensive controls for this survey of common polymorphisms.

Search for polymorphisms by resequencing

Polymerase chain reaction (PCR) amplification was carried out in 50- μl reaction volumes using Expand Long Template PCR System (Roche, Indianapolis, IN, USA); see Appendix, Table 1 data for primer sequences. Each reaction contained 100ng of genomic DNA, 350 μM deoxyribonucleoside triphosphates (dNTPs), 0.2 μM of each PCR primer, 1X reaction buffer #2, and 2.6 U of Taq/Pwo polymerase mix. Cycling conditions included an initial denaturation at 94°C for 2min, 10 cycles of 94°C for 10s, 55°C for 10s, and 68°C for 2min, followed by 20 cycles of 94°C for 10s, 55°C for 10s, and 68°C for 2min + 20s/cycle. Residual primers and dNTPs were removed from PCR products with a Millipore glass fiber filter. The sequence-ready templates were eluted in 70 μl of sterile water. Five microliters of each template was aliquoted to a 384-well sequence dish and evaporated to dryness in a speed-vac.

Cycle sequencing was carried out in 2- μl reaction volumes using ABI BigDye Terminator v.3.0 chemistry (ABI, Foster City, CA, USA). Cycling conditions included an initial denaturation at 96°C for 30s, followed by 45 cycles of 96°C for 10s, 50°C for 5s, and 60°C for 4min. On completion of cycle sequencing, 8 μl of 62.5% ethyl alcohol/1M potassium acetate, pH 4.5, was added to each reaction and the sequence plates were centrifuged at 4000 rpm at 4°C for 45min. The samples were resuspended in 10 μl of formamide and electrophoresed on an ABI 3700 DNA analyzer (ABI) prepared with a POP-5 capillary gel matrix.

Sequence trace files were evaluated using the Phred, Phrap, and Consed programs (Ewing et al. 1998). Potential heterozygotes were identified by using the PolyPhred version 3.5 program (Nickerson et al. 1997). Polymorphisms were verified by manual evaluation of the individual sequence traces.

Reverse transcription-PCR (RT-PCR) and rapid amplification of cDNA ends (RACE)

5' RACE reactions were performed with the SMART II RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Total RNA was purchased from Clontech (Catalog numbers 64096-1, 64097-1) and Stratagene (Cedar Creek, CA, USA; Catalog numbers 735014, 735474). First-strand cDNA synthesis was performed with PowerScript reverse transcriptase (Clontech) on 1 µg of total RNA using the 5' RACE (Clontech) primer and the addition of SMART II A oligonucleotide (Clontech). Following reverse transcription, the first-strand cDNA was used directly in 5' RACE or RT-PCR reactions. Gene-specific PCR products were obtained by using a nested PCR strategy (see Appendix, Table 2 for primer sequences). Products were subcloned into pCR2.1 with the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Standard plasmid sequencing procedures were performed on the subclones, and RT-PCR templates were also sequenced directly.

Quantitative analysis of mRNA

Human kidney, adrenal gland, and liver mRNA, purchased from Clontech, were used for quantitative analysis of mRNA. First-strand cDNA for 1 µg total RNA was prepared using reverse transcriptase (Power Script Reverse Transcriptase, Clontech) by following the manufacturer's standard protocol. Quantitative real-time PCR (Q-PCR) was performed by monitoring the fluorescence of SYBR Green (Molecular Probes, Eugene, OR, USA) with the ABI PRISM 7700 sequence detection system (Applied Biosystems). Oligonucleotide primers were designed to span at least one intron and to minimize primer-dimer formation. PCR reaction products were electrophoresed to verify specific amplification and the absence of primer-dimer formation. All PCR reactions were performed in triplicate with primers that were specific for the corresponding human genes and spanned at least one intron: 5' cct aaa tga gac gtc tcg cat ttg ag 3' (human N4L Ex1 up), 5' agc tgg cgg aga cca gga ttt 3' (human NEDD4L Ex2a UP), 5' ccg cta cgt aca atg aaa gtt tca c 3' (human NEDD4L Ex3 RP), 5' gaa ggt gaa ggt cgg agt c 3' (human glyceraldehyde-3-phosphate dehydrogenase [hGAPDH] UP), 5' gaa gat ggt gat ggg att tc 3' (hGAPDH RP). Amplification was performed during 35 cycles of 94°C for 10s, 60°C for 10s, and 72°C for 20s. Water and genomic DNA served as negative controls. Cloned human cDNAs were used to generate standard curves. Expression of both exon 1–2–3 isoform and exon 2a–3 isoform was relative to hGAPDH expression.

Results

Polymorphism discovery in NEDD4L

Genomic resequencing targets were defined by a detailed analysis of the available NEDD4L (18q21) genomic sequence. KIAA0439 (GenBank accession no. AB007899) is a random full-length human cDNA sequence that maps to the *NEDD4L* locus on 18q21. It was shown that the predicted translation product of KIAA0439 is more similar to the *Xenopus* NEDD4 transcript, AJ000085 (Rebhun and Pratt 1998), than to human (15q), mouse, or rat NEDD4 transcripts (Harvey et al. 2001b). Human *NEDD4* (15q) and *NEDD4L* (18q21) are thus paralogs, and the paralogous split preceded the divergence of the mammalian–amphibian vertebrate lineages. Protein domain analysis of *Xenopus* NEDD4 AJ000085 indicates the presence of an evolutionarily conserved C2 domain, in contrast to the human NEDD4L transcript defined by KIAA0439, which contains only a partial C2 domain sequence that is appended to a 5' sequence lacking an initiating methionine codon. To search for undetected 5' genomic exons that may contain the missing evolutionarily conserved C2 domain sequence, we used the *Xenopus* NEDD4 AJ000085 C2 domain sequence as a translated query in a TBLASTN analysis of human 18q21 genomic sequence. Putative exons 1 and 2 are predicted from the TBLASTN alignments, and they are located 152kb and 136kb upstream of exon 3, respectively. The TBLASTN analysis of exons 1 and 2 is confirmed by EST sequences BF965237 and BF678906. Exon 3 is predicted to begin the partial C2 domain sequence observed in KIAA0439, and genomic cDNA alignment predicts an exon 2a, located 92kb upstream of exon 3, that corresponds to the 5' sequence found in KIAA0439. The location of the predicted *NEDD4L* exon – intron junctions and the genomic target coordinates for resequencing are in Table 1.

Common variants of *NEDD4L* were discovered by sequencing PCR products spanning exons 1, 2, 2a, and 3 through 31 from 48 unrelated individuals. We surveyed 5524 nucleotides in exons, and 23108 nucleotides overall, for polymorphisms by resequencing. Table 2 shows the position, alleles, allele frequency, and functional implication of the 34 single nucleotide and 4 insertion/deletion polymorphisms detected. One polymorphism, variant 13 at nucleotide 82723, occurred at the last nucleotide of exon 1 and is common in Caucasians (70% G, 30% A). This variant has the potential to disrupt exon 1 splicing because G is the most common nucleotide at this position in consensus 5' splice donor sites (Stephens and Schneider 1992). Variants in this position are known to alter splice site selection in numerous human mutations (Nakai and Sakamoto 1994; Rogan et al. 1998). This prompted us to evaluate the effect of variant 13 on exon 1 splice site selection in human kidney and adrenal RNA.

Table 1. Summary of NEDD4L genomic resequencing targets and exon coordinates

Resequencing target ^a				Exon coordinates		
Begin	End	Length (nt.)	Exon	Begin	End	Length (nt.)
80827	82835	2008	1	82481	82723	242
98708	99315	607	2	98954	99027	73
127963	128583	630	2a	143659	143902	243
234613	235239	626	3	234885	234966	81
238062	238691	629	4	238357	238395	38
241163	241797	634	5	241459	241512	53
305150	305786	636	6	305439	305489	50
311752	312995	1243	7	311882	311943	61
			8	312689	312791	102
314181	314805	624	9	314453	314619	166
318155	318757	602	10	318452	318584	132
319956	320592	636	11	320195	320371	176
323037	323666	629	12	323275	323349	74
324733	325219	486	13	324935	324994	59
330241	331513	1272	14	330495	330626	131
			15	331135	331254	119
332149	332780	631	16	332363	332560	197
338719	339341	662	17	338994	339071	77
340213	340849	636	18	340448	340592	54
346361	346992	631	19	346651	346709	58
353435	345067	632	20	353725	353790	65
355258	355899	641	21	355456	355685	229
356971	357592	621	22	357293	357324	121
359495	360059	564	23	359854	359924	70
362367	362997	630	24	362631	362726	95
372427	373048	621	25	372703	372776	73
374717	375346	629	26	374983	375043	60
376607	377237	630	27	376896	376955	59
378280	378918	638	28	378542	378649	107
379855	380491	636	29	380103	380199	96
380678	381313	635	30	380944	381016	72
385395	387844	2449	31	385624	387615	1991
Total: 23108 nt.				5224 nt.		

nt., Nucleotide

^a Begin and end coordinates are from the human genome draft assembly hg8 06 Aug 2001 freeze (www.genome.ucsc.edu, University of California, Santa Cruz). The table coordinates can be indexed to the chromosome 18 hg8 draft coordinates by adding 65 000 000 to each value. The resequencing target is the number of base pairs covered by high quality (phrap > = 20) sequence

Transcript analysis by RT-PCR, RACE, and Q-PCR

RT-PCR analysis was performed on human kidney and adrenal RNA preparations with exon 1- and exon 3-specific primers. Two sources of RNA from each tissue were used: kidney (source 1) = normal, whole kidneys pooled from 6 male/female Caucasians; kidney (source 2) = two female donors; adrenal (source 1) = normal, whole adrenal glands pooled from 62 male/female Caucasians; adrenal (source 2) = one female. Direct sequencing of RT-PCR products from kidney RNA displayed a mixture of sequences beginning at the exon 1–2 junction; therefore, the products were subcloned into a plasmid vector, and independent transformants were sequenced. Figure 1 shows that two distinct exon 1–2 splice junctions were found: splice product 1 corresponds to the predicted sequence of exon 1, with variant 13 as the last exon 1 nucleotide, whereas splice product 2 is generated by splicing 10 nt 3' of variant 13, and results in a transcript that disrupts the predicted C2 domain translated reading frame. The variant 13 G allele displays leaky splice site selection with a mixture of splice product 1 (35/51 in kidney, 11/20 in adrenal) and splice product 2 (16/51 in

kidney, 9/20 in adrenal), whereas the variant 13 A allele is detected only in splice product 2 (87/87 in kidney and adrenal).

Further characterization of the representation and semiquantitative abundance of 5' splice isoforms were performed with 5' RACE reactions with primers specific for exons 2, 2a, 2c, and 3, using the same RNA preparations used in the RT-PCR analysis. RACE products were subcloned into a plasmid vector and 96 clones were sequenced for each RACE reaction. Figure 2 shows the exon structure of the RACE products and the number of unique 5' ends seen with each primer. Exon 3 RACE primers generated products corresponding to the exon 1–2–3 product (isoform I) that were similar to the exon 1–3 RT-PCR products, exon 2a–3 products (isoform III) that were similar to the KIAA0439 transcript, and exon 2a–2d–3 and exon 2b–3 products. Only exon 1-containing forms have a potential initiating methionine codon upstream of exon 3. Using RACE primers within exon 2, approximately 50% of the subcloned RACE products from kidney and adrenal showed splicing to exon 1. The exon 1–2 splice junction for RACE clones containing the G allele of variant 13 were

Table 2. SNP discovery in human NEDD4L exons and intron flanks

Variant	Coordinate ^a	Implication	Alleles	Frequency
1	80950	5' Flank	T/C	0.16
2	80986	5' Flank	C/G	0.01
3	81117	5' Flank	C/A	0.47
4	81144	5' Flank	G/A	0.27
5	81189	5' Flank	A/G	0.22
6	81479	5' Flank	C/-	0.47
7	81543	5' Flank	C/T	0.32
8	81756	5' Flank	G/A	0.49
9	82261	5' Flank	G/A	0.31
10	82369	5' Flank	A/G	0.21
11	82541	5' Flank	G/A	0.01
12	82578	5' Flank	G/A	0.01
13	82723	Splice junction	G/A	0.30
14	98767	Intron 1	A/G	0.01
15	98830	Intron 1	C/T	0.50
16	142069	Intron 2	G/A	0.01
17	142182	Intron 2	C/T	0.01
18	142210	Intron 2	A/C	0.24
19	143720	Exon2a	C/T	0.01
20	143784	Exon2a	G/A	0.22
21	238195	Intron 3	C/G	0.06
22	305555	Intron 6	T/C	0.44
23	305589	Intron 6	A/G	0.19
24	318797	Intron 10	C/T	0.38
25	320155	Intron 10	TTCT/-	0.22
26	330373	Intron 13	G/A	0.42
27	331039	Intron 14	T/C	0.01
28	331053	Intron 14	GTT/-	0.24
29	339121	Intron 17	A/C	0.01
30	359469	Intron 22	G/A	0.02
31	359494	Intron 22	G/A	0.22
32	359713	Intron 22	T/C	0.17
33	360074	Intron 23	A/G	0.21
34	362468	Intron 23	TTAAA/-	0.41
35	362874	Intron 24	C/T	0.04
36	375143	Intron 26	G/C	0.01
37	378727	Intron 28	G/A	0.01
38	386046	3' UTR	T/C	0.05

SNP, Single-nucleotide polymorphism; UTR, untranslated region

^a Coordinates are from the human genome draft assembly hg8 06 Aug 2001 freeze (www.genome.ucsc.edu, University of California, Santa Cruz). The table coordinates can be indexed to the chromosome 18 hg8 draft coordinates by adding 65 000 000 to each value

either splice product 1 or splice product 2, and only splice product 2 was seen when the A allele was present, similar to the results of RT-PCR analysis. The other RACE products terminated in exon 2 or spliced to unaligned genomic sequence. Race experiments with both exon 2a and 2c primers resulted in no detectable 5' sequence spliced to these exons, implying that these exons may be adjacent to promoters expressed in these tissues. RACE experiments with exon 3 primers confirmed the presence of the isoform I transcript and the isoform III transcript. These experiments also revealed two distinct exons expressed in kidney: 2b and 2d, with 2b represented as a 5' exon without an in-frame AUG codon spliced to exon 3, similar to exons 2a and 2c, and exon 2d detected as an alternative splice form consisting of exon 2a–2d–3, each with consensus splice sequences at their 5' donor and 3' acceptor sites.

The relative level and tissue-specific expression of isoform I versus the isoform III was analyzed by Q-PCR.

Figure 3 shows the results of four experiments, each in triplicate, for the quantitative analysis of NEDD4L mRNA from kidney, adrenal gland, and liver, normalized to the expression of hGAPDH in each tissue. Expression of GAPDH in these tissues is not significantly different (data not shown). Expression of isoform I is significantly higher in both kidney and adrenal than isoform III. These findings suggest that isoform I (exon 1–2–3) is expressed in a tissue-specific manner, and that it is more abundant than isoform III (exon 2a–3) in kidney and adrenal gland.

Refinement of NEDD4L predicted genomic structure and transcript isoform pattern

Figure 4A combines cDNA, EST, RT-PCR, and RACE data and suggests six different transcript isoforms spliced to exon 3. Isoforms I and II splice an in-frame AUG codon to exon 2, adding 8 and 16 amino acids, respectively. Isoform I spans 303 kb of genomic DNA (draft sequence with 54 gaps) and consists of 30 exons. Both isoforms are capable of producing a translated protein containing the evolutionarily conserved C2 domain beginning in exon 2. The similarity of this C2 domain to consensus Simple Modular Architecture Research Tool (SMART) C2 domain sequence (Schultz et al. 2000) is shown in Fig. 4B, as well as the location of this domain across exon 2 through exon 6 of *NEDD4L*. The sequence 5' of the exon 2 splice junction in isoform II is not represented in the current version of the draft human genome sequence, and we postulate it may reside 5' of exon 1, in an unfinished region of chromosome 18. This isoform was not detected in the 5' RACE analysis, but is detected by RT-PCR from both kidney and adrenal RNA (data not shown).

Isoforms III and IV splice exons 2a and 2c to exon 3, respectively. Functional studies of mammalian NEDD4L protein have used the exon 2a–3 isoform typified by KIAA0439 (GenBank accession no. AB007899), which lacks an intact N-terminal C2 domain. Neither exon 2a nor exon 2c contains AUG codons in any frame, and the first AUG start codon in these isoforms occurs in exon 7. These isoforms are predicted to produce a form of NEDD4L protein that completely lacks the C2 domain. Isoforms V (exon 2a–2d–3) and VI (exon 2d–3) also lack AUG start codons and are also predicted to initiate translation in exon 7.

Analysis of mouse cDNA, EST, and draft genomic assemblies reveals conserved exon–intron junctions for exons 2, 2a, 2b, 2c, and 3. Mouse exon 2a–3 splice form is represented by the following ESTs: AW106584, BI687556, BB621848, AW228518, BI218843, AW226920, BB846219, AI527754, AI227149, AI931594, AW910412, BI690367, BG971715, BI650832, BI559036. Mouse exon 2b–3 splice isoform is represented by cDNA AK004969, and mouse exon 2c splice isoform is represented by ESTs BG404747 and BG294676. The mouse exon 2–3 splice form is represented by ESTs BB569841, BB611456, BB635343, BB639757, BB640919, BB847602, and BB863379. These seven ESTs form five sequence clusters, and two of these clusters match draft assemblies with a consensus GT dinucleotide at the predicted 5'

Splice Product 1

M R R L A F E Q G E S R I L R V K V V S G I D ...
ATGAGACGTCGCGATTTGAGCAG GGAGAGTCCCGTATTCTCAGAGTAAAGTTGTTTCTGGAATTGA

Splice Product 2

M R R L A F E Q V T L G R V P Y S Q S K S C F W N *
ATGAGACGTCCTCGCATTGAGCAG GTAACACTCG GGAGAGTCCCGTATTCTCAGAGTAAAAGTTGTTTCTGGAATTGA

Kidney

Adrenal

Splice Product

Splice Product

		1	2
variant	G	35	16
	A	0	17

$$p < 10^{-6}$$

		1	2
v a r i a n t	G	11	9
	A	0	70

$$p < 10^{-8}$$

Fig. 2. 5' rapid amplification of cDNA ends (RACE) analysis of NEDD4L in kidney and adrenal gland. The exonic start location of the most 5' end for each transcript is shown, as well as the total number of clones and the number of unique 5' ends found for each transcript. The exonic coordinates locate the 5' end in the human genome draft assembly hg8 06 Aug 2001 freeze by addition of 6500000 bases. *nt.*, Nucleotide

Fig. 3. Quantitative PCR analysis of exon 1-2-3 isoform I versus exon 2a-exon 3 isoform III in human kidney (*hK*), adrenal gland (*hA*), and liver (*hL*). Standard curves from a two-fold dilution series of cloned PCR target for both NEDD4L isoforms and human glyceraldehyde-3-phosphate dehydrogenase (*hGAPDH*) were used to calculate the rela-

tive ratios of NEDD4L/GAPDH. The NEDD4L/GAPDH ratios are calculated based on predicted molar amounts of target mRNA. The probability that the expression levels are significantly different is shown with *P* values calculated with a student's *t*-test

Fig. 4A,B. Exon-intron structure of the 5' end of *NEDD4L* on human chromosome 18q. The exons (A) were defined by searching GenBank Human expressed sequence tag (EST) entries and nr databases with NEDD4L exon 3 as the query (CAGTGATCCGTATGTGAAACTTTCATTGTACGTAGCGGATGAGAATAGAGAACTTGCTTTGGTCCAGACAAAACAATTAAG). ESTs and cDNAs that have exons spliced 5' to exon 3 are shown. AF21730 sequence 5' of exon 2 is not represented in the current draft sequence of the human genome. The sequences were compared with human genome draft assembly hg8

06 Aug 2001 freeze to define exon-intron boundaries; all exons conformed to consensus splice sites (5'-AG, 3'-GT). The exonic location and conservation of the C2 domain (B) is shown for NEDD4L transcripts. The alignment of human NEDD4L is shown versus the Simple Modular Architecture Research Tool (SMART) C2 (SM0239) consensus sequence. The alignments of predicted C2 domains of mouse and *Fugu* NEDD4L orthologs, and the human NEDD4 paralog, are also shown

splice donor site. None of these clusters displays significant similarity to human exon 1, although BB863379 does have 71 nt 5' of exon 2 that match the 5' end of human isoform II at 89% identity. However, this EST is only 96% identical to the other mouse exon 2–3 ESTs; thus, it is likely that this EST represents a pseudogene. TBLASTN analysis of isoform I and II against the draft *Fugu rubripes* (puffer fish) genome revealed a draft assembly (Scaffold_826, 55 kb with nine gaps) spanning 26 kb containing exons 2 through 30 (exons 10, 11, and 12 are missing from the assembly) and displaying conserved exon–intron junctions for the 26 exons detected. Translation of the *Fugu* NEDD4L exons yields a protein that is 87% identical and 94% similar to human NEDD4L isoform II.

Figure 4B shows that there are no amino acid substitutions between the predicted C2 domains of human and mouse NEDD4L, and that there are fewer substitutions between *Xenopus*, *Fugu*, chicken, mouse, and human NEDD4L than between human NEDD4L and NEDD4. The *Fugu* NEDD4L gene also contains an exon 1.2 kb upstream of exon 2 that is predicted to splice an in-frame AUG codon to exon 2 with the addition of 15 N-terminal amino acids. That translated product displays detectable sequence similarity with the translated product of isoform II (Fig. 4B), which is the isoform whose sequence 5' of exon 2 is missing in current human finished and draft sequence databases. Although orthologous 2a, 2b, and 2c exons are detected in the mouse draft sequence, there is no detectable similarity in *Fugu* NEDD4L intron 2 using either TBLASTN or BLASTN analysis. This suggests that isoform II may reflect the most ancestral form of NEDD4L, and that the sequence of the N-terminal C2 domain has been conserved by natural selection.

Discussion

This resequencing survey of NEDD4L exons and flanking introns for polymorphic variants in 48 Caucasian individuals detected 35 flanking/intronic variants, and 3 exonic variants. Variant 13-A occurs at an allele frequency of 0.30, and its location in exon 1 results in a synonymous CAG/A glutamine codon substitution, but also changes the 5' splice donor site from a strong to a weak consensus: CAG-GT to CAA-GT. Analysis of the NEDD4L exon 1–2 splice junction from RT-PCR and RACE products from human and adrenal RNA reveals leaky splice site selection, of which only one form is predicted to lead to in-frame translation of the evolutionarily conserved N-terminal C2 domain. The identity of variant 13 changes the ratio of splice site selection from preferred splicing of the in-frame product with the G variant, to no detectable splicing of the in-frame product with the A variant. Numerous human mutations have been described that have G-to-A alterations at this position in 5' splice donor sites (Rogan et al. 1998). Also, the human cyclin D1 (*CCND1*) gene contains a common G/A polymorphism at this 5' splice donor site consensus position in *CCND1* exon 4, and this polymorphism modulates mRNA splicing to

produce two transcripts (Betticher et al. 1995). This common polymorphism has recently been implicated in altering the age of onset of hereditary nonpolyposis colorectal cancer (Bala and Peltomaki 2001; Kong et al. 2000).

RT-PCR and RACE analysis from human and adrenal RNA revealed six NEDD4L transcript isoforms. Functional studies of mammalian NEDD4L protein have used a spliced form of the human NEDD4L protein typified by KIAA0439 (GenBank accession no. AB007899), which corresponds to isoform III resulting from exon 2a–3 splicing and lacking an intact N-terminal C2 domain. This is in contrast to the founding, and defining, member of the NEDD4L family, a *Xenopus laevis* Nedd4-like protein that has an intact C2 domain (Rebhun and Pratt 1998). Our analysis of genomic, cDNA, and EST sequence reveals a conserved exon 2 in *Fugu*, mouse, and human that corresponds to the orthologous sequence in *Xenopus*. RACE analysis using exon 2 and exon 3 primers demonstrates that isoform I of human NEDD4L is an abundant 5' transcript isoform in adrenal tissue, whereas in kidney there is evidence for multiple isoforms including I, III, V, and VI. Isoforms III through VI all generate transcripts in which the first potential AUG start codon is in exon 7, distal to the C2 domain. RACE experiments from exon 2a and 2c did not detect any sequence spliced 5' of these exons, and exon 1–3 RT-PCR revealed only exon 1–2–3 splice forms, with no splicing detected to exons 2a, 2b, 2c, or 2d. Therefore, isoforms containing exons 2a, 2b, 2c, or 2d are expected to begin translation from the AUG codon in exon 7, which is the first AUG codon in these transcripts. This exon 7 AUG codon is conserved between human, mouse, chicken, *Xenopus*, and *Fugu*, and, as shown in Fig. 4B, it is located at the C-terminal junction of the C2 domain. Q-PCR analysis of isoform I versus isoform III in total RNA from human kidney, adrenal, and liver indicated tissue-specific expression patterns for isoform I, and suggested that this is an abundant isoform in kidney and adrenal. It should be pointed that not only does variant 13-A affect a previously unreported isoform of human NEDD4L, but this isoform I is also significantly more abundant in kidney and adrenal gland than the better investigated, C2-less isoform III corresponding to the KIAA0439 transcript.

The conserved NEDD4L C2 domain translated from exon 2 through exon 6 suggests that this sequence has been maintained through functional selection. The observed average amino acid identity in large-scale comparisons of orthologous mouse and human protein sequences is 85.4% (Makalowski et al. 1996). The translated, 101 amino acid C2 domain of human NEDD4L is 100% identical to mouse C2, 98.0% identical to chicken C2, 96.0% identical to *Xenopus* C2, and 94.1% identical to *Fugu* C2 domains. This level of sequence conservation suggests that the NEDD4L C2 domain is functional and under strong selective constraints. The comparison of the N-terminal sequence prior to the C2 domain of human isoforms I and II with *Fugu* and *Xenopus* indicates that isoform II is conserved for 9 of 16 amino acid residues at the N terminus (Fig. 4B). The genomic sequence corresponding to the 5' end of isoform II has not yet appeared in the human genome assembly, but we can

postulate that it may be located upstream of exon 1 of isoform I. The conserved residues at the N terminus of isoform II suggest it may represent the most ancestral isoform of NEDD4L, and that the isoform I promoter and exon 1 sequences may be more recently evolved. The existence of multiple isoforms that result in either C2 or C2-less forms of NEDD4L protein, some of which are conserved between human and mouse, suggests that these two forms may have different functional roles in ubiquitination and endocytosis of protein targets such as ENaC, and may have evolved under different selective constraints.

Although no experiments have yet been reported with a human or mouse NEDD4L protein containing an intact C2 domain from either isoform I or II, there are several studies using C2-less NEDD4L and Nedd4/Rsp5 proteins in mammalian, amphibian, and yeast systems. The ~110 amino acid C2 domain is a eukaryotic protein module that has Ca^{2+} -dependent interactions with phospholipids, inositol polyphosphates, and intracellular proteins. The human Nedd4 C2 domain has been shown to cause Ca^{2+} -dependent plasma membrane localization in polarized Madin-Darby canine kidney (MDCK) cells (Plant et al. 1997). This localization of endogenous Nedd4 occurred preferably to apical and lateral membranes in these polarized cells, and heterologous expression of a C2 deletion construct showed that the C2 domain was required for Ca^{2+} -dependent membrane localization. Further studies have shown that annexin XIIIa and b are the protein-binding partners of Nedd4 in this Ca^{2+} -dependent membrane localization assay (Plant et al. 2000). The Ca^{2+} -dependent binding to annexin XIIIb further targets the complex to lipid rafts, which are membrane cholesterol and sphingolipid microdomains involved in endocytosis (Ikonen 2001).

Several experiments have investigated the effect of the Nedd4 C2 domain on regulation of ENaC activity. In both *Xenopus* oocytes and rat thyroid epithelia, removal of the Nedd4 C2 domain in expression constructs caused a stronger down-regulation of ENaC activity (Snyder et al. 2001). This result has also been seen in a comparison of human NEDD4 and NEDD4L in down-regulating ENaC activity in *Xenopus* oocytes (Kamynina et al. 2001a, 2001b). In this case, removal of Nedd4 C2 domain also resulted in stronger down-regulation of ENaC activity, and expression of an NEDD4L isoform III construct (KIAA0439) displayed more potent inhibition of ENaC than did NEDD4 constructs. These experiments also attempted to convert isoform III of NEDD4L to a C2-containing form by adding an AUG codon in-frame at the beginning of exon 2a. This construct had no effect on ENaC regulation in *Xenopus* oocytes; however, with the identification of isoform I, it is now apparent that this recombinant protein restored a truncated C2 domain rather than the full-length C2 domain encoded by exons 2 through 6.

Experiments with the RSP5 yeast homolog of the NEDD4/NEDD4L family have demonstrated that it is involved in the ubiquitination and endocytosis of several membrane proteins. C2-less constructs of Rsp5 had no effect on the ubiquitination and internalization of the yeast alpha-factor receptor. However, the C2-less construct

displayed defects in transport of fluid-phase markers through the endocytotic pathway to the vacuole, suggesting that Nedd4 proteins are internalized along with their ubiquitinated protein targets and that the C2 domain may also function postendocytosis (Dunn and Hicke 2001). This study also provided evidence that Rsp5 protein forms homomeric complexes, both in vitro and in vivo. Given these results, as well as the recent recognition that NEDD4L, rather than NEDD4, is the major regulator of ENaC in mammalian cells, the exact role of the NEDD4L C2 domain in both localization and activity of this protein requires further investigation.

Selective constraints on NEDD4L activity independent of ENaC interactions may be derived from evidence that NEDD4L is also involved in viral-associated processes of enveloped virus budding and viral latency. Recent evidence suggests that Nedd4 family proteins interact with coat proteins of enveloped viruses late in the process of viral budding, implicating ubiquitination in the function of viral protein late domains in retrovirus assembly (Hicke 2001; Patnaik et al. 2000; Strack et al. 2000; Vogt 2000). Using the late assembly domain of Rous sarcoma virus (RSV) Gag protein as a peptide probe on a chicken embryo expression library has resulted in the cloning of the C2 and WW domains of the chicken NEDD4L ortholog (Kikonyogo et al. 2001). In a human cell assay of RSV Gag budding, overexpression of the chicken NEDD4L WW domains resulted in dominant-negative inhibition of Gag budding, suggesting the overexpressed chicken NEDD4L WW domains inhibited Gag budding by competing with endogenous human NEDD4L. It has also been shown that the PY domain of ENaC can substitute for the RSV Gag late assembly domain in a virus-like particle assay in HeLa cells (Strack et al. 2000), implicating NEDD4L function in the budding of enveloped viruses containing PY motifs that include retroviruses. The protein produced from KIAA0439 has also been shown to form physiological complexes with the PY motifs in latent membrane protein 2A of Epstein-Barr virus (Winberg et al. 2000). It has been recently suggested that the molecular basis phenomenon of viral pseudotyping, the result of a mixed infection with two different enveloped viruses that results in phenotypic mixing of envelope proteins in the virus progeny, may be due to the sequestration of viral maturation components in lipid rafts (Pickl et al. 2001). As mentioned earlier, the Ca^{2+} -dependent binding of Nedd4 to annexin XIIIb, mediated by the NEDD4 C2 domain, targets Nedd4 to lipid rafts in the apical surface of polarized MDCK epithelial cells (Plant et al. 2000). A plausible hypothesis is that sequestration of Nedd4 proteins in lipid rafts mediated by Ca^{2+} -dependent binding interactions of the C2 domain may expose Nedd4 proteins to selective forces acting at the level of viral infectivity.

It has also been recently shown that ENaC activity is down-regulated after influenza virus infection of mouse tracheal epithelia, contributing to the accumulation of edema fluid in the airway (Kunzelmann et al. 2000; Matthay et al. 2002). This down-regulation is dependent on viral hemagglutinin binding to a cell surface receptor, which was shown to activate phospholipase C and protein kinase C (PKC).

PKC inhibition of ENaC activity has also been demonstrated *in vitro* by coexpression of rat ENaC and rat PKC in *Xenopus* oocytes (Awayda et al. 1996). Regulation of ENaC activity by phosphorylation of mouse Nedd4-2 via aldosterone-dependent activation of serum and glucocorticoid-regulated kinase has also been shown to control the cell surface density of ENaC (Debonneville et al. 2001b). Absorptive colonic epithelial cells are also a site for Na⁺ reabsorption via ENaC, and pathological conditions, such as infectious diarrhea, lead to alterations of ENaC-mediated ion transport (Kunzelmann and Mall 2002). Given the importance of ENaC in maintaining fluid balance by Na⁺ transport in numerous epithelial tissues, and the effects of numerous viral and bacterial pathogens on ENaC activity, it can be postulated that these pathogens may be a source of selective pressure for alterations in ENaC regulation. The presence of NEDD4L transcripts that lack the conserved C2 domain may be an adaptation to regulating ENaC activity in response to a pathogenic challenge, and a putative effect of multiple forms of NEDD4L on orthostatic hypotension could be a secondary consequence of such selective adaptation.

In summary, a common variant in NEDD4L has been shown to affect splice site selection in a major transcript isoform expressed in kidney and adrenal gland. The variety of transcripts detected in human leading to either inclusion or exclusion of the C2 domain suggests that expression of different isoforms may lead to functional differences. Indeed, the presence of the C2 domain, by targeting such isoforms to cell membranes or membrane microdomains such as lipid rafts, may contribute to substrate specificity and the proposed interactions with ENaC. Individuals ho-

mozygous for the A allele variant are predicted to have a decrease in the amount of C2-domain NEDD4L produced from expression of isoform I. The common frequency of this allele suggests that any functional effect will be subtle, as small effects are expected for common variants in common disease. Variant 13 of NEDD4L is therefore a likely candidate single-nucleotide polymorphism for association with phenotypes relevant to orthostatic hypotension, hypertension, enveloped viral infections, and/or pathological conditions involving Na⁺ absorption.

Acknowledgments List of HyperGEN participating institutions and principal staff: Network Center/University of Utah Field Center: Steven C. Hunt, Roger R. Williams (deceased), Hilary Coon, Paul N. Hopkins, Janet Hood, Nona Gallacher, Michael McGinty, Karen Nielsen, Lily Wu, Jan Skuppin. University of Alabama at Birmingham Field Center: Albert Oberman, Cora E. Lewis, Michael T. Weaver, Phillip Johnson, Randi Gilinson, Christie Oden. Boston University/Framingham Field Center: R. Curtis Ellison, Richard H. Myers, Yuqing Zhang, Luc Djoussé, Jemma B. Wilk, Greta Lee Splansky. University of Minnesota Field Center: Donna Arnett, Aaron R. Folsom, Larry D. Atwood, Gregory Feitl, Jim Pankow, Barb Lux. University of North Carolina Field Center: Gerardo Heiss, Barry Freedman, Dee Posey, Kathryn Rose, Amy Haire. Data Coordinating Center, Washington University: D.C. Rao, Michael A. Province, Ingrid B. Borecki, Yuling Hong, Avril Adelman, Derek Morgan, Karen Schwander, David Lehner, Aldi Kraja, Stephe Mandel. Central Biochemistry Lab, University of Minnesota: John H. Eckfeldt, Ronald C. McGlennen, Michael Y. Tsai, Catherine Leindecker-Foster, Greg Rynders. Molecular Genetics Laboratory, University of Utah: Mark Leppert, Steven C. Hunt, Jean-Marc Lalouel, Robert Weiss. National Heart, Lung, & Blood Institute: Stephen Mockrin, Susan E. Old, Millicent Higgins (retired), Peter Savage, Cashell Jaquish.

This hypertension network is funded by cooperative agreements (U10) with NHLBI: HL54471, HL54472, HL-54473, HL54495, HL54496, HL54497, HL54509, HL54515.

Appendix

Appendix, Table 1. Oligonucleotide sequences for PCR amplification and sequencing of human NEDD4L

NEDD4L exon	PCR primers (5'–3')	Sequencing primers (5'–3')
1	TCCGAGCAGAGCTCATGTAA GGCCAGCAAGAGAGATAGGA	AGAGGCATCGAAGTACACGC AGGTTGCAGTGAGCCAAGAT GCTGCCATAACAAATACCCA TCTAGCCCCCTGGAATGTGAC ATATTGGATTAGGGCCACC TATGAACCTGCCACTGGAC GAAAGTGGCAGAGAGGAAGC AGATGAATTAACAGCTCCAGCA TGGCAGGAGTAATTGATCTTAATG AGAGCCATAGTCCCACACTG GGTGTGTTGTTTGTGCAAGG AAATGTTTTCACTGTGCCGC TGTAAGTCTGAACAAATCTGTGTG GCTGAGGCAGGAGAATCACT CTTGTTAGAATAATCATATCAATGCT CGTCTCATAGAATTAACCAATTCTCA TCTACAGTCAAGGAATGCCTTG GCCTACCGTTCTGTATTCCC CAGCTGTGCCCTCAAACC GCCTGAGTGAAGGAGGTGTC CGTGGACTGTGAGGTACCAA TCTAAAGAGAAATCTCTCCGAGG GTGTCACCGACTGTGCAGAT GTGACTCAAAGCAATCGCT GCAAATGGGCAGAGGACTT CCACCTATACTGTCAAATACGGC TGATTCACATCATGGCCCTA CCAAGAAAGTGTAGTGGTATAAGG
2	GAGGAGAGTTCCAGCCACTG TGACTTCACGGTCAAGCAAG	
2a	GCCTGCTTTCTTCACTTCTGA GACTGCACCACCTGAAGA	
3	GGCTGAGGGTGTCCATAGAA CATGGAGATAGGCAATTAAGACA	
4	TGGAGGCTTTATTGAAGATGC GCTTTTCAGTCTCCACAGG	
5	GAGCTGATAGATGCTGTTGGC TGCACTGAATTATAGTGTCACTTCTG	
6	GGCAGCGTGATTAGGTCTGT AAGACCAGGTGCTGCTCACT	
7, 8, 9	AAACCCGGTGTGGATAGTGA TCAGCTCATATTGAAAACAGCA	
10, 11	GTTCTTCAGACCCCGGCT TTGGATGAGCCACTTAAGCA	

Appendix, Table 1. Continued

NEDD4L exon	PCR primers (5'–3')	Sequencing primers (5'–3')
12	TCAAGGGGAAGAACATGAGC	TTCTGAAAGTTTGGTCTCCTC
13	TGTAAGGCAGGTGACGTGAG	TCAAAGTACAGACTGGAGCA
14, 15, 16	TGGAAACTGGTGATTCTCTCC	TACCCAGGTCAAGGATGAGC
	ATGCAATTCCTGTGGCTTCT	TGCACTTAACCAAAGCCTGA
	CCAAGCTGTGTTACCTTCA	GGCAGCAACCCTTTACGTT
	ACTTTGTGAGACGGGATCAA	GGCAGGGAAGAAGCTACACA
		TGGAAACTGGTGATTCTCTCC
		ATGCAATTCCTGTGGCTTCT
		CTGAGTAGCTACCAAAGGATGG
17, 18	AGTCAGCTCCTGGACTCTGG	GAGCTTCCCAGGAGAAAAGG
	ATGAAGAACATGGCACCTCC	CACTGCTGGGACTCTCTTCC
		GCCACTTAAATTGTACCTGC
		TTTTGAGCTAGCAGCCATT
		GCACTGTTACAGATTTTGGACT
19	TGCACATTAACCTCACTTGGAAGA	AAATCCTTAAAAGAACAGGGGTTT
20, 21	GATGGAAGAACCAATGGTGG	AGGAAGGGAGGGAATTTTGA
	TCAATTCCTGAGCCCTCAAC	CACCTCATATCTTGACAA
	AGCCTGGCAATGATGAAATC	TTGTGACTAGGGTCTGAATTATTT
		CATCGTAGTGGATGCCG
		CCATGAACCCAATAGACAAGAA
22, 23	ATTTGGCTGAACCATATGAAA	CTTCGTAGGTCAAAAACAGTCAA
	CATGGTTCAGGAAATGTGA	TTGCTTTCACTAAAGTTATTCCAGA
		TCTGGCCTGTCTACCCGC
		AGATCAATGAACTTTCAAGCAA
24	ACTGCTGGTTTGCCAGTCAT	TTTTGTAGACCAAGTATGGCAGC
	GTGACACCTGCATCCACAG	AACCGAGGCACAAATGAAAC
25, 26	TTTCTGGAGTCTTGATGGGTG	GTTTCCTGATAAATGAATAGCAGAG
	GGGACTGCGCTTCATCAT	TCTTAGTTGATATCCACATATTCCAA
		AAAGCATTTTAAAGCAGTTAAGCA
		GTACCGCTGAAACTCCAAG
27, 28	CACTGCCCAGATCGAACTTT	GCACTGACTCACAGCAGTGTT
	CACAGATCAAAATATTTGTTGGAA	CAGCTCTGCTCAGTGGCCT
		CTAGAGGGGTAGGGAGGACG
		GGAGGACCAAGTCCCTCAAAT
29, 30	ATTATCTTAGGGCCACTGTTTG	TGGTGCCATCAGAGAGTTTG
	TGACTTGGACACCATTGTGA	CGACCATATAAATGACCTACAA
		GCCCAGCTGAGAGGCTGT
		CAGGTTGAGAGCTGCCTTATC
31	ATTGAGCCCTTGCTGTATGC	CTCCGAGATCTGCATCTGACT
	CAAGATGACAGAAGAATCCAG	TTAATTCCATTTCATTGTCATTAGAAA
		ACCTGGTCCCAGCTTGAGTT
		TCGGATGAGAAATGGGAGTC
		CCATACTCAGAAATGAAAACTGGA
		TGAAGCATGCAACAGGTCAT
		TCAATTGTGAATCTGGCTGC
		GAAAAAGGAACCAACACTCAGC
		AAGTTGTGGTTTGGGGAGGT
		GGTTAGAATTGGATTCTCCCTC

PCR, Polymerase chain reaction

Appendix, Table 2. Oligonucleotide sequences for RT-PCR and RACE experiments

Location	Primer sequence
RT-PCR	
Exon 1	CCATTTCCAGAGAGGAACAACCGTG
Exon 3	GCGCCGGCTCCATGGCGACC
	AGCAAGTTCTCTATTCTCATCCGCT
RACE	
Exon 2	CTGGCTCCAAAGATGTCCTTTTGG
	CGAGATCAATTCAGAAACAAC
Exon 2a	GAGTGGGAGGTGCCGTGCTGG
	GGAAGAGCTCGTCTGAAGTGG
Exon 2c	GCTGCTGGAATCTACCTTGG
	GGCTGGAAAGTGTTTACGCTGG
Exon 3	CCGCTACGTACAATGAAAGTTTAC
	AGCAAGTTCTCTATTCTATCCGCT

RT-PCR, Reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends

References

- Atwood LD, Samollow PB, Hixson JE, Stern MP, MacCluer JW (2001) Genome-wide linkage analysis of blood pressure in Mexican Americans. *Genet Epidemiol* 20:373–382
- Awayda M, Ismailov I, Berdiev B, Fuller C, Benos D (1996) Protein kinase regulation of a cloned epithelial Na⁺ channel. *J Gen Physiol* 108:49–65
- Bala S, Peltomaki P (2001) CYCLIN D1 as a genetic modifier in hereditary nonpolyposis colorectal cancer. *Cancer Res* 61:6042–6045
- Betticher DC, Thatcher N, Altermatt HJ, Hoban P, Ryder WD, Heighway J (1995) Alternate splicing produces a novel cyclin D1 transcript. *Oncogene* 11:1005–1011
- Corvol P, Persu A, Gimenez-Roqueplo AP, Jeunemaitre X (1999) Seven lessons from two candidate genes in human essential hypertension: angiotensinogen and epithelial sodium channel. *Hypertension* 33:1324–1331
- Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraïbi A, Pratt JH, Horisberger JD, Pearce D, Löffing J, Staub O (2001a) Phosphorylation of Nedd4-2 by Sgk1

- regulates epithelial Na(+) channel cell surface expression. *EMBO J* 20:7052–7059
- Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraïbi A, Pratt JH, Horisberger J-D, Pearce D, Loffing J, Staub O (2001b) Phosphorylation of Nedd4-2 by Sgkl regulates epithelial Na⁺ channel cell surface expression. *EMBO J* 20:7052–7059
- DeStefano AL, Baldwin CT, Burzstyn M, Gavras I, Handy DE, Joost O, Martel T, Nicolaou M, Schwartz F, Streeten DH, Farrer LA, Gavras H (1998) Autosomal dominant orthostatic hypotensive disorder maps to chromosome 18q. *Am J Hum Genet* 63:1425–1430
- Dunn R, Hicke L (2001) Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. *Mol Biol Cell* 12:421–435
- Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8:175–185
- Harvey KF, Dinudom A, Cook DI, Kumar S (2001a) The Nedd4-like protein KIAA0439 is a potential regulator of the epithelial sodium channel. *J Biol Chem* 276:8597–8601
- Harvey KF, Dinudom A, Cook DI, Kumar S (2001b) The Nedd4-like protein KIAA0439 is a potential regulator of the epithelial sodium channel. *J Biol Chem* 276:8597–8601
- Hicke L (2001) A new ticket for entry into budding vesicles–ubiquitin. *Cell* 106:527–530
- Ikonen E (2001) Roles of lipid rafts in membrane transport. *Curr Opin Cell Biol* 13:470–477
- Kamynina E, Debonneville C, Bens M, Vandewalle A, Staub O (2001a) A novel mouse Nedd4 protein suppresses the activity of the epithelial Na⁺ channel. *FASEB J* 15:204–214
- Kamynina E, Tauxe C, Staub O (2001b) Distinct characteristics of two human Nedd4 proteins with respect to epithelial Na(+) channel regulation. *Am J Physiol Renal Physiol* 281:F469–477
- Kikonyogo A, Bouamr F, Vana ML, Xiang Y, Aiyar A, Carter C, Leis J (2001) Proteins related to the Nedd4 family of ubiquitin protein ligases interact with the L domain of Rous sarcoma virus and are required for gag budding from cells. *Proc Natl Acad Sci USA* 98:11199–11204
- Kong S, Amos CI, Luthra R, Lynch PM, Levin B, Frazier ML (2000) Effects of cyclin D1 polymorphism on age of onset of hereditary nonpolyposis colorectal cancer. *Cancer Res* 60:249–252
- Kristjansson K, Manolescu A, Kristinsson A, Hardarson T, Knudsen H, Ingason S, Thorleifsson G, Frigge ML, Kong A, Gulcher JR, Stefansson K (2002) Linkage of essential hypertension to chromosome 18q. *Hypertension* 39:1044–1049
- Kunzelmann K, Beesley AH, King NJ, Karupiah G, Young JA, Cook DI (2000) From the cover: influenza virus inhibits amiloride-sensitive Na⁺ channels in respiratory epithelia. *Proc Natl Acad Sci USA* 97:10282–10287
- Kunzelmann K, Mall M (2002) Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiol Rev* 82:245–289
- Lemmon SK, Traub LM (2000) Sorting in the endosomal system in yeast and animal cells. *Curr Opin Cell Biol* 12:457–466
- Liddle GW, Bledsoe T, Coppage WS Jr (1963) A familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion. *Trans Assoc Am Physicians* 76:199–213
- Lifton PR, Gharavi AG, Geller DS (2001) Molecular mechanisms of human hypertension. *Cell* 104:545–556
- Luft FC (2000) Molecular genetics of human hypertension. *Curr Opin Nephrol Hypertens* 9:259–266
- Makalowski W, Zhang J, Boguski MS (1996) Comparative analysis of 1196 orthologous mouse and human full-length mRNA and protein sequences. *Genome Res* 6:846–857
- Matthay MA, Folkesson HG, Clerici C (2002) Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol Rev* 82:569–600
- Nakai K, Sakamoto H (1994) Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene* 141:171–177
- Nickerson DA, Tobe VO, Taylor SL (1997) PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res* 25:2745–2751
- Pankow JS, Rose KM, Oberman A, Hunt SC, Atwood LD, Djousse L, Province MA, Rao DC (2000) Possible locus on chromosome 18q influencing postural systolic blood pressure changes. *Hypertension* 36:471–476
- Patnaik A, Chau V, Wills JW (2000) Ubiquitin is part of the retrovirus budding machinery. *Proc Natl Acad Sci USA* 97:13069–13074
- Pickl WF, Pimentel-Muinos FX, Seed B (2001) Lipid rafts and pseudotyping. *J Virol* 75:7175–7183
- Plant PJ, Yeager H, Staub O, Howard P, Rotin D (1997) The C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca²⁺-dependent plasma membrane localization. *J Biol Chem* 272:32329–32336
- Plant PJ, Lafont F, Lecat S, Verkade P, Simons K, Rotin D (2000) Apical membrane targeting of Nedd4 is mediated by an association of its C2 domain with annexin XIIIb. *J Cell Biol* 149:1473–1484
- Rebhun JF, Pratt JH (1998) Molecular cloning of Nedd4 from *Xenopus laevis*. *DNA Seq* 9:295–306
- Rogan PK, Faux BM, Schneider TD (1998) Information analysis of human splice site mutations. *Hum Mutat* 12:153–171
- Schild L, Canessa CM, Shimkets RA, Gautschi I, Lifton RP, Rossier BC (1995) A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system. *Proc Natl Acad Sci USA* 92:5699–5703
- Schultz J, Copley RR, Doerks T, Ponting CP, Bork P (2000) SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res* 28:231–234
- Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR Jr, Ulick S, Milora RV, Findling JW, Canessa CM, Rossier BC, Lifton RP (1994) Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell* 79:407–414
- Snyder PM, Olson DR, McDonald FJ, Bucher DB (2001) Multiple WW domains, but not the C2 domain, are required for inhibition of the epithelial Na⁺ channel by human Nedd4. *J Biol Chem* 276:28321–28326
- Snyder PM, Olson DR, Thomas BC (2002) Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial Na⁺ channel. *J Biol Chem* 277:5–8
- Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na⁺ channel detected in Liddle's syndrome. *EMBO J* 15:2371–2380
- Stephens RM, Schneider TD (1992) Features of spliceosome evolution and function inferred from an analysis of the information at human splice sites. *J Mol Biol* 228:1124–1136
- Strack B, Calistri A, Accola MA, Palu G, Gottlinger HG (2000) A role for ubiquitin ligase recruitment in retrovirus release. *Proc Natl Acad Sci USA* 97:13063–13068
- Vogt VM (2000) Ubiquitin in retrovirus assembly: actor or bystander? *Proc Natl Acad Sci USA* 97:12945–12947
- Williams RR, Rao DC, Ellison RC, Arnett DK, Heiss G, Oberman A, Eckfeldt JH, Leppert MF, Province MA, Mockrin SC, Hunt SC (2000) NHLBI family blood pressure program: methodology and recruitment in the HyperGEN network. *Hypertension genetic epidemiology network. Ann epidemiol* 10:389–400
- Winberg G, Matskova L, Chen F, Plant P, Rotin D, Gish G, Ingham R, Ernberg I, Pawson T (2000) Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases. *Mol Cell Biol* 20:8526–8535