Functional Characterisation of the Ubiquitin-Protein Ligase, Nedd4.

by

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Contents

Abstract

Ubiquitination of proteins is a mechanism used by the cell to regulate the function and abundance of a variety of proteins involved in many different cellular processes, including the cell cycle, transcription, signalling and ion transport. Ubiquitin is attached to protein substrates by the coordinated action of three classes of enzymes; ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin protein ligases (E3). E3s are largely responsible for substrate recognition in the ubiquitin pathway. A major subfamily of E3s are the hect proteins which share a conserved domain required for interaction with E2s and an absolutely conserved Cys residue which binds ubiquitin. Among the hect E3s, a number share similar protein domain structure in that they contain an amino terminal C2 domain, 2 to 4 WW domains and a hect domain at the carboxyl terminus. These proteins are known as the Nedd4 family of proteins, as they share homology with the first identified member of this family, Nedd4. The function of these proteins is poorly understood but their unique domain structure suggests that they target membrane proteins for ubiquitin-mediated turnover. The focus of this study was to determine the cellular function of Nedd4.

Caspase-mediated cleavage of proteins during programmed cell death is central to the execution of this cellular process. Only a select group of proteins appear to be cleaved by caspases during programmed cell death. Therefore it is important to identify all caspase substrates so that a clearer understanding of the significance of caspase-mediated proteolysis during cell death can be achieved. Several potential caspase cleavage sites were identified in the Nedd4 protein and therefore its behaviour in apoptotic cells was studied. Nedd4 was shown to be cleaved into two major fragments in a variety of cell lines and in response to a number of apoptotic stimuli. Cleavage of Nedd4 by caspases during apoptosis is likely to be an energy conserving mechanism.

The first protein to be implicated as a ubiquitination target of Nedd4 was the epithelial Na^+ channel (ENaC). ENaC has three subunit types (α, β and γ), each of which possesses a PY motif in the intracellular carboxyl terminus that is capable of interaction with the WW domains of Nedd4. To determine whether Nedd4 mediates ubiquitin-dependent downregulation of ENaC, dominant negative Nedd4 and ubiquitin molecules were tested for their ability to interrupt Na^+-dependent feedback of ENaC in mouse mandibular duct cells. Nedd4 was found to mediate Na^+-dependent feedback of ENaC in a ubiquitin-dependent manner. Additionally it was shown that Nedd4 mediates regulation of ENaC downstream of the G protein, G_o. These findings are of particular importance as Nedd4-dependent regulation of ENaC is likely to be disrupted in the familial hypertensive disorder, Liddle's Syndrome.

ENaC assembles in the membrane as a multimeric complex consisting of 4 to 8 subunits (and therefore contains 4 to 8 PY motifs), while murine Nedd4 contains three WW domains. The requirement of specific Nedd4 WW domains for interaction with ENaC complexes *in vivo* was unknown however. To investigate Nedd4/ENaC interaction, individual ENaC subunits and Nedd4 WW domains were examined for their ability to interact with each other *in vitro* and to interrupt Na^+-dependent feedback of ENaC in mouse mandibular duct cells. All 3 WW domains of murine Nedd4 were shown to be required for regulation of ENaC, although only WW domains 2 and 3 physically interacted with ENaC *in vitro*. This suggested that another, as yet unidentified protein, is involved in ENaC regulation that binds to WW domain 1 of Nedd4 and serves to recruit Nedd4 to ENaC and/or stabilise the Nedd4/ENaC complex.

To determine which ENaC subunits were involved in the regulation of activity of this channel, the ability of the PY motif-containing regions of α, β and γ ENaC to inhibit Na^+-dependent regulation of ENaC was tested. Overexpression of β and γ ENaC, but not α ENaC

was shown to inhibit the Na^+- feedback pathway in mouse mandibular duct cells. This suggests that when cytoplasmic Na^+ levels are increased, the PY motifs of β and γ ENaC, but not α ENaC are required to bind to a negative regulatory protein, presumably Nedd4. The carboxyl termini of β and γ ENaC were also shown to have an important role in positive regulation of ENaC function when Na^+ import is required by the cell. The carboxyl termini of β and γ ENaC therefore appear to be important sites for interaction with protein(s) that both positively and negatively influence channel activity.

Statement

This thesis contains no material which has been accepted for any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge contains no material previously published or written by another person, except where due reference has been made. I give consent to my thesis, when in the University Library, being available for loan and photocopying.

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Publications

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

Harvey, K. F., Harvey, N. L., Michael, J. M., Parasivam, G., Waterhouse, N., Alnemri, E.S., Watters, D., and Kumar, S. (1998). Caspase-mediated cleavage of the ubiquitin-protein ligase Nedd4 during apoptosis. J Biol Chem *273*(22): 13524-30.

Dinudom, A., **Harvey, K. F.**, Komwatana, P., Young, J. A., Kumar, S., and Cook, D. I. (1998). "Nedd4 mediates control of an epithelial Na+ channel in salivary duct cells by cytosolic Na+." Proc Natl Acad Sci U S A *95*(12): 7169-73.

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Harvey, K. F., and Kumar, S. (1999). Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. Trends Cell Biol *9*(5): 166-9.

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Chapter 1: Introduction

1.1 Background

This thesis describes studies carried out to functionally characterise the novel murine protein, Nedd4. The Nedd4 protein is 120kDa in size and has three types of conserved protein domains; the C2 domain which is a Ca^{2+}-dependent lipid binding domain, three WW protein-protein interaction domains and a hect ubiquitin-protein ligase domain. When this project commenced, initial expression analysis of Nedd4 had been performed but the physiological function of Nedd4 was obscure. Preliminary characterisation of the *Saccharomyces cerevisiae* homologue of Nedd4, Rsp5/Npi1, had begun and this work, coupled with the homology that Nedd4 shared with proteins such as E6-associated protein (E6-AP), suggested that Nedd4 was a ubiquitin-protein ligase. The proteins that Nedd4 targeted for ubiquitination had not been discovered however, and hence its cellular role was unknown.

Nedd4 was originally identified as a developmentally regulated mouse gene highly expressed in early embryonic central nervous system (Kumar *et al.*, 1992). Further analysis revealed that the expression of *Nedd4* is not restricted to the embryonic central nervous system and that it is expressed at varying levels in different embryonic tissues (Kumar *et al.*, 1997). *Nedd4* is expressed widely in adult tissues and cell lines and localisation studies showed that Nedd4 protein is expressed throughout the cytoplasm but not in the nucleus (Kumar *et al.*, 1997). The estimated size of the protein encoded by the murine *Nedd4* gene is 103 kDa but migrates in polyacrylamide gels at a size of 120 kDa (Kumar *et al.*, 1997).

1.2 Structure of the Nedd4 protein

1.2.1 C2 domain

The C2 domain was originally identified in protein kinase C and was subsequently named protein kinase C domain 2 (C2 domain) (Coussens *et al.*, 1986; Knopf *et al.*, 1986). It is approximately 130 amino acids long and is responsible for binding to phospholipid

membranes in response to Ca^{2+}. The C2 domain is found in a number of proteins involved in signalling (eg. phosphatidylinositol 3-kinases, Phospholipases and GTPases) and membrane traffic (eg. synaptotagmins). The C2 domain in signalling proteins such as protein kinase C is thought to mediate relocalisation to membranous areas of the cell upon changes in Ca^{2+} concentration. Synaptotagmin is a synaptic vesicle protein that is believed to play a role in the translocation of synaptic vesicles to the plasma membrane (Elferink *et al.*, 1993). It is also believed to be involved in the fusion of synaptic vesicles with the plasma membrane, a process which is required for neurotransmitter release (Elferink *et al.*, 1993). Synaptotagmin contains an amino terminal intravesicular domain, a transmembrane domain which anchors it to the synaptic vesicle and a cytoplasmic domain containing two C2 domain repeats (Elferink *et al.*, 1993). The first C2 domain of synaptotagmin 1 is responsible for the cooperative binding of calcium ions and negatively charged membrane phospholipids (Popov and Poo, 1993). The C2 domain shows no sequence homology to other calcium binding proteins and its structure, as defined by X-ray crystallography, is distinct from any other calcium binding motif (Sutton *et al.*, 1995). The crystal structure of the C2 domain of synaptotagmin 1 has been shown to be comprised of a compact 8-stranded sandwich surrounding a conserved 60-residue core, which has been labelled the C2 key (Sutton *et al.*, 1995). Calcium binds to the C2 domain in a cup-shaped depression between two polypeptide loops located at the amino and carboxyl termini of the C2 key (Sutton *et al.*, 1995). The C2 domain of Nedd4 shows highest homology to the C2 domain of synaptotagmin and can mediate relocalisation of Nedd4 from the cytoplasm to the extracellular membrane in a calcium-dependent manner (Plant *et al.*, 1997). The C2 domain of Nedd4 may also be responsible for relocating Nedd4 to other membranous regions of the cell such as the nuclear membrane or membrane-bound organelles such as mitochondria. The C2 domain has also been shown to mediate protein-protein interactions. The C2 domain of the Ras GTPase activating protein, P120GAP, can interact with several

proteins *in vitro* (Davis *et al.*, 1996), whilst the C2 domain of murine Nedd4 has recently been shown to interact with the Grb10 protein (Morrione *et al.*, 1999).

1.2.2 WW domains

The WW domain is a compact protein module that mediates protein-protein interactions. It was originally identified as a 38 amino acid-long repeat sequence in the YES-associated protein kinase (YAP) (Bork and Sudol, 1994). The WW domain is characterised by the presence of two conserved tryptohan (W) residues and a conserved proline (P) residue. It was independently identified by two other groups who named it the WWP (Andre and Springael, 1994) or RSP5 domain (Hofmann and Bucher, 1995) (as there are 3 copies in the RSP5 protein). WW domains are found in a large number of unrelated signalling, structural and regulatory proteins, often in multiple copies (Figure 1). Interestingly a proportion of WW domain-containing proteins appear to be implicated in genetic diseases such as Muscular Dystrophy, Alzheimer's Disease and Liddle's Syndrome (Sudol, 1996).

The solution structure of the WW domain of human YAP, in complex with a peptide containing a PY motif-containing peptide, was determined using nuclear magnetic resonance (NMR) (Macias *et al.*, 1996). The domain was shown to have a slightly curved, three-stranded, antiparallel β-sheet. The convex side of the sheet has a hydrophobic buckle that is caused by two Pro residues packing against a Trp residue. The concave side of the WW domain has three exposed hydrophobic residues (Tyr, Trp and Leu) which form the ligand binding pocket of the WW domain (Macias *et al.*, 1996) (Figure 2). The WW domain is one of the smallest known protein-protein interaction domains at 38 amino acids, in contrast to other well characterised protein binding modules such as the SH3 domain which is approximately 120 amino acids long.

WW domains are classed in five groups based on ligand specificity. Group I WW

Figure 1. Modular structure of selected WW domain-containing proteins.

WW domains are found in a number of functionally distinct proteins from a variety of species. Symbols and abbreviations used are as follows: black box with white W - WW domain; C2 - calcium-lipid binding domain; Hect - ubiquitin-protein ligase domain; PPIase - peptidyl-prolyl isomerase domain; A - actin binding domain; Cys - cysteine-rich region; TM - transmembrane domain; PTB - phosphotyrosine binding region; Y - domain of unknown function found in yeast and nematode; PH - pleckstrin homology domain; BCR - breakpoint cluster region; Mp - putative protein module sharing homology with a protein region in the fly protein, Mp20. Proteins mentioned above are described in the following references: mouse Nedd4 (Kumar *et al.*, 1992), rat Nedd4 (Staub *et al.*, 1996), human Nedd4 (Kumar *et al.*, 1992), mouse YAP (Sudol *et al.*, 1994), chicken YAP (Sudol *et al.*, 1994), human YAP (Sudol *et al.*, 1994), human pin1 (Lu *et al.*, 1996), *Saccharomyces cerevisiae* ess1 (Hanes *et al.*, 1989), *Drosophila melanogaster* dodo (Maleszka *et al.*, 1997), human Dystrophin (Genbank accession no. ABO37493), rat Dystrophin (Tinsley *et al.*, 1993), mouse Dystrophin (Koenig *et al.*, 1987), chicken Dystrophin (Genbank accession no. AF082741), ray Dystrophin (Yeadon *et al.*, 1991), mouse CD45AP (Takeda *et al.*, 1994), human CD45AP (Takeda *et al.*, 1994), human FE65 (Bressler *et al.*, 1996), *C.elegans* 38D4, human ORF1.

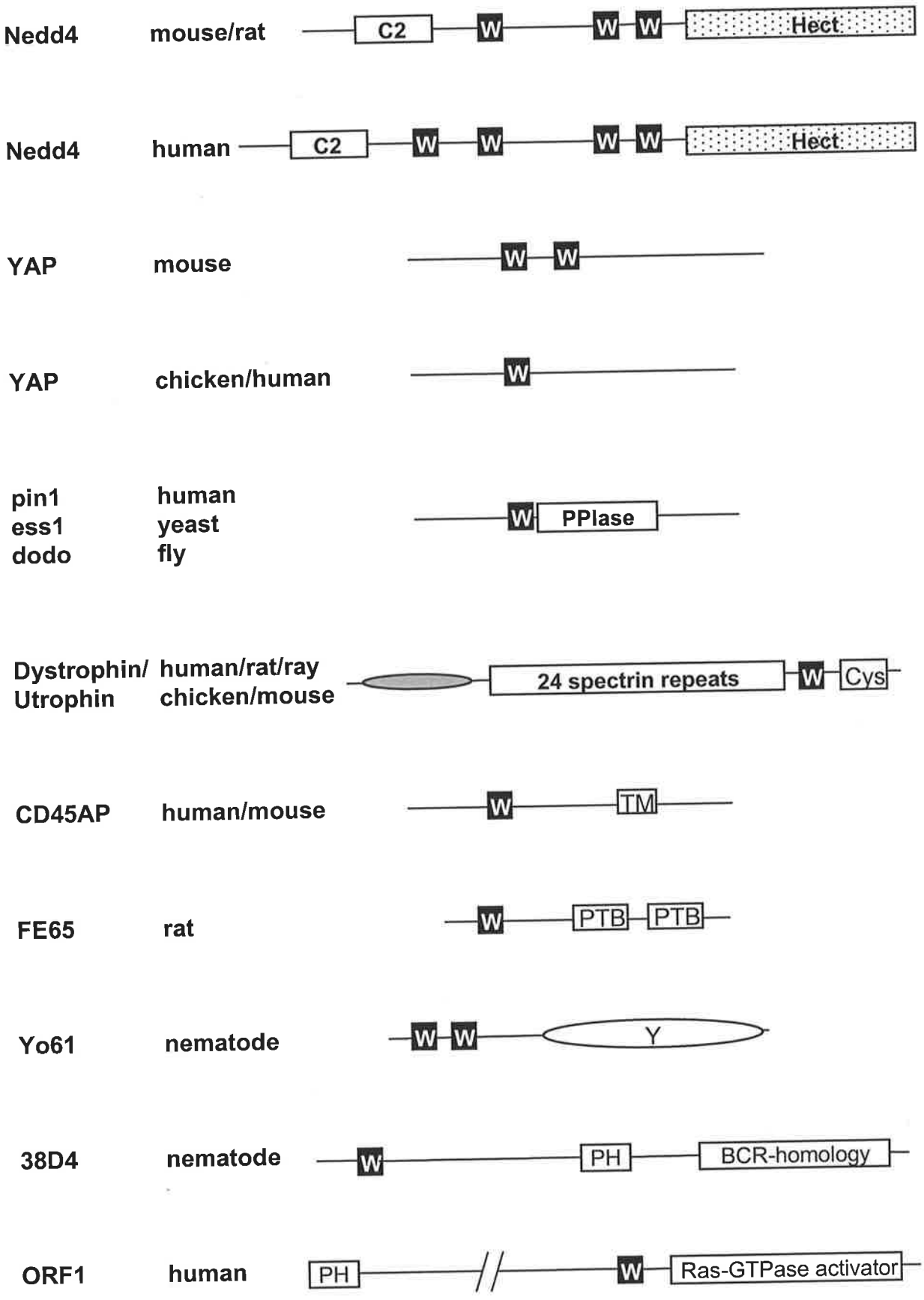

Nedd4
mouse/rat
C2
W
W
W
Hect
Nedd4
human
C2
W
W
W
W
Hect
YAP
mouse
W
W
YAP
chicken/human
W
pin1
ess1
dodo
human
yeast
fly
W
PPIase
Dystrophin/
Utrophin
human/rat/ray
chicken/mouse
24 spectrin repeats
W
Cys
CD45AP
human/mouse
W
TM
FE65
rat
W
PTB
PTB
Yo61
nematode
W
W
Y
38D4
nematode
W
PH
BCR-homology
ORF1
human
PH
W
Ras-GTPase activator

Figure 2. Key structural residues of the WW domain.

The structure of the WW domain of human YAP in complex with its ligand was elucidated by Macias *et al.*, (1997). Indication of the residues involved in ligand binding, well-defined turns and the formation of β-sheets are indicated in the legend. The corresponding residues in WW domain1, 2 and 3 of murine Nedd4 are also shown.


β1
β2
β3
hYAP WW VPLPAGWEMAKTSSGQRYFLNHIDQTTTWQDPRKA
β1
β2
β3
mNedd4 WW1 SPLPPGWEERQDVLGRTYYVNHESRRTQWKRPSPD
β1
β2
β3
mNedd4 WW2 SGLPPGWEEKQDDRGRSYYVDHNSKTTTWSKPTMQ
β1
β2
β3
mNedd4 WW3 GPLPPGWEERTHTDGRVFFINHNIKKTQWEDPRLQ

domains interact with the peptide sequence Pro, Pro, any amino acid, Tyr (PPxY), known as the PY motif. This was the first identified WW domain binding sequence and appears to be the most common binding motif for WW domains to date. The PY motif was discovered by Chen and Sudol (1995), who screened a protein expression library with radioactively labelled YAP WW domain and identified two unrelated binding partners. The only homology these proteins shared was a short stretch of amino acids: PPPPY. They subsequently showed that this sequence was necessary for interaction with the WW domain of YAP and in particular showed that the second and third Pro residues and the Tyr residue were absolutely required (Chen and Sudol, 1995). Group II WW domains, such as those found in formin-binding protein (FBP) 11 and FE65 bind to a long string of Pro residues often interrupted by a Leu residue, known as the PPLP motif (Chan *et al.*, 1996). Group II WW domains differ from group I WW domains by having three, instead of two hydrophobic residues in the second β-sheet of the domain. The importance of this additional hydrophobic residue was demonstrated by Espanel *et al.* (1999), who showed that the substrate specificity of a group I WW domain could be converted to that of a group II WW domain by introduction of a third hydrophobic residue into the second β-sheet. Group III WW domains bind to a Pro-rich sequence containing Gly and Met residues, known as the PMG motif. WW domains belonging to this class include FBP21 WW domains 1 and 2 (Bedford *et al.*, 1998). The WW domain of the nuclear protein, Npw38, appears to represent a fourth class of WW domain as it prefers a Pro-rich sequence containing Gly and Arg residues (Komuro *et al.*, 1999). Recently it has been discovered that some WW domains can bind to both phosphoserine and phosphothreonine residues (Lu *et al.*, 1999). The WW domain of the mitotic prolyl isomerase Pin1, as well as the WW domains of Nedd4, were shown to bind phosphoserine and phosphothreonine residues of several proteins at specific times of the cell cycle (Lu *et al.*, 1999). WW domains that bind phosphorylated Ser and Thr residues may constitute a fourth group of

WW domain that may overlap in binding specificity with group I, II or III WW domains. The frequency of PY, PPLP and PMG motifs is much lower than that of single Ser and Thr residues. Therefore the findings of Lu *et al.* (1999), have significant implications as they greatly increase the potential targets of WW domain-containing proteins.

1.2.3 Hect domain

The hect domain is a large protein module consisting of approximately 350 amino acids, that was first characterised in the E6-associated protein (E6-AP) (Huibregtse *et al.*, 1995). E6-AP was identified based on its ability to form stable complexes with the tumour suppressor protein p53 and the human papilloma virus (HPV) oncoprotein, E6 (Huibregtse *et al.*, 1991). The E6/E6-AP complex was subsequently shown to possess ubiquitin-protein ligase activity and stimulate ubiquitin-mediated degradation of p53 (Scheffner *et al.*, 1993). The carboxyl terminal region of E6-AP is highly homologous to a number of proteins from different species and hence this protein region was termed the hect domain (for homology to the E6-AP carboxyl terminus) (Huibregtse *et al.*, 1995). All of the hect domain proteins identified to date are large proteins (731-4836 amino acids) and in each case the hect domain resides at the carboxyl terminus (Zhu *et al.*, 1999; Lehman *et al.*, 1998). There are 5 hect domain proteins in the yeast *S. cerevisiae* and at least 20 human hect domain proteins in the Genbank database to date. The hect domain contains an absolutely conserved Cys residue towards the carboxyl end of the domain that is required for its catalytic activity (Huibregtse *et al.*, 1995). This Cys residue is required for formation of thioesters with ubiquitin, a process which is critical for protein ubiquitination (Scheffner *et al.*, 1995).

1.3 The ubiquitin system

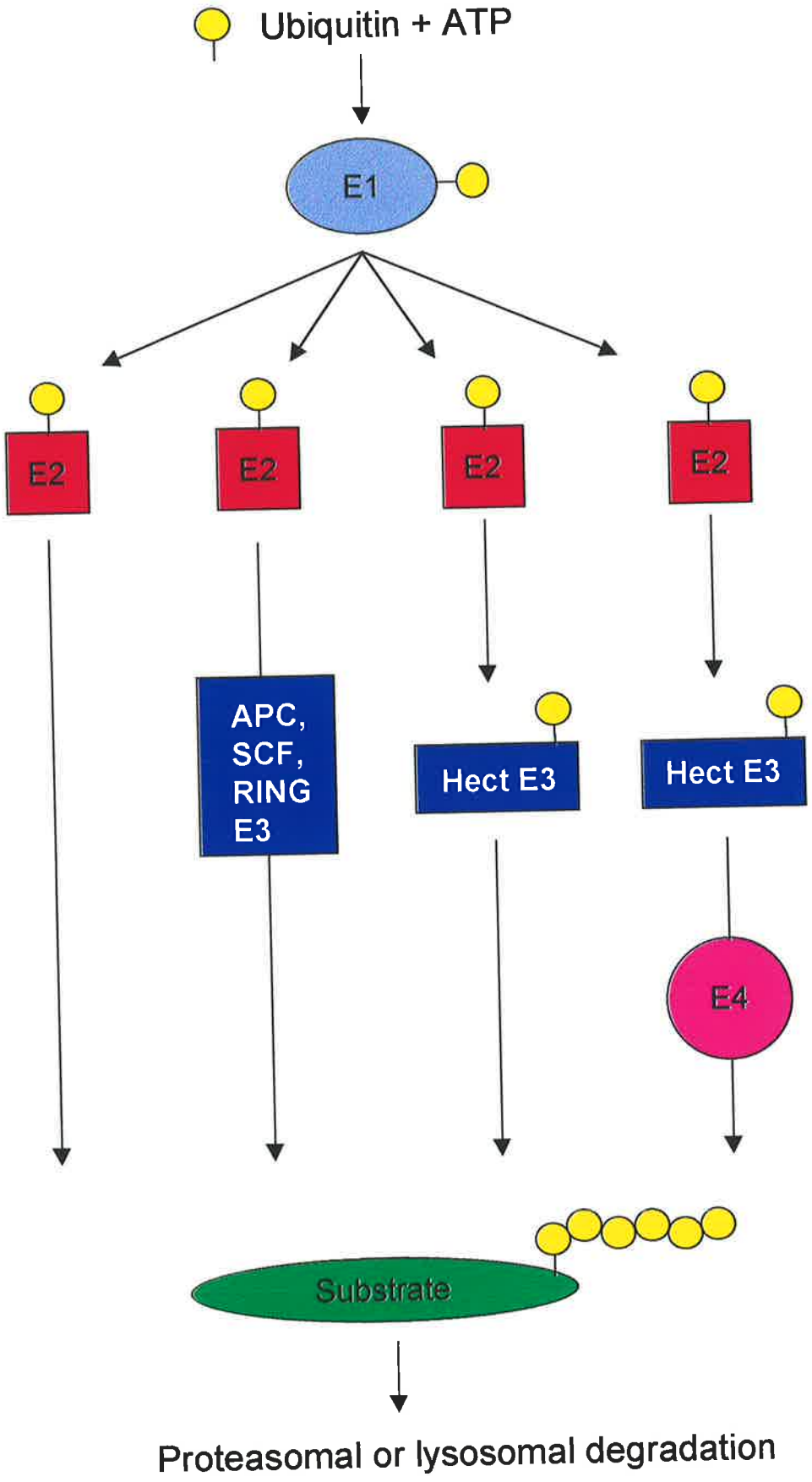
Ubiquitin is an evolutionarily conserved 76 amino acid protein that is expressed in

all eukaryotic cells. The covalent linkage of ubiquitin to protein substrates is one of the major cellular post-translational mechanisms (Hochstrasser, 1996). Ubiquitination is used to remove damaged or abnormal proteins and to regulate the stability of a wide number of proteins involved in diverse functions. Several transcription factors, cell cycle regulators, tumour suppressors, cytokine receptors and ion channels are known to be selectively ubiquitinated (Jentsch, 1992). The majority of cytoplasmic and nuclear proteins that are ubiquitinated are degraded by the 26S proteasome (Jentsch, 1992). This is a multicatalytic protein complex which processes ubiquitinated proteins into small peptides and recycles ubiquitin. In contrast, ubiquitinated transmembrane proteins are often endocytosed and degraded by the endosomal pathway or recycled to the membrane (Hicke, 1999).

The conjugation of ubiquitin to a protein substrate is a complex process involving a number of enzymes. Initially, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent fashion (Haas *et al.*, 1982). Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which either directly ubiquitinates a substrate protein or passes ubiquitin onto a ubiquitin-protein ligase (E3) (Jentsch, 1992) (Figure 3). E3s recognise their substrates and catalyse the formation of an isopeptide bond between the carboxyl terminal Gly residue of ubiquitin and an internal Lys residue of the substrate protein (Jentsch, 1992). Ubiquitin itself can be a substrate for ubiquitination and often large ubiquitin conjugates are formed on a substrate protein. These conjugates are linked through specific Lys residues of ubiquitin; mainly Lys 48, but also through Lys 63 and Lys 29 (Jentsch, 1992). It is these ubiquitin conjugates that serve to signal proteins for degradation. Recently an enzyme responsible for efficient multiubiquitination of proteins was discovered and termed E4 (Koegl *et al.*, 1999). This enzyme binds to the ubiquitin moiety of preformed ubiquitin-protein conjugates and, in concert with E1, E2 and E3, catalyzes polyubiquitination.

Figure 3. The ubiquitin pathway.

Initially, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent fashion. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which either directly ubiquitinates a substrate protein, with or without the aid of docking ubiquitin-protein ligases (E3) such as the anaphase-promoting complex (APC), Skp1-cullin-F box-protein complex (SCF) or the RING family of proteins. Alternatively, ubiquitin is passed onto an E3 that is capable of forming a thioester with ubiquitin (eg. the hect family of E3s), which then ubiquitinates its substrates. E4 enzymes may also be involved in catalysis of polyubiquitin chains on substrates. Ubiquitinated proteins are degraded by the proteasome or the lysosome and ubiquitin is recycled.


Ubiquitin + ATP
E1
E2
E2
E2
E2
APC,
SCF,
RING
E3
Hect E3
Hect E3
E4
Substrate
Proteasomal or lysosomal degradation

1.3.1 Ubiquitin-activating enzymes (E1s)

E1s have been cloned from a number of species and range in size from 110 to 126 kDa. They have 5 conserved Cys residues, one of which is necessary for conjugation to ubiquitin, and hence enzymatic action (McGrath *et al.*, 1991). In *S. cerevisiae* there are three known E1 proteins, at least two of which are essential for cell viability (Jentsch, 1992). In humans the cloning of at least two E1 enzymes has been reported. The importance of mammalian E1 has been demonstrated by several groups, using the mouse cell line ts85 which has a temperature sensitive E1 protein (Finley *et al.*, 1984). At the non-permissive temperature these cells have defects in the degradation of short-lived proteins (Ciechanover *et al.*, 1984), lysosomal protein degradation (Gropper *et al.*, 1991), DNA replication and cell cycle progression (Mori *et al.*, 1993) and UV resistance and mutagenesis (Ikehata *et al.*, 1997). The first human E1 was cloned by Handley *et al.* (1991), who showed that it had 70% similarity to *S. cerevisiae* E1 and exhibited ubiquitous tissue expression. The mouse homologue of this protein was shown to play a role in nuclear DNA replication and spermatogenesis (Kay *et al.*, 1991; Mitchell *et al.*, 1991). A second human E1 has been discovered, but as is the case in yeast, there are likely to be relatively few mammalian E1 proteins (Kok *et al.*, 1993).

1.3.2 Ubiquitin-conjugating enzymes (E2s)

In contrast to E1, there are many members of the E2 enzyme family. In *S. cerevisiae* there are 12 E2 proteins and there are at least 17 E2s in humans (Hochstrasser, 1996). E2s possess a conserved domain (Ubc domain) which is approximately 130 amino acids long and contains the active site cysteine residue that forms a thioester with ubiquitin (Jentsch, 1992). E2s belong to at least 3 classes. Class I E2s are small proteins containing minimal amino acid sequence outside the Ubc domain. This class of E2s can not ubiquitinate proteins in vitro without the assistance of E3s, which may be important for

substrate recognition (Jentsch, 1992). Class II and III E2s are larger proteins, possessing the Ubc domain and either carboxyl or amino terminal extensions respectively (Jentsch, 1992). These extra protein sequences may allow these classes of E2s to recognise substrates independent of E3s. In yeast, E2s are involved in a variety of functions (Table 1) and their importance is underlined by the various defects that arise when they are mutated individually. Mutations in E2s have been shown to affect cell cycle progression, DNA repair, transcription and the stress response (Jentsch, 1992). Often, multiple defects are caused by mutation of just one E2, suggesting that each E2 may target a number of proteins for ubiquitination. The physiological function of only a few mammalian E2s has been investigated. Two human homologues of Rad6, HHR6A and HHR6B have been cloned and were shown to be capable of complementing the DNA repair and mutagenesis functions of Rad6 in yeast (Koken *et al.*, 1991). Gene inactivation of the mouse homologue of HHR6B showed that this gene is required for spermatogenesis as male mice lacking this gene were sterile (Roest *et al.*, 1996). The human homologue of cdc34 was cloned based on its ability to complement a yeast G2 phase checkpoint mutation (Plon *et al.*, 1993). Subsequently both human cdc34 and HHR6B have been shown to target repressors of cAMP-induced transcription for proteolysis (Pati *et al.*, 1999), and human cdc34 has been implicated in the degradation of IκBα (Gonen *et al.*, 1999).

1.3.3 Ubiquitin-protein ligases (E3s)

E3s are generally thought to be most important for imparting substrate specificity to the ubiquitin pathway. E3s can be divided into two groups based on their mode of action. One group of E3s appears to act as docking proteins that bind to both a substrate and an E2, thereby allowing the E2 to ubiquitinate a substrate protein. E3s of this class include the Skp1-cullin-F box-protein (SCF) complex and the cyclosome, or anaphase promoting complex (APC). Both the APC and SCF complexes are comprised of multiple

Table 1. Functions of selected ubiquitin conjugating enzymes (E2).

Protein names, species and physiological functions and processes that each E2 are involved in are indicated. References for the discovery of the above proteins are as follows: *S. cerevisiae* Ubc1 (Seufert *et al.*, 1990), *S. cerevisiae* Ubc2/Rad6 (Jentsch *et al.*, 1987), Human hHR6B (Koken *et al.*, 1991), Mouse mHR6B (Roest *et al.*, 1996), *S. cerevisiae* Ubc3/cdc34 (Goebl *et al.*, 1988), Human cdc34 (Plon *et al.*, 1993), *S. cerevisiae* Ubc4 (Seufert and Jentsch, 1990), *S. cerevisiae* Ubc5 (Seufert and Jentsch, 1990), *S. cerevisiae* Ubc6/Doa2 (Sommer and Jentsch, 1993), *S. cerevisiae* Ubc7 (Chen *et al.*, 1993), *S. cerevisiae* Ubc8 (Qin *et al.*, 1991), *S. cerevisiae* Ubc9 (Seufert *et al.*, 1995), Human Ubc9 (Wang *et al.*, 1996; Yasugi and Howley, 1996), *S. cerevisiae* Ubc10/Pas2 (Wiebel and Kunau, 1992), *S. cerevisiae* Ubc11 (Townsley and Ruderman, 1998), *S. cerevisiae* Ubc12 (Liakopoulos *et al.*, 1998), *S. cerevisiae* Ubc13 (Hofmann and Pickart, 1999).

Protein	Species	Functions
Ubc1	S.cerevisiae	endocytosis of membrane proteins and required for growth from spores
Ubc2/Rad6	"	DNA repair, induced mutagenesis, sporulation
hHR6B	human	degradation of repressors of cAMP induced transcription
mHR6B	mouse	Gene inactivation causes male sterility associated with chromatin modification
Ubc3/Cdc34	S.cerevisiae	Essential for viability, G1 to S cycle progression, DNA replication and spindle pole body separation
Cdc34	human	G1 to S phase transition, degradation of repressors of cAMP induced transcription and Iκbα
Ubc4 Ubc5	S.cerevisiae "	Degradation of abnormal proteins, sporulation, resistance to stress, endocytosis of membrane proteins
Ubc6/Doa2	"	Degradation of MATα2 and membrane proteins
Ubc7	"	Cadmium resistance, Degradation of MATα2 and membrane proteins
Ubc8	"	Not essential for viability
Ubc9	"	Smt3p/Sumo1 conjugation. Essential for viability, G2 to M cycle progression, cyclin degradation
Ubc10/Pas2	"	Peroxisome biogenesis
Ubc11	"	Unknown, not essential for growth
Ubc12	"	Rub1/Nedd8 conjugation
Ubc13	"	Mutant is UV sensitive. Required for poly-ubiquitin chain formation through lysine 63 of ubiquitin.

proteins that each has a special role (Koepp *et al.*, 1999). For example, in the SCF complex, Skp1 binds to the appropriate F box protein, cullin serves to recruit the E2, cdc34 to the Skp1/F box complex, while a variety of different F box proteins contact the substrate that is to be ubiquitinated and degraded (Koepp *et al.*, 1999). There are approximately 400 F-box proteins in the Genbank database to date across many different species and it is these proteins that confer substrate specificity to the SCF complex (Koepp *et al.*, 1999). SCF and APC regulate progression through the cell cycle and targets of these complexes include cyclin-dependent kinase inhibitors, cyclins, anaphase inhibitors and spindle-associated proteins (Peters *et al.*, 1996).

Recently a new family of E3s have been described which bind to a substrate and an E2 and facilitate the transfer of ubiquitin from the E2 to the substrate. This family of proteins all contain RING finger domains which are essential for them to interact with E2s and to function as E3s (Lorick *et al.*, 1999; Joazeiro *et al.*, 1999; Moynihan *et al.*, 1999; Martinez-Noel *et al.*, 1999). The most well-defined RING finger E3 is c-Cbl which negatively regulates many receptor protein tyrosine kinase receptors by ubiquitination including the platelet-derived growth factor receptor (PDGF), epidermal growth factor receptor and colony-stimulating factor-1 receptor (Lee *et al.*, 1999; Miyake *et al.*, 1998; Levkowitz *et al.*, 1996). RING finger proteins are very abundant (several hundred in the Genbank database to date) and therefore it is likely that this family of proteins are involved in the ubiquitination of a large number of proteins.

The other class of E3s directly ubiquitinates protein substrates after forming a thioester with ubiquitin that has been transferred to it by an appropriate E2 (Hochstrasser, 1996). The first characterised E3 of this class was E3α. This protein was purified from rabbit reticulocyte lysate and is the likely homologue of the yeast protein, Ubr1 (Bartel *et al.*, 1990). The major subclass of E3s known to date are the hect domain proteins. As mentioned earlier, the prototype hect domain protein, E6-AP, was discovered based on its

ability to interact with the HPV protein E6. In HPV infected cells, E6-AP, together with E6, can form a stable complex with the tumour suppressor protein p53 (Huibregtse *et al.*, 1991). E6-AP can then function as an E3 to catalyse the transfer of ubiquitin onto p53 (Scheffner *et al.*, 1993). This acts as a degradation signal, resulting in the removal of p53 from the cell. This is advantageous for HPV as the life of the infected cell is prolonged due to the loss of the growth-suppressive and apoptosis-inducing effects normally imparted by p53. Hect domain proteins have a modular structure with the hect domain always residing at the carboxyl terminus (Huibregtse *et al.*, 1995). Generally hect domain proteins do not share significant homology at their amino termini. This portion of the protein is likely to be responsible for substrate recognition, whereas the Hect domain is required for ubiquitin ligase activity. In this way hect domain proteins are likely to recognise a different subset of proteins and impart substrate specificity to the ubiquitin pathway.

1.3.4 Additional regulators of ubiquitination

Recently another enzyme involved in protein ubiquitination was discovered. The *S.cerevisiae* protein, UFD2, was shown to be necessary for efficient multiubiquitination of proteins and was termed E4 (Koegl *et al.*, 1999). It is presently unknown whether E4 enzymes are involved in poly-ubiquitination of all or a sub-population of ubiquitinated proteins (Koegl *et al.*, 1999). The importance of UFD2 in yeast is emphasised by the finding that it is involved in stress tolerance (Koegl *et al.*, 1999). Additionally, a Dictyostelium UFD2 homologue, called NOSA, is required for fruiting body development (Pukatzki *et al.*, 1998). Currently there are 2 human homologues of UFD2, which have unknown functions.

Another group of enzymes involved in protein ubiquitination are the deubiquitinating enzymes. These enzymes are divided into two groups: ubiquitin C-

terminal hydrolases and isopeptidases, or ubiquitin specific proteases (UBPs). Deubiquitinating enzymes serve two important functions. One is to release ubiquitin conjugates from substrate proteins, thereby preventing their degradation by the 26S proteasome. The other is ubiquitin biosynthesis. UBPs can catalyse the removal of ubiquitin from various proteins. They are extremely divergent and are represented by multiple family members, with at least 16 UBPs in *S. cerevisiae* (Hochstrasser, 1996). The abundance and sequence divergence of UBPs suggest that they negatively regulate the ubiquitin-dependent degradation of many proteins (Hochstrasser, 1996). The precise role that UBPs play in *S.cerevisiae* is unclear at present as the majority of UBP mutants do not show phenotypic abnormalities (Hochstrasser, 1996). The importance of UBPs in higher organisms was underlined by Huang *et al.*, (1995), who showed that the Drosophila UBP, fat facets, was required for normal eye development in the fly. Deubiquitinating enzymes can also positively regulate protein ubiquitination by generating free ubiquitin molecules. The majority of ubiquitin is synthesised as a head to tail polymer which is subsequently cleaved into free molecules by deubiquitinating enzymes. Additionally, deubiquitinating enzymes have been implicated in the recycling of ubiquitin monomers from proteins that have been degraded by the proteasome (Papa and Hochstrasser, 1993).

1.3.5 The Proteasome

The majority of ubiquitinated nuclear and cytoplasmic proteins are degraded by the proteasome (Hochstrasser, 1996). The proteasome is a large multi-catalytic protein complex almost 2000 kDa in size, that consists of 3 major subunits (Tanaka, 1998). The central portion of the proteasome contains a 20S particle which possesses the enzymatic activity of the complex. It is barrel-shaped and contains two chymotrypsin-like, two trypsin-like and two caspase-like proteolytic activities that dismantle proteins into small peptides between 3 and 20 amino acids in size (Hochstrasser, 1996). The proteasome also

contains two 19S particles at either end of the 20S barrel which serve as regulatory and substrate specificity domains (Ciechanover and Schwartz, 1998). These particles have binding sites that recognise poly-ubiquitin chains and enzymes that strip ubiquitin from proteins before they enter the 20S barrel. They also contain six ATPases that serve to unfold proteins destined for degradation (Tanaka, 1998). Proteasomes are abundant in all cell types and are freely diffusible between the cytoplasm and the nucleus (Reits *et al.*, 1997). The importance of the proteasome is emphasised in yeast where almost all of the individual proteasome subunits are required for viability (Hochstrasser, 1996).

1.3.6 The Lysosome

The lysosome was the first identified cellular proteolytic machinery. It is formed when a membrane vesicle merges with an endosome, which contains a number of acidic proteases. The lysosome is responsible for the degradation of a number of membrane proteins that in most cases are targeted to it by ubiquitination (Hicke, 1999). The first known membrane proteins to be ubiquitinated were the platelet-derived growth factor receptor (Yarden *et al.*, 1986) and the growth hormone receptor (Leung *et al.*, 1987). Several membrane proteins that serve diverse functions are now known to be ubiquitinated. In yeast these include the general amino acid and uracil permeases and the pheromone receptors, Ste6p and Ste2p (Hicke, 1999). In mammals several ion channels including the cystic fibrosis transmembrane conductance regulator (CFTR) and the epithelial sodium channel (ENaC) are ubiquitinated as well as many signalling receptors such as the c-kit receptor and the epidermal growth factor receptor (Bonifacino and Weissman, 1998). In most cases ubiquitination of membrane proteins enables them to be endocytosed (Hicke, 1999). They can then either be recycled to the cell surface or degraded in the lysosome, or as is the case with some membrane proteins, such as CFTR, degraded in the proteasome (Ward *et al.*, 1995). Why all ubiquitinated membrane proteins

are not degraded by the proteasome is unclear at present. One possible explanation comes from the type of ubiquitin modification that occurs on many membrane proteins. To efficiently recognise ubiquitinated proteins, the proteasome requires ubiquitin chains that are at least 4 subunits in length (Deveraux *et al.*, 1994). Many membrane proteins eg. the *S. cerevisiae* a-factor receptor (Roth and Davis, 1996), and the mammalian ζ subunit T-cell receptor appear to be only di- or mono- ubiquitinated (Cenciarelli *et al.*, 1996). These short ubiquitin chains may be insufficient for recognition by the proteasome. The type of ubiquitin chain formed also appears important. The majority of ubiquitin chain formation occurs through Lys 48 of ubiquitin. Efficient endocytosis of the yeast uracil permease however requires ubiquitin chains that are joined through Lys 63 (Galan and Haguenauer Tsapis, 1997). It is also unclear how ubiquitination acts as a signal for endocytosis. Short ubiquitin chains may be recognised by the endocytosis machinery or ubiquitination of a membrane protein may alter its conformation to allow such recognition. Alternatively, it has been suggested that ubiquitination of membrane proteins may stimulate them to move to regions of the cell membrane that more readily undergo endocytosis (Hicke, 1999).

1.4 The Epithelial sodium channel

When this project commenced, the physiological function of Nedd4 was unknown. The domain structure of Nedd4 suggested that it would target a select group of proteins for ubiquitin-mediated turnover, but no potential substrates had been identified. In 1996, Staub *et al.*, identified rat Nedd4 as a protein that interacted with the carboxyl terminus of the β subunit of the rat epithelial sodium channel (ENaC). They subsequently showed that Nedd4 also interacted with the α and γ ENaC subunits and that all of these interactions were dependent on the WW domains of Nedd4 and the PY motifs of ENaC (Staub *et al.*, 1996).

ENaC plays a major role in fluid and electrolyte homeostasis and hence is an

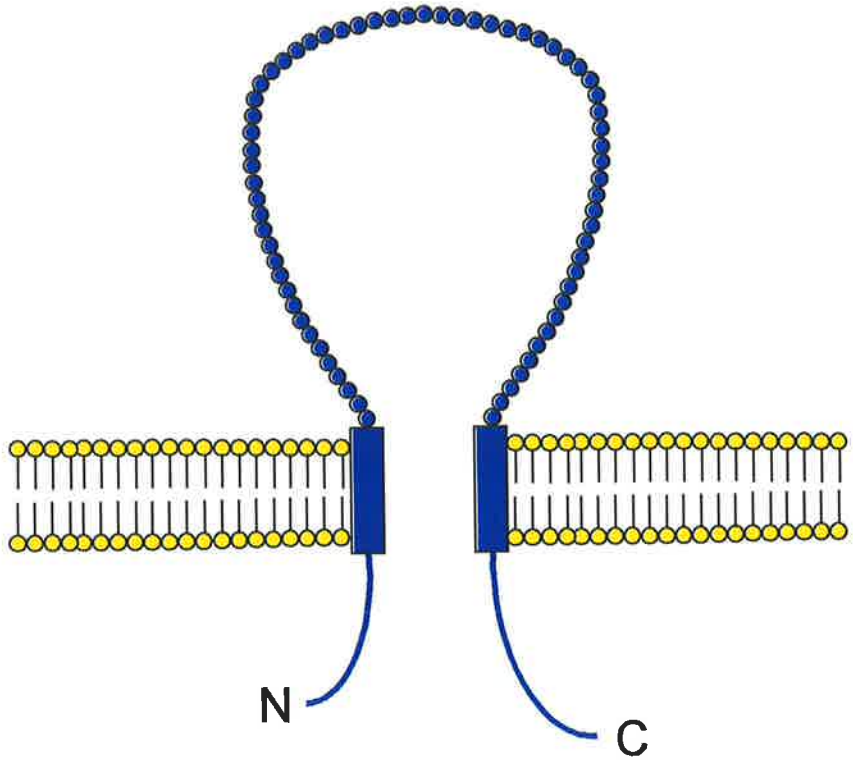
important regulator of blood pressure. ENaC is responsible for active Na^+ reabsorption in many epithelial tissues, such as the renal collecting duct, distal colon, lung and sweat and salivary ducts (Garty and Palmer, 1997). In the kidney ENaC regulates urinary Na^+ excretion which has a major role in establishing the balance of Na^+ and other electrolytes in the body. A similar role is carried out by ENaC in the distal colon where it reduces the amount of Na^+ lost in stools. ENaC plays a major role in fluid clearance in the neonatal lung and prevents salt loss by reabsorbing Na^+ in sweat and salivary ducts (Garty and Palmer, 1997). Prior to the molecular cloning of ENaC these channels were classified according to their biophysical and pharmacological properties. It was known that these channels lined the apical membranes of salt-reabsorbing epithelia and facilitated the first step of transepithelial Na^+ transport (Rossier *et al.*, 1994). They were characterised by their ability to transport Na^+ into the cell by electrodiffusion without requirement for ATP or coupled transport to other solutes (Hummler and Horisberger, 1999). Additionally they were shown to be highly selective for Na^+ over K^+ and their activity could be blocked by the K^+ sparing diuretic, amiloride (Rossier *et al.*, 1994).

1.4.1 Molecular cloning of ENaC

Functional ENaC is composed of three subunits, α, β and γ. ENaC subunits share considerable homology with each other and belong to a newly described superfamily of cation channels called DEG/ENaC. Members of this family have been discovered in the nematode *Caenorhabtidis elegans*, the fly *Drosophila melanogaster*, the snail *Helix aspersa* and also in several vertebrate species (Mano and Driscoll, 1999). DEG/ENaC channels possess a highly conserved secondary structure with each member containing two membrane spanning domains, a large extracellular loop and short intracellular amino and carboxyl termini (Barbry and Hofman, 1997) (Figure 4). DEG/ENaC proteins are implicated in many diverse functions including mechanosensation, pain sensation,

Figure 4. Schematic representation of the structure of members of the DEG/ENaC family of proteins.

Members of this family of proteins have two membrane spanning regions and a large extracellular loop, while the amino and carboxyl termini reside in the cytoplasm.



Extracellular loop

Membrane spanning domains

Cytoplasmic amino and carboxyl termini

gametogenesis and cation transport (Mano and Driscoll, 1999).

The α subunit of ENaC was first isolated in 1993 by two independent groups. Canessa *et al.* (1993), and Lingueglia *et al.*, (1993) both used a functional expression screen to isolate messenger RNA species prepared from rat colon that induced a Na^+ current when injected into *Xenopus* oocytes. Both groups isolated genes that encoded for a 698 amino acid protein that at the time only shared significant homology with two *C. elegans* proteins that were isolated in a genetic screen for touch-insensitive mutants. These proteins, DEG-1 and MEC-4, were the first identified family members of the aforementioned DEG/ENaC superfamily (Driscoll and Chalfie, 1991). The amiloride sensitive Na^+ current observed when α ENaC RNA was injected into oocytes was only small however, suggesting that either additional ENaC subunits or other interacting proteins were required for maximal ENaC activity (Canessa *et al.*, 1993). The human homologue of rat α ENaC was subsequently cloned and shown to have channel properties in *Xenopus* oocytes similar to rat α ENaC (Voilley *et al.*, 1994; McDonald *et al.*, 1994). All groups concluded that α ENaC could produce amiloride sensitive Na^+ current but probably required other subunits either for channel maturation, regulation or ion permeation.

The cloning of the β and γ subunits of rat ENaC were published later in 1994 by Canessa *et al.* They were cloned by functional complementation of the α subunit of ENaC using the *Xenopus* oocyte system. They showed that the β and γ subunits of ENaC share 34% and 37% identity with the α subunit respectively. Coinjection of either β or γ ENaC with α ENaC produced a 3-5-fold increase in channel activity over α alone. When all three subunits were expressed together a 100-fold increase in channel activity was observed and was equal in activity to native ENaC (Canessa *et al.*, 1994). The human subunits of ENaC were also cloned and shown to be 83-85% identical to their rat counterparts (McDonald *et al.*, 1995). The human channel subunits showed identical

channel gating properties in *Xenopus* oocytes as rat ENaC. Human subunits could also substitute for the corresponding rat subunits to form working Na^+ channels, suggesting conservation of function between species (McDonald *et al.*, 1995).

1.4.2 ENaC expression

Rat α ENaC is expressed as a 3.5kb message in the cortex and medulla of the kidney, the lung and the distal colon (Canessa *et al.*, 1993). Expression appears in the embryo a few days before birth and increases significantly after birth (Vehaskari *et al.*, 1998). Human α ENaC is expressed in the kidney, colon and lung, as well as the thryroid, pancreas and liver (Voilley *et al.*, 1994; McDonald *et al.*, 1994). The rat β ENaC subunit is expressed as a 2.2kb message in the cortex and medulla of the kidney, the lung and the distal colon (Canessa *et al.*, 1994). Its human counterpart is expressed in these same tissues as well as the placenta, pancreas and a small amount in skeletal muscle (McDonald *et al.*, 1995). Rat γ ENaC mRNA is 3.2kb in size and is expressed in lung, kidney and colon (Canessa *et al.*, 1994), while human γ ENaC is found in lung, kidney, colon, pancreas and brain (McDonald *et al.*, 1995). All three ENaC subunits are expressed in sweat and salivary ducts (Duc *et al.*, 1994). As expected, all three ENaC subunits are expressed in common epithelial tissues, however the reason for expression of some subunits (eg. high expression of β in the placenta, α in liver and γ in brain) is unknown.

1.4.3 ENaC stoichiometry

There is some debate about the stoichiometry of functional ENaC in the extracellular membrane. One report suggested that ENaC assembled in the membrane as a nonamer, composed of three of each ENaC subunit (Snyder *et al.*, 1998). In contrast to this, two groups independently claimed that membrane bound ENaC was a tetramer, consisting of 2 α, 1 β and 1 γ subunit (Firsov *et al.*, 1998; Kosari *et al.*, 1998). Most

recently, Eskanderi *et al.* (1999), used freeze-fracture electron microscopy to visualise channels inserted into the plasma membrane of *Xenopus* oocytes. They showed that ENaC was composed of either 8 or 9 subunits with no less than two of these being γ subunits. They also showed that the ENaC complex exhibited a near square geometry and therefore suggested that ENaC is composed of 8 subunits with a stoichiometry of 4α, 2β and 2γ subunits (Eskandari *et al.*, 1999).

1.5 Hormonal control of ENaC

Na^+ transport in renal epithelia is regulated by a number of hormones including aldosterone, insulin and vasopressin (Garty and Palmer, 1997).

1.5.1 Aldosterone

Aldosterone is a steroid hormone that increases Na^+ absorption in the cortical collecting tubule of the kidney (Barbry and Hofman, 1997). It is thought to have a dual stimulatory effect on ENaC activity. In the short term (as rapid as 30 to 45 minutes) aldosterone stimulates Na^+ channel activity by modulating the rate of transcription of specific target genes (Chen *et al.*, 1999). These gene products presumably activate pre-existing inactive membrane channels as transcription of ENaC genes is not altered by aldosterone until later (approximately 8 hours) (Champigny *et al.*, 1994). The gene targets of aldosterone in epithelial tissues have been the subject of much study. Spindler *et al.* (1997), used differential display PCR to identify genes that were upregulated by aldosterone in *Xenopus* A6 epithelial cells. Using this approach they identified the small G protein K-Ras2A, and suggested that K-Ras2A increased the activity of ENaCs expressed at the cell surface (Spindler *et al.*, 1997). Subsequently, K-Ras2A has been shown to increase Na^+ absorption 2.2-fold in *Xenopus* A6 cells (Stockand *et al.*, 1999). Two groups independently identified the serum glucocorticoid kinase (SGK) as an aldosterone target in A6 cells (Chen *et al.*, 1999), and in rabbit cortical collecting duct kidney cells (Naray

Fejes Toth *et al.*, 1999). The transcription of the *sgk* gene in A6 cells increased approximately 20-fold after 60 minutes treatment with the aldosterone like molecule, dexamethasone (Chen *et al.*, 1999). SGK protein levels were also increased and when *sgk* and *ENaC* RNA were coinjected into *Xenopus* oocytes, a 7-fold increase in ENaC activity was observed (Chen *et al.*, 1999). The mode of action of SGK on ENaC is still unknown but one possibility is that SGK directly activates ENaC by phosphorylating it. In support of this hypothesis, Shimkets *et al.*, (1998), discovered that β and γ ENaC, but not α ENaC subunits were phosphorylated on Ser and Thr residues in response to aldosterone. It is also possible that SGK stimulates the activity of another protein by phosphorylation that in turn activates ENaC. One such candidate protein is the carboxymethyl transferase. The β subunit of *Xenopus* ENaC has been shown to be carboxylmethylated in response to aldosterone, a process which may stimulate ENaC activity (Rokaw *et al.*, 1998). It is unknown as yet however whether the activity of carboxymethyl transferase is stimulated by phosphorylation.

Aldosterone has been shown to affect the transcription of ENaC subunits in the longer term (around 8 hours) in a variety of tissues. For example, in cultured rat lung cells, transcription of α ENaC was increased significantly with treatment of aldosterone for up to 10 hours (Champigny *et al.*, 1994). In kidney, α ENaC is expressed at constant levels, while transcription of β and γ ENaC subunits is upregulated by aldosterone. This increase in β and γ ENaC transcription occurs after the initial increase in aldosterone-stimulated Na^+ channel activity (Barbry and Hofman, 1997).

1.5.2 Insulin

Insulin is a peptide hormone which is believed to directly stimulate ENaC activity, independent of transcription (Barbry and Hofman, 1997). It functions by binding to the Insulin receptor and stimulates signalling through the intracellular portion of the receptor.

It has been suggested that insulin-dependent activation of Na^+ channels is mediated by protein kinase C (Civan *et al.*, 1998), and by PI-3 kinase (Record *et al.*, 1998). Treatment of Madin-Darby canine kidney (MDCK) cells with insulin for 15 minutes has been shown to stimulate the phosphorylation of β and γ subunits of ENaC but it is unclear how ENaC activity is increased (Shimkets *et al.*, 1998). One report suggests that the open probability (P_o) of existing channels is increased (Marunaka *et al.*, 1992), while other reports claim that either pre-existing dormant channels are activated or that more channels are delivered to the membrane from an intracellular pool (Erlij *et al.*, 1994; Blazer Yost *et al.*, 1998). Aldosterone and insulin appear to influence Na^+ channel activity independently as they have an additive effect when added to cells in vitro (Barbry and Hofman, 1997).

1.5.3 Vasopressin

Vasopressin is a fast acting hormone that increases Na^+ channel activity in approximately 5 minutes and is synergistic to the effect of aldosterone (Garty and Palmer, 1997). Like insulin, vasopressin influences Na^+ channel activity without the requirement of synthesis of new proteins. The activity of vasopressin is mediated through the molecule cyclic AMP (cAMP), which is produced by adenylate cyclase (Garty and Palmer, 1997). cAMP is a known second messenger and it is thought that to activate protein kinases which in turn activate channel activity by phosphorylating either a channel regulatory protein or ENaC itself (Garty and Palmer, 1997).

1.6 Non-hormonal control of ENaC

A number of reports have suggested that different molecules and solutes can directly influence Na^+ channel activity, a process known as feedback inhibition. Extracellular Na^+ and intracellular Na^+, Ca^{2+} and pH have all been implicated in the control of Na^+ channel activity in various experimental systems. By blocking the activity

of the basolateral Na^+/K^+ ATPase in rat cortical collecting tubule cells, Silver *et al.* (1993), showed that intracellular Na^+ and Ca^{2+} were increased and that this led to a 20% decrease in open Na^+ channels. The hypothesis that increased intracellular Na^+ negatively impacts on Na^+ channel activity was strengthened by Komwatana *et al.* (1996), who showed that increases in intracellular Na^+ in mouse mandibular duct cells decreased Na^+ channel activity significantly. They also showed that this Na^+-dependent feedback inhibition occurred independently of changes in Ca^{2+} concentration, pH and Cl^- (Komwatana *et al.*, 1996). They also showed that increases in intracellular Cl^- negatively impacted upon Na^+ channel activity. Kellenberger *et al.* (1998), confirmed the inhibitory effect of intracellular Na^+ on ENaC activity in the *Xenopus* oocyte system and showed that it was independent of Ca^{2+} concentration.

1.6.1 G proteins

G proteins have been widely implicated in the control of ENaC. Ohara *et al.* (1993), showed that G proteins in a GTP-bound state inhibit Na^+ channel activity by demonstrating in A6 cells that GTP and GDPγS decreased Na^+ channel activity by five-fold. In the same system they showed that Na^+ channel activity was increased seven-fold by GDPβS, a molecule which deactivates G proteins by competing for GTP (Ohara *et al.*, 1993). Komwatana *et al.* (1996), showed that different classes of G proteins were responsible for mediating downregulation of Na^+ channel activity in response to increases in intracellular anions and Na^+ in mouse mandibular duct cells. They showed that ENaC activity was decreased in response to rises in intracellular Na^+ concentration and that this Na^+-dependent feedback inhibition was abolished by the addition of GDPβS or pertussis toxin (which ADP ribosylates G proteins, thus preventing their activation by receptor proteins). These data were confirmed by showing that antibodies to the α subunit of the G protein, G_o, also inhibited Na^+-dependent feedback inhibition of ENaC activity.

Additionally, they showed that Cl^- dependent inhibition of ENaC was mediated by the G protein, G_{i2}, as antibodies to this protein neutralised the inhibitory effect of raised intracellular Cl^- on Na^+ channel activity (Komwatana *et al.*, 1996). Collectively, these data suggest that G proteins are important signalling proteins involved in the downregulation of Na^+ channel activity in response to important changes in the concentration of Na^+ and Cl^-.

1.6.2 Cystic fibrosis transmembrane conductance regulator

As mentioned earlier, ENaC has a major role in fluid control in the lung. In the lung and other organs ENaC comes under control of the cystic fibrosis transmembrane regulator (CFTR), which is responsible for Cl^- secretion. Mall *et al.* (1998), showed that CFTR negatively regulates ENaC activity in the lung and colonic epithelium. In patients suffering from cystic fibrosis (CF), CFTR is mutated and lacks the ability to secrete Cl^-. CF patients also have increased Na^+ absorption in the lung which leads to fluid retention and the characteristic increase in lung mucous viscosity. Mutant CFTR can not downregulate ENaC activity in patch clamp experiments and it is thought that this is the reason why ENaC activity is increased in CF patients (Mall *et al.*, 1998). The mode of this regulation is unknown but it has been suggested that the two channel complexes physically interact (Kunzelmann *et al.*, 1997). It was also suggested that CFTR may interfere with the ubiquitin-dependent negative regulation of ENaC, but this was ruled out by Hopf *et al.* (1999), who showed that CFTR could downregulate both wildtype and mutant ENaC that lacked the ubiquitin-dependent negative regulatory domains.

1.7 ENaC subunit knockout mice

α ENaC - Mice lacking the α subunit of ENaC die shortly after birth from the inability to clear fluid from their lungs (Hummler *et al.*, 1996). ENaC has an important role in the lung epithelium just before birth when the lung undergoes transition from a

fluid-filled to an air-filled organ. In rats it has been demonstrated that α ENaC mRNA is greatly increased just before birth (Vehaskari *et al.*, 1998). The increase in ENaC stimulates the lung to switch from secreting Cl^- to reabsorbing Na^+, which assists in clearance of the pulmonary fluid.

β ENaC - Two independent groups published findings on inactivation of the β subunit of ENaC in mice. McDonald *et al.* (1999), found that β ENaC knockout mice developed during embryogenesis normally but died within 2 days of birth. Knockout mice showed increased urinary Na^+, decreased urinary K^+ and elevated serum aldosterone. Unlike mice lacking the α subunit of ENaC, fluid clearance in the lung was normal, suggesting that the β subunit is required for ENaC function in the renal collecting duct, but not in the lung (McDonald *et al.*, 1999). Pradervand *et al.* (1999), generated mice that expressed low levels of β ENaC (mRNA levels were < 4% of wildtype) when attempting to generate mice with truncated β ENaC protein. On a normal salt diet they found that these mice had decreased ENaC activity in the colon, increased serum aldosterone and a slight delay in fluid clearance. On a low salt diet, mice exhibited a severe pseudohypoaldosteroism type 1 (PHA-1) phenotype (weight loss, hyperkalemia and decreased blood pressure) (Pradervand *et al.*, 1999). They concluded that β ENaC is required during salt deprivation but not for fluid clearance.

γ ENaC - γ ENaC knockout mice also exhibit a severe PHA-1 phenotype and died 24-36 hour after birth with metabolic and electrolyte disturbances (Barker *et al.*, 1998). γ ENaC knockout mice also had a minor defect in lung fluid clearance.

1.8 Liddle's syndrome

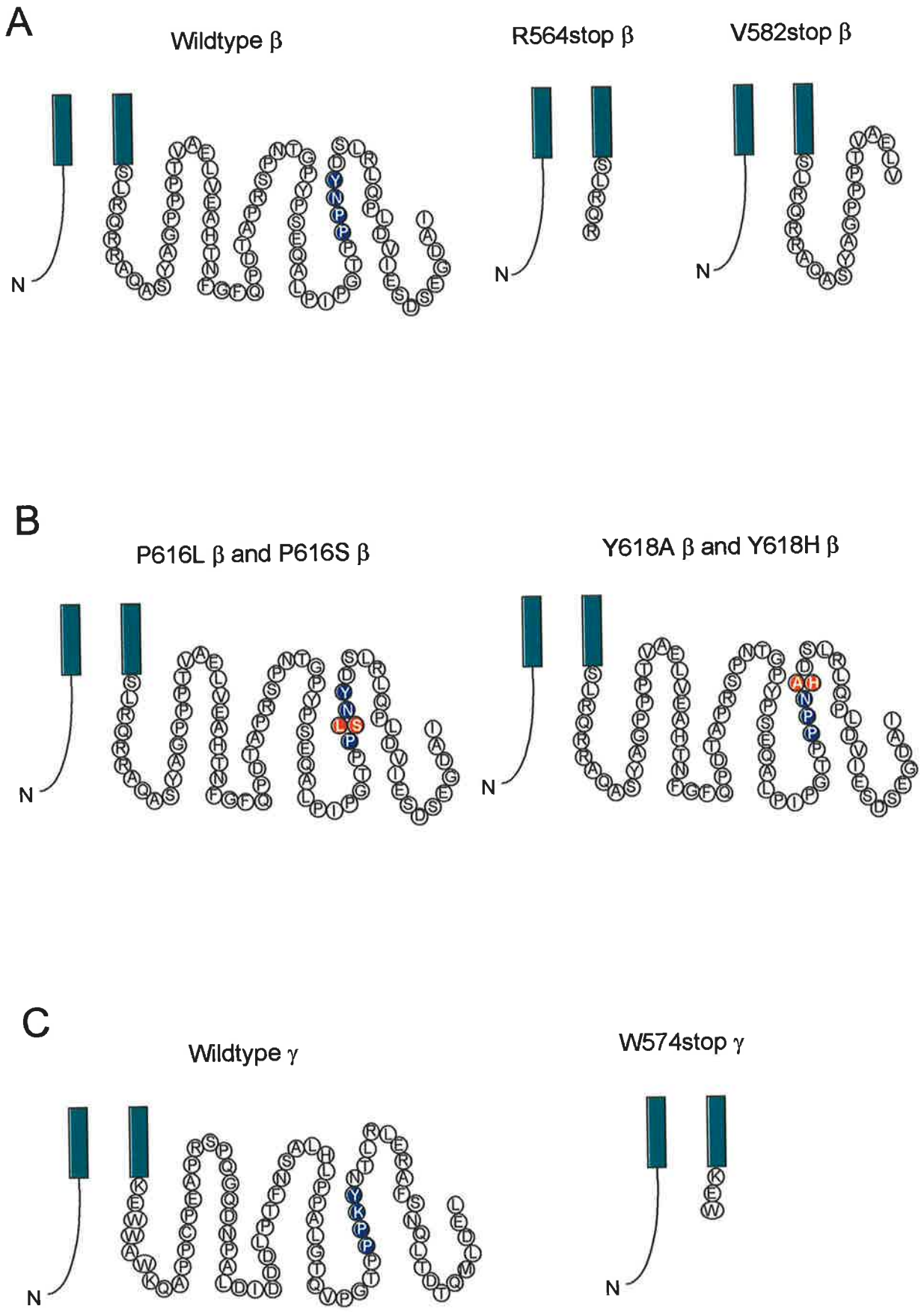
Liddle's Syndrome is an autosomal dominant disease where patients suffer from hypertension. It was first characterised in 1963 by Liddle who showed that affected patients had increased blood pressure, hypokalemia and suppressed levels of serum

aldosterone (Liddle *et al.*, 1963). Patients had a reduced life expectancy but their condition could be controlled with a low salt diet and treatment with antagonists of Na^+ absorption such as triamterine and amiloride (Botero-Velez *et al.*, 1994). Patients did not respond to antagonists of the mineralocorticoid receptor indicating that this disorder did not result from excessive levels of aldosterone (Liddle *et al.*, 1963). In 1994 the molecular cause of Liddle's syndrome was identified by Shimkets *et al.*, who showed that Liddle's syndrome patients carried mutations in the gene encoding for the β subunit of ENaC. These mutations caused truncation of the intracellular carboxyl terminus of β ENaC. In particular, a Pro-rich region of β ENaC was deleted that was hypothesised to normally bind to a negative regulatory protein (Shimkets *et al.*, 1994).

In 1995 another group of Liddle's patients was identified that carried truncations in the intracellular carboxyl terminus of the γ subunit of ENaC (Hansson *et al.*, 1995a). A Pro-rich region that showed homology to the one deleted in β ENaC was also deleted in γ ENaC. Additionally they showed that Na^+ channels containing the same truncations of β or γ ENaC as those seen in Liddle's syndrome patients were much more active than wildtype channels. Subsequently, truncation of β ENaC was shown to increase channel activity without altering its biophysical or pharmacological properties (Schild *et al.*, 1995). Snyder *et al.* (1995), identified the common consensus sequence PPPXYXXL in the carboxyl termini of all three ENaC subunits. They showed that when this motif was deleted, as in Liddle's syndrome, or altered by site-directed mutagenesis, increased ENaC activity was observed that was associated with increased cell-surface expression of ENaC (Snyder *et al.*, 1995). The importance of this motif for regulation of ENaC was emphasised by Hansson *et al.* (1995), who showed that a group of patients suffering Liddle's Syndrome had a missense mutation in the gene encoding for β ENaC that altered Pro 616 to Leu (Figure 5). *Xenopus* oocytes expressing channels containing this mutant had a markedly increased activity compared to wildtype channels (Hansson *et al.*, 1995b).

Figure 5. Mutations found in Liddle's syndrome ENaCs.

A representation of wildtype β and γ ENaCs, and of mutated ENaCs found in Liddle's syndrome patients is depicted. For wildtype and missense mutated channels the carboxyl terminal amino acids that reside in the cytoplasm are shown using the single letter amino acid code. PY motifs are shown in white, with a blue background and missense mutations are shown in white, with an orange background. Amino acids in truncated channels are shown up to the point of the truncation.


A
Wildtype β
R564stop β
V582stop β
N
N
N
B
P616L β and P616S β
Y618A β and Y618H β
N
N
C
Wildtype γ
W574stop γ
N
N

Pro 616 of β ENaC corresponds to the third Pro in the conserved PPPXYXXL motif identified by Snyder *et al.* (1995). This motif is a consensus binding site for WW domains known as the PY motif. This suggested that a WW domain-containing protein normally negatively regulates ENaC, and that this process is facilitated by binding of this protein to the carboxyl terminal PY motifs of one or more ENaC subunit. Although ENaC containing Liddle's syndrome mutations have been widely reported as having increased activity, the exact mechanism for this increase is unclear. Some groups have reported an increased half-life of channels at the surface without a change in P_o, while other groups suggest an increase in both surface expression and P_o.

1.9 ENaC and ubiquitination

More recently, the α and γ subunits of ENaC were shown to be ubiquitinated on several Lys residues in their cytoplasmic tails (Staub *et al.*, 1997). Mutation of some of these Lys residues increased the half-life of these proteins and also increased Na^+ channel activity, demonstrating that ubiquitination normally negatively regulates ENaC function (Staub *et al.*, 1997). These findings, coupled with the findings of Staub *et al.* (1996), suggested that Nedd4 and ENaC may interact and that Nedd4 might actively ubiquitinate ENaC. A direct role for Nedd4 in ubiquitin-mediated control of ENaC was not yet established however. We were the first to demonstrate that Nedd4 negatively regulates ENaC in response to increases in intracellular Na^+, and does so in a ubiquitin-dependent fashion (Chapter 4 of this thesis; Dinudom *et al.*, 1998). Additionally we showed that Nedd4-mediated regulation of ENaC functions downstream of the G protein, G_o (Chapter 4 of this thesis; Dinudom *et al.*, 1998). These results were in part confirmed by Goulet *et al.* (1998), who showed that Nedd4 could downregulate ENaC activity in the *Xenopus* oocyte system, a phenomenon that was dependent on the conserved Cys residue in the Hect domain of Nedd4. Subsequently, we showed that all three WW domains of murine

Nedd4 are required for Na^+-dependent regulation of ENaC, despite the fact that *in vitro*, only WW domains 2 and 3 can bind ENaC subunits (Chapter 5 of this thesis; Harvey *et al.*, 1999). As mentioned previously, ubiquitination of membrane proteins often causes them to be endocytosed and degraded in the lysosome. Support for this process applying to ENaC came from Shimkets *et al.* (1997), who used an inhibitor of clathrin-mediated endocytosis to show that this process is required for negative regulation of ENaC. Staub *et al.* (1997), also showed that the half-life of ENaC was increased by an inhibitor of lysosomal protein degradation. Together these data suggest that ENaC is downregulated by Nedd4-dependent ubiquitination, which is a signal for ENaC to be endocytosed and degraded in the lysosome.

1.10 Nedd4-like proteins

Recently, a number of proteins have been discovered that share the same modular structure as Nedd4 and appear to be part of a family of ubiquitin-protein ligases (Figure 6) (Harvey and Kumar, 1999). These proteins contain a C2 domain located towards the amino terminus, 2-4 copies of the WW domain and a hect domain. All the WW domains present in Nedd4-like proteins appear to belong to the group I subclass, making it likely that these proteins will contact substrates containing PY motifs and possibly phosphorylated Ser and Thr residues. WW domains from the same Nedd4-like protein possess differential substrate specificity *in vitro* (Sudol, 1996), making it is plausible that each of the Nedd4-like proteins interacts with a number of different proteins via their WW domains *in vivo*. Although little is known about the physiological roles of these proteins, studies in both mammals and yeast are providing evidence that members of this family might be involved in diverse cellular functions such as regulation of membrane channels and permeases, the cell cycle and transcription (Table 2).

Figure 6. The Nedd4 family of proteins.

Members of this family are characterised by a C2 domain (black boxes) located at the amino terminus, multiple WW domains located in the middle part of the protein (white boxes), and a ubiquitin-protein ligase domain (grey boxes) at the carboxyl terminus. Some family members are predicted proteins derived from the Genbank database (accession numbers are indicated). The sequence of WWP1/AIP5 is incomplete at both ends, while the sequence of AIP4 is incomplete at the amino terminus. Proteins mentioned above are described in the following references: Human Nedd4 (Genbank accession no. KIAA0093), Human KIAA0439 (Genbank accession no. KIAA0439), Human WWP2/AIP2 (Pirozzi *et al.*, 1997; Wood *et al.*, 1998), Human WWP1/AIP5 (Pirozzi *et al.*, 1997; Wood *et al.*, 1998), Human AIP4 (Wood *et al.*, 1998), Human Smurf1 (Genbank accession no. ACOO4893), Human KIAA0322 (Genbank accession no. KIAA0322), Mouse Nedd4 (Kumar *et al.*, 1992), Mouse Itch (Perry *et al.*, 1998), Rat Nedd4 (Staub *et al.*, 1996), *Xenopus* AJ000085 Nedd4 (Genbank accession no. AJ000085), *Xenopus* Smurf1 (Zhu *et al.*, 1999), *Drosophila* Suppressor of Deltex [Su(Dx)] (Cornell *et al.*, 1999), *Drosophila* AF216521 (Genbank accession no. AF216521), *Drosophila* Nedd4 (Genbank accession no. AI533013), *S. cerevisiae* Rsp5p/Npi1p (Hein *et al.*, 1995), *S. pombe* Pub1p (Nefsky and Beach, 1996), *S. pombe* Z99579 (Genbank accession no. Z99579).

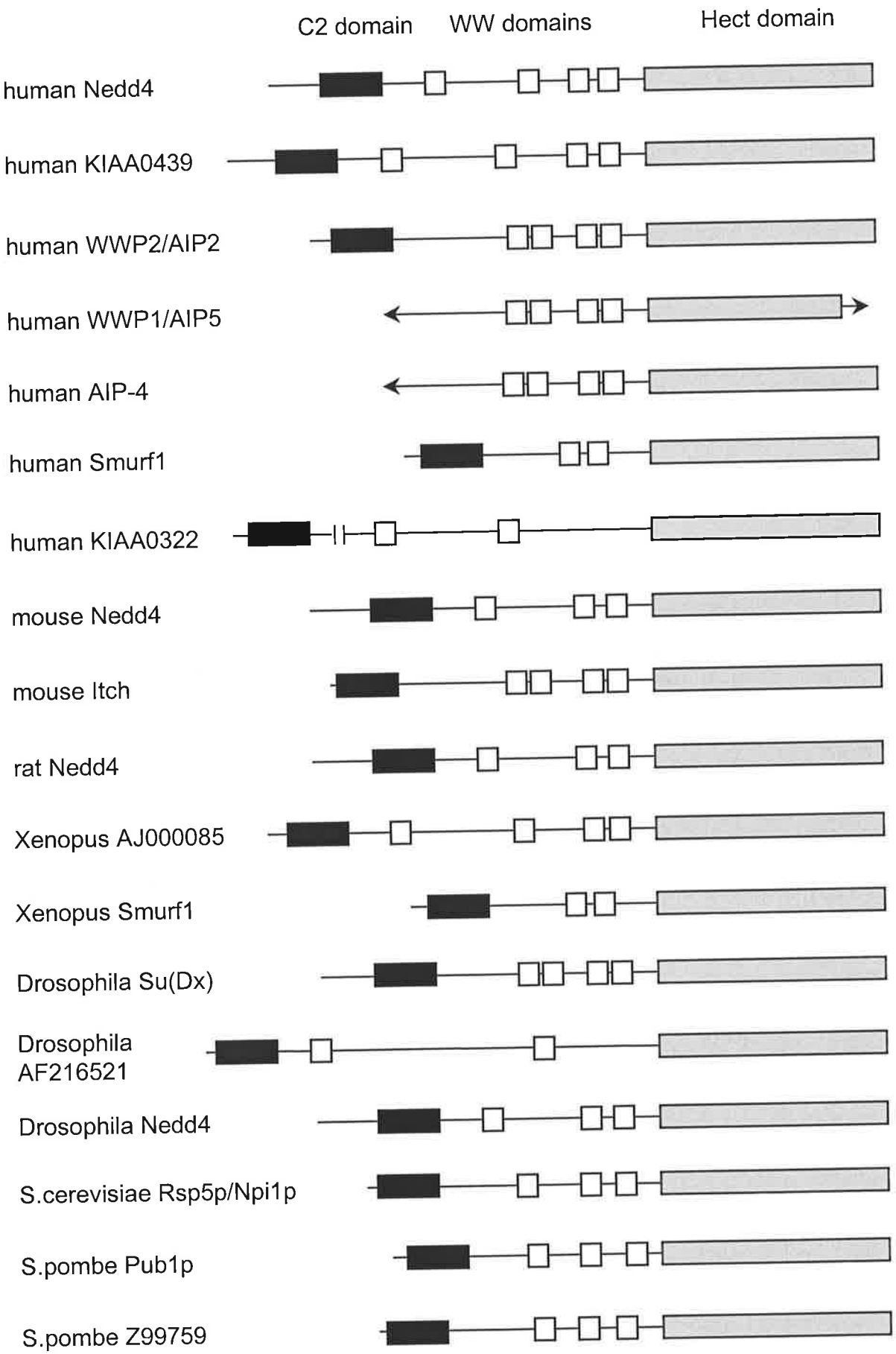

C2 domain
WW domains
Hect domain
human Nedd4
human KIAA0439
human WWP2/AIP2
human WWP1/AIP5
human AIP-4
human Smurf1
human KIAA0322
mouse Nedd4
mouse Itch
rat Nedd4
Xenopus AJ000085
Xenopus Smurf1
Drosophila Su(Dx)
Drosophila AF216521
Drosophila Nedd4
S.cerevisiae Rsp5p/Npi1p
S.pombe Pub1p
S.pombe Z99759

Table 2. Known function of some Nedd4 family members.

Protein names, biochemical activity and physiological functions are indicated. References for the discovery of the above proteins are as follows: Mouse Nedd4 (Kumar *et al.*, 1992), Mouse Itch (Perry *et al.*, 1998), *Xenopus* Smurf1 (Zhu *et al.*, 1999), *Drosophila* Su(Dx) (Cornell *et al.*, 1999), *S. cerevisiae* Rsp5p/Npi1p (Hein *et al.*, 1995), *S. pombe* Pub1p (Nefsky and Beach, 1996).

Protein	Biochemical activity	Physiological function
Nedd4	Ubiquitin-dependent downregulation of epithelial Na^+ channel	Regulation of intracellular Na^+ concentration
Itch	Regulation of unknown protein	Role in inflammatory response
Smurf1	Ubiquitin-dependent regulation of Smad1 and Smad5 proteins	Specification of cell fate during development
Su(Dx)	Regulation of the Deltex protein	Cell fate-specification by Notch Receptor signalling
Rsp5p	Ubiquitination of permeases (uracil permease, general amino acid permease and maltose permease) and RNA polymerase II	Control of import of metabolites and nutrients and regulation of transcription
Pub1p	Ubiquitin-mediated turnover of cdc25 phosphatase and membrane permeases	Regulation of cell cycle, membrane transport and pH tolerance

1.10.1 Yeast orthologues of Nedd4

To date, Nedd4 orthologues have been found in yeast, mouse, rat and human (Kumar *et al.*, 1992; Kumar *et al.*, 1997; Staub *et al.*, 1996). While yeast, mouse and rat proteins have a similar structure, human Nedd4 has an additional WW domain that might allow it to interact with additional proteins. There are Nedd4 homologues in both *S. cerevisiae* and *Schizosaccahromyces pombe*. The *S. cerevisiae* protein Rsp5p/Npi1p was identified originally as a suppressor of mutations in the *SPT3* gene, which encodes for a transcription factor that interacts with a TATA-binding protein (Eisenmann *et al.*, 1992). More recently, it has been identified in numerous screens and implicated in a plethora of apparently unrelated functions. It is required for the ubiquitin-mediated turnover of at least three membrane proteins: the general amino acid permease (GAP) (Hein *et al.*, 1995), the uracil permease (Hein *et al.*, 1995; Galan *et al.*, 1996; Galan and Haguenauer Tsapis, 1997) and the maltose transporter (Lucero and Lagunas, 1997). Rsp5p/Npi1p is also believed to play a role in minichromosome maintenance (Yashiroda *et al.*, 1998), mitochondrial/cytoplasmic protein distribution (Zolladek *et al.*, 1997) and is required for vegetative growth, sporulation and stress response (Kanda, 1996). Rsp5p and human Nedd4 have been shown to potentiate hormone-dependent activation of transcription by progesterone and glucocorticoid receptors, in a manner apparently independent of ubiquitin-protein ligase function and interactions via the WW domains (Imhof and McDonnell, 1996). Rsp5p/Npi1p also mediates ubiquitin-dependent degradation of the single-stranded-DNA binding protein, Rfa1 (Erdeniz and Rothstein 2000), and of RNA polymerase II in response to DNA damage (Huibregtse *et al.*, 1997; Beaudenon *et al.*, 1999). A recent report shows that WW domains 2 and 3 and the hect domain are indispensable for the essential function of Rsp5p/Npi1p (Wang *et al.*, 1999). The C2 domain and WW domain 1 do not appear to be required for viability but might be required for a non-essential function of Rsp5p/Npi1p. In fact recently, the C2 domain of Rsp5p was

shown to be required for endocytosis of the GAP permease, although it was not required for ubiquitination of this protein (Springael *et al.*, 1999). Pub1p, the *S. pombe* homologue of Rsp5p/Npi1p, targets the mitotic activating tyrosine phosphatase cdc25 for degradation by the ubiquitin pathway (Nefsky and Beach, 1996). This suggests that Pub1p has a role in cell cycle regulation in yeast. Like Rsp5p, Pub1p is also implicated in the regulation of membrane permeases (Saleki *et al.*, 1997; Karagiannis *et al.*, 1999). Another *S. pombe* Nedd4 family member (Genbank accession no. Z99759) also exists but has not been functionally characterised.

1.10.2 Other Nedd4 family members

The protein most closely related to human Nedd4 is encoded by a human gene of unknown function (Genbank accession no. KIAA0439). This putative protein shares approximately 78% similarity with human Nedd4 and has a *Xenopus* homologue (Genbank accession no. AJ000085) (Rebhun and Pratt, 1998). Recently, a splice variant of the human KIAA0439 gene, encoding for only three of the four WW domains seen in KIAA0439 was also isolated (Genbank accession no. AL134769). Three human Nedd4-like proteins, WWP2/AIP2, WWP1/AIP5 and AIP4, have recently been cloned and share a high degree of homology with each other (Pirozzi *et al.*, 1997; Wood *et al.*, 1998). WWP2/AIP2 and WWP1/AIP5 were identified based on their ability to bind to a PY motif peptide bait (Pirozzi *et al.*, 1997). WWP2/AIP2, WWP1/AIP5 and an additional member, AIP4, were also cloned as molecules that interacted with atrophin-1, a protein containing five PY motifs (Wood *et al.*, 1998). Certain WW domains from WWP2/AIP2 and WWP1/AIP5 possess binding specificity for peptides representing the PY motifs of several proteins, including RasGAP, ENaC and β-dystroglycan (Pirozzi *et al.*, 1997). Whether these proteins or atrophin-1 are physiological targets of any of these Nedd4-like proteins is yet to be demonstrated. The likely murine homologue of the *AIP-4* gene, the

sequence of which is incomplete at the amino terminus, is *Itch*. *Itch* is disrupted in mice with the genetic disease non-agouti-lethal 18H, which is characterized by a number of inflammatory disorders (Perry *et al.*, 1998). The protein target(s) of Itch are not yet known, but, since many cytokine receptors are involved in the inflammatory response and are regulated by the ubiquitin pathway, it is possible that its substrates include one or more cytokine receptors.

The *Drosophila* gene, *Suppressor of Deltex [Su(Dx)]*, was recently discovered as a negative regulator of Notch signalling (Cornell *et al.*, 1999). Notch is an evolutionarily conserved transmembrane signalling protein that regulates many cell fate decisions during development. Su(Dx) is most closely related to WWP1/AIP5, WWP2/AIP2 and AIP4 which suggests that one or more of these proteins may play a role in the regulation of Notch signalling and hence mammalian development. The most recently characterised member of the Nedd4 family of proteins is the *Xenopus* protein, Smurf1. Smurf1 and its human homologue were shown to regulate ubiquitin-mediated degradation of the signalling proteins, SMAD 1 and 5 (Zhu *et al.*, 1999). SMAD 1 and 5 transduce signals from type 1 TGF-β receptors that have been stimulated by bone morphogenic protein (BMP). TGF-β signalling regulates many important biological processes such as cell growth, differentiation and specification of cell fate during development. Zhu *et al.* (1999), have identified Smurf1 as a key regulator of SMADs and hence of TGF-β signalling. Two additional *Drosophila* Nedd4 family members have recently been submitted to Genbank. Lack (lethal with a checkpoint kinase) is 1061 amino acids long and is most closely related to Smurf1, but its function has not been characterised at present (Genbank accession no. AF216521). The other Drosophila Nedd4 family member appears to be the Drosophila homologue of Nedd4 as it most closely related to this gene (Genbank accession no. AI533013). The remaining member of the Nedd4 family is a predicted human protein (Genbank accession no. KIAA0322) which has not yet been characterised.

1.10.3 Role of multiple WW domains in defining target specificity

The WW domains of Nedd4-like proteins appear to mediate interactions with target proteins. Although *in vitro* data suggest that WW domains from different Nedd4-like proteins can bind to the same PY motif, the validity of these observations *in vivo* and the physiological significance of the *in vitro* interactions remain unknown. A likely scenario is that each WW domain of a Nedd4-like protein binds to a specific set of proteins. Support for this comes from recent studies with Nedd4/ENaC interactions. Only WW domains 2 and 3 of mouse Nedd4 interact *in vitro* with the three ENaC subunits, but all 3 WW domains are required for downregulation of ENaC activity *in vivo* (Chapter 5 of this thesis; Harvey *et al.*, 1999). This suggests that WW domain 1 binds to a protein other than ENaC and that this interaction is required for Nedd4-mediated regulation of ENaC activity. In *S. cerevisiae*, a single Nedd4-like protein appears to regulate the ubiquitination of a variety of different proteins. The presence of multiple Nedd4-like proteins in mammals (seven in human) suggests that many mammalian proteins are likely to be modified via ubiquitination by Nedd4 family members. Interaction with a large number of protein targets might be achieved by means of multiple WW domains in individual Nedd4 family members, each interacting specifically with a small subset of target proteins. Outside the C2, WW and hect domains, Nedd4-like proteins do not share significant homology. In addition to WW domains, other, as-yet-undefined regions of Nedd4-like proteins might be important for binding to factors that further regulate substrate specificity.

1.11 Project aims

When this project commenced only the initial studies describing the cloning, expression and genomic localisation of murine Nedd4 had been carried out while its physiological function was totally obscure (Kumar *et al.*, 1992; Kumar *et al.*, 1997). Owing to the homology that Nedd4 shared with proteins of known function, such as E6-AP, and the investigation of its yeast homologue, Rsp5/Npi1, we hypothesised that Nedd4 was a ubiquitin-protein ligase. We therefore wanted to know which proteins were regulated by Nedd4-mediated ubiquitination. Additionally we wanted to investigate the significance of several potential caspase-cleavage sites in Nedd4, which suggested a possible role in the cell death process.

Aim 1: To determine whether Nedd4 is cleaved by caspases during cell death and whether such cleavage plays an important role in cell death.

Aim 2: To investigate whether Nedd4 is responsible for ubiquitin-dependent regulation of ENaC.

Aim 3: To determine which WW domains of Nedd4 are involved in binding to, and regulation of ENaC in response to increased cytosolic Na^+.

Aim 4: To determine which channel subunits are required for Na^+-dependent regulation of ENaC.

Chapter 2:
Materials and Methods

2.1 Tissue Culture

2.1.1 Cell lines and culture conditions

The human Jurkat T-lymphoblast leukaemia cell line was obtained from Prof. A. Lopez (Hanson Centre for Cancer Research, Adelaide) and was maintained in suspension culture in RPMI 1640 (GibcoBRL) supplemented with 10% foetal bovine serum (FBS). The adherent human HeLa cervical carcinoma cell line, obtained from Dr J. Carr (Division of Virology, IMVS, Adelaide), was cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) which was modified by the addition of 7.5% sodium bicarbonate and supplemented with 10% FBS. The adherent murine fibroblast NIH-3T3 cell line was grown in DMEM supplemented with 10% FBS.

All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained in log phase (1-10 x 10^5 cells/ml) at greater than 95% viability. Cell density was calculated using a haemocytometer and cell viability assessed by trypan blue exclusion. In the case of adherent cell line passage, medium was removed by aspiration, cells were washed with phosphate-buffered-saline (PBS) and incubated briefly in trypsin [0.054% w/v trypsin (Difco), 0.54 mM EDTA in Hank's Balanced Salt Solution (HBSS)] to release them from the flask surface. Medium containing 10% FBS was then added to inactivate trypsin and cells were counted and subcultured.

2.1.2 Cryopreservation of cells

Cells were harvested in log phase and resuspended at 5 x 10^6 cells/ml in medium/10% FBS. An equal volume of cryoprotectant [30% heat-inactivated FBS, 20% dimethyl sulphoxide (DMSO) (BDH, Merck), 50% RPMI 1640] was added and cell suspension dispensed in 1 ml aliquots into 1 ml cryotubes (Nunc). Cells were then frozen by controlled rate freezing and stored in liquid nitrogen-filled tanks.

2.1.3 Thawing cryopreserved cells

Cells were thawed rapidly in a 37 °C water bath, transferred to a 10 ml conical tube and washed once in 10 ml medium. Cells were then seeded into an appropriately sized tissue culture flask in medium supplemented with 10% FBS.

2.2 DNA manipulations

2.2.1 Restriction endonuclease digestion

DNA was digested with the desired restriction endonucleases (Pharmacia or New England Biolabs) in a 10-50 µl total volume comprising DNA, 1-5 units restriction enzyme/µg DNA, 1x digestion buffer (specific for each restriction enzyme) and sterile H_2O for 1-3 hours at 37 °C. Reactions were terminated by either heat inactivation at 65 °C or 85 °C or by the addition of DNA loading dye which was added immediately prior to analysis by gel electrophoresis.

2.2.2 Analysis and separation of DNA fragments

DNA was analysed by electrophoresis in agarose gels of the appropriate percentage to retain fragments of the expected size [0.8-2% agarose dissolved in Tris-Acetate-EDTA buffer (TAE, 40 mM Tris-Acetate, 1 mM EDTA)]. Prior to electrophoresis, 10x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to a final concentration of 1x and the DNA then loaded onto gels immersed in TAE buffer. Routine electrophoresis was performed at 100 Volts. Following electrophoresis, DNA was visualised by staining in ethidium bromide solution (2 µg/ml) and analysis under ultra-violet light.

2.2.3 Purification of DNA fragments from agarose or solution

A) BRESA-CLEAN™

In the case of DNA in solution, 3 volumes of BRESA-SALT™ solution was added and mixed well with DNA solution. In the case of DNA that was excised from agarose following ethidium bromide staining and low energy ultra violet irradiation, the weight of the excised band was measured and a volume of BRESA-SALT™ solution corresponding to 3 volumes of gel was added. The agarose was then dissolved at 55 °C for 5 min and mixed into solution.

Following vigorous vortexing of BRESA-BIND™, 5 µl plus 1 µl/ µg DNA was added to the DNA solution and incubated for 5 min at room temperature. Regular mixing of the solution was performed to maintain BRESA-BIND™ in suspension. The BRESA-BIND™/DNA complex was then pelleted and the supernatant removed. The pellet was washed with BRESA-WASH™ solution in a volume equivalent to that of the BRESA-SALT™ used in step 1 and then pelleted again. Supernatant was again aspirated and all traces of BRESA-WASH™ removed by air-drying. DNA was recovered by resuspension of the BRESA-BIND™/DNA complex in a volume of sterile H_2O equivalent to twice the volume of the BRESA-BIND™ matrix used earlier, followed by incubation at 50 °C for 5 min. BRESA-BIND™ was then pelleted for 1 min and the supernatant containing the DNA was removed.

B) Phenol/choroform extraction

In the case of very small amounts of plasmid DNA or PCR product, 1 µl of glycogen (Boehringer Mannheim) was added prior to extraction to facilitate minimal DNA loss. Generally, the volume of DNA solution was increased to 100 µl and an equal volume

of phenol/choroform (1:1) was added and shaken vigorously. The aqueous and solvent layers were separated by centrifugation at 9000g for 5 min. The upper, aqueous layer was then removed and extracted with an equal volume of chloroform as described above. DNA was precipitated by the addition of 0.1 volume 3M sodium acetate (NaAc) pH 4.6 and 2.5 volumes of 100% ethanol and incubation at -20°C for 60 min. DNA was recovered by centrifugation at 9000g, 15 min, 4 °C, then washed with 70% ethanol and resuspended in sterile H_2O.

2.2.4 End-filling DNA fragments

Restriction digested DNA with 3' overhangs was end-filled to create blunt-ended DNA using Klenow (Pharmacia) or T4 DNA Polymerase (New England Biolabs). Reactions were performed in solution containing DNA, 1x One-Phor-All Buffer Plus (Pharmacia), 0.2 mM dNTPs, 1 unit Klenow or T4 DNA Polymerase and sterile H_2O, at 37 °C for 30 min. Following end-filling, DNA was purified by either phenol/chloroform extraction or BRESA-CLEAN™.

2.2.5 Kinase treatment of DNA

DNA fragments were phosphorylated by treatment with T4 polynucleotide kinase (T4 PNK) (Pharmacia). Reactions were performed in a solution containing DNA, 6 units T4 PNK, 0.5 mM ATP and sterile H_2O, at 37 °C for 30 min. Following kinase treatment, DNA was purified by either BRESA-CLEAN™ or phenol/chloroform extraction and ethanol precipitation.

2.2.6 Dephosphorylation of DNA

To remove 5' phosphate groups from restriction endonuclease digested DNA, DNA was treated with calf intestinal phosphatase (CIP) (New England Biolabs).

Phosphatase treatment was performed in a 50 µl reaction volume comprising DNA, Buffer 3 (New England Biolabs), 1 unit CIP and sterile H_2O. DNA was treated at 37 °C for 30 min and purified by phenol/chloroform extraction and ethanol precipitation.

2.2.7 Ligation of DNA into plasmid vectors

Ligation reactions were performed using a ratio of molecules of insert DNA:vector DNA 3:1. Ligation reactions were done in a 10 µl volume comprising vector DNA, insert DNA, 1x ligation buffer (50 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 50 µg/ml BSA, 1 mM ATP), 1 Weiss unit T4 ligase (Pharmacia) and sterile H_2O, at 4 °C overnight.

2.2.8 Transformation of competent *Escherichia coli*

Chemically competent *E. coli* were thawed on ice, transferred to 10 µl ligation reactions, mixed gently and incubated a further 60 min on ice. Reactions were then heat shocked at 42 °C for 60 sec prior to the addition of 150 µl SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl in 1000 ml sterile H_2O + 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0). Cells were then incubated at 37 °C for 60 min before plating an appropriate volume onto agar plates containing the required vector resistance antibiotics (ampicillin 100 µg/ml, kanamycin 25 µg/ml, chloramphenicol 37 µg/ml). In the case of vectors encoding the genes required for α-complementation, isopropylthio-β-D-galactoside (IPTG) (4 µl of 1M stock) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (40 µl of 20 mg/ml stock), both from Progen, were spread onto plates prior to plating of the transformants. Plates were then incubated at 37 °C overnight.

2.2.9 Colony cracking

To analyse large numbers of putative positive transformants in vectors which did not allow for blue/white selection, colony cracking was employed to screen for colonies

containing the desired clones. Transformant colonies were picked and touched onto a master plate before being dispersed into 15 µl cracking solution (50 µM NaOH, 0.5% SDS, 5 µM EDTA, 0.1% bromophenol blue) and heated at 65 °C for 15 min. Reaction volumes were then loaded onto 0.8% agarose gels and electrophoresed at 40 Volts with the level of TAE just below the level of the wells until DNA had entered into the gel, after which the volume of TAE was increased to cover the gel and electrophoresis was performed at 100 Volts. Colonies containing putative positive clones based on the size of plasmid DNA were then further analysed.

2.2.10 Small scale plasmid purification

1.5 ml aliquots of prospective positive *E. coli* cultures were pelleted and resuspended in 100 µl Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). 100 µl Buffer P2 (200 mM NaOH, 1% SDS) was then added and mixed, followed by the addition of 100 µl Buffer P3 (3M KAc pH 5.5). Lysates were then centrifuged at 9000g for 5 min and supernatants removed to new tubes. DNA was precipitated by the addition of 600 µl 100% ethanol and centrifugation at 9000g, 15 min, 4 °C. DNA was resuspended 100 µl sterile H_2O.

2.2.11 Large scale plasmid DNA purification

50 ml aliquots of *E. coli* cultures were pelleted at 5000g and pellets were resuspended in 4 ml Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). 4 ml Buffer P2 (200 mM NaOH, 1% SDS) was then added, mixed gently and incubated at room temperature for 5 min. 4 ml chilled Buffer P3 (3M KAc pH 5.5) was added, mixed gently and incubated on ice 15 min. Lysates were then centrifuged at 20,000g, 30 min, 4 °C and supernatants removed and applied to QIAGEN-tip 100 cartridges which had been pre-equilibrated with 4 ml Buffer QBT (750 mM NaCl, 50 mM

MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100). Following flow through of the plasmid DNA solution, the columns were washed with 2x 10 ml Buffer QC (1M NaCl, 50 mM MOPS pH 7.0, 15% ethanol) and DNA was then eluted with 5 ml Buffer QF (1.25M NaCl, 50 mM Tris-HCl pH 8.5, 15% ethanol). 3.5 ml isopropanol was added to precipitate DNA and DNA was pelleted by centrifugation at 15 000g for 30 min at 4 °C. Following removal of the supernatant, DNA pellets were air-dried and resuspended in 400 µl sterile H_2O. DNA solution was then transferred to 1.5 ml Eppendorf tubes and precipitated by the addition of 0.1 volume NaAc (3M pH 4.6) and 2.5 volumes ethanol and incubation at -20 °C for 60 min. DNA was centrifuged at 9000g, 15 min, 4 °C, washed with 70% ethanol and resuspended in 100 µl TE pH 8.0. To calculate the concentration of plasmid DNA in solution, the optical density at 280 nm was measured (OD_{280} 1 = 50 µg/ml DNA).

2.2.12 Polymerase chain reaction (PCR)

A) Primer Purification

Oligonucleotide primers were purified by extraction with butanol. 100 µl deprotected oligonucleotide solution was vortexed vigorously with 1 ml butanol, then centrifuged at 9000g, 1 min. After removal of the supernatant, DNA was air-dried and resuspended in 100 µl sterile H_2O. The optical density at 280 nm was then measured to calculate primer concentration (OD_{280} 1 = 20 µg/ml).

B) PCR Primers

D237G 5' ACC AAC CAG GTG CTG CCA 3'

D288G 5' ACC TCA CGG GTG AAG ACA 3'

D293G 5' ACA ATG ATG GA TGC AGC 3'

D316G 5' ATG GCC CTG GCA ACC GGG 3'

Nedd4c 5' CCG GAA TTC ATG GAT GCT GCC ACT CAT TTG 3'

Nedd43' 5' CCG GAA TTC CTA ATC AAC CGC CAT CAA 3'

mWW1 5' GAG GAG GGA TCC CAA CCA GAT GCT GCC ACT 3'

mWW2 5' GAG AGA GAA TTC TCT TGT AAT CTC TGG AGT A 3'

hWW3-A 5' GCG GGA TCC AGT GAT TCA GGA CAG 3'

hWW3-B 5' CCG GAA TTC CAA GTG ATG TCT TTC C 3'

hαENaC-A 5' CGC GGA TTC CCT CCT TCC CAC TTC TGC 3'

hαENaC-B 5' CCG GAA TTC TCA GGG CCC CCC CAG A 3'

hβENaC-A 5' CGC GGA TTC CAC ACC AAC TTT GGC TTC 3'

hβENaC-B 5' CGC GGA TTC TTA GAT GGC ATC ACC CTC 3'

hγENaC-A 5' CGC GGA TTC CCA GCC CTG GAT ATA GAC 3'

hγENaC-B 5' CCG GAA TTC TCA GAG CTC ATC CAG CAT C 3'

mαENaC-A 5' CGC GGA TTC TTC CCT TCC CGT TTC 3'

mαENaC-B 5' CCG GAA TTC TCA GAG TGC CAT GGC 3'

mβ-10 5' CCG GAA TTC CTA GGT GTC CAG CGG CTG C 3'

mγENaC-A 5' CGC GGA TCC CCA GCC CTG GAT AC 3'

mγ-19 5' CCG GAA TTC CTA CAA GGT ATT GTA TCT G 3'

mγPY-1 5' CAC CCC CCA GAG CCA ATA CCT TGC G 3'

mγPY-2 5' CGC AAG GTA TTG GCT CTG GGG GGT G 3'

K48R1 5' GAT CTT TGC CGG TAG GCA GCT AGA AGA CG 3'

K48R2 5' CGT CTT CTA GCT GCC TAC CGG CAA AGA TC 3'

Restriction sites are underlined

GGATTC - Bam HI

GAATCC - Eco RI

CTCGAG - Xho I

C) Taq amplification

In standard PCR amplification, DNA was synthesised in a 100 µl volume comprising DNA template (5 µl first strand cDNA or 10-50 ng plasmid DNA), 1x amplification buffer, 2.5 mM $MgCl_2$, 100 ng each forward and reverse primer, 200 µM dNTPs, 0.5 µl AmpliTaq polymerase (Perkin Elmer) and sterile H_2O. After addition of all components to PCR tubes (Treff), light mineral oil was overlaid and the reaction was amplified according to the required conditions. Generally, reactions were denatured at 95 °C for 5 min, then subjected to 25-30 cycles of denaturation at 94 °C 1 min, primer annealing at 40-55 °C (depending on primer composition) 1 min, and primer extension at 72 °C 1 min. Finally, 10 min extension at 72 °C was performed.

D) Amplification using Pfu polymerase

Pfu DNA polymerase (Stratagene) was employed for amplification of DNA when high fidelity was required for subcloning. DNA was amplified in a 100 µl reaction

comprising 200 μM dNTPs, 100ng forward and reverse primers, 20 ng template DNA, 1x reaction buffer [20 mM Tris-HCl pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2$ SO_4, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/ml BSA] and 2.5 units *Pfu* polymerase. Amplification conditions were initial denaturation at 95 °C for 5 min, followed by 25-30 cycles of denaturation at 95 °C, primer annealing at 40-55 °C (dependent on primer composition) for 1.5 min and primer extension at 72 °C for 1 min per kilobase of DNA. Reactions were then held at 4 °C.

2.2.13 Site directed mutagenesis

A) Kunkel mutagenesis

Kunkel mutagenesis was performed essentially as described by Kunkel *et al.* (1987), with minor modifications. The plasmid DNA template for mutation was first transformed into the chemically competent *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$ F') and transformants were grown overnight on ampicillin containing agar plates. Transformant colonies were then inoculated into 3 x 1.5 ml Luria Bertani Broth cultures containing ampicillin (100 μg/ml) and were grown at 37 °C for 6 h with rapid agitation. 10^7 - 10^8 M13 phage were then added and cultures were grown a further 2 h at 37 °C with agitation prior to the addition of kanamycin to a final concentration of 70 μg/ml and overnight growth at 37 °C.

To recover phage, bacterial cultures were pooled and bacteria pelleted at 5000g, 4 °C, 15 min. 4 ml supernatant, containing phage, was then removed and phage were precipitated by the addition of 0.6 ml 20% PEG/2.5 M NaCl and incubation on ice 60 min. Phage were then pelleted at 5000g, 4 °C, 20 min and resuspended in 1 ml 0.3 M NaAc pH 6.0/1 mM EDTA. Single stranded DNA (ssDNA) was then purified from phage by phenol/chloroform extraction and ethanol precipitation. One ml phenol/chloroform was

added, the solution vortexed and phases separated by centrifugation at 9000g, 5 min. The aqueous phase was removed and DNA was precipitated by the addition of 0.1 volume 3M NaAc and 2.5 volumes of 100% ethanol. DNA was pelleted at 9000g, 4 °C, 20 min and resuspended in 100 µl TE pH 8.0. DNA recovery was analysed by gel electrophoresis.

ssDNA (500 ng) was hybridised to phosphorylated primer containing the required mutation in a 20 µl volume containing 1x EcoRI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100), a 10-50 molar excess of phosphorylated primer and sterile H_2O. The reaction was heated to 70 °C and then cooled gradually to room temperature. Second strand DNA was then synthesised in a 100 µl reaction containing 20 µl annealing mix, 1 x reaction buffer (20 mM HEPES-NaOH pH7.8, 2 mM DTT, 10 mM $MgCl_2$), 200 µM dNTPs, 0.5 mM ATP, 0.5 µl Gene 32 protein (Pharmacia), 1 µl T4 DNA polymerase (Pharmacia), 1 unit T4 DNA ligase (Pharmacia) and sterile H_2O, at 37 °C for 60 min.

The mutated plasmid DNA was transformed into chemically competent XL1-Blue *E. coli* as previously described and colonies grown were overnight at 37 °C on agar plates containing ampicillin (100 µg/ml). Putative positive transformants were then inoculated into 1.5 ml cultures containing ampicillin, grown overnight with vigorous agitation and plasmid DNA was isolated as in 2.2.10. DNA was then sequenced to confirm that the required mutation had been correctly incorporated.

B) Quikchange site-directed mutagenesis

The Quikchange Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocol. Briefly, plasmid DNA template for mutagenesis was amplified in a 50 µl reaction comprising 1x reaction buffer [10 mM KCl, 10 mM $(NH_4)SO_4$, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml

nuclease-free bovine serum albumin (BSA)], 50 ng plasmid DNA template, 125 ng of each mutagenic primer, 2 mM dNTP mix, 2.5U *Pfu* DNA polymerase and sterile H_2O. Reactions were overlaid with sterile mineral oil and amplified according to the following protocol: one cycle of denaturation at 95 °C for 30 sec, followed by 12 cycles of denaturation at 95 °C, 30 sec, primer binding at 55 °C, 1 min and extension at 68 °C for 2 min/kb of plasmid length. Reactions were cooled prior to the addition of 1 µl *Dpn* I restriction enzyme and were then incubated at 37 °C for one hour. 5 µl of mutated DNA was then transformed into competent bacterial cells as described previously. Putative positive clones were grown in liquid culture prior to the extraction and sequencing of their plasmid DNA to confirm that the desired mutation had been incorporated.

2.2.14 Sequencing

A) T7 Superbase sequencing

The T7 Superbase Sequencing Kit (Geneworks) was used according to manufacturer's instructions. Briefly, to 2 µg plasmid DNA, 2 µl 2M NaOH was added and the volume increased to 10 µl with sterile H_2O. DNA was incubated at room temperature 10 min before being precipitated by the addition of 3 µl 3 M NaAc pH 4.6, 7 µl sterile H_2O and 60 µl 100% ethanol and incubation at -70 °C 15 min. DNA was pelleted at 9000g, 15 min, 4 °C, washed with 70% ethanol, dried and resuspended in 7 µl sterile H_2O. 2 µl 5x annealing buffer and 50 ng sequencing primer were then added and incubated at 37 °C for 30 min, then at room temperature for 20 min.

To the DNA/primer mix, 2 µl 1x labelling/extension mix, 1 µl 100 mM DTT, 1 µl sterile H_2O, 1 µl α-^{32}P-dATP and 2 units T7 DNA polymerase were added and incubated at room temperature 5 min. 3.5 µl of each labelling/extension reaction was then transferred into 2.5 µl of each A, C, G, T termination mix (pre-warmed to 37 °C) and incubated at 37

°C for 5 min. 4 µl Stop/Loading buffer was added to each reaction and heated at 95 °C 5 min prior to electrophoresis on 8% polyacrylamide gels. Following electrophoresis, gels were dried under vacuum at 80 °C and exposed to X-ray film (Kodak) overnight.

B) Dye Terminator sequencing

The ABIPRISM Dye Terminator Cycle Sequencing Reaction Kit was used to sequence alkaline lysis purified plasmid DNA. In a sequencing reaction, 8 µl Terminator Ready Reaction Mix (A, C, G, T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris-HCl pH9.0, $MgCl_2$, thermal stable pyrophosphatase, AmpliTaq polymerase) was added to 500 ng template DNA and 3.2 pmol sequencing primer. The volume was then made up to 20 µl with sterile H_2O. The reaction was then cycled using a PERKIN ELMER GeneAmp PCR System 9600 according to the following program; [96 °C 10 sec, 50 °C 5 sec, 60 °C 4 min] x 25 cycles, followed by a 4 °C hold. Following cycle sequencing, DNA was precipitated by the addition of 2 µl 3M NaAc pH 4.6 and 50 µl 100% ethanol and incubation on ice 10 min. DNA was then pelleted at 9000g, 15 min, 4°C, washed in 70% ethanol and air dried. DNA was then sequenced using a Perkin Elmer automated sequencer.

2.2.15 DNA constructs generated

pBS-Nedd4 was generated by subcloning the open reading frame of Nedd4 from a full length Nedd4 cDNA clone in pBluescript (ML-4C7.4) (Kumar *et al.*, 1997). The Nedd4 open reading frame was released using HaeII and SapI, then treated with T4 DNA polymerase and cloned into the SmaI site of pBluescript SK (Stratagene) in both T7 and T3 orientations.

pBS-Nedd4t was created by digesting pBS-Nedd4 with HindIII and subcloning the 1.8kb fragment corresponding to the 5' end of the Nedd4 gene into the HindIII site of pBluescript (Stratagene) in the T7 orientation.

pBS-Nedd4D237G was generated by Kunkel mutagenesis using pBS-Nedd4(T7) as a template. Primer D237G was used to mutate Asp-237→Gly.

pBS-Nedd4D288G was generated by Kunkel mutagenesis using pBS-Nedd4(T7) as a template. Primer D288G was used to mutate Asp-288→Gly.

pBS-Nedd4D293G was generated by Kunkel mutagenesis using pBS-Nedd4(T7) as a template. Primer D293G was used to mutate Asp-293→Gly.

pBS-Nedd4D316G was generated by Kunkel mutagenesis using pBS-Nedd4(T7) as a template. Primer D316G was used to mutate Asp-316→Gly.

pBS-Nedd4D237Gt was created by digesting pBS-Nedd4D237G with HindIII and subcloning the 1.8 kb fragment corresponding to the 5' end of the Nedd4 gene into the HindIII site of pBluescript (Stratagene) in the T7 orientation.

pBS-Nedd4D288Gt was created by digesting pBS-Nedd4D288G with HindIII and subcloning the 1.8 kb fragment corresponding to the 5' end of the Nedd4 gene into the HindIII site of pBluescript (Stratagene) in the T7 orientation.

pBS-Nedd4D293Gt was created by digesting pBS-Nedd4D293G with HindIII and subcloning the 1.8 kb fragment corresponding to the 5' end of the Nedd4 gene into the HindIII site of pBluescript (Stratagene) in the T7 orientation.

pBS-Nedd4D316Gt was created by digesting pBS-Nedd4D316G with HindIII and subcloning the 1.8 kb fragment corresponding to the 5' end of the Nedd4 gene into the HindIII site of pBluescript (Stratagene) in the T7 orientation.

pCXN2-Nedd4 was generated by subcloning the open reading frame of Nedd4 from a full length Nedd4 cDNA clone in pBluesript (ML-4C7.4) . The Nedd4 open reading frame was released using HaeII and SapI, then treated with T4 DNA polymerase and cloned into pCXN2 (Niwa *et al.*, 1991) treated with EcoRI and T4 DNA polymerase.

pCXN2-Nedd4D237G was generated by subcloning the open reading frame of Nedd4 from pBS-Nedd4D237G. The Nedd4 open reading frame was released using NotI, XhoI and BglI, then treated with T4 DNA polymerase and cloned into pCXN2 treated with EcoRI and T4 DNA polymerase.

pCXN2-Nedd4c was generated by PCR amplification of the three WW domains and the hect domain of murine Nedd4 using primers Nedd4c and Nedd43' and pBS-Nedd4 as a template. The PCR product was digested with EcoRI and cloned into the EcoRI site of pCXN2.

pGEX-2TK–mWW1-3 was created by PCR amplification of the 3 WW domains in mouse Nedd4 using primers mWW1 and mWW2 and pBS-mNedd4 as a template. The PCR product was digested with BamH1 and EcoR1 and cloned in frame into the BamH1 and EcoR1 sites of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–hWW3 was created by PCR amplification of the third WW domain in human Nedd4 using primers hWW3-A and hWW3-B and pBS-hNedd4 (a gift from Dr. N. Nomura) as a template. The PCR product was digested with BamHI and EcoRI and cloned in frame into the BamHI and EcoRI sites of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–hαENaCc was generated by PCR amplification of the carboxyl terminal 40 amino acids of the α subunit of human ENaC using primers hαENaC-A and hαENaC-B and pMT3-hαENaC (a gift from Prof. M. Welsh) as a template. The PCR product was

digested with BamHI and EcoRI and cloned in frame into the BamHI and EcoRI sites of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–hβENaCc was generated by PCR amplification of the carboxyl terminal 40 amino acids of the α subunit of human ENaC using primers hβENaC-A and hβENaC-B and pMT3-hβENaC (a gift from Prof. M. Welsh) as a template. The PCR product was digested with BamHI and cloned in frame into the BamHI site of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–hγENaCc was generated by PCR amplification of the carboxyl terminal 40 amino acids of the γ subunit of human ENaC using primers hγENaC-A and hγENaC-B and pMT3-hγENaC (a gift from Prof. M. Welsh) as a template. The PCR product was digested with BamHI and EcoRI and cloned in frame into the BamHI and EcoRI sites of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–mαENaCc was generated by PCR amplification of the carboxyl terminal 40 amino acids of the α subunit of human ENaC using primers mαENaC-A and mαENaC-B and pBS-mENaC (supplied by M. Day and D. I. Cook) as a template. The PCR product was digested with BamHI and EcoRI and cloned in frame into the BamHI and EcoRI sites of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–mβ–10ENaCc was generated by PCR amplification of amino acids - of the β subunit of mouse ENaC using primers mαENaC-A and mβ-10 and pBS-mβENaC (supplied by M. Day and D. I. Cook) as a template. The PCR product was digested with BamHI and EcoRI and cloned in frame into the BamHI and EcoRI sites of pGEX-2TK (Amersham Pharmacia Biotech).

pGEX-2TK–mγENaC PPRA was generated by Quickchange mutagenesis using pGEX-2TK -mγENaCc as a template. Primers K29R-1 and K29R-2 were used to mutate Lys-29→Arg.

pGEX-2TK–K48R ubiquitin was generated by Quickchange mutagenesis using pGEX-2TK ubiquitin as a template. Primers K48R-1 and K48R-2 were used to mutate Lys-48→Arg.

2.2.16 Transient transfection of adherent mammalian cells

A) Transfection using Superfect™

The day prior to transfection, cells were plated out at 8-10 x 10^5 cells/60 mm dish in 5 ml growth medium. On the day of transfection, 5 μg DNA was mixed with 150 μl of serum and antibiotic free medium and 20 μl-30 μl Superfect™ reagent was then added and mixed. DNA/Superfect complexes were then allowed to form during a 15 min incubation at room temperature. Medium was aspirated from cells and monolayers were washed with 5 ml PBS. 1 ml complete medium containing serum and antibiotics was then added to DNA/Superfect™ complexes, mixed and added immediately to cells. After incubating at 37 °C for 3 h, medium was aspirated, cells were washed with 5 ml PBS and were then re-fed with 3 ml fresh, complete medium. Cells were harvested using cell lifters (COSTAR) at the required time following transfection.

For cells plated out in 6-well trays, cells were seeded at a density of 2.5 x 10^5 cells/well in 3 ml growth medium. The day of transfection, 2.5 μg DNA was added to 100 μl of serum and antibiotic-free medium and 10 μl Superfect™ reagent was then added, mixed and incubated 15 min. 600 μl complete medium was then added to the transfection mix. DNA/Superfect™ complexes were added to cells immediately following a PBS wash

of the monolayers and were incubated at 37 °C for 3 h. Cells were then washed with 2 ml PBS and re-fed with 3 ml complete medium.

B) Transfection using FuGENE6

The day prior to transfection, cells were plated out at 2 x 10^5 cells per 35 mm dish in 2 ml medium or 8-10 x 10^5 cells per 60 mm dish in 3 ml medium. On the day of transfection, 3 µl or 6 µl FuGENE 6 (Boehringer Mannheim) reagent was diluted into 100 µl or 150 µl respectively, of serum and antibiotic-free medium, and incubated for 5 min at room temperature. The diluted FuGENE 6 reagent was then added dropwise to 1 µg or 2 µg plasmid DNA and incubated for 15 min at room temperature. The FuGENE6/DNA mix was then added dropwise to cells with gentle mixing and incubated for 12-18 hours prior to expression analysis.

2.3 Protein analysis

2.3.1 SDS-PAGE and protein transfer

Polyacrylamide gels of the appropriate percentage were cast and layered with 5% stacking gel. Gels were assembled into Hoefer gel tank apparatus and immersed in protein electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). Samples were prepared for electrophoresis by the addition of an equal volume of protein loading dye (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and were boiled for 5 min. Samples were then loaded and electrophoresed at 100 volts through the stacking gel and 200 volts through the resolving gel. Following electrophoresis, the gel was prepared for protein transfer by equilibration in protein transfer buffer (49 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol). A sheet of polyvinylidine difluoride (PVDF) ("Polyscreen" Dupont) or nitrocellulose (Schleicher & Schuell) and 4 sheets of Whatman cut to the exact size of the gel were also equilibrated in protein transfer buffer.

To assemble the gel for semi-dry transfer, 2 sheets of Whatman were placed on the cathode (+), followed by the PVDF filter, the gel and the final 2 sheets of Whatman. Protein was transferred at 130 mAmp for 1.5 h.

2.3.2 Coomassie staining

To visualise protein, gels were stained in Coomassie Brilliant Blue R-250 (Biorad) (0.25% w/v in 5:4:1 H_2O:MeOH:HAc). Staining was performed for 1 h, followed by destaining in 5:4:1 H_2O:MeOH:HAc for 1 h with 2 changes of destain solution. Gels were then dried under vacuum at 80 °C for 60 min.

2.3.3 Immunoblotting

In preparation for Immunoblotting, protein filters were blocked in 5% skim milk (Diploma) PBS-T (1x PBS, 0.05% Tween 20) solution for 1 h at room temperature or overnight at 4 °C. Primary antibodies were diluted in 5% skim milk PBS-T solution as follows; N4ab1 rabbit polyclonal antibody 1/4000, N4ab2 affinity purified rabbit polyclonal antibody, GFP mouse monoclonal antibody (Boehringer Mannheim) 1/1000 for 4 h. Secondary antibodies used were anti-rabbit-IgG conjugated with horseradish peroxidase (HRP) (Amersham Corp.) to detect rabbit polyclonal antisera or anti-mouse-IgG conjugated with HRP (Amersham Corp.) to detect mouse monoclonal antibodies. Both secondary antibodies were used at a dilution of 1/2000 in 5% skim milk PBS-T solution and were incubated for 1 h at room temperature. Following incubation in primary and secondary antibodies, filters were washed, 3 x 5 min and 2 x 10 min in PBS-T solution. Signals were visualised by using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Briefly, filters were exposed to ECL reagents mixed in a 1:1 ratio for 1 min and then exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for the appropriate time.

2.3.4 Immunoblot stripping

To strip protein from Western Blots in preparation for re-probing, filters were washed 2 x 10 min in PBS-T and then incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) for 10 min at 50 °C. Following stripping, filters were washed 2 x 10 min in PBS-T and blocked in 5% skim milk PBS-T solution as described above.

2.3.5 Protein expression in *Escherichia coli*

Overnight cultures of *E. coli* DH5α harbouring the appropriate pGEX expression plasmid (Amersham Pharmacia Biotech) were diluted 1/50, grown for 2 hr at 37° C, induced with 1 mM isopropyl β-D-thiogalactoside and grown for an additional 5 hr at 37° C. Bacterial cell pellets were resuspended in phosphate buffered saline (PBS), lysed by sonication and clarified by centrifugation at 10,000 g for 10 minutes at 4° C. Glutathione Sepharose (Amersham Pharmacia Biotech) was incubated with the cleared lysate for 60 min at room temperature, and then the beads were washed three times with PBS. Bound fusion protein was eluted by incubating with glutathione elution buffer five times for 15 minutes each. Protein concentration was measured using a BCA kit (Pierce). Briefly, several dilutions of protein eluates were prepared, along with serial dilutions of the protein standard bovine serum albumin (BSA) ranging from 2 mg/ml-0.125 mg/ml. 10 µl of each protein sample was then incubated with 200 µl of the kit reagents A and B mixed together in a 50:1 ratio, for 30 min at 37 °C. The optical density at 562 nm for each protein sample was then measured and a standard curve constructed for the BSA measurements. The protein concentration of the protein eluates was calculated by comparison with the standard curve.

2.3.6 Protein expression by in vitro translation

cDNAs were transcribed and translated *in vitro* using the Promega TNT™ Coupled Reticulocyte Lysate System. In a standard reaction, ^{35}S-Met labelled protein was generated in a 50 µl reaction volume comprising 25 µl TNT Rabbit Reticulocyte Lysate, 2 µl TNT Reaction Buffer, 1 µl T7 or SP6 polymerase, 1 µl amino acid mixture minus methionine, 4 µl ^{35}S-methionine (ICN), 1 µl RNasin Ribonuclease inhibitor, 1 µg DNA template and sterile H_2O. Reactions were incubated at 30 °C for 1.5 h and were used immediately or stored at -20 °C for no more than one week.

2.3.7 Far-Western blotting

^{32}P-labelled protein probes were produced by directly labelling the appropriate GST fusion protein (described in 2.3.5) using protein kinase A (New England Biolabs). Glutathione beads containing bound fusion protein were incubated with 5 units protein kinase A and γ^{32}P -ATP in a buffer containing 20mM Tris-HCl (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$ and 1mM DTT for 60 minutes at 4 °C. Beads were washed 5 times in PBS and labelled protein was eluted with glutathione buffer. To prepare WW domain protein filters, approximately 2 µg of each GST fusion protein was resolved on SDS-PAGE gels and transferred to nitrocellulose membrane (Schleicher and Schuell). Membranes were blocked in Hyb 75 (20 mM HEPES, pH 7.4, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40) overnight at 4 °C and then hybridised with ^{32}P-labelled protein probes for 4 hr at 4 °C in Hyb 75. Membranes were washed three times in Hyb75 and exposed to X-ray film.

2.4 Antibody production

2.4.1 Protein purification

GST-WW fusion protein was produced as in 2.3.5

2.4.2 Protein inoculation

Purified GST-WW protein (0.5mg) was mixed in Freund's complete adjuvant (Sigma) and inoculated intramuscularly into a male rabbit. Two subsequent booster inoculations consisting of 0.5 mg GST-WW protein in Freund's incomplete adjuvant (Sigma) were performed at monthly intervals, before the rabbit was sacrificed and serum collected.

2.4.3 Antibody affinity purification

CNBr activated sepharose (0.3 g) (Amersham Pharmacia Biotech) was washed 4 times in 1mM HCl, pH 3.0 and twice in coupling buffer (0.1 M $NaHC0_3$, 0.5 M HCl, pH 8.0) before resuspension in 5ml of coupling buffer. The CNBr activated sepharose was then incubated with 5mg of GST-WW protein overnight at 4 °C. Following coupling to GST-WW protein, the sepharose was washed once in coupling buffer and then incubated for 2 hours at room temperature with 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0. The sepharose was then washed alternatively in 0.1 M Na Acetate, 0.5 M NaCl, pH 4.0 and coupling buffer 4 times. The sepharose was then packed into a column and washed with 0.1 M phosphates buffer (0.1 M Na_3PO_4, Na_2HPO_4, pH 8.2). 1.5 ml of serum from a rabbit immunised with Nedd4 GST-WW protein was diluted in an equal volume of phosphates buffer and passed through the column 3 times. The column was then washed 5 times with phosphates buffer. To elute the affinity purified antibody 0.2 M Gly, pH 3.0 was added to the column and 0.5 ml fractions collected into 0.5 ml of 1 M Tris-HCl, pH 8.0. The absorbance of the fractions was measured at a wavelength of 280nm and the protein-rich

fractions pooled. The pooled affinity purified antibody was then dialysed overnight at 4 °C against PBS and tested for its affinity by immunoblotting cell and organ lysates and recombinant GST-WW protein.

2.5 Apoptosis assays

2.5.1 Transient transfection apoptosis assay

The day prior to transfection, NIH-3T3 cells were plated in 6 well tissue culture plates at a density of 2.5 x 10^5 cells per well. The following day, cells were co-transfected with 2 μg of the appropriate gene contained in the pCXN2 vector (Niwa *et al.*, 1991) and 0.5 μg of the β-galactosidase expression vector (pEF-βgal) (Kumar *et al.*, 1994) as described above (2.2.16). 24 h post-transfection, cells were analysed for β-galactosidase expression by rinsing with PBS, fixing with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min, rinsing twice with PBS and staining with 0.1% X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS for 3 h. After rinsing in PBS, positively stained blue cells were analysed microscopically for apoptotic morphology. The extent of apoptosis was represented as the % of morphologically apoptotic cells among the total number of blue stained transfected cells.

2.5.2 Treatment of cells with apoptosis-inducing agents

Cells were treated with the following stimuli for various times to induce apoptosis; etoposide (Calbiochem) at a final concentration of 40 μM, γ-irradiation (30 Gy from a ^{137}Cs source), α-Fas antibody at a final concentration of 200 ng/ml (kindly provided by Dr Andreas Strasser) or 100 ng/ml (Upstate Biotechnology), tumour necrosis factor-α (TNFα) (R&D Systems) at a final concentration of 10 ng/ml together with 10 μg/ml cycloheximide (Sigma).

2.5.3 Analysis of apoptotic morphology by nuclear staining

Cytosmears of control cells and of cells treated with apoptotic stimuli were prepared by centrifuging 5 x 10^4 cells per slide at 1500g, 5 min and resuspending them in 100 µl FBS per 5 x 10^4 cells. Cells were then centrifuged onto slides at 28g, 5 min, using a Cytospin 3 centrifuge (Shandon Scientific Ltd.). Cytosmears were stored at 4 °C prior to fixing and analysis. Cells were fixed in Carnoy's fixative (6:1 ethanol:acetic acid) for 10 min, washed 2x 5 min in PBS, rinsed in sterile H_2O and air-dried. Cells were then stained in 4,6-diamino-2-phenylindole (DAPI) (8 µM) for 1 min, rinsed 3 x in sterile H_2O, air dried, mounted in anti-fade medium (1% propylgalate, 86% glycerol) and visualised by fluorescence microscopy using an Olympus BH2-RFCA fluorescence microscope. Apoptotic cells were scored by the analysis of nuclear morphology which appears condensed and/or fragmented in cells committed to apoptosis.

2.5.4 Preparation of cytoplasmic extracts

Cytoplasmic extracts were prepared essentially as described by Martin *et al.* (1995a) with minor modifications. Following different apoptotic stimuli, cells were harvested at the appropriate time points, pelleted at 200g and washed twice in ice cold PBS. Cell pellets were resuspended in 100 µl ice cold cell extraction buffer [50 mM 1,4-piperazinediethanesulfonic acid (PIPES) pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] per 5 x 10^6 cells. Cells were allowed to swell on ice for 20 min and were then lysed by two cycles of freeze/thawing using dry ice and a chilled water bath. Cell lysis was confirmed by trypan blue uptake. Lysates were centrifuged at 200g for 5 min at 4 °C and supernatants then removed and centrifuged at 9000g for 15 min at 4 °C. The clarified cytosol was removed and either used immediately or stored at -70 °C.

2.5.5 Proteolysis assays

In a standard cleavage assay, 5 µl of ^{35}S-Met (ICN) labelled *in vitro* translated protein was incubated for 5 h at 37 °C in the presence of either cytoplasmic extracts, purified proteases or bacterially expressed caspases. All assays were performed in cell extraction buffer in a final volume of 15 µl or 20 µl. Inhibitors were used at the following concentrations; YVAD-CMK (Bachem) 100 nM, DEVD-CHO (Bachem) 100 nM. Inhibitors were pre-incubated with cell extracts or isolated proteases for 30 min at 37 °C prior to the addition of *in vitro* translated protein. Reactions were terminated by the addition of an equal volume of 2x protein loading buffer comprising 100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol. Samples were boiled for 5 min, centrifuged at 9000g for 5 min and analysed by SDS-PAGE electrophoresis. Following electrophoresis, proteins were transferred to nitrocellulose membrane (Schleicer & Schuell) and visualised by autoradiography.

2.6 Whole cell patch clamping

2.6.1 Isolation and preparation of granular duct cells

Male mice were sacrificed and mandibular glands removed and finely chopped. Single mandibular duct cells were isolated by placing chopped glands into a physiological salt solution, containing 50 units/ml collagenase for 40 min. These cells were then placed into a standard bath solution (pH 7.4) containing 145 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 7.5 mM HEPES, and 10 mM glucose. Pipettes were placed on the membrane of single mandibular duct cells and the whole-cell configuration was established. The bath solution was then replaced with one containing 145 mM Na^+-glutamate, 5 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA and 10 mM glucose (pH 7.4). The standard (zero Na^+) pipette solution (pH 7.2) contained 150

mM N-methyl-D-glucamine (NMDG)-glutamate, 1mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA and 10 mM glucose. In the 70-72 mM Na^+ pipette solution, Na^+ concentration was adjusted by substitution of Na^+-glutamate for NMDG-glutamate.

2.6.2 Measurement of amiloride-sensitive Na^+ current

Pipettes were pulled from borosilicate tubes so as to have resistances of 1-3MΩ. The reference electrode that was placed into the bath solution was a Ag/AgCl electrode. An Axopatch-1D patch-clamp amplifier was used to measure whole cell currents. To determine whole cell I/V relations, a MacLab-4 data acquisition interface (ADInstruments, Sydney, Australia) attached to a Macintosh-IIci computer was used to generate command voltages and measure whole cell currents. Amiloride-sensitive current (which represents ENaC activity) was measured as the difference between the whole-cell current before and after the addition of 100 μM amiloride to the bath solution (. Whole-cell I/V relations were by applying voltage pulses of 200 msec duration from a resting potential of 0 mV. Steady-state currents were calculated as the average current between 100 and 200 msec after the start of the voltage pulse. Chord conductances were calculated as the slope of the line joining the current at –80 mV and the reversal potential of the amiloride-sensitive or $NMDG^+$-sensitive current as appropriate. Chord conductances were expressed in pico-Siemens (pS or 1/Ohm). Results are presented as mean ± S.E.M. Statistical significance was assessed using Student's unpaired t-test. All experiments were performed at 20-22 °C.

Chapter 3: Caspase-mediated cleavage of the ubiquitin-protein ligase Nedd4 during apoptosis

3.1 Introduction

Cell death by apoptosis is characterised by a distinct set of morphological changes in the dying cell including chromatin condensation, the formation of apoptotic bodies, DNA fragmentation, plasma membrane blebbing and cellular shrinkage (Kerr *et al.*, 1972). Since dead cells are rapidly removed by phagocytosis, apoptosis occurs without causing inflammation, in direct contrast to necrotic cell death which is characterised by cell swelling, lysis and leakage of the cellular contents, which causes swelling. Apoptosis is a fundamental process during embryogenesis, where large amounts of cell death occur. It is also required for the maintenance of homeostasis in adult organisms, the importance of which is emphasised by diseases that occur when apoptosis is dysregulated (reviewed in Thompson, 1995). Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Spinal Muscular Atrophy are caused by excessive apoptosis, while some autoimmune disorders and cancers can result from an inhibition of apoptosis. Two major pathways of apoptosis have been described in mammals: 1) those involving the activation of the tumour necrosis factor (TNF) receptor family (eg. TNF and Fas) or 2) those activated via cellular insults such as toxins, DNA damaging agents and γ-irradiation.

Research on apoptosis in the nematode, *Caenorhabditis elegans*, has helped to identify the molecular machinery responsible for regulating cell death. To date 14 genes have been isolated that are involved in various stages of the cell death pathway (reviewed in Horvitz *et al.*, 1994). Three of these genes, egl-1, ced-4 and ced-3 are absolutely required for cell death to occur (Ellis and Horvitz, 1986; Conradt and Horvitz, 1998). The product of the ced-9 gene prevents cell death in *C. elegans* and genetic studies have shown that it functions upstream of CED-3 and CED-4 in the apoptotic pathway (Hengartner *et al.*, 1992). CED-9 is homologous to the mammalian Bcl-2 oncoprotein, which also protects cells from apoptosis (Vaux *et al.*, 1992). In mammals there are several Bcl-2 family members, which include both pro-apoptotic and anti-apoptotic proteins. The EGL-1

protein shares homology with a family of mammalian pro-apoptotic molecules, including Bik, Bid and Harakiri (Conradt and Horvitz 1998). These proteins all share a BH3 (Bcl-2 homology region 3) domain which mediates their association with Bcl-2-like proteins and their ability to induce cell death. EGL-1 is believed to function by sequestering CED-4 away from CED-9, thus allowing CED-4 to interact with and activate CED-3 (Conradt and Horvitz 1998). CED-3 and CED-4 are required for developmental cell death in *C. elegans* (Ellis and Horvitz, 1986). There is at least one mammalian homologue of CED-4, called APAF-1, which is also pro-apoptotic (Zou *et al.*, 1997). CED-4 is thought to function by directly interacting with and activating CED-3 (Chinnaiyan *et al.*, 1997). When the ced-3 gene was cloned it was shown to be homologous to interleukin-1β converting enzyme (ICE), a cysteine protease required for processing of the inflammatory cytokine, interleukin-1β (Yuan *et al.*, 1993). Currently, 14 human CED-3 homologues have been identified, many of which are involved in the execution of cell death.

Mammalian CED-3 homologues, or caspases [for **asp**artate specific **c**ysteine prote**ases** (Alnemri *et al.*, 1996)] are key components of the proteolytic cascade that is activated during apoptosis. Numerous studies have shown that activation of caspases is central to the execution of apoptosis and that inhibition of caspases can suppress apoptosis in a variety of situations (reviewed in Ekert *et al.*, 1999). Caspases are synthesised as zymogens, which undergo proteolytic cleavage and processing prior to activation (Kumar, 1995; Nicholson and Thornberry, 1997). Based on their hierarchical position in the apoptotic cascade, caspases can be divided into two subclasses, upstream and downstream, depending on the presence or absence of an amino terminal prodomain. Upstream caspases, such as caspase-2, -8 -9 and –10 possess prodomains, which are thought to be important for recruitment of these caspases to activated death receptors or death signalling complexes. Once recruited to a death complex upstream caspases are activated by a mechanism that involves proximity-induced autocatalytic processing (Kumar and Colussi,

1999; Kumar, 1999). Activated upstream caspases cleave and activate downstream caspases such as caspase-3, -6 and –7, that then mediate the cleavage of cellular substrates.

Caspases cleave after an Asp residue in their substrate: the minimum substrate being a tetrapeptide. The caspase-1 subfamily, comprising caspases -1, -4 and -5, prefers the substrate sequence (W/L)EHD (Thornberry *et al.*, 1997). The caspase-3 subfamily, comprising caspases-3, -6, -7, -8, -9 and -10, can be divided into two groups based on substrate specificity. Caspase-3 and -7 have a preference for DEXD sequences, whereas caspases-6, -8 and -9 optimally recognise (I/L/V)EXD sequences (Thornberry *et al.*, 1997). Caspase-2 has unique substrate specificity and prefers the pentapeptide sequence, VDVAD ((Talanian *et al.*, 1997)).

Active caspases cleave a range of cellular substrates during apoptosis. The DNA repair enzyme poly(ADP-ribose) polymerase (PARP) was one of the first identified cellular substrates cleaved in apoptosis (Kaufmann, 1989). Caspase-3 was subsequently shown to cleave PARP with high efficiency (Tewari *et al.*, 1995; Nicholson *et al.*, 1995). At least 70 proteins of diverse cellular functions are cleaved during apoptosis by caspases (reviewed in Nicholson, 1999) and include the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (Song *et al.*, 1996; Casciola Rosen *et al.*, 1996), U1-70 kDa ribonucleoprotein (Casciola Rosen *et al.*, 1994), heteronuclear ribonucleoproteins C1 and C2 (Waterhouse *et al.*, 1996), α-fodrin (Martin *et al.*, 1995), nuclear lamins (Takahashi *et al.*, 1996; Orth *et al.*, 1996), Gas2 (Brancolini *et al.*, 1995), D4-GDI (Na *et al.*, 1996), PITSLRE kinases (Beyaert *et al.*, 1997), Rb (Janicke *et al.*, 1996; Tan *et al.*, 1997), PKC-δ (Emoto *et al.*, 1995), MDM2 (Erhardt *et al.*, 1997), PAK2 (Rudel and Bokoch, 1997), the large subunit of replication factor C (Song *et al.*, 1997), huntingtin (Goldberg *et al.*, 1996), transcription factors SREBP-1 and SREBP-2 (Wang *et al.*, 1996), focal adhesion kinase (Crouch *et al.*, 1996; Wen *et al.*, 1997), DNA fragmentation factor (Liu *et al.*, 1997), Bcl-2 (Cheng *et al.*, 1997), MEKK-1 (Cardone *et al.*, 1997), the inhibitor of caspase-activated

DNase (ICAD) (Sakahira *et al.*, 1998) and gelsolin (Kothakota *et al.*, 1997). Cleavage of these proteins and other possible caspase targets is likely to result in changes that occur in a cell undergoing apoptosis by mediating events such as the abrogation of DNA repair mechanisms, detachment of the apoptotic cell from surrounding cells/tissue, disruption of the cytoskeleton, initiation of DNA fragmentation, and the formation and engulfment of apoptotic bodies (Nicholson and Thornberry, 1997).

Apoptosis is not accompanied by random cleavage of a large number of proteins. Two-dimensional electrophoresis of apoptotic and non-apoptotic protein extracts has shown that less than 200 proteins appear to be cleaved during apoptosis (Brockstedt *et al.*, 1998). Additionally, the specificity of caspases involved clearly argues against a large number of proteins being degraded during apoptosis. Thus the proteins that are targeted specifically for degradation by caspases are likely to play some vital role in the apoptotic process. Therefore, it is necessary to identify all caspase substrates that are cleaved during apoptosis so that a clearer picture can emerge about the significance of caspase-mediated proteolysis in apoptosis.

We identified several potential caspase cleavage sites in Nedd4 and investigated whether Nedd4 was a target of caspase-mediated cleavage. The results in this chapter show that Nedd4 is indeed cleaved during apoptosis, a phenomenon that occurs in a number of cell types, and in cells undergoing apoptosis in response to various agents. The data presented in this chapter have been published (Harvey *et al.*, 1998).

Results

3.2 Production of affinity purified Nedd4 antibody

To investigate whether Nedd4 is cleaved during apoptosis we used two rabbit polyclonal antibodies raised against different portions of the murine Nedd4 protein. N4ab1 was raised against the hect domain and the WW domains of Nedd4 (Figure 6) as described in Kumar *et al.* (1997). To generate N4ab2, the three WW domains of murine Nedd4 were expressed as a glutathione-S transferase-fusion protein (GST-WW) in *E. coli* (Figure 1A). Serum was collected from rabbits that had been inoculated with GST-WW protein and affinity purified against the immunising GST-WW antigen. The affinity-purified N4ab2 was then tested for its ability to detect GST-WW protein (Figure1B), endogenous Nedd4 in murine tissues and cell lines (Figure 1C) and endogenous Nedd4 in human cell lines (Figure 1D) by immunoblotting. As can be seen in Figure 1C, murine Nedd4 was detected as a 120kDa protein, whilst human Nedd4 was detected at a slightly larger size of 125kDa. Although the predicted size of mouse Nedd4 is 103 kDa, while human Nedd4 is approximately 5 kDa larger than the mouse protein due to the presence of an additional WW domain, in SDS polyacrylamide gels these proteins migrate more slowly giving a larger apparent molecular weight (Kumar *et al.*, 1997). In some human cell lines, a smaller band of approximately 110 kDa is also detected by both antibodies, which we believe is derived from alternative splicing or represents a proteolytic fragment of Nedd4 (Figures 1C, 2 and 3). This smaller band has also been noted in rat tissues using two independently raised antibodies against rat Nedd4 protein (Staub *et al.*, 1997).

3.3 Cleavage of Nedd4 protein during Fas mediated apoptosis in Jurkat cells

To check whether Nedd4 cleavage occurs *in vivo* during apoptosis mediated by a physiological stimulus, we exposed Jurkat T-cells to an apoptosis-inducing Fas antibody and monitored the cleavage of Nedd4 by immunoblotting. For immunoblotting we

employed both polyclonal Nedd4 antibodies (N4ab1 and N4ab2), both of which detect the mouse and the human Nedd4 protein of 120~125kDa in size. Both antibodies detected identical Nedd4 cleavage products during Fas-mediated apoptosis in Jurkat cells (Figure 2). Within two hours of Fas antibody treatment, disappearance of both Nedd4 bands was clearly visible concomitant with the appearance of a 95 kDa band. By 4 hours no intact Nedd4 protein was visible.

The time course of Nedd4 cleavage in Jurkat cells was similar to that of a well known caspase substrate, PARP (Harvey *et al.*, 1998). Cleavage of Nedd4 and PARP was completely inhibited when Jurkat cells were treated with anti-Fas antibody in the presence of the broad spectrum cell-permeable caspase inhibitor zVAD-fmk (Figure 2 and Harvey *et al.*, 1998). Cleavage of Nedd4 also correlated with the activation of caspase -2 and caspase-3 (Harvey *et al.*, 1998).

3.4 Cleavage of Nedd4 occurs during apoptosis induced by etoposide

To check whether Nedd4 cleavage occurs in response to treatment by other apoptosis inducing agents, we subjected Jurkat T cells to 40 μM etoposide treatment. Proteolytic fragments identical to those released in response to anti-Fas treatment were evident, although the kinetics of cleavage were slower, reflecting slower apoptotic induction (Figure 3). For example, at 8 hr after treatment with etoposide, 43 % of cells showed apoptotic morphology by DAPI staining, as compared to 72 % observed for Fas antibody treated cells at the same timepoint. Again, both N4ab1 and N4ab2 antibodies detected identical Nedd4 cleavage products during the time course (Figure 3). The time course of activation of both these caspases was similar to the time course of Nedd4 and PARP cleavage suggesting that cleavage of Nedd4 is dependent on the onset of apoptosis and is likely to be mediated by caspases (Harvey *et al.*, 1998).

3.5 Cleavage of Nedd4 occurs in various cell types in response to a variety of apoptotic stimuli

We further analysed whether Nedd4 cleavage occurs in other cell types undergoing apoptosis. In HeLa cells treated with TNF-α, significant Nedd4 cleavage was evident by 4 hours when only a fraction of cells (< 15%) appeared apoptotic as assessed by nuclear staining with DAPI (Figure 4). The size of the Nedd4 cleavage products generated in apoptotic HeLa cells were identical to those seen in Jurkat cells. In addition, in the Burkitt's lymphoma cell line BL30A treated with either C-8 ceramide or γ-radiation and in BM13674 cells treated with γ-irradiation, Nedd4 cleavage similar to that seen in Jurkat and HeLa cells was clearly evident (Figure 4). In the Burkitt's lymphoma cell line, BL30K, that is resistant to apoptosis induced by γ-irradiation (Khanna *et al.*, 1997), no cleavage of Nedd4 was evident (Figure 4). Collectively, these results suggest that Nedd4 cleavage is a general apoptosis-related phenomenon and is not restricted to a particular cell type or to a specific apoptosis-inducing agent.

3.6 *In vitro* cleavage of Nedd4 by various caspases

An examination of the Nedd4 protein sequence showed that it contains several DxxD [consensus sequence for cleavage by downstream caspases such as caspases-3 and -7,(Thornberry *et al.*, 1997)] sequences which are conserved in mouse, rat and human proteins (Kumar *et al.*, 1997). We therefore investigated whether Nedd4 protein can be cleaved by individual caspases *in vitro*. As shown in Figure 5, both human and mouse Nedd4 proteins were cleaved by caspases-1, -3, -6 and -7, and extracts from apoptotic cells, but not by caspase-2. The major cleavage products in all cases were roughly similar in size suggesting that the recombinant caspases, and caspases activated in apoptotic Jurkat cells cleave Nedd4 either at the same sites or in the same vicinity. Mouse Nedd4 generated fragments of 90 kDa and a doublet of around 25 kDa when treated with

caspases-1, -7, and apoptotic cell extracts, while caspases-3 and -6 produced a single band around 25 kDa in addition to the 90 kDa fragment (Figure 5A). Cleavage of mouse Nedd4 by caspases and cell extracts also generated a cleavage intermediate of around 115 kDa. In the case of human Nedd4, the major cleavage products were approximately 95 kDa and 20 kDa in size (Figure 5B). The intermediate corresponding to the 115 kDa mouse Nedd4 product was not evident and a doublet of approximately 20 kDa was seen with caspase-1 and caspase-6. These results showed that there is at least one caspase cleavage site common to both mouse and human proteins. Incubation of apoptotic cell extracts with the tetrapeptide caspase inhibitors, 100 nM DEVD-CHO, but not 100nM YVAD-CMK abolished their Nedd4 cleaving activity, suggesting that a caspase-3-like protease in cell extracts mediates the cleavage of Nedd4.

3.7 Mapping of the caspase cleavage sites in Nedd4

The two antibodies used in this study were raised against residues 210-720 (N4ab1) and residues 235-511 (N4ab2) of the mouse Nedd4 protein (Figure 6). Both detected the same 95 kDa human Nedd4 cleavage product which was identical in size to that seen in *in vitro* cleavage experiments. The smaller fragments of Nedd4 (20-25 kDa) seen in *in vitro* cleavage assays were not detected by either antibody. Therefore we predicted that the major cleavage site may be located 20-25 kDa from either the amino- or carboxyl- terminus of the Nedd4 protein. Although there are three DxxD sequences conserved in mouse, human and rat Nedd4 sequences (Figure 6), only one ($DQPD^{237}$ in mouse or $DQPD^{206}$ in the known human sequence) would generate the expected size fragments. Therefore a truncated form of mouse Nedd4 protein lacking the two carboxyl terminal DxxD sites ($DVND^{774}$ and $DGVD^{887}$) was generated. When subjected to digestion by recombinant caspases, the truncated Nedd4 protein was cleaved, generating fragments of about 25 kDa, possibly representing the amino terminus of the protein, and 50 kDa from

the region downstream of the 25 kDa fragment (Figure 7A). In the mouse Nedd4 sequence, besides the $DQPD^{237}$, three other DxxD sequences, $DLTD^{288}$, $DNDD^{293}$ and $DGPD^{316}$ are located in the vicinity of the putative cleavage site (Figure 6). All four putative cleavage sites were mutated by replacing the P1 Asp with Gly. While D288G, D293G and D316G had no effect on cleavage of mouse Nedd4 by various caspases (data not shown), the D237G mutation in the mouse protein abolished the generation of both 50kDa and 25 kDa fragments by caspase-1, -3, -7 and apoptotic cell extracts, without affecting cleavage by caspase-6 (Figure 7B). These results suggest that *in vitro* cleavage by caspases-1, -3 and -7 occurs at D^{237} in the mouse protein [D^{238} in rat and D^{206} in the incomplete human sequence lacking the amino terminus (Kumar *et al.*, 1997)]. In all mutants, the generation of the 75 kDa intermediate (equivalent to the 115 kDa intermediate of full length Nedd4) was not affected (Figure 7B). We believe that this fragment arises due to cleavage of mouse Nedd4 at the $DVTD^{41}$ site. No corresponding site is present in the known human sequence, and accordingly, the intermediate product is not seen in human Nedd4 incubated with various caspases or apoptotic cell extracts (Figure 7B).

3.8 Nedd4 cleavage products do not induce apoptosis

To check whether Nedd4 cleavage can alter the apoptotic response in cells, we attempted to generate mammalian cells stably expressing high levels of transfected wild-type and D237G mutant Nedd4. Despite several attempts, we were unable to generate such cells, which led us to conclude that constitutive high level expression of Nedd4 may be cytotoxic, and that such cells are probably deleted during G418 selection of transfectants. In further attempts to understand the significance of Nedd4 cleavage in apoptosis, full length Nedd4 and its cleavage fragments were analysed for their ability to induce cell death. The rationale for this was based on the knowledge that caspase cleavage of proteins

such as PAK-2, Bcl-2 and gelsolin can generate products which are able to promote apoptotic changes (Goldberg *et al.*, 1996; Cheng *et al.*, 1997; Kothakota *et al.*, 1997). NIH-3T3 cells were transiently transfected with various Nedd4 expression constructs. While a caspase-2 expression construct efficiently killed cells 24 hours after transfection, neither the wild-type, Cys mutant, or a truncated version of Nedd4 showed any significant cell killing activity (Figure 8). It therefore appears that Nedd4 cleavage is a consequence of the induction of apoptosis and does not play a role in amplifying the apoptotic cascade.

3.9 Discussion

Several proteins have recently been shown to be cleaved by caspases during apoptosis. The results presented in this chapter demonstrate that Nedd4 is another target of caspases and is cleaved during apoptosis in different cell types induced by a variety of apoptotic stimuli. From mutagenesis studies, one of the cleavage sites in the Nedd4 protein was mapped as a DQPD sequence, present in both mouse and human proteins. This sequence is also conserved in rat Nedd4 (Staub *et al.*, 1996). As DxxD is the preferred cleavage sequence for caspase-2, -3 and -7 (Thornberry *et al.*, 1997), it would appear that *in vivo*, one or more of these caspases mediate the cleavage of Nedd4 during apoptosis. *In vitro* cleavage data suggests that both mouse and human Nedd4 proteins are cleaved by caspase-1, -3, -6 and -7, but not caspase-2. Caspase-2 is an upstream protease based on its ability to autoprocess following homodimerization (Thornberry *et al.*, 1997), and thus it was not surprising that it did not cleave Nedd4. Inhibitor studies indicated that caspase-1 is unlikely to be involved, at least in Jurkat cells, and one or more caspase-3-like proteases are involved (Figure 6). Caspase-6 does not appear to cleave at the DQPD↓ sequence as the D237G mutation did not affect cleavage by this caspase (Figure 7). Moreover, DxxD is not a preferred cleavage sequence for caspase-6 (Thornberry *et al.*, 1997). Caspase-6 may thus cleave Nedd4 at another Asp residue in the vicinity of Asp^{237}. One likely candidate is Asp^{216} in mouse Nedd4 (or the corresponding Asp^{186} in human protein), cleavage at which would generate a fragment slightly smaller than the 25 kDa, which appears to be the case (Figure 6). Based on *in vivo* and *in vitro* cleavage and inhibitor studies, most of the proteins known to be degraded in apoptotic cells are predicted to be cleaved by the downstream "effector" caspases which include caspase-3, caspase-6 and caspase-7 (Kumar, 1999; Nicholson and Thornberry, 1997; Nicholson, 1999). It is thus reasonable to assume that *in vivo* cleavage of Nedd4 is also mediated by one or more of these caspases.

Interestingly, the cleavage of Nedd4 removes the amino terminal C2 domain from the rest of the protein without disrupting the WW domains and the ubiquitin-protein ligase domain of Nedd4. As evident from studies on the epithelial Na^+ channel, the WW domains in Nedd4 are responsible for binding to target proteins [chapters 5 and 6 of this thesis; (Harvey *et al.*, 1999)], while a region between the WW domains and the *hect* domain binds the ubiquitin-conjugating enzyme (E2) (Hatakeyama *et al.*, 1997). This would suggest that cleavage of Nedd4 by caspases during apoptosis is unlikely to disrupt substrate recognition and enzymatic activity of Nedd4. The C2 domain of Nedd4 expressed in *E. coli* binds phospholipid vesicles in a calcium dependent manner (Plant *et al.*, 1997). *In vivo*, the C2 domain is responsible for the Ca^{2+}-dependent redistribution of Nedd4 from cytoplasm to membrane in canine kidney cells, possibly bringing it near its membrane associated targets, such as ENaC (Plant *et al.*, 1997). Therefore, the removal of C2 domain during apoptosis would render Nedd4 protein unable to redistribute and bind to some of its physiological targets. We noticed that in later stages of apoptosis, the 95kDa Nedd4 cleavage product is also degraded. Thus it is also possible that removal of the amino terminal region of Nedd4 makes it unstable and that the cleaved product is degraded. Abrogation of Nedd4 function during the induction of apoptosis may be an energy-saving exercise, or alternatively, Nedd4 may normally be required to mediate the turnover of a protein(s), which is required for the apoptotic function. Although the significance of Nedd4 cleavage during apoptosis is not entirely clear at present, the results in this chapter show that an enzyme of the ubiquitin pathway is cleaved by caspases during apoptosis.

Figure 1. Characterisation of the affinity purified Nedd4 antibody, N4ab2.

The GST-WW domain fusion protein used to inoculate rabbits was purified as a 67kDa protein (A). Affinity purified N4ab2 was assayed for its ability to detect the immunising GST-WW antigen (B), murine Nedd4 in murine tissues and cell lysates (C) and human Nedd4 in human cell lysates (D). N4ab2 did not detect any proteins in control *E. Coli* lysates (not shown).

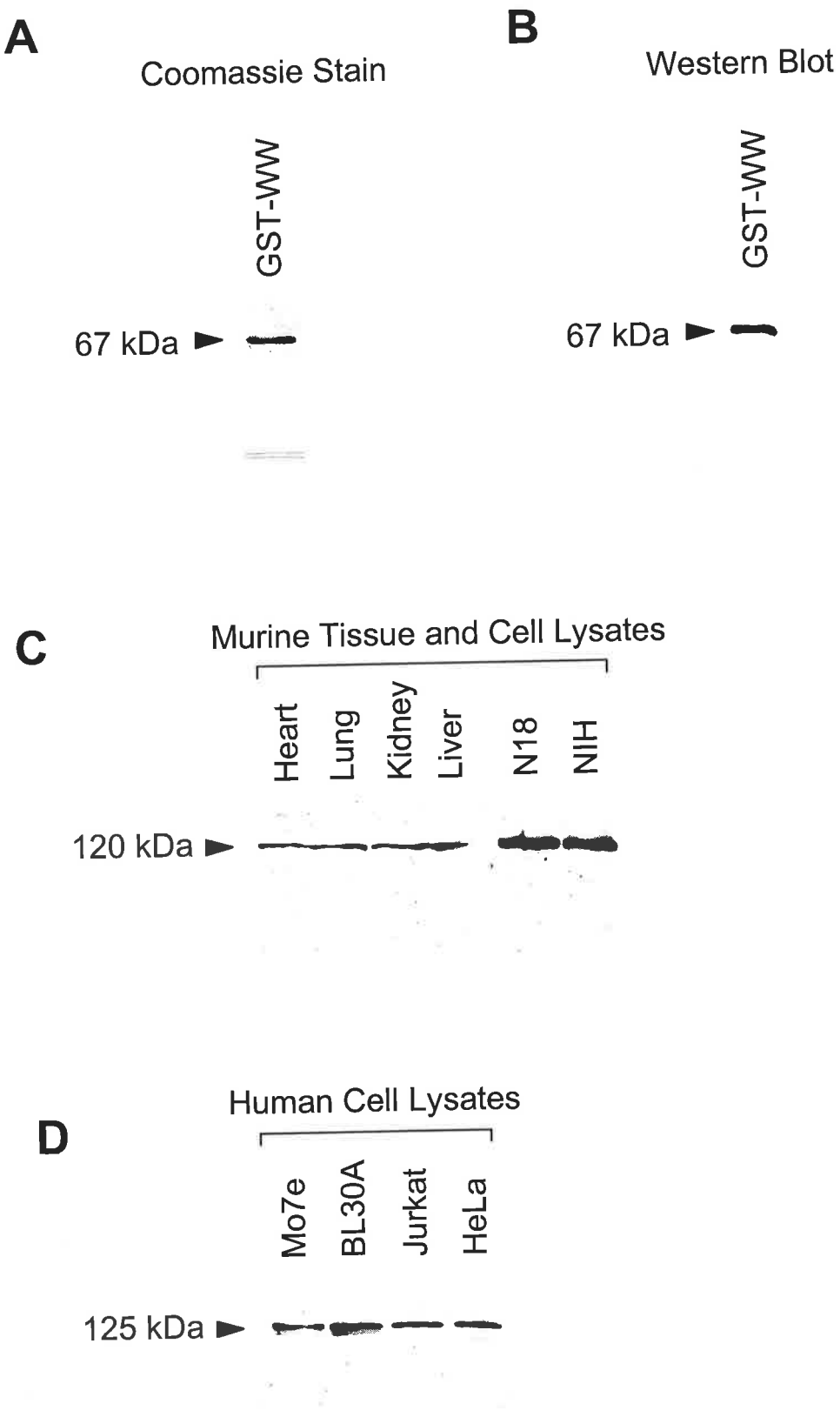
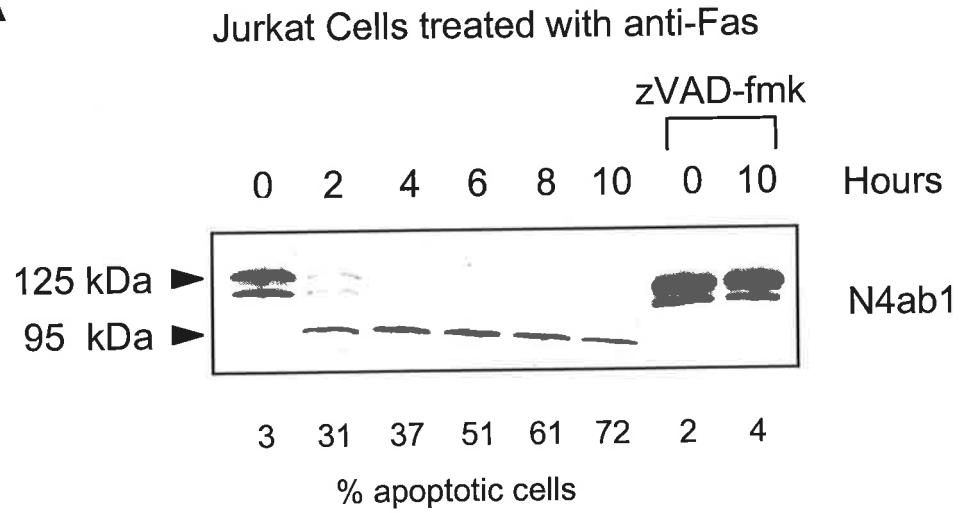

A
Coomassie Stain
GST-WW
67 kDa
B
Western Blot
GST-WW
67 kDa
C
Murine Tissue and Cell Lysates
Heart
Lung
Kidney
Liver
N18
NIH
120 kDa
D
Human Cell Lysates
Mo7e
BL30A
Jurkat
HeLa
125 kDa

Figure 2. Nedd4 protein undergoes cleavage during Fas-mediated apoptosis in Jurkat T-cells.

Jurkat T-cells were treated with an anti-Fas monoclonal antibody at a concentration of 200 ng/ml for the indicated duration of time. Cell extracts were subjected to immunoblot analyses using two separate antibodies against Nedd4 (A, N4ab2 and B, N4ab1). % apoptotic cells as assessed by 4,6-diamino-2-phenylindole (DAPI) staining of the sample used for immunoblotting are indicated. Cells pre-treated with zVAD-fmk before incubation with anti-Fas antibody were also assayed for Nedd4 cleavage by immunoblotting.

A


Jurkat Cells treated with anti-Fas
zVAD-fmk
0 2 4 6 8 10 0 10 Hours
125 kDa
95 kDa
N4ab1
3 31 37 51 61 72 2 4
% apoptotic cells

B

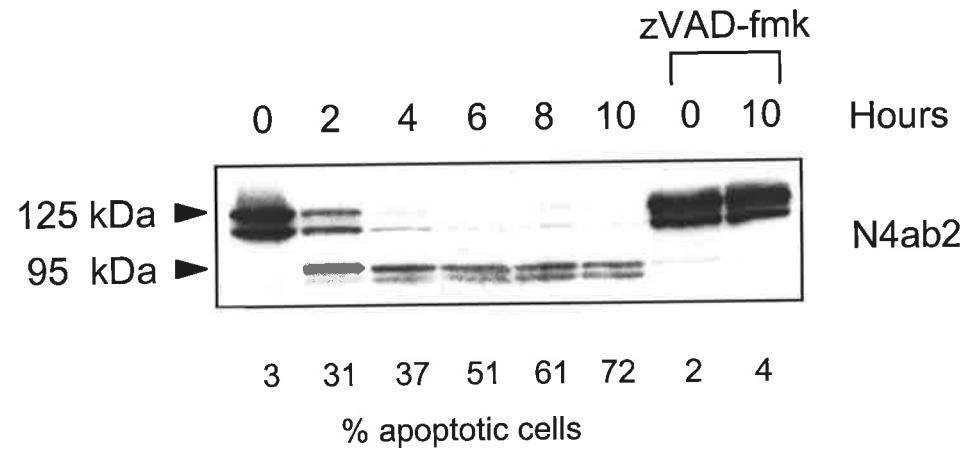
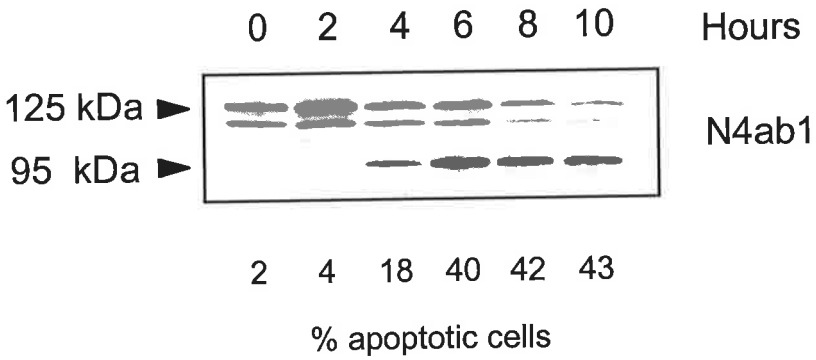

Jurkat Cells treated with anti-Fas
zVAD-fmk
0 2 4 6 8 10 0 10 Hours
125 kDa
95 kDa
N4ab2
3 31 37 51 61 72 2 4
% apoptotic cells

Figure 3. Cleavage of Nedd4 in Jurkat T cells following treatment with 40 μM etoposide.

A, B: Jurkat T-cells were treated with 40 μM etoposide and at the indicated times following treatment, cell extracts were subjected to immunoblot analysis using two separate Nedd4 antibodies (A, N4ab2 and B, N4ab1). % apoptotic cells as assessed by DAPI staining of the sample used for immunoblotting are indicated.

A

Jurkat Cells treated with etoposide



B

Jurkat Cells treated with etoposide

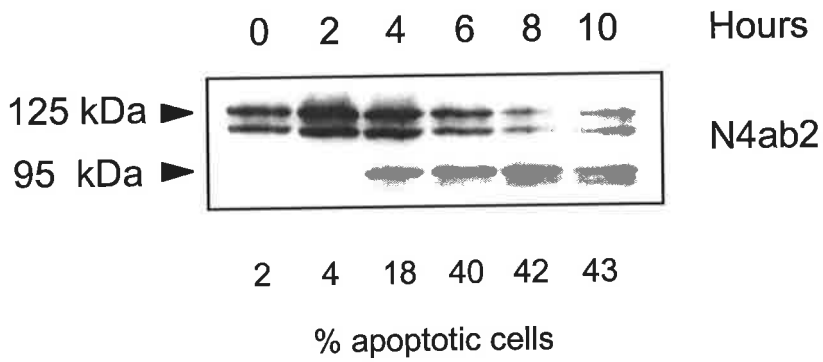
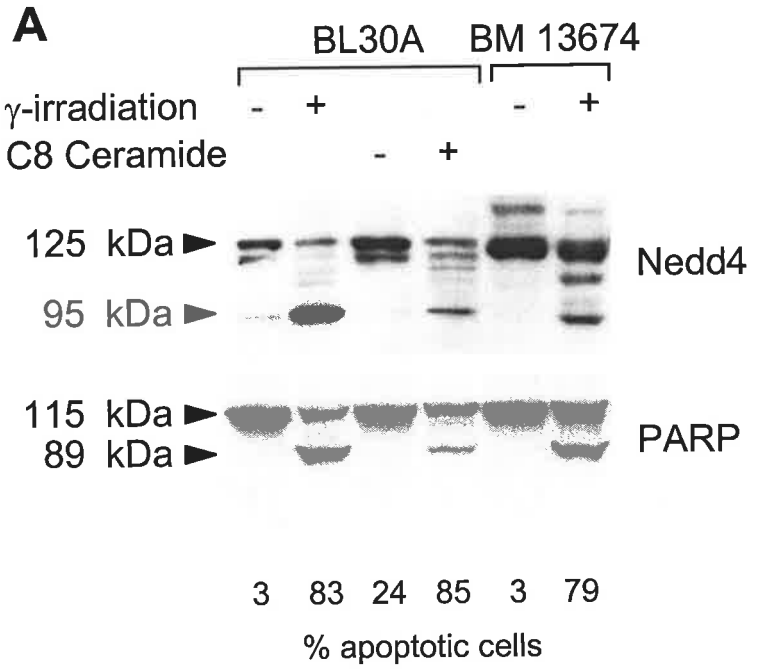
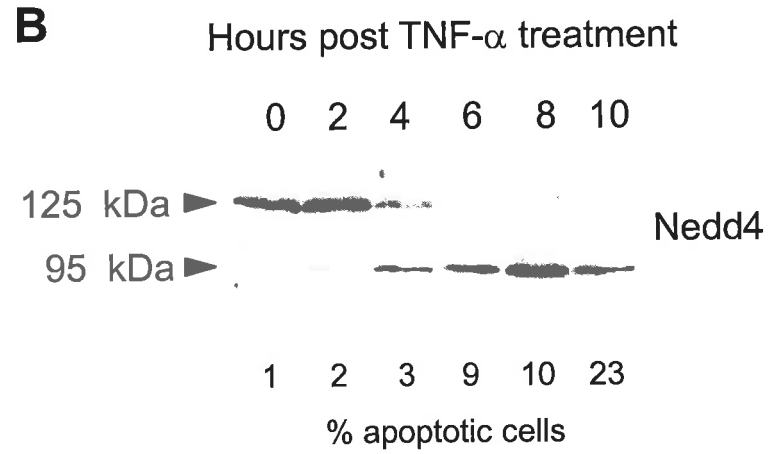


Figure 4. Nedd4 cleavage occurs in different cell types and in response to various apoptosis inducing signals.

Various cultured cells, as indicated, were exposed to either 20 Gy of γ-radiation and harvested 8 h post-treatment (A and C), 20 μM C-8 ceramide for 24 h (A), or 10 ng/ml TNF-α and 10 μg/ml cycloheximide for the indicated duration of time (B). Three Burkitt's lymphoma cell lines which are resistant to apoptosis induced by γ-irradiation did not show any appreciable cleavage of Nedd4 protein (C). Cell extracts were subjected to immunoblotting using N4ab2. % apoptotic cells as assessed by DAPI staining of the sample used for immunoblotting are indicated. Note that BL29 cells express considerably lower amounts of Nedd4 protein compared to other cell lines. Immunoblots in A and C of this figure were performed by Nigel Waterhouse and Julie Michael from the laboratory of Dianne Watters, Queensland Institute of Medical Research.


A
BL30A
BM 13674
γ-irradiation
- + - +
C8 Ceramide
- +
125 kDa
95 kDa
Nedd4
115 kDa
89 kDa
PARP
3 83 24 85 3 79
% apoptotic cells


B
Hours post TNF-α treatment
0 2 4 6 8 10
125 kDa
95 kDa
Nedd4
1 2 3 9 10 23
% apoptotic cells

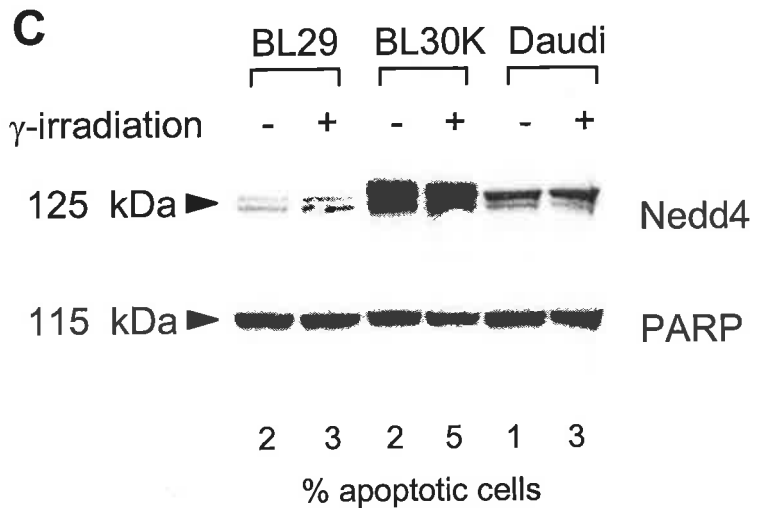

C
BL29
BL30K
Daudi
γ-irradiation
- + - + - +
125 kDa
Nedd4
115 kDa
PARP
2 3 2 5 1 3
% apoptotic cells

Figure 5. *In vitro* cleavage of mouse and human Nedd4 protein by various caspases.

^{35}S-met labelled mouse (A) and human (B) Nedd4 proteins were generated by *in vitro* translation and incubated with recombinant caspases or extracts prepared from apoptotic Jurkat cells. Where indicated, cell extracts (CE) were incubated with 100 nM of either YVAD-cmk or DEVD-CHO for 30 min prior to the addition of Nedd4 protein. Major cleavage products are shown on the left-hand side of the panels. Minor cleavage bands and intermediates are indicated by open arrowheads on the right hand side.

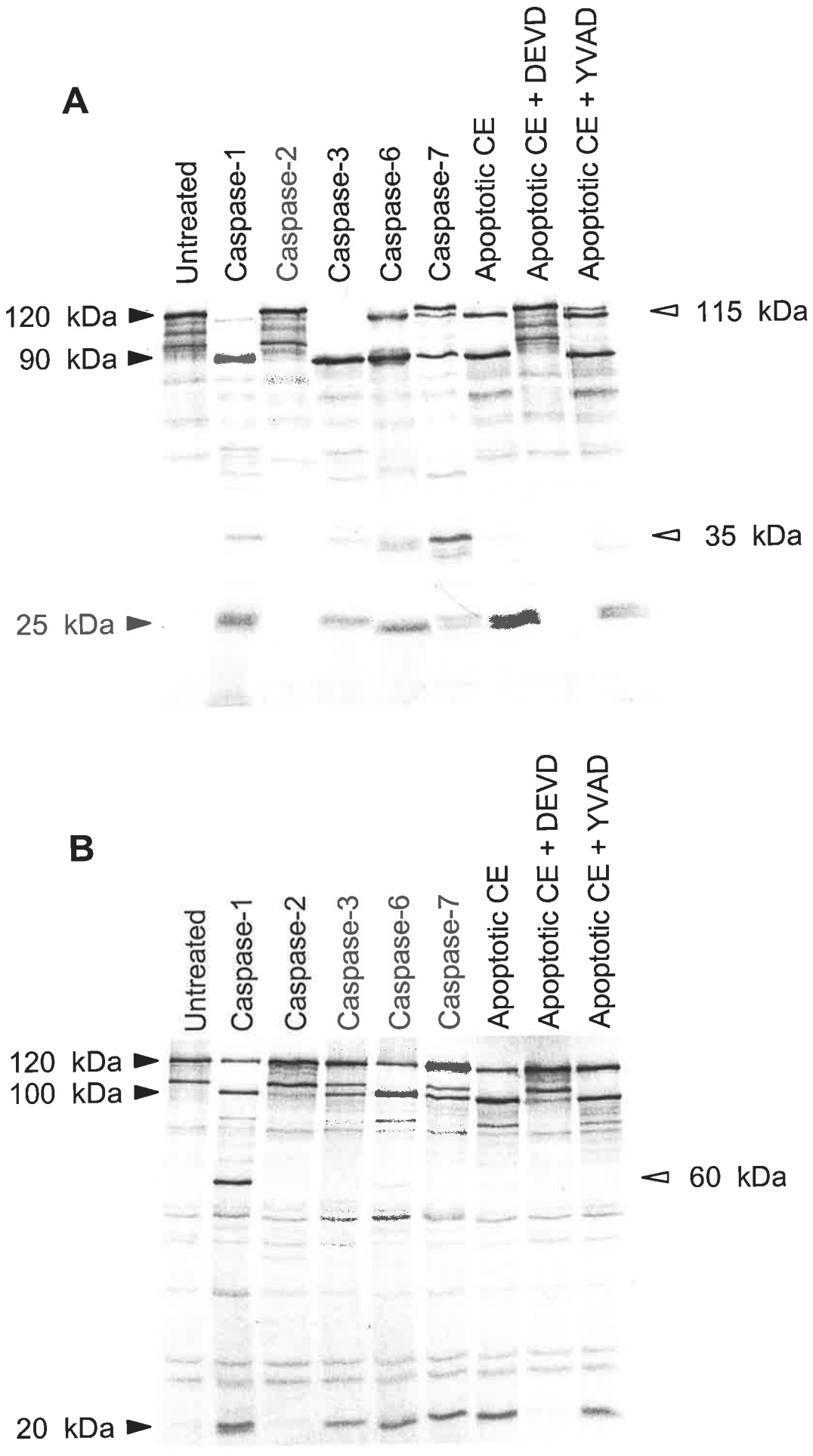

A
Untreated
Caspase-1
Caspase-2
Caspase-3
Caspase-6
Caspase-7
Apoptotic CE
Apoptotic CE + DEVD
Apoptotic CE + YVAD
120 kDa
90 kDa
25 kDa
115 kDa
35 kDa
B
Untreated
Caspase-1
Caspase-2
Caspase-3
Caspase-6
Caspase-7
Apoptotic CE
Apoptotic CE + DEVD
Apoptotic CE + YVAD
120 kDa
100 kDa
20 kDa
60 kDa

Figure 6. Structure of Nedd4 proteins used for *in vitro* translation and the location of various putative caspase cleavage sites.

Calcium/lipid binding domain (C2), WW domains (numbered 1-4) and ubiquitin-protein ligase domain (Hect) are indicated. The three DxxD sequences conserved between mouse, human and rat Nedd4 are shown (D206, 815 and 927 in human and D237, 774 and 887 in mouse). The position of DxxD sites mutated in mouse (D237, D308, D313 and D336) are indicated as well as D41 which is believed to be cleaved *in vitro* and is only present in mouse Nedd4. The regions of mouse Nedd4 protein that were used to raise the two polyclonal rabbit antisera (N4ab1 and N4ab2) are indicated by bars.

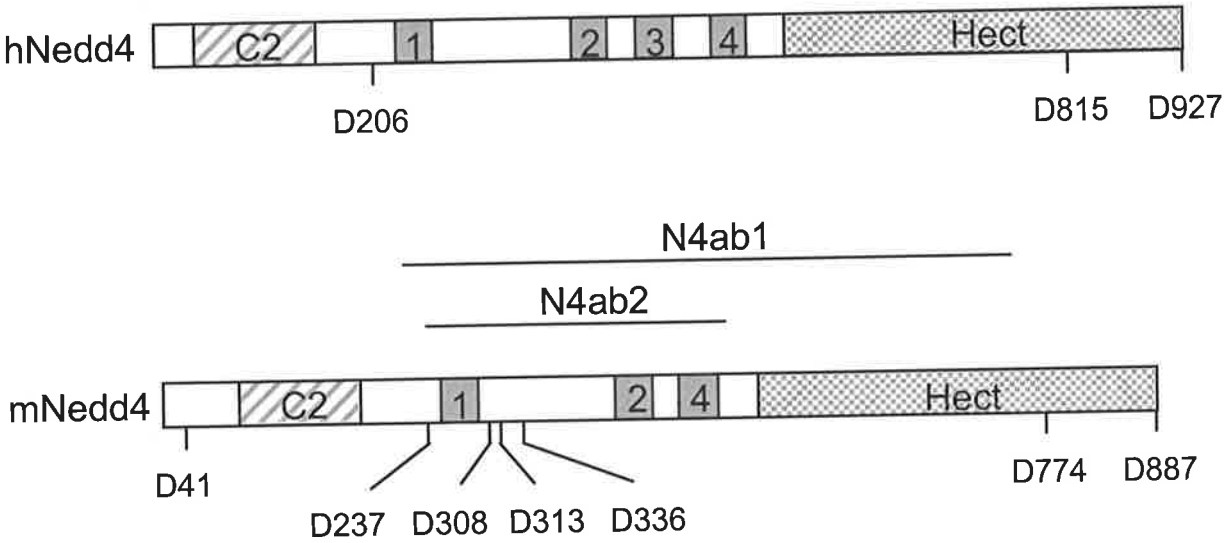
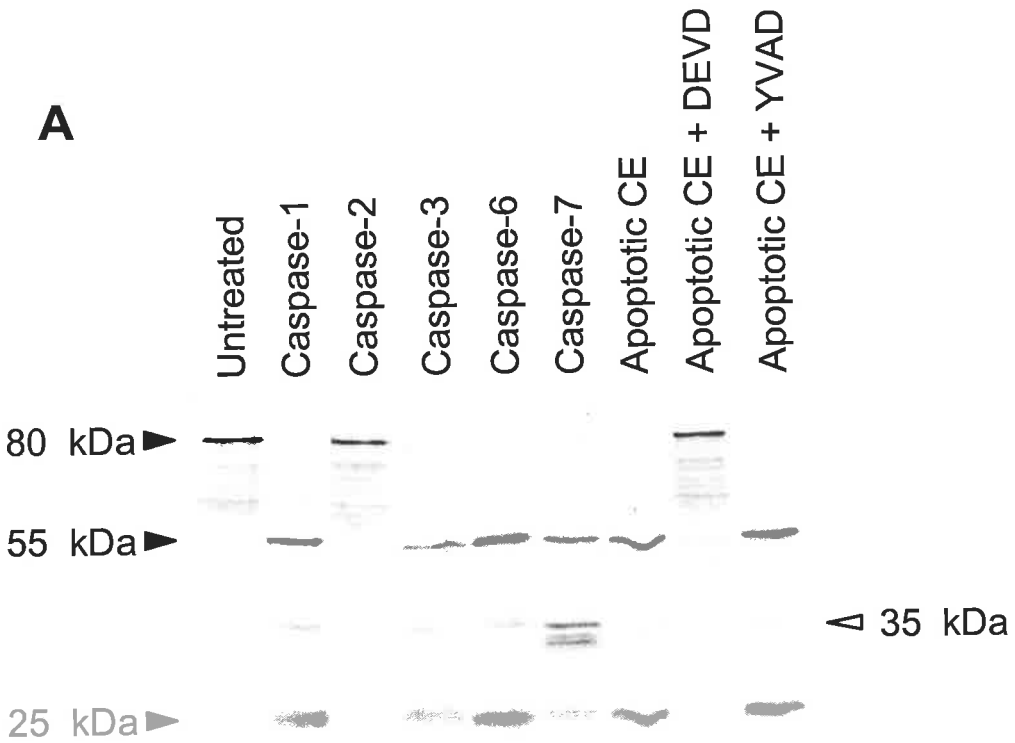

hNedd4
C2
1
2
3
4
Hect
D206
D815
D927
N4ab1
N4ab2
mNedd4
C2
1
2
4
Hect
D41
D237
D308
D313
D336
D774
D887

Figure 7. Mapping of the caspase cleavage sites in the Nedd4 protein.

Truncated versions of the wild type (A) or D237G mutant (B) Nedd4 proteins were translated from cDNA constructs lacking the coding region for the carboxyl terminal 352 residues of the mouse Nedd4 protein. ^{35}S-met labelled proteins were exposed to various caspases and extracts from apoptotic Jurkat cells (CE) as described in Figure 5. Major cleavage products are shown on the left-hand side of the panels. Minor cleavage bands and intermediates are indicated by open arrowheads on the right hand side.


A
Untreated
Caspase-1
Caspase-2
Caspase-3
Caspase-6
Caspase-7
Apoptotic CE
Apoptotic CE + DEVD
Apoptotic CE + YVAD
80 kDa
55 kDa
25 kDa
35 kDa

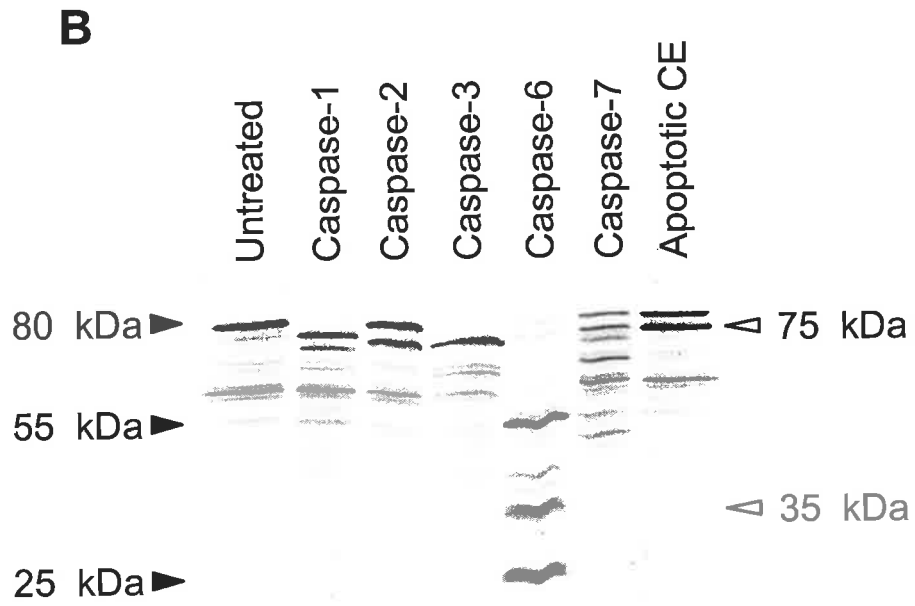
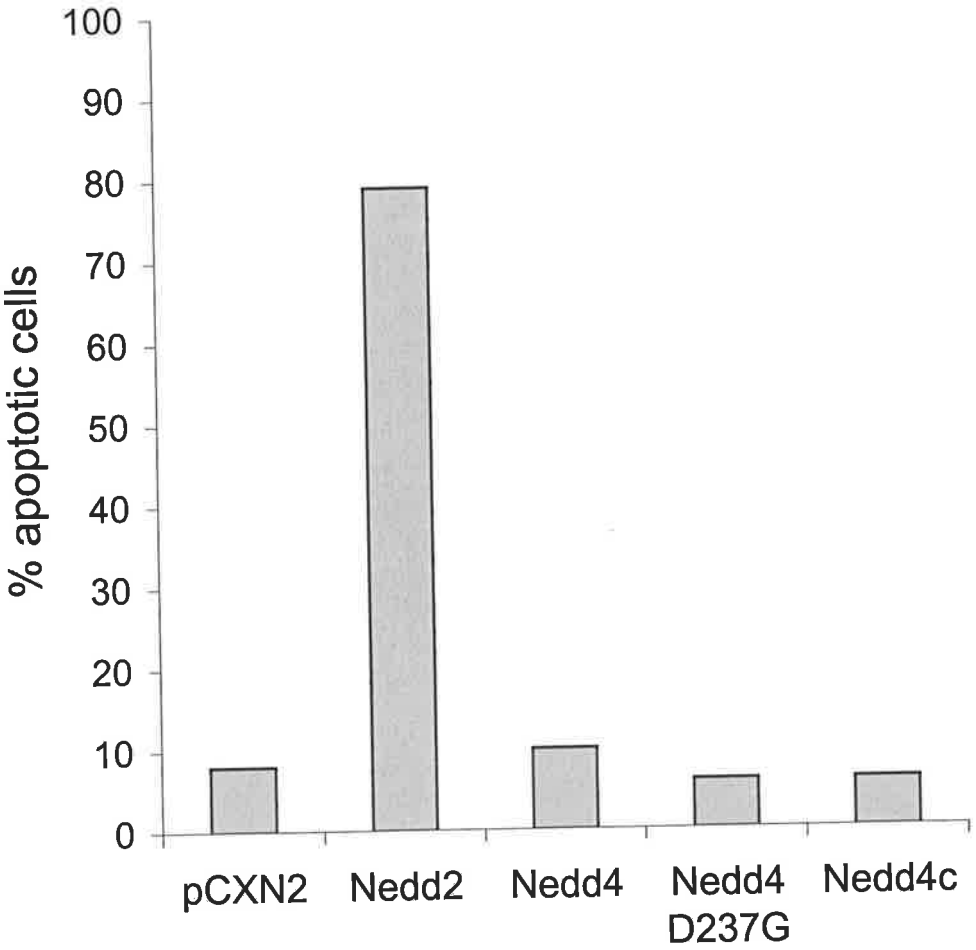

B
Untreated
Caspase-1
Caspase-2
Caspase-3
Caspase-6
Caspase-7
Apoptotic CE
80 kDa
55 kDa
25 kDa
75 kDa
35 kDa

Figure 8. Expression of wild type and mutant Nedd4 does not induce apoptosis.

Various expression constructs in the pCXN2 vector were co-transfected with a β-galactosidase expression vector. At 18 hours post-transfection, cells were washed, fixed and stained with X-gal. β-galactosidase positive blue cells were scored for apoptotic morphology. Data, derived from two separate experiments performed in duplicate, are presented as mean % morphologically apoptotic cells in the total β-galactosidase positive cells. Nedd4c represents the construct containing the coding region for an amino-terminally truncated mouse Nedd4 protein (amino acids 238-887).


% apoptotic cells
100
90
80
70
60
50
40
30
20
10
0
pCXN2
Nedd2
Nedd4
Nedd4 D237G
Nedd4c

Chapter 4: Nedd4 mediates downregulation of the epithelial Na^+ channel in response to increased intracellular Na^+

4.1 Introduction

The discovery that Nedd4 is cleaved during apoptosis suggested that it might be important to deactivate Nedd4 during cell death to conserve energy, but did not help to elucidate the physiological function of Nedd4. The presence of a carboxyl terminal hect domain suggests that Nedd4 is a ubiquitin-protein ligase. One characteristic feature of hect domain proteins is the ability to form a thioester with ubiquitin *in vitro*, an interaction that is mediated through a conserved Cys residue in the hect domain (Huibregtse *et al.*, 1995). The first biochemical evidence supporting the hypothesis that Nedd4 was a ubiquitin-protein ligase came from Hatakeyama *et al.* (1997). Their studies showed that bacterially expressed Nedd4 could stimulate non-specific ubiquitination of bacterial proteins in the presence of a particular subclass of ubiquitin-conjugating enzymes (Hatakeyama *et al.*, 1997). Therefore it appeared that Nedd4 was a bone-fide ubiquitin-protein ligase, although no substrates of Nedd4 had been identified.

The presence of a C2 domain capable of redistributing Nedd4 to the cytoplasmic membrane suggested that Nedd4 might target one or more membrane proteins for ubiquitination. The first protein identified as a potential substrate of Nedd4 was the epithelial sodium channel (ENaC). Staub *et al.* (1996), identified rat Nedd4 as an interacting partner of the carboxyl terminus of the β subunit of rat ENaC using the yeast 2-hybrid system. They subsequently showed that both α and γ ENaC subunits could also interact with Nedd4 and that these interactions were dependent on the WW domains of Nedd4 and on PY motifs present in each ENaC subunit (Staub *et al.*, 1996). This finding was of great significance because the carboxyl terminus of both β and γ ENaC subunits had been shown to be mutated in Liddle's Syndrome, a heritable form of hypertension caused by increased Na^+ absorption in the distal nephron. Specifically, Liddle's syndrome patients were identified that carried genes encoding for truncated β and γ ENaC subunits (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a). These truncations removed a Pro-rich

region that was thought be a negative regulatory domain because when it was removed, ENaC activity increased (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a). It was postulated that the Pro-rich regions in the ENaC carboxyl termini may bind to an SH3 domain-containing protein, which recognise the motif, PXXP (McDonald and Welsh, 1995). The carboxyl terminus region of α ENaC was found to bind *in vitro* to SH3 domains from a number of proteins including c-abl, grb2 and fyn, but no candidate negative regulatory proteins were identified (McDonald and Welsh, 1995). In 1995 a new Liddle's syndrome mutation was discovered in the β subunit of ENaC. Specifically, a missense mutation at position 616 of β ENaC resulted in Tyr being substituted to Leu (Hansson *et al.*, 1995b). This mutation destroyed a PY motif recognised by WW domains, suggesting that the hypothesised negative regulatory protein may in fact be a WW domain-containing protein and not an SH3 domain-containing protein as first thought.

Subsequent to the discovery that the WW domains of Nedd4 could bind to ENaC *in vitro*, Staub *et al.* (1997), showed that the abundance and activity of ENaC was regulated by ubiquitination. They showed that α and γ, but not β ENaC subunits were ubiquitinated *in vivo* on several cytoplasmic Lys residues and that mutation of some of these residues increased the stability and surface expression of ENaC (Staub *et al.*, 1997). Additionally, when channels carrying these Lys mutations were expressed in *Xenopus* oocytes, a higher than normal Na^+ conductance was observed (Staub *et al.*, 1997). Ubiquitination of membrane proteins often causes them to be endocytosed. They can then either be recycled back to the membrane or undergo lysosomal or proteasomal degradation (Hicke, 1999). Staub *et al.* (1997), showed that inhibitors of both of these proteolytic activities increased the stability of ENaC, suggesting that ENaC is endocytosed and degraded in the lysosome following ubiquitination, but may also be degraded by the proteasome. Shimkets *et al.* (1997), confirmed the finding that ENaC activity is regulated by endocytosis by showing that dominant negative molecules of dynamin, which is

required for clathrin-mediated endocytosis, increased ENaC activity in *Xenopus* oocytes.

These studies demonstrated that Nedd4 could interact with ENaC *in vitro* and that ENaC is ubiquitinated, endocytosed and degraded in the lysosome, but no direct evidence had been produced demonstrating that Nedd4 negatively regulates ENaC activity by ubiquitination. In order to determine whether Nedd4 was involved in ENaC regulation, a collaboration was initiated with David Cook's laboratory at the University of Sydney. They had extensive experience in measuring ENaC activity in mouse mandibular duct cells using the whole cell patch-clamp technique. Mandibular duct cells are present in the salivary glands where both amiloride-sensitive ENaC and Nedd4 are expressed (Dinudom *et al.*, 1998; Duc *et al.*, 1994), making it an ideal system to study the possible involvement of Nedd4 in the regulation of ENaC activity. Briefly, the whole cell patch-clamp technique involves isolation of a single mandibular duct cell, which is placed into a balanced salt solution (Figure 1). A pipette attached to the cell that acts as an electrode, and another electrode is placed into the balanced salt solution. First, the whole-cell current is recorded and then amiloride is perfused into the bath solution and the whole-cell current is recorded again. The current recorded in the presence of amiloride is subtracted from the first current reading to give a value for amiloride-sensitive current, which is representative of ENaC activity (for more detail see Materials and Methods, section 2.6).

Cook and colleagues have shown previously that changing the cytoplasmic concentration of solutes such as Na^+ and Cl^- can alter the activity of ENaC (Komwatana *et al.*, 1996; Komwatana *et al.*, 1998). This can be done by altering the concentration of solutes in the pipette solution. High intracellular concentrations of either Na^+ or Cl^- negatively impact upon Na^+ channel activity in mouse mandibular duct cells, presumably by initiating a feedback pathway which deactivates ENaC (Komwatana *et al.*, 1996; Komwatana *et al.*, 1998). The involvement of specific proteins in this regulation can also be studied by inclusion in the pipette solution of dominant negative molecules or purified

active proteins. For example, downregulation of ENaC activity has been shown to be mediated by G proteins (G_o in the case of Na^+ and G_{i2} in the case of Cl^-) by inclusion of active G proteins and dominant negative G proteins in pipette solution containing different concentrations of Na^+ and Cl^- (Komwatana *et al.*, 1996). The mechanism by which G proteins regulate the activity of ENaC is obscure. It is unlikely that they interact with ENaC and directly alter channel activity or numbers so presumably they activate one or more proteins which ultimately downregulate ENaC activity.

The aim of the studies described in this chapter was to establish a direct involvement of Nedd4 in the regulation of ENaC, using the whole cell patch clamp technique. The results of this chapter have been published (Dinudom *et al.*, 1998).

Results

4.2 Nedd4 does not influence Na^+ channel activity when the Na^+ and Cl^- loops are inactive

It has been previously reported by Komwatana *et al.* (1996 and 1998), that when mouse mandibular duct cells are studied using the whole-cell patch clamp method with a Na^+-rich bath solution (containing 72 mM Na^+-glutamate) and a Na^+-free/low Cl^- pipette solution (containing 150 mM NMDG-Glutamate) that the major conductance seen is an amiloride sensitive Na^+ current. This conductance is not voltage-activated and is permeable to Li^{2+} but not K^+, properties that are characteristic of ENaC, which is known to be expressed in these cells (Duc *et al.*, 1994).

It has been widely reported that cells expressing ENaC subunits containing mutations which abolish or alter PY motifs in their intracellular carboxyl termini have increased ENaC activity phenotype (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a; Hansson *et al.*, 1995b; Tamura *et al.*, 1996; Jeunemaitre *et al.*, 1997). Additionally, these PY motifs are known to be capable of binding to the WW domains of Nedd4 (Staub *et al.*, 1996). This suggested that Nedd4 normally negatively regulates ENaC by contacting it via its PY motifs. This hypothesis was tested by attempting to prevent an interaction between Nedd4 and ENaC in mandibular duct cells, and analysing the resultant effect on ENaC activity. To do this, Na^+ channel activity was studied in mouse mandibular duct cells in a high Na^+ bath solution (containing 72 mM Na^+-glutamate) and a low Na^+ pipette solution (containing 150 mM NMDG-glutamate). In order to inhibit any interaction between Nedd4 and ENaC either: 1) the three WW domains of Nedd4 fused to GST (GST-WW, final concentration 300 μg/ml) or 2) purified IgG directed against the hect domain and three WW domains of Nedd4 (N4ab1, final concentration 1 μg/ml) were added to the pipette solution. It was predicted that the high concentration of GST-WW domain protein in the pipette solution would bind to the PY motifs of ENaC and exclude endogenous

Nedd4 from binding, and that N4ab1 would bind and sequester endogenous Nedd4, thereby inhibiting its interaction with ENaC. It was found that neither GST-WW nor N4ab1 increased amiloride-sensitive Na^+ current above the level observed with 150 mM Na^+-glutamate in the pipette solution (Figure 2A). Furthermore we showed that neither GST-WW nor N4ab1 caused an increase in amiloride-sensitive Na^+ conductance above that of the control protein, GST, or pre-immune serum IgG in the 150 mM Na^+-glutamate pipette solution (Figure 2A). It was concluded from these experiments that Nedd4 does not have an inhibitory effect on ENaC activity when the Na^+ and Cl^- feedback systems are inactive.

4.3 Nedd4 mediates the Na^+ feedback loop

It has been shown previously that when the Na^+ concentration of the pipette solution is increased from 0 mM to 72 mM [a value within the physiological range for cytosolic Na^+ concentration in exocrine tissues (Xu *et al.*, 1995; Poronnik *et al.*, 1995)], the amiloride-sensitive Na^+ conductance of mouse mandibular duct cells is abolished (Komwatana *et al.*, 1996; Komwatana *et al.*, 1998). This suggests that a feedback system is in place in these cells that is activated when cytosolic Na^+ levels increase and the requirement for Na^+ import is reduced. To investigate whether Nedd4 is involved in inhibition of Na^+ channel activity by increased cytoplasmic Na^+ we added GST-WW (final concentration 300 μg/ml) to pipette solution containing 72 mM Na^+-glutamate. It was found that amiloride-sensitive Na^+ conductance was restored to a level similar to that observed when using pipette solution containing 0 mM Na^+, thus completely abolishing the inhibitory effect of cytosolic Na^+ (Figure 2B). Inclusion of the control GST protein failed to prevent Na^+-dependent inhibition of amiloride-sensitive Na^+ conductance (Figure 2B). These findings were confirmed by showing that inclusion of N4ab1 in pipette solution containing 72 mM Na^+-glutamate also completely restored amiloride-sensitive

Na^+ conductance, whereas addition of control pre-immune serum IgG had no effect (Figure 2B). These experiments provided the first evidence that Nedd4 is indeed involved in the downregulation of ENaC, and functions under high Na^+ conditions, when the requirement of ENaC to import Na^+ is reduced.

4.4 Nedd4 does not mediate the Cl^- feedback loop

It has also been reported that Na^+ conductance in mandibular duct cells can be inhibited by the presence of anions such as Cl^-, Br^- and NO^{3-} in the cytosol of these cells (Dinudom *et al.*, 1993). To test whether Nedd4 is also involved in anion-dependent feedback inhibition of ENaC, GST-WW (final concentration 300 μg/ml) or N4ab1 (final concentration 1μg/ml) was added to pipette solution containing NMDG-NO^{3-}. It was found that addition of neither GST-WW nor N4ab1 in the pipette solution reversed the inhibitory effects of NMDG-NO^{3-} on amiloride-sensitive Na^+ conductance. Therefore it was concluded that Nedd4 is not involved in anion-dependent feedback inhibition of ENaC (Figure 2C).

4.5 Nedd4 mediates the effect of G_o on Na^+ channels

As mentioned earlier, (Komwatana *et al.*, 1996), showed that the α subunit of the G protein, G_o, mediates the inhibitory effect of cytosolic Na^+ on ENaC in mandibular duct cells. Therefore it was decided to test whether Nedd4 is involved in G_o-mediated inhibition of ENaC in mandibular duct cells. Initially it was re-confirmed that inclusion of the activated α subunit of G_o (at a final concentration of 0.2 μM) in pipette solution containing 0 mM Na^+-glutamate and 150 mM NMDG-glutamate almost completely inhibited amiloride-sensitive Na^+ conductance (Figure 3). Subsequently it was found that inclusion of N4ab1 (1 μg/ml) in the pipette solution completely overcame the inhibitory effect of adding activated G_o to pipette solution containing NMDG-glutamate (Figure 3).

In contrast, the control pre-immune serum IgG did not overcome G_o-dependent inhibition. This demonstrates that Nedd4 mediates G_o-dependent inhibition of ENaC in mandibular duct cells. The inhibitory effect of G_{i2} [which mediates the effect of the anion feedback system on Na^+-channel activity (Komwatana *et al.*, 1996)], was not inhibited by N4ab1 (Figure 3). This result was expected as it had been shown in section 4.4 above, that Nedd4 was not involved in anion-dependent feedback inhibition of ENaC.

4.6 Downregulation of Na^+ channel activity by intracellular Na^+ requires ubiquitin

ENaC activity and stability has been shown to be regulated by ubiquitination of the α and γ subunits (Staub *et al.*, 1997). This, coupled with the findings in this chapter (section 4.3) that Nedd4 mediated Na^+-dependent feedback inhibition of ENaC in mandibular duct cells led us to investigate the possibility that ubiquitination was involved in this control pathway. To do this, a dominant negative ubiquitin molecule fused to GST was produced that had the Lys residue at position 48 mutated to Arg (K48R). Lys 48 is required for the formation of polyubiquitin chains on protein substrates and the introduction of K48R ubiquitin to cells *in vitro* has been shown to increase the stability of proteins that are normally regulated by the ubiquitin system (Chau *et al.*, 1989), (Ward *et al.*, 1995). Inclusion of the GST-K48R ubiquitin mutant (300 μg/ml) in pipette solution containing 72 mM Na^+-glutamate completely overcame the inhibitory effects of cytoplasmic Na^+ on ENaC activity (Figure 4). In contrast, wildtype GST-ubiquitin did not overcome this inhibition (Figure 4). Additionally K48R ubiquitin was shown to have no effect on amiloride-sensitive Na^+ conductance when the Na^+-feedback system was inactive (Figure 4).

4.7 Discussion

In this chapter dominant negative molecules of Nedd4 and antibodies directed against Nedd4 were employed to investigate ENaC regulation in mouse mandibular duct cells. The principal findings of these studies are: 1) Nedd4 mediates the inhibitory effect of increased cytosolic Na^+ on amiloride-sensitive Na^+ channels, and does so downstream of the G protein, G_o, 2) The Na^+ feedback pathway is dependent on ubiquitin, 3) Nedd4 does not alter the activity of ENaC when Na^+ import is required and 4) Nedd4 does not mediate the negative feedback pathway that is exerted on ENaC by increased cytosolic anions.

Prior to this study the WW domains of Nedd4 had been shown to interact with the PY motifs of ENaC and it had been postulated that Nedd4 might negatively regulate ENaC activity by ubiquitin-mediated proteolysis (Staub *et al.*, 1996). The finding that Nedd4 WW domains and Nedd4 antibodies can interfere with the Na^+ feedback pathway provided the first evidence that Nedd4 does indeed negatively regulate ENaC activity. This, coupled with the observation that dominant negative molecules of ubiquitin interfered with Na^+-dependent feedback inhibition of ENaC suggests that Nedd4 downregulates ENaC by ubiquitinating it. This suggestion is supported by the finding that the α and γ subunits of ENaC are ubiquitinated *in vivo* (Staub *et al.*, 1997). Ubiquitination of membrane proteins is often a signal for them to be endocytosed and either degraded by the lysosomal or proteasomal pathways or recycled to the membrane (Hicke, 1999). ENaC has been shown to be internalised by clathrin-mediated endocytosis, a process that is inhibited by mutation or truncation of the PY motifs of β and γ ENaC, but not α ENaC (Shimkets *et al.*, 1997). Together these findings suggest that under high Na^+ conditions, when Na^+ import is not required, ENaC is ubiquitinated by Nedd4 and endocytosed, resulting in its deactivation.

It has previously been shown that the G protein, G_o, mediates the Na^+ feedback pathway in mouse mandibular duct cells (Komwatana *et al.*, 1996). The data presented

here show that Nedd4 functions downstream of G_o in the Na^+ feedback pathway. This suggests that in response to increased cytosolic Na^+, G_o is activated by some unknown mechanism and then stimulates Nedd4 to ubiquitinate ENaC, leading to its endocytosis and deactivation. The exact function of G_o in this process is unknown however. G_o may directly interact with and activate Nedd4 or activate an intermediate protein(s) that stimulates Nedd4-dependent ubiquitination of ENaC (Figure 5). Alternatively, in response to increased cytosolic Na^+, G_o may alter the conformation of ENaC in such a way that it can recognised and ubiquitinated by Nedd4. Further experiments are required to explore these possibilities. An alternative G protein, G_{i2}, mediates anion-dependent inhibition of ENaC (Komwatana *et al.*, 1996). The results presented here show that Nedd4 does not mediate this pathway, indicating that G_o and G_{i2} inhibit Na^+ channel activity by different mechanisms.

The findings presented here provide for the first time an explanation for the increase in Na^+ channel activity that is seen in Liddle's syndrome patients which lack the PY motifs that are responsible for interaction with Nedd4 *in vitro*. Similar results have subsequently been published by other groups (Abriel *et al.*, 1999; Goulet *et al.*, 1998).

Figure 1. Schematic diagram of the whole-cell patch-clamp technique

A single mandibular duct cell is placed in a balanced salt solution and a thin glass pipette is attached to the membrane. The pipette acts as an electrode and another electrode is placed in the balanced salt solution. The potential difference across the cellular membrane of the mandibular duct cell can then be measured which provides a readout of ENaC activity. Different proteins and chemicals can then be directly added to the pipette solution to investigate their effect on ENaC activity.

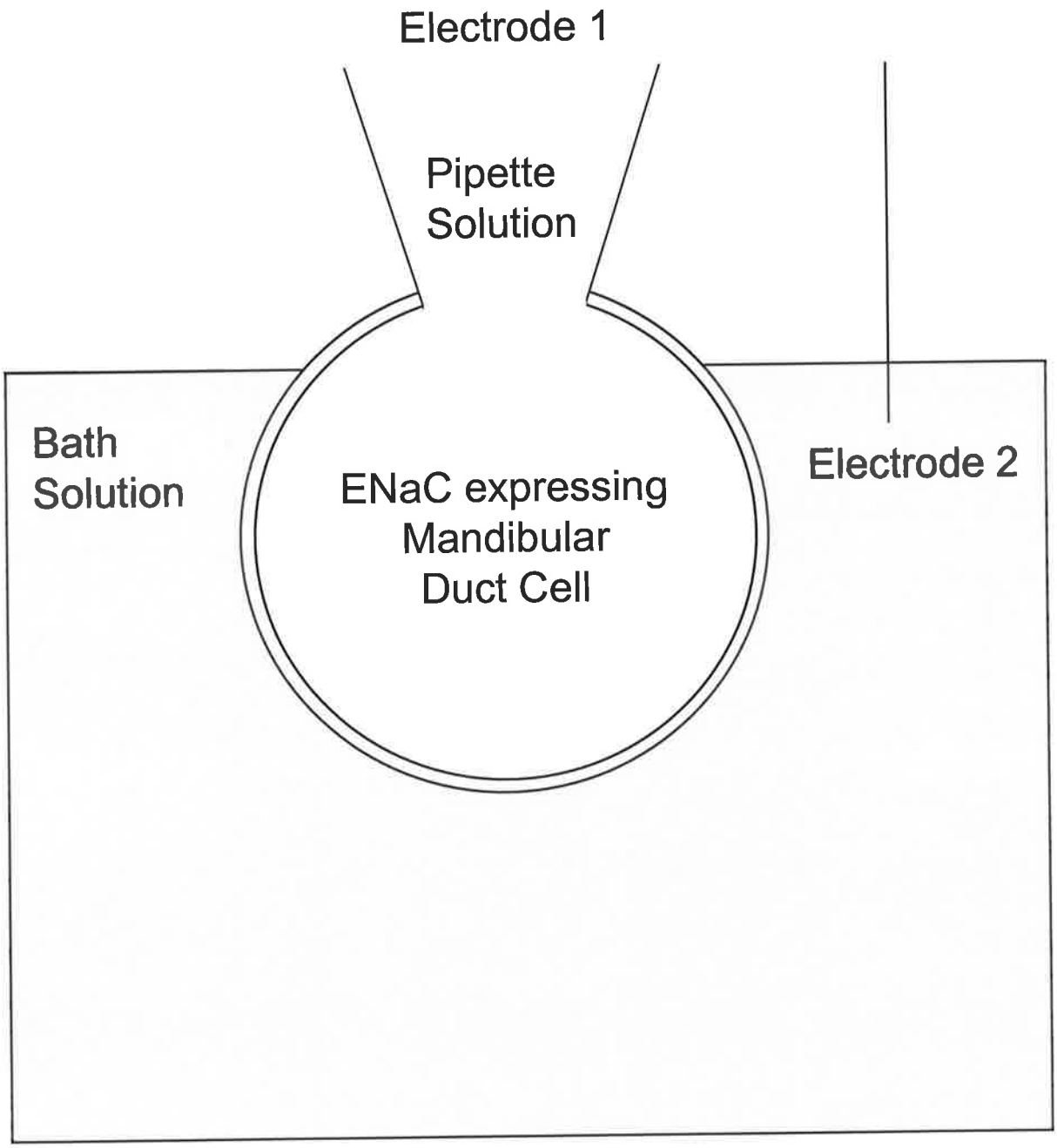

Electrode 1
Pipette
Solution
Bath
Solution
ENaC expressing
Mandibular
Duct Cell
Electrode 2

Figure 2. Nedd4 mediates the Na^+ feedback loop but not the Cl^- feedback loop

A, The effects of the inclusion of the inclusion in NMDG-glutamate pipette solution of GST-WW fusion protein (G-W), control GST protein (G), purified anti-Nedd4 IgG (A-N4), or control IgG purified from pre-immune serum (IgG) on the chord conductance measured at –80 mV of the amiloride-sensitive Na^+ conductance. The number of experiments performed under each condition is shown in brackets above the appropriate error bar.

B, The effects of the inclusion in 72 mM Na^+ pipette solution of control GST protein, GST-WW fusion protein, control IgG purified from pre-immune serum (IgG) or purified anti-Nedd4 IgG (A-N4) on the chord conductance measured at –80 mV of the amiloride-sensitive Na^+ conductance. The number of experiments performed under each condition is shown in brackets above the appropriate error bar.

C, The effects of the inclusion in NMDG-NO_3 pipette solution of GST-WW fusion protein or purified anti-Nedd4 IgG (A-N4) on the chord conductance measured at –80 mV of the amiloride-sensitive Na^+ conductance. The number of experiments performed under each condition is shown in brackets above the appropriate error bar.

The chord conductance observed using NMDG-glutamate solution is shown in each panel as a dotted line.

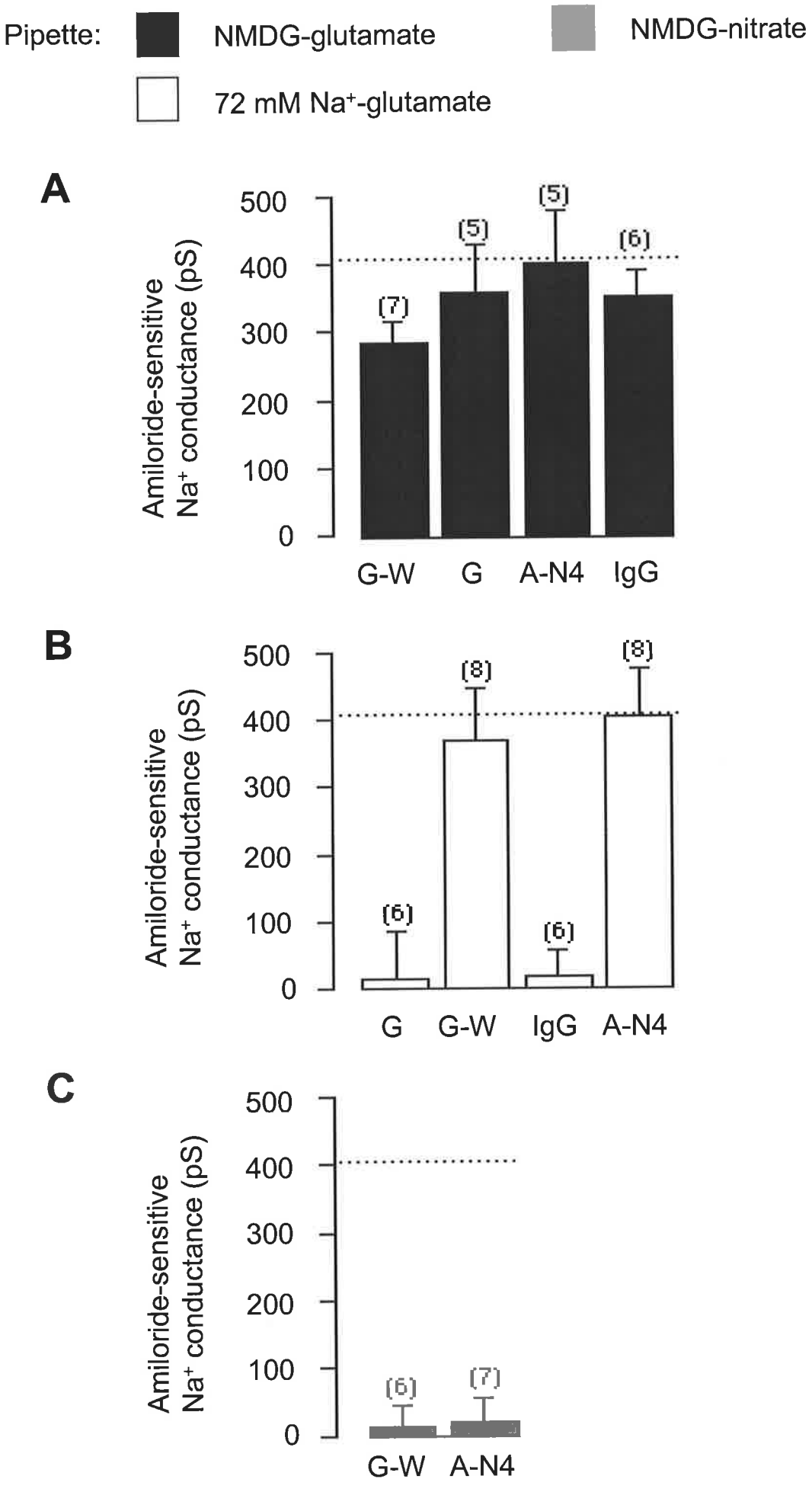

Pipette:
NMDG-glutamate
NMDG-nitrate
72 mM Na+-glutamate
A
Amiloride-sensitive Na+ conductance (pS)
500
400
300
200
100
0
(7)
(5)
(5)
(6)
G-W
G
A-N4
IgG
B
Amiloride-sensitive Na+ conductance (pS)
500
400
300
200
100
0
(6)
(8)
(6)
(8)
G
G-W
IgG
A-N4
C
Amiloride-sensitive Na+ conductance (pS)
500
400
300
200
100
0
(6)
(7)
G-W
A-N4

Figure 3. Nedd4 mediates the effects of G_o on Na^+ channels

The effects of the inclusion in NMDG-glutamate pipette solution of the activated α subunit of G_o (G_o), activated α subunit of G_o and control IgG purified from pre-immune serum (G_o + IgG), activated α subunit of G_o together with purified anti-Nedd4 IgG (G_o + A-N4), or activated α subunit of G_{i2} together with purified anti-Nedd4 IgG (G_{i2} + A-N4) on the chord conductance measured at –80 mV of the amiloride-sensitive Na^+ conductance. The chord conductance observed using NMDG-glutamate solution is shown as a dotted line. The number of experiments performed under each condition is shown in brackets above the appropriate error bar.

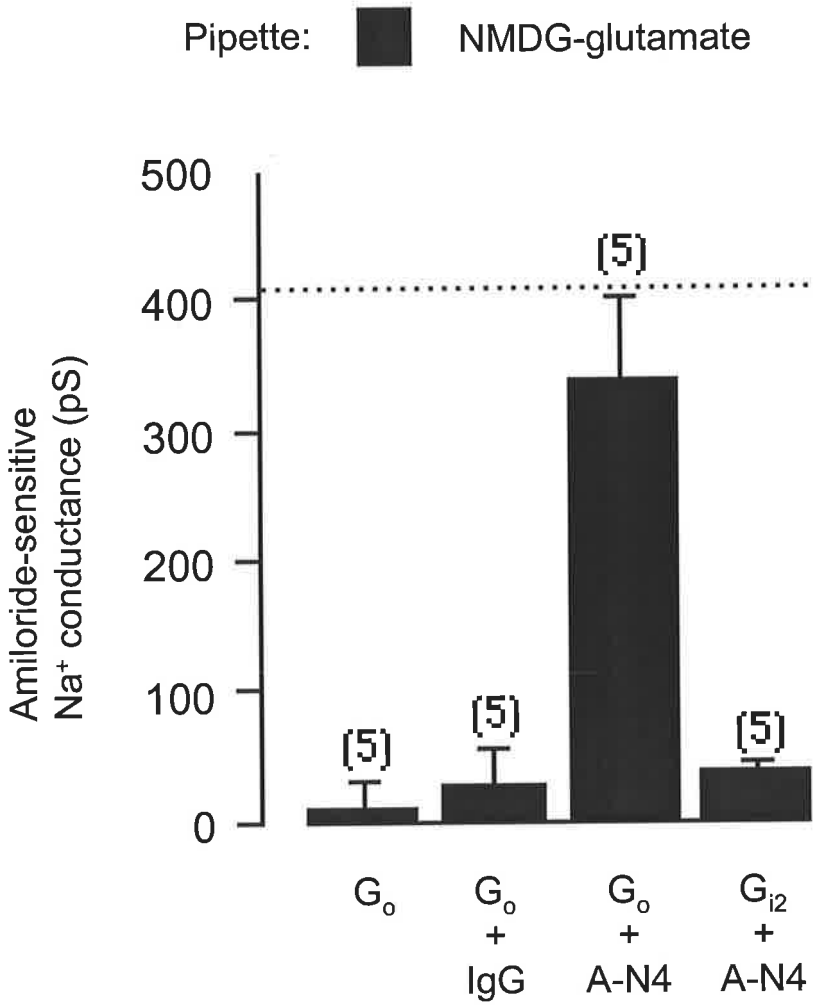

Pipette:
NMDG-glutamate
Amiloride-sensitive
Na+ conductance (pS)
500
400
300
200
100
0
(5)
(5)
(5)
(5)
Go
Go
+
IgG
Go
+
A-N4
Gi2
+
A-N4

Figure 4. Downregulation of Na^+ channel activity by intracellular Na^+ requires ubiquitin

The effects of the inclusion in 72 mM Na^+ pipette solution or in the NMDG-glutamate pipette solution of dominant negative GST-K48R ubiquitin (dn) or wildtype GST-ubiquitin (wt) on the chord conductance measured at –80 mV of the amiloride-sensitive Na^+ conductance. The number of experiments performed under each condition is shown in brackets above the appropriate error bar.

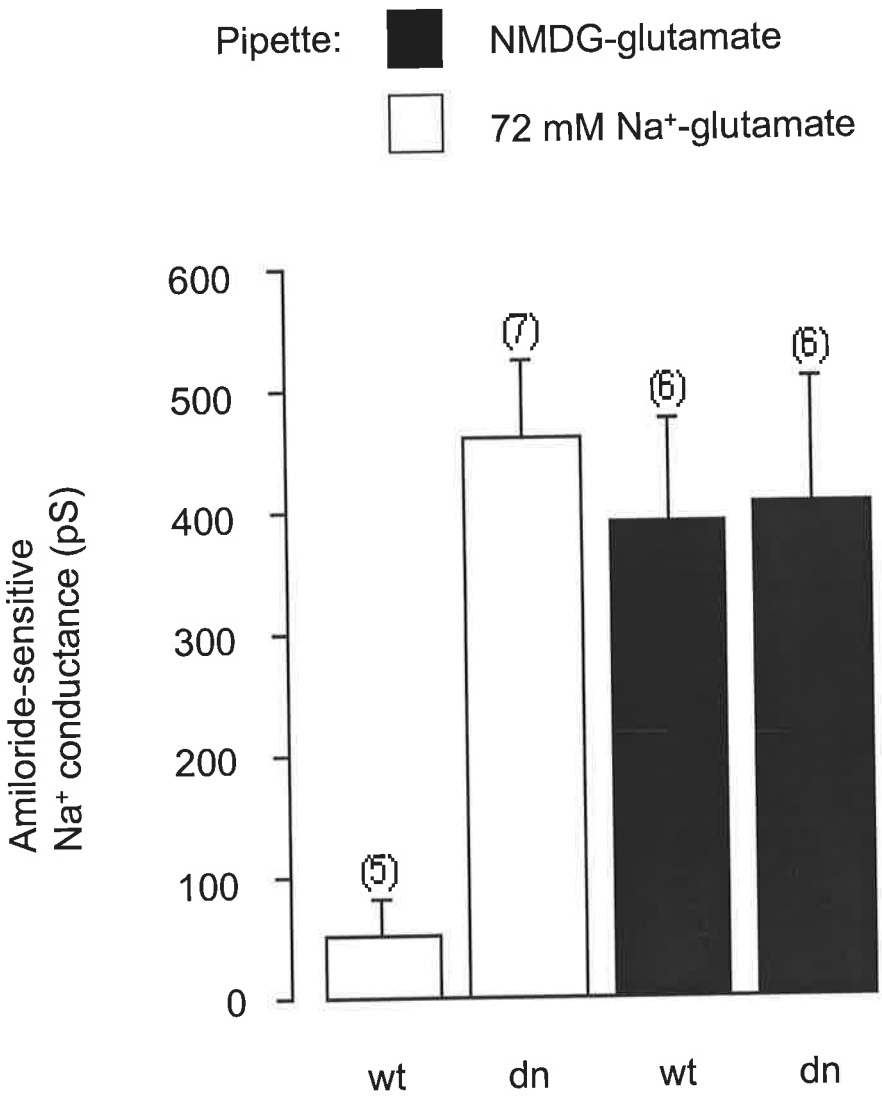
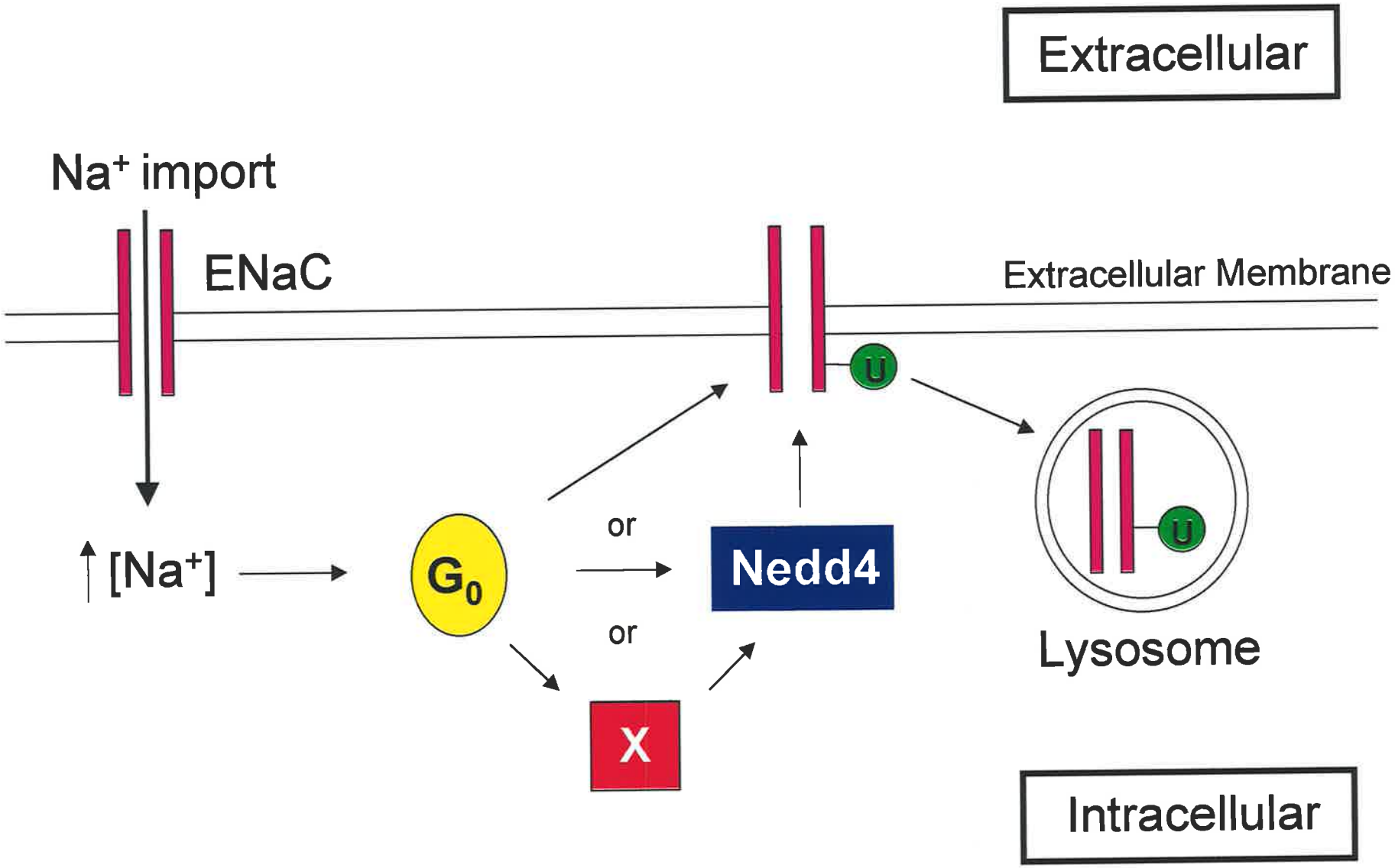

Pipette:
NMDG-glutamate
72 mM Na+-glutamate
Amiloride-sensitive
Na+ conductance (pS)
600
500
400
300
200
100
0
(5)
(7)
(6)
(6)
wt
dn
wt
dn

Figure 5. Model of Na^+-dependent feedback regulation of Na^+ channels acting through G proteins and Nedd4.

Increased intracellular Na^+ concentrations stimulate Nedd4 to associate with ENaC in a G_o-dependent manner. Nedd4 then catalyses ubiquitination of ENaC with leads to its endocytosis and degradation in the lysosome. It is unclear how G_o is activated by increased levels of intracellular Na^+ and whether it directly stimulates Nedd4 to associate with and ubiquitinate ENaC, stimulates an intermediate protein (X), which in turn activates Nedd4, or directly alters the conformation of ENaC to allow association with Nedd4.


Extracellular
Na+ import
ENaC
Extracellular Membrane
U
[Na+]
G0
or
Nedd4
or
X
U
Lysosome
Intracellular

Chapter 5:
All three WW domains of murine Nedd4 are involved in the regulation of the epithelial Na^+ channel by intracellular Na^+

5.1 Introduction

Results presented in the previous chapter demonstrated that Nedd4 is responsible for mediating downregulation of ENaC in response to increased cytosolic Na^+ in mouse mandibular duct cells. These results suggested that Nedd4 downregulates ENaC by ubiquitin-mediated proteolysis. Further support for this hypothesis was provided by Goulet *et al.* (1998), who showed that the catalytic Cys residue of Nedd4 that is essential for ubiquitin-protein ligase activity, was required for downregulation of ENaC activity in *Xenopus* oocytes (Goulet *et al.*, 1998). Additionally, in chapter 4 of this thesis, Nedd4 was shown to mediate Na^+ dependent inhibition of ENaC downstream of the G protein, G_o. However, the precise mechanisms by which Nedd4 is recruited to and interacts with ENaC remained obscure. Until recently, ENaC was thought to assume a tetrameric structure in the cytoplasmic membrane consisting of one β, one γ and two α subunits (Firsov *et al.*, 1998; Kosari *et al.*, 1998). More recently the native ENaC complex has been shown to consist of eight subunits with the same stoichiometry as previously reported (Eskandari *et al.*, 1999). The question that we wanted to ask is: how many WW domains of Nedd4 are required to interact with a single ENaC complex?

The carboxyl terminus of each ENaC subunit contains a PY motif, which when mutated or deleted in either the β or γ ENaC subunits leads to Liddle's Syndrome, an autosomal dominant form of hypertension (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a; Hansson *et al.*, 1995b; Tamura *et al.*, 1996; Jeunemaitre *et al.*, 1997). Therefore, mutating the PY motif of a single type of subunit (either β or γ) of the octameric ENaC complex presumably abolishes association with Nedd4 and is sufficient to lead to a disease phenotype. In *in vitro* systems, identical mutations to those that cause Liddle's Syndrome, have been shown to increase amiloride sensitive Na^+ current (Hansson *et al.*, 1995a; Hansson *et al.*, 1995b; Snyder *et al.*, 1995; Schild *et al.*, 1995; Schild *et al.*, 1996; Firsov *et al.*, 1996; Tamura *et al.*, 1996). This increase is believed to result from the presence of

increased numbers of active Na^+ channels in the cell membrane (Firsov *et al.*, 1996; Shimkets *et al.*, 1997; Kellenberger *et al.*, 1998; Goulet *et al.*, 1998), although an increase in channel open probability may also contribute (Firsov *et al.*, 1996; Awayda *et al.*, 1997; Kellenberger *et al.*, 1998).

Both mouse and rat Nedd4 proteins contain 3 WW domains, while human Nedd4 has an additional WW domain (Staub *et al.*, 1996; Kumar *et al.*, 1997). It has previously been reported that WW domains 1, 2 and 3 from rat Nedd4 can bind to the PY motifs of the β and γ ENaC subunits *in* (Staub *et al.*, 1996). It is not known however whether all, or only a subset, of these three WW domains are required for Nedd4 to regulate Na^+ channel activity. In this chapter a combination of *in vitro* binding assays and whole cell patch clamp analysis have been used to investigate the role of individual WW domains of Nedd4 in mediating the feedback inhibition of Na^+ channels by raised intracellular Na^+. The results of this chapter have been published (Harvey *et al.*, 1999).

Results

5.2 Production of GST-WW fusion proteins in *E. coli*

To investigate the specificity of the interaction between the WW domains of Nedd4 and the subunits of ENaC, a dual approach involving far-Western blotting and patch-clamp analysis was used. To do this, GST-fusion proteins comprising the three WW domains of murine Nedd4 with one domain mutated or all three domains in their wild-type configuration were produced as well as wild-type and mutant versions of each individual WW domain (Figure 1). Mutated WW domains had the second conserved Trp altered to Phe and the conserved Pro changed to Ala. These mutations were designed to abolish WW domain binding activity without significantly altering the tertiary structure (M. Sudol, personal communication).

The human Nedd4 protein contains 4 WW domains of which domains 1, 2 and 4 correspond to domains 1, 2 and 3 respectively of the mouse protein (Kumar *et al.*, 1997). These three domains are highly conserved between mouse and human Nedd4 (Kumar *et al.*, 1997) and are thus expected to have similar ligand binding specificity. However, WW domain 3 in human Nedd4 is not found in the mouse protein. Therefore a GST fusion protein containing WW domain 3 of the human protein was generated for our binding and patch clamp assays.

5.3 α, β and γ ENaC subunits selectively interact with Nedd4 WW domains

The PY motifs of α, β and γ subunits of ENaC have been shown to be required for interaction with Nedd4 WW domains (Staub *et al.*, 1996), and therefore the intracellular carboxyl terminus of each ENaC subunit containing the PY motif was cloned into the pGEX-2TK vector and used for production of ^{32}P-labelled protein probes. Various GST-WW domain fusion proteins were immobilised on nitrocellulose membranes by electroblotting following electrophoresis on polyacrylamide gels, and were probed with

^{32}P-labelled GST-ENaC subunits. As shown in Figure 2B, the α subunit of ENaC did not bind to the control, GST, (lane 1) but bound with equal strength to the wild type (wt) protein (lane 2) and the WW domain 1 mutant, m1 (lane 3) suggesting that it does not interact with WW domain 1. The α subunit of ENaC showed reduced binding ability to mutant WW domain 2 (m2) and even less affinity for mutant WW domain 3 (m3) suggesting that both WW domains 2 and 3 can bind to the α subunit, although the α subunit has a preference for WW domain 3. The β ENaC subunit has very similar binding characteristics to the α subunit as it bound with similar strength to wt and m1 but with reduced affinity to m2 and m3 (Figure 2C). This suggests that the β subunit can bind to WW domains 2 and 3 but not to WW domain 1 and may also have a slight preference for WW domain 3. The γ ENaC subunit bound efficiently to both wt and m1, once again suggesting that it does not interact with WW domain 1 (Figure 2D). The γ ENaC subunit bound weakly to m2 and m3 indicating that it has affinity for both WW domains 2 and 3.

To explore the binding specificity of ENaCs further, individual WW domains were fused to GST and used for far-western analysis. As depicted in Figure 3B, the α ENaC subunit bound to WW domains 2 and 3 with similar affinity, but not to WW domain 1, confirming the result in Figure 2B. The α subunit also bound to human WW domain 3, which is not found in mouse Nedd4. As was the case in Figure 2, the β subunit showed similar binding characteristics to the α subunit by binding to murine WW domains 2 and 3 and human WW domain 3, but not to WW domain 1 (Figure 3C). The γ ENaC subunit displayed a higher affinity for murine WW domain 2 and human WW domain 3 and reduced affinity for murine WW domain 3, but like the α and β ENaC subunits, showed no affinity for WW domain 1 (Figure 3D).

5.4 Binding specificity of murine ENaC subunits for murine Nedd4 WW domains

The binding analysis presented in Figures 2 and 3 was open to objection that interactions between human ENaC carboxyl termini and murine Nedd4 WW domains may differ from those that we would observe if we were to use murine ENaC carboxyl termini. Therefore GST-fusion proteins of murine α, β and γ subunits ENaC subunits were produced. When used in far-Western analyses, GST-fusion proteins generated from all three subunits of murine ENaC carboxyl termini gave results similar to those shown in Figures 2 and 3 (Figure 4). This was not surprising given the close homologies evident in and around the PY motifs of human and mouse ENaC subunits (Figure 5). The PY motifs in the α and β subunits (PPAY and PPNY, respectively) of mouse and human ENaC are identical, while the in the γ subunit, the sequence differs by a single conservative change (PPRY in mouse compared with PPKY in human) (Figure 5).

The observation that none of the mouse or human ENaC subunits bound to WW domain 1 that was immobilised on nitrocellulose membrane raised the possibility that WW domain 1 may need to be in a fully native conformation in order to recognise binding partners. Therefore the binding assay was performed in reverse with ^{32}P-labelled WW domain 1 used to probe immobilised ENaC-GST fusion proteins. A positive control probe, containing all three WW domains (wt), bound strongly to all wildtype mouse and human ENaC subunits (but not to a negative control β ENaC containing a mutated PY motif), whereas WW domain 1 did not exhibit significant binding to any of the ENaC subunits even after long exposures (Figure 6), further confirming the results shown in Figures 2, 3 and 4.

5.5 All Three WW Domains of mouse Nedd4 need to be occluded to inhibit the Na^+ feedback loop

As mentioned in the introduction (5.1), Nedd4 mediates the feedback inhibition of

Na^+ channel activity produced by an increase in intracellular Na^+. Previously it has been shown using the whole cell patch-clamp technique that Na^+ channel activity of single mouse mandibular granular duct cells can be inhibited by increasing the pipette solution Na^+ to 70 mM [Chapter 4 of this thesis; Komwatana *et al.*, 1996; Komwatana *et al.*, 1998; Dinudom *et al.*, 1998)]. This feedback inhibition by raised intracellular Na^+ can be inhibited by inclusion in the pipette solution of: (i) an antibody directed against the WW domains of Nedd4 (Dinudom *et al.*, unpublished), (ii) an antibody directed against the ubiquitin-protein ligase domain of Nedd4 [(Chapter 4 and (Dinudom *et al.*, 1998)], or (iii) a GST-Nedd4 WW domain fusion protein that presumably acts as a dominant negative mutant by displacing endogenous Nedd4 from ENaC subunits [Chapter 4 and (Dinudom *et al.*, 1998)].

The ability of the GST-WW domain fusion protein (wt in Figure 1) to block Na^+ feedback inhibition of Na^+ channels led to an investigation of the roles that individual WW domains of Nedd4 play in regulating ENaC activity. First the concentration-dependency of the ability of the wt protein to block Na^+ feedback inhibition of Na^+ channels in mouse granular duct cells was investigated. Inclusion of 100 μg/ml GST-wt in pipette solution containing 70 mM Na^+-glutamate, but not 50 μg/ml GST-wt, was found to be sufficient to overcome feedback inhibition of Na^+ channels (Figure 7). In subsequent studies 100 μg/ml was therefore used as the reference concentration of the GST-wt protein.

To test whether all three WW domains of Nedd4 are required to inhibit the Na^+ dependent feedback pathway, GST fusion proteins containing 2 intact WW domains and one mutant WW domain (m1, m2 and m3 in Figure 1), were examined. It was found that none of these proteins were able to overcome the inhibition of Na^+ channel activity produced by inclusion of 70 mM Na^+ in the pipette solution (Figure 8A). These results suggested that each of the 3 WW domains of murine Nedd4 binds to a distinct, non-

interchangeable site and that to prevent Na^+ feedback control of Na^+ channels it is necessary to occlude all three of these sites. This interpretation was further tested by examining whether inclusion in pipette solution of a mixture of individual WW domains of murine Nedd4 (s1, s2 and s3 in Figure 1, each at 100 μg/ml) could block Na^+ feedback. Indeed, as shown in Figure 8B, the effect of adding the three individual WW-GST proteins to the pipette solution was similar to the wt WW-GST fusion protein containing the three WW domains in sequence. Furthermore, omission of any of these isolated domains from the mixture lead to the loss of this blocking effect despite the adjustment of the concentration of each of the two remaining WW domain proteins to 150 μg/ml to maintain the total concentration of GST-WW domain fusion proteins in the pipette solution at 300 μg/ml (Figure 8B). Finally the WW domain unique to human Nedd4 (h3 in Figure 1) was assayed for its ability to replace any of the 3 murine WW domains. It was found that it was unable to do so (Figure 8C). Thus it was concluded that the additional WW domain in human Nedd4 does not substitute for any of the 3 WW domains that are present in both murine and human Nedd4.

5.6 Discussion

This study has shown that in order to block Na^+-dependent feedback inhibition of epithelial Na^+ channels, distinct binding sites for all 3 WW domains of murine Nedd4 must be occluded. It also suggests that *in vivo,* each of these WW domains binds one of these sites and is unable to bind to the other two. *In vitro,* the WW domains of Nedd4 were found to have varying specificity for the PY motifs of different ENaC subunits. Murine WW domains 2 and 3, and human WW domain 3 show varying affinity for all 3 ENaC subunits whereas WW domain 1 failed to bind to any of the three ENaC subunits. This suggests that WW domain 1 either prefers a different Pro-rich motif to WW domains 2 and 3 or different residues surrounding the PY motif. There is a precedence for WW domains preferring Pro-rich motifs other than PPxY; the WW domain of Npw38 prefers a proline-rich motif containing glycine and arginine residues (Komuro *et al.*, 1999) and the WW domains of some formin binding proteins (FBPs) prefer a PPLP motif (Chan *et al.*, 1996; Bedford *et al.*, 1998)), whereas other FBPs prefer a Pro, Met and Gly rich motif (Lehman *et al.*, 1998). Nedd4 WW domain 1 is much more homologous to Nedd4 WW domains 2 and 3 than to the WW domains of FBPs however, so it is reasonable to expect that WW domain 1 will bind to a PPxY motif-containing protein. Additionally, colleagues in our laboratory have demonstrated interaction of WW domain 1 of murine Nedd4 with PY motifs of other proteins (Jolliffe *et al.*, unpublished). Staub *et al.* (1996), reported that WW domain 1 of rat Nedd4, like WW domains 2 and 3, bound *in vitro* to the PY motif of both the β and γ ENaC subunits. Since mouse and rat Nedd4 WW domain 1 sequences are highly homologous (95% identical), the possibility of different ligand specificities is highly unlikely. Our data show that, similar to mouse, human Nedd4 WW domain 1 does not interact with any one of the three ENaC subunits. We are therefore unsure about the reason for discrepancy between our results and those reported by Staub *et al.* (1996). It is worth noting that several other proteins including p45/NF-E2 and RNA polymerase II,

which interact with Nedd4 through their PY motifs, also do not bind WW domain 1 (Gavva *et al.*, 1997; Jolliffe and Kumar, unpublished) suggesting that WW domain 1 of Nedd4 may have very restricted ligand-binding specificity. The finding that WW domain 1 of Nedd4 does not interact with any of the ENaC subunits was recently confirmed by Farr *et al.*, (2000).

In light of our binding assays it may have been reasonable to expect that Nedd4 WW domains 2 and 3 alone would be capable of exerting a dominant negative effect on the Na^+ feedback pathway as measured by patch clamp analysis. This was not the case however as an intact WW domain 1 was required, in concert with WW domains 2 and 3, to restore Na^+ channel activity under high Na^+ conditions. We therefore suggest that WW domain 1 must play a role in ENaC regulation that is not dependent on direct binding of an ENaC subunit PY motif. We postulate that WW domain 1 binds to an additional, as yet unidentified molecule that is activated under high salt conditions, possibly downstream of G_o. This molecule may recruit Nedd4 to the ENaC complex and /or stabilise the ENaC/Nedd4 complex. We postulate that a stable Nedd4/ENaC complex will comprise one ENaC octamer, four molecules of Nedd4 and four molecules of the unknown recruitment/stabilisation factor (Figure 9). The possibility also exists that fewer molecules of Nedd4 bind to a single ENaC octamer, and that not all ENaC PY motifs are required for interaction with Nedd4. Further experiments are required to explore these possibilities. *In vitro,* human WW domain 3 showed affinity for all three subunits of ENaC, comparable to the binding characteristics shown by murine WW domains 2 and 3 (Figures 3 and 4), but it was unable to complement either of these WW domains, or WW domain 1 *in vivo*. This suggests that although human WW domain 3 recognises the PY motifs of ENaC subunits *in vitro* it does not play a role in the Na^+ dependent feedback inhibition of Na^+ channels in mouse mandibular duct cells.

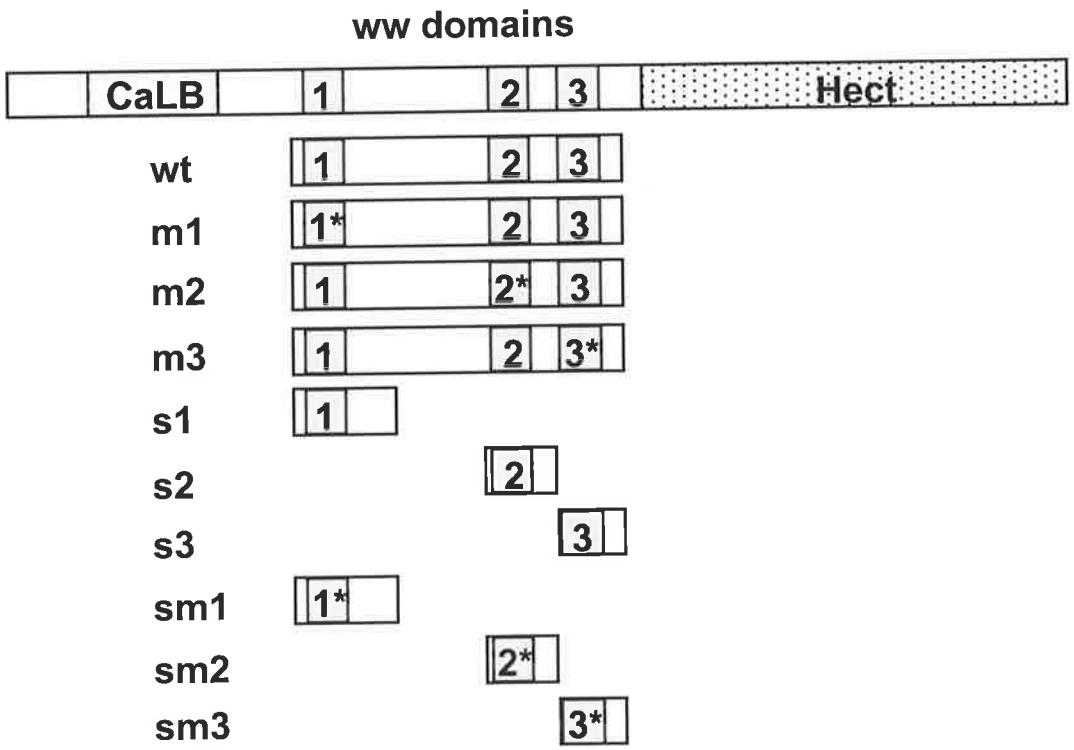
The finding that all three WW domains of murine Nedd4 must be occluded to

inhibit the Na^+ feedback loop suggests that more than one WW domain/PY motif contact needs to occur for stable complex formation between Nedd4 and ENaC. This is supported by the molecular understanding of the hypertensive disorder, Liddle's syndrome, where mutation in a single ENaC subunit (β or γ) is sufficient to cause a disease phenotype (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a; Hansson *et al.*, 1995b; Tamura *et al.*, 1996; Jeunemaitre *et al.*, 1997). As ENaC is an octamer, presumably 6 subunits with intact PY motifs will still be present in Liddle's syndrome patients. This would seem insufficient to form a stable complex with Nedd4 however, as hypertension results, presumably as a consequence of lack of negative regulation of ENaC by Nedd4.

Figure 1. Schematic representation of the regions of Nedd4 that were fused to GST for protein production.

All three WW domains of mouse Nedd4 (wt) along with mutants in either WW domain 1 (m1), WW domain 2 (m2) or WW domain 3 (m3) were fused to GST. Single WW domains were also fused to GST in wild-type and mutant forms and are labelled s1, s2 and s3 (wild-type WW domains 1, 2 and 3 respectively) and sm1, sm2 and sm3 (mutant WW domains 1, 2 and 3 respectively). The third WW domain of human Nedd4 (h3) was also fused to GST.

mNedd4



hNedd4

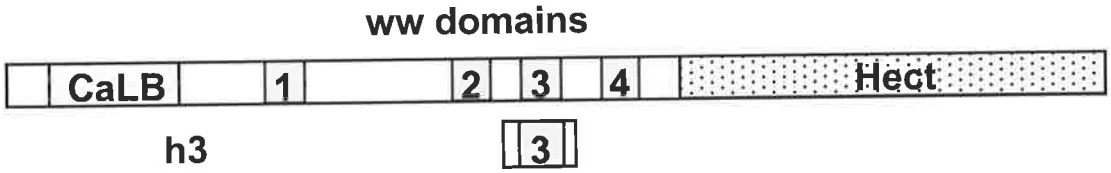


Figure 2. Varying specificity of α, β and γ ENaC for Nedd4 WW domains.

A, Coomassie stained gel of GST fusion proteins as outlined in Figure 1 and indicated on top of the gel. Lane 1 contains GST alone and molecular weight markers in kDa are indicated on the right hand side of the gel. B, C, and D, are far-Western blots of the above gel probed with ^{32}P-labelled α, β or γ ENaC protein probes respectively.

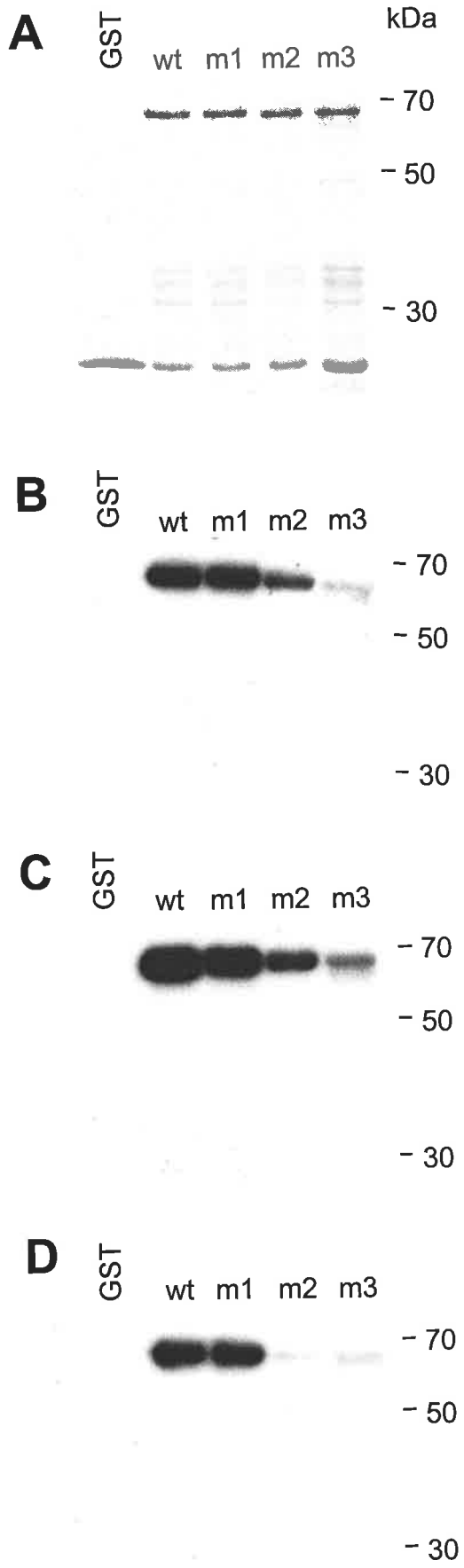

A
GST
kDa
wt m1 m2 m3
70
50
30
B
GST
wt m1 m2 m3
70
50
30
C
GST
wt m1 m2 m3
70
50
30
D
GST
wt m1 m2 m3
70
50
30

Figure 3. Specificity of α, β and γ ENaC for individual Nedd4 WW domains.

A, Coomassie stained gel of GST fusion proteins as outlined in Figure 1 and indicated on top of the gel. Molecular weight markers in kDa are indicated on the right hand side of the gel. B, C, and D, are far-Western blots of the above gel probed with ^{32}P-labelled α, β or γ ENaC protein probes respectively.

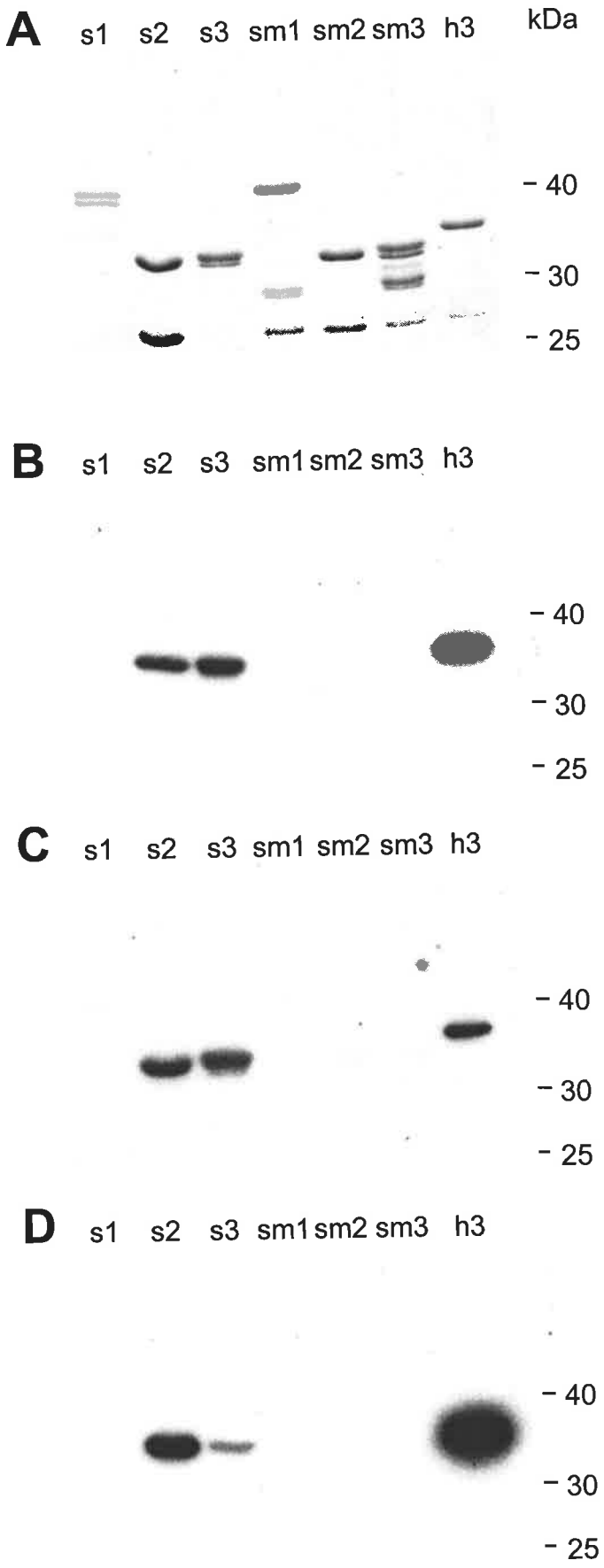

A
s1 s2 s3 sm1 sm2 sm3 h3
kDa
40
30
25
B
s1 s2 s3 sm1 sm2 sm3 h3
40
30
25
C
s1 s2 s3 sm1 sm2 sm3 h3
40
30
25
D
s1 s2 s3 sm1 sm2 sm3 h3
40
30
25

Figure 4. Varying specificity of murine α, β and γ ENaC for Nedd4 WW domains.

A, Coomassie stained gel of GST fusion proteins as outlined in Figure 1 and indicated on top of the gel. Lane 1 contains GST alone and molecular weight markers in kDa are indicated on the right hand side of the gel. B, C, and D, are far-Western blots of the above gel probed with ^{32}P-labelled murine α, β or γ ENaC protein probes respectively.

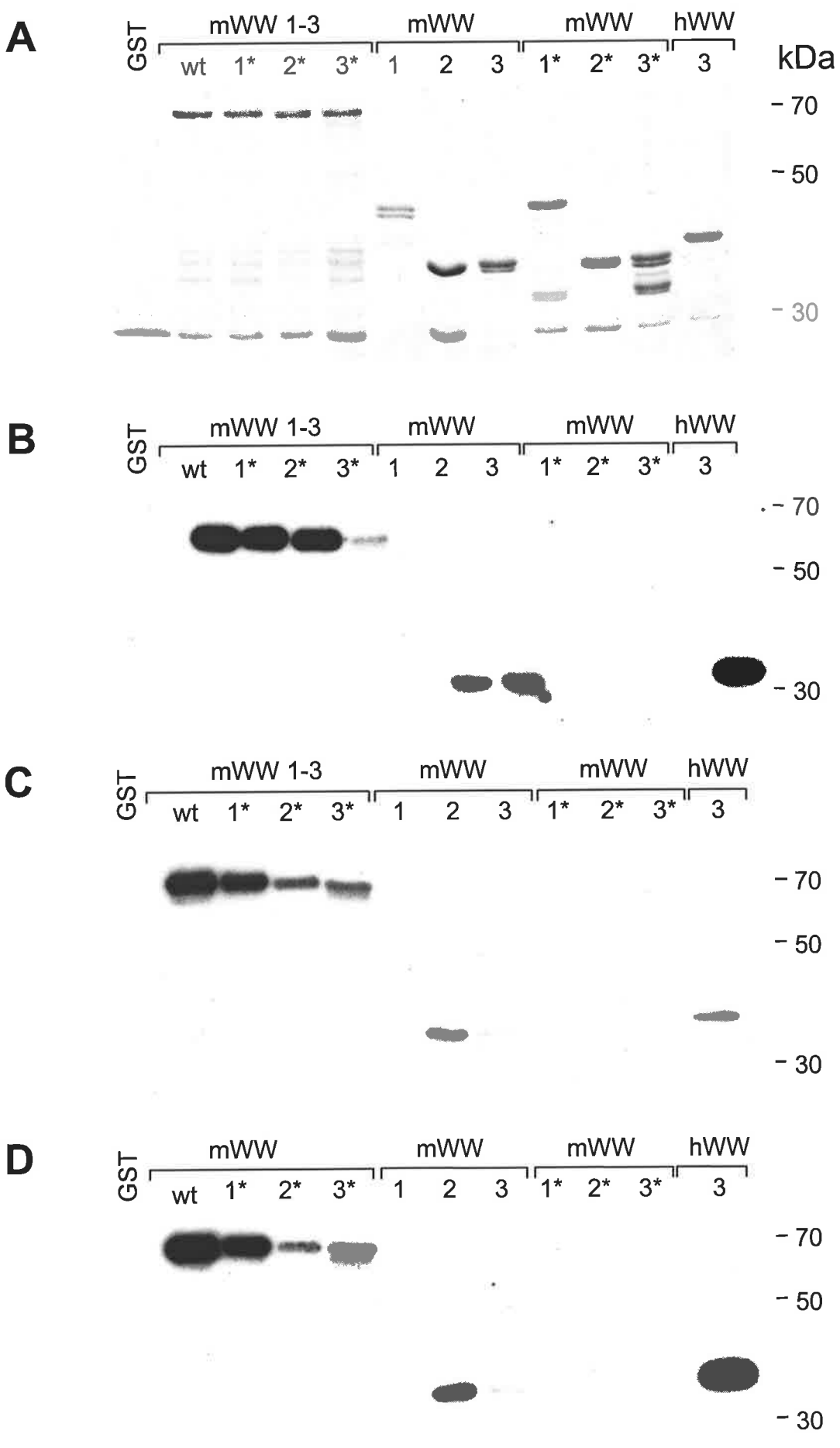

A
B
C
D
GST
mWW 1-3
mWW
mWW
hWW
wt
1*
2*
3*
1
2
3
1*
2*
3*
3
kDa
70
50
30

Figure 5. A comparison of the carboxyl terminal amino acid sequences derived from α, β, and γ subunits of mouse and human ENaC.

Approximately the last 50 amino acid residues from the carboxyl termini of the three ENaC subunits are shown. The PPxY motifs present in all three subunits are underlined. The residues identical between the human and mouse proteins are shown in bold.

α ENaC

mouse SPPPSLPQQGTTPPLALTAPPPAYATLGPSASPLDSAVPGSSACAPAMAL*

human S..LSLSQPGPAPSPALTAPPPAYATLGPRPSPGGSAGASSSTCPLGGP*

β ENaC

mouse PDTTSCRPHGEVYPDQQTLPIPGTPPPNYDSLRLQPLDTMESDSEVEAI*

human PDTAPRSPNTGPYPSEQALPIPGTPPPNYDSLRLQPLDVIESDSEGDAI*

γ ENaC

mouse DLPTFTSAMRLPPAPEAPVPGTPPPRYNTLRLDSAFSSQLTDTQLTNEF*

human DLPTFNSALHLPPSLGTQVPGTPPPKYNTLRLERAFSNQLTDTQMLDEL*

Figure 6. Varying specificity of Nedd4 WW domains for α,β and γ ENaC.

A, Coomassie stained gel of human and murine GST-ENaC fusion proteins as indicated on top of the gel. Lane 1 contains GST alone and molecular weight markers in kDa are indicated on the right hand side of the gel. B, and C, are far-Western blots of the gel in A, probed with ^{32}P-labelled murine WW domains 1-3 (3 hour exposure) or WW domain 1 alone (48 hour exposure), respectively.

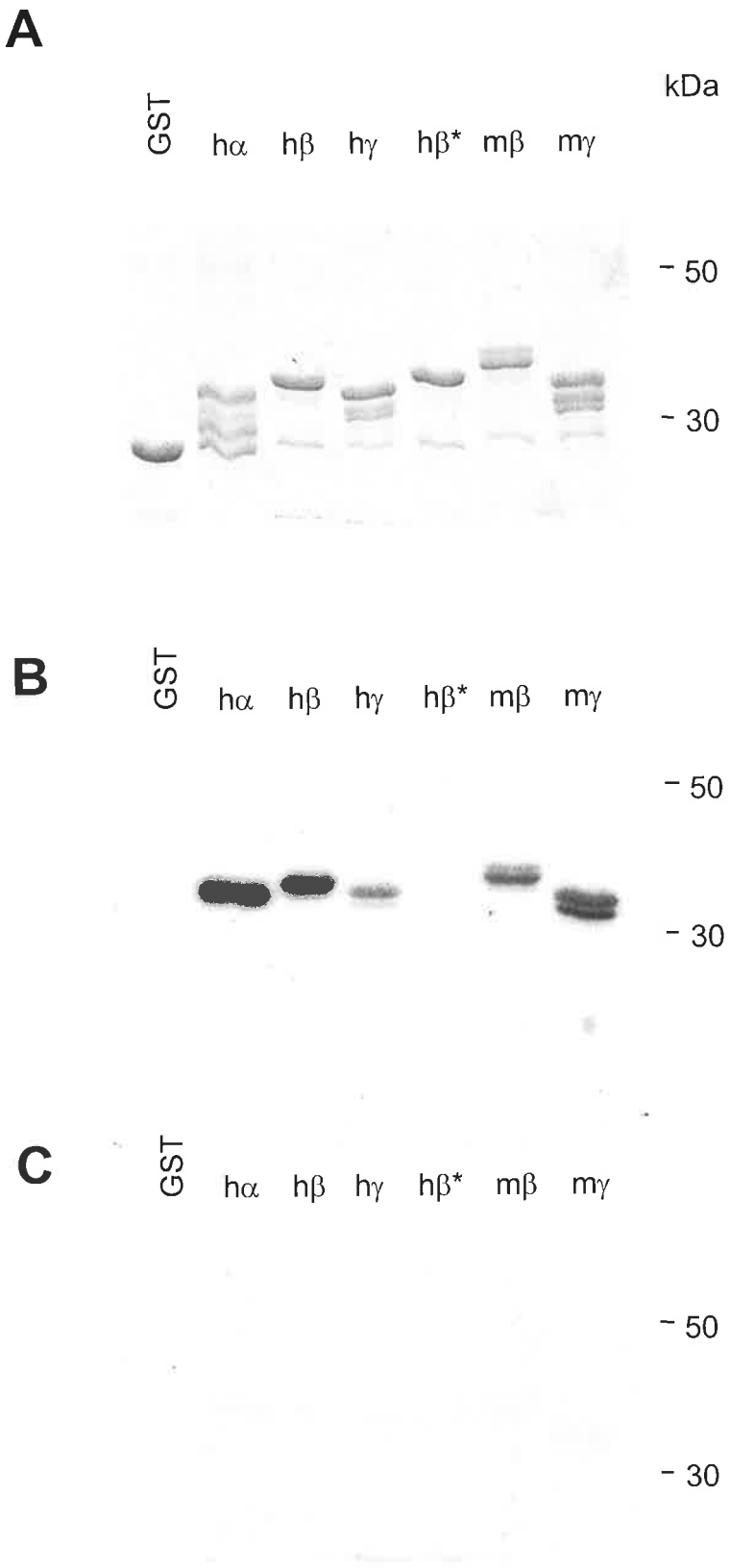

A
kDa
GST
hα
hβ
hγ
hβ*
mβ
mγ
50
30
B
GST
hα
hβ
hγ
hβ*
mβ
mγ
50
30
C
GST
hα
hβ
hγ
hβ*
mβ
mγ
50
30

Figure 7. Dose response curve of inhibition of the Na^+ dependent feedback pathway by the GST-WW protein.

Concentration response relation for the effects of inclusion in the pipette solution of the GST-WW fusion protein on the chord conductance of the amiloride-sensitive current when the pipette contained 70 mM Na^+. The broken line indicates the mean amiloride-sensitive Na^+ conductance when the zero Na^+ pipette solution was used. A minimum of 4 recordings were taken for each pipette concentration of GST-WW fusion protein.

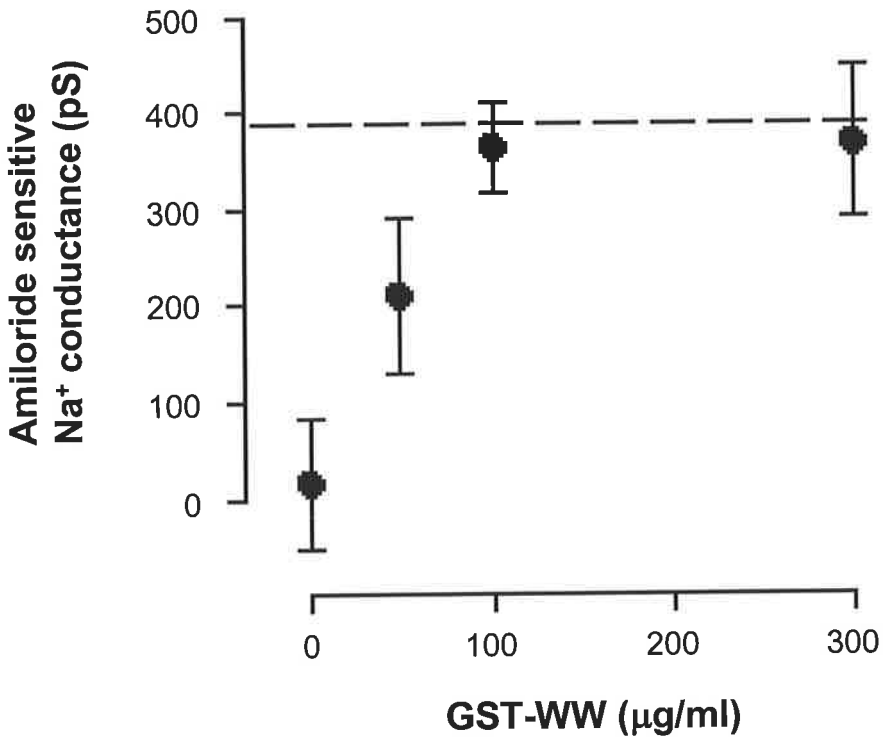

Amiloride sensitive
Na+ conductance (pS)
500
400
300
200
100
0
0
100
200
300
GST-WW (μg/ml)

Figure 8. All three WW domains of Nedd4 are required to inhibit the Na^+ dependent feedback pathway.

(A) The effects on the chord conductance of the amiloride-sensitive Na^+ current of the inclusion in the 70 mM pipette solution of the control GST-WW fusion protein (100 μg/ml) and of variants of this fusion protein (100 μg/ml) in which one WW domain has been mutated leaving the other two intact (m1, m2, m3). (B) The effects on the chord conductance of the amiloride-sensitive Na^+ current of the inclusion in the 70 mM pipette solution of various mixtures of fusion proteins containing individual WW domains of murine Nedd4 (s1, s2, s3) in equal concentrations totalling 300 μg/ml. (C) The effects on the chord conductance of the amiloride-sensitive Na^+ current of the inclusion in the 70 mM pipette solution of the h3 construct carrying the WW domain 3 of human Nedd4 (100 μg/ml) with pairs of constructs of single WW domains of murine Nedd4 (s1, s2 and s3, 100 μg/ml each). A minimum of 5 recordings were taken for each experiment. Details of the proteins used in this figure are given in Figure 1.

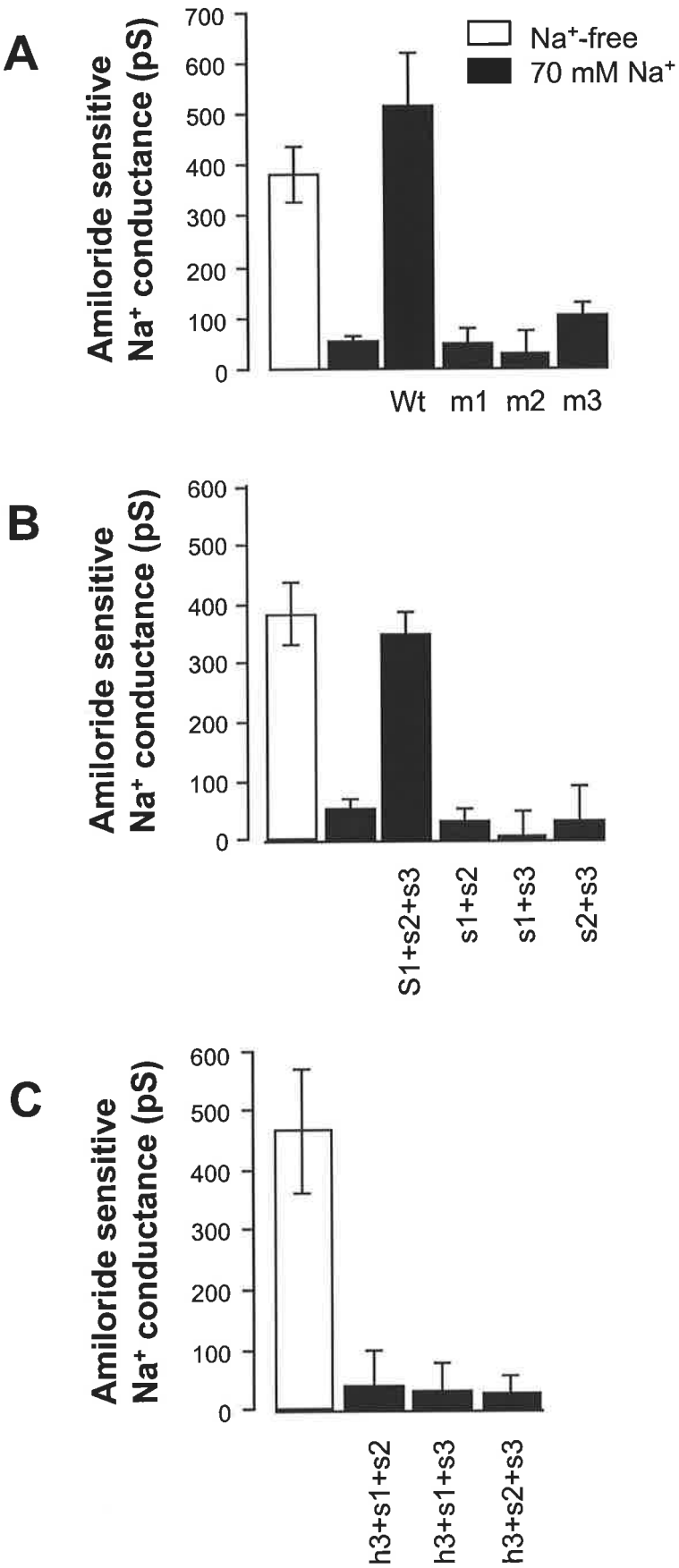
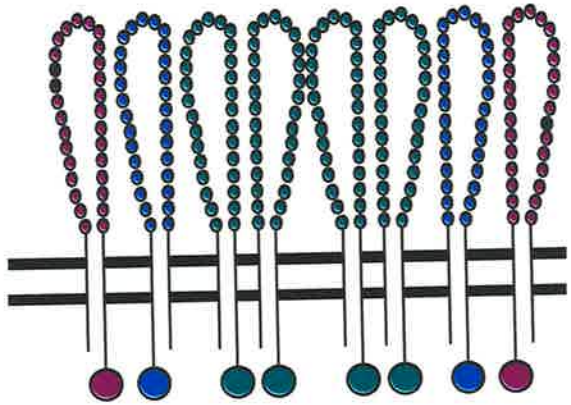

A
Amiloride sensitive
Na+ conductance (pS)
700
600
500
400
300
200
100
0
Na+-free
70 mM Na+
Wt
m1
m2
m3
B
Amiloride sensitive
Na+ conductance (pS)
600
500
400
300
200
100
0
S1+s2+s3
s1+s2
s1+s3
s2+s3
C
Amiloride sensitive
Na+ conductance (pS)
600
500
400
300
200
100
0
h3+s1+s2
h3+s1+s3
h3+s2+s3

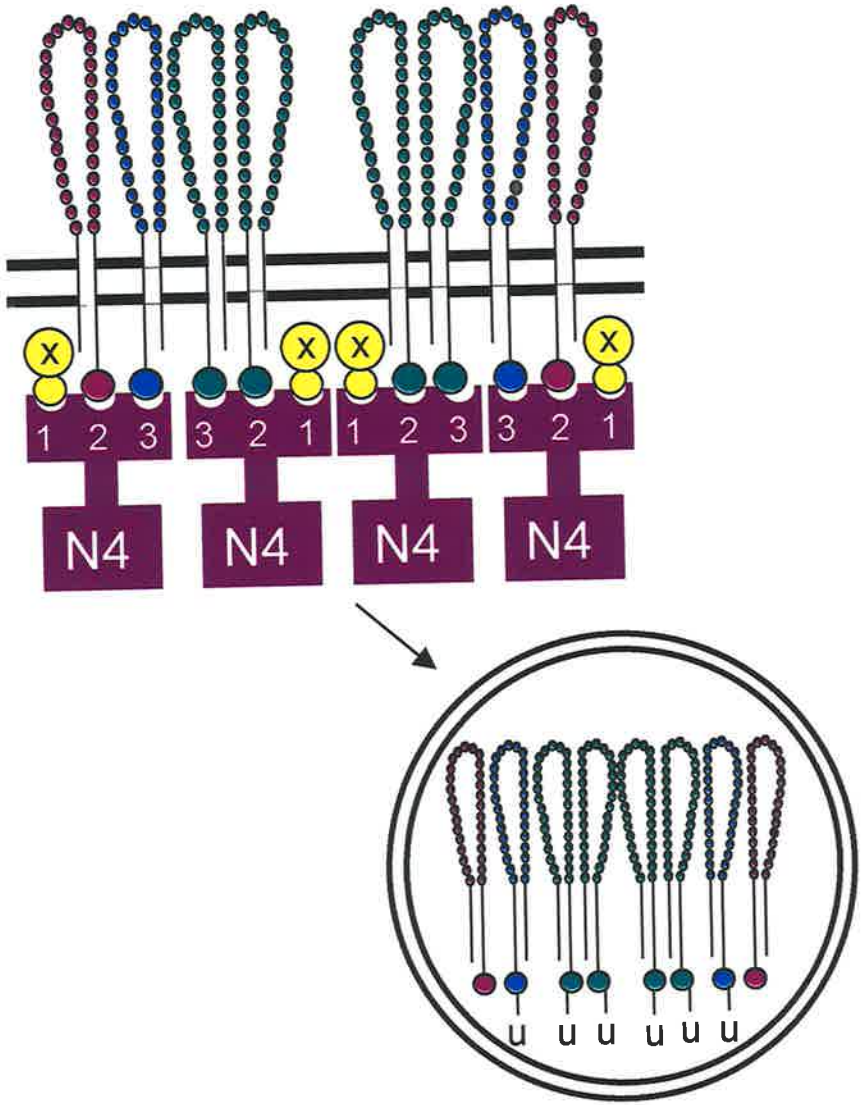
Figure 9. A possible model for interaction of Nedd4 with ENaC.

In response to high intracellular Na^+ an as yet unidentified factor (X) is activated and binds to Nedd4 via WW domain 1. This serves to recruit Nedd4 to ENaC and/or stabilise the binding of Nedd4 WW domains 2 and 3 to the α, β and γ subunits of ENaC. We postulate that a stable Nedd4/ENaC complex will comprise one ENaC octamer, four molecules of Nedd4 and four molecules of factor X. Once stably bound, Nedd4 ubiquitinates ENaC and stimulates its internalisation and/or subsequent degradation.

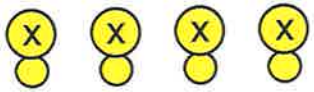
Low Na^+



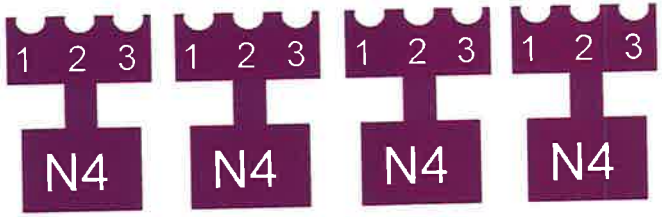
High Na^+



Protein X



Nedd4



Chapter 6:
The carboxyl termini of β and γ, but not α epithelial Na^+ channel subunits are important for regulation of channel activity

6.1 Introduction

In the previous chapter, all three WW domains of Nedd4 were shown to be required for Na^+-dependent feedback of ENaC. *In vitro*, only WW domains 2 and 3 were capable of interaction with ENaC subunits, while WW domain 1 failed to bind to any of the ENaC subunits. Recently, mature membrane associated ENaC has been shown to consist of eight subunits in total (4α, 2β and 2γ subunits) (Eskandari *et al.*, 1999). It was therefore postulated that four molecules of Nedd4 would interact with one ENaC octamer through WW domains 2 and 3, and four molecules of an unknown recruitment or stabilisation protein through Nedd4 WW domain 1 (Figure 9, chapter 5).

The WW domains of Nedd4 interact with ENaC via the PY motifs contained in the carboxyl termini [Chapter 4 of this thesis; (Harvey *et al.*, 1999; Staub *et al.*, 1996)]. The results presented in the previous two chapters showed that under high Na^+ conditions, the addition of large amounts of free Nedd4 WW domains to the cell inhibited Na^+-dependent feedback of ENaC. Therefore it was reasoned that addition of excess amounts of free PY-motif containing regions of the ENaC subunits should also inhibit Na^+-dependent feedback of ENaC. This experiment would provide confirmation that Nedd4 and ENaC interact under high Na^+ conditions and also test the hypothesis that all three types of ENaC subunit (α, β and γ) are required for interaction with Nedd4 when cytoplasmic Na^+ levels are elevated. To test this hypothesis, recombinant proteins containing the PY motif and surrounding amino acids of the α, β and γ subunits were tested for their ability to block Na^+-dependent feedback of ENaC.

Results

6.2 The last 40 amino acids of β and γ ENaC, but not α ENaC influence Na^+ channel activity when the Na^+ and Cl^- loops are inactive

In section 4.3 of this thesis, overexpression of the WW domains of Nedd4 were shown to inhibit Na^+-dependent downregulation of ENaC, presumably by interfering with the interaction of endogenous Nedd4 and ENaC. To confirm this finding we examined whether overexpression of the PY motif-containing portion of ENaC subunits could also modulate Na^+ channel activity. To do this, proteins were generated consisting of the carboxyl terminal 40 amino acids of human or murine α, β and γ ENaC, fused to GST.

Initially, the effect of ENaC carboxyl termini on Na^+ conductance was measured when the Na^+ and Cl^- feedback loops were inactive. In section 4.2 of this thesis it was shown that Nedd4 does not mediate the activity of ENaC when the Na^+ and Cl^- feedback loops are inactive and therefore it was expected that inclusion of GST-α, β or γ ENaC would have no effect on Na^+ conductance under these conditions. The addition of GST-α ENaC at a final concentration of 300 μg/ml to pipette solution containing 150 mM NMDG-glutamate, did not alter Na^+ conductance (Figure 1). Unexpectedly, when GST-β ENaC (final concentration, 300 μg/ml) was added to pipette solution containing 150 mM NMDG-glutamate, Na^+ conductance was almost totally abolished (Figure 1). When GST-γ ENaC (final concentration, 300 μg/ml) was added to pipette solution containing 150 mM NMDG-glutamate, Na^+ conductance was significantly reduced, but not to the same level as when GST-β ENaC was included in the pipette solution (Figure 1).

6.3 The inhibitory effect of the carboxyl terminus of β ENaC on Na^+ conductance is dependent on the last 10 amino acids and is independent of the PY motif

The results observed in 6.2 raised the possibility that either: 1) the carboxyl termini

of β and γ ENaC interact with a protein that normally activates ENaC when Na^+ import is required, and when large amounts of free β or γ ENaC are added to the cell, such a protein is sequestered, rendering native ENaC inactive; or alternatively 2) the carboxyl termini of β and γ ENaC act as a "plug" which block channel activity [as suggested by (Awayda *et al.*, 1997)].

There are several potential protein interaction motifs in the carboxyl termini of β and γ ENaC and therefore the first possibility was investigated. The PY motif found in the β and γ ENaC carboxyl termini is a known WW domain-binding motif and is the site of interaction with the negative regulatory protein, Nedd4, under high Na^+ conditions [chapters 4 and 5 of this thesis; (Dinudom *et al.*, 1998; Harvey *et al.*, 1999)]. We considered the possibility that under low Na^+ conditions, the PY motifs of β and γ ENaC may recruit a protein that positively influences ENaC activity and that inclusion of GST-β ENaC or GST-γ ENaC in the low Na^+ pipette solution could inhibit the interaction of β and γ ENaC with such a protein. To test this hypothesis, Tyr 616 of the PY motif in hβENaC was mutated to Ala and this protein (hβENaC PPXA) was expressed and purified from *E. coli*. The Tyr residue of PY motifs is absolutely required for PY motif-WW domain interaction and therefore mutation of Tyr 616 in hβENaC should abolish any potential interaction of WW-domain containing proteins with hβENaC. When included into pipette solution containing 150 mM NMDG-glutamate (final concentration, 300 μg/ml), hβENaC PPXA reduced Na^+ conductance with the same potency as hβENaC, demonstrating that the PY motif of βENaC is dispensable for the inhibitory effect of βENaC on Na^+ conductance under low Na^+ conditions (Figure 2).

It had been previously reported that the last 10 amino acids of β ENaC could attenuate amiloride-sensitive Na^+ channel activity in lymphocytes obtained from Liddle's syndrome patients (Ismailov *et al.*, 1996). To determine if the last 10 amino acids of β

ENaC are critical for downregulation of Na^+ channel activity under low Na^+ conditions a construct was generated that was identical to GST-β ENaC except that it lacked the last 10 amino acids (βmENaC-10). When βmENaC-10 was included in pipette solution (at a final concentration of 300 μg/ml) containing 150 mM NMDG-glutamate, Na^+ conductance was unaltered (Figure 2), demonstrating that the last 10 amino acids of β ENaC are required for the carboxyl terminus of β ENaC to inhibit Na^+ conductance under low Na^+ conditions. To confirm this finding a peptide was produced that was identical to the last 10 amino acids of mouse β ENaC (βmC10). When this peptide (at a final concentration of 20 nM) was added to pipette solution containing 150 mM NMDG-glutamate, the Na^+ conductance was reduced to levels observed when hβENaC was added to pipette solution (Figure 3).

6.4 Ser 635 is required for the inhibitory effect of the carboxyl terminus of β ENaC on Na^+ conductance

In section 6.2 the carboxyl termini of β and γ ENaC were shown to have an inhibitory effect on Na^+ conductance when ENaC is normally active, and in section 6.3 this property was shown to be dependent on the last 10 amino acids of βENaC. The mechanism underlying this effect was still mysterious however. The hypothesis that the last 10 amino acids of βENaC bound to a protein responsible for activation of ENaC appeared the most likely explanation for this observation and so this region of βENaC was examined further. A possible PDZ domain-binding site and two possible Ser phosphorylation sites were identified in the carboxyl terminus of βENaC. PDZ domains (originally identified in postsynaptic density-95, discs large, and ZO-1) mediate protein-protein interactions and are found in a large number of multifunctional proteins (Craven and Bredt, 1998). There is a short consensus sequence at the carboxyl terminus of some membrane proteins that is critical for interaction with PDZ domains; one consensus sequence consists of the amino acids (D/E)(T/S)XV (Short *et al.*, 1998). A sequence in the

carboxyl terminus of βENaC fits this consensus (DSEV in mouse and rat and DSEG in human).

The carboxyl termini of β and γ ENaC but not α ENaC are phosphorylated under basal conditions on Ser and Thr residues (Shimkets *et al.*, 1998). In the βENaC carboxyl terminus this phosphorylation site has been narrowed down to one of three potential Ser residues at positions 620, 632 or 634 (621, 633 and 635 in mouse) (Shimkets *et al.*, 1998). Two of these residues, Ser 633 and 635, are found in the last 10 amino acids of βENaC. These residues may be important for recruitment of a protein kinase, which phosphorylates ENaC and directly activates it or stimulates recruitment of a channel activating protein.

To investigate the importance of the potential PDZ domain-binding site and potential Ser phosphorylation site 635, a peptide was produced that was identical to βmC10 except that Asp 634 and Ser 635 were replaced with Gly. When this peptide was included at a concentration of 20 nM in pipette solution containing 150 mM NMDG-glutamate, Na^+-conductance was unaltered, demonstrating that either or both Asp 634 and Ser 635 were required for the channel inhibitory effect of βmC10 (Figure 3). A peptide was then produced where only Ser 635 was replaced by Gly and this too had no effect on Na^+-conductance when included at a concentration of 20 nM in pipette solution containing 150 mM NMDG-glutamate (Figure 3). This showed that Ser 635 is necessary for the channel inhibitory effect of the β ENaC carboxyl terminus.

6.5 The inhibitory effect of the carboxyl terminus of γ ENaC on Na^+ conductance is dependent on the last 19 amino acids

An attempt was then made to identify the Na^+ channel inhibitory element of the γ ENaC carboxyl terminus. A protein identical to mγENaC, except that it lacked the last 10 amino acids of γ ENaC, was expressed and purified from *E. coli*. When mγENaC-10 was included in pipette solution containing 150 mM NMDG-glutamate at a final concentration

of 300 μg/ml, Na^+ conductance was still reduced (Figure 4). A protein was then produced that lacked the last 19 amino acids of γ ENaC (mγENaC-19). When this protein was included in pipette solution containing 150 mM NMDG-glutamate at a final concentration of 300 μg/ml, Na^+ conductance was unaltered (Figure 4), demonstrating that the Na^+-conductance inhibitory property of γ ENaC lies between residues 631 and 639.

6.6 The PY motif regions of β and γ ENaC are important for Na^+-dependent regulation of ENaC

The original aim of using the PY motif-containing carboxyl termini of the ENaC subunits was to examine whether, like the WW domains of Nedd4, these proteins could inhibit Na^+-dependent downregulation of ENaC. When GST-αENaC (final concentration, 300 μg/ml) was added to pipette solution containing 70 mM Na^+-glutamate, amiloride-sensitive Na^+ conductance was unaltered (Figure 5). To examine the effect of the PY motif-containing region of βENaC, GST-βENaC could not be used as it reduced Na^+ conductance under low Na^+ conditions. Therefore the effect of βmENaC-10 on Na^+ conductance under high Na^+ conditions was studied. When βmENaC-10 (final concentration, 300 μg/ml) was included in pipette solution containing 70 mM Na^+-glutamate, amiloride-sensitive Na^+ conductance was restored to a level similar to that observed when using pipette solution containing 0 mM Na^+, thus completely abolishing the inhibitory effect of cytosolic Na^+ (Figure 5). When γmENaC-19 (final concentration, 300 μg/ml) was included in pipette solution containing 70 mM Na^+-glutamate, amiloride-sensitive Na^+ conductance was restored to a level similar to that observed when using pipette solution containing 0 mM Na^+, thus completely abolishing the inhibitory effect of cytosolic Na^+ (Figure 5). These results suggest that the carboxyl termini of β and γ, but not α ENaC are important for negative regulation of ENaC under high Na^+ conditions.

6.7 Discussion

The principal findings of the studies presented in this chapter are: 1) The PY motif-containing regions of β and γ ENaC, but not α ENaC appear to be involved in negative regulation of ENaC activity under high Na^+ conditions; 2) The carboxyl terminal 10 amino acids of β ENaC, and in particular serine 635, are important for regulation of ENaC activity when Na^+ channels are functionally active; 3) Residues 631-639 of the carboxyl terminal of γ ENaC are important for regulation of ENaC activity when Na^+ channels are functionally active.

The importance of the PY motifs of ENaC for channel regulation was confirmed by the finding that overexpression of the PY motif-containing portions of β and γ ENaC (βmENaC-10 and γmENaC-19) interfered with the Na^+ feedback pathway. Presumably, βmENaC-10 and γmENaC-19 sequestered a negative regulatory protein that normally renders ENaC inactive under high Na^+ conditions. The experiments performed in this chapter do not reveal the identity of this negative regulatory protein, but taken together with the observations in chapters 4 and 5 of this thesis, suggest that this protein is Nedd4. The involvement of an additional WW domain-containing negative regulatory protein can not be discounted however. Overexpression of αmENaC did not interfere with Na^+-dependent inhibition of ENaC, suggesting that although α ENaC has a PY motif and interacts with WW domains 2 and 3 of Nedd4 *in vitro,* it is not required for negative regulation of ENaC under high Na^+ conditions. This finding appears to contrast with the observation that the α subunit of ENaC is ubiquitinated *in vivo*, but may be explained by the fact that ENaC is present in the cytosolic membrane as a complex. The PY motifs of the β and γ ENaC subunits may recruit Nedd4 which may then be in close enough proximity to catalyse ubiquitination of the α subunit of ENaC. With these results in mind the model of Nedd4/ENaC association has been reassessed. It is now hypothesised that in response to increased intracellular Na^+, two molecules of Nedd4 will associate with two

molecules of Protein X and one ENaC octamer (Figure 6).

When investigating the effect of the ENaC carboxyl termini on Na^+ channel activity, the carboxyl termini of both β and γ ENaC were found to have an inhibitory effect on Na^+ conductance under low Na^+ conditions. Two explanations were suggested to explain these observations. Previously, Awayda *et al.*, (1997) had shown that the last 30 amino acids of β and γ ENaC inhibit Na^+ channel activity in cells that express ENaC with truncated carboxyl termini (as in Liddle's syndrome). They suggested that the last 30 amino acids of β and γ ENaC normally act as a plug that negatively regulates the activity of ENaC, and that when excess free β and γ ENaC carboxyl termini are added to cells, Na^+ conductance is abolished as a result of ENaC being constitutively plugged. The results observed in this chapter differ from those of Benos' laboratory (Ismailov *et al.*, 1996; Awayda *et al.*, 1997; Ji *et al.*, 1999) however, as the studies presented here have been carried out on cells that express fully intact native ENaC, whereas Benos *et al.*, have studied Liddle's syndrome truncated ENaC where the carboxyl terminus of β ENaC has been removed.

The other explanation to account for the observations in section 6.2 that was considered, was that the carboxyl termini of β and γ ENaC are responsible for binding a protein that normally binds to and activates ENaC, and that when excess free β and γ ENaC are added to cells, ENaC activity is reduced because such a protein has been sequestered. Proteins that interact with ENaC and positively regulate its function have been identified. For example, the vesicle traffic regulatory protein, syntaxin 3 was recently shown to associate with the γ subunit of ENaC and increase activity of ENaC as well as expression of ENaC at the cell surface (Saxena *et al.*, 1999). Syntaxins have been implicated in the regulation of a number of other ion channels(Saxena *et al.*, 1999). The *Xenopus* protein, Apx, was identified by Staub *et al.* (1992), as a protein that associates with the native ENaC complex in A6 epithelial cells. Apx was shown to be required for

ENaC activity as injection of antisense Apx oligonucleotides into ENaC-expressing *Xenopus* oocytes, abolished ENaC activity (Staub *et al.*, 1992; Zuckerman *et al.*, 1999). The method by which Apx stimulates ENaC activity is unknown, although Apx is expressed on the apical membrane of epithelial cells and may recruit ENaC to the same region of the cell, where Na^+ import occurs (Zuckerman *et al.*, 1999).

This second hypothesis was tested by mutating possible protein binding and modification sites in the carboxyl termini of β and γ ENaC, and examining their effect on Na^+ conductance. Initially, the PY motif of β ENaC was ruled out, as PY motif mutant carboxyl termini were found to decrease Na^+ channel activity as effectively as wildtype carboxyl termini. The inhibitory effect of β and γ ENaC on Na^+ conductance was then narrowed down to the last 10 amino acids of β ENaC and residues 631-639 of γ ENaC. This portion of β ENaC contains a putative PDZ domain-binding site, which was shown to be necessary for the inhibitory effect of the β ENaC carboxyl termini. A similar PDZ domain-binding site has been identified in CFTR and is required for interaction of scaffolding proteins that anchor CFTR to the cell matrix (Short *et al.*, 1998; Moyer *et al.*, 1999). The ability of the carboxyl terminus of βENaC to bind to PDZ domain-containing proteins has not been investigated as yet.

Within the last 10 amino acids of the β ENaC carboxyl terminus, one residue in particular, Ser 635, was shown to be required for the inhibitory effect of β ENaC. This Ser residue is a candidate amino acid for phosphorylation by protein kinases. β and γ ENaC, but not α ENaC carboxyl termini are phosphorylated in the basal state (Shimkets *et al.*, 1998). A protein kinase may require Ser 635 of β ENaC to recognise ENaC and subsequently phosphorylate it. Such phosphorylation may activate ENaC or allow β ENaC to recruit a protein that directly activates its function. Clearly, further investigation is required to explore these possibilities and to determine the mechanism by which β and γ

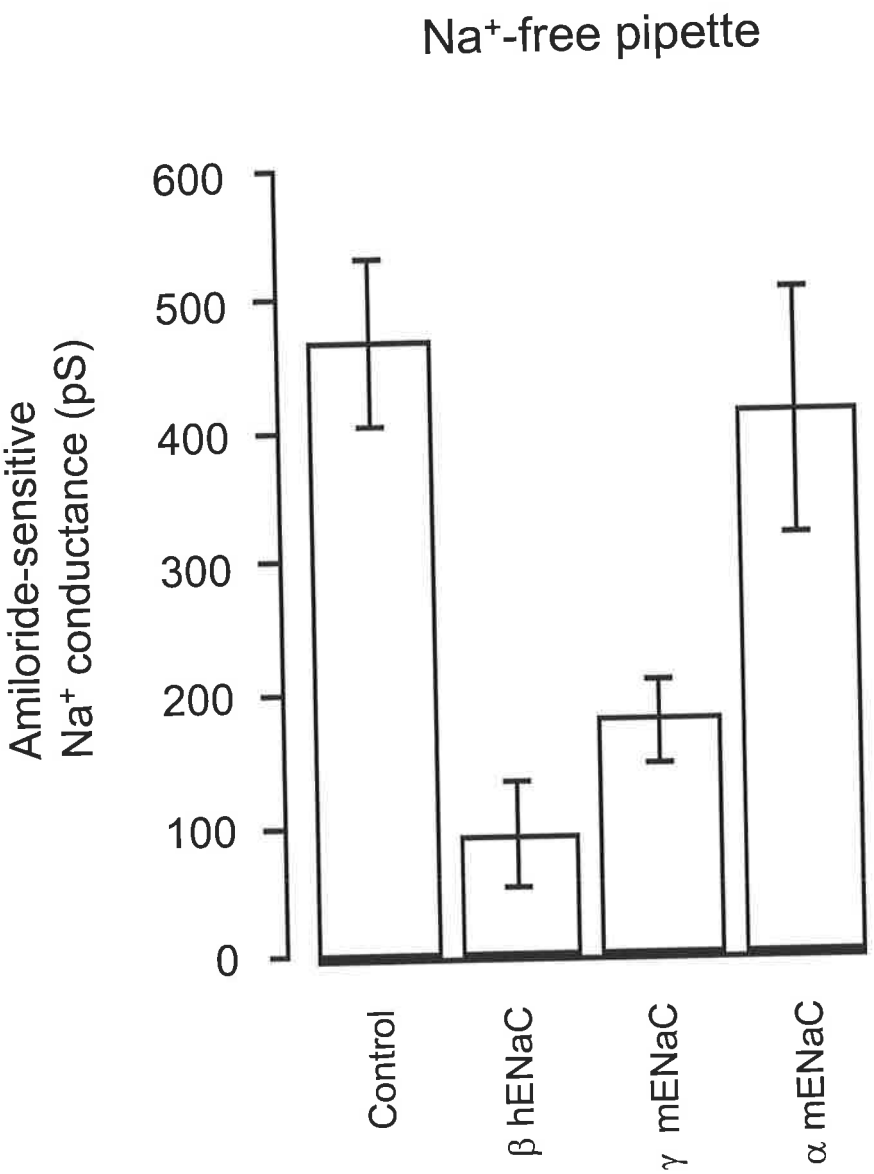
ENaC regulate channel activity.

One obvious question is how do the results from these experiments relate to the hypertensive disease, Liddle's Syndrome? Why do patients with truncations in their β or γ ENaC subunits have increased channel activity? In Liddle's syndrome patients it may be expected that the loss of Nedd4-mediated negative control will be balanced by a loss of positive control, due to the lack of a positive regulatory protein binding to the last 10 amino acids of β ENaC or residues 631-639 of γ ENaC. Also why do ENaC complexes carrying missense Liddle's syndrome mutations expressed in *Xenopus* oocytes not have a greater current than Liddle's syndrome truncated channels in the same system? This may be expected as missense mutated ENaC will have lost Nedd4-mediated negative control but will still possess the positive regulatory Ser 635 residue in the β ENaC carboxyl termini and residues 631-639 of γ ENaC. One explanation for these findings is that the β and γ carboxyl termini can each impart positive regulatory effects on ENaC by the same mechanism. Therefore, if the carboxyl terminus of either β or γ ENaC is truncated, the positive regulatory element in the remaining intact subunit can still influence ENaC activity. This hypothesis is supported by the finding that inclusion of the carboxyl terminus of either β ENaC or γ ENaC individually in the low Na^+ pipette solution abolished ENaC activity. If the carboxyl termini of β and γ ENaC regulated channel activity by independent means then it would be expected that inclusion of both β and γ ENaC carboxyl termini in the low Na^+ pipette solution would be required to inhibit ENaC activity.

The results in this chapter suggest that the carboxyl termini of β and γ ENaC are important sites for interaction with protein(s) that both positively and negatively influence channel activity.

Figure 1. The carboxyl termini of β and γ ENaC but not α ENaC inhibit Na^+ channel activity when the Na^+ and Cl^- loops are inactive

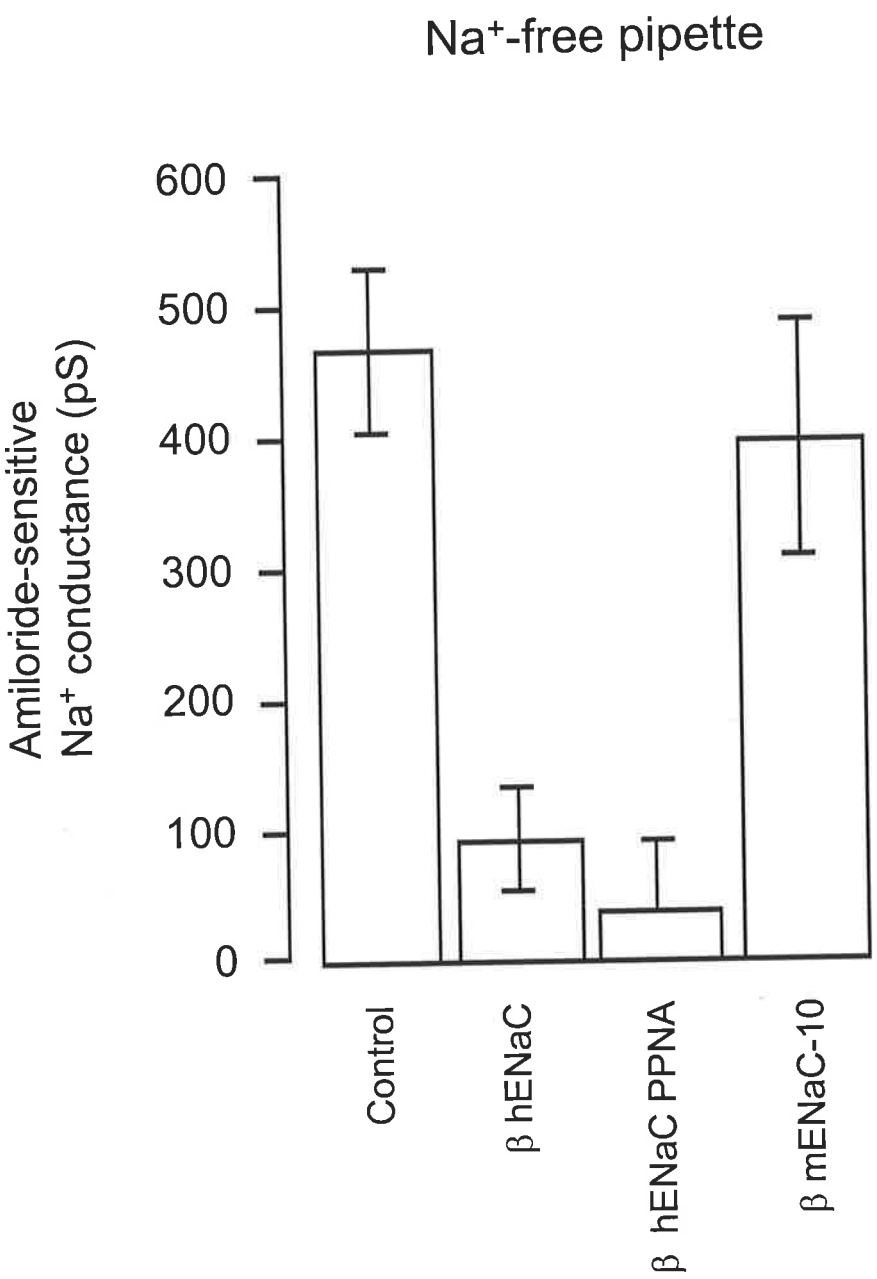
The effects on the amiloride-sensitive Na^+ current of the inclusion in the Na^+-free pipette solution of the the carboxyl terminal 40 amino acids of either human β ENaC (βhENaC) (300 μg/ml), mouse α ENaC (αmENaC) (300 μg/ml) or mouse γ ENaC (γmENaC) (300 μg/ml) fused to GST. A minimum of 5 recordings were taken for each experiment.



βhENaC	TGPYPSEQAL PIPGTP**PPNY** DSLRLQPLDY ESDSEGDAI
αmENaC	TTPPLALTAP **PPAY**ATLGPS ASPLDSAVPG SSACAPAMAL
γmENaC	RLPPAPEAPV PGTP**PPRY**NT LRLDSAFSSQ LTDTQLTNEF

Figure 2. The inhibitory effect of the carboxyl terminus of β ENaC on Na^+ conductance is dependent on the last 10 amino acids and is independent of the PY motif

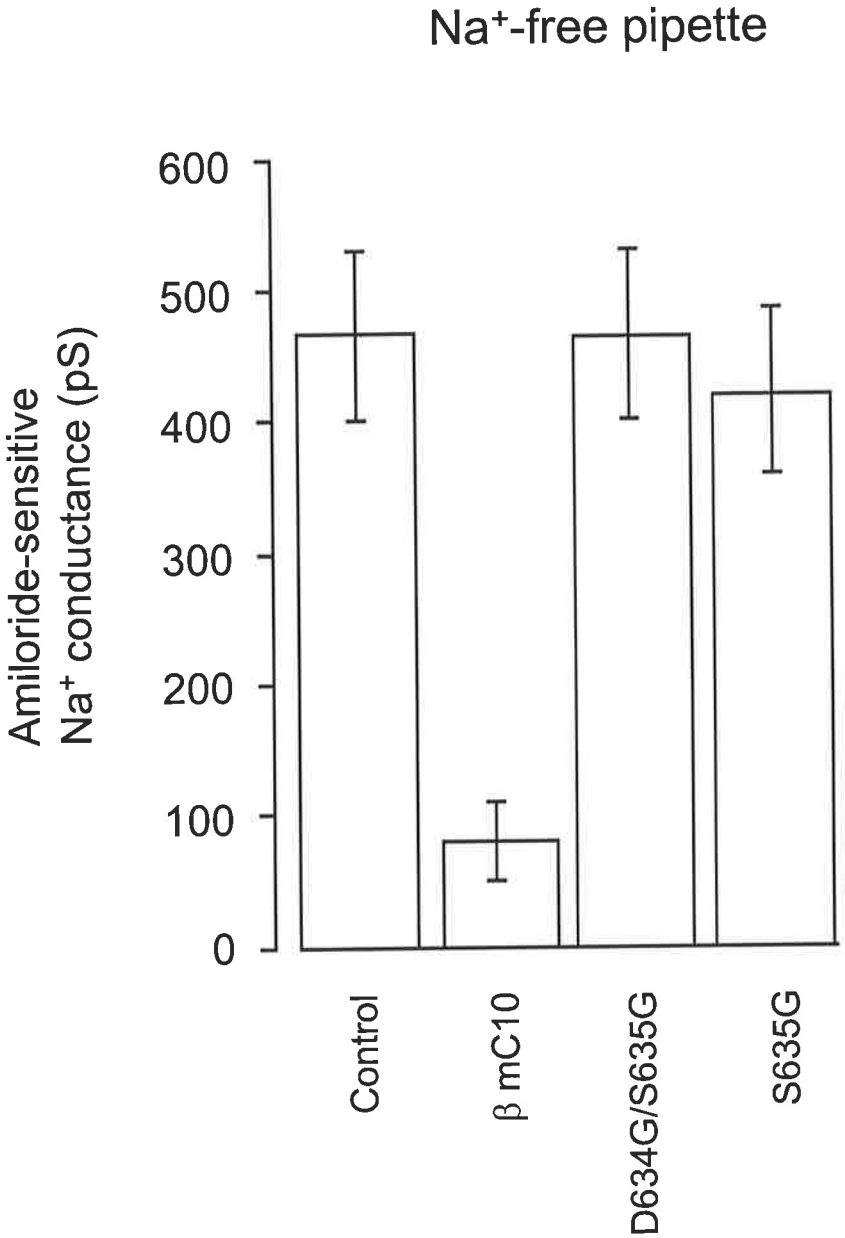
The effects on the amiloride-sensitive Na^+ current of the inclusion in the Na^+-free pipette solution of βhENaC (300 μg/ml), a PY motif-mutant version of βhENaC (300 μg/ml) or mouse β ENaC lacking the last 10 amino acids (βmENaC-10) (300 μg/ml). A minimum of 5 recordings were taken for each experiment.



βhENaC	TGPYPSEQAL PIPGTP**PPNY** DSLRLQPLDY ESDSEGDAI
βhENaC PPNA	TGPYPSEQAL PIPGTP**PPNA** DSLRLQPLDY ESDSEGDAI
βmENaC	GEVVPDQQTL PIPGTP**PPNY** DSLRLQPLDT MESDSEVEAI
βmENaC-10	GEVVPDQQTL PIPGTP**PPNY** DSLRLQPLDT

Figure 3. Ser 635 is required for the inhibitory effect of the carboxyl terminus of β ENaC on Na^+ conductance

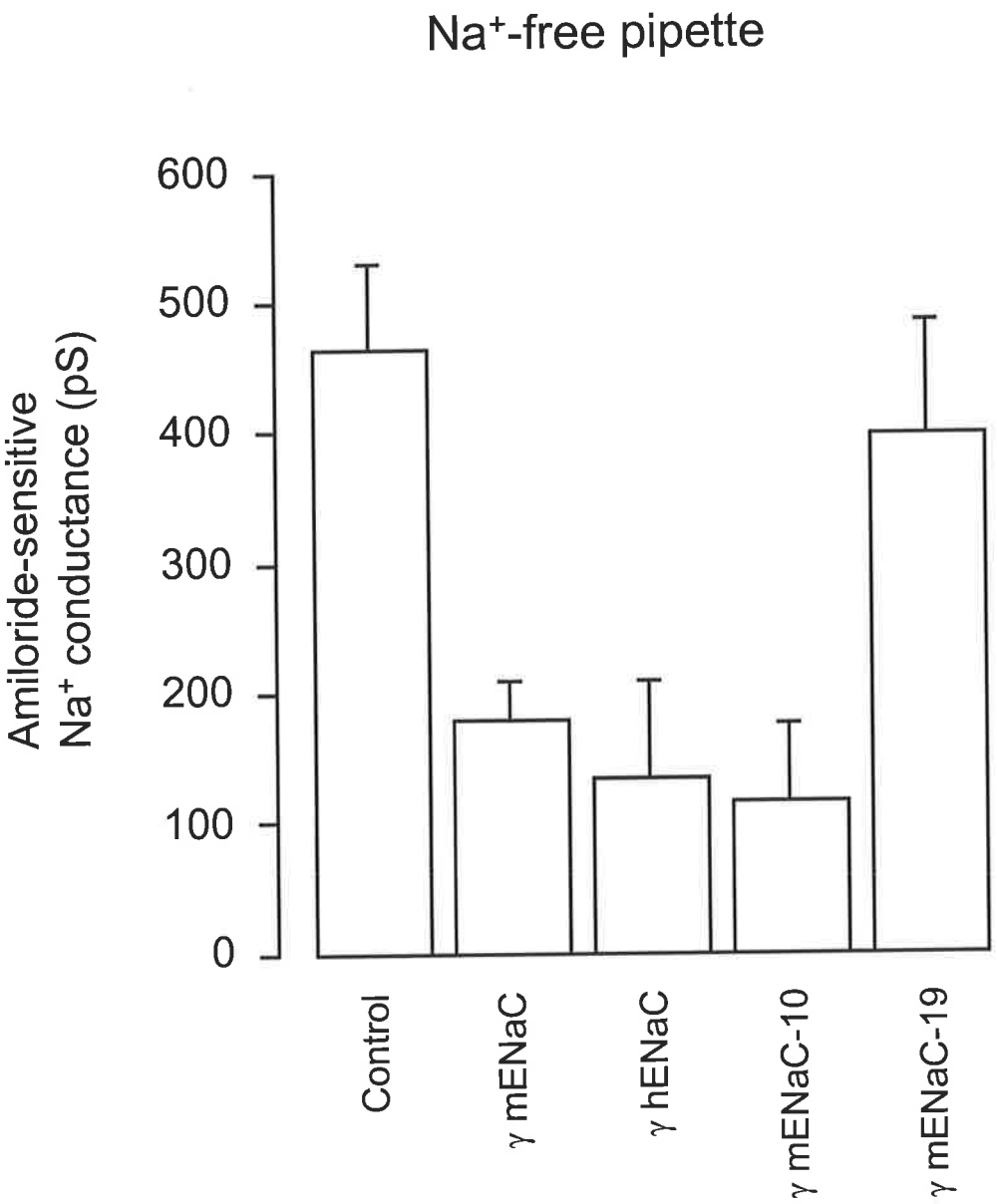
The effects on the amiloride-sensitive Na^+ current of the inclusion in the Na^+-free pipette solution of a peptide consisting of the last 10 amino acids of mouse β ENaC (βmC10) (20 nM), βmC10 except that residues Asp 634 and Ser 635 were altered to Gly (D634G/S635G) (20 nM),βmC10 except that residues Ser 635 was altered to Gly (S635G) (20 nM). A minimum of 5 recordings were taken for each experiment.



βmENaC	GEVVPDQQTL PIPGTP**PPNY** DSLRLQPLDT MESDSEVEAI
βmC10	MESDSEVEAI
D634G/S635G	MEGGSEVEAI
S635G	MEGDSEVEAI

Figure 4. The inhibitory effect of the carboxyl terminus of γ ENaC on Na^+ conductance is dependent on the last 19 amino acids

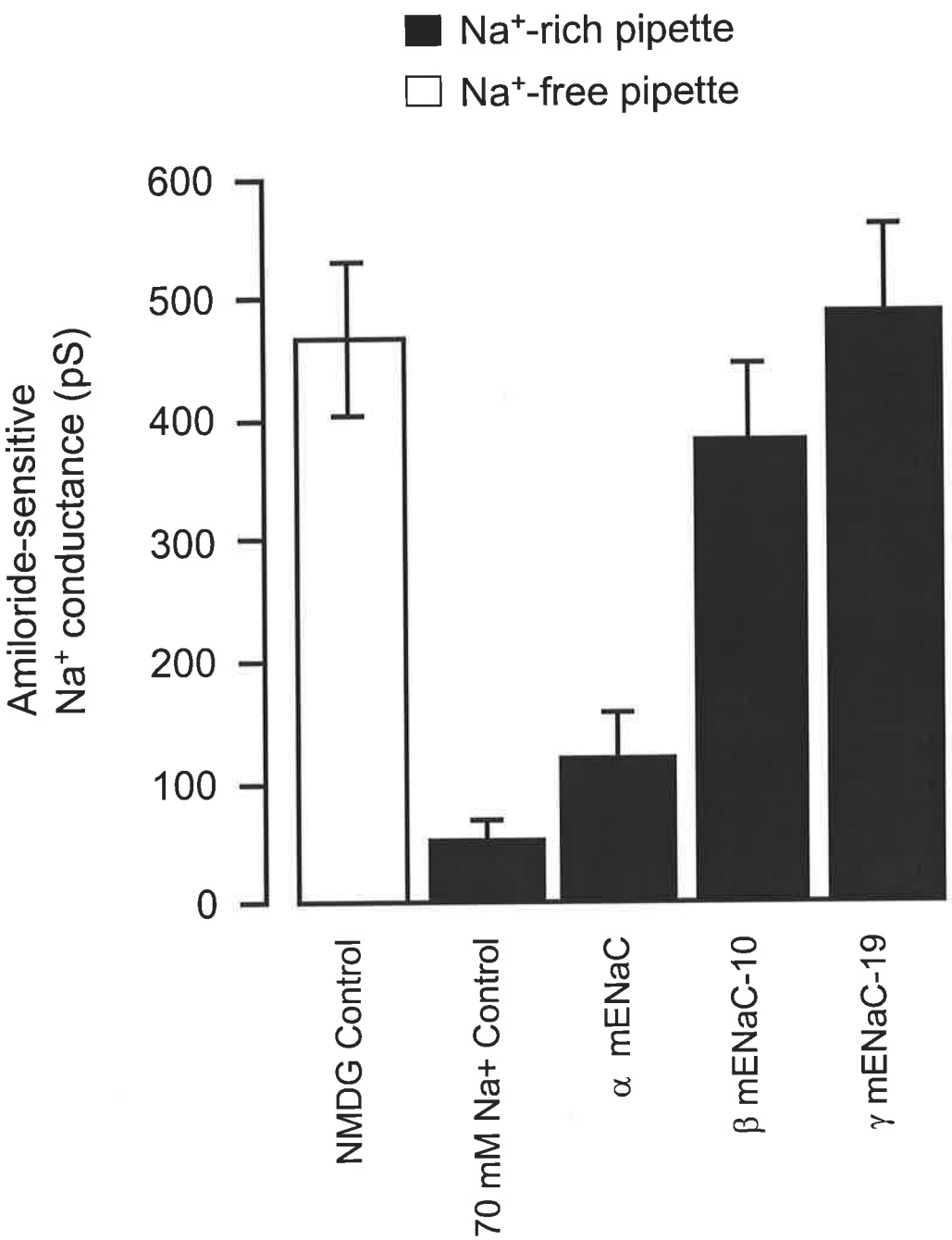
The effects on the amiloride-sensitive Na^+ current of the inclusion in the Na^+-free pipette solution of γmENaC (300 μg/ml), the carboxyl terminal 40 amino acids of human γ ENaC fused to GST (γhENaC) (300 μg/ml), γmENaC lacking the last 10 amino acids (γmENaC-10) (300 μg/ml) or γmENaC lacking the last 19 amino acids (βmENaC-10) (300 μg/ml). A minimum of 5 recordings were taken for each experiment.



γmENaC	RLPPAPEAPV PGTP**PPRY**NT LRLDSAFSSQ LTDTQLTNEF
γhENaC	HLPPAPEAPV PGTP**PPRY**NT LRLDSAFSSQ LTDTQLTNEF
γmENaC-10	RLPPAPEAPV PGTP**PPRY**NT LRLDSAFSSQ
γmENaC-19	RLPPAPEAPV PGTP**PPRY**NT L

Figure 5. β and γ ENaC, but not α ENaC carboxyl termini are required for Na^+-feedback inhibition of ENaC

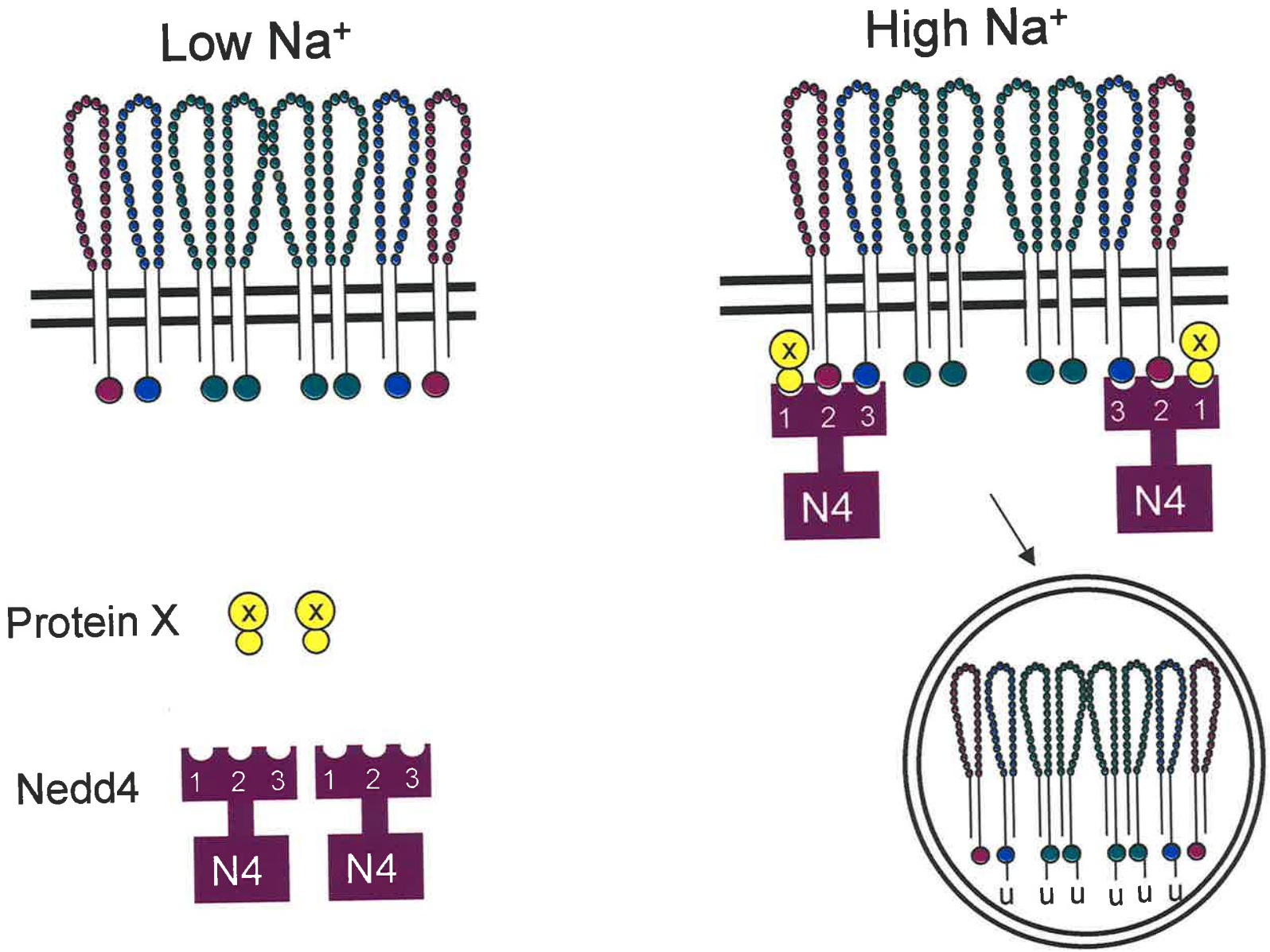
The effects on the amiloride-sensitive Na^+ current of the inclusion in the 70 mM Na^+ pipette solution of either αmENaC (100 μg/ml), βmENaC-10 (100 μg/ml), or γmENaC-19 (100 μg/ml). A minimum of 5 recordings were taken for each experiment.



αmENaC	TTPPLALTAP **PPAY**ATLGPS ASPLDSAVPG SSACAPAMAL
βmENaC	RLPPAPEAPV PGTP**PPRY**NT LRLDSAFSSQ LTDTQLTNEF
βmENaC-10	GEVVPDQQTL PIPGTP**PPNY** DSLRLQPLDT
γmENaC	RLPPAPEAPV PGTP**PPRY**NT LRLDSAFSSQ LTDTQLTNEF
γmENaC-19	RLPPAPEAPV PGTP**PPRY**NT L

Figure 6. An updated model for interaction of Nedd4 with ENaC.

In response to high intracellular Na^+ an as yet unidentified factor (X) is activated and binds to Nedd4 via WW domain 1. This serves to recruit Nedd4 to ENaC and/or stabilise the binding of Nedd4 WW domains 2 and 3 to the α, β and γ subunits of ENaC. We postulate that a stable Nedd4/ENaC complex will comprise one ENaC octamer, two molecules of Nedd4 and two molecules of protein X. Once stably bound, Nedd4 ubiquitinates ENaC and stimulates its internalisation and/or subsequent degradation.


Low Na+
High Na+
X
1 2 3
3 2 1
N4
N4
Protein X
X
X
Nedd4
1 2 3
1 2 3
N4
N4
u u u u u u

Chapter 7:
General Discussion

The discovery of the human E6-associated protein (E6-AP) as an essential factor for ubiquitin-mediated degradation of the tumor suppressor protein, p53, defined a new class of enzymes involved in the ubiquitin system. Several enzymes with homology to E6-AP have since been identified (hect proteins) and appear to represent a group of ubiquitin-protein ligases (E3s) that are important for targeting many of the proteins that are regulated by ubiquitination (Hein *et al.*, 1995; Huibregtse *et al.*, 1995; Rosa *et al.*, 1996; Dinudom *et al.*, 1998; Galan *et al.*, 1996; Huibregtse *et al.*, 1997; Lehman *et al.*, 1998; Malzac *et al.*, 1998; Perry *et al.*, 1998; Saleh *et al.*, 1998). Hect proteins are different from other E3 proteins in that they form a thioester with ubiquitin, which is subsequently transferred to the substrate protein, rather than acting as a bridging molecule between an E2 enzyme and a substrate (Scheffner *et al.*, 1995). Few substrates of E3 proteins have been identified at present and as a result the function of this class of proteins is not well understood. At the commencement of this project the function of the Nedd4 protein was obscure and only initial tissue expression, genomic localisation and subcellular protein localisation studies had been performed (Kumar *et al.*, 1992; Kumar *et al.*, 1997). The aim of this project therefore was to attempt to characterise the function of Nedd4.

At the commencement of this project it was postulated that the most direct means of elucidating the function of Nedd4 was to create a mouse lacking the Nedd4 gene, or to identify interacting proteins that may be ubiquitinated and therefore regulated by Nedd4. The first protein that was identified to interact with Nedd4 was the β subunit of the epithelial Na^+ channel (ENaC) (Staub *et al.*, 1996). It was subsequently shown that all three ENaC subunits could interact with Nedd4 and that this interaction was dependent on the WW domains of Nedd4 and PY motifs contained in the carboxyl termini of the ENaC subunits (Staub *et al.*, 1996). This finding was particularly interesting as the β and γ subunits of ENaC had been shown to be mutated in the genetic hypertensive disorder, Liddle's Syndrome. In particular, the PY motif present in the β and γ subunits of ENaC

was truncated or mutated in patients with this disease (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a; Hansson *et al.*, 1995b). This region of ENaC was therefore thought to be a negative regulatory domain and a possible site of interaction with a protein that may downregulate the activity of ENaC. In chapter 4 of this thesis, the discovery that antibodies specific for Nedd4 could interfere with Na^+-dependent downregulation of ENaC activity was the first evidence that Nedd4 may indeed negatively regulate ENaC function. This was strengthened by the finding that overexpression of the WW domains of Nedd4 could also interfere with normal downregulation of ENaC activity, presumably by disallowing interaction between the WW domains of wildtype Nedd4 and ENaC [chapter 4 of this thesis and (Dinudom *et al.*, 1998)]. The observation that dominant negative ubiquitin also interfered with Na^+-dependent inhibition of ENaC [chapter 4 of this thesis; (Dinudom *et al.*, 1998)] did not provide direct evidence that Nedd4 ubiquitinated ENaC, but along with the finding that ENaC is ubiquitinated (Staub *et al.*, 1997) shows that ubiquitination of ENaC is important for downregulating its activity.

Additionally, results presented in chapter 4, demonstrate that Nedd4 downregulates ENaC downstream of the G protein, G_o. The method by which G proteins impart control on ENaC activity is unknown. G proteins normally regulate protein function after being recruited to activated receptors. They can then either initiate signalling cascades that stimulate synthesis of new proteins, or directly alter the function of existing proteins (reviewed in Hamm, 1998, and Gutkind, 1998). ENaC-expressing cells might possess a sensor of increased cytosolic Na^+, which might stimulate the activity of particular G proteins. Na^+-dependent inhibition of ENaC in mouse mandibular duct cells is unlikely to require synthesis of new proteins as inhibition of ENaC by increased intracellular Na^+ occurs very rapidly (A. Dinudom *et al.*, unpublished). Therefore it is likely that G_o activates Nedd4 itself or initiates a signalling pathway which results in Nedd4-mediated ubiquitination and endocytosis of ENaC.

One possible clue to the question of how Nedd4 is stimulated to ubiquitinate ENaC arose from studies carried out in chapter 5 of this thesis, which investigated the role of individual WW domains of Nedd4 in Na^+-dependent downregulation of ENaC. It was found that only WW domains 2 and 3 of murine Nedd4 bound to the α, β and γ ENaC subunits *in vitro*, whereas WW domain 1 did not bind to any of the subunits. In mouse mandibular duct cells however all three WW domains were required to inhibit Na^+-dependent feedback of ENaC. This led to the suggestion that WW domains 2 and 3 of Nedd4 interact with the PY motifs of ENaC while WW domain 1 is responsible for binding to an as yet unknown protein that may act to stimulate recruitment to ENaC or serve to stabilise the Nedd4/ENaC complex once it has formed. Such a protein may interact with Nedd4 WW domain 1 through a PY motif or possibly through a phosphoserine or phosphothreonine residue, both of which have been shown to be capable of interaction with WW domains from Nedd4 (Lu *et al.*, 1999). This is an attractive possibility as many signaling cascades employ selective interaction of phosphoproteins to carry out their function.

At the time the studies in chapter 5 of this thesis were carried out, .the stoichiometry of ENaC was believed to be tetrameric, consisting of 1β, 1γ and 2α subunits (Firsov *et al.*, 1998; Kosari *et al.*, 1998). The mode of association with Nedd4 was therefore based on ENaC residing in the cytosolic membrane as a tetramer. It was postulated that two molecules of Nedd4 would interact with one ENaC tetramer and 2 molecules of protein X (Harvey *et al.*, 1999). Contrary to this hypothesis, experiments done in Chapter 6 of this thesis suggested that the α subunit of ENaC is not involved in Na^+-dependent regulation of ENaC. This finding is supported by the observation that no Liddle's syndrome patients have been identified that carry mutations in the α subunit of ENaC. In chapter 6 of this thesis, the ENaC β and γ subunits were both found to be required for Na^+-downregulation of ENaC and correspondingly several different groups of

Liddle's syndrome patients have been identified with mutations in either the β or γ ENaC subunits. Strong evidence was recently presented claiming that membrane-bound ENaC actually consists of eight subunits, still with the same stoichiometry of 1β, 1γ and 2α subunits (Eskandari *et al.*, 1999). In light of these results the model of Nedd4/ENaC interaction was amended. As shown in Figure 6 (chapter 6), still two molecules of Nedd4 are hypothesised to interact with two molecules of protein X and one ENaC complex, however this model assumes that ENaC contains eight subunits (4α, 2β and 2γ). It is postulated that only the β and γ subunits of ENaC will interact with Nedd4 while the α subunits remain unbound. This is suggested despite the fact that α ENaC contains a conserved PY motif that is capable of interacting with Nedd4 WW domains 2 and 3 *in vitro* [chapter 5 of this thesis and (Harvey *et al.*, 1999)]. It is possible that *in vivo,* the α subunit of ENaC is unable to interact with Nedd4 as it is physically associated with other proteins [e.g. α-spectrin is known to associate with α ENaC *in vivo* (Rotin *et al.*, 1994)]

The findings in chapters 5 and 6 of this thesis appear to agree with the present understanding of the hypertensive disorder, Liddle's syndrome. In Liddle's syndrome the mutation of a single type of channel subunit (either β or γ ENaC) appears to be sufficient to abrogate interaction with Nedd4 as ENaC activity is increased, presumably as a result of a lack of Nedd4 and ubiquitin-mediated control. This suggests that both intact β and γ ENaC are required to stably recruit Nedd4 to the ENaC complex. If Nedd4 only interacted with either β or γ ENaC subunits, then ENaC complexes in Liddle's syndrome patients would presumably still be capable of association with Nedd4 through the remaining intact ENaC subunit. This appears not to be the case however.

The possibility exists that additional proteins that are involved in Na^+-dependent downregulation of ENaC are mutated in Liddle's syndrome patients. Nedd4 is one candidate protein, although one might expect multiple defects in people carrying Nedd4

mutations as it is likely that Nedd4 will regulate the activity of proteins other than ENaC. ENaC has a restricted tissue expression in adults and is expressed at very low levels during development, whereas Nedd4 is expressed highly during development and in almost every adult tissue. Coupled with this, immunoprecipitations of tissue extracts with Nedd4 antibodies consistently pull down a number of proteins that are different sizes to ENaC (K. Harvey and S. Kumar, unpublished).

The discovery that Nedd4 is cleaved by caspases during apoptosis [chapter 3 of this thesis and (Harvey *et al.*, 1998)] was an important observation but the significance of this finding is unclear at present. The activation of caspases is central to the execution of apoptosis, as inhibitors of these proteases are known to prevent cell death (Ekert *et al.*, 1999). From two-dimensional electrophoresis of apoptotic and non-apoptotic protein extracts it appears that less than 200 proteins are cleaved during apoptosis (Brockstedt *et al.*, 1998). Approximately 70 of these proteins have been identified to date. The cleavage of these proteins induces structural disassembly of the cell and cell cycle arrest, inactivates cellular repair enzymes and inhibitors of apoptosis, and flags the cell for phagocytosis. Caspase-mediated cleavage affects protein function in a number of ways: eg. cleavage of structural proteins such as lamins, fodrin and gelsolin stimulates organised dismantling of the cell; cleavage of repair enzymes such as DNA-dependent protein kinase and poly(ADP-ribose) polymerase inactivates them; cleavage of proteins such as protein kinase C, cytosolic phospholipase A_2 and the caspase-activated DNAse (CAD) are activated by removal of a regulatory domain or inhibitory protein; whereas the function of some proteins, e.g. Bcl-2 and Bcl-X_L is converted from being anti-apoptotic to pro-apoptotic (reviewed in Nicholson, 1999). The simplest explanation for Nedd4 cleavage during apoptosis is that it is an energy-conserving process as it prevents protein ubiquitination, which is an energy-expensive process. The significance of caspase-mediated cleavage of Nedd4 may become more obvious when the function of Nedd4 is

better understood.

A growing number of membrane proteins have been found to be regulated by ubiquitin-stimulated endocytosis. In yeast, a number of nutrient transporters have been found to be ubiquitinated and internalised while in mammals several growth factor receptors are ubiquitinated and endocytosed following ligand binding (reviewed in Hicke, 1999). The unique domain structure of the Nedd4 family of ubiquitin-protein ligases makes them strong candidates for being responsible for targeting multiple membrane proteins for ubiquitin-mediated regulation. The C2 domain of Nedd4 has been shown to be responsible for redistribution of Nedd4 to the extracellular membrane in response to ionymicin plus Ca^{2+} treatment (Plant *et al.*, 1997). While Rsp5, a *S. cerevisiae* Nedd4 family member, has been implicated in ubiquitin-mediated regulation of a number of membrane proteins including the general amino acid permease (Hein *et al.*, 1995), the uracil permease (Hein *et al.*, 1995; Galan *et al.*, 1996; Galan and Haguenauer Tsapis, 1997) and the maltose transporter (Lucero and Lagunas, 1997). Pub1, a *S. pombe* Nedd4 family member, appears to regulate at least one membrane protein as it is required for tolerance of low pH (Saleki *et al.*, 1997).

The identification of substrates of Nedd4 is an ongoing project in our laboratory and several candidate proteins that were isolated on their ability to interact with the WW domains of Nedd4 are being examined at present. If these proteins prove to be physiological substrates of Nedd4 then the precise function of Nedd4 will become clearer. A more direct method to identify bona fide targets of Nedd4 is to immunoprecipitate Nedd4-interacting proteins from cultured cell or organ lysates and microsequence them. We have generated antibodies that consistently precipitate Nedd4 and proteins other than Nedd4 and such experiments are planned for the near future. Another approach to identify proteins that are regulated by Nedd4 is to use a simple organism such as *Drosophila*

melanogaster, which is relatively easy to manipulate genetically and has its full genomic sequence available. Nedd4 is conserved throughout evolution, with homologues present as far back as yeast. A *Drosophila* homologue of Nedd4 also exists which is 70% similar to mouse Nedd4 and is likely to regulate similar proteins as its mammalian homologue. *Drosophila* that have mutations in Nedd4 will be valuable for analysis of Nedd4 function in this organism and will allow us to test the hypothesis that targeting membrane proteins for ubiquitin-mediated turnover is the evolutionary conserved function of Nedd4. Additionally, targets of *Drosophila* Nedd4 could be identified using transgenic flies that overexpress Nedd4 in the eye. If such flies carry a distinguishable eye phenotype (such as rough eyes) then they can be used for screens to identify proteins that genetically interact with Nedd4. Flies that have an alteration of the Nedd4 eye phenotype can be identified and the gene that is responsible for the suppression or enhancement of the eye phenotype isolated, and its relation to Nedd4 characterised. If mammalian homologues of such genes exist then their possible regulation by mammalian Nedd4 can be examined.

The generation of Nedd4 knockout mice should provide a valuable reagent for determining the function of Nedd4 and is currently underway in collaboration with Prof. P. Hertzog (Monash University, Melbourne). One might expect that Nedd4 null mice will be hypertensive due to a lack of negative regulation of ENaC, as occurs in Liddle's syndrome. If so these mice will provide a valuable reagent for the study of this disease. The possibility exists that Nedd4 null mice will have multiple defects if Nedd4 is required for regulation of a number of proteins involved in different physiological functions. Nedd4 null mice may die during development if Nedd4 is important for regulation of proteins that are required for embryogenesis to occur. Alternatively, Nedd4 knockout mice may be normal due to compensation by other Nedd4 family members, which may possess the ability to regulate the same substrates as Nedd4.

In summary, the studies presented in this thesis have identified Nedd4 as a key protein involved in Na^+-dependent downregulation of ENaC. All three WW domains of Nedd4 and the β and γ subunits of ENaC have been shown to be required for this regulation to occur. Additionally, an unknown Nedd4 WW domain 1-interacting protein has been implicated in this process. These studies have aided in the molecular understanding of the familial hypertensive disorder, Liddle's syndrome, and the regulation of blood pressure in general. Nedd4 has also been identified as a protein that undergoes caspase-mediated cleavage in apoptotic cells, although the significance of this finding is not fully understood at present. Current and future studies will aim to further characterise the function of the Nedd4 protein by analysing mice and *Drosophila* that lack the Nedd4 gene and by identifying additional proteins that are regulated by Nedd4-mediated ubiquitination.

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Addenda

1. Chapter 1, Figure legend for Figure 2 should read: **Figure 2. Key structural residues of the WW domain.** The structure of the WW domain of human YAP in complex with its ligand was elucidated by Macias *et al.*, (1997). Residues involved in ligand binding (shown in black boxes), well-defined turns (underlined) and the formation of β-sheets (β1, β2 and β3) are indicated. The corresponding residues in WW domain 1, 2 and 3 of murine Nedd4 are also shown.

2. Chapter 1, Figure legend for Figure 6: Accession numbers for the following genes are as follows: Human Nedd4: P46934, Human KIAA0439: AB007899, Human KIAA0322: AB002320.

3. Chemically competent *E. coli* cells described in Materials and Methods were prepared as described in Sambrook *et al.*, (1989).

4. Chapter 3, page 35: Preparation of caspases derived for experiments performed in sections 3.6 and 3.7 were described in Harvey *et al.*, (1998).

5. Error bars from figures 2 to 4 from chapter 2; 7 and 8 from chapter ; and 1 to 5 from chapter 4 represent standard error of the mean as derived by Student's unpaired t-test.

6. Missing reference from Bibliography: Huang, Y., Baker, R.T. and Fischer-Vize, J.A. (1995). Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene. Science. (1995) *270*, 1828-31.