Ubiquitin ligases play a pivotal role in substrate recognition and ubiquitin transfer, yet little is known about the regulation of their catalytic activity. Nedd4 (neural-precursor-cell-expressed, developmentally down-regulated 4) is an E3 ubiquitin ligase composed of a C2 domain, four WW domains (protein–protein interaction domains containing two conserved tryptophan residues) that bind PY motifs (L/PPXY) and a ubiquitin ligase HECT (homologous with E6-associated protein C-terminal) domain. In the present paper we show that the WW domains of Nedd4-2 bind (weakly) to a PY motif (LPXY) located within its own HECT domain and inhibit auto-ubiquitination. Pulse–chase experiments demonstrated that mutation of the PY motif of Nedd4-2 decreases the stability of Nedd4-2, suggesting that it is involved in stabilization of this E3 ligase. Interestingly, the HECT PY-motif mutation does not affect ubiquitination or down-regulation of a known Nedd4-2 substrate, ENaC (epithelial sodium channel).

Key words: epithelial sodium channel (ENaC), HECT (homologous with E6-associated protein C-terminal) domain, protein stability, self-ubiquitination, ubiquitin ligase, WW domain.

INTRODUCTION

Ubiquitination plays a critical role in many cellular processes, primarily regulating protein stability and trafficking/endocytosis [1–3]. The ubiquitination cascade is performed by the sequential action of E1, E2 and E3 enzymes. E3 ubiquitin ligases, which belong primarily to the RING (really interesting new gene) or HECT (homologous with E6-associated protein C-terminal) families, impart selectivity on the ubiquitination process by recognizing target substrates and promoting the transfer of activated ubiquitin from the E2 enzymes (RING family) or themselves (HECT family) to the substrate, lysine residues [1].

The Nedd4 (neural-precursor-cell-expressed, developmentally down-regulated 4) family of HECT E3 ligases (including Nedd4-1 and -2) are composed of a C2 domain, 3–4 WW domains (protein–protein interaction domains containing two conserved tryptophan residues) that interact with the PY motifs (L/PPXY) of target proteins, and a C-terminal ubiquitin ligase HECT domain [3]. The best characterized Nedd4-2 substrate is the ENaC (epithelial sodium channel). Nedd4-2 WW domains bind to ENaC PY motifs, regulating the cell-surface stability of the channel by targeting it for endocytosis and lysosomal degradation [4–10]. Nedd4-2-mediated down-regulation of the channel is critical, since mutations in the PY motif of ENaC that disrupt Nedd4-2 ENaC ubiquitination, in turn, appears to promote Nedd4-2 self-ubiquitination. These results support a model in which the intra- or inter-molecular WW-domain–HECT PY-motif interaction stabilizes Nedd4-2 by preventing self-ubiquitination. Substrate binding disrupts this interaction, allowing self-ubiquitination of Nedd4-2 and subsequent degradation, resulting in down-regulation of Nedd4-2 once it has ubiquitinated its target. These findings also point to a novel mechanism employed by a ubiquitin ligase to regulate itself differentially compared with substrate ubiquitination and stability.

EXPERIMENTAL

Plasmids

The human Nedd4-2 isoform lacking the C2 domain was cloned into the V5 expression vector, pCDNA3.1-nV5, and the human Nedd4-2 isoform with C2 domain was cloned into a GST (glutathione transferase) vector for bacterial expression (pDEST15 with an engineered PreScission protease (GE Healthcare) site) using the Gateway system (Invitrogen). The bacterial expression plasmids pGEX-6P1-HECT, pQE-30-WW1, pQE-30-WW2, pQE-30-WW3 and pQE-30-WW4 were constructed by standard PCR and restriction-enzyme cloning procedures using Xenopus Nedd4-2 as a DNA template, and pGEX-KG-βPY (where βPY binding cause Liddle’s syndrome, a hereditary hypertension caused by elevated ENaC stability/activity [11].

Despite their importance, little is known about the regulation of catalytic activity of the E3 ligases, including that of Nedd4-2, and it was thought previously to be constitutively active. In the present paper we show that Nedd4-2 regulates its own stability through self-ubiquitination, which is inhibited by interaction between Nedd4-2 WW domains and a PY motif located within its own HECT domain.
is a PY motif from βENaC) was constructed using rat βENaC as a DNA template. Construction of pSDeasy plasmids encoding ENaC subunits and Xenopus Nedd4-2 was performed as described previously [12]. All Nedd4-2 and HECT mutations were made using the QuikChange® site-directed mutagenesis kit (Stratagene).

Transfections and antibodies

Transient transfections in HEK-293T cells (human embryonic kidney cells expressing the large T-antigen of simian virus 40) were performed using the calcium phosphate precipitation method with a total of 20 μg of DNA per 10 cm-diameter dish. The antibodies used for immunoblotting were: anti-RGS-His (where RGS is arginine–glycine–serine) antibody (1:1000 dilution; Qiagen), anti-ubiquitin antibody (1:1000 dilution; Covance), anti-FLAG antibody (1:10000 dilution, Sigma), anti-haemagglutinin antibody (1:10000 dilution, Sigma), anti-Myc antibody (1:5000 dilution; Innotech), anti-ubiquitin antibody (1:1000 dilution; Covance), anti-FLAG antibody (1:5000 dilution; Chemicon), anti-V5 antibody (1:5000 dilution; Invitrogen) and a rabbit polyclonal anti-Nedd4-2 antibody (1:500 dilution) which has been described previously [13]. Incubation with primary antibodies was followed by either horseradish-peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (both at 1:10000 dilution; Jackson ImmunoResearch) and detected by ECL® (enhanced chemiluminescence; GE Healthcare).

Far Western blotting

GST fusion proteins were produced in bacteria and purified on glutathione–agarose beads (Sigma). MRGS (methionine–RGS)–His–tagged WW domains were expressed and purified as described previously [14]. GST fusion proteins (50 μg) were resolved by SDS/PAGE (10% gels), transferred on to nitrocellulose (GE Healthcare), incubated with 50 μg of purified WW domains and analysed by immunoblotting with an anti-RGS-His antibody.

Ubiquitination assays

For in vitro ubiquitination assays, bacterially expressed Xenopus Nedd4-2–HECT domain, full-length human Nedd4-2 (~1 μg) cleaved of its GST tag (using PreScission protease) or human Nedd4-2 immunopurified from transfected HEK-293T cells was incubated in reaction mixtures containing 100 nM yeast E1 ubiquitin-activating enzyme (BostonBiochem, Cambridge, MA, U.S.A.), 500 nM E2 ubiquitin-conjugating enzyme (UbcH7; BostonBiochem), 2 μg of ubiquitin (Sigma) and 4 mM ATP in a reaction buffer [25 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 μM dithiothreitol and 4 mM MgCl₂]. Reactions were incubated for 1 h at room temperature (22°C) unless otherwise noted and analysed by immunoblotting with an anti-ubiquitin antibody. For human Nedd4-2 and ENaC ubiquitination in HEK-293T cells, cells were treated with 20 μM MG101 (Boston Biochem), 2 μg of ubiquitin (Sigma) and 4 mM ATP in a reaction buffer [25 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 μM dithiothreitol and 4 mM MgCl₂]. Reactions were incubated for 1 h at room temperature (22°C) unless otherwise noted and analysed by immunoblotting with an anti-ubiquitin antibody. For human Nedd4-2 and ENaC ubiquitination in HEK-293T cells, cells were treated with 20 μM MG101 (Boston Biochem) for 12 h at 37°C before lysis, or with 10 μM clasto-lactacystin-β-lactone (Boston Biochem) for 3 h at 37°C before lysis. Cells were lysed 48 h post-transfection in a lysis buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% (v/v) glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml apro tin, 1 μg/ml pepstatin A and 1 mM PMSF] supplemented with 50 μM L-lysine (N-acetyl-L-lysyl-L-lysyl-L-norleucinal; Sigma) and 0.4 mM chloroquine (Sigma). Cell lysates were denatured with 2% (w/v) SDS and boiled for 5 min, then diluted eleven times with lysis buffer to dilute the SDS prior to immunoprecipitation.

Pulse–chase experiments

Cells were washed three times with methionine/cysteine-deficient medium (Invitrogen) in a 30 min period and then incubated in methionine/cysteine-deficient medium with 0.1 mCi/ml [35S]methionine/cysteine (ProMix; GE Healthcare) for 2 h at 37°C. Cells were washed three times with chasing medium [Dulbecco’s modified Eagle’s medium (Invitrogen) and 10% (v/v) fetal bovine serum with 10 mM unlabelled methionine and cysteine] and then chased for the indicated times. Before lysis (performed as detailed above), cells were washed three times with PBS. Nedd4-2 was immunoprecipitated from denatured lysates using an anti-V5 antibody (1:10000 dilution) as described above. Gels were dried and exposed to X-ray film. The amount of Nedd4-2 was quantified from the X-ray film using an Alphalmager and the spot-density-analysis function of the AlphaEase software (Alpha Innotech).

Electrophysiological measurements of ENaC activity in Xenopus oocytes

Plasmids encoding the three ENaC subunits (3.3 ng each) and Xenopus Nedd4-2, either WT (wild-type) or the Y971A mutant (2.5 ng each), were linearized, in vitro transcribed and injected into Xenopus laevis oocytes. Electrophysiological measurements for amiloride (5 μM)-sensitive Na+–channel activity were carried out after overnight incubation at 19°C, as described previously [15].

RESULTS

The Nedd4-2 WW domains bind to the HECT PY motif and regulate the catalytic activity of the HECT domain

The HECT domain of Nedd4/Nedd4-2 and other HECT-containing proteins contain a PY (LPXY) motif (Figure 1A and [16]) located adjacent to the conserved catalytic cysteine residue (Figure 1B). This suggests that the catalytic activity of Nedd4 family members may be regulated through intra- or inter-molecular interactions between the WW domain(s) and the HECT domain. To test for such binding, we performed an in vitro binding assay with a GST-tagged HECT domain and His₆–tagged individual WW domains of Nedd4-2, but could not detect an interaction between the two proteins (results not shown). Since the LPXY motif is in a turn, rather than the normally extended conformation (Figure 1B), it may be inaccessible to the WW domains until a conformational change occurs, which is expected to occur on ubiquitin transfer from the E2 enzyme to the HECT active-site cysteine residue [17,18]. To ensure the accessibility of the HECT PY motif, we performed Far Western analysis, in which GST–HECT was denatured prior to incubation with the His₆–WW domains. Strips of nitrocellulose bound with denatured GST–HECT WT, or GST–HECT Y971A, in which Tyr⁹⁷¹ had been mutated to an alanine residue, were incubated with individual purified His₆–Nedd4-2 WW domains. Interactions were detected by probing the nitrocellulose with an anti-RGS-His antibody. Figure 2(A) (and Supplementary Figure S1 at http://www.BiochemJ.org/bj/415/bj4150155add.htm) shows that His₆–Nedd4-2 binds to the catalytic HECT PY motif of W1 and W2 domains, bound denatured HECT WT, but not HECT Y971A. WW domain bound denatured HECT domain very weakly (seen in only some of our experiments). Overall, the binding to the HECT PY motif appeared much weaker than binding to βPY (Figure 2A), a known Nedd4-2 substrate. βPY binds Nedd4-2 WW1 and WW3 domains with a Kₘ of ~10 μM and ~50 μM
respectively [14]. Our measurements of the affinity of interaction between the Ned4-2 WW domains and a Ned4-2 HECT PY peptide using intrinsic tryptophan fluorescence (the same method used to measure the affinity between Ned4-2 WW domains and βPY [14]) revealed unsaturable binding for up to 1 mM HECT PY motif peptide incubated with 1 μM WW domains, suggesting that binding is very weak ($K_d > 400 \mu M$). Note, however, that this interaction is measured with the Ned4-2 WW domains and the HECT PY motif peptide as separate molecules. In the natural protein, the WW domains and the HECT domain are part of the same polypeptide chain. The Ned4-2 WW domains and HECT PY motif could be involved in an intra-molecular interaction within Ned4-2 and, therefore, a high-affinity interaction may not be necessary for binding.

We next investigated whether this Ned4-2 WW-domain–HECT PY-motif interaction affects the catalytic activity of the HECT domain. Using purified recombinant Ned4-2 HECT (after removal of its GST tag), we set up an in vitro ubiquitination assay, in which Ned4-2 HECT catalytic activity was measured by self-auto-ubiquitination and detected using an anti-ubiquitin antibody. Self-Nedd4-2 HECT catalytic activity was measured by self-ubiquitination (Figure 2B); however, the WW4 domain and GST alone (used as a negative control) did not affect ubiquitination. We also observed inhibition of HECT catalytic activity by the WW3 domain in the context of the full-length Ned4-2 protein (Figure 2C). These results show that increasing amounts of Ned4-2 WW1, WW2 and WW3 domains significantly inhibited Ned4-2 HECT self-ubiquitination (Figure 2B); however, the WW4 domain and GST alone (used as a negative control) did not affect ubiquitination. We also observed inhibition of HECT catalytic activity by the WW3 domain in the context of the full-length Ned4-2 protein (Figure 2C). These results suggest that the interaction between the Ned4-2 WW domain and the HECT domain may play a role in regulating the catalytic activity of Ned4-2. The discrepancy between WW4-domain binding and inhibition may be the result of the method used to test binding. We had demonstrated previously that the WW4 domain does not bind well to PY motifs with a leucine residue in the first position (i.e. LPXY), due to steric restraints between this residue and a bulky histidine residue in the WW4 domain [19]; therefore it is not surprising that the WW4 domain was not able to inhibit HECT activity. In the Far Western blotting experiment (Figure 2A), the HECT domain is denatured prior to binding to the WW domains, which may have allowed weak binding that is not observed when both proteins are in their native state.

To demonstrate the dependence of WW domain inhibition on the HECT PY motif, we attempted to repeat this experiment using the HECT Y971A mutant, with the expectation that HECT Y971A activity would not be inhibited by the WW domains, since they cannot bind. However, we observed that the HECT Y971A mutant was not active in the presence or absence of WW domains (results not shown). Although CD spectroscopy revealed that the HECT Y971A mutant was folded, the CD spectra were not identical with those of HECT WT (results not shown), suggesting there may be slight structural changes affecting activity. Interestingly, the Y971A mutation in the context of full-length Ned4-2 (Ned4-2 Y971A) was active, but its activity is somewhat reduced relative to Ned4-2 WT (see below). This difference in catalytic activity precluded the quantitative comparison of WW inhibition of Ned4-2 WT compared with the Y971A mutant.

**Ned4-2 stability is regulated by self-ubiquitination and subsequent degradation**

We have frequently observed higher expression levels of catalytically inactive Ned4-2 [Ned4-2 CS (where CS is cysteine to serine substitution)] than of Ned4-2 WT transfected into HEK-293T cells, suggesting that Ned4-2 may destabilize itself through self-ubiquitination. To examine this in more detail, we studied ubiquitination levels and the stability of Ned4-2 both in vitro and in vivo. First, we compared the ability of full-length V5-tagged Ned4-2 WT (WT, CS or Y971A constructs) expressed and immunopurified from HEK-293T cells to perform ubiquitination in an in vitro assay. As seen in Figure 3, both Ned4-2 WT and Ned4-2 Y971A, but not Ned4-2 CS, exhibited ubiquitination of Ned4-2 and possibly of other putative associated proteins that co-precipitated with it.
Figure 2  The Nedd4-2 HECT PY motif binds its WW domains and regulates catalytic activity

(A) Weak binding of HECT PY motif to the WW domains of Nedd4-2. Upper panel: strips of nitrocellulose containing denatured GST–HECT WT or the Y971A (YA) mutant domain (arrow) were incubated with purified His6–WW domains (His–WW1, His–WW2, His–WW3 and His–WW4) from Nedd4-2, and immunoblotted with an anti-RGS-His antibody (anti-His6) to detect binding of the WW domains to the HECT domain. The nitrocellulose strips also contained GST–PY motif from βENaC (βPY, *) as a positive control. Lower panel: Coomassie staining to demonstrate the amount of WW domain inputs (50% of the total protein incubated with nitrocellulose strips). The amount of the GST fusion proteins used for the assay is shown in Supplementary Figure S1.

(B) In vitro ubiquitination of the Nedd4-2 HECT domain is inhibited by its WW domains. Purified Nedd4-2 HECT was incubated with increasing amounts of His6–Nedd4-2 WW1, WW2, WW3 or WW4 domains (0, 2.5, 5 and 10 μg), or GST alone, together with E1 and E2 (UbcH7) enzymes and ATP. Reaction mixtures were analysed by immunoblotting with an anti-ubiquitin antibody.

(C) In vitro ubiquitination of full-length Nedd4-2 is also inhibited by its WW domains. Purified Nedd4-2 was incubated with increasing amounts of His6–Nedd4-2 WW3 (0, 2.5, 7.5 and 15 μg), together with E1 and E2 (UbcH7) enzymes and ATP, and analysed by blotting with an anti-ubiquitin (anti-Ub) antibody. A Western blot using an anti-Nedd4-2 antibody shows that an equal amount of Nedd4-2 was present in all lanes (lower panel).

To test specifically for Nedd4-2 ubiquitination, HEK-293T cells were transfected with human V5-tagged Nedd4-2 (WT, CS or Y971A constructs). Cell lysates were then boiled in SDS to dissociate Nedd4-2-interacting proteins, and Nedd4-2 was subsequently immunoprecipitated from the boiled lysates using an anti-V5 antibody and immunoblotted with an anti-ubiquitin antibody to detect conjugation of ubiquitin. As shown in Figure 4(A), a high-molecular-mass smear representing self-ubiquitinated Nedd4-2 is observed for Nedd4-2 WT, but not for Nedd4-2 CS, demonstrating that the ubiquitination observed is indeed self-ubiquitination. Notably, Nedd4-2 Y971A was also able to self-ubiquitinate.

To determine whether self-ubiquitination results in protein degradation, we carried out pulse–chase experiments and compared the stability of Nedd4-2 WT with that of the Nedd4-2 mutants. As seen in Figures 4(B) and 4(C), Nedd4-2 CS, which cannot ubiquitinate itself, is much more stable than Nedd4-2 WT (t½ of ~13 h compared with ~7 h for WT), suggesting that self-ubiquitination results in protein degradation. Notably, despite the decreased catalytic activity exhibited by the Nedd4-2 Y971A mutant, it nevertheless displayed a reduced stability relative to Nedd4-2 WT (t½ of 5 h). These results suggest that the WW-domain–HECT interaction stabilizes Nedd4-2 by inhibiting self-ubiquitination, in agreement with the observed inhibition of HECT activity on WW domain binding (Figures 2B and 2C).

The HECT PY motif regulates self ubiquitination and stability, but not substrate ubiquitination and stability

We next examined the effects of the Nedd4-2 HECT PY mutation, in the context of the full-length protein, on the ability of Nedd4-2 to regulate a known substrate, ENaC, in a physiological assay. Using a Xenopus oocyte-expression system, we measured the ability of Nedd4-2 Y971A to down-regulate ENaC. Voltage-clamp experiments were performed in which I Na (amiloride-sensitive Na+ currents) were measured in Xenopus oocytes expressing ENaC in the absence or presence of co-expressed Xenopus Nedd4-2 WT or Nedd4-2 Y971A. Surprisingly, we observed that Nedd4-2 Y971A could down-regulate ENaC similarly to Nedd4-2 WT (Figure 5A), suggesting that the reduction in activity exhibited by Nedd4-2 Y971A is not sufficient to affect ENaC down-regulation, at least in this system.

We then investigated the ability of the Nedd4-2 Y971A mutant to ubiquitinate specifically its known substrate, ENaC. Figure 5(B) demonstrates that this Nedd4-2 Y971A mutant can ubiquitinate ENaC (and destabilize the channel) almost as effectively as Nedd4-2 WT, and unlike the catalytically inactive Nedd4-2 CS mutant that was used as a negative control. Figure 5(B) depicts the ubiquitination of γENaC, the ENaC subunit shown previously to be most important for ENaC ubiquitination [5]. We also found Nedd4-2-mediated ubiquitination of εENaC (results not shown), which is also known to contribute to channel ubiquitination [5].
A PY motif within Nedd4-2 regulates self-ubiquitination

Figure 3 Both Nedd4-2 WT and the HECT PY Y971A mutant display ubiquitination activity in an in vitro assay

Nedd4-2 Y971A (YA), purified from transfected HEK-293T cell lysates, is active in vitro. V5-tagged Nedd4-2 WT, CS or Y971A constructs were immunopurified from transfected HEK-293T cell lysates and incubated with ATP, ubiquitin and E1 enzyme with (+) or without (−) E2 (UbcH7) enzyme, as indicated. Reaction mixtures were analysed by immunoblotting with an anti-ubiquitin antibody (anti-Ub) (upper panel). Lower panel: Western blot analysis with an anti-V5 antibody to show that equal amounts of V5–Nedd4-2 (WT, CS and Y971A constructs) lysates (and immunoprecipitates (IP)) were used in all treatments.

These results suggest that mutation of the HECT PY motif does not affect the ability of Nedd4-2 to ubiquitinate its substrates, in agreement with the ability of this mutant to down-regulate ENaC properly.

Since substrate binding is likely to disrupt any inhibitory interaction between the Nedd4-2 WW domains and the HECT domain, we hypothesized that substrate binding might also promote Nedd4-2 self-ubiquitination. To test this hypothesis, we repeated the Nedd4-2 ubiquitination experiment, comparing ubiquitination of Nedd4-2 immunoprecipitated from HEK-293T cells that were transfected with Nedd4-2 alone (WT or mutants) or co-transfected with Nedd4-2 plus ENaC. In the presence of ENaC, Nedd4-2 WT displayed greater self-ubiquitination than when expressed alone (Figure 6, top panel). In the case of the Nedd4-2 Y971A mutant, although it is intrinsically less active, nevertheless the increase in self-ubiquitination observed on ENaC co-expression is less drastic than that seen in Nedd4-2 WT, and this is likely to be because substrate binding is not required to relieve an inhibitory interaction and promote self-ubiquitination in this case.

A model depicting the binding surface between the Nedd4-2 WW3 domain and the HECT PY motif based on previously solved structures of the WW-domain–PY-motif complexes is shown in Figures 7(A) and 7(B). Although the core PY motif residues (LPPY) residues adopt a conformation and mediate interactions similar to those seen for other PY-motif-containing peptides bound to WW domains, unexpectedly, the C-terminal phenylalanine residue does not bind to the WW domain (Figure 7A). This is in contrast with our previously solved Nedd4 WW4-domain–βENaC PY-motif and Nedd4 WW3-domain–comissureless PY-motif complexes, in which the analogous residue bound to the cognate WW domain (Figure 7B). On the basis of this model and our previously published results [8,19], we predicted that replacing the HECT PY-motif with the higher-affinity PY motif from βENaC (PPYDSDL) would strengthen intra- or inter-molecular interactions with the Nedd4-2 WW domains and prevent substrate ubiquitination. Although such a substitution did indeed strengthen the interaction (Figure 8A), unfortunately, it also abolished HECT activity (results not shown). A less drastic mutation was then employed, substituting the phenylalanine residue at the tyrosine +3 position with a leucine residue (LPPYDSF to LPPYDSL). According to our WW3–HECT PY-motif model (Figures 7A and 7B), the bulky phenylalanine residue at the tyrosine +3 position was likely to be responsible, at least in part, for the low-affinity interaction observed with the WW domain; indeed, mutation of this phenylalanine to a leucine residue led to a modest

Figure 4 Mutation of the HECT PY motif affects Nedd4-2 stability

(A) Nedd4-2 Y971A (YA) displays similar self-ubiquitination to Nedd4-2 WT. V5–Nedd4-2 WT, CS or Y971A constructs were transfected into HEK-293T cells. Cells were lysed and the lysates boiled in SDS to remove Nedd4-2-interacting proteins. Lysates were then immunoprecipitated (IP) with an anti-V5 antibody (to precipitate Nedd4-2) and immunoblotted with an anti-ubiquitin antibody to detect ubiquitinated Nedd4-2. (B) HEK-293T cells expressing V5–Nedd4-2 WT, CS or Y971A (YA) constructs were labelled with [35S]methionine/cysteine (pulse) and chased with non-radioactive methionine/cysteine for the indicated times. Cell lysates (500 μg from each sample) were immunoprecipitated with an anti-V5 antibody, followed by SDS/PAGE. The experiment was performed in triplicate, with a representative experiment shown. (C) Half-life (t½) of Nedd4-2 WT and the Y971A (YA) and CS mutants. The amount of Nedd4-2 was quantified from the film by spot-density analysis. Results are means ± S.D. (n = 3, except n = 2 for Nedd4-2 CS). The t½ of the Y971A mutant was significantly shorter than that of Nedd4-2 WT (∗∗P < 0.01, unpaired t test).
increase in the strength of interactions (Figure 8A). Although this phenylalanine to leucine substitution did not affect the activity of the full-length protein, as evidenced by its ability to self-ubiquitinate (Figure 8B), it also did not affect its stability (results not shown), suggesting that the modest increase in affinity of the phenylalanine to leucine residue substitution mutant was not sufficient to compete with substrate binding.

**DISCUSSION**

The present paper suggests a novel mode of regulation of the catalytic activity of the E3 ligase Ned4-2, via an intra- or intermolecular interaction between its WW domains and an internal PY motif within its HECT domain, leading to regulation of self-ubiquitination, but not regulation of substrate ubiquitination (Figure 9). This report differs from previous reports describing regulation of HECT-containing E3s. Smurf2, a member of the Ned4 family involved in TGF-β (transforming growth factor-β) signalling, is regulated through binding of an adaptor protein Smad7, promoting E2 binding to the HECT domain [20]. Similar to what we observed for Ned4-2, Smurf2 self-ubiquitination results in degradation and is enhanced by Smad7 binding. More recently, Smurf2 catalytic activity was also shown to be regulated by an auto-inhibitory interaction between the C2 and HECT domains [21]. Itch, another Ned4 family member, is regulated by JNK (c-Jun N-terminal kinase)-mediated phosphorylation, which enhances the catalytic activity by disrupting an inhibitory interaction between the central region (including a proline-rich region and Itch WW domains) and the HECT domain [22]; this previous study, however, did not identify or implicate the HECT PY motif in the observed intra-molecular interaction. Interestingly, in this case, phosphorylation was required to disrupt the inhibitory interaction. Contrary to what was observed with Smurf2 and Ned4-2, there was no evidence to suggest that activated (phosphorylated) Itch was less stable than the unphosphorylated form [22]. Activity of the yeast Ned4 homologue, Rsp5, was previously shown to be modulated by an association
A PY motif within Nedd4-2 regulates self-ubiquitination

Figure 7 Homology model of a Nedd4-2 WW3-domain–HECT PY-motif complex

(A) An ensemble of the 100 lowest-energy models (from a total of 500). (B) Comparison of the human Nedd4-2 WW3-domain–HECT PY-motif complex (Nedd4 WW3 – Hect PY Motif) with the Nedd4 WW4-domain–βENaC PY-motif complex (Nedd4 WW4 – βENaC PY Motif). The WW domain is shown in blue for the backbone and cyan for the side chains. For clarity, only the side chains of the canonical tryptophan (W523 and W545) and proline (P548) residues are shown. The HECT PY motif is shown in orange for the backbone and yellow for the side chains. The canonical PY motif residues [Leu968 (L968), Pro969 (P969) and Tyr971 (Y971)] are shown, as well as Phe974 (F974).

Figure 8 Nedd4-2 FL (phenylalanine to leucine residue mutant) displays an increased affinity for the HECT PY motif, but exhibits the same stability as Nedd4-2 WT

(A) Increased binding of the mutant HECT PY motif to Nedd4-2 WW domains. Strips of nitrocellulose containing denatured GST–HECT domain [WT, Y971A (YA) mutant, βENaC PY motif (PPNYDSL mutant) or phenylalanine to leucine residue mutant (LPPYDSL mutated to LPPYDSL)] were incubated with purified His6–WW domains (WW1–WW4 domains) from Nedd4-2, and immunoblotted with an anti-RGS-His (anti-His) antibody to detect binding of the WW domains to the mutated HECT domain. Note the strong increase in binding of WW2, WW3 and WW4 domains to the βENaC PY motif, and the modest increase in binding to the phenylalanine to leucine residue mutant, relative to HECT WT. (B) Nedd4-2 phenylalanine to leucine residue mutant self-ubiquitinated similarly to Nedd4-2 WT and Nedd4-2 Y971A (YA). V5–Nedd4-2 (WT, CS, Y971A or phenylalanine to leucine residue mutant) was immunoprecipitated (IP) from transfected HEK-293T cell lysates using an anti-V5 antibody, boiled in SDS to remove Nedd4-2-interacting proteins. Subsequently, ubiquitinated Nedd4-2 was observed by immunoblotting with an anti-ubiquitin (anti-Ub) antibody, with blotting with an anti-V5 antibody performed as a control.
its own de-stabilization. Of the inhibitory WW–HECT interaction, Nedd4-2 now becomes self-ubiquitinated, leading to down-regulation. (Figure 9) The interaction between Nedd4-2 WW domains and its own HECT domain stabilizes the protein by preventing Nedd4-2 self-ubiquitination. (A) On arrival of a substrate (e.g. ENaC), the inhibitory WW–HECT interaction is disrupted by the substrate WW motif binding to Nedd4-2 WW domains, since this interaction is of a higher affinity. This leads to substrate ubiquitination and down-regulation. (B) Following (or concurrent with) substrate ubiquitination and the disruption of the inhibitory WW–HECT interaction, Nedd4-2 now becomes self-ubiquitinated, leading to its own de-stabilization.

with a deubiquitinating-enzyme complex [23]. Notably, this association affects only ubiquitination of Rsp5 substrates, since Rsp5 has not been shown to undergo auto-ubiquitination in vivo and has the same half-life as that of the catalytically inactive Rsp5. Additionally, Mule/ARF-BP1 [ARF (ADP-ribosylation factor)-binding protein 1], a HECT E3 ligase for p53, is negatively regulated by its binding partner ARF, which binds directly to the HECT domain [24]. The present paper and these previous reports collectively suggest that although some similarities in HECT activity regulation exist, each HECT ligase seems to exhibit its own unique mode of regulation, tailored to fit the needs of the proteins they regulate.

For Nedd4-2 (and possibly other Nedd4 family members), the PY motif within the HECT domain regulates low-affinity intra- or inter-molecular interactions and self-ubiquitination. This is likely to maintain the HECT domain in an inactive state, preventing self-ubiquitination, thereby stabilizing the protein. When the enzyme encounters a real substrate (e.g. ENaC), to which it binds with a higher affinity [14], the intra- or inter-molecular WW-domain–HECT PY-motif interaction is disrupted. Our results suggest that, once substrate ubiquitination is complete, self-ubiquitination becomes prominent, either subsequent to or simultaneously with substrate ubiquitination (Figure 9), leading to destabilization of Nedd4-2 itself.

Finally, it is curious that the HECT PY motif is also conserved in HECT E3s that do not contain WW domains (e.g. E6-AP) (Figure 1), raising the possibility that they may be regulated by inter-molecular interactions with Nedd4-family members in cells that express such family members in the same compartment.

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A PY motif within Nedd4-2 regulates self-ubiquitination


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SUPPLEMENTARY ONLINE DATA

Regulation of Nedd4-2 self-ubiquitination and stability by a PY motif located within its HECT-domain

M. Christine BRUCE*,†, Voula KANELIS*,†, Fatemeh FOULADKOU*,†, Anne DEBONNEVILLE‡, Olivier STAUB‡ and Daniela ROTIN*†1

*Programs in Cell and Structure Biology and Biochemistry, The Hospital for Sick Children, University of Toronto, 101 College Street, Toronto, ON, Canada, M5G 1L7, †Department of Biochemistry, University of Toronto, 101 College Street, Toronto, ON, Canada, M5G 1L7, and ‡Pharmacology Department, University of Lausanne, Rue du Bugnon 27, CH-1005, Lausanne, Switzerland

Figure S1 Ponceau-S staining control for Figure 2(A) of the main manuscript, depicting equal amounts of GST–HECT WT, GST–Y971A (YA) and GST–PY motif from βENaC (βPY) loaded on to the nitrocellulose membranes prior to incubation with the His6-tagged WW domains (WW1, WW2, WW3 and WW4) of Nedd4-2 in a Far Western blot experiment

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1 To whom correspondence should be addressed (email drotin@sickkids.ca).

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