Oligomerization of the HECT ubiquitin ligase NEDD4-2/NEDD4L is essential for polyubiquitin chain assembly

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The NEDD4-2 (neural precursor cell–expressed developmentally down-regulated 4-2) HECT ligase catalyzes polyubiquitin chain assembly by an ordered two-step mechanism requiring two functionally distinct E2–ubiquitin–binding sites, analogous to the trimeric E6AP/UBE3A HECT ligase. This conserved catalytic mechanism suggests that NEDD4-2, and presumably all HECT ligases, requires oligomerization to catalyze polyubiquitin chain assembly. To explore this hypothesis, we examined the catalytic mechanism of NEDD4-2 through the use of biochemically defined kinetic assays examining rates of 125I-labeled polyubiquitin chain assembly. To explore this hypothesis, we examined the catalytic mechanism of NEDD4-2 through the use of biochemically defined kinetic assays examining rates of 125I-labeled polyubiquitin chain assembly and biophysical techniques. The results from gel filtration chromatography and dynamic light-scattering analyses demonstrate for the first time that active NEDD4-2 is a trimer. Homology modeling to E6AP revealed that the predicted intersubunit interface has an absolutely conserved Phe-823, substitution of which destabilized the trimer and resulted in a \( \approx 10^4 \)-fold decrease in \( k_{\text{cat}} \) for polyubiquitin chain assembly. The small-molecule Phe-823 mimic, N-acetylphenylaminoamide, acted as a noncompetitive inhibitor \( (K_I = 8 \pm 1.2 \text{ mM}) \) of polyubiquitin chain elongation by destabilizing the active trimer, suggesting a mechanism for therapeutically targeting HECT ligases. Additional kinetic experiments indicated that monomeric NEDD4-2 catalyzes only HECT–ubiquitin thioester formation and monoubiquitination, whereas polyubiquitin chain assembly requires NEDD4-2 oligomerization. These results provide evidence that the previously identified sites 1 and 2 of NEDD4-2 function in trans to support chain elongation, explicating the requirement for oligomerization. Finally, we identified a conserved catalytic ensemble comprising Glu-464 and Arg-604 that supports HECT–ubiquitin thioester exchange and isopeptide bond formation at the active-site Cys-922 of NEDD4-2.

The HECT\(^3\) family of ubiquitin ligases consists of 28 function-specific paralogs in humans \((1–3)\). Compared with the ~600-member superfamily of RING (really interesting new gene) ligases, the relatively small number of HECT ligases plays integral but disproportionate roles in diverse cellular signaling pathways, disruption of which results in a spectrum of patho logical conditions \((1, 4, 5)\). Members of the HECT family are approximately 100 kDa in molecular mass and are defined by the presence of a highly conserved 350-residue C-terminal domain responsible for binding its cognate E2–ubiquitin thioester and catalyzing the formation of a high-energy HECT–ubiquitin thioester intermediate prior to conjugation of the activated ubiquitin to the target protein \((1, 3)\). Formation of the HECT–ubiquitin covalent intermediate distinguishes this family of conjugating enzymes from the RING ligases that attach ubiquitin to protein targets directly from the E3-bound E2–ubiquitin thioester co-substrate \((1, 2, 4)\).

The HECT superfamily can be subdivided according to differences in the N-terminal domain architecture \((1, 3)\). The NEDD4 family of HECT ligases consists of nine members characterized by an N-terminal C2 domain responsible for membrane anchoring and 2–4 WW domains that bind PY motifs present on target proteins to recruit the latter to the catalytic domain \((1)\). The NEDD4 ligases are key mediators in protein trafficking of transmembrane receptors and ion channels \((1, 6–9)\). Their role in facilitating vesicular transport has been exploited by the Ebola and Marburg viruses to promote viral egress \((1, 10–12)\). The NEDD4-2 (neural precursor cell–expressed developmentally down-regulated 4-2) paralog is the best characterized member of the family and is noted for catalyzing Lys-63-linked polyubiquitination of the amelioride-sensitive epithelial sodium channel (ENaC) in the distal nephrons to promote endocytic uptake and lysosomal degradation \((6, 7, 13, 14)\). Disruption of NEDD4-2–dependent ENaC targeting results in Liddle’s syndrome, a rare autosomal dominant salt-sensitive hypertension \((6, 7, 15–17)\). The NEDD4-2 ligase also targets voltage-gated sodium channels \((Na_v)\) in vitro and in vivo, disruption of which is linked to Na\(_v\)-dependent hyperexcitability and neuropathic pain, along with the development of mesial temporal lobe epilepsy \((18–21)\). In contrast, knockdown of NEDD4-2 in murine lung epithelial cells results in a cystic fibrosis–like disease, consistent with its role in regulating ENaC and \(\Delta F508-CFTR\) protein levels at the membrane \((22, 23)\). The morbidity and mortality associated with the disruption of NEDD4-2–dependent cell signaling highlight the importance of the NEDD4 family in cell homeostasis.

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2 The abbreviations used are: HECT, homologous to E6AP C terminus; E6AP, ENaC, epithelial sodium channel; GST, glutathione S-transferase; IsOT, isopeptidase T.

3 The tilde symbol (\(\sim\)) denotes a high-energy thioester bond.
Polyubiquitin assembly requires NEDD4 oligomerization

Unfortunately, our knowledge of the roles the HECT ligases assume in cellular regulation exceeds our understanding of the mechanism(s) by which these enzymes function. Early structural and biochemical studies supported a standard model for HECT ligase polyubiquitin chain assembly through a distal sequential addition mechanism as a reiterative extension of substrate monoubiquitination (24–26). In this model, the cognate E2–ubiquitin co-substrate binds to a single site at the small N-terminal subdomain of the HECT catalytic module prior to transsthioleolation to the HECT active-site cysteine, suggested by the crystal structure of the isolated E6AP HECT domain in complex with UbcH7 solved by Huang et al. (24). The resulting HECT-linked ubiquitin thioester is then transferred to lysine side chain(s) present on the target protein to form an isopeptide bond in a step termed monoubiquitination (24). Subsequent chain elongation is thought to proceed by the addition of ubiquitin moieties to the distal end of the growing polyubiquitin chain through an iterative sequence of steps (24, 25).

The E6AP HECT–UbcH7 structure reveals an approximately 41-Å gap between the donor and acceptor sites even though the two sulfur atoms are required to approach within atomic distance to support nucleophilic attack during thioester exchange (24). Kamadurai et al. (25) attempted to resolve this paradox by solving the crystal structure of the NEDD4-2HECT domain in complex with a Ubc5BL3S/T98K double mutant covalently linked to ubiquitin to simulate the otherwise labile thioester bond. The latter structure demonstrated similar E2 binding at the small N-terminal subdomain identified for E6AP, and rotation of the C-terminal subdomain harboring the HECT active-site cysteine reduced the donor-acceptor distance to 8 Å, seemingly resolving the topological barrier to facile transsthioleolation (25). Crystal structures of other HECT domains possess similar architectures, with the HECT C-terminal subdomain adopting a variety of orientations relative to the N-terminal subdomain (24, 25, 27–30). These observations have led to the gradual acceptance of a mechanism predicated on large conformational changes of the HECT C-terminal subdomain to close the gap required for thioester exchange (24, 25, 28). Verdecia et al. (28) observed that modifying the flexible linker connecting the N- and C-terminal subdomains by substitution of prolyl residues significantly decreases the activity of the WWP1 HECT ligase, interpreted as restricting the required mobility proposed for the C-terminal subdomain.

In contrast, more recent studies by Ronchi et al. (31–33) for the first time employing biochemically defined kinetic assays of full-length E6AP-catalyzed polyubiquitin chain formation have revealed marked inconsistencies that directly rule out the standard model. These new observations proffer a two-site proximal indexation mechanism that is distinguished by the coordinated sequential binding of E2–ubiquitin at functionally distinct E2-binding sites that assemble Lys-48–linked polyubiquitin chains in an “inside-out” manner on the HECT active-site cysteine prior to stochastic transfer of the preassembled degradation signal en bloc to a target substrate or competing nucleophile such as water (31–33). According to this model, the initial binding of E2–ubiquitin at a cryptic site 1, not observed in the original Huang et al. (24) structure but required by the more recent kinetic data, is associated with thioester exchange to yield the HECT–ubiquitin intermediate followed by binding of a second E2–ubiquitin at the canonical E2-binding site (site 2) to support polyubiquitin chain elongation (24, 32, 33). More recently, parallel studies have demonstrated this two-site kinetic mechanism in the paralogous full-length NEDD4-2HECT ligase that assembles Lys-63–linked polyubiquitin chains, suggesting conservation across the superfamily (34). A concurrent observation that the evolutionarily unrelated IpaH and SspH2 families of bacterial ubiquitin ligases exhibit an analogous mechanism suggests convergent evolution, which provides strong circumstantial support for the conservation of the proximal indexation mechanism (35). These observations expand on an original observation by Wang and Pickart (36) suggesting that the isolated E6AP HECT domain might assemble polyubiquitin chains on the active-site cysteine based on single-turnover studies in the formation of diubiquitin.

In addition to reconciling the topological restrictions on polyubiquitin chain assembly imposed by the standard model, Ronchi et al. (31, 33) have demonstrated that the proximal indexation mechanism requires full-length E6AP oligomerization as an integral aspect of the model. Huang et al. (24) originally noted that the E6AP HECT domain formed a radially symmetric trimer, but this structure was dismissed as an artifact of crystal packing forces. Analysis of the trimer subunit interface revealed a hydrophobic pocket into which an absolutely conserved Phe-727 (E6AP numbering) intercalated to stabilize adjacent subunits, mutation of which resulted in dissociation of the oligomer (24). Because the E6AP monomer retained the ability to form a HECT–ubiquitin thioester, assumed to represent the immediate donor for isopeptide bond formation to the target protein, the monomer was accepted as the active form of the enzyme (24). More recent studies by Ronchi et al. (31–33) confirm a central role of Phe-727 in stabilizing the full-length E6AP trimer and the ability of the E6AP monomer to undergo 125I-ubiquitin thioester transfer and target protein monoubiquitination. However, E6AP oligomerization is absolutely required for the assembly of polyubiquitin chains because sites 1 and 2 function in trans across adjacent subunits during chain elongation.

The present studies demonstrate that full-length NEDD4-2 is a trimer stabilized in part by an absolutely conserved Phe-823 paralogous to the Phe-727 of E6AP, disruption of which ablates Lys-63–linked polyubiquitin chain assembly. Therefore, the mechanism of polyubiquitin chain assembly is likely independent of linkage specificity. Consistent with the model, we show for the first time that the regulation of mono- versus polyubiquitination depends on the oligomerization state of the HECT ligase, reconciling observations that these enzymes can form mono- versus polyubiquitinated protein targets depending on the cellular context. Finally, we identify a conserved catalytic ensemble comprising Glu-646 and Arg-604 that is essential for polyubiquitin chain assembly, substrate targeting, and thioester exchange.

Results
The active form of NEDD4-2 is a trimer
Full-length bacterial expression–optimized opt.NEDD4-2 was analyzed by Superose 12 size exclusion chromatography
as described under “Materials and methods.” Recombinant opt.NEDD4-2 elutes as a broad absorbance peak with a relative solution molecular mass of 190 kDa (Fig. 1A, closed circles), ∼1.7-fold larger than the predicted 110-kDa monomer. The polydisperse absorbance peak is consistent with a mixture of monomeric and oligomeric species in solution. When NEDD4-2 activity in each fraction was assayed as the initial rate of 125I-polyubiquitin chain assembly, two apparent peaks were observed with relative molecular masses of 350 and 80 kDa (Fig. 1A, open circles). Although these relative molecular masses are consistent with a trimer and a monomer, respectively, this interpretation is unlikely correct, as these species are expected to exist in equilibrium and elute as a single peak of intermediate size. The peak of protein absorbance at 190 kDa coincides with the activity minimum between the 350- and 80-kDa peaks (Fig. 1A). We resolved each fraction by 10% SDS-PAGE and visualized protein by Coomassie staining. Fig. 1B, lane 10, demonstrates that the absorbance peak at 190 kDa predominantly consists of full-length opt.NEDD4-2 and a mixture of co-eluting lower molecular mass protein bands. Orbitrap fusion tribrid MS verified these latter bands to be NEDD4-2 fragments and not an alternative contaminating protein (not shown). We have shown previously that similar E6AP fragments inhibit full-length oligomeric E6AP activity (31). By analogy, we reasoned that the NEDD4-2 fragments were eluting from the sizing column in complex with full-length protein and might result in a similar inhibition of opt.NEDD4-2 activity (Fig. 1B). Therefore, the two activity peaks at 350 and 80 kDa could be interpreted to reflect co-elution of the inhibitory fragments superimposed over a broad polydispersed peak of opt.NEDD4-2 activity (Fig. 1, A and B).

To test this prediction, we first examined full-length opt.NEDD4-2 by dynamic light scattering to avoid sample dilution, as described under “Materials and methods.” Our results indicate a peak corresponding to 290 kDa (Fig. 1C, solid line), in agreement with our gel filtration activity assay (Fig. 1A). We next analyzed the fraction eluting at 11.5 ml corresponding to the 80-kDa species (Fig. 1B, lane 12) by dynamic light scattering and observed a single peak corresponding to 330 kDa (not shown), consistent with reoligomerization of the eluting monomeric species under our assay conditions.

To test directly whether NEDD4-2 fragments can inhibit the activity of the full-length protein, presumably by incorporation into the oligomer, we generated a truncated GST—NEDD4-2/HECT (residues 1–596) mutant by inserting a stop codon at residue 597 to remove the catalytic HECT domain. The recombinant truncated protein was then added to full-length GST–NEDD4-2 at increasing concentrations, and the resulting activity was assayed by initial rates of 125I-polyubiquitin chain assembly (Fig. 2A). If active NEDD4-2 is a monomer, one would expect no effect on WT NEDD4-2 activity with the addition of the GST-linked NEDD4-2 truncation protein. In contrast, trimeric GST–NEDD4-2 is expected to incorporate the truncated GST–NEDD4-2/HECT subunits into the multimer at equilibrium, resulting in a loss of activity (Fig. 2B). Kinetic analysis demonstrates that the addition of GST–NEDD4-2/HECT results in a hyperbolic concentration dependence for inhibition.
of WT GST–NEDD4-2–catalyzed $^{125}$I-polyubiquitin chain assembly tending to $\sim$9% limiting activity (Fig. 2). The residual activity corresponds to the low rate of E3-independent Ubc5B-catalyzed $^{125}$I-polyubiquitin chain formation reported previously for this paralog (37). In the kinetic experiment of Fig. 2A, the GST moiety was left unprocessed because we had observed previously that its removal has no effect on the activity of full-length NEDD4-2 (34), suggesting that the full-length protein forms a stable oligomer in the absence of contributions from the GST moiety. Nonlinear regression analysis of the data results in a $K_i = 5.0 \pm 1.4 \, \mu M$, in good agreement with the $K_i = 12 \pm 3 \, \mu M$ for inhibition of E6AP by the analogous GST–E6AP495 mutant (31) (Fig. 2A). These data support a model requiring oligomerization of NEDD4-2 for full activity and suggest that the HECT domains must work in concert to support polyubiquitin chain assembly.

**Phe-823 is required for NEDD4-2 oligomerization and polyubiquitin chain assembly**

By sequence analysis and homology modeling, we identified along the “backside” of the NEDD4-2HECT domain Phe-823 as the paralogous stabilizing residue to Phe-727 of trimeric E6AP (24, 31). Similarly, residues constituting a hydrophobic pocket into which Phe-823 could intercalate was also identified. We hypothesized that active NEDD4-2, and likely the remaining HECT ligases, forms a similar trimeric quaternary structure, because these residues are conserved across the HECT ligase superfamily (24, 31). To test this hypothesis, we mutated Phe-823 to aspartate and tested the ability of the point mutant to assemble $^{125}$I-polyubiquitin chains. In the presence of 2 nM WT GST–NEDD4-2, robust unanchored free $^{125}$I-polyubiquitin chain formation is observed at relative molecular weights that failed to enter the stacker gel (Fig. 3, lane 5), as we had observed earlier for E6AP and NEDD4-2 (31–34). In addition, a small amount of anchored $^{125}$I-polyubiquitin chains conjugated to GST–NEDD4-2 is observed at the top of the resolving gel (34) (Fig. 3, lane 5). Also present in the lanes are E3-independent bands representing monoubiquitinated Uba1 at 110 kDa and monoubiquitinated Ubc5B at 19 kDa (32, 34, 38). In contrast, no detectable free or anchored $^{125}$I-polyubiquitin chain assembly was observed at 2 nM GST–NEDD4-2F823D (not shown) nor at a $10^3$-fold higher concentration of Phe-823 mutant (Fig. 3, lane 6), allowing an estimation for an upper limit to the first-order rate constant of $\sim 5.6 \times 10^{-7} \, s^{-1}$ (Table 1). The significant reduction in $k_{cat}$ observed for the GST–NEDD4-2F823D mutant prompted us to generate GST–NEDD4-2F823A, which we predicted would show a less dramatic effect on activity. Kinetic analysis revealed measurable activity at 2 $\mu M$ GST–NEDD4-2F823A.

**Figure 2. GST–Nedd4-2ΔHECT quantitatively inhibits GST–Nedd4-2 activity.** A, kinetic analysis of $^{125}$I-polyubiquitin chain assembly rates under E3-limiting initial velocity conditions in reactions containing 100 nM Uba1, 100 nM Ubc5B, 0.5 nM GST–Nedd4-2, and increasing concentrations of GST–Nedd4-2ΔHECT (0–160 $\mu M$) as described under “Materials and methods.” The solid line represents the nonlinear inverse hyperbolic regression fit of the data using GraFit 5.0.8, schematic diagram illustrating the model for inhibition by the GST–Nedd4-2ΔHECT truncation mutant.

**Figure 3. Mutation of Phe823 ablates Nedd4-2–catalyzed polyubiquitination.** Shown is an autoradiogram of 15-min $^{125}$I-polyubiquitin conjugation reactions containing 100 nM Uba1 (E1), 200 nM Ubc5B (E2), and 2 nM WT GST–Nedd4-2 or the indicated GST–Nedd4-2F823 mutant (2 $\mu M$) carried out as described under “Materials and methods.” The positions of the mobility standards are shown on the left, and the position of free $^{125}$I-ubiquitin is shown on the right. The position of the stacker gel is denoted by a bracket.
Polyubiquitin assembly requires NEDD4 oligomerization

Table 1
Summary of kinetic constants for NEDD4-2F823D mutants

|          | $K_m$    | $k_{cat}$ |
|----------|----------|-----------|
| Nedd4-2  | 37 ± 2   | 1.2 ± 0.02 × 10^{-2} |
| Nedd4-2F823A | 36 ± 11 | 8.8 ± 0.7 × 10^{-6} |
| Nedd4-2F823D |        | ≤5.6 × 10^{-7} |

NEDD4-2F823A (Fig. 3, lane 7) but not at 2 nm (not shown). When the Ubc5B-dependent kinetics of the GST–NEDD4-2F823A mutant were examined, an intermediate catalytic phenotype with a 10^{4}-fold reduction in $k_{cat}$ (8.8 ± 0.7 × 10^{-6} s^{-1}) was observed (Fig. 3 and Table 1). The absence of an effect on $K_m$ (36 ± 11 nm) for Ubc5B–125I-ubiquitin binding is consistent with the mutation not significantly affecting native protein folding of the ligase; however, the marked $k_{cat}$ effect is consistent with the importance of Phe-823 in NEDD4-2–catalyzed 125I-polyubiquitin chain assembly through stabilization of the active oligomer (Fig. 3 and Table 1), as noted previously for WT E6AP versus E6APF727D (31). Accordingly, when opt.NEDD4-2F823D was analyzed by dynamic light scattering, mutation of Phe-823 resulted in quantitative dissociation of the trimer to a peak at 127 kDa, in good agreement with the predicted molecular weight of the monomer (Fig. 1C, dashed line).

To further validate that trimeric NEDD4-2 represents the functional form of the enzyme, we employed a small-molecule phenylalanine analog, Ac-Phe-NH$_2$, to serve as a mimic of Phe-823 to block binding at the postulated hydrophobic pocket. We hypothesized that Ac-Phe-NH$_2$ would destabilize the NEDD4-2 trimer and result in the loss of 125I-polyubiquitin chain assembly activity, similar to the effect observed previously with E6AP (31). Dynamic light-scattering analysis of 6.3 μM opt.NEDD4-2 in 7% (v/v) DMSO was carried out in the absence (Fig. 4A, solid line) and presence (dashed line) of 100 mM Ac-Phe-NH$_2$, resulting in a quantitative shift in molecular mass from 330 to 105 kDa, respectively. To test the effects of the Ac-Phe-NH$_2$ analog on activity, we next analyzed the dependence of the initial velocity for NEDD4-2–catalyzed 125I-polyubiquitin chain assembly on increasing Ac-Phe-NH$_2$ concentrations (not shown). The Ac-Phe-NH$_2$ analog inhibited NEDD4-2 activity with a hyperbolic concentration dependence tending to zero velocity ($K_i = 25 ± 13$ mM; not shown). A kinetic analysis of the dependence of initial velocity on [Ubc5B], in the absence or presence of 15 mM Ac-Phe-NH$_2$ demonstrates noncompetitive inhibition with a $K_i = 8.0 ± 1.2$ mM (Fig. 4B), which is in good agreement with the $K_i = 12 ± 3$ mM reported for E6AP (31). Binding of the Ac-Phe-NH$_2$ analog to a single homogeneous site, presumably the hydrophobic pocket, is suggested by the linearity of the corresponding double reciprocal plot (Fig. 4B). These data are consistent with the $K_{cat}$ effect observed for the GST–NEDD4-2F823D mutant and serve as proof-of-principle experiments for targeting HECT ligase oligomerization to regulate activity.

Oligomerization of the HECT domain is required for polyubiquitin chain assembly

Truncation of the N-terminal protein substrate-targeting region of full-length NEDD4-2 to yield the isolated GST–NEDD4-2HECT catalytic domain (residues 597–955) significantly destabilizes the oligomerization observed with full-length enzyme and results in an $10^{4}$-fold reduction in $k_{cat}$ (1.9 ± 0.2 × 10^{-6} s^{-1}) for 125I-polyubiquitin chain assembly compared with full-length GST–NEDD4-2 (34). Interestingly, the isolated GST–NEDD4-2HECT domain exhibits cooperative allostERIC kinetics ($n_H = 2.6 ± 1$), requiring a priori that the active enzyme function as an oligomer with respect to 125I-polyubiquitin chain formation (34). The main reaction product shifts from high-molecular-weight unassembled 125I-polyubiquitin chains located in the stacker for full-length GST–NEDD4-2 to predominantly 125I-labeled monoubiquitin autoconjugates observed as an adduct band at 70 kDa for the truncated HECT domain (34, 38), Fig. 5A. Other bands observed in Fig. 5A correspond to those identified in Fig. 3. Rates for assembly of free 125I-polyubiquitin chains present in the stacker exhibit substrate inhibition at low micromolar Ubc5B–ubiquitin concentrations, similar to full-length enzyme (34), Fig. 5A. Observation of substrate inhibition requires that the enzyme function with a minimum of two ordered E2–ubiquitin binding sites of different affinities (32, 34, 35). Kinetic analysis of the GST–NEDD4-2HECT–catalyzed 125I-polyubiquitin chain assembly in a parallel experiment demonstrates an excellent fit to the mathematical model for ordered cooperative substrate binding described kinetically by Equation 1 (32–35).

\[ V_0 = \frac{V_{max}[S]^{n_H}}{K_{app} + [S]^{n_H}} \times \frac{V_{max}[S]^{n_H}}{K_{app} + [S]^{n_H}} \]  

(Eq. 1)

The apparent [S]^{n_H} for the activation ($K_{+1app}^{n_H}$) and inhibition ($K_{-2app}^{n_H}$) sites were fit to values of 210 ± 100 nM and 2.0 ± 0.8 μM, respectively, corresponding to sites 1 and 2 described previously (34), $k_{cat} = 2.1 ± 0.3 × 10^{-6}$ s$^{-1}$ was determined for the leading substrate, in agreement with prior studies (34). In contrast to full-length NEDD4-2, the mathematical model describing substrate inhibition for GST–NEDD4-2HECT requires modification of the terms representing sites 1 and 2 by the inclusion of a Hill coefficient ($n_H$), observed only at site 2 for the full-length ligase (34). This is consistent with the observed cooperativity at site 1 for the isolated GST–NEDD4-2HECT.
domain but not full-length NEDD4-2 (34). The Hill coefficients for site 1 \((n_{H1})\) and site 2 \((n_{H2})\) were fit to values of \(1.8 \pm 0.3\) and \(1.6 \pm 0.3\), respectively.

Interestingly, substrate inhibition was not observed for \(^{125}\)I-monomoubiquitin autoconjugation (Fig. 5A), suggesting that the cross-talk between sites 1 and 2 required for \(^{125}\)I-polyubiquitin chain elongation is absent for \(^{125}\)I-monomoubiquination, in agreement with previous observations (31–34). By analogy to E6AP, we predicted that the distribution of mono-versus polyubiquitin reaction products likely reflects the oligomeric state of the enzyme, with the monomer capable only of monoubiquination. This hypothesis was supported by the observation of monomeric and trimeric species in solution by dynamic light-scattering analysis of \(200 \mu M\) GST–NEDD4-2HECT, corresponding to peaks at 60 and 180 kDa, respectively (not shown).

Processing the GST moiety to yield NEDD4-2HECT results in \(^{125}\)I-monomoubiquitin autoconjugation seen as an adduct band at 43 kDa and complete loss of signal in the stacker representing \(^{125}\)I-polyubiquitin chains (Fig. 6A). Dynamic light-scattering analysis of \(125 \mu M\) NEDD4-2HECT reveals a distinct peak at 46 kDa, consistent with a monomer (not shown). In contrast to full-length GST–NEDD4-2, we hypothesize that the GST moiety of GST–NEDD4-2HECT promotes oligomerization of an unstable complex (34). This observation supports the prediction that full-length NEDD4-2 forms a more stable oligomer supported by N-terminal interactions (31, 34). Kinetic analysis of NEDD4-2HECT–catalyzed \(^{125}\)I-monomoubiquitin autoconjugation reveals hyperbolic Michaelis-Menten kinetics \((K_m = 39 \pm 7 \, \text{nM}; k_{cat} = 6.0 \pm 0.2 \times 10^{-6} \, \text{s}^{-1})\) and loss of substrate inhibition consistent with dissociation of the oligomer (Fig. 6B). Parallel experiments extending Ubc5B concentrations to \(8 \mu M\) confirmed the plateau effect (not shown). These data demonstrate that NEDD4-2–catalyzed \(^{125}\)I-polyubiquitin chain elongation requires oligomerization of the HECT catalytic module, as described for E6AP (31–33). Additionally, the \(k_{cat}\) for NEDD4-2HECT \(^{125}\)I-monomoubiquination \((6.0 \pm 0.2 \times 10^{-6} \, \text{s}^{-1})\) is in good agreement with the \(k_{cat}\) for GST–NEDD4-2HECT \(^{125}\)I-polyubiquitin chain assembly \((1.9 \pm 0.2 \times 10^{-6} \, \text{s}^{-1})\), demonstrating that isopeptide bond formation is rate-limiting and does not discriminate between lysine residues present on the target protein or ubiquitin (34).

Polyubiquitin chain assembly requires sites 1 and 2 to function in trans

The observation that titration of the C-terminally truncated NEDD4-2 into full-length enzyme quantitatively inhibits \(^{125}\)I-polyubiquitin chain assembly with a hyperbolic concentration dependence (Fig. 2) is consistent with two alternate interpretations. Either sites 1 and 2 function in trans, for which oligomerization is absolutely required for polyubiquitin chain assembly, or sites 1 and 2 function in cis, but some conformational shift, such as C-terminal subdomain rotation, must be sterically restricted in the oligomer. To distinguish between these models, we generated a dominant-negative GST–NEDD4-2C922A active-site mutant. If sites 1 and 2 function in trans, the addition of the GST–NEDD4-2C922A mutant is predicted to reach an end point in which only one of three interfaces corresponds to site 2 being adjacent to an active WT site 1 (Fig. 7B), resulting in an end point of 33% activity compared with WT alone. In contrast, if sites 1 and 2 function in cis and the subunits of the trimer are simply required for conformational stabilization, there should be no change in activity upon the addition of GST–NEDD4-2C922A.

Fig. 7A shows that the addition of recombinant GST–NEDD4-2C922A to the WT enzyme results in a hyperbolic concentration-dependent decrease in rates of \(^{125}\)I-polyubiquitin chain assembly with a \(K_I = 4.7 \pm 0.5 \, \mu M\). More impor-
Polyubiquitin assembly requires NEDD4 oligomerization

Figure 6. Nedd4-2HECT exhibits 125I-labeled monoubiquitination and loss of substrate inhibition. A, autoradiogram of 20-min 125I-monoubiquitin conjugation reactions containing 100 nM Uba1 (E1), 860 nM Nedd4-2HECT, and increasing Ubc5B concentrations (0–1 μM) carried out at 37 °C as described under “Materials and methods.” B, concentration dependence of initial velocity for 125I-monoubiquitin conjugation on [Ubc5B] (0–2 μM) in a parallel assay. The solid line represents the theoretical nonlinear regression fit of the data to the Michaelis-Menten equation for $K_m = 39 \pm 7$ nM and $k_{cat} = 6.0 \pm 0.2 \times 10^{-6}$ s$^{-1}$. The positions of the mobility standards are shown on the left, and the position of free 125I-ubiquitin is shown on the right. The position of the stacker gel is denoted by a bracket.

Figure 7. Polyubiquitin chain assembly requires sites 1 and 2 to function in trans. A, kinetic analysis of 125I-polyubiquitin conjugation reactions under E3-limiting initial velocity conditions in the presence of 100 nM Uba1, 100 nM Ubc5B, 1 nM GST–Nedd4-2, and increasing concentrations of GST–Nedd4-2C922A (0–4 μM) as described under “Materials and methods.” The solid line represents the nonlinear inverse hyperbolic regression fit of the data using GraFit 5.0. B, schematic diagram illustrating the model of inhibition by the GST–Nedd4-2C922A active-site mutant.

A conserved catalytic triad supports NEDD4-2 [125I]polyubiquitin chain assembly

Thioester exchange and aminolysis are critical to a variety of biologically important processes and are central components of the ubiquitin conjugation pathways (39–41). The kinetics of thioester hydrolysis and thioalkane exchange have been characterized extensively under biological conditions, and it is well understood that the relevant species consists of the corresponding thiolate nucleophile (40, 42, 43). Recent findings with thioalkanes suggest that HECT–ubiquitin thioester exchange and subsequent isopeptide bond formation might proceed through general base catalysis to facilitate formation of the thiolate nucleophile (33). A candidate general base group for proton abstraction was identified previously for the active site of E6AP (33). Analysis of the original E6AP HECT structure
revealed a potential catalytic ensemble consisting of Glu-550, Arg-506, and the active-site Cys-820 (33). Ronchi et al. (33) note that the carboxyl group of Glu-550 hydrogen bonds to the thiol of Cys-820 potentially to serve as the required general base, whereas the guanidinium group of Arg-506 forms a stabilizing hydrogen bond with Glu-550 ensuring proper orientation of the catalytic base and potentially neutralizing the oxanion transition state during thioester exchange and isopeptide bond formation.

Sequence analysis reveals that Glu-550 and Arg-506 are highly conserved among the HECT ligase family members and correspond to Glu-646 and Arg-604 in NEDD4-2, respectively (Fig. 8B). We hypothesized that these paralogous residues constitute a conserved catalytic triad with the active-site Cys-922 in the context of the full-length NEDD4-2 oligomer. However, the active-site Cys-922 is not located in proximity to Glu-646 in the NEDD4-2HECT domain structure of Kamadurai et al. (25) because of rotation of the C-terminal subdomain but can assume the correct orientation if the C-terminal subdomain is rotated to a position approximating that in E6AP.

To test the model of general base catalysis, we first mutated Glu-646 to alanine to yield GST–NEDD4-2E646A and analyzed the effect on initial rates of 125I-polyubiquitin chains in the stacker and the accumulation of low-molecular-weight 125I-polyubiquitin conjugates in the resolving gel at increased protein concentrations (Fig. 8A, lanes 3 and 6). Quantitative analysis of the signal in the resolving gel for the GST–NEDD4-2E646D mutant demonstrates an ~1,200-fold reduction in $k_{\text{cat}}$ compared with WT GST–NEDD4-2 (Table 2), illustrating the importance of geometry in positioning of the Glu-646 carboxylate rather than the presence of the charged group in supporting 125I-polyubiquitin chain assembly (Fig. 8A, lanes 3 and 6).

Finally, we mutated Arg-604 to alanine and measured the rates of 125I-polyubiquitin chain assembly for the resulting GST–NEDD4-2R604A. The latter point mutant showed a similar loss of 125I-polyubiquitin conjugates in the stacker as seen for the other mutants and an ~150-fold reduction in $k_{\text{cat}}$ (Fig. 8, lanes 4 and 7, and Table 2). Interestingly, a reduction in $K_m$ was observed for each putative catalytic mutant compared with WT GST–NEDD4-2 (Table 2). The significantly reduced $k_{\text{cat}}$ accompanied by the decrease in $K_m$ appears counterintuitive but is consistent with NEDD4-2 functioning by a Briggs-Haldane steady-state kinetic mechanism, as opposed to rapid equilibrium kinetics, in which a decrease in $k_{\text{cat}}$ will result in a lower apparent $K_m$. This finding suggests that the $k_{\text{cat}}$ for 125I-polyubiquitin chain assembly is significantly greater than the off-rate of enzyme–substrate dissociation for the WT

Table 2

| Mutant          | $K_m$ (nM) | $k_{\text{cat}}$ (s$^{-1}$) |
|-----------------|------------|----------------------------|
| Nedd4-2         | 37 ± 2     | 1.2 ± 0.2 × 10$^{-5}$      |
| Nedd4-2E646A    | 11 ± 1.5   | 2.9 ± 0.1 × 10$^{-5}$      |
| Nedd4-2E646D    | 4.6 ± 0.9  | 9.8 ± 0.2 × 10$^{-6}$      |
| Nedd4-2R604A    | 5.3 ± 0.3  | 8.3 ± 0.1 × 10$^{-5}$      |

* Wildtype kinetic data from Table 1.
Polyubiquitin assembly requires NEDD4 oligomerization

enzyme and that the $K_m$ represents a pseudo equilibrium constant representing the difference between the steady-state enzyme–substrate complex concentration and the equilibrium concentration (44). Overall, these data suggest a catalytic role for Glu-646 and Arg-604 in 125I-polylubiquitin chain assembly. Importantly, these results preclude rotation of the NEDD4-2 C-terminal subdomain, as envisioned in the standard model, as a viable step in the catalytic cycle, as such rotations would disrupt the catalytic interaction between Glu-646 and Cys-823 (24, 33, 34, 45). In addition, C-terminal domain rotation is sterically precluded for a trimeric Nedd4-2 structure paralogous to that of E6AP.

If Glu-646 and Arg-604 are essential catalytic residues, they should be conserved across the HECT ligase superfamily. We conducted a ClustalW multiprotein sequence alignment of the 28 human HECT ligase paralogs using the DNASTAR MegAlign software suite (version 5.06). We chose to analyze isoform 1 of each paralog except for NEDD4-2, for which we used isoform 3, as this is used throughout the present functional studies. The alignment was carried out using full–length protein sequences obtained from the NCBI protein databank. Sequence analysis of the HECT ligases demonstrates conservation of the putative catalytic glutamate and arginine residues across all classes except class I and class IV (Fig. 8B), the most divergent of the families according to the phylogenetic classification of Grau-Bové et al. (5). The paralogous arginine residue is absent in all class I and IV family HECT ligases, whereas the paralogous glutamate is absent in the class I KIAA0614 ligase and the class IV UBR5 ligase (Fig. 8B). The histidine residue located at the expected glutamate position for KIAA0614 may be a sequencing error because it corresponds to a single cystosine–to–guanine base substitution at the first position in the codon for aspartate (Fig. 8B). Alternatively, the deprotonated imidazole of the histidine may serve as the requisite general base. Additionally, the class I ligases HERC1 and HECTD3 contain a conservative aspartate substitution in place of the catalytic glutamate (Fig. 8B). However, slight structural adjustments may allow aspartate to substitute for these paralogs while resulting in the loss of function for the NEDD4-2 and E6AP mutants (33) (Fig. 8A and Table 2). These findings suggest that the purported class I and class IV ligases may deviate functionally from the more highly conserved HECT ubiquitin ligases (classes II, III, V, and VI), consistent with the observation that the large HERCs (HERC1 and HERC2) of class I are paraphyletic to the small HERCs of class III and exhibit an evolutionarily independent acquisition of their domain architecture (5, 46, 47). Additionally, Brooks et al. (48) suggest that the class IV HECT ligase G2E3 contains an inactive HECT domain, as evidenced by the failure of the intact HECT domain to support polyubiquitin chain assembly when the N-terminal PHD/RING-like domains are mutated. Further mechanistic studies of these ligases may illuminate the functional implications of these distinct sequence variations. Altogether, our analysis demonstrates exceptional conservation of the Glu-646 and Arg-604 residues across the HECT superfamily (Fig. 8B), consistent with a conserved mechanism of action.

The significant reductions in $k_{cat}$ observed for the Glu-646 and Arg-604 mutants must reflect contributions to the step of thioester exchange, isopeptide bond formation during chain assembly, or both. To distinguish between these two half-reactions, we chose to extend the present kinetic studies to the effects of each mutant on thioester exchange. We are unable to observe the steady-state formation of the full-length GST–NEDD4-2–125I-ubiquitin thioester, presumably reflecting a slow rate of formation compared with the rapid rates of removal during conjugation of the polyubiquitin chain, hydrolysis by transfer of the thioester-linked chain to water, or transfer to another nucleophile such as DTT (29, 34, 49). However, the HECT–125I-ubiquitin thioester intermediate is observed with the isolated GST–NEDD4-2 HECl domain truncation because of the significantly reduced $k_{cat}$ for chain assembly (34). This observation led us to ask whether we could isolate the HECT–125I-ubiquitin thioester for the Glu-646 and Arg-604 mutants for which $k_{cat}$ is similarly ablated (Table 2). At 10 nM full-length GST–NEDD4-2 (Fig. 9, lane 1), GST–NEDD4-2E646A, GST–NEDD4-2E646D, or GST–NEDD4-2R604A (not shown), 125I-ubiquitin thioester formation was not observed in 1-min end point reactions resolved under nonreducing conditions. In contrast, we observed robust HECT–125I-ubiquitin thioester formation for each of the catalytic mutants at 10 μM (Fig. 9, lanes 2–4), WT GST–NEDD4-2 at 10 μM catalyzes the assembly of high-molecular-weight 125I-polyubiquitin chains that become imbedded in the stacker but fails to demonstrate HECT–125I-ubiquitin thioester formation (not shown) (34). The observation of thioester formation by the Glu-646 and Arg-604 mutants presumably reflects the significantly ablated $k_{cat}$ for chain assembly compared with thioester exchange as described previously for GST–NEDD4-2HECl (34). These studies indicate that Glu-646 and Arg-604 are essential in supporting isopeptide bond formation during 125I-polyubiquitin chain elongation but probably have a reduced role in general base catalysis during the initial thioester exchange reaction.

To test the role of the catalytic ensemble in thioester exchange, we mutated the putative general base residue Glu-
Table 3

| Reaction | \(k_{\text{m}}\) | \(k_{\text{cat}}\) | \(V_{\text{max}}\) |
|----------|-----------------|-----------------|-----------------|
| GST–NEDD4-2HECT \(a\) | 20 ± 5 \(\mu\text{M}\) | 3.5 ± 0.2 \(\times 10^{-4}\) \(\text{s}^{-1}\) | \(3.5 \times 10^{-4}\) \(\text{s}^{-1}\) |
| GST–NEDD4-2HECTE646A | 107 ± 72 \(\mu\text{M}\) | 6.8 ± 1.5 \(\times 10^{-5}\) \(\text{s}^{-1}\) | \(6.8 \times 10^{-5}\) \(\text{s}^{-1}\) |

* Kinetic data from Todaro et al. (34).

646 of GST–NEDD4-2HECT to alanine to yield GST–NEDD4-2HECTE646A, allowing us to kinetically examine the effect on thioester formation (34). Quantitative analysis of GST–NEDD4-2HECTE646A–\(^{125}\)I-ubiquitin thioester exchange under E3-limiting initial velocity conditions demonstrated a modest 5-fold decrease in \(k_{\text{cat}}\) (6.8 ± 1.5 \(\times 10^{-5}\) \(\text{s}^{-1}\)) compared with that for WT GST–NEDD4-2HECT (3.5 ± 0.2 \(\times 10^{-4}\) \(\text{s}^{-1}\)) (34). The observed \(K_{\text{m}}\) (107 ± 72 \(\mu\text{M}\)) was in good agreement with the \(K_{\text{m}}\) (20 ± 5 \(\mu\text{M}\)) and \([S]\) \(\text{half}\) (105 ± 20 \(\mu\text{M}\)) values reported previously for GST–NEDD4-2HECT–\(^{125}\)I-ubiquitin thioester formation and free \(^{125}\)I-polubiquitin chain assembly, respectively (34). The modest 5-fold reduction in \(k_{\text{cat}}\) observed for GST–NEDD4-2HECTE646A–catalyzed \(^{125}\)I-ubiquitin thioester transilation (Table 3) compared with the 400-fold decrease in \(k_{\text{cat}}\) for 125I-polubiquitin chain assembly (Table 2) suggests that Glu-646 of the putative catalytic ensemble plays a critical role in catalyzing isopeptide bond formation between ubiquitin moieties during polubiquitin chain assembly while providing only modest support for the initial HECT–\(^{125}\)I-ubiquitin thioester exchange reaction (34).

**NEDD4-2 catalyzes S5a/Rpn10 polubiquitination with hyperbolic kinetics**

Many E3 ubiquitin ligases are capable of assembling free polubiquitin chains in the absence of a cognate target protein (38). This property allowed us to dissect the mechanism of the HECT family ligases using biochemically defined kinetic assays measuring rates of free \(^{125}\)I-polubiquitin chain assembly as a functional readout (31–34, 38). In the present study, we extended our analysis to examine the final step in the ubiquitin cascade, conjugation to target proteins. Previously, Kim and Goldberg (50, 51) demonstrated that the pro teaseal subunit S5a/Rpn10, a ubiquitin-interacting motif (UIM)–containing protein, is ubiquitinated by a broad range of E3 ligases including the U-box CHIP ligase, Mdm2 and MuRF1 RING ligases, the RBR ligase Parkin, and the HECT ligases E6AP and NEDD4, among others. Additionally, Isasa et al. (52) demonstrate that the yeast NEDD4 ortholog Rsp5 monoubiquitinates Rpn10 in vitro, inhibiting its ability to recruit Lys-48–linked polubiquitinated substrates to the proteasome. Whether S5a/Rpn10 ubiquitination arises through its ability to bind ubiquitin or as a bona fide substrate is unclear; nonetheless, these observations demonstrate that S5a/Rpn10 can be utilized as a universal in vitro substrate, allowing us to test whether the mechanistic insights gained from the surrogate \(^{125}\)I-polubiquitin chain assembly reaction hold for the complete catalytic cycle of target protein conjugation.

The autoradiogram shown in Fig. 10A shows the concentration-dependent \(^{125}\)I-polubiquitination of S5a/Rpn10. Interestingly, a concentration-dependent decrease in unanchored \(^{125}\)I-polubiquitin conjugates in the stacker is observed (Fig. 10A). Fig. 10A (lane 13) also demonstrates a decrease in S5a/Rpn10 polubiquitin conjugates and an increase in S5a/Rpn10 monoubiquitin conjugates at saturating S5a/Rpn10 concentrations. Presumably, the concentration-dependent shift in reaction products reflects en bloc transfer of preassembled polubiquitin chains from the active-site cysteine to the target protein predicted by the proximal indexation mechanism described for E6AP (33). Kinetic analysis of the signal in the stacker demonstrates a hyperbolic concentration-dependent decrease in \(^{125}\)I-polubiquitin chain assembly to ~30% limiting activity with a \(K_{i} = 265 ± 150 \text{ nM}\) (Fig. 10B).

The ubiquitin–specific protease isopeptidase T (IsoT), which disassembles unanchored polubiquitin chains from the free C-terminal end, was employed to determine whether the remaining signal in the stacker represents free or anchored \(^{125}\)I-polubiquitin chains (34, 35, 38), Fig. 10A. The \(^{125}\)I-polubiquitin conjugation assays were carried out as described under “Materials and methods” in the absence or presence of 1.5 \(\mu\text{M}\) S5a/Rpn10. Following a 20-min incubation period, reactions were quenched with apyrase followed by the addition of 10 mM DTT to reduce all remaining thioesters. A final concentration of 34 \(\mu\text{M}\) IsoT was added to each reaction, and aliquots were taken at the indicated times and quenched by the addition of sample buffer (Fig. 10C). A second aliquot of IsoT was added at 50 min followed by incubation for an additional 20 min to ensure the reaction had reached the end point (Fig. 10C). Fig. 10C demonstrates a reduction of the signal in the stacker to ~35% limiting activity in the absence or presence of S5a/Rpn10, suggesting that the remaining signal represents NEDD4-2–anchored \(^{125}\)I-polubiquitin conjugates (32, 34, 35, 38). This observation likely accounts for the remaining signal observed in the stacker at saturating S5a/Rpn10 concentrations in Fig. 10, A and B. Incubation with IsoT results in a 35% reduction of the signal in the resolving gel above 50 kDa in the presence of 1.5 \(\mu\text{M}\) S5a/Rpn10, consistent with the remaining signal representing anchored S5a/Rpn10 conjugates (Fig. 10C). Kinetic analysis of NEDD4-2–catalyzed S5a/Rpn10 \(^{125}\)I-ubiquitin conjugation under E3-limiting initial velocity conditions demonstrates hyperbolic Michaelis–Menten kinetics with a \(K_{\text{m}} = 300 ± 88 \text{ nM}\) and \(k_{\text{cat}} = 2.3 ± 0.1 \text{ s}^{-1}\) (Fig. 10D). These results are in good agreement with the \(K_{i} = 265 ± 150 \text{ nM}\) observed for S5a/Rpn10-dependent inhibition of free \(^{125}\)I-polubiquitin chain assembly, consistent with the model requiring stochastic transfer of polubiquitin chains en bloc to the target substrate. Additionally, the \(k_{\text{cat}}\) observed for S5a/Rpn10 \(^{125}\)I-polubiquitination \((2.3 ± 0.1 \times 10^{-2} \text{ s}^{-1})\) (Fig. 10D) is in good agreement with the \(k_{\text{cat}}\) value observed for S5a/Rpn10 \(^{125}\)I-monoubiquitination \((1.6 ± 0.1 \times 10^{-2} \text{ s}^{-1})\) (not shown) and for free \(^{125}\)I-polubiquitin chain assembly \((1.2 ± 0.02 \times 10^{-2} \text{ s}^{-1})\) (Table 1), consistent with our previous observation that isopeptide bond formation is rate-limiting and does not discriminate between lysine residues.

**Glu-646 and Arg-604 are essential for S5a/Rpn10 ubiquitination**

Because NEDD4-2 does not discriminate between the source of lysine residues during isopeptide bond formation, we hypothesized that Glu-646 and Arg-604 might be required for substrate conjugation. To test this, we examined the ability...
of GST–NEDD4-2E646A, GST–NEDD4-2E646D, and GST–NEDD4-2R604A to support S5a/Rpn10 125I-ubiquitin conjugation (Fig. 11). Kinetic analysis of 15-min 125I-ubiquitin conjugation reactions containing WT GST–NEDD4-2 or the indicated catalytic mutant was carried out in the absence or presence of 1.5 μM S5a/Rpn10 (Fig. 11). WT GST–NEDD4-2 catalyzed S5a/Rpn10 125I-ubiquitin conjugation as described previously (Fig. 10). In contrast, mutation of Glu-646 and Arg-604 results in the loss of S5a/Rpn10 125I-ubiquitination (Fig. 11, lanes 6–11). The upper limit of detection for the first-order rate constant for S5a/Rpn10 conjugation was calculated to be approximately ±3.0 × 10⁻³ s⁻¹ (Fig. 11), ~8-fold lower than the $k_{cat}$ observed for WT GST–NEDD4-2–catalyzed S5a/Rpn10 125I-ubiquitination (Fig. 10D). These results indicate that Glu-646 and Arg-604 are essential residues in supporting substrate conjugation, in addition to their previously demonstrated role in supporting isopeptide bond formation during 125I-polyubiquitin chain assembly.

**Discussion**

Marked conservation of sequence and structure among the HECT ligase catalytic domains suggests conservation in their mechanisms of target protein conjugation. In earlier papers we demonstrated that E6AP, the archetype of this ligase class, assembles Lys-48–linked polyubiquitin chains through a proximal indexation mechanism that markedly diverged from previous models for these enzymes (31–33). More recent work with the extensively studied Nedd4-2 paralog suggests that it also assembles Lys-63–linked polyubiquitin chains through an analogous mechanism. Thus, the mechanism of HECT-catalyzed polyubiquitin chain assembly appears to be linkage-independent and likely conserved throughout the ligase superfamily (34). Our present kinetic and biophysical studies now demonstrate that oligomerization is required for NEDD4-2 catalyzed assembly of polyubiquitin chains (Figs. 1A and 2A), satisfying an important prediction of the proximal indexation model in functionally linking the two E2–ubiquitin–binding sites (34).
Polyubiquitin assembly requires NEDD4 oligomerization

Figure 11. Glu-646 and Arg-604 are essential for S5a/Rpn10 \(^{125}\text{I}\)-ubiquitin conjugation. Shown is an autoradiogram of 20-min \(^{125}\text{I}\)-polyubiquitin conjugation reactions containing 100 nM Uba1 E1, 100 nM Ubc5B (E2), and 1 nM WT GST–Nedd4-2 or the indicated catalytic mutant in the absence or presence of 1.5 \(\mu\text{M} S5a/Rpn10. The positions of the mobility standards are shown on the left, and the position of free \(^{125}\text{I}\)-ubiquitin is shown on the right. The position of the stacker gel is denoted by a bracket.

Other evidence empirically demonstrates that the oligomer is a trimer under the conditions of the assay and that sites 1 and 2 required by the proximal indexation model function in trans across subunits (Figs. 1C and 7).

The structure of the active NEDD4-2 oligomer likely corresponds to the paralogous E6AP trimer, as Phe-727 and the hydrophobic pocket into which it intercalates to stabilize the structure are conserved among all members of the HECT superfamily (31, 53). The shift in molecular weight of full-length NEDD4-2 to that of the monomer (Fig. 1C) and the corresponding loss of activity (Fig. 3 and Table 1) for the Nedd4-2F823D point mutant are also consistent with the paralogous E6AP trimer structure (31). Finally, the quantitative effect of Ac-Phe-NH\(_2\) on \(k_{\text{cat}}\) as a noncompetitive inhibitor that antagonizes binding of Phe-823 in the adjacent hydrophobic pocket also supports conservation of the trimeric structure (Fig. 4).

The large conformational differences in orientation of the C-terminal subdomains that have been noted previously among the Nedd4-like HECT ligase structures are unlikely to be a feature of the NEDD4-2 catalytic cycle because such motions are sterically hindered for the E6AP trimer (25, 28). In addition, significant mobility of the C-terminal subdomain would disrupt the hydrogen bond between Cys-823 and Glu-646 that is critical for general base catalysis, as evidenced by the marked decrease in \(k_{\text{cat}}\) accompanying mutation of the latter residue (Table 2).

Recognition that rates of \(^{125}\text{I}\)-polyubiquitin chain formation can serve as a facile surrogate assay for ligase function provides an important tool for probing the mechanisms of these enzymes (38). However, chain assembly assays do not allow us to address the conjugation of the cognate target proteins for the ligases. Recombinant S5a/Rpn10 provides a useful but artificial alternative target protein for such assays. The data in Fig. 10A show that S5a/Rpn10 is readily conjugated by Nedd4-2 to yield both mono- and polyubiquitinated species, reflecting the equilibrium presence of monomeric and oligomeric ligase species, respectively. Because conjugation of S5a/Rpn10 competes with polyubiquitin chain elongation at the HECT~ubiquitin active site in the proximal indexation mechanism, one expects complex kinetics. Therefore, the hyperbolic decrease in unanchored \(^{125}\text{I}\)-polyubiquitin chain accumulation in the stacker gel with increasing [S5a/Rpn10]~ is predicted by the proximal indexation mechanism and supported by the good agreement between the \(K_i\) for loss of unanchored chains (265 \(\pm\) 150 \text{nM}) and the \(K_m\) for substrate conjugation (300 \(\pm\) 88 \text{nM}) (Fig. 10, B and D). Finally, the good agreement among the \(K_m\) values for \(^{125}\text{I}\)-polyubiquitin chain addition to S5a/Rpn10 (2.3 \(\pm\) 0.1 \text{M} \text{s}^{-1} \text{M}^{-1}\)) indicates that Nedd4-2 does not readily distinguish between the sources of the lysine nucleophile, consistent with transfer through the common HECT~\(^{125}\text{I}\)-ubiquitin intermediate.

The present studies provide an important advance in our understanding of the mechanism of this class of ubiquitin ligases and supports conservation of the proximal index mechanism among the HECT paralogs. The shift in product from monoubiquitination to polyubiquitination with the oligomerization state of the enzymes reconciles a longstanding question regarding these enzymes, provides a mechanistic rationale for the regulation of enzyme activity through changes in oligomerization state. Finally, we note that positioning the chain elongation step in trans provides a mechanism for directing chain linkage specificity.

Materials and methods

Rabbit muscle creatine phosphokinase (C3756) and bovine erythrocyte ubiquitin (U6253) were purchased from Sigma- Aldrich. The ubiquitin was further purified to apparent homogeneity by FPLC and radioiodinated using the chloramine-T method and carrier-free Na\(^{125}\text{I}\) (PerkinElmer Life Sciences) (54). The N-acetyl-L-phenylalanyl-amide (Ac-Phe-NH\(_2\); E-1160) was purchased from Bachem Americas. Human ubiquitin-activating enzyme (Uba1) was purified from outdated human erythrocytes, and activity was quantified by the stoichiometric formation of \(^{125}\text{I}\)-ubiquitin thioester (55–57). Human recombinant Ubc5B (UBE2D2) was that described previously (58). Active Ubc5B was determined by the Uba1-dependent stoichiometric formation of its respective \(^{125}\text{I}\)-ubiquitin thioester (38). All recombinant proteins were stored at \(-80^\circ\text{C}\) in small aliquots to minimize the loss of activity with successive freeze-thaw cycles (38, 59).

Generation and purification of recombinant NEDD4-2

Human recombinant NEDD4-2 (isoform 3; GenBank\textsuperscript{TM} ID NM015277.5) and optimized NEDD4-2 (opt.NEDD4-2) was that used previously (34). The opt.NEDD4-2 protein was used for all experiments requiring removal of the GST moiety to
preclude processing artifacts arising from a cryptic internal thrombin cleavage site (34). Recombinant NEDD4-2F823A, NEDD4-2F823D, NEDD4-2E646A, NEDD4-2E646D, NEDD4-2R604A, and NEDD4-2C922A were generated from pDEST15-NEDD4-2 using the QuickChange site-directed mutagenesis kit (Agilent) and sequenced to confirm the desired mutation and to preclude any cloning artifacts (34). The NEDD4-2HECT (residues 597–955), NEDD4-2HECTE646A, and NEDD4-2ΔHECT (residues 1–596) constructs were subcloned into pGEX4T1, sequenced to ensure the integrity of the clone, and expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen). All cultures were grown at 37 °C to an *A*$_{600}$ of 0.4, and then the temperature was lowered to 15 °C and cultures allowed to reach an *A*$_{600}$ of 0.6. Protein expression was induced by adding L-arabinose to a final concentration of 0.2% (w/v) (BL21 AI cells) or isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM (BL21(DE3) cells), and then the mixture was incubated for 20 h at 15 °C. All other steps were conducted at 4 °C as described previously (34). Total recombinant protein was determined spectrophotometrically from the theoretical 280 nm extinction coefficient. Typical yields were ~5 mg/liter of culture. Total NEDD4-2 protein was used to calculate enzyme concentrations due to the inability to observe the 125I-ubiquitin thioester for the stoichiometric determination of active protein, as discussed previously (34).

### Generation and purification of recombinant S5a/Rpn10

Human S5a/Rpn10 cDNA, a generous gift from Dr. George DeMartino (Department of Physiology, University of Texas Southwestern Medical Center), was subcloned into a pGex-4T1 expression vector to yield pGex-4T1-S5a/Rpn10. Recombinant GST–S5a/Rpn10 was expressed in *E. coli* BL21(DE3) cells (Invitrogen) initially grown at 37 °C. When cell cultures reached an *A*$_{600}$ of 0.4, they were placed on ice for 30 min and then grown to an *A*$_{600}$ of 0.6 at 15 °C. Protein expression was induced with isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM (BL21(DE3) cells), and then the mixture was incubated for 20 h at 15 °C. All other steps were conducted at 4 °C as described previously (34). Total recombinant protein was determined spectrophotometrically from the theoretical 280 nm extinction coefficient. Typical yields were ~5 mg/liter of culture. Total NEDD4-2 protein was used to calculate enzyme concentrations due to the inability to observe the 125I-ubiquitin thioester for the stoichiometric determination of active protein, as discussed previously (34).

### NEDD4-2–catalyzed [125I]ubiquitin conjugation assay

The activity of NEDD4-2 proteins was quantitated in kinetic assays analyzing rates of 125I-polyubiquitin chain assembly under E3-limiting initial velocity conditions (38). Reactions were carried out as described previously, and 125I-ubiquitin conjugates were resolved from free 125I-ubiquitin by 12% (w/v) SDS-PAGE at 4 °C; then gels were dried and reaction products visualized by autoradiography (38, 59, 60). Unless otherwise specified, 125I-polyubiquitin chain formation was measured by exciting product from the stacker, representing predominantly unanchored polyubiquitin chains, and quantifying 125I-ubiquitin by associated γ-counting (34, 38). Absolute rates were calculated using the corrected specific activity for the 125I-ubiquitin (34, 38, 59). Kinetic data were analyzed by nonlinear regression analysis using GraFit 5.0 (Erithacus Software Ltd.).

### GST–NEDD4-2HECTE646A [125I]ubiquitin transthiolesterification assay

Analysis of GST–NEDD4-2HECTE646A thioester exchange kinetics was carried out as described previously for GST–NEDD4-2HECT (34). Reactions containing 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 10 mM MgCl$_2$, 1 mM DTT, 10 mM creatine phosphokinase, 4 μM 125I-ubiquitin, 40 nm human Uba1, 10 μM GST–NEDD4-2 HECTE646A (total protein), and increasing concentrations of Ub5B were carried out for 20 s at 37 °C. Reactions were quenched with 25 μl of 2X SDS sample buffer and resolved at 4 °C under nonreducing conditions (34, 38). Gels were dried and visualized by autoradiography, and then reaction rates were determined as described above for the 125I-ubiquitin conjugation assays except that the corresponding HECT–125I-ubiquitin thioester band was excised and quantitated (34, 38).

### Dynamic light-scattering analysis

The solution molecular weights of the indicated recombinant proteins were determined by dynamic light scattering at 37 °C using a DynaPro NanoStar light-scattering spectrometer (Wyatt Technologies) at 663 nm wavelength. Spectra were collected at the indicated protein concentrations in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT. Examination of species was restricted to a range of 0.5 to 15 nm to prevent skewing of the data toward large protein aggregates (~1500 kDa) and/or small protein fragments (~15 kDa). The effect of Ac-Phe-NH$_2$ on NEDD4-2 was determined at 100 mM Ac-Phe-NH$_2$ and 7% (v/v) carrier DMSO. Data were collected as 10 measurements of 5-s acquisitions and analyzed by Dynamics® software.

### Size exclusion chromatography

One hundred microliters of 63 μM opt.NEDD4-2 was analyzed by gel filtration chromatography using a Superose 12 (10/30) analytical column (Amersham Biosciences) equilibrated in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM DTT at 4 °C. Protein was resolved at 0.5 ml/min and absorbance measured at 280 nm. 500-microliter aliquots were collected, and NEDD4-2 activity was analyzed by 125I-polyubiquitin chain assembly. The column was calibrated using Bio-Rad standards (catalog No.
Polyubiquitin assembly requires NEDD4 oligomerization

Sequence homology and structural modeling

DNASTAR MegAlign™ software, version 5.06, was employed for multiple protein sequence alignments and homology modeling using the ClustalW program and a Gonnet 250 matrix (61). Protein sequences were obtained from the National Center for Biotechnology Information protein databank using isoform 1 for each protein, except for isoform 3 with NEDD4-2, because this was used in the present functional studies. Full-length protein sequences were used in the alignment analysis. The PyMOL Molecular Graphics System (version 1.8), Schrödinger, LLC., was used to visualize structural models (62).

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