In silico modeling of the cryptic E2~ubiquitin~binding site of E6-associated protein (E6AP)/UBE3A reveals the mechanism of polyubiquitin chain assembly

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To understand the mechanism for assembly of Lys48-linked polyubiquitin degradation signals, we previously demonstrated that the E6AP/UBE3A ligase harbors two functionally distinct E2~ubiquitin~binding sites: a high-affinity Site 1 required for E6AP Cys820~ubiquitin thioester formation and a canonical Site 2 responsible for subsequent chain elongation. Ordered binding to Sites 1 and 2 is here revealed by observation of UbcH7~ubiquitin~dependent substrate inhibition of chain formation at micromolar concentrations. To understand substrate inhibition, we exploited the PatchDock algorithm to model in silico UbcH7~ubiquitin bound to Site 1, validated by chain assembly kinetics of selected point mutants. The predicted structure buries an extensive solvent-excluded surface bringing the UbcH7~ubiquitin thioester bond within 6 Å of the Cys820 nucleophile. Modeling onto the active E6AP trimer suggests that substrate inhibition arises from steric hindrance between Sites 1 and 2 of adjacent subunits. Confirmation that Sites 1 and 2 function in trans was demonstrated by examining the effect of E6APC820A on wild-type activity and single-turnover pulse-chase kinetics. A cyclic proximal indexation model proposes that Sites 1 and 2 function in tandem to assemble thioester-linked polyubiquitin chains from the proximal end attached to Cys820 before stochastic en bloc transfer to the target protein. Non-reducing SDS-PAGE confirms assembly of the predicted Cys820~linked 125S-polyubiquitin thioester intermediate. Other studies suggest that Glu550 serves as a general base to generate the Cys820 thiolate within the low dielectric binding interface and Arg506 functions to orient Glu550 and to stabilize the incipient anionic transition state during thioester exchange.

The E6AP/UBE3A protein is a 100-kDa ubiquitin ligase that participates in a diverse subset of regulatory pathways in eukaryotes (1). Association of E6AP with aggresomes and its induction in response to various stressors, in addition to targeting polyglutamine expansion proteins for proteasomal degradation, suggest that the ligase functions as part of the cellular protein quality control response (2, 3). Human E6AP also serves regulatory roles as a dual-function co-activator of steroid hormone receptors through targeted degradation of transcriptional complexes (for a review, see Ref. 4). Other observations identify E6AP as one of several ligases that temporally regulate steady-state levels of p27 during mitotic progression, ablation of which leads to cell cycle arrest (5). Most recently, E6AP is shown to drive oscillations in levels of the core transcription factor of the mammalian circadian clock BMAL1/ARNTL and to regulate steady-state cellular concentrations of PPRPγ/C/EBPα, a key transcription factor in adipocyte differentiation, diabetes, and metabolic syndrome (6, 7). However, E6AP is best known for its role in epithelial cell transformation in response to human papillomavirus-16/18 infection, during which association of E6 viral protein is thought to redirect ligase specificity to target p53 and other key cell components for 26S proteasomal degradation (8–12) (for reviews, see Refs. 13–15). A parallel strategy is exploited by hepatitis C virus NS5B protein in redirecting accelerated retinoblastoma tumor suppressor protein degradation through E6AP in the etiology of liver cirrhosis and hepatocellular carcinoma (16). Most recently, E6AP has been identified as the Angelman syndrome gene, whose ablation of function by chromosomal deletion or mutation in the maternally inherited 15q11–13 region results in a congenital neuropathy leading to severe mental retardation, speech impairment, ataxia, and behavioral uniqueness (17–21). The related Prader–Willi syndrome is associated with similar loss-of-function mutations/deletions within paternally inherited 15q11–13 regions of the brain and leads to neuro-cognitive disorders, endocrinological insufficiency, and behavioral man-

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3 The abbreviations used are: E6AP, E6-associated protein (gene name UBE3A); E1, generic term for activating enzymes of class 1 ubiquitin-like proteins; E2/Ubc, generic name for ubiquitin carrier protein/ubiquitin-conjugating enzyme; Hect, homologous to E6AP C terminus; PDB, Protein Data Bank; rmUb, reductively methylated ubiquitin; Uba1, human ubiquitin-activating enzyme (gene name UBE1); UbcH7, human ubiquitin carrier protein (gene name UBE2L2).

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The mechanism of E6AP

The E6AP ligase is the founding example of the Hect (homologous to E6AP C terminus) domain superfamily of ubiquitin-conjugating enzymes (1). The Hect ligases share a common domain architecture comprising an N-terminal region harboring one or more protein interaction motifs required for target protein recruitment and a highly conserved ~350-residue C-terminal catalytic Hect domain (1, 23). The Hect domain is responsible for appending polyubiquitin chains to target proteins that, depending on linkage type, subsequently serve as degradation signals for 26S proteasome recognition (Lys48 linkages) or scaffolds for assembly of multimeric complexes required for non-proteolytic functions (Lys63 linkages) (reviewed in Refs. 1 and 23). The Hect domain folds into two lobes that form an L shape, with subdivision of the N-terminal lobe into large and small subdomains and a C-terminal lobe that contains the essential Cys820 (E6AP isoform 1 numbering) active site residue required for target protein conjugation (24, 25). The Hect ligases catalyze a thiol exchange reaction (transthiolestion) in which activated ubiquitin is transferred from its bound E2−ubiquitin3 thioester co-substrate to Cys820 before target protein ligation (23, 25). Transthiolestion requires that Cys820 and the Gly76 carbonyl of the E2− ubiquitin thioester approach within atomic distance to allow the ensuing nucleophilic attack and thiol exchange. The original structure for the human E6AP Hect domain reveals a putative UbcH7/E2-binding site within the small N-terminal subdomain from which activated ubiquitin is thought to be transferred from bound UbcH7−ubiquitin thioester to Cys820 (24). However, this structure is problematic, because the cysteine residues of the donor and acceptor sites are separated by 41 Å, precluding facile nucleophilic attack (24). Because the extant structures for a number of Hect domains show considerable variation in the orientation of the C-terminal subdomain, conformational changes during the catalytic cycle have been proffered to close some of the distance required for nucleophilic attack during transthiolestion because point mutations that presumably restrict mobility of the C-terminal domain also ablate ligase activity (26, 27). In support of this model, the crystal structure for a “high-affinity” Ubc5BL3S/T98K−ubiquitin double mutant thioester bound to the small N-terminal subdomain of the Nedd4−2/Nedd4L Hect ligase brings the cysteine residues within 8 Å and has been proposed to model the Michaelis complex that precedes intramolecular thiol exchange with the Hect domain active site cysteine (26).

Marked conservation among the sequences of the Hect domain superfamily suggests that the mechanism for target protein conjugation and polyubiquitin chain assembly should also be conserved (28, 29). To this end, a standard model of sequential addition has been proposed in which cyclic rounds of E2−ubiquitin thioester binding at the canonical small N-terminal subdomain site precedes transthiolestion to form the Hect−ubiquitin intermediate, which in turn transfers the activated polypeptide to specific target protein lysine residue(s) or sequentially to the distal end of the elongating polyubiquitin chain anchored to the target protein (24, 26, 30). However, among other questions, the sequential model fails to provide a mechanistic rationale for the Hect−ubiquitin thioester intermediate, the function of which is not required for other classes of ligases.

We have recently reported the first comprehensive kinetic characterization of E6AP using the innate ability of the enzyme to assemble polyubiquitin chains in the absence of substrate as a functional readout of ligase activity (31, 32). The results of these studies are inconsistent with a single site for binding of the E2−ubiquitin thioester substrate on E6AP and subsequent polyubiquitin chain assembly by the earlier sequential addition model (32). In contrast, biochemically defined rate studies indicate that the E6AP Hect domain harbors two independent and functionally distinct binding sites for UbcH7−ubiquitin (32). More recently, we have demonstrated that assembly of the polyubiquitin degradation signal additionally requires E6AP oligomerization and that the active structure probably corresponds to the trimer originally reported by Huang et al. (24, 33). The trimer buries an extensive surface area of 7508 Å and is stabilized by a network of radially symmetric conserved side-chain interactions at the subunit interfaces, mutation of which destabilizes the oligomer and ablates catalytic activity in chain assembly but not Cys820−linked ubiquitin thioester exchange (33).

The minimum kinetic model that emerges from these kinetic and biophysical observations posits a two-step ordered burst-like mechanism (Scheme 1) for polyubiquitin chain assembly involving a cryptic high affinity Site 1 proximal to the active site Cys820 through which rapid transthiolestion (kcat > 9 s−1) of the bound UbcH7−ubiquitin forms the Hect−ubiquitin thioester intermediate and a canonical lower-affinity Site 2, represented in the original crystal structure of Huang et al. (24), that catalyzes rate-limiting polyubiquitin chain elongation before en bloc transfer of the chain to the target protein (32). The burst mechanism, defined by rapid formation of a covalent intermediate (in this case, the E6AP−ubiquitin thioester) followed by slower rate-limiting product formation (corresponding to polyubiquitin chain assembly), requires that the kcat determined from saturation kinetics reflects binding of UbcH7−ubiquitin to Site 1, whereas the kcat corresponds to the Site 2−catalyzed step of polyubiquitin chain elongation (32). The reported inhibition patterns observed for inert substrate versus product analogs additionally require that the cryptic Site 1 harbor significantly greater binding affinity for the UbcH7−ubiquitin intermediate than for either UbcH7 or ubiquitin alone, potentially accounting for the absence of Site 1 in the original crystal structure. Substrate inhibition observed earlier for UbcH7−ubiquitin requires mutually exclusive ordered binding at Sites 1 and 2, consistent with the predicted large difference in

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4 The sequence for human E6AP isoform 3 used in the present studies differs from isoform 1, from which the original crystal structure was determined (24), by the presence of an additional 20 amino acids at the N terminus. To facilitate comparison with the crystal structure, residues for isoform 3 will be referenced to the paralogous position of isoform 1 (i.e. by subtracting 20 from the isoform 3 residue number).

5 The symbol − denotes a high-energy thioester bond.
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affinities (32). Remarkably, we find that the catalytic cycles for a family of Shigella flexneri IpaH ubiquitin ligases and the related SspH2 ubiquitin ligase of Salmonella enterica can also be described by Scheme 1, suggesting convergence among evolutionarily disparate enzyme families in the mechanism of polyubiquitin chain assembly (34).

We believed that understanding the structure of UbcH7–ubiquitin bound to the cryptic Site 1 could inform the mechanism of polyubiquitin chain assembly and its requirement for oligomerization. However, because it is not always possible to obtain relevant structures for all enzyme intermediates and unfettered of spatial restrictions imposed by the earlier standard model for E2–ubiquitin binding to the canonical site (24, 35), we have used in silico docking and energy minimization refinement to model the Site 1 predicted from the empirical kinetic data. The resulting structure provides unique insights into the catalytic cycle of E6AP and explicated the observed kinetic behavior of the ligase in addition to suggesting a mechanism for polyubiquitin chain assembly and target protein conjugation that reconciles the requirement for oligomerization of the enzyme.

Results

In silico modeling of Site 1

In *silico* modeling of the cryptic E2–ubiquitin–binding site of E6AP is simplified by the mechanistic requirement that the active site Cys^820 approach the UbcH7–ubiquitin thioester bond within atomic distance for the exchange reaction to proceed (32). We chose to exploit the unique features of the PatchDock algorithm to model the protein–protein interactions required for binding of UbcH7–ubiquitin thioester to E6AP. The PatchDock program divides interacting molecules into segments (“patches”) and iteratively parses the resulting surfaces for shape complementarity, much as one would perform physically with CPK models in real space, after which the quality of the resulting fit is scored and a binding energy is approximated based on desolvation of the interface (36). PatchDock or adaptations of the algorithm have been used successfully in similar applications to predict multimolecular assemblies of several regulatory complexes (37), the effects of mutations on the predicted association of the phosphoinositide 3-kinase with p85α (38), complex formation between NFκB and the proapoptotic ASPP2 protein (39), and subunit assembly of multiketide synthases (40), among others. We docked UbcH7–ubiquitin onto a subunit of the E6AP trimer (PDB entry 1C4Z) because we have previously shown that the catalytically active form of the enzyme is the oligomer, and C-terminal conformational variability observed with other Hect domain structures is sterically precluded by subunit association (33).

The most important decision in our initial work centered on choosing a structural model for UbcH7–ubiquitin thioester. Recent NMR studies indicate that E2–ubiquitin thioesters are dynamic structures for which the ubiquitin moiety exhibits considerable flexibility of motion, ranging from an “open” solvent accessible form to a more compact “closed” conformer in which the polypeptide binds to a defined E2 surface (41). From the limited number of examples to date, structures for E2–ubiquitin bound to E3 suggest that ligases selectively sample the closed conformer (42–44). Reasoning that thioester binding to E6AP might similarly favor the more stable closed conformer, which is the preferred solution structure of UbcH7–ubiquitin (41, 45, 46), we threaded human UbcH7 onto the closed crystal structure of Ubc1–ubiquitin thioester (PDB entry 1FXT) reported by Hamilton et al. (47). Docking of the resulting UbcH7–ubiquitin thioester to E6AP was constrained by defining an arc of residues across E6AP that included the active site Cys^820 as the receptor-binding site and residues involved in the UbcH7–ubiquitin thioester and adjacent residues on the E2–ubiquitin structure as the ligand-binding site, as described under “Materials and methods.”

Fig. 1 displays the final docking model for UbcH7–ubiquitin bound to Site 1 after refinement in explicit solvent to relieve steric hindrances and relax side-chain interactions in the docked complex. The active site Cys^820 of E6AP and the UbcH7 Cys^86-linked thioester bond are separated by 6 Å within an extensive binding interface between E6AP and UbcH7–ubiquitin (Fig. 1A). Because the PatchDock algorithm treats the interacting molecules as rigid surfaces, final structural energy minimization achieved by minor conformational adjustments that would occur in real space are precluded without manual adjustment to the docking solution, which we avoided as not to introduce bias into the model. Thus, the final docking model lacks the structural refinement typical of conventional crystal structures but informs regarding the general features predicted for Site 1. Nonetheless, the irregularly shaped UbcH7–ubiquitin closed conformer (orange and red, respectively) fits into a complementary surface that forms a ledge created by packing of the C-terminal subdomain (dark blue) onto the large N-terminal subdomain (marine), Fig. 1A. Examination of the model suggests that a slight shift in the orientation of UbcH7 relative to the ubiquitin moiety would further relax the structure and bring the active site cysteines into the optimal proximity required for facile nucleophilic attack.

The predicted structure buries an extensive surface totaling 1600 Å², of which 1100 Å² are contributed by the interface between E6AP and UbcH7 (−4.3 kcal/mol desolvation energy; $K_{d,calc} = 0.7 \text{ mM}$), and 500 Å² are contributed by the interface between E6AP and ubiquitin (−3.8 kcal/mol desolvation energy; $K_{d,calc} = 1.3 \text{ mM}$). The observed $K_d$ for UbcH7–ubiquitin thioester binding to E6AP determined as $K_m$ from the kinetics of $^{125}$I-polyubiquitin chain formation is 58 ± 6 mM and represents a binding energy of −9.8 kcal/mol (32). Thus, the upper limit for $K_d$ estimated by PatchDock based on solvent exclusion alone is consistent with the empirically observed $K_m$ for Site 1, exclusive of additional ionic and hydrogen-bonding interactions. We have previously speculated that Site 1 is not observed in the crystal structure of Huang et al. (24) for uncharged UbcH7 bound to E6AP because neither the E2 nor ubiquitin has sufficient binding affinity alone without the significant entropically linked increase expected to accompany thioester formation (32). The markedly ablated binding affinities for the individual UbcH7 and ubiquitin moieties estimated from desolvation compared with that of UbcH7–ubiquitin calculated from the $K_m$ are thus consistent with this expectation (32). The position of ubiquitin in the docked structure overlaps that for
ubiquitin in the Nedd4-2 Hect domain−ubiquitin thioester modeled by Maspero et al. (48) if the ubiquitin moiety is rotated 40° about the nascent Nedd4-2 Hect−ubiquitin thioester bond (Fig. 1B, red and salmon structures, respectively). Therefore, there is little expected change in the position of ubiquitin during thioester exchange. In addition, ubiquitin in the PatchDock-derived structure is positioned to interact with a cluster of conserved residues within the C-terminal subdomain identified previously to interact with the ubiquitin moiety (49, 50) (Fig. 1, C and D, grey residues).

We were reassured to observe that the final docking solution identified a number of side-chain interactions independently determined to affect the catalytic activity of the ligase. Asparagine 78 of UbcH7 is positioned to form a hydrogen bond with His818 in the C-terminal subdomain, and Lys82 of UbcH7 forms an ionic bond with the side-chain carboxyl of Glu539 in the large N-terminal subdomain of E6AP (Fig. 1, C and D). Interestingly, Glu539 of E6AP is an absolutely conserved residue within the Hect domain superfamily and an Angelman syndrome loss-of-function mutation that forms a directed ionic/hydrogen bond with the guanidinium side chain of Arg506 (not shown), another highly conserved Hect domain residue and Angelman syndrome mutation site (24, 49, 51). In addition to these conserved sites, Lys96 of UbcH7 forms an ionic bond with Asp563 of E6AP in the docking model (Fig. 1, C and D). In contrast, Phe63 of UbcH7 does not interact with E6AP in the Site 1 docking model, consistent with the marginal effect on the affinity for binding of UbcH7F63A-ubiquitin oxyester relative to wild-type UbcH7−ubiquitin thioester to Site 1 versus Site 2 noted earlier (32) (Fig. 1, C and D).

Validation of the Site 1 docking model

As with crystal structures, the docking model shown in Fig. 1 was validated by examining the effect of selected point mutants on the kinetics of E6AP-catalyzed 125I-polyubiquitin chain formation (32). Initial rates of 125I-polyubiquitin chain formation were determined at various concentrations of UbcH7 under E3-limiting conditions, confirmed by the independence of rate on [Uba1]o (31, 32). The latter criterion assures that UbcH7 is stoichiometrically present as its Cys86-linked ubiquitin thioester, the actual substrate for E6AP (31, 32). As reported previously (32), the data displayed hyperbolic kinetics (not shown), from which values of $K_m$ and $k_{cat}$, defined as $V_{max}/[E6AP]_o$, were determined by non-linear regression analysis as described under “Materials and methods.” Earlier studies demonstrate that the resulting $K_m$ reflects the intrinsic $K_d$ for binding of the UbcH7−ubiquitin thioester to the cryptic Site 1 of E6AP, whereas the $k_{cat}$ reflects the rate-limiting Site 2−dependent chain elongation step in the minimum kinetic mechanism (32) (Scheme 1).

We chose not to test the predicted interactions involving Asn78, Lys82, or Gln84 because the crystal structure reported by Kamadurai et al. (26) for the “high-affinity” Ubc5BL3S/T98K−ubiquitin bound to the canonical site of the truncated Nedd4-2 Hect domain identified these positions among a sub-
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Figure 2. *In silico* model for E6AP Site 1 highlighting residues used to validate the model. The same coloring scheme is used as in Fig. 1 except that highlighted residues are rendered in CPK coloring. A, the overall docking model with selected residues labeled. B, detail of the Asn561–Asn94 hydrogen bond between Ubc7 and E6AP, respectively. C, rotation of A 90° to highlight the tripartite interaction between Ubc7 and E6AP. D, details of the tripartite interaction shown in C.

Table 1
Summary of kinetic constants for Site 1 mutants

| E3             | E2       | K  | kcat | kcat/Km |
|----------------|----------|----|------|---------|
|                | gM       | s-1| s-1  | s-1    |
| E6AP           | Ubc7     | 83 | 1.4  | 1.0 × 10^{-2} | 1.3 × 10^{2} |
| E6APN561A      | Ubc7     | 41 | 8.3  | 1.0 × 10^{-2} | 2.0 × 10^{3} |
| E6AP           | Ubc7K64A | 63 | 1.8  | 1.0 × 10^{-2} | 2.9 × 10^{3} |
| E6AP           | Ubc7K67A | 49 | 2.0  | 1.0 × 10^{-2} | 2.9 × 10^{3} |
| E6AP           | Ubc7K64/67A | 220 | 1.5 | 1.0 × 10^{-2} | 4.0 × 10^{4} |
| E6APH508A      | Ubc7     | 360 | 3.0 | 1.0 × 10^{-2} | 8.3 × 10^{4} |
| E6APH508A      | Ubc7K64/67A | 650 | 1.7 | 1.0 × 10^{-2} | 2.6 × 10^{6} |
| E6APR505A      | Ubc7     | 97 | 2.6 | 1.0 × 10^{-2} | 2.7 × 10^{4} |
| E6APH508AR505A | Ubc7     |    |     | <2.0 × 10^{2} |

set of paralogous UbcH5 residues predicted to interact with the C-terminal subdomain of the ligase. Instead, we sought predicted interactions unique to the present docking model that were removed from the canonical small N-terminal subdomain. The current docking model reveals that among the non-ionic interactions predicted to orient substrate binding in Site 1, the side-chain amide nitrogen of Asn561 within the large N-terminal subdomain of E6AP hydrogen bonds to the side-chain carbonyl oxygen of Ubc7 Asn94 (Fig. 2, A and B). Asparagine 561 is highly conserved among members of the Hect domain superfamily whose activity is supported by Ubc7, Ubc8, and/or Ubc5, such as Nedd4-1/2 (52–55), Smurf1/2 (56, 57), and Herc5 (58), among others. Within the E2 superfamily, Asn94 is conserved only in Ubc7, Ubc8, and Ubc6, all of which function with E6AP, although the latter with a significantly lower efficiency that indicates that other features also contribute to E2 specificity (32). Interestingly, the three human Ubc5 isoforms possess a conservative residue substitution to glutamine at the position paralogous to Asn94. Therefore, this position may serve as a major, but not exclusive, specificity determinant for cognate E2 binding to E6AP.

Mutation of Asn561 to alanine abrogates hydrogen bonding to Asn94 and yields a 17-fold reduction in the kcat for 125I-polyubiquitin chain formation but no effect on the Kcat for Ubc7–ubiquitin thioester binding to E6AP (Table 1). Although initially counterintuitive, this result could be expected because the binding affinity contributed by the extensive interface stabilized principally by desolvation would be only marginally affected by disrupting a single hydrogen bond interaction unless that position made a substantial contribution to the desolvation-independent binding energy. However, such a mutation would be expected to markedly affect kcat if ablation of such an interaction altered the overall geometry and catalytic competence of the resulting transition state. An analogous scenario is thought to account for the reduced ability of Ubc5 isoforms to support E6AP-catalyzed polyubiquitin chain formation compared with their structurally related Ubc7 and Ubc8 paralogs (32). The kcat effect reported for mutation of Asn561 is not due to differences in specific activity of the wild-type versus mutant enzyme preparations because concentrations of Ubc7 (defining Kcat) and E6AP (defining kcat) are independently quantitated for each experiment by stoichiometric 125I-ubiquitin thioester activity assays, as described under “Materials and methods.” Importantly, similar small effects for single point mutations have been reported for the binding of Ubc5–ubiquitin to Nedd4-2 by Kamadurai et al. (26) and, thus, appear to be a general property of such modifications. However, this interpretation is complicated by the fact that kcat reports on Site 2–catalyzed 125I-polyubiquitin chain elongation, the significance of which was not initially apparent.

The docking model predicts a more complex and unique set of hydrogen bonds between the side-chain imidazole of His508 within the large N-terminal subdomain of E6AP and the

![Image](image-url)
e-amino groups of Lys\textsuperscript{64} and Lys\textsuperscript{67} within UbcH7 (Fig. 2, C and D). Mutation of either hydrogen-bonded lysine to alanine has no effect on the kinetics of \textsuperscript{125}I-polyubiquitin chain formation, presumably because the other hydrogen bond remains intact and any effect is below our limit of kinetic resolution (Table 1); however, mutation of both lysines to alanine results in an increase in \(K_m\) from 83 ± 14 nM for wild-type UbcH7 to 220 ± 83 nM (\(\Delta G_{\text{binding}} = 0.58\) kcal/mol) (Table 1). The complementary mutation of His\textsuperscript{508} to alanine has much the same effect as loss of both lysine residues, with the \(K_m\) increasing to 360 ± 220 nM (\(\Delta G_{\text{binding}} = 0.86\) kcal/mol), but is additionally accompanied by a 46-fold decrease in \(k_{\text{cat}}\) (Table 1). Again, mutation of the predicted hot spot interactions has more significant effects on the catalytic competence of chain elongation than binding affinity at Site 1.

We noted that Arg\textsuperscript{505} (as opposed to the vicinal Arg\textsuperscript{506} mentioned earlier) is adjacent to His\textsuperscript{508} and is in an excellent position to form hydrogen bonds to Lys\textsuperscript{64} and Lys\textsuperscript{67} upon mutation of His\textsuperscript{508} to alanine (Fig. 1D). Such a compensatory effect would be expected to shift the position of UbcH7−ubiquitin within the resulting Michaelis complex and to ablate the resulting \(k_{\text{cat}}\). In support of this interpretation, the kinetics of E6AP/H508A-catalyzed \(\textsuperscript{125}\text{I}-\text{polyubiquitin}\) chain formation in the presence of the UbcH7K64A/K67A double mutant exhibits no change in \(k_{\text{cat}}\) beyond that of the His\textsuperscript{508} mutant alone but increases \(K_m\) to 650 ± 280 nM compared with wild-type UbcH7, resulting in a \(\Delta G_{\text{binding}}\) (0.36 kcal/mol) of a magnitude similar to that observed for UbcH7K64A/K67A alone (Table 1). However, when the latter experiment is repeated with a E6AP/H508A/R505A double mutant, no detectable \(\textsuperscript{125}\text{I}-\text{polyubiquitin}\) chain formation is observed, and we can only estimate the upper limit for the activity of the E6AP/H508A/R505A as a \(k_{\text{cat}}/K_m\) of \(<2 \times 10^2\) M\textsuperscript{-1} s\textsuperscript{-1} (Table 1). Because the E6AP/R505A single mutant exhibits kinetics for \(\textsuperscript{125}\text{I}-\text{polyubiquitin}\) chain formation identical to wild-type ligase (Table 1), dramatic loss of activity for simultaneous mutation of His\textsuperscript{508} and Arg\textsuperscript{505} must be a specific effect.

The kinetic data of Table 1 suggest that the His\textsuperscript{508}-Lys\textsuperscript{64}-Lys\textsuperscript{67} triad is an important determinant for UbcH7−ubiquitin binding to Site 1. Among the Hect ligases, arginine or lysine are invariant residues at the position paralogous to that of Arg\textsuperscript{505}, whereas the His\textsuperscript{508} position is found among six Hect ligase paralogs, including E6AP, HeCW1/2, Huwe1, HectD2, and Herc5. Tandem lysines are only observed among UbcH7, paralogs, including E6AP side-chain inter-

6 The observed rates for these mutants approach our limit of detection for \(\textsuperscript{125}\text{I}-\text{polyubiquitin}\) chain formation and result in a substantial increase in the S.E. values of the measurements.

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**Figure 3. E6AP exhibits substrate inhibition with UbcH7−\textsuperscript{125}\text{I}-ubiquitin thioester.** Initial rates of E6AP-catalyzed \(\textsuperscript{125}\text{I}-\text{polyubiquitin}\) chain formation were determined as described under “Materials and methods” in the presence of 175 nM Uba1, 0.5 nM E6AP, and the indicated concentrations of UbcH7. Solid line, nonlinear regression fit of the data to Equation 1. Inset, double-reciprocal plot of data below 0.5 \(\mu\)M UbcH7.

**Structural basis of UbcH7−ubiquitin substrate inhibition**

The concentration dependence of UbcH7 on the initial rate of unanchored \(\textsuperscript{125}\text{I}-\text{polyubiquitin}\) chain assembly displays hyperbolic kinetics in the nanomolar range and substrate inhibition above 2–5 \(\mu\)M (32). When such a study is extended to higher UbcH7 concentrations, hyperbolic kinetics are again observed below 2 \(\mu\)M, as evidenced by the linearity of the corresponding double reciprocal plot (inset) but substrate inhibition above 2 \(\mu\)M that tends to zero rate at infinite [UbcH7]\textsubscript{c} (Fig. 3). Because the rate assays of Fig. 3 are conducted under E3-limiting conditions, for which the activity of Uba1 is sufficient to maintain UbcH7 quantitatively as its corresponding \(\textsuperscript{125}\text{I}-\text{ubiquitin}\) thioester (31), the observed substrate inhibition is not due to binding of uncharged UbcH7 to E6AP or change in rate-limiting step. As noted previously (32), the data are consistent with ordered binding to two kinetically independent, functionally distinct sites having significantly different binding affinities, the higher affinity of which corresponds to the cryptic Site 1 (\(K_m = 56 ± 6\) nM) required for Cys\textsuperscript{820} thioester formation and the lower affinity of which corresponds to the canonical Site 2 (\(K_d = 7 ± 0.7\) \(\mu\)M) (32). The initial rate kinetics for such a two-site hyperbolic system can be modeled by Equation 1,

\[
V_o = \frac{V_{\text{max}}[\text{UbcH7}]_0}{K_1 + [\text{UbcH7}]_0} - \frac{V_{\text{max}}[\text{UbcH7}]_0}{K_2 + [\text{UbcH7}]_0}
\]

(Eq. 1)

for which \(K_1\) is the \(K_m\) for Site 1, and \(K_2\) is the \(K_m\) for Site 2. Non-linear regression analysis of the data conforms to the prediction of Equation 1 (Fig. 3, solid line) and yields a \(K_1\) of 14 ± 7 \(\mu\)M, in good agreement with the \(K_d\) of 7 ± 0.7 \(\mu\)M for binding of
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Figure 4. The structural basis of UbcH7–\(^{125}\)I-ubiquitin substrate inhibition. The closed structure for UbcH7–ubiquitin used in the docking study was modeled onto the 1D5F trimeric E6AP structure of Huang et al. (24). Subdomains of the reference E6AP subunit are colored as in Fig. 1, whereas the other two subunits are colored in shades of gray. The UbcH7 moiety is shown in orange, whereas ubiquitin is shown in red. A, sites 1 and 2 of the reference subunit are occupied with UbcH7–ubiquitin. B, sites 1 and 2 of adjacent subunits are occupied with UbcH7–ubiquitin. C, model of Hect–ubiquitin thioester at Site 1 and UbcH7–ubiquitin occupying Site 2. Proposed trajectory of Lys\(^{48}\) on transition to the open conformer is shown with a yellow arrow.

the product analog UbcH7C86A to Site 2 determined kinetically (32) or 5.0 ± 0.5 μM determined previously by fluorescence polarization (59). The good correspondence among the \(K_d\) values is consistent with substrate inhibition arising by binding of UbcH7–ubiquitin to the canonical Site 2 and with the ubiquitin moiety not contributing to the apparent affinity at this site.

Modeling of Site 1 allowed us to examine the structural basis for the predicted steric hindrance between Sites 1 and 2 that results in substrate inhibition. No obvious steric hindrance is observed between adjacent UbcH7–ubiquitin moieties when they occupy Sites 1 and 2 within the same subunit of the E6AP trimer (PDB entry 1D5F) (Fig. 4A). In the simulation of Fig. 4A, UbcH7–ubiquitin bound to Site 1 is modeled from the PatchDock solution of Fig. 1, and UbcH7–ubiquitin bound to Site 2 was generated by superimposing the threaded structure of UbcH7-ubiquitin thioester onto the existing bound UbcH7 of the original Huang et al. (24) structure. In contrast, when Sites 1 and 2 of adjacent subunits of the trimer are occupied by UbcH7–ubiquitin, significant steric hindrance is found between the α-helix of ubiquitin at Site 2 and the C-terminal helix of UbcH7 bound at Site 1 (Fig. 4B). Such steric hindrance potentially explicates substrate inhibition observed at micromolar UbcH7–\(^{125}\)I-ubiquitin concentrations (Fig. 3), corresponding to the \(K_d\) for binding of charged UbcH7 to Site 2 in the range of 5–7 μM (32, 59). Moreover, steric hindrance would be enhanced if Site 2-bound UbcH7–ubiquitin reversibly shifts between the closed and open conformers (44). In support of substrate inhibition in trans, we have earlier shown that the kinetics for monoubiquitination of the truncated E6AP Hect domain, which is unable to oligomerize at the enzyme concentrations employed in our functional assays, shows hyperbolic kinetics without subsequent substrate inhibition in the micromolar range, in contrast to full-length enzyme, consistent with the steric hindrance modeled in Fig. 4B (32).

As noted earlier in Fig. 1B, deletion of UbcH7 from the Site 1 docking model simulates the E6AP–ubiquitin thioester intermediate and, as noted earlier, approximates the structure of the Nedd4-1–UbG76C thioester mimic reported by Maspero et al. (48) (Fig. 4C). Analogous product release of UbcH7 from Site 1 following thioester exchange relieves the steric hindrance imposed by the E2 moiety. We noted, as a consequence, that the UbcH7 thioester-linked ubiquitin bound at Site 2 of the adjacent subunit could then pivot out, much as occurs in the open E2–ubiquitin conformer (41), to a position that exactly juxtaposes Lys\(^{48}\) of the ubiquitin with the E6AP Cys\(^{820}\)–ubiquitin thioester bond for nucleophilic attack (Fig. 4C). In such a model, specific contacts at the interface between the mobile ubiquitin moiety, the adjacent C-terminal domain, and the orientation of the small N-terminal subdomain to which the E2 is bound could coordinately direct the correct orientation of Lys\(^{48}\) for facile nucleophilic attack. Interestingly, the C-terminal subdomain face predicted to contact the Site 2–anchored ubiquitin following the pivot corresponds to a transposable 60-residue segment previously demonstrated to direct chain linkage specificity for yeast Rsp5 and human Nedd4-2 (60) (Fig. 4C). This insight suggests a facile cyclic mechanism for polyubiquitin chain elongation that we have termed proximal indexation.

A proximal indexation model for E6AP-catalyzed polyubiquitin chain assembly

The overall mechanism we propose for E6AP-catalyzed polyubiquitin chain assembly is summarized in Scheme 2. Each subunit of the ligase is assumed to harbor two independent and functionally distinct E2–ubiquitin–binding sites (32): the
cryptic Site 1 modeled in silico in Fig. 1 and the canonical E2–ubiquitin Site 2 reported by Huang et al. (24) (intermediate 1). The cognate E2–ubiquitin thioester binds to Site 1 to form the corresponding Michaelis complex (intermediate 2, \( n = 1 \)), followed by thioester exchange to form the E6AP–ubiquitin thioester intermediate and dissociation of the now uncharged E2 (intermediate 3, \( n = 1 \)). Release of the uncharged E2 product relieves trans steric hindrance and allows binding of the second E2–ubiquitin thioester to Site 2 on the adjacent subunit to form the corresponding Michaelis complex (intermediate 4, \( n = 1 \)), followed by pivot of the thioester-bound ubiquitin, nucleophilic attack of Lys48 on the E6AP–ubiquitin thioester of Site 1, and formation of a Site 2–bound E2–diubiquitin (intermediate 5, \( n = 1 \)). Translocation of the Site 2–bound E2–diubiquitin to Site 1 reconstitutes intermediate 2 (\( n = 2 \)) and initiates a new cycle of Site 2 binding, pivot, nucleophilic attack, and translocation to assemble the elongating polyubiquitin chain. We have termed this mechanism proximal indexation.

The animation provides a more compelling case for the good fit of the topology present in the extant structures and provides a more complete visualization of the spatial considerations for chain elongation proposed by the proximal indexation mechanism.

Proximal indexation explicates a number of experimental observations noted previously for E6AP-catalyzed polyubiquitin chain formation, as detailed under “Discussion.” Equally important, the proximal indexation mechanism is robust in making specific testable predictions to validate the model, as shown below.

**Sites 1 and 2 function in trans**

The proposed chain assembly mechanism requires sites 1 and 2 to act in trans from adjacent subunits. We have previously demonstrated that the addition of increasing concentrations of truncated enzyme lacking the Hect domain results in non-competitive inhibition of full-length E6AP-catalyzed polyubiquitin chain formation, with the rate tending to zero at high concentrations of the mutant (33). This behavior requires the active enzyme to function as an oligomer and is consistent with Sites 1 and 2 acting in trans but not cis to assemble polyubiquitin chains (33). To further test the trans mechanism for chain formation shown in Scheme 2, we performed a variation of our earlier study by titrating dominant negative forms of full-length E6AP into wild-type native enzyme and then monitoring rates of \(^{125}\)I-polyubiquitin chain assembly (Fig. 5). The active site Cys820 of GST–E6AP was mutated to alanine to yield an inactive full-length ligase (33). The addition of the resulting recombinant GST–E6APC820A to wild-type enzyme yielded a hyperbolic decrease in the initial rate of \(^{125}\)I-polyubiquitin chain formation with increasing concentrations of the point mutant (\( K_i = 0.6 \pm 0.3 \) \( \mu M \)) (Fig. 5A). Whereas the addition of C-terminal truncated E6AP results in complete loss of activity (33), Scheme 2 predicts that the addition of GST–E6APC820A should result in a limiting 33% residual activity at an end point in which a single wild-type Cys820 Site 1 is adjacent to a neighboring Site 2 for the proposed trimeric structure. The observed 50% inhibition suggests that E6AP is dimeric at the concentration used in the kinetic experiment of Fig. 5A, although it is trimeric at higher concentrations monitored by dynamic light
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scattering and gel filtration chromatography (33). This conclusion is consistent with the observation that only about 5% of active GST-E6AP is oligomeric under these assay conditions when viral E6 protein is titrated into identical assays to promote oligomerization (33). Moreover, the radially symmetric subunit interactions stabilizing the trimeric structure is compatible with subunit dissociation to yield an active dimer (33). The trend in loss of activity with increasing concentration of the dominant negative form is inconsistent with Sites 1 and 2 acting in cis because the latter alternative predicts the rate of chain assembly to be unaffected by the addition of the inactive GST-E6APC820A mutant.

Because recombinant bacterially expressed GST-E6AP contains trace amounts of truncated fragments arising during expression and purification that can also inhibit chain formation when incorporated into the oligomer, we repeated the study with iodoacetamide-modified full-length E6AP that is substantially free of such fragments (33) (Fig. 5). Incubation of baculovirus-expressed full-length His6-E6AP with iodoacetamide resulted in a first-order loss in the rate of 125I-polyubiquitin chain formation ($t_{1/2} = 2.5$ min) (Fig. 5B). Rigorous first-order kinetics for loss of activity over at least four half-lives is consistent with inactivation resulting from modification of a specific site, presumably Cys820 (33). Following quenching with DTT, the addition of the inactive E6AP to wild-type enzyme resulted in a concentration-dependent decrease in the initial rates of 125I-polyubiquitin chain formation to a final value of 50% of initial activity ($K_{i} = 57$ nM) (Fig. 5C). The difference in $K_{i}$ for GST-E6APC820A versus iodoacetamide-modified E6AP probably reflects the markedly higher specific enzyme activity of the baculovirus-expressed enzyme and sets an upper limit to the intrinsic $K_{i}$ for E6AP subunit association (33). General agreement in the two experimental approaches demonstrates that Sites 1 and 2 function in trans from adjacent subunits to assemble polyubiquitin chains, accounting for the earlier reported requirement for oligomerization for chain assembly (33).

125I-polyubiquitin chains are assembled as E6AP-linked thioesters

The mechanism of Scheme 2 proposes that 125I-polyubiquitin chains are assembled on Cys820 before en bloc transfer to the target protein and predicts that one should observe high-molecular weight E6AP-125I-polyubiquitin thioesters under non-reducing conditions. The autoradiogram of Fig. 6A (right) shows that under non-reducing conditions, the predicted high-molecular weight 125I-polyubiquitin chains are observed at the top of the stacker gel. Loss of this signal when a parallel sample is resolved under reducing conditions, along with corresponding thioester bands for Uba1, UbcH7, and E6AP, confirms that the high-molecular weight 125I-polyubiquitin chains are linked by thioester bonds (Fig. 6A, left). The thiol-sensitive autoradiographic density observed under non-reducing conditions between the migration positions of UbcH7-125I-ubiquitin and Uba1-125I-ubiquitin is consistent with transient intermediates of increasing chain length bound to UbcH7 (Fig. 6A, lanes 8 and 4). These thiol-sensitive high-molecular weight intermediates must be present at very low steady state levels because they are not observed under the E3-limiting conditions used in our standard rate assays but only at the high concentrations of E6AP employed in the experiment of Fig. 6A for which rates become Uba1-limiting.

That the high molecular weight signal in Fig. 6A represents 125I-polyubiquitin chains is demonstrated by the ExTerm assay shown in Fig. 6B. Under E3-limiting conditions, the ExTerm assay has been used as a facile method to identify the linkage specificity of 125I-polyubiquitin chains using a chain termination scheme similar to the Sanger DNA sequencing protocol (34, 61). Radiolabeled ubiquitin is diluted with either wild-type

![Image](image-url)

Figure 6. 125I-polyubiquitin chains are assembled as E6AP-linked thioesters. A, initial rates of E6AP-catalyzed 125I-polyubiquitin chain formation were determined as described under “Materials and methods” but in the presence, where indicated, of 19 nM Uba1 (E1), 126 nM UbcH7 (E2), and 96 nM GST-E6AP (E3) for 1 min and then resolved under reducing (lanes 1–4) or non-reducing (lanes 5–8) conditions before autoradiography. B, assays were conducted as in A in the presence of either 4 μM 125I-ubiquitin (lane 1) or 3.2 μM 125I-ubiquitin and a 0.8 μM concentration of either wild-type ubiquitin (lane 3), UbK0 in which all lysines have been mutated to arginine (lane 2), or the indicated single-lysine point mutants (lanes 4–10) before resolution under non-reducing conditions and autoradiography. Migration of molecular weight markers is shown on the left of each panel, and migration of assay components is shown on the right.
ubiquitin as a control (lane 3) or with each of the single lysine-to-arginine point mutants of ubiquitin (lanes 4–10) (Fig. 6B). A loss of signal due to chain termination by the point mutant identifies the linkage type (34). However, the experiment of Fig. 6B is complicated by the requirement of excess E6AP to detect the high molecular weight thiolel-sensitive intermediates so that the results also reflect potential rate effect(s) on Uba1 activation and E2 transthiolation steps. It is clear from the loss of signal in the presence of the K48R point mutant (lane 9), compared with that of the K0 positive control in which all of the lysines of the polypeptide have been mutated to arginine (lane 2), that the high-molecular weight thioester-anchored 125I-polyubiquitin chains present in the stacker gel and top of the resolving gel are linked through Lys48 (Fig. 6B).

125I-polyubiquitin chain assembly occurs by proximal addition

Fig. 7A emphasizes that polyubiquitin chains can be assembled by the addition of thioester-activated ubiquitin to the distal Lys48 of a chain or by the proximal addition of Lys48 to the thioester-activated C terminus of a chain. To test the proximal addition mechanism proposed in Scheme 2, we utilized reductively methylated 125I-ubiquitin (125I-rmUb) to preclude the potential for distal addition in the pulse-chase experiment schematically outlined in Fig. 7B (62–64). The method for in situ generation and use of UbcH7~ubiquitin depends on the ability to manipulate this intermediate by exploiting trapping of the intermediate through reversal of the Uba1-catalyzed ubiquitin activation step (65, 66). As described under “Materials and methods,” UbcH7 was loaded with 125I-rmUb, and then ATP remaining from the charging step was removed by the addition of hexokinase and glucose (Fig. 7B). Inclusion of AMP in the ATP depletion step and successive incubation with inorganic pyrophosphate followed by a brief pulse with inorganic pyrophosphate back-traps residual Uba1 ternary complex without removing UbcH7~125I-rmUb thioester (65), as shown by loss of the Uba1 thioester band in Fig. 7C but full retention of the E2 thioester (lanes 3 and 6). The absence of thioester formation in a parallel control lane for which hexokinase and glucose were added before the addition of 125I-rmUb confirmed the efficacy of ATP depletion (lane 5). The subsequent addition of E6AP to a final concentration of 44 nM results in stoichiometric transthiolation to form E6AP~125I-rmUb thioester within 10 s (Fig. 7, C lanes 7 and E). That the band represents E6AP~125I-rmUb, along with the minor E6AP truncation at 55 kDa, is shown by their ability to reduce conditions. The E6AP charging step was quenched by back-trapping the remaining UbcH7~125I-rmUb through Uba1 with a 1-min pulse of inorganic pyrophosphate (Fig. 7C, lane 8). High-molecular weight signal at the top of the resolving gel in lanes 7 and 8 of Fig. 7C (left) represents residual oligomeric E6AP~125I-rmUb thioester not completely denatured by the addition of the SDS sample buffer because it is labile to reducing conditions (lanes 7 and 8 of Fig. 7C right) and can be dissipated by brief incubation at 100 °C (not shown). The inability of the inorganic pyrophosphate pulse to trap E6AP~125I-rmUb thioester is probably a consequence of the negligible binding affinity of Site 1 for uncharged UbcH7 (32).

Following a 2-min chase of the E6AP~125I-rmUb with 317 nM unlabeled UbcH7~ubiquitin thioester, the radiolabel is stoichiometrically found in high-molecular weight polyubiquitin chains at the top of the resolving gel and in the stacker that are stable to reducing conditions (Fig. 7, C and E). The results are consistent with the prediction of Scheme 2 for chase of label through intermediate 4 by unlabeled UbcH7~ubiquitin to form a UbcH7~ubiquitin~125I-rmUb diubiquitin adduct by proximal addition before subsequent chain elongation supported by the remaining unlabeled UbcH7~ubiquitin. That the chase of label to polyubiquitin chains occurs in trans through oligomeric E6AP is consistent with the marked ablation in chain formation in the presence of N-AcPheNH2, which blocks chain assembly by dissociating the trimer (33) (Fig. 7C, lanes 10 versus 11, left and right). The absence of high-molecular weight label when E6AP~125I-rmUb is chased with UbcH7~ Ub(K0) in which all lysines have been mutated to arginine demonstrates that the signal results from ongoing chain formation. Collectively, the results of Fig. 7 provide strong validation for the proximal indexation model.

Site 1 is the donor for Cys820 transthiolation

Scheme 2 requires that UbcH7~ubiquitin bound to Site 1 serve as the immediate donor in the transthiolation step to form Cys820~ubiquitin thioester, and current kinetic evidence is consistent with this prediction (32). Earlier studies demonstrated that Phe63 is a critical residue in defining the affinity for binding of UbcH7 to the small N-terminal subdomain identified in the crystal structure of Huang et al. (24) and that mutation to alanine shifts the $K_d$ from 5 μM for wild-type protein to 810 μM when monitored by fluorescence polarization (59). We have shown that UbcH7F63A fails to support measurable E6AP-catalyzed 125I-polyubiquitin chain assembly under rigorous E3-limiting conditions in the presence of 17 nM E6AP and up to 32 μM E2 for 10 min, setting an upper limit to the $k_{cat}$ for 125I-polyubiquitin chain formation supported by UbcH7F63A of 5.2 × 10⁻⁵ s⁻¹ (32). The absence of activity is not due to a defect in charging of UbcH7F63A with 125I-ubiquitin because parallel Uba1-catalyzed transthiolation kinetics yielded a $K_m$ of 130 ± 30 nM and $k_{cat}$ of 3.3 ± 0.4 s⁻¹ compared with 103 ± 20 nM and 2.1 ± 0.1 s⁻¹ for wild-type UbcH7 (not shown). The good correspondence in Uba1-catalyzed transthiolation kinetics additionally argues against marked changes in structure accompanying the point mutation.

As demonstrated previously, wild-type UbcH7 is capable of stoichiometrically forming E6AP~125I-ubiquitin within 15 s, corresponding to a $k_{cat}$ of >9 s⁻¹ (32) (Fig. 8, lanes 2–6). In contrast, the rate of E6AP~125I-ubiquitin thioester formation supported by UbcH7F63A is slower and, upon quantification of associated radioactivity, corresponds to a $k_{cat}$ of 0.6 s⁻¹. Therefore, although UbcH7F63A-supported thioester exchange to form E6AP~125I-ubiquitin is slower than wild-type E2, the difference does not account for the quantitative loss of polyubiquitin chain formation. More importantly, the results of Fig. 8 demonstrate that Site 1 is the immediate donor for E6AP~125I-ubiquitin thioester exchange, as kinetically inferred previously (32). In the docking model of Fig. 1, Phe63 of UbcH7 does not interact with E6AP, although its mutation to alanine ablates the

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**A**

![Diagram of E6AP mechanism](image)

**B**

![Diagram of E6AP mechanism](image)

**C**

![Non-reducing (NR) gel](image) ![Reducing (R) gel](image)

**D**

![Non-reducing (NR) gel](image)

**E**

![Graph](image)

Figure 7. 125I-polyubiquitin chain formation occurs by proximal addition. A, ubiquitin can be added from either the proximal or distal end of an E6AP–ubiquitin thioester to form a polyubiquitin chain. B, a schematic outline of the pulse-chase experiment, as detailed under “Materials and methods.” Numbers within the circles refer to the corresponding lanes in Fig. 7C. 107 nM Ubch7 was loaded with 125I-rmUb (1.7 μM; filled circles) in the presence of Uba1 (4 nM) for 1 min. Remaining ATP was removed by the addition of hexokinase (1 IU/ml) and glucose (8 mM) with AMP (2 mM) added to back-trap Uba1–125I-rmUb ternary complex during a 1-min incubation. Residual Uba1 ternary complex was removed by the addition of yeast inorganic pyrophosphatase (2 mM) for 1 min followed by a 1-min pulse with inorganic pyrophosphate (2 mM). The in situ generated Ubch7–125I-rmUb was incubated with E6AP (44 nM) for 10 s and then quenched by the addition of pyrophosphate (2 mM) for 1 min to remove remaining Ubch7–125I-rmUb. The resulting in situ generated E6AP–125I-rmUb was used in the subsequent chase incubations (open circles): incubation for 2 min with unlabeled Ubch7–ubiquitin prepared in parallel (317 nM final concentration), the same incubation in the presence of 8% (v/v) carrier DMSO, carrier DMSO containing 112 mM final concentration of N-AcPheNH2 to dissociate the E6AP trimer, or Ubch7–Ub(K0) (317 nM final concentration) in which all lysines have been mutated to arginine. C, non-reducing (left) and reducing (right) autoradiogram of SDS-PAGE resolution of the incubations outlined in B. Lane numbers correspond with the steps in Fig. 7B. Lane 5, control for ATP depletion in which hexokinase and glucose were added before125I-ubiquitin for formation of Ubch7–ubiquitin; lane 14, parallel incubation with tracer125I-ubiquitin to confirm formation of Ubch7–ubiquitin for the chase incubations. D, control incubations for ATP depletion and unlabeled Ubch7–ubiquitin formation. Lane 13, control for ATP depletion in which hexokinase and glucose were added before125I-ubiquitin for formation of Ubch7–ubiquitin; lane 14, parallel incubation with tracer125I-ubiquitin to confirm formation of Ubch7–ubiquitin for the chase incubations. Relative molecular weight markers are shown on the left. Mobilities of selected components are shown on the right. D, control incubations for ATP depletion and unlabeled Ubch7–ubiquitin formation. Lane 13, control for ATP depletion in which hexokinase and glucose were added before125I-ubiquitin for formation of Ubch7–ubiquitin; lane 14, parallel incubation with tracer125I-ubiquitin to confirm formation of Ubch7–ubiquitin for the chase incubations. Relative molecular weight markers are shown on the left. Mobilities of selected components are shown on the right. E, radioquantitation of products from Fig. 7C. The lanes were excised, and associated 125I radioactivity was quantified to determine corresponding 125I-rmUb for the selected regions of the non-reducing (NR) or reducing (R) panels (31). Each lane corresponds to 25 μl of the original incubation.

affinity of Ubch7 for Site 1 (32) and the $k_{cat}$ for thioester exchange (Fig. 8). These observations are consistent with a topology for full-length enzyme in which some portion of the N-terminal region not represented in the crystal structure for the Hect domain folds to interact with Site 1 through Phe63.

Identification of an E6AP catalytic ensemble

The docking model of Fig. 1 predicts that nucleophilic attack of Cys820 on the Ubch7–ubiquitin thioester bond occurs within a water-excluded binding interface. This is germane to
consistent with this catalytic model, mutation of Glu550 to alanine has no effect on the kinetic of 125I-polyubiquitin chain formation, decreasing the $k_{cat}$ by 4400-fold without altering $K_m$ (Table 2). Although the effect of mutating Arg506 is consistent with a role in orienting the side chain of Glu550, the data do not rule out the possibilities that the guanidinium moiety of Arg506 additionally serves to stabilize the general base-derived Cys820 thiolate or the predicted oxanion transition state during thioester exchange, both enhanced by the localized low dielectric microenvironment at the binding interface of Site 1.

Discussion

We were surprised when our earlier kinetic studies of E6AP-catalyzed 125I-polyubiquitin chain assembly appeared to refute the standard model for transthiolation through the canonical E2-binding site proposed by Huang et al. (24) and the subsequent sequential distal chain addition of activated ubiquitin catalyzed by E6AP (32, 33). As described earlier, our rate studies conform to a minimum kinetic mechanism summarized by Scheme 1, whose defining feature is the coordinated action of two functionally distinct E2−ubiquitin−binding sites: one to rapidly form the initial Hect−ubiquitin thioester through an E6AP-catalyzed exchange reaction (Site 1) and a second to catalyze rate-limiting polyubiquitin chain elongation (Site 2) (32, 33). Scheme 1 accounts for the ability of specific point mutations to severely ablate binding to the canonical Site 2 and assembly of polyubiquitin chains yet fail significantly to affect the rate of Hect−125I-ubiquitin thioester formation (32, 59) (Fig. 8). In fact, the data of Fig. 8 provide the most direct empirical evidence that Site 1 rather than the canonical Site 2 described by Huang et al. (24) is the direct donor for activated ubiquitin in E6AP−ubiquitin thioester formation. The mechanism of Scheme 1 also explicates the competitive inhibition observed for a stable UbcH7C86S-ubiquitin oxyester substrate mimic with respect to the wild-type UbcH7−125I-ubiquitin substrate yet non-competitive inhibition for a UbcH7C86A product analog (32). Finally, the mechanism potentially reconciles distance constraints on the geometry of transthiolation implicit in the standard model by proposing a cryptic Site 1 not represented in the original crystal structure (32). In an effort to understand the topology of Site 1 and the steric hindrance predicted by Site 2, we turned to in silico modeling using the well-established PatchDock algorithm (36). The latter results provide unexpected insights into general features of Site 1 in addition to suggesting a mechanism for E6AP-catalyzed polyubiquitin chain formation and target protein conjugation.

The energy-minimized final docking model shown in Fig. 1 exhibits excellent complementarity for the binding interface between E6AP and the thermodynamically favored closed conformer of UbcH7−ubiquitin. The model brings the Cys820 nucleophile and UbcH7−ubiquitin thioester bond within 6 Å, with additional closure presumably achievable by relaxation of the structure not available in the docking algorithm (36). The position of the ubiquitin moiety before thioester exchange is nearly identical to that of the Nedd4−1−ubiquitin thioester model structure reported by Maspero et al. (48) and is in contact with a cluster of previously reported ubiquitin-interacting...
residues in the C-terminal subdomain (48–50). The extensive water-excluded binding surface of the final docking solution is consistent with the nanomolar \( K_d \) for UbcH7–\( \sim^{125}\text{I-} \)ubiquitin binding determined kinetically, whereas the millimolar affinities predicted for the separate UbcH7 and ubiquitin moieties satisfy earlier predictions in accounting for the inability of neither molecule alone to competitively inhibit \( ^{125}\text{I-} \)polyubiquitin chain assembly (32).

To validate the docking model, we chose uniquely predicted side-chain interactions outside of the canonical small N-terminal subdomain that were not paralogous to residues tested in the only other E2–ubiquitin binding model, that of Ubc5BL35S/T98K–ubiquitin bound to Nedd4-2 (26) (Fig. 2). All of the mutations tested represent conserved cognate Hect–E2 interactions and affect \( K_m \), \( k_{cat} \), or both (Table 1). Because the enzyme follows burst kinetics, differences in \( K_m \) reflect binding of UbcH7–\( \sim^{125}\text{I-} \)ubiquitin or its point mutants to Site 1 (32). The relatively modest increases in \( K_m \) probably reflect small contributions of the specific interactions to the larger entropic binding contribution of the water-excluded interface revealed by the docking model in Fig. 1. In contrast, changes in \( k_{cat} \) must reflect alterations in the kinetics of chain elongation contributed by Site 2 (Scheme 1), the interpretation of which was not immediately apparent. However, with additional data and expansion of the model to that of Scheme 2, the effect of Site 1 mutations on the \( k_{cat} \) for chain elongation emerge as the consequence of the cyclic nature of the E6AP mechanism. We propose that following translocation of the UbcH7–\( \sim^{125}\text{I-} \)ubiquitin intermediate from Site 2 to Site 1, the UbcH7 moiety occupies the same position as in the original Michaelis complex. This accounts for the effects of mutation on \( k_{cat} \) as suboptimal positioning of the intermediate for facile thioester exchange. Overall, the kinetic effects observed for mutation of the novel tripartite interactions among Lys\(^{64}\), Lys\(^{57}\), and His\(^{508}\) in addition to that of Asn\(^{561}\) provide strong validation for the binding model (Table 1), especially in satisfying the predictions of Scheme 2. In addition, it is notable that the binding model predicts a side-chain interaction between Lys\(^{32}\) of UbcH7 and the absolutely conserved Glu\(^{539}\) Angelman syndrome loss-of-function mutation site in the large N-terminal subdomain (51), potentially accounting for the phenotype of this sequence change. Remarkably, the functional link between \( K_d \) for UbcH7–\( \sim^{125}\text{I-} \)ubiquitin binding to Site 1 and \( k_{cat} \) for chain elongation extends to the UbcH7F63A mutation, for which no obvious interaction between the E6AP Hect domain and Phe\(^{63}\) is evident (Fig. 8). This suggests that the N-terminal region of E6AP folds back on the catalytic domain to allow interaction with Phe\(^{63}\).

Substrate inhibition typically reflects the presence of two functionally distinct binding sites of different affinities, observation of which in the case of E6AP provided corroborating evidence for Scheme 1 in our earlier study (32). The expanded data set of Fig. 3 demonstrates that rates of \( ^{125}\text{I-} \)polyubiquitin chain formation tend to zero at high concentrations of the substrate, indicating absolutely ordered addition for Sites 1 and 2. The excellent fit of the rate data to the two-site model of Equation 1 provides strong support for the model and allowed estimation of the \( K_d \) for UbcH7–\( \sim^{125}\text{I-} \)ubiquitin binding to Site 2. That substrate inhibition results from steric hindrance at Site 1 upon occupancy of Site 2 is indicated by the excellent agreement between the \( K_d \) (\( K_2 \)) of 14 ± 7 \( \mu \text{M} \) estimated by non-linear regression of the rate data of Fig. 3 to Equation 1 versus the \( K_d \) of 7 ± 0.7 \( \mu \text{M} \) determined from non-competitive inhibition of \( ^{125}\text{I-} \)polyubiquitin chain assembly by the UbcH7C86A product analog (32) and the \( K_d \) of 5.0 ± 0.5 \( \mu \text{M} \) determined earlier by Eletr and Kuhlman using fluorescence polarization (59).

Modeling simultaneous occupancy of Sites 1 and 2 by UbcH7–\( \sim^{125}\text{I-} \)ubiquitin shows that only when the substrate molecules bind to neighboring subunits of the catalytically active E6AP trimer is substrate inhibition observed (Fig. 4, A and B). Ordered binding at adjacent subunits during the catalytic cycle is guided by the concerted contributions of steric hindrance

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**Figure 9. Identification of a potential catalytic ensemble within Site 1.** A, a side-on view of the in silico docking model for Site 1 similar to that of Fig. 1C but identifying a potential catalytic ensemble comprising Cys\(^{520}\), Glu\(^{550}\), and Arg\(^{506}\). B, close-up of A rendered in stick structures to show the catalytic ensemble relative to the thioester bond between ubiquitin (red) and UbcH7 (orange).

**Table 2** Summary of kinetic constants for Glu550 and Arg506 mutants

|        | \( K_m \) (M) | \( k_{cat} \) (s\(^{-1}\)) | \( k_{cat(WT)/k_{cat(mut)}} \) |
|--------|--------------|----------------------------|-----------------------------|
| E6AP   | 58 ± 6       | 3.1 ± 0.9 \( \times 10^{-2} \) |                             |
| E6APE550A | 52 ± 10     | 2.0 ± 0.1 \( \times 10^{-5} \) | 1500                        |
| E6APE550D | 35 ± 42     | 1.0 ± 0.1 \( \times 10^{-3} \) | 31                          |
| E6APR506A | 21 ± 6      | 7.0 ± 0.1 \( \times 10^{-6} \) | 4400                        |
and affinity differences between Sites 1 and 2. This conclusion agrees with earlier observations that substrate inhibition is found with trimeric E6AP but not with the monomeric enzyme (32). Dissociation of uncharged UbcH7 from Site 1 following thioester exchange with Cys820 to form the E6AP–ubiquitin intermediate relieves the steric hindrance and allows binding of UbcH7–ubiquitin to Site 2 on the adjacent subunit. We noted that on binding of UbcH7–ubiquitin to Site 2, the thioester-bound ubiquitin is free to pivot and juxtapose its Lys48 adjacent to the Cys820–ubiquitin thioester bond on the adjacent subunit, Fig. 4C. This insight provides an elegant mechanism for processive chain assembly if one invokes nucleophilic attack of Lys48 on the Cys820–ubiquitin thioester bond to form Site 2–bound UbcH7–diubiquitin followed by translocation of the latter to Site 1, driven by the ~250-fold difference in affinities (Scheme 2). Translocation resets the mechanism and is topologically required in the assembly of repeating structures, such as polyubiquitin, when the assembly machinery is unable to translate along the growing chain, as encountered with stationary E2–ubiquitin–binding sites present on E6AP. Translocation also yields a structure analogous to the initial Michaelis complex (intermediate 2) that we propose then undergoes thioester exchange to generate a Hect–diubiquitin thioester (intermediate 3),7 thus explicating effects on \( k_{cat} \) due to mutation of the predicted binding sites (Table 1).

The expanded mechanism of Scheme 2, which we term proximal indexation, satisfies the known features of the enzyme and proposes chain assembly by an “inside-out” addition of ubiquitin moieties to the proximal end of the chain prior to en bloc transfer to the target protein. Such a mechanism accounts for the ability of E6AP rapidly to assemble the very long polyubiquitin chains that we observe under optimal catalytic conditions and the absolute requirement for oligomerization in polyubiquitin chain formation (33). Scheme 2 additionally provides a mechanistic rationale for the Hect–ubiquitin thioester intermediate as a platform for chain assembly that is not adequately addressed by models involving distal addition to target protein-anchored polyubiquitin chains (1, 32, 33, 73). In addition, occupancy of Site 2 with E2–Ub, for intermediate 4 suggests that en bloc transfer of chains to the target protein principally occurs stochastically through intermediate 3 of Scheme 2.

The model posits that polyubiquitin linkage specificity is a consequence of side-chain interactions between the ubiquitin moiety of the Site 2-bound E2–ubiquitin (intermediate 4) and the C-terminal subdomain in addition to potential contributions due to orientation of the E2 at the latter site within the small N-terminal subdomain. This feature is consistent with observations by Kim and Huijbregts (60) that the last 60 residues of the C-terminal domain are required for linkage specificity with Lys63–linked polyubiquitin assembly by yeast Rsp5. Finally, Scheme 2 and the requirement for oligomerization for polyubiquitin chain formation explains how a Hect ligase can append target proteins with either polyubiquitin chains or monoubiquitin under different cellular contexts.

Scheme 2 is robust and makes specific predictions regarding E6AP-catalyzed polyubiquitin chain assembly. One prediction unique to the proximal indexation model is that titration of a full-length active site E6AP mutant into wild-type enzyme should result in a hyperbolic loss of activity to a final value of 33% because, in the limit, there will be only a single instance of Site 2 trans to a wild-type Site 1 in the trimer. In contrast, if Sites 1 and 2 function in cis or oligomerization is not required for \( 125^I \)-polyubiquitin chain formation, then no effect should be observed upon the addition of the mutant. The data of Fig. 5 confirms that Sites 1 and 2 function in trans, corroborated by the ablation of chain formation in the single-turnover studies of Fig. 7 in the presence of N-\text{AcPheNH2}. The limiting rate of 50% observed in these studies requires that under the conditions of the assay, E6AP exists as a dimer, consistent with the plasticity of E6AP oligomerization described earlier (33) or functional asymmetry in the trimer subunits. A second prediction is that E6AP should form Cys820–\( 125^I \)-polyubiquitin chains under non-reducing conditions, as is shown in Fig. 6A (lane 8). That the signal present in the stacker gel of Fig. 6A (lane 8) represents Lys48-linked \( 125^I \)-polyubiquitin chains is demonstrated by the results of the parallel ExTerm assay of Fig. 6B. The collective results of Figs. 5–7, predicted by Scheme 2, provide strong and unambiguous support for the proximal indexation mechanism.

The docking model of Fig. 1 with its extensive water-excluded binding interface places Cys820 within an apolar microenvironment upon binding of UbcH7–ubiquitin to Site 1. Whereas the low dielectric microenvironment at the binding interface is expected to enhance nucleophilic reactions, such as thioester exchange, it is also predicted to increase the \( k_{cat} \) of Cys820 from its typical value of 9.0 (70, 71). Recent model studies demonstrate that alkyl thiols are not sufficiently reactive to undergo thioester exchange in the absence of general base catalysis to generate the corresponding thiolate (72); therefore, the inherently low reactivity of the Cys820 thiol is expected to be further compromised by the increased \( k_{cat} \) of Cys820 in the water-excluded microenvironment (71, 74, 75). The original crystal structure of E6AP reveals that Cys820 is hydrogen-bonded to Glu550, which in turn is hydrogen-bonded to Arg506 (24). Such a hydrogen bonding network is predicted by the PROPKA algorithm of Li et al. to raise the \( k_{cat} \) values of Cys820 and Glu550 to 14.8 and 7.1 (71), respectively. Within this structural context, Glu550 is an excellent candidate for the requisite general base catalyst for proton abstraction from Cys820 to generate the corresponding thiolate nucleophile. The 1500-fold decrease in \( k_{cat} \) for E6APE550A is consistent with such a role (Table 2). However, because the mutation affects the \( k_{cat} \) for chain elongation, Glu550 also formally serves as a general base catalyst during the symmetric step of polyubiquitin transhistidination (intermediate 5) (Scheme 2).

The 31-fold reduction in \( k_{cat} \) for the conservative E6APE550D mutation demonstrates that the geometry of the carboxyl group is important because substitution of glutamate with the shorter side chain of aspartate will disrupt the hydrogen-bonding network (Table 2). The latter insight has important implications because many structures of Hect domains, *The mechanism of E6AP*
The mechanism of E6AP

with the exception of E6AP and Smurf2 (24, 45, 76), reveal a C-terminal subdomain rotated away from the paralogous absolutely conserved Glu$^{550}$ residue, disrupting its interaction with and predicted general base contribution to the active site cysteine (26, 27). Verdecia et al. (27) have observed that mutation of the flexible linker region between the large N-terminal and the C-terminal subdomains of the WWP1 Hect domain, presumably imparting rigidity, results in marked loss of activity and has been interpreted as evidence that conformational flexibility is required for activity. In contrast, we have argued that the skewed conformers are artifacts of including an additional amphipathic helix in the expression constructs, not present in the E6AP structure that binds to and occludes the normal trimerization interface between subunits, resulting in catalytically inactive monomers and adoption of a skewed C-terminal subdomain conformation otherwise sterically excluded in the trimer (33). The data of Table 2 are consistent with the latter conclusion and suggest an alternative interpretation in which the C-terminal skewed structures are inactive conformers rather than requisite intermediates in the respective ligase catalytic cycles.

The E6APR506A mutant has the greatest effect on the $k_{\text{cat}}$ for $^{125}\text{I}$-polyubiquitin chain elongation (Table 2) and probably reflects its contribution to orienting Glu$^{550}$ relative to Cys$^{820}$ (Fig. 9). However, within the low dielectric microenvironment predicted for UbcH7−ubiquitin bound at Site 1 (Fig. 1), one cannot exclude additional contributions from stabilization of the incipient anionic transition state accompanying thioester exchange. These catalytic contributions potentially account for the absolute conservation of Glu$^{550}$ and Arg$^{506}$ within the Hect superfamily and the loss-of-function phenotype on mutation in Angelman syndrome. Glutamate 539 is a third absolutely conserved Glu$^{550}$ residue, disrupting its interaction with Arg$^{506}$, presumably also serving to orient the guanidine group of the side chain in addition to hydrogen bonding with Lys$^{82}$ of UbcH7 in the Site 1 docking model (Fig. 1, C and D).

The present studies represent the first use of in silico approaches to model E2−ubiquitin binding to a ligase. Structural insights from the docking model and additional kinetic studies of E6AP-catalyzed $^{125}\text{I}$-polyubiquitin chain formation combine to generate an expanded proximal indexation model for the enzyme (Scheme 2). Proximal indexation accounts for the radially symmetric structure of E6AP, the requirement for oligomerization in polyubiquitin chain formation, and a mechanistic rationale for the Cys$^{820}$−ubiquitin thioester as a platform for chain assembly. Our recent demonstration that the two-site burst mechanism of Scheme 1 is also employed by the evolutionarily unrelated S. flexneri IpaH family and the S. enterica SspH2 bacterial ubiquitin ligases, presumably through convergent evolution, provides compelling evidence that proximal indexation represents an optimal solution to the catalytic and topological challenges of polyubiquitin chain assembly and target protein ligation.

Materials and methods

Bovine ubiquitin was purchased from Sigma and purified to apparent homogeneity by FPLC and then quantitated spectro-

photometrically (77). A portion of the ubiquitin was radioiodinated by the chloramine-T procedure to yield specific radioactivities of ~15,000 cpm/pmol using carrier-free Na$^{125}\text{I}$ purchased from either GE Healthcare or PerkinElmer Life Sciences (78). A portion of the $^{125}\text{I}$-ubiquitin was used to produce $^{125}\text{I}$-rmUb (64). Human erythrocyte Uba1 was purified to apparent homogeneity from outdated human blood (78), and active enzyme was quantitated by the stoichiometric formation of $^{125}\text{I}$-ubiquitin thioester (65, 79). Yeast hexokinase (Type F300) and inorganic pyrophosphatase (I1643) were obtained from Sigma-Aldrich, as was the cytological grade DMSO (D2438). The N-acetylphenylalanyl amide (Bachem, E-1160) was that used previously (33).

Generation and purification of recombinant E2 proteins

Human recombinant E2 protein UbcH7 and UbcH7F63A were those described previously (32, 80). The UbcH7 point mutants were generated from pGEX4T1-HsUbcH7 using the QuikChange mutagenesis kit (Stratagene) (32). Coding regions for all clones were sequenced to preclude cloning artifacts and, where relevant, to ensure the correct point mutation. Recombinant wild-type and mutant proteins were expressed in Escherichia coli BL21 (DE3) cells harboring the desired pGEX-E2 plasmid, purified to apparent homogeneity, and processed with thrombin to remove the GST tag as described (32, 81). Active E2 concentrations were quantitated by the Uba1-dependent stoichiometric formation of $^{125}\text{I}$-ubiquitin thioester and compared with total E2 protein determined spectrophotometrically using their calculated 280-nm extinction coefficients (82). The E2 proteins were stored at −80°C in small aliquots that were stable for >6 months although subject to differential rates of activity loss with successive freeze-thaw cycles (31, 82).

Generation and purification of recombinant E6AP

Human E6AP isoform 3 (UBE3A; IMAGE clone NM000462.2) was subcloned into the BamHI/NotI sites of pGEX4T1 to yield pGEX4T1-E6AP (32). The point mutants listed in Tables 1 and 2 were generated from pGEX4T1-E6AP using the QuikChange protocol of Stratagene to yield the corresponding expression plasmids (58). The coding regions for all clones were sequenced to preclude cloning artifacts and to verify the desired mutation. Recombinant protein was expressed at 16°C in E. coli BL21 (DE3) cells harboring the desired pGEX-E6AP plasmid after induction with 0.4 mm isopropyl-1-thio-β-D-galactopyranoside. The recombinant GST-E6AP fusion proteins were purified from the resulting supernatant as described previously (32, 33) by glutathione-Sepharose affinity chromatography (32). The GST moiety was not processed from recombinant E6AP fusion protein because the GST moiety enhanced the stability of the enzyme (32). As reported previously, the purification protocol consistently yielded ~4 mg of GST-E6AP protein per liter of medium. The activities of GST-E6AP and its mutants were quantitated by stoichiometric $^{125}\text{I}$-ubiquitin thioester formation (32, 82) and compared with protein of the full-length band, estimated densitometrically using BSA as a protein standard. Typically, the E6AP preparations exhibited ~1% active enzyme based on total full-length protein (32, 33).
E6AP-catalyzed $^{125}$I-ubiquitin conjugation assay

The E3 ligase activity of recombinant E6AP was quantitated under initial velocity conditions (31–33). Rates of E6AP-catalyzed $^{125}$I-polyubiquitin chain formation were measured at 37 °C in incubations of 25-μl final volume containing 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 10 mM MgCl₂, 1 mM DTT, 10 mM creatine phosphate, 1 IU of creatine phosphokinase, 5 μM $^{125}$I-ubiquitin ($\sim 1.5 \times 10^8$ cpm/μmol), 50 nM human Uba1, and the indicated concentrations of E2 and E6AP (32, 82, 83). Reactions were initiated by the addition of $^{125}$I-ubiquitin and then quenched after 10 min by the addition of 25 μl of 2× SDS sample buffer containing 0.3% (v/v) β-mercaptoethanol and heating of the samples to 100 °C for 3 min. The $^{125}$I-polyubiquitin conjugates were resolved from free $^{125}$I-ubiquitin by 12% (w/v) SDS-PAGE under reducing conditions at 4 °C and visualized by autoradiography of the dried gels (82, 83). Radioiodinated unanchored polyubiquitin chain formation was measured by exciting stacker lanes and quantitating associated $^{125}$I-ubiquitin by γ-counting (32, 82, 83). Active Uba1, UbcH7, and E6AP were independently determined in parallel for each experiment by their stoichiometric formation of $^{125}$I-ubiquitin thioester (82).

In situ generation and use of E2–$^{125}$I-ubiquitin

In the presence of 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 10 mM MgCl₂, 1 mM DTT, 4 mM Uba1, and 1.7 μM $^{125}$I-rmUb, 107 nM UbcH7 was stoichiometrically loaded to generate UbcH7–$^{125}$I-rmUb. Residual ATP was removed by incubation for 1 min in the presence of 1 IU/ml yeast hexokinase and 8 mM glucose. Inclusion of 2 mM AMP in the incubation removed Uba1–$^{125}$I-rmUb by back-trapping (65, 66). Incubation for an additional 1 min in the presence of 8 IU/ml yeast inorganic pyrophosphatase removed any residual Uba1 thioester without loss of UbcH7–$^{125}$I-rmUb by the same reversal of the activation step (82). The resulting in situ generated UbcH7–$^{125}$I-rmUb could then be added to E6AP to form the corresponding E6AP–$^{125}$I-rmUb thioester. A brief pulse with 2 mM inorganic pyrophosphate removed any residual UbcH7–$^{125}$I-rmUb by back-trapping through the Uba1 present from the initial charging step before depletion by the inorganic pyrophosphatase. In applications involving chase with unlabeled UbcH7–ubiquitin, the intermediate was prepared by an identical sequence of steps, and formation was confirmed by a parallel incubation in which $^{125}$I-ubiquitin was substituted for the unlabeled polypeptide. Depletion of ATP following the charging step was confirmed in parallel incubations for which hexokinase and glucose were added for 1 min before radioiodinated ubiquitin.

In silico docking simulation of Site 1

The sequence of human UbcH7 was built onto the structure of Saccharomyces cerevisiae Ubc1–ubiquitin thioester (PDB entry 1FXT) using Modeler 9v7 (47, 84). The resulting UbcH7–ubiquitin thioester homology model was docked to the Hect domain of E6AP (PDB entry 1CAZ) from which the bound uncharged UbcH7 had been deleted using the online variant of PatchDock (24, 36, 85). To constrain the docking simulation, Arg$^{506}$, Glu$^{507}$, His$^{818}$, and Cys$^{820}$ were defined as receptor-binding site residues, whereas Cys$^{86}$ and Leu$^{121}$ of UbcH7 and Gly$^{75}$ and Gly$^{76}$ of ubiquitin were defined as ligand-binding site residues. The top-scoring docking pose (PatchDock score = 12,346) placed the active site Cys$^{820}$ within −6 Å of the UbcH7–ubiquitin thioester bond (buried interface = 1600 Å²) and was close to the final refined fit shown in Fig. 1. The model was relaxed with GROMACS (86) in a cubic box of explicit water (a 6-Å shell beyond the solvent was used to size the box) using the AMBER03 potential (87) and a TIP3P water model. A particle-mesh Ewald treatment of the electrostatics was used with a dielectric constant of 1.0. After the addition of sodium counterions to neutralize the system, a Broyden-Fletcher-Goldfarb-Shanno minimization algorithm was run for 5000 steps, followed by 500 steps of steepest-descent minimization. Side-chain interactions and buried surface area for the final binding solution were subsequently analyzed using PISA (88).

Structural modeling

Structures were visualized using the PyMOL Molecular Graphics System version 1.8 (Schrödinger, LLC, New York).

Author contributions—The experiments were conceived by V. P. R., E. D. K., and A. L. H.; experiments and data analyses were performed by V. P. R., E. D. K., and J. M. K.; E. D. K. conceived and developed the methodology for in situ E2–$^{125}$I-ubiquitin thioester generation and utilization; in silico docking calculations were performed by C. M. S.; and the manuscript was written by V. P. R., E. D. K., and A. L. H.

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