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Ubiquitin C-terminal electrophiles are activity-based probes for identification and mechanistic study of ubiquitin conjugating machinery

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Abstract

Protein modification by ubiquitin (Ub) and ubiquitin-like modifiers (Ubl) requires the action of activating (E1), conjugating (E2), and ligating (E3) enzymes and is a key step in the specific destruction of proteins. Deubiquitinating enzymes (DUBs) deconjugate substrates modified with Ub/Ubls and recycle Ub inside the cell. Genome mining based on sequence homology to proteins with known function has assigned many enzymes to this pathway without confirmation of either conjugating or DUB activity. Function-dependent methodologies are still the most useful for rapid identification or assessment of biological activity of expressed proteins from cells. Activity-based protein profiling (ABPP) uses chemical probes that are active-site directed for the classification of protein activities in complex mixtures. Here we show that the design and use of an expanded set of Ub-based electrophilic probes allowed us to recover and identify members of each enzyme class in the ubiquitin-proteasome system, including E3 ligases and DUBs with previously unverified activity. We show that epitope-tagged Ub-electrophilic probes can be used as activity-based probes for E3 ligase identification by *in vitro* labeling and activity studies of purified enzymes identified from complex mixtures in cell lysate. Furthermore, the reactivity of our probe with the HECT domain of the E3 Ub ligase ARF-BP1 suggests that multiple cysteines may be in the vicinity of the E2-binding site and are capable of the transfer of Ub to self or to a substrate protein.

Introduction

The addition of ubiquitin (Ub), a 76 amino acid polypeptide, or a ubiquitin-like modifier (Ubl) to proteins via a primary amino group serves to modulate protein function in a variety of ways. Mono- and multi-ubiquitination play a critical role in transcriptional activation(1), as well as receptor internalization and trafficking(2-4), while poly-ubiquitination typically leads to protein degradation by the proteasome and is therefore important for regulation of many cellular functions(5). Dysregulation of Ub/Ubl substrate modification has been implicated in a growing number of human diseases, including cancer(6,7) and neurodegenerative disorders (8-11).

Ub/Ubls are installed on substrate proteins in a complex cascade involving Ub/Ubl-activating enzymes (E1 enzymes), Ub/Ubl-conjugating enzymes (E2 enzymes), and Ub/Ubl-ligating enzymes (E3 enzymes)(12,13). E1 enzymes activate the C-terminus of Ub/Ubl for nucleophilic

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attack via ATP-dependent thioester formation with an active site cysteine. This activated Ub/Ubl intermediate is then transferred from E1 to E2 as a thioester and is finally installed on a recipient lysine by an E3 ligase, which contains a substrate binding region and ultimately confers specificity. The action of the ligation machinery is balanced by the activity of deubiquinating enzymes (DUBs) and Ubl-specific proteases (ULPs) — proteases that remove Ub/Ubl from modified substrates, thereby rescuing proteins from proteasomal degradation (14,15). DUBs/ULPs themselves may be key players in disease pathology, as they are known regulators of the cell cycle machinery and can act as either oncoproteins(16) or tumor suppressors(17,18). The identification of members of this pathway is important for understanding substrate specificity, regulation of the Ub-proteasome system, and ultimately for the development of specific inhibitors for possible use in treatment of disease(19,20).

Activity-based protein profiling (ABPP) has proved an essential complement to *in silico* efforts for the identification of DUBs/ULPs(14). Novel members of this class of proteases were previously identified by Ub-based active site-directed probes(21). The recombinant expression of an epitope-tagged Ub fusion protein allowed the installation of electrophilic groups at the C-terminus via intein-based chemical ligation. Incubation of these first generation probes with cell lysate led to the isolation and identification of 23 known DUBs by MS/MS analysis(21). Additionally, an OTU domain-containing protein was isolated on the basis of probe reactivity and was shown to have DUB-like activity *in vitro*. An ABPP-based approach was also used to identify DUBs and ULPs encoded by both viral(22,23) and bacterial genomes(24,25), as well as eukaryotic pathogens like *Plasmodium falciparum*(26) and *Toxoplasma gondii*(27). This strategy proved essential for the identification of the M48^{USP} — a DUB within the N-terminal 500 residues of the large tegument protein of the herpes simplex virus 1 (HSV1), which bears no homology to any known host DUBs(28). UbVME was confirmed to be a mechanism-based suicide inhibitor as it was crystallized covalently bound to the active site cysteine of UCH-L3 (29) and M48(28).

Since earlier campaigns using the C-terminal electrophilic probes identified no more than half of the ~100 proposed mammalian DUBs (21,30,31), we postulated that incorporation of “warheads” with greater electrophilicity should retrieve a larger number of DUBs. Here we report the synthesis and reactivity of an expanded set of Ub-based chemical probes. Enzymes modified by the probes were isolated from whole cell lysate and identified by tandem mass spectrometry. While several new DUBs were recovered, including two members of the Josephin-domain subfamily (JOS1 and JOS2)(32), we were surprised to recover numerous members of the Ub/Ubl conjugating machinery including a gene product containing a HECT (homologous to E6-AP carboxy terminus) domain, but with no reported ligase activity. Previously, only recombinantly expressed and purified E1, E2 or E3 enzymes had shown any reactivity with electrophilic Ub derivatives(21,33). One E3 ligase we identified, ARF-BP1, was particularly intriguing due to recent interest in one of its target proteins, Mcl-1, as a potential tumor marker(34). Further characterization of the binding interaction between hemagglutinin (HA)-tagged UbVME and the HECT domain of ARF-BP1 revealed that the probe modified cysteines present in this domain. Mutagenesis of probe-labeled cysteines indicates that several residues in the HECT domain may participate in the transfer of Ub to self or substrate proteins, with, as expected, a dominant role in the reaction for the catalytic cysteine.

Results and Discussion

Synthesis and Characterization of Second Generation HAUb-Electrophilic Probes

Known Ub-based active site-directed probes were synthesized as previously reported (Figure 1A)(21). Three new glycine-based electrophiles were synthesized (see the Supporting Information available with this article online for schemes of chemical syntheses and compound characterization) and installed using an intein-based chemical ligation on a recombinantly

expressed HAUb lacking the C-terminal glycine (G76). The product of each ligation reaction was purified using cation-exchange chromatography and characterized by mass spectrometry (LC-ESI-MS, see Supporting Information). These second generation glycine derivatives, including a vinylthioethersulfone (OEtVS), a β -lactone (Lac) and a 2,6-trifluoromethylbenzylloxymethylketone (TF₃BOK) were chosen for increased electrophilicity as compared to compounds synthesized earlier(21), and the likelihood to retain specificity for cysteine over serine proteases in reactions with complex mixtures of proteins(35). All three HAUb probes should react at the position corresponding to the C-terminal carbonyl group of the G76, as this is the position of conjugation between Ub and peptide substrates. HAUbVME, which is the most reactive first generation probe in retrieval of diverse DUBs, was used as a comparison for probe reactivity.

Profiling and Identification of Enzymes Modified by Newly Synthesized HAUb-Electrophilic Probes in EL-4 and HMLE Cell Lysate

To identify potentially uncharacterized DUBs using an ABPP approach, our new set of probes was used to label whole-cell lysate. The EL-4 mouse thymoma cell line was initially chosen (21) since it is known to express a diverse set of DUBs/ULPs(36), and was selected for sake of comparison with previous ABPP experiments. For identification of human orthologs of particular DUBs/ULPs reactive with our HAUb electrophilic probes, we chose the immortalized human mammary epithelial cell line (HMLE)(37). Cell lysate was incubated with HAUb-derived probes and enzymes modified were visualized by anti-HA immunoblotting (Figure 2). Titrations and time courses were conducted to determine the optimal concentrations of each probe and the optimal time for labeling enzymes present in EL-4 cell lysate (data not shown). Significant enrichment of probe-reactive material was observed by immunoblotting of samples incubated with 0.2 μ g of each HAUb electrophile per 30 μ g cell lysate for more than 3h. Extended reaction times or increased quantities of probe beyond 0.2 μ g per 30 μ g cell lysate, however, did not affect the number of distinct polypeptides retrieved. HAUb-derived probes have different activities and specificities using otherwise identical labeling conditions and probe concentrations (Figure 2). Furthermore, the pattern of labeled DUBs/ULPs differs between the two cell types — as expected, EL-4 and HMLE cells contain diverse and differentially expressed DUBs/ULPs. In all cases, labeling could be blocked by preincubation of lysate with the alkylating agent N-ethylmaleimide (NEM) (Supplementary Figure 1), consistent with active site cysteine modification by the HAUb-derived probes. The increased electrophilicity of our newly synthesized probes did not translate into greater reactivity with proteins in cell lysates — the first generation HAUbVME probe was still the most broadly reactive (Figure 2). The increased chemical reactivity of these newly synthesized probes likely means they are also more quickly hydrolyzed during the labeling reaction, which may explain their seemingly lower reactivity with cell lysates.

To isolate the proteins modified by each of the HAUb probes, we immunoprecipitated labeled proteins from either EL-4 or HMLE cell lysate using agarose-conjugated anti-HA antibody as described(21). Precipitated proteins were separated by reducing SDS-PAGE (10%) and visualized by silver staining (data not shown). Polypeptides were excised from the gel, trypsinized and analyzed by MS/MS. MS/MS data were subjected to database searches using the US NCBI expressed sequence tag (EST) databases. Proteins identified were corrected for nonspecific interactions with the antibody and agarose against untreated, immunoprecipitated cell lysate using the MScomp program(38). 29 DUBs were identified from EL-4 cell extracts and 23 DUBs and one NEDD8-specific ULP were identified from HMLE cell extracts (see Supplementary Table 1).

We observed selective reactivity with DUBs for each HAUb-electrophilic probe, with HAUbVME being the most diversely reactive probe. HAUbTF₃BOK was the next most DUB-

reactive probe and showed significant overlap of labeled species with HAUbVME in EL-4 lysate. Ubiquitin C-terminal hydrolases, including UCH-L1, -L3, and -L4 displayed broad reactivity with all probes in EL-4 lysate, while UCH-L3 and -L5 had similar reactivity in HMLE extracts. Several DUBs not previously identified using this ABPP approach were recovered: Otubain-domain containing DUB Cezanne 2 (OTUD-7A) and U2af1-rs1, an uncharacterized peptidase C19 family member with putative DUB activity toward polyubiquitinated peptides, were identified in EL-4 cell lysate using HAUbVME and HAUbLac respectively. Otubain-domain containing DUBs OTUD4 (isoform 3) and OTUD5 were identified from HMLE extracts using HAUbVME, as were Josephin domain (JD)-containing DUBs JOS1 and JOS2 (32). Josephin-type DUBs represent a new class of DUBs to be identified using a chemical biology approach. Finally, the identification of one ULP, SENP8 — a NEDD8 specific protease, in HMLE extracts using HAUbVME was not surprising considering the fact that Ub and NEDD8 contain the same 4 C-terminal residues and share ~60% sequence identity(39). Reactivity of HAUbVME toward a different subset of DUBs, as compared to those identified previously, could be attributed to either altered DUB expression particular to this EL-4 cell culture, or to the longer incubation times with probe and increased probe concentrations used here, which allows the retrieval of enzymes that contain more weakly nucleophilic active site cysteines.

Recovery of diverse members of the Ub/Ubl conjugation machinery was also observed (Table 1). Since previous experiments failed to retrieve any such enzymes from cell lysate, this observation was surprising, but not altogether unexpected — most Ub/Ubl conjugating enzymes also contain an active site cysteine that participates in the conjugation of these modifiers to substrate peptides. Ub/Ubl installation on substrate lysines via the E1 to E2 to E3 thioester cascade hinges on reversible interaction of the active site cysteines in these enzymes with the Ub/Ubl C terminus. In fact, the pK_a of the active site cysteine residue of several E2 enzymes is approximately 2 pH units above that of a free cysteine, likely preventing these residues from reacting non-specifically with other thiol-reactive groups in the cell(40). The increased potency of the newly synthesized HAUb probes should encourage labeling of more weakly nucleophilic cysteine residues; indeed, HAUbTF₃BOK was the most reactive probe toward these types of proteins. Representative members of all three classes of conjugating enzymes, including E1s and E2s for both Ub and Ubls and both HECT and RING domain E3 Ub ligases, were identified in EL-4 and HMLE lysate. One protein we recovered, Trip 12, contains both WWE and HECT domains characteristic of a Ub E3 ligase, but its activity as such had not been verified either *in vivo* or *in vitro*. We expressed the HECT domain of Trip12 (expressed as a GST fusion to improve solubility) and confirmed that it has autoubiquitination activity that is E1/E2 dependent (Supplementary Figure 2)(41,42).

While active site cysteines capable of being modified by HAUb-electrophilic probes do exist in most classes of conjugating enzymes, some proteins might be retrieved as part of larger multi-protein complexes containing only a single probe-reactive species. This may apply in particular to RING ligases, which do not utilize an active site cysteine, but rather activate Ub/Ubls using two zinc ions coordinated in the active site by eight conserved cysteine and histidine residues(13). For example, the RING ligases containing CUL-4A and CUL-4B consist of a multi-subunit complex containing the cullin scaffold with C-terminally associated substrate binding proteins and an N-terminally associated RING-domain protein, which recruits the E2-conjugating enzyme to form the active ligase complex(43). While the cullin scaffold itself is not capable of reacting with Ub-based electrophiles, reaction of the E2 protein with probe could lead to recovery of these associated scaffold proteins in our immunoprecipitations as performed using non-denaturing conditions. Indeed, this is the case for one example RING E3, UBAC1, identified in EL-4 lysate by immunoblotting, but unreactive with HAUbVME as indicated by the presence of a single unmodified species following incubation with probe (Supplementary Figure 3). UBC9 and other Ubl-conjugating enzymes may be retrieved either due to cross

reactivity with Ub(44) under the *in vitro* labeling conditions, or as part of a complex with its interacting E1, which in the case of UBC9 was also recovered from EL-4 lysate (Aos1).

HAUb-Electrophilic probes are activity-based probes for Ub E3 ligases

After demonstrating the capability of our panel of probes to identify Ub conjugating machinery, we were interested in validating the utility of these proteins for mechanistic study of purified enzymes. The HECT Ub E3 ligase that was recovered with the most diverse panel of electrophilic probes using both types of cell lysate was the protein ARF-BP1. ARF-BP1 (Mule) is a 482 kD HECT-domain-containing Ub E3 ligase that has several known substrates: p53 (45), Cdc6(46), Mcl-1(47,48), N-Myc(49), C-Myc(50), TopBP1(51), and histones(52). ARF-BP1 is a key player in both p53-dependent and independent functions of the ARF tumor suppressor(45) and regulates DNA damage-induced apoptosis(46-48). ARF-BP1 is interesting not only because its substrates function in multiple pathways, but also because a catalytic mutant of ARF-BP1 in which the active site cysteine is mutated to alanine (C4341A) or serine (C4341S)(50) reduces, but does not abolish ligase activity. We therefore selected this protein for verification of reactivity with HAUbVME and confirmation of enzyme activity as an E3 Ub ligase.

To verify that HAUbVME does indeed bind to the active site-cysteine of ARF-BP1, we incubated HAUbVME for three hours with recombinantly expressed ARF-BP1 HECT domain (residues 4012-4374). The reaction mixture was separated by reducing 10% SDS-PAGE and visualized by coomassie stain. We observed multiple species, each corresponding to covalent modification of ARF-BP1 (Figure 3, panel A) which appear to occur only in the context of a folded HECT domain — modification of ARF-BP1 by HAUbVME following denaturation in 6.4 M urea is nearly abolished. In contrast, incubation of recombinant DUB enzymes with HAUbVME invariably produces a single modified species (53). We identified the nature of the modified species using MS/MS analysis of polypeptides excised from the gel. Three out of the six cysteines within the expressed HECT domain were modified by HAUbVME: C4099, C4341, and C4367 (hereafter called Cys 1, Cys 5, and Cys 6 in reference to their sequential position from the N terminus of the HECT domain). The modification of Cys 5 was expected, as it was identified as the likely catalytic residue based on multiple sequence alignment (54). We reasoned that the modification of the other two residues should correlate with the ability of these cysteines to contribute to product formation. To investigate this possibility, we expressed and purified ARF-BP1 HECT domain mutants (residues 4012-4374) in which cysteines 1, 5, and 6 were mutated to alanine either singly or in combination. We then tested the activity of these proteins *in vitro* using one of two assays: autoubiquitination, or ubiquitination of the anti-apoptotic protein Mcl-1, a substrate of ARF-BP1 (54).

When wild-type ARF-BP1 is incubated for ten minutes at room temperature in the presence of recombinant E1, the E2 Ubch7, [³²P]-Ub, and an ATP-regenerating system, the E3 efficiently catalyzes polyubiquitination of itself (Figure 3, panel B). Following separation of reaction mixture on 10% SDS-PAGE, ubiquitinated products are quantified using phosphoimaging (Figure 3, panel D). Mutation of the catalytic cysteine (Cys 5) to alanine greatly reduces activity but does not abolish it; the mutant forms 8.2% of wild-type product after a 10 minute reaction. Mutation of Cys 6 reduces product formation in the autoubiquitination assay to 56.27% of the wild type level. Finally, the mutation of Cys 1 alone does not affect activity. The mutation of Cys 1 in combination with either Cys 5 or Cys 6 does dramatically alter autoubiquitination activity: in combination with a Cys 6 mutation, activity is restored; in the context of a Cys 5 mutation, ARF-BP1 fails to ubiquitinate itself. Finally, mutation of all three probe-labeled cysteines (1, 5, and 6) abolishes all activity.

Although we cannot exclude that these cysteine mutations affect binding to Ub or Ubch7, these results suggest that mutation of probe-labeled cysteines affects activity of the ARF-BP1 HECT

domain. We next asked whether this holds true for ARF-BP1-catalyzed ubiquitination of its substrate protein Mcl-1. In this case, ARF-BP1 is incubated with recombinant E1, the E2 Ubch7, Ub, an ATP-regenerating system, and [32 P]-Mcl-1 for one hour at 37°C as described (47). Reaction mixtures are separated on SDS-PAGE and quantified as described above. Wild-type ARF-BP1 catalyzes multi-ubiquitination of Mcl-1 as reported (Figure 1, panel C). We observed that the cysteine-to-alanine mutants of ARF-BP1 show a similar pattern of reduced activity as seen in the autoubiquitination assay; however, the relative contributions of cysteines 5 and 6 toward total activity differ in this assay as compared to the autoubiquitination assay. As in the autoubiquitination assay, mutation of Cys 1 has no effect on activity toward Mcl-1. More significantly, mutation of either Cys 5 or Cys 6 reduces activity to a similar extent (30 % and 40.7 %, respectively) after a 1 h incubation, suggesting that both of these residues participate in ubiquitination of Mcl-1. The pattern of activity of the double mutants in the autoubiquitination assay resembles the pattern for substrate activity: the 1,6 mutant resembles wild-type ARF-BP1, whereas the 1,5 mutant is inactive. Again, the triple mutant lacking cysteines 1, 5, and 6 shows no activity.

Multiple Cysteines in ARF-BP1 form Thioesters with Ub

The mechanism of catalysis by HECT E3 ligases involves several steps. The E3 enzyme binds Ub-loaded E2, followed by Ub transfer from the E2 catalytic cysteine to the E3 catalytic cysteine. The E3 then catalyzes isopeptide bond formation between Ub and a recipient lysine residue on substrate, which may be the E3 itself, Ub, or another protein. A crucial determinant in isopeptide bond formation is the presence of a conserved phenylalanine located four amino acids from the C terminus of most HECT E3s (55). Truncations which remove this residue abolish Ub transfer to substrate, trapping the E3 with the catalytic cysteine in a thioester linkage to Ub (55). We reasoned that if more than one cysteine is capable of receiving Ub from the associated E2, we should be able to detect the presence of multiple Ub-thioesters in an ARF-BP1 HECT domain truncation mutant (ARF-BP1 Δ 4) lacking the conserved phenylalanine.

We therefore expressed and purified two versions of the ARF-BP1 HECT domain lacking the terminal four amino acids: wild-type (WT Δ 4) and a Cys5 to Ala mutant (C5A Δ 4) and assayed these mutants, along with the full-length ARF-BP1 HECT domain (WT FL) for Ub thioester formation. In this assay, ARF-BP1 is incubated with recombinant E1, the E2 Ubch7, Ub, and an ATP regenerating system for 10 minutes at room temperature. The reaction is quenched by urea denaturation followed by addition of SDS-PAGE loading buffer with or without β -mercaptoethanol, separated on 10% SDS-PAGE, and analyzed by anti-Ub immunoblot. Under these conditions, WT FL ARF-BP1 efficiently catalyzes autoubiquitination (Figure 4, panel A). In contrast, WT Δ 4 ARF-BP1 forms a mono-ubiquitylated species when the reaction is quenched with reducing sample buffer, indicating that the activity of this protein is compromised by the truncation. The ability of the WT Δ 4 ARF-BP1 to form a monoubiquitylated species depends on Cys 5 (Figure 4). Interestingly, quenching the reaction with non-reducing sample buffer preserves multiple Ub-thioesters on WT Δ 4 ARF-BP1. We detect the persistence of a single Ub-thioester in the C5A Δ 4 ARF-BP1 mutant, which is likely due to Ub binding to a cysteine in the HECT domain. The apparent thioester formation catalyzed by both WT Δ 4 and C5A Δ 4 ARF-BP1 is E1/E2 dependent (Figure 4, panel B). These results indicate that at least two cysteines in ARF-BP1 simultaneously can form Ub-thioesters and that one of these cysteines is Cys 5.

We predicted that the Δ 4 truncation would abolish the ability of ARF-BP1 to ubiquitinate Mcl-1, and indeed we find this to be the case (Figure 4, panel C). WT Δ 4 ARF-BP1 catalyzes monoubiquitylation of Mcl-1 in a manner dependent on Cys 5 and the presence of E1 and E2 enzymes (Figure 4, panel D). As this small amount of ligase activity persists after complete denaturation of samples in 4 M urea, it must correspond to covalent lysine modification.

Given that our probes were designed to act as electrophiles capable of reaction with local nucleophiles following specific binding by Ub-reactive proteins, it is likely that the probe's reactivity with cysteines other than the catalytic residue means that these residues are close in proximity to the electrophilic warhead within the tertiary structure of the ARF-BP1 HECT domain. We proceeded to construct a threaded structure based on the structures available for the HECT domains of WWP1(56) and SMURF2(57), the HECT domains with highest homology to ARF-BP1 (> 40% identity, data not shown). In our threaded structure, Cys 6 appears to be equidistant to Cys 5 in proximity to the E2 binding site and all three reactive cysteines are along a single face of the domain. We next ask if these three residues from the human ARF-BP1 sequence are conserved across eukaryotes (Figure 5). As expected, Cys 5 is conserved across all species examined, and Cys 6 is conserved within the animal kingdom. Cys 1, the most weakly reactive residue toward our Ub-based electrophiles, is conserved only through bony fishes (*Danio rerio*), but is also observed in *Hydra*. Altogether, these data link the ability of our probes to identify residues with the potential to react with Ub, either due to catalytic activity or proximity.

Conclusions

The ubiquitin-proteasome system is critical for the regulation of protein function in eukaryotes. Here we extend an earlier effort to allow the isolation and identification of members of all enzyme classes that comprise the ubiquitin-proteasome system. While we continue to find novel enzymes of the DUB family, we have also demonstrated the utility of Ub-based electrophiles as activity-based probes for certain HECT domain E3 ligases. In studying the covalent modification of the HECT domain of E3 ligase ARF-BP1 with HAUbVME, we observed that the probe covalently labels three out of the six cysteines found in the ARF-BP1 HECT domain (C4099 (Cys 1), C4341 (the canonical catalytic residue, Cys 5), and C4367 (Cys 6)). We provide evidence for the formation of multiple thioester-linked Ub molecules during catalysis by wild-type ARF-BP1 HECT domain, supporting the notion that the HAUbVME probe is capable of identifying residues that are competent nucleophiles for Ub transfer. We are eager to see our hypotheses about the positioning of nucleophilic residues in the ARF-BP1 HECT domain confirmed with structural data. The utility of Ub electrophilic probes as mechanism-based chemical tools to study E3 ligase structure and function will likely provide further insights into the mechanism employed by ARF-BP1 and other HECT E3 Ub ligases.

Methods

Synthesis and Purification of HAUb-electrophilic Probes

Electrophilic glycine analogs 4-amino but-1-enyl ethoxysulfonate, serine- β -lactone, and 2,6-trifluoromethylbenzyloxy glycine methyl ketone were synthesized according to literature procedures(58-60) (see Supporting Information for synthesis details and compound characterization). HAUb₇₅-MESNa (500 μ L, ~10-15 mg/mL), synthesized as previously reported(21) from an Ub-intein-chitin domain fusion protein, was treated with 1 mM N-hydroxysuccinimide, immediately followed by 0.5 mM of the desired electrophilic glycine analog in 1.1 mL (total volume) of a 1 M NaHCO₃ (pH 8.0) solution. These conjugation reactions were incubated overnight at 37°C. The resulting products were dialyzed and purified as previously reported(21). Each purified HAUb-electrophilic probe was characterized by LC/MS using a Michrom Paradigm MS4 HPLC equipped with a Waters Symmetry C8 2.1 mm $\times$ 50 mm column using standard reverse-phase gradients. The effluent from the column was mass analyzed using a Waters LCT Electrospray Time-OF-Flight Mass Spectrometer (see Supporting Information for MS characterization).

Preparation of EL-4 and HMLE Cell Lysate and Labeling with HAUb-electrophilic Probes

EL-4 cells (cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO₂) were harvested, washed once with culture media and once with PBS. Cell pellets were lysed with glass beads as previously reported(21). 30 µg of protein extract was incubated with 0.2 µg of each HAUb-electrophilic probe (2 µM, 10 µL total volume per sample) for 5 h at room temperature. After terminating the reactions with reducing SDS-PAGE sample buffer and boiling 10 min, reaction mixtures were separated by SDS-PAGE (10%) and analyzed by immunoblotting with anti-HA antibody (3F10, Roche).

The immortalized, nontransformed HMLE line, expressing the SV40 large-T oncogene, and hTERT, was cultured as previously described(37) using a 2:1:1 mixture of mammary epithelial growth medium (MEGM, Clonetics), DMEM, and F12 medium supplemented with EGF (10 ng/ml), insulin (10 µg/ml), and hydrocortisone (1 µg/ml). Cells were harvested at a confluence of 90% and washed once with PBS. Cell pellets were lysed with glass beads as previously reported(21). 7.5 mg of cell lysate were incubated with 1 µg of each HAUb-electrophilic probe (0.2 µM) 5 h at room temperature. A sample of labeled proteins (30 µg protein extract) were separated by SDS-PAGE (8%) and analyzed by immunoblotting with anti-HA antibody (3F10, Roche).

Anti-HA Immunoprecipitation of Labeled Proteins and Identification by Tandem Mass Spectrometry

EL-4 and HMLE lysate were prepared as above. 7 mg EL-4 protein extract or 7.5 mg HMLE protein extract were incubated with 1 µg (0.2 µM) of each HAUb-electrophilic probe for 5 h at room temperature in 50 mM Tris (pH 8.0), 150 mM NaCl. Anti-HA agarose (3F10 antibody, Roche) was incubated with the samples overnight at 4°C. The immunoprecipitations were washed extensively with NET buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% NP40) and the bound proteins were eluted by boiling for 10 min with reducing SDS-PAGE sample buffer, separated by SDS-PAGE (10%), and visualized by silver stain using standard conditions. Bands were excised, reduced, alkylated and digested with trypsin at 37°C overnight. The resulting peptides were extracted, concentrated and injected onto a Waters NanoAcquity HPLC equipped with a self-packed Jupiter 3 µm C18 analytical column (0.075 mm by 10 cm, Phenomenex). Peptides were eluted using standard reverse-phase gradients. The effluent from the column was analyzed using a Thermo LQT linear ion trap mass spectrometer (nanospray configuration) operated in a data dependent manner. The resulting fragmentation spectra were correlated against the known database using SEQUEST. Bioworks browser was used to provide consensus reports of the proteins identified.

ARF-BP1 Labeling with HAUbVME

Recombinantly expressed and purified ARF-BP1 HECT domain (5 µg) was incubated with HAUbVME (2.0 µM) in 50 mM Tris, 150 mM NaCl, pH 8.0 for 3h at room temperature. The specificity of probe labeling was tested by first denaturing the ARF-BP1 HECT domain with 6.4 M urea (30 min incubation at 30°C) and then diluting the sample to 0.6 M urea before the addition of HAUbVME (2.0 µM). Activity of HAUbVME was tested with ARF-BP1 in 50 mM Tris, 150 mM NaCl, pH 8.0 containing 0.6 M urea to ensure that probe reactivity was not compromised in dilute concentrations of urea. All of the above labeling reactions were quenched by addition of reducing sample buffer, boiled for 10 min and separated by SDS-PAGE (10%). Labeled and unlabeled species were visualized by coomassie staining and bands were excised and treated as above for MS/MS analysis. Modified cysteine residues were identified by the presence of the mass change corresponding to the C-terminus of the VME probe (= 172.08 Da).

ARF-BP1 Autoubiquitination and Substrate Ubiquitination Assays with Wild-type and Mutant HECT Domain proteins

Autoubiquitination activity was tested in a 20 μ L reaction by incubating ARF-BP1 HECT domain (10 μ g) with 100 ng human E1 (Ube1, Boston Biochem), 1 μ g Ubch7, and 10 μ g [32 P]-Ub (see Supporting Information for plasmid construction, protein expression and purification conditions, and [32 P]-labeling) with an ATP regenerating system (50 mM Tris [pH 7.6], 5 mM MgCl₂, 5 mM ATP, 10 mM creatine phosphate, 3.5 U/mL creatine kinase) for 10 min at room temperature. Substrate ubiquitination activity was tested by adding 1 μ g [32 P]-Mcl-1 (see Supporting Information for plasmid construction, protein expression and purification conditions, and [32 P]-labeling) to the above reaction containing 100 μ g non-radiolabeled Ub and incubating the mixture for 1 h at 37°C. A control reaction was run without ARF-BP1 HECT domain, but containing all other reaction components for each case. After terminating the reactions with reducing SDS-PAGE sample buffer and boiling 10 min, reaction mixtures were separated by SDS-PAGE (10%) and analyzed by phosphorimaging. Product formation by ARF-BP1 HECT domain mutants was calculated as a percentage of total (background corrected) [32 P] counts attributable to ubiquitinated product in the wild type HECT domain. The average and standard deviation of 9 experiments is shown.

Ub-thioester assays

To test ARF-BP1 thioester formation, reaction mixtures (10 μ L) were set up in duplicate containing 100 ng human E1 (Ube1, Boston Biochem), 1 μ g Ubch7, 10 μ g ARF-BP1 Δ 4 HECT domain, 10 μ g Ub, 50 μ M DTT, and an ATP regenerating system, and incubated for 10 min at room temperature. Reactions were terminated with 10 μ L 8M urea and incubated 15 min at 30°C. One set of samples received reducing SDS-PAGE sample buffer, the other set received non-reducing SDS-PAGE sample buffer, and were then boiled 10 min, separated on 10% SDS-PAGE, and analyzed by immunoblotting with anti-Ub antibody (Sigma). To test the activity of the ARF-BP1 Δ 4 proteins against substrate, reactions were set up as above except with 100 μ g Ub and 1 μ g Flag-Mcl-1 and were incubated 1 hr at 37°C. Reactions were terminated as above, separated on 10% SDS-PAGE, and analyzed by immunoblotting with anti-Flag antibody (Sigma).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ABPP approach for identifying DUBs, Ub/Ubl activating enzymes, conjugating enzymes and ligases. (a) Synthesis of HA-Ub-derived probes using an intein-based chemical ligation. Recombinant HA-tagged Ub was expressed as a C-terminal fusion with an intein-chitin binding domain (CBD) for purification and introduction of reactive groups. Addition of β -mercaptoethane sulfonic acid (MESNa) to the chitin agarose (CA) with bound HA-Ub fusion protein results in elution of a species containing a thioester capable of chemical ligation. (b) A table of new glycine-based electrophiles synthesized for attachment to the C-terminus of HA-Ub. HA-UbVME, a first generation probe, was used for comparison of second generation probe reactivity. Figure adapted with permission from *Nature Chemical Biology*, ref. 14.

Figure 2.

Different HAUb-derived probes show distinct activities and labeling profiles. (a) EL4 cell lysates (30 μ g) were incubated with 0.2 μ g of each HAUb (2 μ M) probe for 5 h at room temperature (22°C). The labeled proteins were resolved by SDS-PAGE (10%) under reducing conditions and immunoblotted with anti-HA antibody. The HAUbVME probe is the most reactive as visualized by labeling intensity in this immunoblot. Labeling is not observed using HAUbLac at the concentration used. (b) HMLE cell lysates (7.5 mg) were incubated with 1 μ g of each HAUb probe (0.2 μ M) for 5 h at room temperature (22°C). A sample of labeled proteins (30 μ g protein extract) were resolved by SDS-PAGE (8%) under reducing conditions and immunoblotted with anti-HA antibody. The amount of sample loaded onto the gel and the exposure times of this immunoblot were varied to account for differences in the reactivity and decomposition of the C-terminal electrophiles in each Ub-based probe.

Figure 3.

Biochemical analysis of probe-labeled cysteines in ARF-BP1. (a) Incubation of HAUbVME with recombinant ARF-BP1 HECT domain covalently labels multiple cysteines as identified using tandem M/S analysis. 5 μ g recombinant ARF-BP1 HECT domain was incubated with 2.0 μ M HAUbVME for 3 h at 22°C. Labeled species were resolved by 10% SDS-PAGE and detected by coomassie staining. The labeled peptides were excised from the gel, trypsinized, and subject to MS/MS analysis. Residues C4099 (Cys 1), C4341 (Cys 5), and C4367 (Cys 6) were modified by probe. Probe labeling is specific for these sites, as HECT domain denatured in 6.4 M urea for 30 minutes at 30°C and then diluted with reaction buffer to 0.6 M urea before probe addition no longer efficiently binds HAUbVME. ARF-BP1 HECT domain and

HAUbVME retain activity when incubated initially with 0.6 M urea. Ub ligase activity of wild-type (wt) or cysteine-to-alanine mutant ARF-BP1 HECT domains was assayed using (b) [^{32}P]-Ub for autoubiquitination or (c) [^{32}P]-Mcl-1 for substrate ubiquitination. Reaction components contained recombinant UBE1, UbcH7, an ATP regenerating system, and either no ligase (N), WT, or a mutant ligase as indicated. Ligation reaction mixtures were separated by 10% SDS-PAGE and visualized by phosphorimaging. (d) Product formation by wild type or mutant ARF-BP1 in the autoubiquitination assay or Mcl-1 ubiquitination assay was calculated by averaging the percent wild-type activity quantified using phosphorimaging data from nine independent experiments.

Figure 4.

Detection of Ub-thioesters in ARF-BP1 HECT domain. (a) Ub-thioester assay with the indicated ARF-BP1 HECT domain proteins. Purified ARF-BP1 HECT domain proteins were incubated with recombinant E1 and E2 (UbcH7), an ATP regenerating system, and Ub. Reactions were stopped with 4 M urea and either reducing (left panel) or non-reducing (right panel) SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed by immunoblot with anti-Ub antibody. (b) Ub thioester assay with the indicated ARF-BP1 truncation proteins was performed with all necessary components; without UBE1; or without UbcH7. (c) Substrate ubiquitination assay using the indicated ARF-BP1 HECT domain proteins and recombinant Mcl-1 as substrate. Reactions contained recombinant UBE1, UbcH7, Ub, an ATP regenerating system, and 1 μ g recombinant Flag-labeled Mcl-1. Reaction mixtures were quenched as above, separated on SDS-PAGE and analyzed by anti-Flag immunoblot. (d) Substrate ubiquitination assay using the indicated ARF-BP1 HECT domain with all components; no UBE1; and no UbcH7.

				*						*					*		
Homo sapiens	4086	DRV	TYT	INP	SSH	CNP	NHLS	YFK	4108...4328	DR	STD	RLPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGFGLA	4374				
Macaca mulatta	4069	DRV	TYT	INP	SSH	CNP	NHLS	YFK	4091...4111	DR	STD	RLPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGFGLA	4357				
Bos Taurus	4087	DRV	TYT	INP	SSH	CNP	NHLS	YFK	4109...4329	DR	STD	RLPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGFGLA	4375				
Canis familiaris	4087	DRV	TYT	INP	SSH	CNP	NHLS	YFK	4109...4329	DR	STD	RLPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGFGLA	4375				
Mus musculus	4090	DRV	TYT	INP	SSH	CNP	NHLS	YFK	4112...4332	DR	STD	RLPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGFGLA	4378				
Danio rerio	4072	DRV	TYT	INP	SSH	CNP	NHLS	YFK	4094...4114	DR	STD	RLPSAHTCFNQLDLPAYESFEKLRHMLLLAIQECSEGFGLA	4360				
Tribolium castaneum	3661	DRV	TYM	INS	AHY	PNP	NHLS	LCYK	3683...3903	DR	STD	RLPSAHTCFNQLDLPVYETDYLRSYLLKAIHECSEGFGLA	3949				
Drosophila melanogaster	4858	DRV	TYM	INS	PNP	SH	NHLS	YFK	4880...5100	DR	STD	RLPCAHTCFNQLDLPVYETDYLRSYLLKAIHECSEGFGLA	5146				
Hydra magnipapillata	2002	EK	STY	LP	NQ	SH	CNP	NHLS	YFK	2024...2194	DR	STD	RLPCAHTCFNQLDLPAYETDYLRSYLLKAIHECSEGFGLA	2240			
Aspergillus nidulans	3735	DR	TFH	PN	RL	SG	VN	PEHLM	FFK	3757...3976	YGN	KDR	LPSSHTCFNQLDLPAYETDYLRSYLLKAIHECSEGFGLA	4022			
Pichia guilliermondii	2987	DE	TFH	PN	RL	SG	VN	PEHLM	FFK	3009...3228	YGT	DR	LPSSHTCFNQLDLPAYETDYLRSYLLKAIHECSEGFGLA	3274			

Figure 5.

Multiple sequence alignment of ARF-BP1 HECT domain. Sequence conservation of ARF-BP1 HECT domain across diverse eukaryotes. The region depicted represents two portions of ARF-BP1 C-terminus and includes Cys 1 (C4099), Cys 5 (C4341), and Cys 6 (C4367), each marked above with an asterik. Residues depicted in red are small or hydrophobic, those depicted in blue are acidic, those depicted in magenta are basic, and the remainders are depicted in green. Alignment made with Clustal W(61).

Table 1

Ub/Ubl activating enzymes (E1s), conjugating enzymes (E2s) and ligases (E3s) identified from (a) mouse lymphoma (EL4) and (b) human mammary epithelial cell lysates (HMLE).

A.				
Protein identified	Protein activity	Accession number (NCBI)	Predicted MW (kDa)	HAUb probe used for retrieval
UBE1	E1 Ub-activating enzyme	267190	118	TF ₃ BOK
UBE1-L2	E1 Ub-activating enzyme	27370032	118	TF ₃ BOK
UBE1 (Chromosome X)	E1 Ub-activating enzyme	148878383	118	TF ₃ BOK
UBA2	E1 Ubl-activating enzyme	32493410	70.5	TF ₃ BOK
Aos1	E1 Ubl-activating enzyme	18490720	38.5	TF ₃ BOK
UBC9	E2 Ubl-conjugating enzyme	54039792	18	TF ₃ BOK
UBC12	E2 Ubl-conjugating enzyme	46577656	21	TF ₃ BOK
UBC13	E2 Ub-conjugating enzyme	18017605	17	TF ₃ BOK
UB2V1	E2 Ub-conjugating enzyme	51702141	16	TF ₃ BOK
UB2V2	E2 Ub-conjugating enzyme	51702142	16	TF ₃ BOK
UBE2-L3 (UbcM4)	E2 Ub-conjugating enzyme	54039806	18	TF ₃ BOK
UBE2S	E2 Ub-conjugating enzyme	53734650	24	TF ₃ BOK
UBE2O	E2 Ub-conjugating enzyme	50234896	141	TF ₃ BOK
E6-AP (Ube3A)	HECT E3 Ub ligase	76880500	97.5	VME
Nedd4 (Rsp5)	HECT E3 Ub ligase	32172436	103	OEtVS, TF ₃ BOK
ARF-BP1 (Mule)	HECT E3 Ub ligase	73915354	482	VME, Lac, TF ₃ BOK
Trip12	Putative HECT E3 Ub ligase	91932791	224	VME, TF ₃ BOK
UBAC1 (KPC2)	RING E3 Ub ligase	19527050	45	VME
UBR2	RING E3 Ub ligase	73622074	199	TF ₃ BOK
CUL-4A	RING E3 Ub ligase	108936014	88	OEtVS
TRAF2	RING E3 Ub ligase	83921633	56	OEtVS
B.				
Protein identified	Protein activity	Accession number (NCBI)	Predicted MW (kDa)	HAUb probe used for retrieval
UBE1	E1 Ub-activating enzyme	24418865	117.8	VME, OEtVS, TF ₃ BOK
UBC1	E2 Ub-conjugating enzyme	46577658	22.4	VME, Lac, TF ₃ BOK
UB2E2	E2 Ub-conjugating enzyme	47606201	22.2	VME, Lac, TF ₃ BOK
UBE2N	E2 Ub-conjugating enzyme	46577660	17.1	VME, Lac, TF ₃ BOK

B.

Protein identified	Protein activity	Accession number (NCBI)	Predicted MW (kDa)	HAUb probe used for retrieval
UBE2S	E2 Ub-conjugating enzyme	23829978	23.8	TF ₃ BOK
UBE2C (isoform 4)	E2 Ub-conjugating enzyme	32967289	46.4	TF ₃ BOK
UBE2T	E2 Ub-conjugating enzyme	73622065	22.5	TF ₃ BOK
E6-AP (isoform 3, HUb3A)	HECT E3 Ub ligase	19718764	100	VME
ARF-BP1	HECT E3 Ub ligase	73915353	481.6	VME, TF ₃ BOK
Trip12	Putative HECT E3 Ub ligase	2499839	220.3	TF ₃ BOK
RN138	RING E3 Ub ligase	74733576	28.2	Lac
CUL-4A	RING E3 Ub ligase	108936013	87.6	Lac
CUL-4B (isoform 2)	RING E3 Ub ligase	121114302	102.2	VME, Lac
SH3R2	Putative RING E3 Ub ligase	122070123	79.2	Lac, TF ₃ BOK