Proteolytic Targeting of Transcriptional Regulator TIP120B by a HECT Domain E3 Ligase*

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Ubiquitin-protein ligases (E3s) of the HECT family share a conserved catalytic region that is homologous to the E6-AP C terminus. The HECT domain defines a large E3 family, but only a handful of these enzymes have been defined with respect to substrate specificity or biological function. We showed previously that the C-terminal domain of one family member, KIAA10, catalyzes the assembly of polyubiquitin chains, whereas the N-terminal domain binds to proteasomes in vitro (You, J., and Pickart, C. M. (2001) J. Biol. Chem. 276, 19871–19878). We show here that KIAA10 also associates with proteasomes within cells but that this association probably involves additional contacts with proteasome subunits other than the one (S2/Rpn1) identified in our previous work. We report that the N-domain of KIAA10 also mediates an association with TIP120B (TATA-binding protein-interacting protein 120B), a putative transcriptional regulator. Biochemical and co-transfection studies revealed that TIP120B, but not the closely related protein TIP120A, is a specific substrate of KIAA10 in vitro and within C2C12 myoblasts but not in Cos-1 cells. KIAA10 and TIP120B are both highly expressed in human skeletal muscle, suggesting that KIAA10 may regulate TIP120B homeostasis specifically in this tissue.

Many important intracellular proteins, including cell-cycle regulators, tumor suppressors, oncoproteins, and transcription factors, are targeted for degradation by 26 S proteasomes through conjugation to Ub,1 a highly conserved protein of 76 amino acids (1). This proteolytic targeting function underlies the role of ubiquitin in such processes as cell cycle progression, tumorigenesis, antigen presentation, and cell death (1).

A single Ub is an inefficient signal for degradation by proteasomes (2). Proteasome-bound substrates are rather marked with a polyUb chain in which successive Ubs are joined by Lys48-Gly76 isopeptide bonds (3, 4). This modification is accomplished through the sequential actions of three classes of enzymes (1, 5). Ub-activating enzyme (E1) uses ATP to drive the formation of a thiol ester bond between an E1 active-site Cys and the C-terminal carboxyl of Ub (Gly76). The activated Ub is then transferred to the active site Cys of a Ub-conjugating enzyme (E2). Ub is finally transferred from the E2 to an e-amino group of the substrate in a reaction that requires a specific Ub-protein ligase (E3). In many cases, the polyUb chain is probably assembled by the same E3 that recognizes the substrate, but in some cases chain assembly may require specialized, Ub-dedicated E3s (6, 7). The substrate-linked chain is ultimately recognized by the 19 S regulatory complex of the 26 S proteasome (2–4, 8), resulting in the unfolding, translocation, and hydrolysis of the substrate polypeptide chain (9). PolyUb chains linked through other lysine residues of Ub also occur within cells and in some cases may represent functionally distinct signals (for review, see Ref. 10).

The Ub conjugation system features multiple E2 and E3 enzymes, with a large array of combinatorial E2-E3 pairings allowing for the selective targeting of diverse substrates (1). Substrate recognition usually reflects a direct interaction between the E3 enzyme and a specific ubiquitination signal or degron of the substrate (for review, see Ref. 11). E3 enzymes display a modular construction, with unique domains/subunits mediating substrate interaction and conserved domains/subunits responsible for catalysis. A small number of distinctive catalytic domains defines the known E3 families (for review, see Ref. 5). One family, known as the HECT E3s, employs a covalent catalytic mechanism (12). A second family, known as the RING E3s, recruits a specific E2 enzyme by means of a globular zinc binding domain (13). A subset of the RING E3s, known as SCF E3s, consists of multisubunit enzymes in which the RING and substrate-binding domains are localized to distinct subunits (13).

HECT E3s share a conserved ~350-amino acid region that is defined by its homology to the C terminus of E6-AP. This HECT domain harbors the Cys residue that forms a catalytic thiol ester with Ub (14). The prototypic family member, E6-AP, is a 100-kDa host cell protein that forms a complex with the E6 protein of oncogenic human papilloma viruses, thereby acquiring the ability to bind and ubiquitinate the p53 tumor suppressor (15). In contrast to the conserved catalytic module, HECT E3s display highly variable N-terminal domains. The recognition of p53 is mediated principally by the N terminus of E6-AP in conjunction with the viral E6 protein (16). The current model for HECT E3 structure-function postulates that the divergent N termini of these E3s mediate specific substrate binding, whereas the conserved HECT domain supplies catalytic activ-
ity in ubiquitination (14). In principle, independent substrate binding and catalytic motifs can ensure the selective and efficient ubiquitination of different substrates. However, although databases reveal a large family of mammalian HECT E3s (14), only a handful of these enzymes has been defined with respect to substrate specificity or biological function.

In previous work we used a cognate E2 affinity approach to purify and identify a rabbit E3 enzyme that uses free Ub as the substrate for assembly of unanchored polyUb chains linked through Lys29 or Lys48. This enzyme corresponded to the HECT domain E3 encoded by the human KIAA10 cDNA (17). The C-terminal 420 amino acids of KIAA10, called the C-domain (CD), are necessary and sufficient for assembly of both types of polyUb chains, indicating that the N-terminal domain (ND) is responsible for another function(s). We found that the ND bound proteasomes in vitro (17), suggesting that KIAA10 belongs to the small group of conjugating factors known to associate with proteasomes (18–20). Among these factors is the HUL5-encoded HECT E3 (21), an apparent ortholog of KIAA10 in budding yeast (17). The purpose of E3-proteasome interactions is uncertain, but one possibility is that they provide an independent mechanism (besides the substrate-linked chain) for targeting certain substrates to proteasomes. In the case of KIAA10, we demonstrated a robust interaction with the purified S2/Rpn1 subunit of the 19 S complex of the proteasome (17). However, it was uncertain whether KIAA10 associates with proteasomes in vivo. Nor could we exclude that the ND mediated other functionally significant interactions.

Here we report experiments probing the function of the KIAA10-ND. We show that KIAA10 indeed associates with 26 S proteasomes in mammalian cells but that this interaction is likely to depend on contacts with a subunit(s) besides S2/Rpn1. Most importantly, we provide strong evidence that TIP120B (TBP-interacting protein 120B (22)) is a specific substrate that is targeted for degradation in skeletal muscle through KIAA10-catalyzed polyubiquitination. Our results confirm the utility of affinity capture methods for the identification of E3 substrates and set the stage for achieving a fuller understanding of the functions of TIP120B and KIAA10.

EXPERIMENTAL PROCEDURES

Proteins—E1 (23), UbcH5A (17), several versions of KIAA10 (17), and GST-S2 (17) were expressed and purified as in previous work. Bovine Ub and GSH beads were from Sigma. Sources of other reagents are given below.

Plasmids and Cloning—pGEX* plasmids encoding full-length (FL), 655-residue N-terminal domain (ND), and 428-residue C-terminal domain (CD) of KIAA10 have been described (17). We made pET3a-KIAA10 a template for in vitro transcription/translation and full-length enzyme expression in Escherichia coli. The KIAA10 open reading frame was amplified by PCR to introduce flanking NotI sites and ligated into pET3a. The C1051A mutation was introduced into this plasmid by standard PCR methods. Plasmids pBluescript-TIP120A, pCDNA-TIP120B, and pCDNA-FLAG-TIP120B have been described (22). For mammalian cell expression, the KIAA10 and KIAA10-C1051A coding sequences were amplified by PCR to introduce 5′-SalI and 3′-NotI sites and subcloned in-frame with the Myc tag in pcMV-MYC (Clontech). The sequences of all PCR-derived cDNA constructs were verified. For Northern blotting we probed a human MTN blot (Clontech) with a 32P-labeled probe complementary to the ND coding region.

KIAA10-Proteasome Interaction—A series of N-terminal truncation mutants of KIAA10-ND (see Fig. 1 and "Results") were constructed in pET3a-KIAA10 by standard PCR methods. [35S]Met-labeled wild-type and C1051A-KIAA10 proteins were expressed by in vitro transcription/translation in reticulocyte lysate (Promega). An aliquot of each translation mixture was assayed for activity in K29-Ub2 synthesis (17). The active fractions were pooled and loaded onto a MonoQ fast protein liquid chromatography column (Amersham Biosciences) equilibrated with base buffer. The column was eluted with a 0.6–0.2 M NaCl gradient. The pooled active fractions from this column were used as an E3 source after desalting by repeated dilution with base buffer and concentration (final volume, ~1.7 ml).

Characterization of C1051A-KIAA10—[35S]Met-labeled wild-type and C1051A-KIAA10 proteins were expressed by in vitro transcription/translation and C1051A-KIAA10 was trans-
protein (~0.5 mg) was mixed with 10 μl of anti-FLAG M2 agarose (Sigma) and rotated at 4°C for 2 h. The beads were washed with 4 × 0.5 ml radiolabeled immunoprecipitation assay buffer, and bound proteins were eluted with 40 μl of sample buffer. Aliquots (10 μl) were resolved on an 8% gel. Proteins were transferred to Immobilon-P (Millipore) and blotted with anti-c-Myc antibody (Santa Cruz A-14) to detect the Myc-tagged E3 (ECL detection).

Intracellular Ubiquitination of TIP120B—Myoblast C2C12 cells were from ATCC. C2C12 cells (105 cells) were co-transfected with FuGENE 6 (above) with pCDNA-FLAG-TIP120B together with pCMV-MYC-KIAA10 or pCMV-MYC-KIAA10-C1051A (or an empty vector control). Each transfection employed 3 μg of each vector and was done in duplicate. After 40 h, 50 μM MG-132 (Peptides International) was added to one set of transfections. Cells were harvested 6 h later and lysed (above). TIP120B was immunoprecipitated (above), and the immunoprecipitates were screened by immunoblotting with affinity-purified Ub antibodies (produced by us according to procedures described in Haas and Bright (24)).

Intracellular Destabilization of TIP120B—C2C12 cells (105 cells) were co-transfected with pCDNA-FLAG-TIP120B and pCMV-MYC-KIAA10 or pCMV-MYC-KIAA10 mutant (or vector control) as described above using a total of 2 μg of plasmid DNA (1 μg of each) per transfection. After 40 h, 50 μM MG-132 was added to the indicated transfections. After 6 h more, cells were harvested and lysed (above). Lysates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibody (Sigma) or affinity-purified anti-TIP120B antibody (22).

RESULTS

Studies of the ND-Proteasome Interaction—We have shown that KIAA10 (Fig. 1A) associates via its ND with purified 26 S proteasomes (17). This interaction might be explained by binding of the ND to S2/Rpn1, one of the two largest subunits of the 19 S regulatory complex of the proteasome (Fig. 1B and Ref. 17). As a first step in evaluating the biological significance of this interaction, we carried out deletion mutagenesis to map the region of the ND responsible for its interaction with S2/Rpn1. N-terminally truncated versions of the ND were labeled by in vitro transcription/translation and tested for binding in pull-down assays with a GST-S2 fusion protein (17). Removal of the first 88 residues of the ND had a minimal effect on the S2-ND interaction (Fig. 1, A and C, but mutants truncated by ≥132 residues displayed strongly diminished binding (Fig. 1B, compare lanes 10, 12, and 14, with 6 and 8). Versions of the ND lacking ≥132 N-terminal residues did, however, retain weak binding activity (~18% of the intact ND, Fig. 1C). Thus, a second, minor S2 binding determinant is located elsewhere in the ND. In all cases, minimal binding to GST alone (Fig. 1B) confirmed the specificity of the ND-S2 interaction.

Several considerations suggest that reduced binding of Δ132-ND to S2/Rpn1 reflects the presence of a binding epitope in the first 132 residues of the ND versus improper folding of truncated ND molecules. First, in vitro transcription/translation frequently produces folded proteins. For example, full-length KIAA10 produced by this method quantitatively forms a thiol ester with Ub, indicating full activity (see “Experimental Procedures”) even though full-length KIAA10 produced in bacteria is largely insoluble (17). Second, a GST-Δ132-ND fusion protein binds purified 26 S proteasomes (see the next paragraph) as well as a KIAA10-specific substrate (TIP120B, see Fig. 2 below). These findings show that N-terminal truncation is compatible with proper folding. This conclusion is confirmed by the behavior of Δ132-KIAA10 expressed in Cos-1 cells (see the next paragraph). Finally, the results of a complementary C-terminal deletion study are fully consistent with the result shown in Fig. 1B. In these experiments the ND was successively truncated at its C terminus, and a fragment consisting of residues 1–222 was found to bind efficiently to GST-S2.

* J. Blum and C. Pickart, unpublished experiments.
detectable inhibition of the ND-proteasome interaction as a result of the truncation (data not shown). In a more physiological experiment, we transfected Myc-tagged versions of WT-KIAA10 and Δ132-KIAA10 into Cos-1 cells, immunoprecipitated proteasomes with an antibody against the S8/Rpt6 ATPase subunit of the 19 S complex, and screened the immunoprecipitates for the presence of KIAA10 by Western blotting. Full-length KIAA10 was co-precipitated by the S8 antibody (Fig. 1, middle panel). Although this interaction seemed to be more pronounced with the truncated E3, this effect could be explained by higher expression of the truncated protein (upper panel). A similar result was obtained when we used antibody against a non-ATPase 19 S subunit, S10α/Rpn7, to precipitate proteasomes (Fig. 1D, bottom panel). The agreement between the results of intracellular and in vitro interaction assays strongly suggests that KIAA10 interacts directly with proteasomes. Direct interaction also applies in the case of yeast U64 (18, 25), whereas E6-AP interacts with proteasomes through an accessory factor (20).

These findings show for the first time that KIAA10 associates with proteasomes within cells. The in vitro and intracellular results concur in suggesting that the extreme N terminus of the E3 is not the only determinant of its interaction with proteasomes. We cannot exclude that the residual interaction between Δ132-KIAA10 and S2/Rpn1 (Fig. 1C) is sufficient for interaction with cellular proteasomes, but we consider it more likely that a distal region of the ND (beyond residue 132) also binds to a different, unidentified subunit of the 19 S complex. There is precedent for a given E3 interacting with more than one subunit of the 19 S complex (18, 25).

Interaction of TIP120B with KIAA10—We next addressed whether the KIAA10-ND might be responsible for interacting with a specific substrate. To gain insight into the likely properties of cognate substrates, we conducted a Northern blot analysis of KIAA10 mRNA levels in human tissues (data not shown). The results confirmed a previous report that KIAA10 is highly expressed in skeletal muscle (26). Levels of KIAA10 mRNA in kidney and pancreas were <1% that of the level in muscle, whereas expression in heart, brain, placenta, lung, and liver was nearly undetectable (data not shown). Based on this expression pattern we speculated that KIAA10 might target a muscle protein(s) for degradation in the Ub-proteasome pathway.

In earlier work we found that an affinity-purified preparation of KIAA10 also contained low levels of peptides conserved among members of the TIP120 family (data not shown and Ref. 17), raising the possibility that these proteins interact with UbcH15α or KIAA10. TIP120A, the original family member, was identified during a search for proteins that bound to TBP in vitro (27). TIP120B was later cloned by virtue of its similarity to TIP120A (22). In humans, TIP120B is expressed most highly in skeletal muscle, whereas TIP120A is ubiquitously expressed (22, 28). The two TIP120 proteins are 60% identical to one another (22), and both contain multiple copies of a proposed protein interaction module, the HEAT repeat (29), whose significance for TIP120 function is unknown. Both isoforms interact with TBP under physiological conditions in vitro and associate with TBP in nuclear extracts (22, 27). TIP120A may function as a general transcriptional regulator by virtue of its ability to stimulate all three classes of eukaryotic gene transcription (30). TIP120A also binds to the Cul1 scaffold subunit of SCF E3s in a manner that displaces the Skp1 adaptor subunit and, thus, inhibits Ub ligase activity (31, 32). Based on its tissue-specific expression, TIP120B is likely to have one or more distinct functions relative to TIP120A. Indeed, TIP120B, but not TIP120A, interacts with NOT3, a protein implicated in global gene regulation (28).

To address if the TIP120 proteins bind to KIAA10, GST-KIAA10-FL, -ND, and -CD fusion proteins were immobilized on glutathione beads and tested for their ability to pull down in vitro transcribed/translated TIP120A or TIP120B. KIAA10 strongly preferred to bind TIP120B over TIP120A (Fig. 2A, lanes 1 and 2 versus 5 and 6, a mean difference of 4.5-fold by densitometry). The interaction with TIP120B was mainly mediated by the ND (lane 1 versus 2). To determine whether KIAA10 and TIP120B associate within cells, FLAG-TIP120B was co-transfected with Myc-tagged wild-type or mutant (C1051A) KIAA10 in Cos-1 cells. Wild-type and mutant E3s were both immunoprecipitated with TIP120B, as indicated by the presence of the expected Myc-reactive band in the FLAG immunoprecipitate (Fig. 2B, lanes 1 and 2 versus 3). Thus, KIAA10 and TIP120B interact intracellularly, and the active Cys residue of KIAA10 is dispensable for this interaction. The latter result is as expected since the KIAA10-ND mediates the interaction with TIP120B (Fig. 2A). We did not map the region(s) within the ND that binds TIP120B, but preliminary studies suggest that the Δ132-ND has only a slightly reduced ability to bind TIP120B in vitro (data not shown). The principal TIP120B binding determinants of KIAA10 are, therefore, located downstream of this region.
Targeting of TIP120B by a Specific HECT E3

Fig. 3. KIAA10 E3 ubiquinates TIP120B. A, in vitro ubiquitination of TIP120B by recombinant KIAA10 E3 (autoradiograph). TIP120B produced by in vitro transcription/translation was incubated for the indicated times with purified E1, Ubch5A, and Ub with or without partially purified recombinant KIAA10 (see “Experimental Procedures”). B, KIAA10-dependent ubiquitination of TIP120B in myoblast C2C12 cells (Western blot (IB)). C2C12 cells were co-transfected with pCDNA-FLAG-TIP120B and pCMV-MYC-KIAA10 (WT) or pCMV-MYC-KIAA10-C1051A (CA) or, as a control, an empty vector. After 40 h, one set of transfections was treated with MG-132 (50 μM) for 6 h. FLAG-TIP120B was immunoprecipitated (IP) with anti-FLAG beads. Precipitated proteins were resolved by SDS-PAGE, and Ub conjugates were detected by Ub immunoblot.

TIP120B Is Ubiquitinated and Degraded in a KIAA10-dependent Manner—The N-terminal domains of HECT E3s are generally responsible for substrate binding, whereas the HECT domain functions in catalysis of substrate ubiquitination (5, 18, 33–37). We next asked if full-length KIAA10 catalyzes the ubiquitination of TIP120B. In vitro transcribed/translated TIP120B was incubated with E1, recombinant Ubch5A, and Ub in the absence or presence of recombinant full-length KIAA10. TIP120B was ubiquitinated only in the presence of KIAA10, as indicated by the appearance of high molecular mass radioactive adducts in lane 3 of Fig. 3. These data (Fig. 3A) indicate that KIAA10 is competent to ubiquitinate TIP120B. However, despite this positive in vitro result, KIAA10 did not stimulate the ubiquitination of TIP120B when we co-expressed the two proteins in Cos-1 cells (data not shown). The discrepancy between the data obtained in vitro and in Cos-1 cells could be explained if the KIAA10-catalyzed ubiquitination of TIP120B requires another cellular component(s) that is present in reticulocyte lysate but absent in Cos-1 cells.

KIAA10 and TIP120B are both highly expressed in human skeletal muscle (see above in the “Results” section). We therefore tested the effect of KIAA10 expression on TIP120B ubiquitination in undifferentiated mouse myoblast C2C12 cells. FLAG-TIP120B was co-transfected with wild-type E3, inactive mutant E3 (C1051A), or empty vector (Fig. 3B). In some cases, transfected cells were treated with the proteasome catalytic site inhibitor MG-132 (38). The cells were lysed, and TIP120B (i.e., FLAG) immunoprecipitates were screened by immunoblot analysis with Ub antibodies. In contrast to observations in Cos-1 cells, KIAA10 significantly stimulated TIP120B ubiquitination when the two proteins were co-expressed in C2C12 cells (Fig. 3B, lanes 2 and 6). However, this effect was observed only if proteasomes were inhibited (Fig. 3B, odd- versus even-numbered lanes). We attribute this feature to rapid degradation of polyubiquitinated TIP120B in the absence of MG-132, as shown in Fig. 4. In contrast to results obtained with wild-type KIAA10, expression of the C1051A mutant did not stimulate the ubiquitination of TIP120B (lane 4 of Fig. 3B). Expression of the mutant E3 rather seemed to decrease the basal level of TIP120B ubiquitination (lane 4 versus 6), suggestive of a dominant negative effect. However, further work is needed to confirm this interpretation.

These results (Fig. 3B) provide confidence that the TIP120B ubiquitination observed in vitro (Fig. 3A) can be relevant in a cellular setting. Moreover, the different results obtained in Cos-1 and C2C12 cells again suggest that KIAA10-dependent TIP120B ubiquitination may require a specific component in muscle cells. The nature of this hypothetical component remains to be discerned (see “Discussion”). In conjunction with the binding data (Fig. 2), the ubiquitination and degradation results are in accord with the established model for HECT E3 structure/function (see the Introduction), which assigns substrate binding and catalysis of ubiquitination to the N-terminal and HECT domains, respectively.

KIAA10-dependent ubiquitination of TIP120B was detected only after inhibiting proteasomes (Fig. 3B), suggesting that ubiquitination normally targets TIP120B for rapid degradation by proteasomes. To determine if this was the case, FLAG-TIP120B was co-transfected with wild-type E3, mutant E3 carrying the C1051A mutation, or empty E3 vector (Fig. 4). Cell extracts were screened by immunoblot analysis with antibodies against the FLAG epitope (top panel) or the TIP120B protein (bottom panel). Both methods of detection revealed that the steady-state level of TIP120B was significantly reduced upon co-expression of the wild-type E3 (lanes 3). This destabilization was blocked by MG-132 (lanes 4) or by the inactivating C1051A mutation in the E3 (lanes 5). Similar results were obtained in eight independent co-transfection experiments. These data provide rigorous evidence that KIAA10-dependent ubiquitination of TIP120B targets this substrate for degradation by 26 S proteasomes.

DISCUSSION

KIAA10 emerged from our efforts to identify the activity responsible for Ubch5A-dependent assembly of unanchored Lys52- and Lys48-linked polyUb chains (6, 17). We found that the C-terminal 420 residues of this E3 are necessary and sufficient for this activity (17). We also showed that the ND was necessary and sufficient for interaction of the E3 with intact proteasomes and with the isolated S2/Rpn1 subunit of the 19 S regulatory complex (17). One model to integrate these findings postulates that KIAA10 is a Ub-dedicated E3 whose ND serves to localize chain-assembly activity to proteasomes. A second possibility is that the ND binds a non-Ub substrate, which is then conjugated to Lys52- or Lys48-linked polyUb chains through the activity of the CD. These models are not mutually
exclusive. A principal finding of the present work is that KIAA10 can act as a conventional E3 that conjugates Ub to a non-Ub substrate, namely TIP120B.

Identifying the cognate substrate(s) of an uncharacterized E3 can be a difficult problem. E3s generally bind their specific substrates tightly, however, and in favorable cases this property can be exploited for substrate identification. Huibregtse et al. (34, 35) use E6-AP/Rsp5 affinity chromatography to identify several natural substrates of these HECT E3s, whereas Howley and co-workers (39, 40) use two-hybrid screening for the same purpose. In hindsight, our identification of TIP120B as a substrate of KIAA10 probably resulted from a variant of the affinity chromatography approach. TIP120B was among the proteins that co-eluted with KIAA10 from a column bearing this E3 cognate E2 (17). Presumably, the presence of TIP120B in the eluate reflects the binding of a KIAA10-TIP120B complex to the E2. This interpretation is bolstered by the fact that our affinity eluate contained the S2/Rpn1 subunit of the 19 S complex, which is a bona fide KIAA10-binding protein (Fig. 1B and Ref. 17). The biochemistry of the conjugation reaction requires that there is an E3-E2-substrate complex on the reaction pathway, and the structure of a HECT domain complexed to an E2 also suggests that E2 and substrate are likely to interact with distinct surfaces of the E3 (41). Our success in the present work confirms the utility of E2/E3 affinity capture as a method for substrate discovery.

TIP120B fulfills all of the criteria that should be met by a specific E3 substrate. TIP120B binds tightly to the KIAA10-ND (Fig. 2A), which is the region expected to mediate substrate binding in a HECT E3 (see the Introduction). This interaction is observed both in vitro and in living cells (Figs. 2, A and B). The interaction is specific, as it is much stronger than the interaction observed with the related protein TIP120A (Fig. 2A). Recombinant KIAA10 ubiquitinates in vitro translated TIP120B (Fig. 3A). KIAA10 promotes the ubiquitination of TIP120B when the two proteins are co-expressed in C2C12 cells, and this reaction is strictly dependent on the essential HECT Cys residue of the E3 (Fig. 3B). Moreover, the level of TIP120B protein detected in C2C12 cells is strongly reduced by co-expression of wild-type KIAA10 but not by a mutant version of KIAA10 lacking ubiquitination activity (Fig. 4). Finally, this destabilization is proteasome-dependent (Fig. 4). Together these results show that KIAA10 polyubiquitinates TIP120B in C2C12 cells, leading to TIP120B degradation by 26 S proteasomes. TIP120B and KIAA10 are both highly expressed in human skeletal muscle, so this enzyme-substrate relationship is likely to apply with the endogenous proteins as well. We noticed that in C2C12 cells transfected TIP120B undergoes ubiquitination in the absence of transfected KIAA10 (Fig. 3B, lane 6). This reaction may be catalyzed by endogenous KIAA10. We also found that transfected KIAA10 was ubiquitinated in C2C12 cells (data not shown), suggesting that as seen for many other E3s, the enzyme itself may be a target of the Ub-proteasome pathway (for review, see Ref. 5).

To the extent that TIP120B is a global regulator of transcription (22, 28), our findings suggest that KIAA10 could modulate this role through effects on TIP120B homeostasis. We predict that TIP120A, if it is expressed in human skeletal muscle, would not be subject to such regulation (Fig. 2A). Besides transcriptional regulation, TIP120A binds to several collins and can negatively regulate SCF E3 function (31, 32). Although TIP120B was not found to associate with Cul1 (31, 32), these studies involved cultured cells that may not express significant levels of TIP120B. Thus, it remains unclear if KIAA10 could modulate SCF E3 activity in skeletal muscle through effects on TIP120B homeostasis.

In contrast to results obtained in myoblast C2C12 cells, TIP120B was neither ubiquitinated nor destabilized by KIAA10 when the two proteins were co-expressed in Cos-1 cells. The discrepancy suggests that Cos-1 cells are missing a factor that is required for the KIAA10-catalyzed ubiquitination of TIP120B. There are many examples known in which a lack of substrate ubiquitination reflects purposeful negative regulation of the E3-substrate interaction (for review, see Refs. 11 and 13). However, KIAA10 and TIP120B display a robust association in Cos-1 cells (Fig. 2B). Any block to ubiquitination in Cos-1 cells is, therefore, downstream of substrate binding. One rather uninteresting possibility is that Cos-1 cells express a limiting level of UbcH5A (this UbcH5 isoform partners efficiently with KIAA10 in catalyzing polyUb chain assembly, whereas UbcH5B does not3). We consider this explanation to be unlikely because UbcH5 isoforms are broadly expressed in mammalian tissues (42), and preliminary results suggest that co-expression of UbcH5A with KIAA10 in Cos-1 cells does not promote the ubiquitination or destabilization of TIP120B.4 A different explanation is that Cos-1 cells have excessive deubiquitinating activity such that ubiquitinated TIP120B, although produced, is kept at undetectable levels. Because the activity of other E3s is readily detected in Cos cells (e.g. Wallace and Cidlowski (43)), if this explanation applies, then the relevant deubiquitinating enzyme might show specificity for TIP120B. There is now precedent for substrate-specific deubiquitination (44). Finally, we easily detected the binding of KIAA10 to proteasomes in Cos-1 cells (Fig. 1D), but we could not observe this interaction in C2C12 cells (data not shown). Thus, there is a formal possibility that association of the E3 with proteasomes inhibits KIAA10 Ub-conjugating activity. Although we cannot exclude this model, we consider it most likely that the low level of KIAA10 expression in C2C12 cells explains the failure to detect interaction with proteasomes (data not shown). Low expression is probably due in part to E3 degradation (see above in the “Discussion” section). Understanding the basis of the tissue specificity of KIAA10 activity is a goal of future studies.

As just discussed, besides binding TIP120B, the KIAA10-ND also mediates an association with 26 S proteasomes (17). This interaction is observed in Cos-1 cells (Fig. 1D) and relies on residues other than the first 192 residues of the ND (44). Even though these residues are principally responsible for KIAA10 binding to isolated S2/Rpn1 (Fig. 1, B and C), it is, therefore, likely that the ND interacts with a proteasome subunit(s) besides S2/Rpn1. TIP120A has been detected in a functionally uncharacterized complex that contains multiple ATPase subunits, but not non-ATPase subunits, of the 19 S complex (45). It is not known if TIP120B forms a similar complex. KIAA10, on the other hand, associates with conventional proteasomes because it could be precipitated from Cos-1 cell extracts with antibodies that recognize both ATPase and non-ATPase subunits of the 19 S complex (Fig. 1D).

One of the original goals of this work was to abrogate the interaction of KIAA10 with proteasomes while maintaining competence in TIP120B binding. With this type of mutant E3 we could test whether the KIAA10-proteasome interaction is significant for the ubiquitination and proteolysis of TIP120B. Although we did not achieve this goal with KIAA10, Xie and Varshavsky (25) recently reported the results of such studies with yeast Ufd4. The N-terminal 200 residues of this HECT enzyme interact with two ATPase subunits of the 19 S complex. Remarkably, although abrogating the Ufd4-proteasome interaction had no impact on the ubiquitination of a specific Ufd4

3 J. You and C. Pickart, unpublished data.
4 M. Wang and C. Pickart, unpublished data.
substrate, it significantly impaired substrate degradation (25). Thus, the productive targeting of this substrate for degradation may rely on two sets of interactions with proteasomes, one involving Ufd4 and the other involving the polyUb chain that is linked to the substrate (25). It will be interesting to determine whether this model applies more broadly.

The identification of TIP120B as a specific substrate of KIAA10 and the potential tissue specificity of this targeting set the stage for a fuller analysis of the biological functions of both proteins. The inability of the closely related protein TIP120A to bind KIAA10 should facilitate mapping of the ubiquitination signal in TIP120B and may ultimately lead to additional specific substrates. The fact that KIAA10 has a substrate other than Ub itself indicates that this E3 does not function solely to assemble unanchored polyUb chains for use by other conjugating factors (6, 17), although free chain synthesis may still represent a semi-independent function of KIAA10. The identification of a specific substrate of KIAA10 should ultimately facilitate our efforts to determine whether Lys29-linked polyUb chains are a competent signal for degradation by proteasomes.

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