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Stabilization of the E3 Ubiquitin Ligase Nrdp1 by the Deubiquitinating Enzyme USP8

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Nrdp1 is a RING finger-containing E3 ubiquitin ligase that physically interacts with and regulates steady-state cellular levels of the ErbB3 and ErbB4 receptor tyrosine kinases and has been implicated in the degradation of the inhibitor-of-apoptosis protein BRUCE. Here we demonstrate that the Nrdp1 protein undergoes efficient proteasome-dependent degradation and that mutations in its RING finger domain that disrupt ubiquitin ligase activity enhance stability. These observations suggest that Nrdp1 self-ubiquitination and stability could play an important role in regulating the activity of this protein. Using affinity chromatography, we identified the deubiquitinating enzyme USP8 (also called Ubpy) as a protein that physically interacts with Nrdp1. Nrdp1 and USP8 could be coimmunoprecipitated, and in transfected cells USP8 specifically bound to Nrdp1 but not cbl, a RING finger E3 ligase involved in ligand-stimulated epidermal growth factor receptor down-regulation. The USP8 rhodanese and catalytic domains mediated Nrdp1 binding. USP8 markedly enhanced the stability of Nrdp1, and a point mutant that disrupts USP8 catalytic activity destabilized endogenous Nrdp1. Our results indicate that Nrdp1 is a specific target for the USP8 deubiquitinating enzyme and are consistent with a model where USP8 augments Nrdp1 activity by mediating its stabilization.

Ubiquitination plays central roles in regulating protein stability and activity. The canonical function of ubiquitination is in directing proteins for proteolytic degradation by proteasomes (23). Ubiquitination is also involved in plasma membrane protein internalization and degradation by lysosomes (2) and in regulating the activities of specific proteins (5, 14, 24). Ubiquitin molecules are linked to target proteins through an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and ε-amino groups of lysine residues of the substrate. Ubiquitination requires the activities of three enzymes. E1 enzymes activate ubiquitin and transfer it to E2 ubiquitin-conjugating enzymes through thiolester linkages. E3 ubiquitin ligases bring substrates to the E2 by binding both the E2 enzyme and specific target proteins. HECT domains and RING finger domains of E3 ligases mediate their binding to E2-conjugating enzymes (45), while a variety of domains are responsible for substrate recognition.

Ubiquitin modifications take the form of monoubiquitin, where a single ubiquitin moiety is attached to a single target protein lysine; multiple monoubiquitination, where several target protein lysines are modified with single ubiquitin moieties; or polyubiquitination, where a ubiquitin moiety attached to a target protein is iteratively ubiquitinated through one of its seven lysines (57). The different types of ubiquitin conjugates control different cellular processes. Polyubiquitination through ubiquitin lysine residue K48 is generally thought to direct proteasomal delivery and degradation of cytosolic proteins. Polyubiquitination through ubiquitin lysine residue K63 or K29 regulates nondegradative processes, while multiple monoubiquitination may mediate internalization and trafficking of plasma membrane proteins.

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Deubiquitinating enzymes can reverse protein ubiquitination and are thought to function in processing ubiquitin precursors, recycling ubiquitin, unclogging proteasomes of ubiquitinated proteins, and promoting protein stability. Most known deubiquitinating enzymes are cysteine proteases that fall into two categories (11, 13, 30, 58). Ubiquitin carboxy-terminal hydrolases (UCHs) comprise a subfamily of closely related proteins and contain a catalytic domain of ~200 amino acids that includes blocks containing conserved cysteine and histidine residues. UCHs efficiently remove ubiquitin from peptides and adducts but act less efficiently on ubiquitinated proteins. These enzymes are thought to suppress the accumulation of nonproductive ubiquitin adducts in the cell and to recycle ubiquitin for reuse. Ubiquitin-processing proteases (UBPs) contain a ~400-amino-acid-residue catalytic domain that includes blocks of cysteine and histidine residues similar to those of UCHs, but the highly divergent intervening sequences may contribute to substrate specificity. In addition, sequences unique to the amino or carboxyl side of the catalytic domains of UBPs are likely to mediate specific protein-protein interactions to modulate localization and substrate specificity (34, 35). This class of deubiquitinating enzyme may have evolved to interact with specific targets to mediate their stabilization. Although the human genome encodes at least 63 distinct UBPs, substrate specificities for only a very few mammalian UBPs have been described (8, 31, 33, 52, 53, 55, 58).

Recent studies point to a key role for ubiquitination in the down-regulation and degradation of a variety of plasma membrane proteins (29), including growth factor receptor tyrosine kinases. Upon growth factor binding many receptor tyrosine kinases localize to clathrin-coated pits, become internalized, and are delivered to endosomes. Receptors are sorted in endosomes according to whether they are to be recycled to the cell surface or degraded in lysosomes. Ligand binding stimulates the multiple monoubiquitination of epidermal growth

factor (EGF) receptor and platelet-derived growth factor receptor (20), and it has been demonstrated that monoubiquitination is sufficient to drive EGF receptor internalization and degradation (20, 38). Moreover, growth factor-stimulated monoubiquitination of endosomal sorting accessory proteins may regulate their function as ubiquitin receptors (17, 21), underscoring the central role of protein ubiquitination in receptor trafficking and degradation.

Multiple monoubiquitination of EGF receptor is mediated, at least in part, by the RING finger E3 ubiquitin ligase cbl (38). cbl is recruited to the receptor in an activation-dependent manner by the binding of its tyrosine kinase binding domain to phosphorylated tyrosine 1045 of the EGF receptor (54). Point mutation of Y1045 (32), or oxidant-induced receptor activation that does not result in Y1045 phosphorylation (48), suppresses EGF receptor down-regulation. Likewise, cbl mutants that are unable to mediate EGF receptor ubiquitination also promote receptor stability (32). Hence, escape of receptor tyrosine kinases from cbl-mediated down-regulation has been suggested to promote cellular growth properties associated with oncogenesis (44).

We recently identified a novel RING finger E3 ubiquitin ligase that regulates steady-state levels of ErbB3 and ErbB4 (15, 46), members of the same receptor tyrosine kinase family as the EGF receptor. Since ErbB3 and ErbB4 are binding receptors for the neuregulin subfamily of EGF-like growth factors, we have named this protein Nrdp1 for neuregulin receptor degradation pathway protein 1. Our observations suggest that cellular levels of Nrdp1 may be critical in regulating steady-state levels of receptors (51) and are of particular relevance because the aberrant overexpression and activation of ErbB family receptor tyrosine kinases contribute to tumor malignancy (25). More recently it has been reported that Nrdp1, also called FLRF (1), mediates the ubiquitination and degradation of BRUCE (47), a large membrane-associated inhibitor-of-apoptosis domain-containing protein (22) that inhibits cell death in cultured mammalian cells (10) and in *Drosophila melanogaster* eye (56).

In the present study we demonstrate that autoubiquitination destabilizes the Nrdp1 protein. We employed an affinity chromatography approach to identify Nrdp1-interacting proteins that might modulate its activity and found that the UBP deubiquitinating enzyme USP8 specifically interacts with and stabilizes the Nrdp1 protein.

MATERIALS AND METHODS

Antibodies. Mouse (M2; immunoglobulin G1 [IgG1]) and rabbit antibodies to FLAG epitope were purchased from Sigma. Antibody to V5 epitope (IgG2a) was purchased from Invitrogen, mouse antiactin AC-15 was from Sigma, and rabbit anti-cbl was purchased from Santa Cruz Biotechnologies. Affinity-purified rabbit antibodies to Nrdp1 were described previously (15). Rabbit blotting antibodies to USP8 were generated using a glutathione *S*-transferase (GST) fusion of mouse USP8 amino acid residues 703 to 864. Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Zymed and Chemicon, respectively, and protein A/G agarose was from Oncogene Research Products.

Nrdp1 and USP8 constructs. Construction of FLAG-tagged versions of wild-type human Nrdp1 and the carboxy terminus (clone 32) was described previously (15). The C34S/H36Q mutation of Nrdp1 was also described previously (46). The cDNA encoding mouse USP8 was obtained by reverse transcriptase PCR with Turbo *Pfu* (Stratagene) from total C2C12 myotube RNA. The product was subcloned into the pcDNA3.1$^+$ (Invitrogen) expression vector, and sequence was verified by sequencing both strands. Truncation mutants were generated by PCR and encompassed the following amino acid residues of the mouse sequence: T1, 1 to 92; T2, 1 to 183; T3, 1 to 464; T4, 1 to 612; T5, 1 to 735; T6, 93 to 1080; T7, 184 to 1080; T8, 465 to 1080; T9, 613 to 1080; T10, 736 to 1080; T11, 184 to 735; T12, 308 to 735; T13, 465 to 735. The C748A and C748S mutations were made using the QuickChange site-directed mutagenesis kit (Stratagene), and mutations were verified by sequencing. A plasmid containing the cDNA encoding human USP2 was obtained from the American Type Culture Collection, V5 epitope tagged at the carboxy terminus, and subcloned into pcDNA3.1$^+$ with the use of PCR.

Cell culture, transfections, and blotting. COS7, 293T, and C2C12 cell lines were obtained from the American Type Culture Collection. COS7 and 293T cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum. C2C12 cells were maintained in DMEM containing 15% fetal calf serum and 0.5% chicken embryo extract. To make myotubes, cells at 60 to 70% confluence were switched to DMEM containing 5% horse serum and further incubated for 4 days. Transfections were carried out using Fugene 6 (Roche) according to the directions of the manufacturer, and cells were allowed to express protein for 48 h following transfection. In some experiments cells were treated overnight with or without 2 μM MG132 (Calbiochem) prior to lysis. For experiments where lysates were blotted with anti-FLAG or anti-V5 antibodies, transfected cells in six-well dishes were lysed in 400 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. In some experiments 293T cells were cotransfected with pBABEpuro plasmid and treated for 72 h prior to lysis with 2 μg of puromycin/ml to enrich for the transfected population. For immunoprecipitation experiments, cells in 100-mm-diameter dishes were lysed in 1 ml of coimmunoprecipitation buffer (12), and cleared lysates were immunoprecipitated with 2 μg of anti-FLAG antibody M2, anti-cbl, or anti-Nrdp1 for 3 h at 4°C with protein A/G Sepharose to capture immune complexes. Lysate or immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose; blotted with a 1/1,000 dilution of anti-FLAG M2, anti-V5, anti-Nrdp1, or anti-USP8 or a 1/25,000 dilution of antiactin; and detected with 1/10,000 dilutions of horseradish peroxidase-conjugated secondary antibodies.

Isolation of Nrdp1 binding proteins. GST and GST-32 were expressed in DH5α bacteria and purified as described previously (15). Ten 100-mm-diameter dishes of C2C12 myotubes were lysed in a total of 6 ml of binding buffer (20 mM HEPES [pH 7.4]; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA; 0.2 mM Na_3VO_4; 10 mM sodium pyrophosphate; 1 mM NaF; 5 mM β-glycerophosphate; 0.2 mM phenylmethylsulfonyl fluoride; 4 μg each of pepstatin, leupeptin, and aprotinin/ml), and 15 mg of protein was used for batch-wise binding experiments. Fifty micrograms of GST or GST-32 immobilized on glutathione agarose beads was incubated with binding buffer alone or with cell lysates in a 35-ml total volume in a 50-ml conical tube. Incubation was carried out at 4°C with rocking for 1.5 h. The mix was poured into a 10-ml disposable column (Bio-Rad Laboratories), and beads were washed with 50 ml of binding buffer. Beads were eluted with 200 μl of SDS-PAGE sample buffer, and eluted proteins were resolved by gradient SDS–6 to 10% PAGE. Proteins were stained with Coomassie blue, and bands were excised. The identity of eluted bands was determined by tandem mass spectrometry of trypsin-digested proteins by the W. M. Keck Foundation mass spectrometry protein identification facility at Yale University.

RESULTS

Nrdp1 protein instability is mediated by autoubiquitination. In characterizing the properties of the Nrdp1 E3 ubiquitin ligase, we observed that the protein could not be detected by immunoblotting when transiently expressed in numerous cultured cells of various types (15). For example, Fig. 1B shows that protein expression was undetectable when COS7 cells were transiently transfected with FLAG-tagged wild-type Nrdp1. However, Nrdp1 accumulated to very significant levels when cells were incubated overnight with 2 μM MG132, a proteasome inhibitor. Similar results were obtained when cells were treated with lactacystin and proteasome inhibitor 1, two other proteasome inhibitors, but not with known lysosome inhibitors (data not shown). These observations indicate that Nrdp1 is very efficiently degraded in cells in a proteasome-dependent manner.

Our previous studies indicate that clone 32 (Fig. 1A), a form

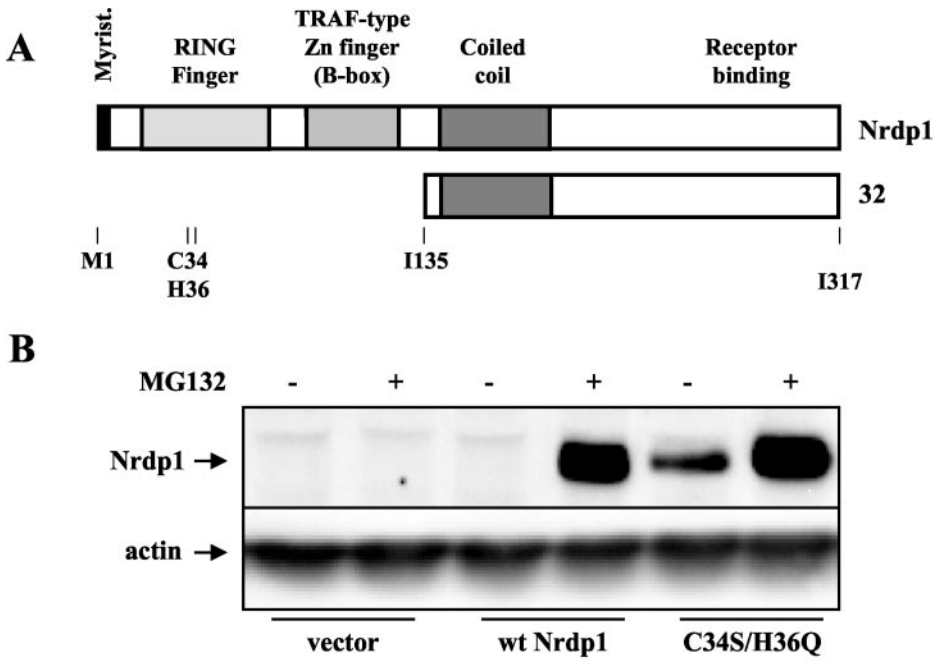

FIG. 1. Mutation of the RING finger domain of Nrdp1 enhances its stability. (A) Domain structure of full-length Nrdp1, C36S/H38Q mutant, and clone 32 used in these studies. (B) COS7 cells were transfected with vector, FLAG-tagged wild-type Nrdp1, or FLAG-tagged C34S/H36Q double mutant in the RING finger domain. Cells were then treated overnight without or with 2 μM MG132, as indicated, and lysates were immunoblotted with anti-FLAG or antiactin antibodies.

of Nrdp1 lacking its RING finger and B-box domains, is intrinsically stable and that this stability is only marginally increased with MG132 (15). However, a form of Nrdp1 consisting of the RING finger and the B box alone is intrinsically unstable, suggesting that RING finger-mediated ubiquitin ligase activity may contribute to Nrdp1 instability. To determine whether Nrdp1 self-ubiquitination could contribute to its instability, we examined the expression of a mutant incapable of autoubiquitination. The C34S/H36Q mutant of Nrdp1 harbors two point mutations in its RING finger domain (Fig. 1A) that are predicted to disrupt its binding to E2 ubiquitin-conjugating enzymes. It has been previously demonstrated that this mutant is incapable of ubiquitinating itself in vitro and incapable of ubiquitinating ErbB3 in vitro and in cells (46). When transiently expressed in COS7 cells, the C34S/H36Q mutant was markedly more stable than wild-type Nrdp1, and its stability was further enhanced if proteasome-mediated protein degradation was entirely blocked by MG132. These observations indicate that Nrdp1 is intrinsically unstable in cells and suggest that autoubiquitination significantly contributes to its instability. The greater extent of Nrdp1 mutant accumulation in the presence than in the absence of proteasome inhibitor suggests that additional proteasome-dependent mechanisms may also contribute to Nrdp1 degradation in these cells.

Identification of USP8 as an Nrdp1 binding protein. The intrinsic instability of Nrdp1 implies that there may exist cellular factors that enhance its stability. To identify proteins that might contribute to Nrdp1 activity, we carried out a screen for binding proteins present in C2C12 myotubes. This cell line was chosen because we have detected Nrdp1 expression by Northern blotting and by immunoblotting (unpublished observations). For our screen we employed a GST fusion of the Nrdp1 clone 32 because it is much more soluble when expressed in bacteria than is the GST fusion of the full-length protein (unpublished observations). GST and GST-32 immobilized on beads were incubated with lysates from myotubes, and associated proteins were eluted with SDS-PAGE sample buffer and visualized by SDS-PAGE followed by Coomassie blue staining.

Figure 2 shows that a number of cellular proteins specifically associated with GST-32 but not GST, including bands with apparent molecular masses of 270, 185, 120, 80, and 75 kDa. Bands were excised, and the identities of some of the Nrdp1 binding proteins were determined by tandem mass spectrometry. The identity of the ~270-kDa band was determined to be BRUCE, consistent with a previous report that this protein is a binding substrate of Nrdp1 (47). The 120-kDa band, p120, was determined to be mouse USP8 or Ubpy, a member of the UBP subfamily of ubiquitin-specific proteases of unknown function. The predicted molecular mass of USP8 is 123 kDa.

To determine whether USP8 and Nrdp1 form a functional complex, we first determined whether the two proteins could be coimmunoprecipitated from lysates of a cell line expressing both. While many cell lines express very low or undetectable levels of endogenous Nrdp1, endogenous protein was detected in 293T human embryonic kidney cells. These cells also express significant levels of endogenous USP8. In the experiment depicted in Fig. 3A we immunoprecipitated Nrdp1 from lysates of 293T cells with an affinity-purified rabbit antibody described previously (15) and blotted precipitates with that antibody or with an antibody to USP8. Endogenous Nrdp1 (~38 kDa) could be detected in these precipitates, along with the 120-kDa

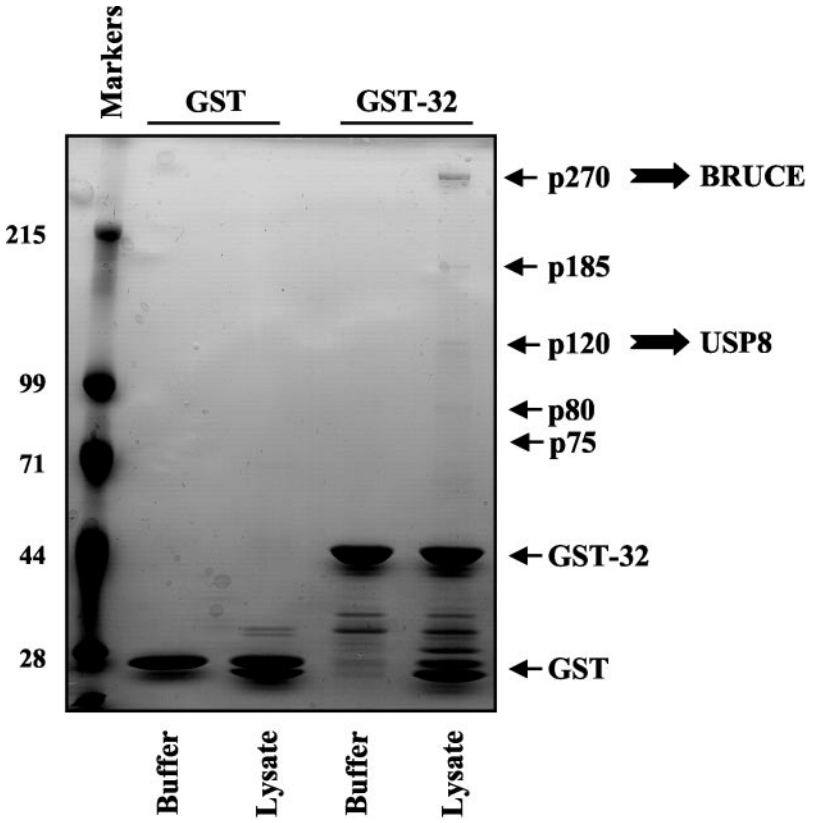

FIG. 2. Identification of Nrdp1 binding proteins by affinity chromatography. Buffer alone or lysates from C2C12 myotubes were incubated with GST or GST-32 bound to glutathione beads. Beads were washed extensively, and bound proteins were eluted with SDS-PAGE sample buffer. Eluted proteins were resolved by gradient SDS–6 to 10% PAGE and visualized by staining with Coomassie blue. Numbers at left are molecular masses in kilodaltons.

USP8, indicating that the two proteins exist in a complex in cells.

To begin to analyze the functional consequence of Nrdp1-USP8 interaction, we obtained the cDNA encoding the full-length mouse USP8 by reverse transcriptase PCR from C2C12 cell RNA and added a V5 epitope tag to its carboxy terminus. When transiently expressed in COS7 cells, V5-tagged USP8 yielded the full-length 120-kDa band as well as several smaller fragments (Fig. 3B). This construct could be coimmunoprecipitated with FLAG-tagged wild-type Nrdp1 (Fig. 3B) or FLAG-tagged 32 (Fig. 3C) when coexpressed in COS7 cells treated with MG132. A catalytically inactive point mutant of USP8 (see below) was also capable of interacting with Nrdp1 (Fig. 3B). However, USP8 could not be coimmunoprecipitated with either endogenous or overexpressed cbl (Fig. 3D). Hence, USP8 specifically interacts with the Nrdp1 E3 ubiquitin ligase.

Mapping of Nrdp1 binding sites in USP8. To further characterize the interaction of USP8 and Nrdp1, we constructed a series of 12 mutants with mutations in USP8 that deleted its various domains (Fig. 4A). The USP8 protein consists of two coiled-coil domains, a rhodanese domain, a putative WW domain, and a carboxy-terminal catalytic domain responsible for deubiquitinating enzyme activity. A cysteine residue, C748, has been previously demonstrated to be required for USP8 enzyme activity (40). Using the coimmunoprecipitation assay, we analyzed the binding of the various V5-tagged USP8 constructs to full-length FLAG-tagged Nrdp1. We observed that constructs T3 through T11 coimmunoprecipitated with FLAG-Nrdp1 but constructs T12 and T13 did not (Fig. 4B and C). No coimmunoprecipitation of USP8 was observed with construct T2; however, we were unable to confirm the presence of this construct in immunoprecipitates (data not shown). Deletion constructs (as shown for T6 and T9 in Fig. 4) did not precipitate with FLAG antibodies unless Nrdp1 was cotransfected. These results map the regions of USP8 responsible for Nrdp1 interaction to two domains, the catalytic domain and the rhodanese domain.

While previous studies have suggested a role for variable regions within the catalytic domain in determining UBP protein target recognition, unique sequences and domains flanking the catalytic domains are also proposed to mediate specific protein-protein interactions (58). Our observations suggest that the rhodanese domain may play a role in the specific binding of USP8 to Nrdp1. To determine whether this domain is sufficient to specifically bind Nrdp1, we created a GST fusion of the rhodanese domain (GST-rho; Fig. 5A) and used this protein to examine rhodanese binding to Nrdp1 and cbl. As illustrated in Fig. 5B, the GST-rho specifically bound to FLAG-tagged Nrdp1 and FLAG-tagged clone 32 but was not able to bind to endogenous cbl. These observations suggest that the rhodanese domain of USP8 is sufficient to mediate its specific interaction with Nrdp1.

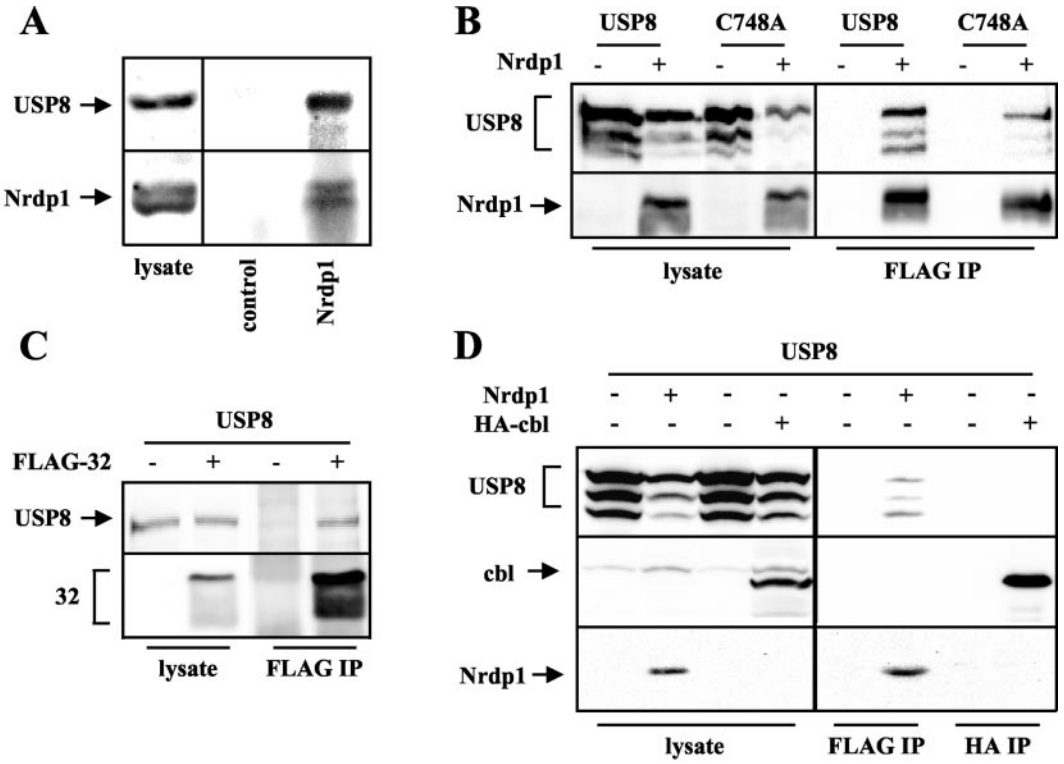

FIG. 3. Specific association of Nrdp1 and USP8 in coexpressing cells. (A) Lysates (left panels) of 293T cells were immunoprecipitated (right panels) with control rabbit antibody to mouse IgM or rabbit anti-Nrdp1 antibody and blotted with rabbit anti-USP8 (upper panels) or anti-Nrdp1 (lower panels). (B to D) COS7 cells were transfected with FLAG-tagged Nrdp1, FLAG-tagged Nrdp1 carboxy terminus (clone 32), hemagglutinin (HA)-tagged cbl, V5-tagged wild-type USP8, or V5-tagged C748A USP8 as indicated and treated with MG132 to stabilize Nrdp1 protein expression. (B and C) Lysates were immunoprecipitated with antibodies to FLAG and blotted with antibodies to V5 (upper panels) or FLAG (lower panels). (D) Lysates were immunoprecipitated with antibodies to FLAG or HA and blotted with antibodies to V5 (top panels), cbl (middle panels), or FLAG (bottom panels).

USP8 mediates Nrdp1 stabilization. Because Nrdp1 autoubiquitination promotes its degradation and because USP8 could potentially deubiquitinate Nrdp1 following its specific interaction with the ligase, we sought to determine whether USP8 could stabilize Nrdp1 protein in cells. Thus, we examined the levels of Nrdp1 protein in cells when coexpressed either with wild-type USP8 or with point mutants where the catalytic cysteine residue is changed to alanine (C748A) or serine (C748S). Figure 6A shows that wild-type Nrdp1 protein could be markedly stabilized by wild-type USP8 but not by either mutant. The C34S/H36Q Nrdp1 mutant was intrinsically stable, and its stability was not significantly enhanced by USP8. These observations indicate that USP8 mimics the C34S/H36Q mutation by stabilizing the Nrdp1 protein and that USP8 deubiquitinating activity is necessary for this stabilization.

Interestingly, expression of the inactive forms of USP8 was lower when they were coexpressed with wild-type Nrdp1 but not with the C34S/H36Q mutant. These observations are consistent with a model where Nrdp1 E3 ligase activity can act on USP8 and USP8 can protect itself from degradation via its deubiquitinating activity. An alternative possibility is that binding of the catalytically inactive form to Nrdp1 is prolonged relative to that of wild-type USP8 and serves as a more efficient substrate. USP2, the mammalian ubiquitin-specific protease that exhibits the highest amino acid sequence homology to USP8 in its catalytic domain, was not able to stabilize Nrdp1 (Fig. 6B), indicating that Nrdp1 stabilization is a property unique to USP8.

Since USP8 deubiquitinating activity is required for Nrdp1 stabilization, it might be predicted that the overexpression of the USP8 C748A mutant could act in a dominant-negative manner to destabilize endogenous Nrdp1. To test this, we transfected 293T cells with vector alone or with vector expressing the mutant and analyzed levels of endogenous Nrdp1 by blotting cell lysates. In this experiment we cotransfected all cells with a plasmid that confers puromycin resistance so that transfectants could be rapidly selected with puromycin. As shown in Fig. 6C, overexpression of USP8 mutant resulted in the loss of endogenous Nrdp1 but not actin. These observations strongly suggest that Nrdp1 levels are maintained by endogenous USP8 in these cells.

To examine the relationship between USP8 rhodanese-mediated binding and Nrdp1 stabilization, we assessed Nrdp1 levels when the ligase was coexpressed with each of the USP8 truncation mutants described above. As shown in Fig. 7, constructs T2 through T5 and T11 through T13 were unable to mediate Nrdp1 stabilization. Constructs T6 and T7 mediated strong Nrdp1 stabilization, and constructs T8 through T10 mediated modest Nrdp1 stabilization. A comparison of the binding and stabilization properties of the USP8 truncation

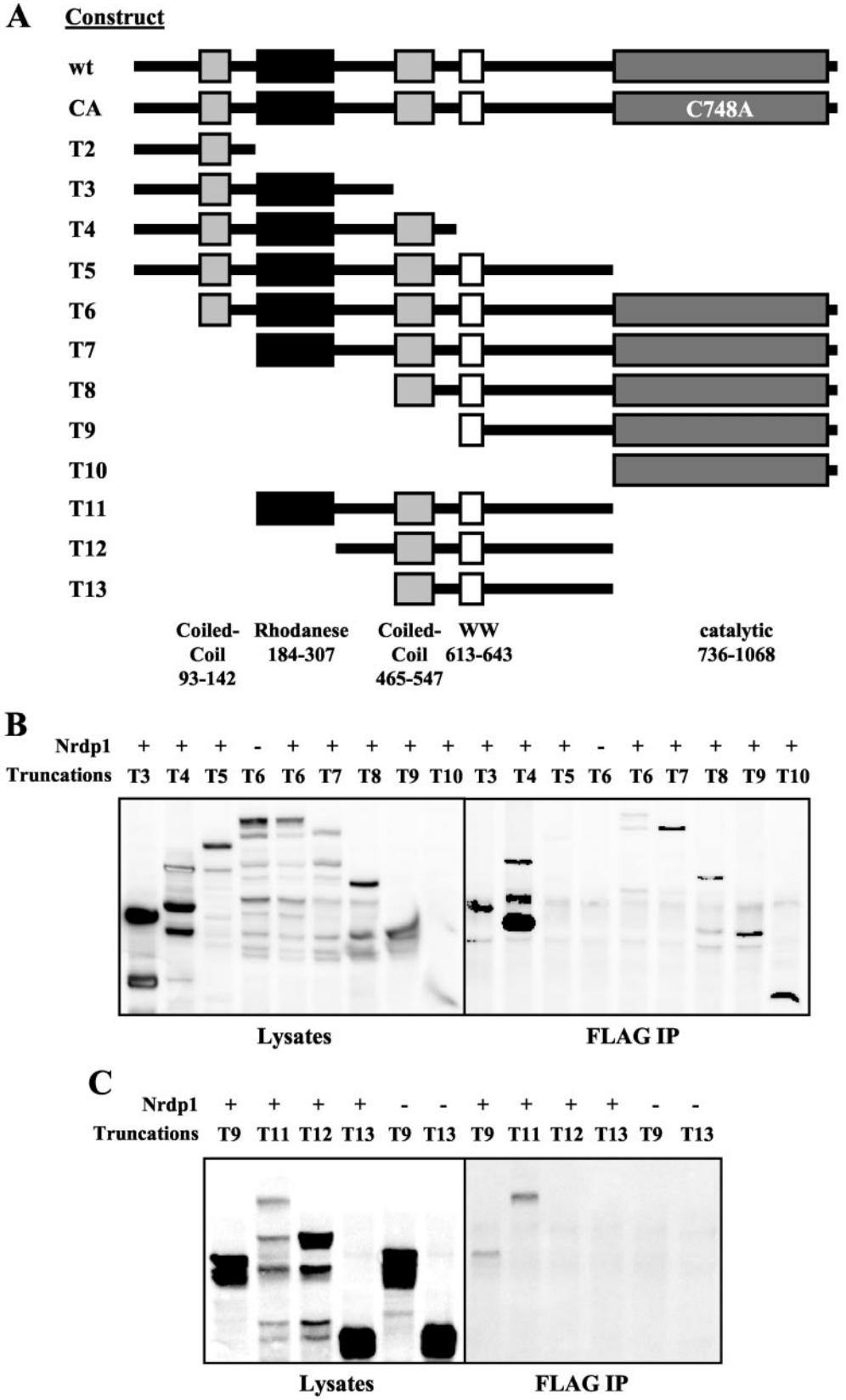

FIG. 4. USP8 binding to Nrdp1 is mediated by the catalytic and rhodanese domains. (A) Domain structure of USP8 mutants. wt, wild type. (B and C) Coimmunoprecipitation of USP8 with Nrdp1. COS7 cells were transfected with wild-type Nrdp1 (FLAG-tagged) and USP8 (V5-tagged) truncation mutants as indicated. Lysates from MG132-treated cells were immunoprecipitated with antibodies to FLAG, and lysates and precipitates were blotted with antibodies to V5.

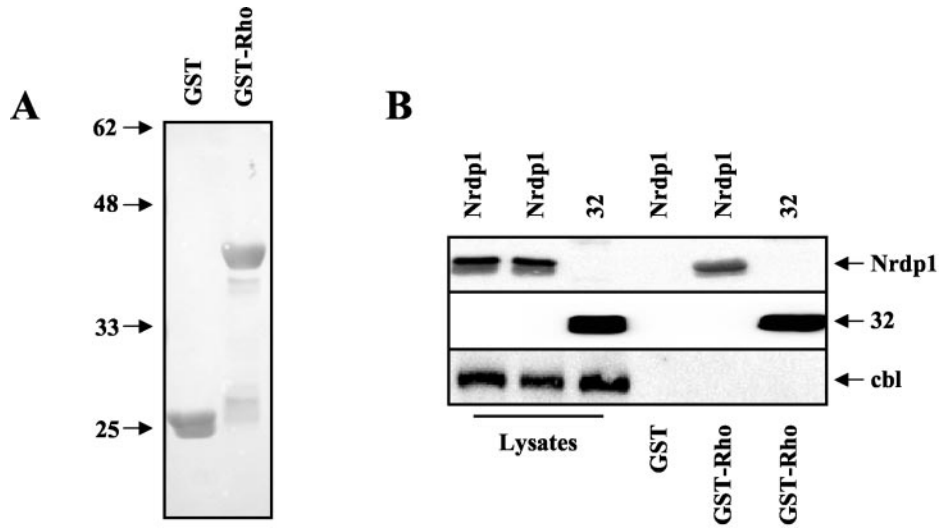

FIG. 5. USP8 rhodanese domain is sufficient to bind Nrdp1. (A) Coomassie blue stain of purified GST and GST-rho. Numbers at left are molecular masses in kilodaltons. (B) USP8 rhodanese domain binds to the carboxy-terminal region of Nrdp1. COS7 cells were transfected with FLAG-tagged wild-type Nrdp1 or clone 32 and treated with MG132 to stabilize Nrdp1 expression, and lysates were incubated with 5 μg of GST or GST-rho immobilized on Sepharose beads, as indicated. Proteins bound to washed beads were eluted, and lysates and eluates were blotted with anti-FLAG or anti-cbl.

mutants is depicted in Table 1. These observations indicate that, while the USP8 catalytic domain itself is moderately capable of mediating Nrdp1 stabilization, full stability requires Nrdp1 association through the rhodanese domain.

DISCUSSION

Previous studies have implicated Nrdp1 as a E3 ubiquitin ligase involved in the ligand-independent maintenance of steady-state levels of the neuregulin receptor tyrosine kinases ErbB3 and ErbB4 (15, 46) and in the maintenance of cellular levels of the antiapoptotic protein BRUCE (47). Here we demonstrate that the Nrdp1 protein is highly labile and that point mutations in Nrdp1 that eliminate its ubiquitin ligase activity also significantly stabilize the protein. This is consistent with previous observations that autoubiquitination of E3 ligases, for example, Mdm2 (59), influences their intrinsic stability in cells. Moreover, we demonstrate that the multidomain UBP deubiquitinating enzyme USP8 specifically interacts with and stabilizes Nrdp1. The simplest explanation for these observations is as follows. At low levels of USP8 binding or activity, constitutive Nrdp1 autoubiquitination efficiently targets the protein for degradation by proteasomes, resulting in low levels of the Nrdp1 protein. High levels of functional USP8 mediate Nrdp1 deubiquitination and stabilization.

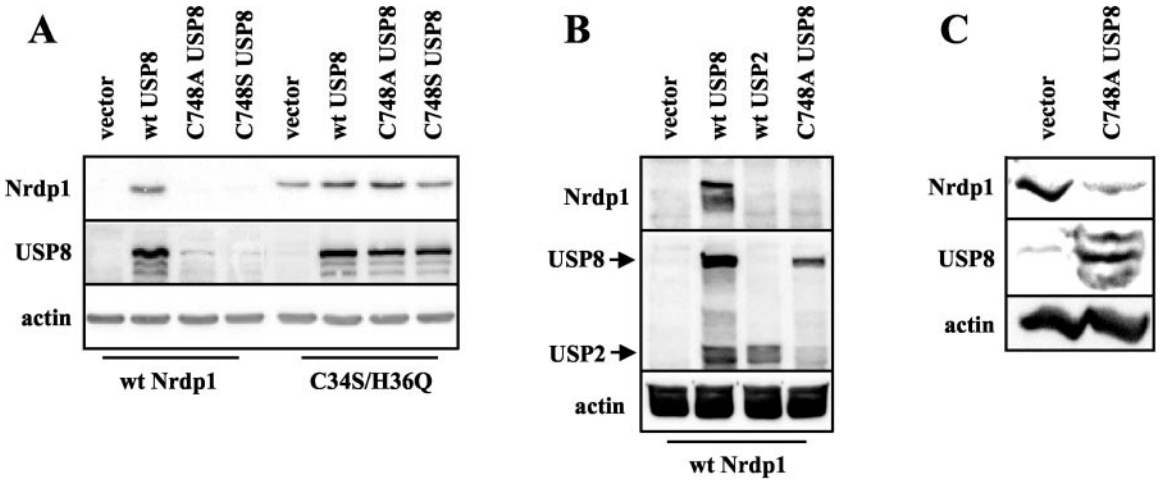

FIG. 6. Stabilization of Nrdp1 by USP8 requires USP8 deubiquitinating activity. (A and B) COS7 cells were transfected with wild-type or C34S/H36Q FLAG-Nrdp1 and cotransfected with the indicated V5-tagged USP constructs. Lysates were immunoblotted with antibodies to FLAG, V5, and actin. (A) USP8 deubiquitinating activity is required for Nrdp1 stabilization. (B) USP2 is incapable of stabilizing Nrdp1. (C) Catalytically inactive USP8 destabilizes endogenous Nrdp1. 293T cells were transfected with vector or USP8CA, and lysates were blotted with antibodies to Nrdp1, USP8, or actin.

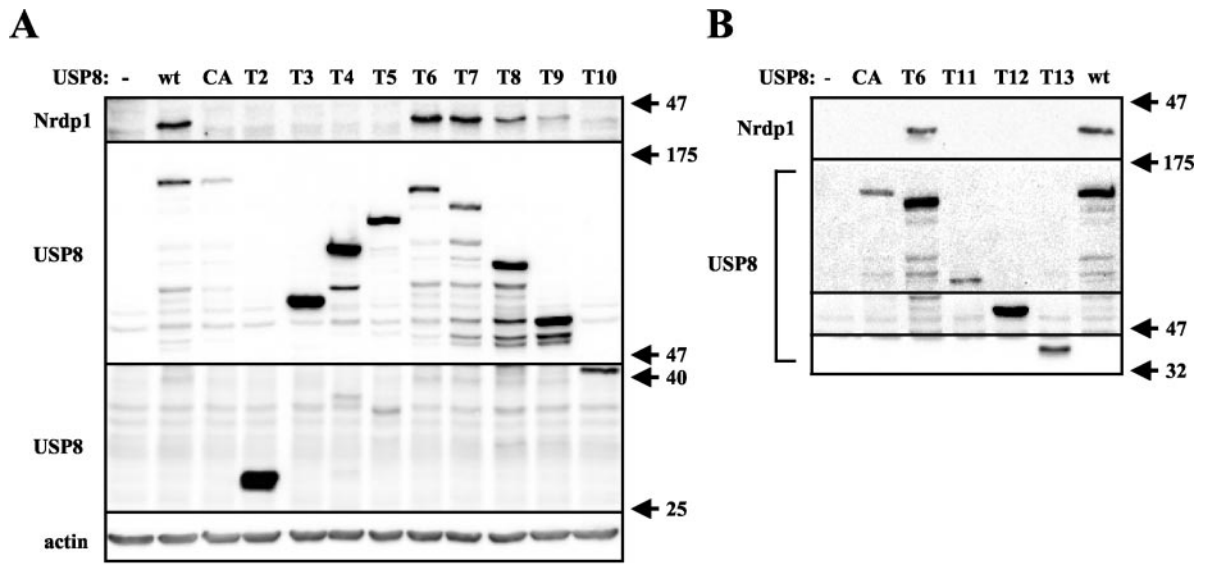

FIG. 7. Nrdp1 stabilization by USP8 is facilitated by rhodanese domain binding. COS7 cells were transfected with FLAG-tagged wild-type Nrdp1 and cotransfected with the indicated V5-tagged USP8 constructs. Lysates were blotted with antibodies to FLAG, V5, and actin. (A) Effect of truncation constructs CA and T2 through T10 on Nrdp1 stability. (B) Effect of constructs T11 through T13. Numbers at right of each panel are molecular masses in kilodaltons.

Interestingly, Nrdp1 may also ubiquitinate USP8 to mediate its destruction, an activity unmasked with the USP8 catalytic cysteine point mutants. Hence, Nrdp1 may be capable of mediating the destruction of itself and USP8 through its ubiquitin ligase activity, and USP8 could be capable of preserving itself and Nrdp1 through its deubiquitinating activity. When coupled with the coimmunoprecipitation data and our unpublished observations that Nrdp1 mediates the subcellular relocalization of USP8, these results underscore the notion that the functional form of the two proteins is a complex.

While our model emphasizes the role of USP8 in determining Nrdp1 levels, the mechanism by which the formation or activity of the Nrdp1/USP8 complex is regulated in cells remains an important question. One mode of regulation of the complex could be at the level of USP8 expression, through transcriptional regulation or through regulation of its half-life or localization. Alternatively, the binding of USP8 to Nrdp1 may be a regulated step that is influenced by posttranslational events such as phosphorylation, ubiquitination, or binding to other cellular factors. Finally, it is possible that the catalytic activity of USP8 is regulated.

TABLE 1. Summary of Nrdp1 binding and stabilization by USP8 mutants[a]

Construct	Nrdp1 binding	Nrdp1 stabilization
WT	+	+
CA	+	−
T2	?	−
T3	+	−
T4	+	−
T5	+	−
T6	+	+
T7	+	+
T8	+	−/+
T9	+	−/+
T10	+	−/+
T11	+	−
T12	−	−
T13	−	−

[a] WT, wild type; +, strong binding or stabilization; −, no binding or stabilization; ?, could not be determined; −/+, weak stabilization.

The catalytic and rhodanese domains of USP8 mediate its interaction with Nrdp1. As catalytic domains in UBPs exhibit marked divergence that may confer substrate specificity (58), this domain of USP8 probably contributes to Nrdp1 recognition as a target for deubiquitination. The USP8 rhodanese domain is sufficient to specifically recognize Nrdp1 and may be responsible for maintaining a stable complex between the two proteins. Rhodanese domains are ubiquitous structural modules conserved from bacteria to humans whose functions at present are unclear. A subset of rhodanese domains possesses sulfurtransferase activity, while noncatalytic versions are found in proteins of various functions including eukaryotic deubiquitinating enzymes and mitogen-activated protein (MAP) kinase phosphatases (7). Our observations suggest that noncatalytic rhodanese domains mediate specific protein-protein interactions. This conclusion is consistent with previous observations indicating that the amino-terminal region of the MAP kinase phosphatase MKP-3 encompassing its rhodanese domain is responsible for binding to MAP kinases Erk1 and Erk2 (39). Fine mapping of the rhodanese binding site on Nrdp1 could shed light on sequence motifs selected by rhodanese domains.

Although the large number and divergence of mammalian UBPs strongly suggest that these enzymes have specific substrates and distinct biological activities, substrates for only a few have been described (58). HAUSP (USP7) mediates the deubiquitination and stabilization of p53 (33), reversing the action of the Mdm2 E3 ubiquitin ligase and enhancing p53 tumor suppressor function. CYLD is a deubiquitinating en-

zyme that is mutated in familial cylindromatosis syndrome, where patients are predisposed to tumors of skin appendages (6). CYLD interacts with the Nemo regulatory component of the Iκ kinase complex and negatively regulates activation of the transcription factor NF-κB in response to specific tumor necrosis factor receptors by suppressing the K63 polyubiquitination and activation of TRAF2 and TRAF6 (8, 31, 55).

fam (USP9), the mouse homolog of the *D. melanogaster* fat facets gene, colocalizes and interacts with AF-6 (52) and β-catenin (53) at sites of cell-cell contact in cultured epithelial cells. Together with data indicating that AF-6 and β-catenin protein levels are suppressed by fam loss in developing embryos (41), these observations suggest that fam-mediated deubiquitination may contribute to the stability of a subset of proteins at cell adhesion sites. Analogous to its function in *Drosophila* (26), fam also mediates the deubiquitination of epsin 1 (9), a protein involved in membrane trafficking. Hence, it appears that a single UBP is capable of mediating the deubiquitination of distinct sets of proteins.

The interaction of deubiquitinating enzymes with E3 ubiquitin ligases has been reported previously, but the functional consequences are unclear. The deubiquitinating enzyme UnpEL was isolated in a screen for proteins that interact with SSA/Ro (16), a putative E3 ubiquitin ligase of unknown function with an overall domain structure similar to that of Nrdp1. Analogous to Nrdp1, SSA/Ro coexpression in cells mediates the redistribution of UnpEL; however, this study made no attempt to assess UnpEL impact on SSA/Ro stabilization. Association of the deubiquitinating enzyme BAP1 with the RING finger domain of the E3 ubiquitin ligase BRCA1 has been demonstrated to enhance the tumor suppressor activity of the BRCA1/BARD1 complex in cells (27). Although autopolyubiquitination enhances the E3 ligase activity of the complex in vitro, BAP1 may not function in the deubiquitination of the complex (37) but target other substrates.

Previous studies have implicated a role for USP8 in cellular growth regulation. USP8 message accumulates upon growth stimulation of serum-starved human fibroblasts, and its levels decrease in response to growth arrest induced by cell-cell contact. Moreover, antisense oligonucleotides prevented fibroblasts from entering S phase, suggesting that USP8 expression is necessary for normal cell cycling (40). Similar to our observations, USP8 has been shown to interact with and stabilize the RING finger E3 ubiquitin ligase GRAIL to mediate T-cell anergy (49). GRAIL is localized to the transferring recycling endocytic pathway (4), suggesting a function for USP8 in membrane protein trafficking. Mouse USP8 has been previously demonstrated to interact with the brain-specific ras guanine nucleotide exchange factor cdc25mm and coexpression of USP8-mediated cdc25mm deubiquitination and stabilization (19). Interestingly, USP8 has been also been demonstrated to interact with the Hrs binding protein Hbp/STAM2A (28), a member of the EAST/STAM/hbp family of adapter proteins. Hbp/STAM2A plays roles in receptor endo- and exocytosis and probably also in the regulation of actin cytoskeleton (36). Hence, while the overall function of USP8 is unclear, several lines of evidence point to its involvement in disparate cellular pathways that contribute to cellular growth regulation.

Finally, the overall domain structure of USP8 is similar to that of a rhodanese domain-containing subfamily of *Saccharomyces cerevisiae* deubiquitinating enzymes that includes yeast Ubp4/Doa4, Ubp5, and Ubp7. Of these only Ubp4/Doa4 has been characterized in any detail. Ubp4/Doa4 associates with proteasomes, is required for efficient proteasome activity, and promotes proteolysis through the removal of ubiquitin from proteolytic intermediates upon substrate breakdown (42, 43). Hence, Ubp4/Doa4 may play a role in ubiquitin homeostasis by recycling ubiquitin for reuse (50). Evidence has also accumulated that Ubp4/Doa4 plays a necessary role in the deubiquitination of plasma membrane proteins prior to their degradation in yeast vacuoles (3, 18). Taken together, these observations indicate that Ubp4/Doa4 plays a central role in cytosolic and membrane protein degradation by mediating the deubiquitination of target proteins prior to full degradation. It should be noted, however, that yeast Ubp5 cannot substitute for Ubp4/Doa4, suggesting that the rhodanese domain-containing deubiquitinating enzymes in yeast harbor distinct functions. Therefore, USP8, the only rhodanese domain-containing deubiquitinating enzyme encoded by the human genome, may be functionally distinct from Ubp4/Doa4.

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