The E2–E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain

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We dissected physical and functional interactions between the ubiquitin-conjugating (E2) enzyme Ubc2p and Ubr1p, the E3 component of the N-end rule pathway in *Saccharomyces cerevisiae*. The binding of the 20 kDa Ubc2p by the 225 kDa Ubr1p is shown to be mediated largely by the basic residue-rich (BRR) region of Ubr1p. However, mutations of the BRR domain that strongly decrease the interaction between Ubr1p and Ubc2p do not prevent the degradation of N-end rule substrates. In contrast, this degradation is completely dependent on the RING-H2 finger of Ubr1p adjacent to the BRR domain. Specifically, the first cysteine of RING-H2 is required for the ubiquitylation activity of the Ubr1p–Ubc2p complex, although this cysteine plays no detectable role in either the binding of N-end rule substrates by Ubr1p or the physical affinity between Ubr1p and Ubc2p. These results defined the topography of the Ubc2p–Ubr1p interaction and revealed the essential function of the RING-H2 finger, a domain that is present in many otherwise dissimilar E3 proteins of the ubiquitin system.

Keywords: E2/E3/N-end rule/proteasome/RING finger/ubiquitin

Introduction

Ubiquitin (Ub) is a 76 residue protein whose covalent conjugation to other proteins, usually in the form of a multi-Ub chain, marks these proteins for processive degradation by the 26S proteasome, an ATP-dependent multisubunit protease (Hochstrasser, 1996; Varshavsky, 1997; Baumeister *et al.*, 1998; Herskho and Ciechanover, 1998; Scheffner *et al.*, 1998). Through either constitutive or conditional degradation of many intracellular proteins, the Ub-dependent proteolytic pathways regulate a multitude of biological processes, including the cell cycle, cell differentiation, apoptosis, DNA transcription, replication and repair, signal transduction, functions of the nervous system and stress responses, including the immune response (Hicke, 1997; Varshavsky, 1997; Peters *et al.*, 1998).

The conjugation of Ub to other proteins involves the formation of a thioester between the C-terminus of Ub and a specific cysteine of the Ub-activating (E1) enzyme. The Ub moiety of E1~Ub thioester is thereafter transesterified to a specific cysteine in one of several Ub-conjugating (E2) enzymes. The Ub moiety of E2~Ub thioester is

conjugated, via the isopeptide bond, to the ϵ -amino group of either a substrate's Lys residue or a Lys residue of another Ub moiety, the latter reaction resulting in a substrate-linked multi-Ub chain (Chau *et al.*, 1989; Pickart, 1997; Scheffner *et al.*, 1998). Most E2 enzymes function in complexes with proteins called E3s. The functions of E3s include the initial recognition of degradation signals (degrons) in the substrate proteins, with different E3s recognizing different classes of degrons. At least some E3s, specifically those containing the HECT domain, accept the Ub moiety from the associated E2~Ub thioester, forming an E3~Ub thioester and acting as a proximal donor of the Ub moiety to substrates selected by the E3 (Scheffner *et al.*, 1995; Nuber and Scheffner, 1999; Wang *et al.*, 1999).

One pathway of the Ub system is the N-end rule pathway (Bachmair *et al.*, 1986; Varshavsky, 1996). Among the targets of this pathway are proteins bearing destabilizing N-terminal residues. In the yeast *Saccharomyces cerevisiae*, Asn and Gln are tertiary destabilizing N-terminal residues, in that they function through their conversion, by the *NTA1*-encoded N-terminal amidase, into the secondary destabilizing N-terminal residues Asp and Glu. The destabilizing activity of N-terminal Asp and Glu requires their conjugation, by the *ATE1*-encoded Arg-tRNA-protein transferase, to Arg, one of the primary destabilizing residues (Balzi *et al.*, 1990; Baker and Varshavsky, 1995). In mammals, the deamidation step is bifurcated, in that two distinct amidases specific, respectively, for N-terminal Asn or Gln, mediate the activity of tertiary destabilizing residues (Stewart *et al.*, 1995; Grigoryev *et al.*, 1996). The primary destabilizing N-terminal residues are bound directly by the *UBR1*-encoded E3 (N-recogin), the recognition component of the N-end rule pathway. In *S.cerevisiae*, Ubr1p is a 225 kDa protein that binds to potential N-end rule substrates through their primary destabilizing N-terminal residues Phe, Leu, Trp, Tyr, Ile, Arg, Lys and His (Bartel *et al.*, 1990). Ubr1p contains at least three substrate-binding sites. The type 1 site is specific for basic N-terminal residues Arg, Lys and His. The type 2 site is specific for the hydrophobic residues Phe, Leu, Trp, Tyr and Ile (Reiss *et al.*, 1988; Gonda *et al.*, 1989; Baker and Varshavsky, 1991). Ubr1p also targets short-lived proteins such as Cup9p and Gpa1p, which lack destabilizing N-terminal residues, and are recognized by Ubr1p through its third substrate-binding site, the exact location and specificity of which remain to be determined (Madura and Varshavsky, 1994; Byrd *et al.*, 1998). Similar but distinct versions of the N-end rule pathway are present in all organisms examined, from bacteria to mammals. For a summary of the currently known functions of this pathway, see Kwon *et al.* (1999a,b).

The roles of E3 proteins in the E2, E3-dependent ubiquitylation of substrates are not understood in detail.

It has been shown that the *UBR1*-encoded N-recognin, the E3 of the N-end rule pathway, functions in a complex with Ubc2p (Rad6p) (Madura *et al.*, 1993; Watkins *et al.*, 1993), one of 13 E2 enzymes in *S.cerevisiae* (Hochstrasser, 1996). The polyacidic C-terminal tail of Ubc2p was found to be essential for the binding of Ubc2p to Ubr1p, but the Ubc2p-binding site of Ubr1p remained unknown. The mechanism of cooperation between E3 (Ubr1p) and E2 (Ubc2p) in forming a substrate-linked multi-Ub chain is unknown for either this or other Ub-dependent pathways.

We report two main results. (i) The Ubr1p–Ubc2p interaction is mediated by the basic residue-rich (BRR) region of Ubr1p and the polyacidic tail of Ubc2p. Interestingly, this high-affinity interaction is not essential for the activity of the N-end rule pathway. (ii) The RING-H2 domain of Ubr1p, adjacent to the BRR region, is strictly required for ubiquitylation of N-end rule substrates, even though this domain is not required for the binding of Ubr1p to either Ubc2p or N-end rule substrates. Because the RING-H2 and similar RING variants are a characteristic feature of many otherwise dissimilar E3 proteins, the ubiquitylation-enabling function of the Ubr1p RING-H2 identified in the present work is likely to be a general property of E3 components in the Ub system.

Terminology

Ubiquitin whose C-terminal (Gly76) carboxyl group is covalently linked to another compound is called the *ubiquityl* moiety, the derivative terms being *ubiquitylation* and *ubiquitylated*. The term Ub refers to both free ubiquitin and the ubiquityl moiety. This nomenclature, which is also recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, brings Ub-related terms in line with the standard chemical terminology (Varshavsky, 1997).

Results

Dissection of the Ubr1p–Ubc2p interaction

To search for Ubc2p-interacting proteins, we employed the two-hybrid technique (Fields and Song, 1989; James *et al.*, 1996), using the 20 kDa Ubc2p as bait. A plasmid thus identified contained a fragment of the *S.cerevisiae* open reading frame (ORF) YLR024C, which was previously termed *UBR2* (Hochstrasser, 1996; Kwon *et al.*, 1998), because the predicted sequence of the 216 kDa Ubr2p was 22% identical and 46% similar to that of the 225 kDa, *UBR1*-encoded N-recognin, the E3 of the N-end rule pathway (Bartel *et al.*, 1990). Ubr2p is not essential for cell viability under standard growth conditions and does not target N-end rule substrates, but does compete with Ubr1p for the binding to Ubc2p (H.Rao, Y.Xie and A.Varshavsky, unpublished data).

The cloned Ubc2p-interacting region of Ubr2p was a 287 residue fragment (positions 1134–1420). Sequence comparisons revealed two distinct motifs conserved between Ubr1p and Ubr2p in this region (Figure 1A). One was a basic residue-rich region, termed BRR, and the other a RING-H2 finger (Saurin *et al.*, 1996; Kwon *et al.*, 1998). The RING-H2 motif is a distinct variant of the previously defined Cys/His-rich RING motif, which is thought to be involved in protein–protein interactions (Borden and Freemont, 1996; Inouye *et al.*, 1997). Two-

hybrid analyses indicated that Ubc2p interacted with the Ubr2p-homologous region of Ubr1p as well (positions 1081–1367) (Figure 1C and D). In addition, two-hybrid assays with a series of Ubr1p fragments fused to the Gal4p transcriptional activation domain demonstrated that the 287 residue region was the only detectable Ubc2p-binding site in Ubr1p (data not shown). We used site-directed mutagenesis to dissect the Ubc2p–Ubr1p interaction in this region of Ubr1p. Two sets of mutants were constructed (Figure 1B). The mutants of one set, termed MB (mutation in BRR), bore a missense mutation(s) in the BRR domain; the mutants of another set, termed MR (mutation in RING-H2) bore either Cys→Ser or His→Ala mutations in the RING-H2 finger. These derivatives of the 287 residue region of Ubr1p were tested for their binding to Ubc2p using a version of the two-hybrid assay that utilized two reporter genes, *ADE2* and *HIS3* (James *et al.*, 1996). This design increased the assay's affinity range, because a strong two-hybrid interaction was required to confer on the tester cells the Ade⁺ phenotype, whereas a weak two-hybrid interaction sufficed to make the same tester cells His⁺ (James *et al.*, 1996).

The replacement of a single basic residue at various positions in the BRR domain of Ubr1p with uncharged residues (e.g. the alleles MB10, MB11 and MB17) significantly weakened the interaction of Ubc2p with the 287 residue fragment of Ubr1p, as indicated by the Ade[−] His⁺ phenotype of the tester strain, in comparison with the Ade⁺ His⁺ phenotype observed with the fragment's wild-type (wt) version (Figure 1C). When two basic residues of the BRR domain were converted to uncharged residues (MB4 allele), the resulting tester strain was still His⁺, but grew more slowly on histidine-lacking medium than did the tester strain bearing single-substitution Ubr1p mutants (Figure 1C; data not shown), suggesting a further weakening of the Ubc2p–Ubr1p interactions. With triple-substitution mutants in the BRR domain of Ubr1p (MB5 and MB6), the affinity of Ubc2p for the 287 residue region of Ubr1p decreased to levels undetectable with the two-hybrid assay (Figure 1C).

In contrast to these findings with the BRR domain, none of the four single Cys→Ser substitutions in the adjacent RING-H2 finger of Ubr1p impaired the interaction of Ubc2p with the 287 residue region of Ubr1p (Figure 1C, mutants MR1–MR3 and MR18). A His→Ala mutation in the RING-H2 finger was also without effect (Figure 1C, mutant MR12). Moreover, even the simultaneous Cys→Ser alterations at two positions of the RING-H2 finger failed to affect the Ade⁺ His⁺ phenotype of the tester strain (Figure 1C, mutants MR7 and MR8). The 287 residue region of Ubr1p was then subcloned into two fusions, which contained, respectively, the BRR domain alone (positions 1081–1220) and the RING-H2 finger alone (positions 1177–1367). The RING-H2 finger alone did not exhibit affinity to Ubc2p (Figure 1D). In contrast, the binding of Ubc2p to the BRR domain alone was readily detectable, but this interaction was weakened in comparison with the interaction between Ubc2p and the 287 residue region of Ubr1p containing both BRR and RING-H2 (Figure 1D).

Taken together, these results indicated that the positively charged BRR domain is the main region mediating the binding of Ubr1p to Ubc2p, through largely electrostatic

Fig. 1. Ubc2p binds to the BRR domain of Ubr1p. (A) Sequence comparison of the Ubc2p-binding domains of *S.cerevisiae* Ubr1p and Ubr2p. Identical residues are highlighted in black; similar residues are shaded. The conserved and similar basic residues are marked by '+'. The Cys and His residues of the RING-H2 motif are denoted by asterisks. (B) Schematic representation of mutations in the BRR and RING-H2 domains of Ubr1p. The 287 residue region of the 1950 residue Ubr1p (positions 1081–1367) was divided into two regions containing the BRR domain and the RING-H2 finger. Mutations of basic residues, termed the MB mutations, in the BRR domain were at positions 1165–1175 and are underlined in bold. Mutations of either Cys or His residues in the RING-H2 finger, termed the MR mutations, were at positions 1220–1323 and are underlined in bold. The positions of mutated Cys and His residues within RING-H2 are listed in parentheses. (C) Summary of the two-reporter (*HIS3* and *ADE2*) two-hybrid results with the MB and MR mutants of Ubr1p versus wt Ubc2p. See the text and Materials and methods for details. (D) wt Ubc2p and its two truncated alleles, Ubc2p₁₋₁₄₉ and Ubc2p₁₀₋₁₇₂, were assayed in the two-reporter/two-hybrid system for their interaction with either the 287 residue Ubr1p fragment, or its BRR domain alone, or its RING-H2 domain alone. The 9 residue N-terminal region and the polyacidic C-terminal tail of Ubc2p are denoted by the black and hatched boxes, respectively.

interactions of the BRR region with the polyacidic C-terminal tail of Ubc2p (see below). These findings also indicated that the presence of the RING-H2 finger near the BRR domain was not essential for the binding of Ubc2p but was required (in an undefined way; but see below) for the high-affinity Ubr1p–Ubc2p interaction (Figure 1B–D). In addition, either single or double Cys→Ser substitutions in the RING-H2 finger did not appear to result in a gross conformational alteration of the BRR/RING-H2 region of Ubr1p, as indicated by the undiminished affinity of mutants in this region for Ubc2p (Figure 1C); further evidence for this is described below.

Previous work has shown that both the N-terminal 9 residue region and the 23 residue polyacidic C-terminal tail of Ubc2p are required for the binding of Ubc2p to Ubr1p (Madura *et al.*, 1993; Watkins *et al.*, 1993). To determine the relative contributions of these Ubc2p regions to the interaction between Ubc2p and the BRR domain of

Ubr1p, we constructed two hybrid fusions containing two truncated alleles of *UBC2* which encoded, respectively, Ubc2p₁₀₋₁₇₂ (lacking residues 1–9) and Ubc2p₁₋₁₄₉ (lacking the polyacidic tail). These derivatives of Ubc2p were tested in the two-hybrid assay for binding to the entire 287 residue region of Ubr1p, or to the BRR domain alone, or to the RING-H2 finger alone. The deletion of the polyacidic C-terminal tail of Ubc2p abolished its interaction with the BRR domain of Ubr1p, whereas the deletion of the first 9 residues of Ubc2p weakened but did not abolish this interaction (Figure 1D). Interestingly, the latter interaction, weakened but readily detectable, was independent of the presence of the RING-H2 finger (Figure 1D). Thus, the bulk of the affinity of Ubc2p for the 287 residue fragment of Ubr1p stems from electrostatic interactions between the polyacidic C-terminal tail of Ubc2p and the positively charged, ~140 residue BRR domain of the 1950 residue Ubr1p. (The size of a

Fig. 2. The BRR domain of Ubr1p is essential for high-affinity binding to Ubc2p. (A) GST pull-down assays with wt Ubr1p and its mutants. FLAG-Ubr1p (denoted as wt UBR1) (lane 2), or FLAG-Ubr1p^{MR1} (lane 3) or FLAG-Ubr1p^{MR2} (lane 4), or FLAG-Ubr1p^{MB6} (lane 5) were overexpressed in AVY301 (*ubr1Δ ubc2Δ*) cells (see Materials and methods). Cells were labeled with [³⁵S]methionine for 30 min at 30°C, and the extracts were assayed using glutathione-agarose beads bound with GST-Ubc2p (top panel) or applied to immunoprecipitation with anti-FLAG monoclonal antibody (lower panel). (B) *In vivo* association of Ubc2p with wt Ubr1p, Ubr1p^{MR1} and Ubr1p^{MR2}, but not with Ubr1p^{MB6}. Cell extracts from transformants expressing FLAG-Ubr1p alone (lane 1) or the N-terminally HA-tagged Ubc2p (HA-Ubc2p) alone (lane 2), or co-expressing HA-Ubc2p with either FLAG-Ubr1p (lane 3) or FLAG-Ubr1p^{MB6} (lane 4), FLAG-Ubr1p^{MR1} (lane 5) or FLAG-Ubr1p^{MR2} (lane 6) were incubated with anti-HA antibody (see Materials and methods). Co-precipitated wt Ubr1p and its mutant derivatives were resolved by SDS-PAGE, followed by immunoblotting with anti-FLAG antibody (top panel). The asterisk marks a cross-reactive band. The expression levels of Ubr1p and Ubc2p in these transformants were examined by immunoblotting with, respectively, anti-FLAG (middle panel) and anti-HA antibodies (bottom panel). SDS-PAGE was carried out in 8% gels.

contiguous region within the BRR domain that is required for the binding of Ubc2p is likely to be significantly less than 140 residues.)

To determine whether basic residues of the BRR domain and the RING-H2 finger were essential for the binding of the full-length Ubr1p to Ubc2p, we first employed a pull-down assay with a fusion of Ubc2p to glutathione *S*-transferase (GST). Specifically, wt FLAG-Ubr1p (tagged at the N-terminus with the FLAG epitope), or FLAG-Ubr1p^{MB6} (a triple-substitution mutant in the BRR domain; Figure 1B), or FLAG-Ubr1p^{MR1} or FLAG-Ubr1p^{MR2} (a single Cys→Ser substitution at Cys1 and Cys5, respectively; Figure 1B) was overexpressed in a *ubr1Δ ubc2Δ S.cerevisiae* strain and labeled with [³⁵S]methionine. The N-terminal FLAG epitope did not impair the function of Ubr1p (A.Webster and A.Varshavsky, unpublished data). The extracts from labeled, Ubr1p-overexpressing cells (and the extract from cells carrying vector alone) were incubated with glutathione-agarose beads loaded with GST-Ubc2p, and the amounts of ³⁵S-labeled FLAG-Ubr1p and Ubr1p mutants recovered from the beads were determined by SDS-PAGE (Figure 2A, top panel). In parallel, the same extracts were immunoprecipitated with a monoclonal anti-FLAG antibody, to verify equality of the initial inputs of FLAG-Ubr1p and its mutants (Figure 2A, lower panel). In agreement with the results of the two-hybrid assays (Figure 1B and C), the binding

of FLAG-Ubr1p^{MB6} to Ubc2p was barely detectable (Figure 2A, top panel, lane 5), whereas the binding of either FLAG-Ubr1p^{MR1} or FLAG-Ubr1p^{MR2} to Ubc2p was comparable to that of wt FLAG-Ubr1p (Figure 2A, top panel, lanes 2–4).

We also examined the *in vivo* association of wt Ubr1p and its mutants with Ubc2p, using co-immunoprecipitation-immunoblot assays. Either wt FLAG-Ubr1p, FLAG-Ubr1p^{MB6}, FLAG-Ubr1p^{MR1} or FLAG-Ubr1p^{MR2} was co-overexpressed in a *ubr1Δ ubc2Δ S.cerevisiae* with Ubc2p bearing the N-terminal hemagglutinin (HA) epitope. Controls included congenic cells expressing FLAG-Ubr1p alone or Ubc2p alone. Proteins were immunoprecipitated from cell extracts with anti-HA antibody, followed by SDS-PAGE and immunoblotting with anti-FLAG antibody (Figure 2B, top panel). The expression levels of Ubr1p and Ubc2p in these transformants were monitored by immunoblotting the initial extracts with either anti-FLAG antibody (Figure 2B, middle panel) or anti-HA antibody (Figure 2B, bottom panel). In agreement with the results of two-hybrid and pull-down assays, the *in vivo* association of FLAG-Ubr1p^{MB6} with Ubc2p was undetectable (lane 4), whereas both FLAG-Ubr1p^{MR1} and FLAG-Ubr1p^{MR2} bound Ubc2p as efficiently as wt FLAG-Ubr1p (lanes 3, 5 and 6). Thus, the BRR domain of Ubr1p is essential for the binding of Ubr1p to Ubc2p, whereas the adjacent RING-H2 finger of Ubr1p is not directly involved (Figures 1B, C and 2).

High affinity between Ubr1p and Ubc2p is not essential for degradation of N-end rule substrates

We asked whether the MB-type point mutations in the BRR domain of Ubr1p that virtually abolished the binding of Ubr1p to wt Ubc2p (Figures 1B, C and 2) affected the activity of the N-end rule pathway. wt Ubr1p and the BRR-domain mutants Ubr1p^{MB4} and Ubr1p^{MB6} (Figure 1C and D) were expressed in *ubr1Δ* cells from a low-copy plasmid and the native P_{UBR1} promoter. The *ubr1Δ* cells also expressed an N-end rule substrate, either Arg-βgal or Leu-βgal, which are short-lived in *UBR1* cells (*t*_{1/2} of ~2 and ~3 min, respectively) (Varshavsky, 1996). Arg-βgal and Leu-βgal were expressed as Ub-Arg-βgal and Ub-Leu-βgal, which were co-translationally deubiquitylated *in vivo* (Varshavsky, 1996). Previous work has also shown that the enzymatic activity of an N-end rule substrate such as X-βgal is a sensitive measure of the substrate's metabolic stability *in vivo* (Madura *et al.*, 1993; Kwon *et al.*, 1999a).

Both Arg-βgal and Leu-βgal were long-lived *in vivo* in the absence of Ubr1p (*t*_{1/2} >20 h) ('vector' bars in Figure 3A; data not shown). As expected, Arg-βgal and Leu-βgal were degraded in the presence of wt Ubr1p (Figure 3A). Surprisingly, the steady-state levels of Arg-βgal and Leu-βgal in the transformants with Ubr1p^{MB4} or Ubr1p^{MB6} were similar to (or only slightly higher than) the levels of these proteins in transformants expressing wt Ubr1p (Figure 3A), indicating that high-affinity binding of Ubr1p to Ubc2p is not essential for the activity of the N-end rule pathway. Control experiments with *ubc2Δ* cells confirmed that the Ubr1p^{MB6}-dependent degradation of N-end rule substrates was Ubc2p-dependent as well (data not shown).

Previous work with the tailless Ubc2p_{1–149} mutant,

Fig. 3. High-affinity interaction between Ubr1p and Ubc2p is not essential for the activity of the N-end rule pathway. (A) The activity of Ubr1p is weakly affected by MB mutations. All *UBR1* alleles were expressed from the P_{UBR1} promoter in a low-copy vector. Constructs expressing wt Ubr1p, Ubr1p^{MB4} or Ubr1p^{MB6}, or the empty vector were transformed into JD55 (*ubr1Δ*) cells expressing Arg-βgal or Leu-βgal. Measurements of βgal activity were used to assay the degradation of Arg-βgal and Leu-βgal. The values shown are the means of duplicate measurements of three independent transformants. Standard deviations are indicated above the bars. (B and C) High-affinity association of Ubr1p and Ubc2p is not essential for activity of the N-end rule pathway. βgal activity assays (B) and immunoprecipitations with anti-βgal antibody (C) were used to compare the degradation of model substrates, either Arg-βgal (B and C) or Tyr-βgal (B). All *UBR1* and *UBC2* alleles were expressed from the native P_{UBR1} and P_{UBC2} promoters, respectively, in a low-copy vector. Different combinations of *UBR1* and *UBC2* alleles were transformed into *ubr1Δ ubc2Δ* cells expressing either Arg-βgal, Tyr-βgal (B) or DHFRha-Ub^{R48}-Arg-βgal (C) (see the text and Materials and methods). The measured ratio of Arg-βgal to DHFR-Ub (the reference protein) in the absence of Ubr1p [(C), lane 1] was set at 1.00 and used to normalize the other ratios.

which did not bind to wt Ubr1p, led to the conclusion that Ubc2p₁₋₁₄₉ did not support the degradation of N-end rule substrates (Madura *et al.*, 1993), and implied that high-affinity binding of Ubc2p to Ubr1p was strictly essential for the pathway's function. However, another study reported a diminished but significant activity Ubc2p₁₋₁₄₉ (Watkins *et al.*, 1993). The latter conclusion was consistent with our findings about the BRR-region mutants of Ubr1p (Figure 3A). To address this discrepancy, we compared the activity of the N-end rule pathway in *ubr1Δ ubc2Δ* cells expressing different combinations of either wt Ubr1p or Ubr1p^{MB6} with either wt Ubc2p or the tailless Ubc2p (Ubc2p₁₋₁₄₉). *UBR1*, *UBC2* and their mutant alleles were expressed from their native promoters and a low-copy plasmid. The relative rates of degradation of Arg-βgal and Tyr-βgal ($t_{1/2}$ of ~2 and ~10 min, respectively) (Bachmair *et al.*, 1986; Varshavsky, 1996) were assayed by measuring the activity of βgal (Figure 3B). With wt Ubc2p, the activity of the N-end rule pathway did not change significantly between the wt Ubr1p and Ubr1p^{MB6} alleles (Figure 3B). Furthermore, in the presence of wt Ubr1p, the pathway's activity decreased significantly but not strongly upon the replacement of wt Ubc2p with its tailless derivative Ubc2p₁₋₁₄₉ (Figure 3B), in agreement with the finding of Watkins *et al.* (1993) but in contrast to the result of Madura *et al.* (1993). Interestingly, the activity of the N-end rule pathway was further decreased, but still not abolished, by combining Ubr1p^{MB6} and Ubc2p₁₋₁₄₉ (Figure 3B).

To measure the relative metabolic stability of Arg-βgal in a different way, we utilized a recently developed UPR (Ub/protein/reference) technique, which provides a reference protein (Lévy *et al.*, 1996, 1999). This method employs a linear fusion such as DHFRha-Ub^{R48}-X-βgal, in which Ub^{R48}, containing Arg instead of Lys at position 48, is placed between a protein of interest (X-βgal) and a reference protein moiety such as DHFRha (mouse

dihydrofolate reductase bearing the HA epitope tag) (Lévy *et al.*, 1996). DHFRha-Ub^{R48}-X-βgal is co-translationally cleaved by Ub-specific deubiquitylating enzymes (DUBs) (Wilkinson and Hochstrasser, 1998) after the last residue of Ub^{R48}, producing equimolar amounts of X-βgal and DHFRha-Ub^{R48}. This way, the relative amounts of X-βgal can be normalized against the (co-immunoprecipitated) DHFRha-Ub^{R48} reference protein in the same sample. The UPR technique can therefore compensate for the scatter of immunoprecipitation yields, sample volumes, and other sources of sample-to-sample variation (Lévy *et al.*, 1996). *ubr1Δ ubc2Δ* cells expressing DHFRha-Ub^{R48}-Arg-βgal, and wt Ubr1p or Ubr1p^{MB6} with wt Ubc2p or Ubc2p₁₋₁₄₉ were labeled with [³⁵S]methionine for 30 min at 30°C. Cell extracts were immunoprecipitated with both anti-βgal and anti-HA antibodies, followed by SDS-PAGE and the determination, using a PhosphorImager, of the ratio of Arg-βgal (test protein) to DHFRha-Ub^{R48} (reference protein) (Figure 3C). The ratio determined for *UBR1*-lacking cells was arbitrarily set at 1.00, and was used to normalize the same ratios from other transformants. The results (Figure 3C) were in agreement with those obtained by measuring the enzymatic activity of βgal (Figure 3A and B). Specifically, in the presence of wt Ubc2 and Ubr1p^{MB6}, ~90% of Arg-βgal was degraded under the assay's conditions, similar to the result with wt Ubc2p and wt Ubr1p. In cells expressing wt Ubr1p and Ubc2p₁₋₁₄₉, ~70% of Arg-βgal was degraded. With Ubr1p^{MB6} and Ubc2p₁₋₁₄₉ the degradation of Arg-βgal was decreased further, to ~50%, but still not abolished (Figure 3C).

Taken together, these results (Figure 3) indicated that high-affinity interactions between the E3 Ubr1p and the E2 Ubc2p are essential for normal levels of the pathway's activity. These data also showed that even much weaker interactions between mutant variants of Ubr1p and Ubc2p, the interactions that could not be detected by either two-hybrid or pull-down assays (Figures 1 and 2), were still

sufficient to sustain a large fraction of activity of the N-end rule pathway (Figure 3). We conclude that a transient interaction between Ubr1p and Ubc2p is functionally suboptimal but still sufficient for the multiubiquitylation of a substrate and for the substrate's delivery to the 26S proteasome.

The RING-H2 finger of Ubr1p is essential for degradation of N-end rule substrates

Given the proximity of the 104 residue RING-H2 finger to the BRR region of Ubr1p that mediates its binding to Ubc2p (Figure 1), we determined the effects of missense mutations in RING-H2 (Figure 1B) on the ability of Ubr1p to target N-end rule substrates for ubiquitylation and degradation. Since the structure of the folded RING-H2 domain is unknown, we constructed single missense mutants throughout this domain (Figure 1B). The sequences RING-H2^{MR1}, RING-H2^{MR18}, RING-H2^{MR3} and RING-H2^{MR2} contained single Cys→Ser substitutions at the Cys1, Cys2, Cys4 and Cys5 positions of RING-H2, respectively (Figure 1A and B). RING-H2^{MR12} contained a His→Ala substitution at the His1 position of RING-H2 (Figure 1A and B). These RING-H2 variants were introduced into wt Ubr1. The resulting mutant proteins, Ubr1p^{MR1}, Ubr1p^{MR2}, Ubr1p^{MR3}, Ubr1p^{MR12} and Ubr1p^{MR18}, were expressed in *ubr1Δ* cells from a low-copy plasmid, the native *P_{UBR1}* promoter, and in the presence of either Arg-βgal or Leu-βgal, as described for the BRR-domain mutants of Ubr1p (Figure 3).

In striking contrast to the negligible effect of mutations in the RING-H2 finger of Ubr1p on the binding of Ubr1p to Ubc2p (Figures 1C, D and 2), all of the tested RING-H2 mutations except MR12 had a major inhibitory effect on the degradation of N-end rule substrates (Figure 4). Specifically, Ubr1p^{MR1}, which bore a Cys→Ser substitution at Cys1 of the RING-H2 finger, was completely inactive as an E3 (N-recognin), in that the steady-state levels of Arg-βgal and Leu-βgal in cells expressing Ubr1p^{MR1} were indistinguishable from the levels of these substrates in *ubr1Δ* cells, which lacked the N-end rule pathway (Figure 4). Other mutations of the RING-H2 finger impaired the N-end rule pathway strongly but incompletely, whereas the MR12 mutation (His→Ala at His1 of RING-H2) had a relatively weak effect (Figure 4).

Previous work has shown that Ubr1p is a rate-limiting component of the N-end rule pathway, in that overexpression of Ubr1p accelerated the degradation of N-end rule substrates (Bartel *et al.*, 1990). We introduced the apparently inactive Ubr1p^{MR1} and the partially active Ubr1p^{MR2} mutations into wt Ubr1p bearing the N-terminal FLAG epitope and expressed the resulting proteins from the *P_{ADH1}* promoter on a high-copy plasmid. Overexpression of the partially active Ubr1p^{MR2} increased the activity of the N-end rule pathway in comparison with cells expressing Ubr1p^{MR2} at wt levels (Figure 4). In contrast, the effect of overexpressing Ubr1p^{MR1} was marginal, in that only ~4% of Leu-βgal and <20% of Arg-βgal were degraded, in comparison with virtually no degradation at physiological levels of Ubr1p^{MR1} (Figure 4). Finally, the ~90 kDa *in vivo* cleavage product of βgal (denoted by an asterisk), which is characteristic of short-lived X-βgal substrates and is not produced in the absence of either

Fig. 4. Proteolysis by the N-end rule pathway requires the RING-H2 finger of Ubr1p. Methods described in the legend to Figure 3 and Materials and methods were used to assess the degradation of test substrates Arg-βgal (A and B) and Leu-βgal (A) in the presence of different *UBR1* alleles (see Figure 1B). All *UBR1* alleles were expressed from the native *P_{UBR1}* promoter in a low-copy vector. The Ubr1p^{MR1} (ADH1MR1) and Ubr1p^{MR2} (ADH1MR2) mutants were also expressed from the *P_{ADH1}* promoter in a high-copy vector. Constructs expressing wt Ubr1p and its mutants were transformed into JD55 (*ubr1Δ*) cells expressing the Arg-βgal or Leu-βgal [(A), βgal activity assays], or DHFRha-Ub^{R48}-Arg-βgal [(B), immunoprecipitation assays]. The activity of βgal in the absence of *UBR1* (vector alone) was set at 100%. The values shown are the means of duplicate measurements of three independent transformants. Standard deviations are indicated above the bars. In (B), the asterisk denotes the 90 kDa βgal cleavage product characteristic of the short-lived X-βgals (Bachmair *et al.*, 1986). Ubr1p co-immunoprecipitated with anti-βgal antibody is also indicated. The measured ratio of Arg-βgal to DHFR-Ub in the absence of Ubr1p [(B), lane vector] was set at 1.00 and used to normalize the other ratios.

Ubr1p or Ubc2p (Dohmen *et al.*, 1991), was nearly absent with Ubr1p^{MR1} (Figures 4B and 5B); this result was consistent with negligible functional activity of Ubr1p^{MR1}.

Previous work has shown that the N-end rule pathway controls the import of peptides through degradation of the homeodomain protein Cup9p, which inhibits the expression of the Ptr2p peptide transporter (Byrd *et al.*, 1998). We examined whether cells expressing Ubr1p^{MR1} instead of wt Ubr1p were perturbed in their ability to import peptides by comparing the growth of *lys2Δ ubr1Δ* cells

Fig. 5. The RING-H2 finger of Ubr1p is required for ubiquitylation of N-end rule substrates. (A) The RING-H2 finger is not required for the binding of N-end rule substrates by Ubr1p. The UPR-based fusion DHFRha-Ub^{R48}-Leu-βgal (see the text and Materials and methods) was co-expressed in AVY301 (*ubr1Δ ubc2Δ*) cells with the FLAG-tagged wt Ubr1p (lane 3), Ubr1p^{MR1} (lane 4), Ubr1p^{MR2} (lane 5) or vector alone (lane 2). The Ubr1p proteins were expressed from the P_{ADH1} promoter in a high-copy vector. Lane 1, AVY301 cells expressing wt Ubr1p alone. Extracts from cells labeled with [³⁵S]methionine were immunoprecipitated with anti-βgal (top panel) or anti-FLAG antibodies (lower panel), followed by SDS-8% PAGE (see Materials and methods). (B) The RING-H2 finger of Ubr1p is required for ubiquitylation of N-end rule substrates. JD55 (*ubr1Δ*) cells expressing Leu-βgal and different amounts of Ubr1p or its mutants were labeled with [³⁵S]methionine for 6 min at 30°C, followed by the addition of NEM to 50 mM and preparation of extracts by heating at 100°C in the presence of SDS (see Materials and methods). The extracts were precipitated with anti-βgal antibody and fractionated by SDS-6% PAGE. Note the virtual absence of the multiubiquitylated Leu-βgal species (denoted Ub_n-βgal on the right) from cells expressing Ubr1p^{MR1} (lane 3). The asterisk denotes the 90 kDa βgal cleavage product characteristic of short-lived X-βgals (Bachmair *et al.*, 1986).

expressing either wt Ubr1p, Ubr1p^{MR1} or Ubr1p^{MB6} on plates containing Lys-Ala dipeptide as the sole source of lysine. Under these conditions, inactivation of the N-end rule pathway is lethal, owing to the inability of cells (auxotrophic for lysine) to import the Lys-Ala dipeptide (Byrd *et al.*, 1998). Cells expressing Ubr1p^{MR1}, which is virtually inactive as N-recogin, failed to grow on Lys-Ala dipeptide plates, whereas cells expressing either wt Ubr1p or Ubr1p^{MB6} survived (data not shown), consistent with the effects of the MR1 and MB6 mutations on the activity of the N-end rule pathway (Figures 3 and 4).

We conclude that at least Cys1 of the RING-H2 finger in Ubr1p is essential for the E3 function of Ubr1p in the

N-end rule pathway, even though this cysteine is not required for the binding of Ubr1p to Ubc2p. Yet another insight from the pulse-immunoprecipitation assays with Arg-βgal was that the anti-βgal antibody co-precipitated the partially active FLAG-Ubr1p^{MR2} and the inactive FLAG-Ubr1p^{MR1} from the extracts of cells that over-expressed these Ubr1p mutants (Figure 4B). Thus, the functionally inactivating Cys1→Ser1 mutation in the RING-H2 finger of Ubr1p^{MR1} may not have impaired the binding of substrates by Ubr1p^{MR1}. This conjecture is confirmed below.

RING-H2 of Ubr1p is not required for the binding of N-end rule substrates by Ubr1p

To compare directly the affinities of Ubr1p and its derivatives for an N-end rule substrate, FLAG-Ubr1p, FLAG-Ubr1p^{MR1} or FLAG-Ubr1p^{MR2} were overexpressed in a *ubr1Δ ubc2Δ* strain, which also expressed Leu-βgal (produced from Ub-Leu-βgal). Owing to the absence of Ubc2p, Leu-βgal was long-lived in this strain, irrespective of the presence of Ubr1p (Dohmen *et al.*, 1991). Controls included congenic cells expressing FLAG-Ubr1p alone or Leu-βgal alone. Cells were labeled with [³⁵S]methionine for 30 min; proteins were immunoprecipitated from extracts with anti-βgal antibody, followed by SDS-PAGE. Anti-βgal co-immunoprecipitated Leu-βgal and comparable amounts of either wt FLAG-Ubr1p, FLAG-Ubr1p^{MR1} or FLAG-Ubr1p^{MR2} (Figure 5A, upper panel, lanes 3–5). This co-immunoprecipitation was specific for Leu-βgal, because no FLAG-Ubr1p was brought down with anti-βgal in the absence of Leu-βgal (Figure 5A, upper panel, lane 1). The expression levels of FLAG-Ubr1p, FLAG-Ubr1p^{MR1} and FLAG-Ubr1p^{MR2} were nearly identical, as could be shown by immunoprecipitating these proteins with anti-FLAG antibody (Figure 5A, lower panel). In contrast to the results with anti-βgal antibody (Figure 5A, upper panel), Leu-βgal was not co-precipitated with the FLAG-tagged Ubr1p proteins by anti-FLAG antibody (Figure 5A, lower panel). The probable explanation is that under these conditions, only free (overproduced) FLAG-Ubr1p proteins could be immunoprecipitated by anti-FLAG, because in the Leu-βgal/FLAG-Ubr1p complex Leu-βgal is bound to the type 2 substrate-binding site of Ubr1p, located in the N-terminal region of Ubr1p (A.Webster, M.Ghislain and A.Varshavsky, unpublished data). As a result, the N-terminus of Ubr1p could be shielded from recognition by the bulky βgal moiety bound to Ubr1p.

Taken together, these results (Figures 1–5) indicated that the MR1 (Cys1→Ser1) mutation in the RING-H2 finger of Ubr1p virtually eliminated the E3 function of Ubr1p, but did not impair its affinity for either N-end rule substrates or the Ubc2p Ub-conjugating enzyme.

The RING-H2 finger of Ubr1p is required for ubiquitylation of N-end rule substrates

To examine ubiquitylation of an N-end rule substrate in cells containing mutant Ubr1p proteins, FLAG-Ubr1p^{MR1}, FLAG-Ubr1p^{MR2}, FLAG-Ubr1p^{MR12}, FLAG-Ubr1p^{MB6} (Figure 1B) and the wt FLAG-Ubr1p were expressed in a *ubr1Δ UBC2* strain, which also expressed Leu-βgal (produced from Ub-Leu-βgal). To minimize the extent of the degradation of Leu-βgal during labeling, the duration

of the [^{35}S]methionine pulse was reduced to 6 min. At the end of the pulse, high concentrations of *N*-ethylmaleimide (NEM), the inhibitor of DUBs (Johnsson and Varshavsky, 1994), were employed to inhibit deubiquitylation after cell lysis. ^{35}S -labeled, immunoprecipitated Leu- β gal was fractionated by SDS-PAGE (Figure 5B).

Multiubiquitylated derivatives of Leu- β gal were observed in the presence but not in the absence of wt Ubr1p, and the extent of ubiquitylation increased upon overexpression of FLAG-Ubr1p (Figure 5B, lanes 1, 2 and 9). However, virtually no ubiquitylated Leu- β gal was observed with Ubr1p^{MR1}, the degradation-inactive RING-H2 mutant of Ubr1p (Figure 5B, lane 3, compare with lanes 1 and 2). Cells overexpressing FLAG-Ubr1p^{MR1} contained trace amounts of multiubiquitylated Leu- β gal, in comparison with much higher levels of these species in cells overexpressing wt FLAG-Ubr1p (Figure 5B, lane 7, compare with lane 9). In agreement with the results of degradation tests (Figure 4), the partially degradation-defective RING-H2 mutant Ubr1p^{MR2} yielded intermediate levels of the multiubiquitylated Leu- β gal, and ubiquitylation was strongly enhanced upon overexpression of FLAG-Ubr1p^{MR2} (Figure 5B, lanes 4 and 8). Ubr1p^{MR12}, whose ability to mediate the degradation of N-end rule substrate was similar to that of wt Ubr1p (Figure 4), yielded the ubiquitylation pattern indistinguishable from that produced by wt Ubr1p (Figure 5B, lane 6). Consistent with the results of β gal activity assays (Figure 3), Ubr1p^{MB6}, which bore three mutations in the BRR domain and was severely impaired in its binding to Ubc2p (Figures 1–3), yielded nearly wt levels of multiubiquitylated Leu- β gal (Figure 5B, lane 5, compare with lane 2). Taken together, these results indicated that the RING-H2 finger of Ubr1p is specifically required for the Ubr1p, Ubc2p-dependent multiubiquitylation of N-end rule substrates, and that Cys1 of the RING-H2 finger is a functionally critical residue of this domain.

Dominant-negative effect of the ubiquitylation-inactive RING-H2 mutant of Ubr1p

The earlier gel filtration data suggested that Ubr1p functions as a monomer (Bartel, 1990). In addition, the immunoprecipitation of FLAG-Ubr1p with anti-FLAG antibody from extracts of cells that overexpressed both FLAG-Ubr1p and Ubr1p-HA did not co-immunoprecipitate Ubr1p-HA, also suggesting the monomeric configuration of Ubr1p (A. Webster and A. Varshavsky, unpublished data). We asked whether the functionally inactive Ubr1p^{MR1} could exert a dominant-negative effect on the N-end rule pathway. Either wt Ubr1p or Ubr1p^{MR1} (in addition to vector alone) was overexpressed from the P_{ADHI} promoter on a high-copy plasmid in *UBR1* cells that also expressed the UPR-based fusions which produced Arg- β gal (or Leu- β gal) and equimolar amounts of DHFR-Ub^{R48}, the reference protein. Cells were labeled with [^{35}S]methionine for 30 min, followed by immunoprecipitation of Leu- β gal (Figure 6A) or Arg- β gal (Figure 6B), as well as the reference protein DHFR-Ub^{R48}. The ratios of X- β gal to DHFR-Ub^{R48} were measured, and thereafter normalized by the same ratios in congeneric *ubr1* Δ cells (Figure 6A and B, lane 1). Whereas overexpression of wt Ubr1p in *UBR1* cells accelerated the degradation of Leu- β gal and Arg- β gal, overexpression of Ubr1p^{MR1} strongly inhibited this degradation (Figure 6A and B, lanes 2 and 3, compare with lanes 4). The dominant-

Fig. 6. Dominant-negative effect of Ubr1p^{MR1}, the RING-H2 mutant of Ubr1p. The P_{ADHI} promoter-based, high-copy vector alone (lane 2), and its derivatives expressing the FLAG-tagged wt Ubr1p (lane 3) or Ubr1p^{MR1} (lane 4) were transformed into JD52 (*UBR1*) cells expressing the UPR-based fusion DHFRha-Ub^{R48}-Leu- β gal (A) or DHFRha-Ub^{R48}-Arg- β gal (B). Cells were labeled with [^{35}S]methionine for 30 min at 30°C, followed by extraction, immunoprecipitation with a mixture of anti- β gal and anti-HA antibodies, and SDS-12% PAGE, and quantitation. Lanes 1 in (A) and (B) show the results of the same tests with JD55 (*ubr1* Δ) cells in the absence of any version of Ubr1p. The measured ratio of the test protein (Leu- β gal or Arg- β gal) to the reference protein (DHFRha-Ub^{R48}) in lanes 1 was set at 1.00. The ratios determined in the other settings and normalized against the lane-1 ratios are shown below the lanes.

negative effect of overexpressed Ubr1p^{MR1} was caused at least in part by the sequestration of endogenous Ubc2p, inasmuch as this effect could be partially reversed through overexpression of Ubc2p (data not shown). The sequestration of N-end rule substrates by Ubr1p^{MR1}, which could bind to substrates indistinguishably from wt Ubr1p (Figure 5), was another likely (and independent) cause of the dominant-negative effect of Ubr1p^{MR1} (Figure 6). The possibility that overexpression of Ubr1p^{MR1} might also interfere with the downstream components of the Ub system, such as the 26S proteasome, was ruled out by the observation that the degradation of Ub-Pro- β gal was not affected by the overexpression of Ubr1p^{MR1} (data not shown). Ub-Pro- β gal is a short-lived protein degraded by the UFD (Ub/fusion/degradation) pathway, which targets the non-removable (or slowly removable) N-terminal Ub moiety of a fusion protein (Johnson *et al.*, 1995; Koegl *et al.*, 1999).

Discussion

We report two main results. First, we identified and dissected the E2 enzyme-binding site of Ubr1p, the E3 component of Ub-protein ligase in the N-end rule pathway of *S.cerevisiae*. This site, the BRR domain, is shown to bind the Ubc2p Ub-conjugating (E2) enzyme mainly through electrostatic interactions between the basic residues of BRR and the polyacidic C-terminal tail of Ubc2p. Although high-affinity interactions between the E3 Ubr1p and the E2 Ubc2p are essential for normal activity of the N-end rule pathway,

even much weaker interactions between mutant variants of Ubr1p and Ubc2p, the interactions which could not be detected by either two-hybrid or pull-down assays, were found to be sufficient for sustaining a large fraction of the pathway's activity. Secondly, we discovered the requirement for the RING-H2 finger of Ubr1p in the ubiquitylation of substrates mediated by the Ubr1p-Ubc2p complex. A single Cys→Ser missense mutation in the RING-H2 finger (which is adjacent to the BRR domain in Ubr1p) severely impairs ubiquitylation and degradation of N-end rule substrates, but does not impair the ability of Ubr1p to bind the substrates and Ubc2p. These findings defined the physical and functional aspects of the Ubc2p-Ubr1p interaction, and revealed a specific essential function of the RING-H2 finger. This domain is present not only in the members of the UBR family of E3s (Kwon *et al.*, 1998) but also in otherwise dissimilar E3 proteins of the Ub system.

Physical and functional aspects of E2-E3 interaction

Ubr1p, the 225 kDa E3 protein, was shown to interact with the 20 kDa Ubc2p E2 enzyme mainly, if not exclusively, through the BRR domain of <15 kDa. This interaction requires the polyacidic C-terminal tail of Ubc2p and has a strong electrostatic component, because conversions of single basic residues in the BRR domain into neutral ones resulted in comparable decreases in the affinity of Ubr1p for Ubc2p. Moreover, this affinity was decreased in rough proportion to the number of mutated basic residues in BRR (Figures 1 and 2). These results are consistent with the reported dependence of the Ubr1p-Ubc2p interaction on the length of the polyacidic tail of Ubc2p. Specifically, the truncated Ubc2p derivative that lacked eight of 23 residues of the polyacidic tail retained high affinity for Ubr1p, but further shortening of the Ubc2p polyacidic tail strongly decreased the affinity (Madura *et al.*, 1993).

Previous work suggested that the 9 residue N-terminal region of Ubc2p was essential for the binding of Ubc2p to Ubr1p (Watkins *et al.*, 1993). Our current data demonstrated that deletion of the first 9 residues of Ubc2p weakened but did not eliminate its interaction, in the two-hybrid assay, with the 287 residue fragment of Ubr1p that contained the BRR domain and the RING-H2 finger (Figure 1). It is not clear how the N-terminal 9 residues of Ubc2p contribute to the interaction with Ubr1p. Inasmuch as Arg6 and Arg8 of the folded Ubc2p are likely to interact through a hydrogen bond (Worthylake *et al.*, 1998), it is possible that the effect of the 9 residue N-terminal deletion is indirect, stemming from a conformational destabilization of Ubc2p. Our finding of the 287 residue Ubr1p fragment that contains the BRR and RING-H2 domains and is sufficient for the high-affinity binding to Ubc2p (Figures 1 and 2) should facilitate the dissection of this E2-E3 interaction at the atomic level through co-crystallization of the previously solved Ubc2p (Worthylake *et al.*, 1998) and the 287 residue fragment of Ubr1p.

Function of the RING-H2 finger of Ubr1p

One of the major results of this work is the discovery of an essential and specific requirement for the RING-H2 finger of Ubr1p in the ubiquitylation of N-end rule substrates mediated by the Ubr1p-Ubc2p complex. The RING-H2 motif is a distinct variant of the previously defined Cys/His-rich RING motif. In several proteins outside the Ub

system, RING-H2 has been shown to function as a site of protein-protein interactions (Borden and Freemont, 1996; Inouye *et al.*, 1997; Kamura *et al.*, 1999). In addition to the UBR family of E3 proteins (Kwon *et al.*, 1998), several other E3 components of the Ub system, including Apc11p, Roc1p (Rbx1p or Hrt1p), Roc2p and their homologs in multicellular eukaryotes, have also been found to contain RING-H2 or other variants of RING (Yu *et al.*, 1998; Zachariae *et al.*, 1998; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Tan *et al.*, 1999).

The RING-H2 of Ubr1p is adjacent to the BRR domain, which mediates the physical interaction between Ubr1p and Ubc2p (Figures 1 and 2). A single Cys→Ser mutation at position 1 of the RING-H2 finger abolished ubiquitylation and degradation of N-end rule substrates, but did not impair the ability of the resulting mutant, Ubr1p^{MR1}, to bind both the substrates and Ubc2p (Figures 4-6). The Cys→Ser mutations at other positions of the RING-H2 finger had similar but partial effects, while the His→Ala mutation at His1 position of RING-H2 had the smallest effect among the mutants tested (Figures 4-6). Thus, even though the Ubc2p E2 enzyme can interact with the E1 enzyme to yield the Ubc2p~Ub thioester, and can also produce isopeptide bond-mediated Ub-protein conjugates in the absence of E3 (Jentsch *et al.*, 1987), the synthesis of a multi-Ub chain by the Ubc2p-Ubr1p complex requires the presence of cysteine at position 1 of the RING-H2 finger in Ubr1p.

The Ub system of a cell contains a number of distinct E2 and E3 proteins (Hochstrasser, 1996; Varshavsky, 1997; Hershko and Ciechanover, 1998). An E3 protein of a Ub-dependent proteolytic pathway was initially presumed to function as a non-catalytic, substrate-recognition component of the corresponding Ub-protein ligase, which consists of an E3 and its associated E2 enzyme. Ubr1p (N-recognin) of the present work was the first E3 that has been shown to bind proteins bearing specific degradation signals (Bachmair *et al.*, 1986; Bartel *et al.*, 1990). More recently, it was found that in addition to recognizing specific substrates, at least some E3s, specifically those that contain the HECT domain (Scheffner *et al.*, 1993), also function as enzymes. An E3 of this class can accept the Ub moiety from an associated E2~Ub thioester, initially forming the E3~Ub thioester confined to a specific (and essential) cysteine of E3. The Ub moiety of this E3~Ub thioester is thereafter conjugated, through the isopeptide bond, either directly to a substrate protein or to the Ub moiety of a growing, substrate-linked multi-Ub chain (Scheffner *et al.*, 1995). Other classes of E3 proteins, including the SCF, APC and UBR families (Hershko and Ciechanover, 1998; Kwon *et al.*, 1998; Tyers and Willems, 1999), lack recognizable HECT domains, and have not thus far been demonstrated to form thioesters with Ub.

The mutations tested in the RING-H2 finger of Ubr1p (including the Cys1 mutation which abolished the ubiquitylation activity of the Ubr1p-Ubc2p complex) did not impair the ability of Ubr1p to bind the Ubc2p enzyme and N-end rule substrates (Figures 1-5). Thus, the specific functional effect of these mutations cannot be due to a global conformational destabilization of Ubr1p. We conclude that the Ubr1p (E3) component of the N-end rule pathway not only recognizes specific degradation signals in substrate proteins, but in addition directly

participates, through a mechanism that requires the RING-H2 domain, in the Ubc2p-mediated synthesis of a substrate-linked multi-Ub chain. In the first of possible models, a specific cysteine of RING-H2, perhaps the essential Cys1 residue (Figure 1), acts as an acceptor of the Ub moiety transesterified from the Ubc2p~Ub thioester, similar to the one previously described mechanism of the HECT-domain E3 called E6AP (Scheffner *et al.*, 1995; Huibregtse *et al.*, 1998). Earlier experiments with an *in vitro* ubiquitylation system containing purified *S.cerevisiae* Ubr1p and other targeting components of the N-end rule pathway suggested the Ubc2p-dependent formation of a Ubr1p~Ub thioester (V.Chau and A.Varshavsky, unpublished data). Attempts to rigorously verify the functionally relevant presence of such a thioester in the completely defined, Ubr1p-based *in vitro* system have not been successful thus far (F.Du and A.Varshavsky, unpublished data). Another possible role of the cysteine(s) of the RING-H2 finger in Ubr1p may be to modify the immediate vicinity of the active cysteine (Cys88) of the Ubr1p-associated Ubc2p in a way that facilitates the multi-Ub synthesis by Ubc2p, in the absence of Ubr1p~Ub thioester formation. Physical proximity between the Ubc2p-binding BRR domain of Ubr1p and its RING-H2 domain (Figure 1A) is consistent with this mechanism. A third model posits the existence of an unidentified factor that binds to the RING-H2 finger of Ubr1p, and is required for the efficient synthesis of multi-Ub by the Ubr1p~Ubc2p complex. Jentsch and colleagues (Koegl *et al.*, 1999) have identified a factor, termed E4, which facilitates the synthesis of a multi-Ub chain linked to a substrate of the *S.cerevisiae* UFD pathway. This factor, encoded by the *UFD2* gene, is required for the *in vivo* degradation of Ub^{V76}-Val-βgal, but is not required for the degradation of a much smaller and monomeric UFD substrate such as Ub^{V76}-Val-DHFR; Ufd2p is also not required for the degradation of βgal-based N-end rule substrates (Johnson *et al.*, 1995). Whether the function of the RING-H2 finger of Ubr1p is mediated by a finger-binding protein analogous to E4 remains to be determined.

The RING-H2 finger and its variants are present in several otherwise dissimilar classes of E3 proteins in the Ub system. For example, the mammalian E3 encoded by the *Mdm2* gene and specific for the p53 substrate contains a putative RING finger (Elenbaas *et al.*, 1996); the conserved Cys464 residue of this domain was proposed to define an active site analogous to the Ub thioester-forming site of the HECT-domain E3 enzymes (Honda *et al.*, 1997). Roc1p (Rbx1p, Hrt1p), a recently identified subunit of the cell cycle-regulating Ub~protein ligase complex called SCF, contains a variant of RING-H2 in which Cys6 is replaced by Asp (Kamura *et al.*, 1999). Mutagenesis-based analyses suggested that the RING finger of Roc1p was required for the function of the SCF complex (Kamura *et al.*, 1999; Ohta *et al.*, 1999). The sequence of Roc1p (Rbx1p, Hrt1p) is highly similar to that of Apc11p, the RING-H2-containing subunit of another cell cycle-regulating Ub~protein ligase, called APC or cyclosome (Yu *et al.*, 1998; Zachariae *et al.*, 1998). The RING-H2 finger and its variants have also been found in other (possibly also E3) components of the Ub system, including Siah-1 and Der3p (Hrd1p), which are required, respectively, for the degradation of the DCC

protein (deleted in colorectal cancer) (Hu and Fearon, 1999) and of the misfolded luminal and integral membrane proteins (Bordallo *et al.*, 1998).

Further dissection of the RING domains in these and other E3 proteins will determine whether the ubiquitylation-enabling function of RING-H2 in Ubr1p of the N-end rule pathway is a general property of the E3 components in the Ub system.

Materials and methods

Strains, media and β-galactosidase assay

The *S.cerevisiae* strains used were JD55 (MATa *trp1-Δ63 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 ubr1Δ::HIS3*); JD52 (MATa *trp1-Δ63 ura3-52 his3-Δ200 leu2-3, 112 lys2-801*); AVY301 (a *ubc2Δ::URA3* derivative of JD55); and PJ69-4A (a gift from P.James and E.Craig) (MATa *trp1-109 leu2-3, 112 ura3-52 his3-Δ200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met::GAL7-lacZ*). Cultures were grown in rich (YPD) or in synthetic media containing standard ingredients (Sherman, 1991) and either 2% glucose (SD medium), 2% galactose (SG medium) or 2% raffinose. Yeast were transformed as described previously (Chen *et al.*, 1992). For the induction of the *P_{CUP1}* promoter, CuSO₄ was added to a final concentration of 0.1 mM (Ghislain *et al.*, 1996). The enzymatic activity of βgal in yeast extracts, from cultures at OD₆₀₀ of 0.8–1.0, was determined as described previously (Baker and Varshavsky, 1991), using *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The *Escherichia coli* strain used was DH5α.

Uses of the two-hybrid system

The *UBC2* ORF was amplified by PCR using pKM1313 (Madura and Varshavsky, 1994) as the template DNA. The two primers were designed so that the PCR products contained a *NcoI* site immediately 5' to the start codon, and a *SalI* site immediately 3' to the stop codon. The *NcoI*-*SalI*-cut PCR fragment was subcloned into *NcoI*-*SalI*-cut PAS2 (Durfee *et al.*, 1993), yielding pAS2-UBC2, which expressed a fusion containing the DNA-binding domain of Gal4p upstream of the Ubc2p moiety. The construct was verified by nucleotide sequencing, and also by immunoblotting, in which the Gal4-Ubc2p protein was detected with an anti-Gal4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Gal4-Ubc2p retained the ability of Ubc2p to rescue the UV-sensitivity defect of *ubc2Δ* cells. The PCR primers used were ggaacatggagatgtccacacacagctagaag and cctgtcgactcagctgtctctgtctgt. The two-hybrid DNA libraries and the host *S.cerevisiae* strain PJ69-4A were gifts from P.James and E.Craig (James *et al.*, 1996). PJ69-4A expressed three reporters from different inducible promoters: *P_{GAL1}-HIS3*, *P_{GAL2}-ADE2*, and *P_{GAL7}-lacZ*. pAS2-UBC2 was transformed into PJ69-4A. Trp⁺ transformants were then transformed with the two-hybrid libraries, with selection directly on SD (–trp, –leu, –ade) plates at 30°C. Ade⁺ colonies were then tested on SD (–trp, –leu, –his) plates. Library plasmids were rescued from the Ade⁺ His⁺ transformants, and re-transformed into PJ69-4A expressing either pAS2-UBC2 or the control vector pAS2. The plasmids that induced *ADE2* and *HIS3* in the presence but not in the absence of pAS2-UBC2-encoded putative Ubc2p-interacting proteins, and were analyzed by partial sequencing. One clone thus identified encoded a 287 residue fragment (position 1134–1420) of the ORF YLR024C, which was previously termed *UBR2* (Hochstrasser, 1996; Kwon *et al.*, 1998), as described in Results.

The two-hybrid system was also used to qualitatively assess the interaction of either Ubc2p or its truncated derivatives with the BRR domain and/or the RING-H2 finger of either wt Ubr1p or its derivatives containing point mutations. *UBC2_{1–149}*, encoding the C-terminally truncated Ubc2p lacking the 23 residue polyacidic tail, was constructed using PCR and the primers ggaacatggagatgtccacacacagctagaag and gctagtcgacccaagatttctctaccgtctc. The PCR products were cut by *SalI* and *BamHI*, and subcloned into *SalI*-*BamHI*-cut pGBDUC1 (James *et al.*, 1996), yielding pGBDUC1UBC2_{1–149}, which expressed a fusion of Ubc2p_{1–149} to the DNA binding domain of Gal4p. Similarly, *UBC2_{10–172}*, encoding Ubc2p lacking the first 9 residues, was constructed using the primers tacggatccatgagagatttaaacgtatg and cctgtcgactcagctgtctctgtctgt. The PCR products were digested with *SalI* and *BamHI*, and subcloned into *SalI*-*BamHI*-cut pGBDUC1 vector, yielding pGBDUC1UBC2_{10–172}. The *UBR1* fragment encoding residues 1081–1367, including the BRR domain and the RING-H2 finger, was produced using PCR and the primers aactggatccggaaggactctataccagaagct and

ttccgtcgacctcagtaaaagggttgaagacct. The PCR products were cut with *Bam*HI and *Sal*I, and subcloned into *Bam*HI-*Sal*I-cut pGADC1 (James *et al.*, 1996), yielding pGADC1UBR1₁₀₈₁₋₁₃₆₇, which encoded a fusion of Ubr1p₁₀₈₁₋₁₃₆₇ to the transcriptional activation domain of Gal4p. UBR1₁₀₈₁₋₁₂₂₀ encoding the Ubr1p fragment containing the BRR domain but not the RING-H2 finger, was constructed using the primers tacggatccatgagagatttaaacatg and ttccgtcgacgcaggttaaatctccgattc, the above procedure, and yielding pGADC1UBR1₁₀₈₁₋₁₂₂₀, which expressed a fusion of Ubr1p₁₀₈₁₋₁₂₂₀ to the transcriptional activation domain of Gal4. UBR1₁₁₇₇₋₁₃₆₇ encoding the Ubr1p fragment containing the RING-H2 finger but not the BRR domain, was constructed using the primers aactggatccgcaaggctcttagccaag and ttccgtcgacgcaggttaaatctccgattc, the above procedure, and yielding pGADC1UBR1₁₁₇₇₋₁₃₆₇, which expressed a fusion of Ubr1p₁₁₇₇₋₁₃₆₇ to the transcriptional activation domain of Gal4. UBR1 alleles containing point mutations in either the BRR domain or the RING-H2 finger were produced using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Briefly, an ~850 bp *Eco*RI-*Bgl*II fragment from pGADC1UBR1₁₀₈₁₋₁₃₆₇ that encoded the BRR domain and the RING-H2 finger was subcloned into the *Eco*RI-*Bgl*II-cut LITMUS28 vector (New England Biolabs, Beverly, MA), yielding LITMUS28UBR1(*Eco*RI-*Bgl*II), which was subjected to site-directed mutagenesis. The resulting plasmids were cut with *Eco*RI and *Bgl*II, and the ~850 bp *Eco*RI-*Bgl*II fragments containing the intended point mutations were subcloned back into *Eco*RI-*Bgl*II-cut pGADC1UBR1₁₀₈₁₋₁₃₆₇, yielding pGADC1UBR1MB10, pGADC1UBR1MB11, pGADC1UBR1MB17, pGADC1UBR1MB4, pGADC1UBR1MB5, and pGADC1UBR1MB6; pGADC1UBR1MR1, pGADC1UBR1MR2, pGADC1UBR1MR3, pGADC1UBR1MR12, pGADC1UBR1MR18, pGADC1UBR1MR7 and pGADC1UBR1MR8. All PCR products and inserts containing point mutations were verified by DNA sequencing on both strands. These mutants belonged to either the MB or the MR groups (Figure 1B).

UPR plasmids encoding N-end rule substrates

UPR plasmids were the yeast vector-based counterparts of the plasmids originally constructed for expression in mammalian cells (Lévy *et al.*, 1996). These plasmids, termed pRβgalUPR and pLβgalUPR, expressed, respectively, DHFR-Ub-Arg-βgal and DHFR-Ub-Leu-βgal from the P_{CUP1} promoter. A *Bam*HI-*Sma*I insert of pBARUPR or pBALUPR was replaced with a 3.8 kb βgal-encoding insert from pUB23-M (Bachmair *et al.*, 1986), using *Bam*HI and *Sca*I. pBARUPR and pBALUPR were derived from the low-copy, pRS314-based pBAM vector (Ghislain *et al.*, 1996). They expressed, respectively, DHFR-Ub-Arg-Ura3p and DHFR-Ub-Leu-Ura3p from the P_{CUP1} promoter (A. Webster and A. Varshavsky, unpublished data), and were a gift from A. Webster.

Plasmids encoding Ubr1p, Ubc2p and their mutant derivatives

UBR1 and its mutant alleles were expressed either from its natural promoter in a low-copy, pRS315-derived vector or from the P_{ADHI} promoter in a high-copy, YEplac181-based plasmid. Briefly, pRS315 (Sikorski and Hieter, 1989) was cut with *Xba*I, blunted by Klenow Pol I, and thereafter self-ligated, yielding pRS315(-*Xba*I) lacking the *Xba*I site. pUBR1 (Bartel *et al.*, 1990) was cut with *Sal*I and *Pst*I. An ~8.1 kb insert containing the ORF and promoter of UBR1 was subcloned into *Sal*I-*Pst*I-cut pRS315(-*Xba*I), yielding pRS315(-*Xba*I)UBR1. To produce mutant *ubr1* alleles, a ~2.9 kb *Bgl*II-*Xba*I fragment of UBR1 from pRS315(-*Xba*I)UBR1 was subcloned into LITMUS28. The resulting LITMUS28UBR1(*Bgl*II-*Xba*I) was digested with *Hpa*I and *Sap*I, and an ~1.4 kb fragment was isolated. LITMUS28UBR1(*Bgl*II-*Xba*I) was also cut by *Sap*I, and an ~3.7 kb band was isolated. The 1.4 and 3.7 kb fragments were ligated with a set of ~600 bp *Sap*I-*Hpa*I fragments from the point mutation-containing pGADC1UBR1MB4, pGADC1UBR1MB6, pGADC1UBR1MR1, pGADC1UBR1MR2, pGADC1UBR1MR3, pGADC1UBR1MR12 or pGADC1UBR1MR18, yielding, respectively, pLITMUS28UBR1MB4, pLITMUS28UBR1MB6, pLITMUS28UBR1MR1, pLITMUS28UBR1MR2, pLITMUS28UBR1MR3, pLITMUS28UBR1MR12 and pLITMUS28UBR1MR18. A 2.9 kb *Bgl*II-*Xba*I fragment from each of these plasmids was subcloned into *Bgl*II-*Xba*I-cut pRS315(-*Xba*I)UBR1, yielding, respectively, pRS315(-*Xba*I)UBR1MB4, pRS315(-*Xba*I)UBR1MB6, pRS315(-*Xba*I)UBR1MR1, pRS315(-*Xba*I)UBR1MR2, pRS315(-*Xba*I)UBR1MR3, pRS315(-*Xba*I)UBR1MR12 and pRS315(-*Xba*I)UBR1MR18. pNTFlagUBR1 expressed the full-length Ubr1p bearing the N-terminal FLAG epitope (MDYKDDDDK) from the P_{ADHI} promoter in a high-copy, LEU2-containing vector. The MB6, MR1 or MR2 mutations (Figure 1B) were introduced into pNTFlagUBR1 by replacing its 2.9 kb

*Bgl*II-*Xba*I insert with its counterparts from pRS315(-*Xba*I)UBR1MB6, pRS315(-*Xba*I)UBR1MR1 or pRS315(-*Xba*I)UBR1MR2, yielding, respectively, pNTFlagUBR1MB6, pNTFlagUBR1MR1 and pNTFlagUBR1MR2.

UBC2 and UBC2₁₋₁₄₉ alleles containing the native P_{UBC2} promoter (~500 bp of the 5' region) were amplified by PCR. The *Sac*I-*Sal*I-cut PCR products were subcloned into *Sac*I-*Sal*I-cut p413GAL1 (Mumberg *et al.*, 1994). Both wt Ubc2p and Ubc2p₁₋₁₄₉ expressed from these vectors were able to complement the UV-sensitivity phenotype of *ubc2Δ* cells (data not shown). The PCR primers for the wt UBC2 and the *ubc2*₁₋₁₄₉ were, respectively, acgtgagctcccctaattgaattgtaagcg and cctgtgctgactgctgtcctgctgctg, and acgtgagctcccctaattgaattgtaagcg and gctagtgcactaccaagattctctaccgtc.

GST binding assays

The purified GST-Ubc2p fusion protein (~1 μg) was diluted into 0.5 ml of the loading buffer (1% Triton X-100, 10% glycerol, 0.5 M NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0), incubated with 10 μl (bed volume) of the glutathione-agarose beads (Sigma, St Louis, MO) for 1 h at 4°C, and washed three times with 1 ml of the binding buffer (10% glycerol, 50 mM NaCl, 50 mM Na-HEPES pH 7.5). Washed beads were incubated with extracts from ³⁵S-labeled cells (~5 × 10⁶ CCl₃COOH-insoluble counts/min/extract) containing the vector alone or a plasmid expressing one of the UBR1 alleles, including wt FLAG-Ubr1p (specifically, pNTFlagUBR1, pNTFlagUBR1MB6, pNTFlagUBR1MR1 or pNTFlagUBR1MR2) at 4°C for 1 h. The incubation buffer contained 1 mg/ml of the ovalbumin carrier (Sigma, St Louis, MO) in the binding buffer. Beads were washed three times with the binding buffer. Bound proteins were eluted with the loading buffer containing 2% SDS and fractionated by SDS-8% PAGE.

Co-immunoprecipitation-immunoblotting

Ubc2p bearing the N-terminal HA epitope was expressed from the P_{GAL1} promoter in a low-copy number vector p416GAL1 (Mumberg *et al.*, 1994). AVY301 cells (*ubr1Δ ubc2Δ*) containing a plasmid expressing HA-tagged Ubc2p or a plasmid expressing FLAG-Ubr1p, or plasmids expressing HA-tagged Ubc2p and one of the UBR1 alleles, including pNTFlagUBR1, pNTFlagUBR1MB6, pNTFlagUBR1MR1 or pNTFlagUBR1MR2 were cultured in raffinose medium to an OD₆₀₀ of 0.8–1.0, then switched to galactose medium for induction of Ubc2p expression for 6 h. Cells were harvested and resuspended in DB buffer (50 mM NaCl, 1 mM EDTA, 50 mM Na-HEPES pH 7.5) containing 1× protease inhibitor mix (Boehringer Mannheim), and thereafter lysed by vortexing with glass beads (Bartel *et al.*, 1990). For the co-immunoprecipitation of Ubc2p with Ubr1p (Figure 2B, top panel), supernatants were incubated with anti-HA antiserum (Babco, Berkeley, CA), followed by SDS-8% PAGE and immunoblotting with a monoclonal anti-FLAG antibody (Sigma, St Louis, MO). The expression levels of Ubr1p and Ubc2p in these transformants were monitored by immunoblotting with anti-FLAG antibody (Figure 2B, middle panel) and anti-HA antibody (Figure 2B, bottom panel), respectively. SDS-8% PAGE was used.

Metabolic labeling and immunoprecipitation

Saccharomyces cerevisiae cells from 10 ml cultures (OD₆₀₀ 0.8–1.2) in SD media containing 0.1 mM CuSO₄ were labeled for 30 min at 30°C with 0.15 mCi of [³⁵S]methionine/cysteine (EXPRESS, New England Nuclear, Boston, MA). The same conditions were used to label FLAG-Ubr1p and its derivatives for the GST pull-down assays, except that no CuSO₄ was added. To assay the *in vivo* ubiquitylation of N-end rule substrates, cells were labeled for 6 min, followed by the addition of 2 M NEM in dimethylformamide to a final concentration of 50 mM (Johnsson and Varshavsky, 1994). Two methods were used to prepare cell extracts. For the GST pull-down assays, cell extracts were prepared as described above for co-immunoprecipitation immunoblots. For the experiments shown in Figures 3C, 4B, 5A and 6, labeled cells were pelleted, and resuspended in 0.8 ml of buffer A (1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 50 mM Na-HEPES pH 7.5) containing protease inhibitors, and were lysed as above. The extracts were centrifuged at 12 000 g for 10 min, and supernatants containing equal amounts of CCl₃COOH-insoluble ³⁵S were used for the GST assays described above or for immunoprecipitations. To detect Ubr1p (Figures 2A and 5A, lower panels), immunoprecipitations were carried out with a monoclonal anti-FLAG antibody conjugated to agarose beads (Kodak, Rochester, NY), followed by SDS-8% PAGE. For the co-immunoprecipitation of Leu-βgal with Ubr1p (Figure 5A, top panel), supernatants were incubated with a monoclonal anti-βgal antibody (Promega, Madison, WI), followed by SDS-8% PAGE. For measurements of X-βgal stability using the

UPR technique (Figures 3C, 4B and 6), a mixture of an anti-HA antiserum (Babco, Berkeley, CA) and the monoclonal anti- β gal antibody was incubated with 35 S-labeled cell extracts. Immunoprecipitated proteins were separated by SDS-12% PAGE. For detection of the *in vivo* ubiquitylation, cells labeled for 6 min were lysed using the SDS-boiling method (Hochstrasser and Varshavsky, 1990). Briefly, a pellet of NEM-treated cells (see above) was mixed with an equal volume of the 2 $\times$ SDS buffer at 100°C (2% SDS, 30 mM dithiothreitol, 90 mM Na-HEPES pH 7.5) plus 50 mM NEM, and incubated at 100°C for 5 min. The extracts were diluted 10-fold with buffer A plus protease inhibitors and 50 mM NEM. CCl_3COOH -insoluble 35 S was determined, and samples containing equal amounts of 35 S were immunoprecipitated with the monoclonal anti- β gal antibody, followed by SDS-6% PAGE.

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