EL5 is a rice ubiquitin-protein isopeptide ligase (E3) containing a RING-H2 finger domain that interacts with *Oryza sativa* (Os) UBC5b, a rice ubiquitin carrier protein. We introduced point mutations into the EL5 RING-H2 finger so that residues that functionally interact with OsUBC5b could be identified when assayed for ubiquitination activity in vitro. The residue positions were selected based on the results of an EL5 RING-H2 finger/OsUBC5b NMR titration experiment. These RING-H2 finger residues form or are adjacent to a shallow groove that is recognized by OsUBC5b. The E3 activity of EL5 is shown to be dependent on a Trp located at the center of the groove. We classified rice RING fingers according to the type of metal-chelating motif, i.e. RING-H2 or RING-HC, and according to the presence or absence of a conserved EL5-like Trp. We discuss the probable relationship between E3 activity and the conserved Trp.

The RING finger motif, found in many functionally distinct proteins, was first identified as the protein product of the human gene *RING1* (Really Interesting New Gene 1) (1). The RING finger motif is defined by the consensus sequence Cys-X$_2$-Cys-X$_{3–9}$-Cys-X$_{1–3}$-His-X$_{2–3}$-(Cys/His)-X$_{2}$-Cys-X$_{4–46}$-Cys-X$_{2}$-Cys, where X is any amino acid and the number of X residues varies in different fingers. Two types of RING finger motifs are distinguished by a cysteine (RING-HC) or histidine (RING-H2) as the fifth metal-chelating residue. A RING finger typically binds two zinc atoms, with its Cys and/or His side chains in a unique “cross-brace” arrangement. The nearly invariant spacing between the second and third pairs of Cys/His residues probably conserves the distance between the two metal-chelating sites (2). RING fingers are commonly found in proteins that are involved in cell growth and differentiation (3). Some RING fingers may be required for protein association, e.g. homo- or heterodimerization, whereas others are needed for ubiquitination (4–6). The ubiquitination product is an isopeptide bond between the C-terminal carboxyl of ubiquitin (Gly$^{55}$) and a substrate lysine e-amino group. Ubiquitination requires three sequential enzymatic reactions: (i) a ubiquitin-activating enzyme (E1) forms a thiol ester between one of its cysteines and the ubiquitin Gly$^{55}$ carboxyl; (ii) then a conjugating enzyme (ubiquitin carrier protein (E2)) transiently carries the ubiquitin (as a thiol ester); and (iii) a ubiquitin-protein isopeptide ligase (E3) helps to transfer the activated ubiquitin from E2 to the substrate Lys. Generally, eukaryotic cells contain a single type of E1, multiple types of E2, and many different E3 enzymes. Efficient and targeted ubiquitination depends on E3. All known E3 enzymes have one of two E2-binding domains: the RING finger domain or the HECT domain (7). The three-dimensional structures of E3-type RING fingers have been determined by NMR spectroscopy (8–13) or x-ray diffraction (14–16). These structures all have a groove formed by the first zinc-binding loop (N-loop; Cys$^{134}$–Cys$^{137}$ of the E3 EL5), the second zinc-binding loop (C-loop; Cys$^{72}$–Cys$^{75}$ of EL5), and the central α-helix (Cys$^{161}$–Leu$^{166}$ of EL5) that is the site for E2/E3 binding.

EL5 is a rice RING-H2 finger protein of 325 amino acids, is structurally related to the *Arabidopsis* ATL family of RING-H2 finger proteins, and is rapidly induced when rice cells are exposed to *N*-acetylmuramoyl-L-alanine-L-acidity (17). The ATL family RING-H2 finger proteins each have a transmembrane domain, a basic domain, a conserved domain, and a RING-H2 finger domain that is upstream of a non-conserved C-terminal region (18). Although some ATL family members resemble EL5 as they are also induced during the early stages of a defense response (19), their biological functions are not well characterized. We have shown that maltose-binding protein (MBP)-EL5 RING-H2 finger fusion constructs are polyubiquitinated in *vitro* when ubiquitin, recombiant mouse E1, and human UbcH4/5A (E2) or rice *Oryza sativa* (Os) UBC5a/b (E2) are incubated together (20). We demonstrated that the EL5 RING-H2 finger domain (residues 129–181) is sufficient for E3-type activity when OsUBC5b is present. Furthermore, an EL5 RING-H2 finger/OsUBC5b NMR titration experiment detected altered environments for certain RING-H2 finger amide groups (8). The chemical shift changes indicate direct contact between the RING finger and OsUBC5b as well as conformational changes for certain RING-H2 finger residues.

To identify unambiguously the specific residues involved in OsUBC5b recognition, we prepared recombiant mutant EL5 RING-H2 fingers each containing a single amino acid substitution and tested them for ubiquitination activity *in vitro*. All of these residues are near or in a shallow groove that binds OsUBC5b. We also classified rice RING finger proteins according to type, i.e. RING-H2 and RING-HC, and according to the presence or absence of a Trp homologous to EL5 Trp$^{165}$ of EL5. We then assayed certain members of each category for ubiquitination activity in the presence of OsUBC5b, a UBC4/5-type E2, and, based on our results, concluded that a Trp homologous to EL5 Trp$^{165}$ is one of the most important recognition features necessary for the activity of a RING-H2 finger/OsUBC5b complex.

**MATERIALS AND METHODS**

Cloning and Mutagenesis of Recombinant MBP-ELS Fusion Proteins—The nucleotide sequence for residues 96–181 of EL5 was PCR-amplified using primers 5′-CGAATTCCGAGGGGCTGCAGCCG-3′ and 5′-GGAATTCGACCGATCGATCGGT-3′. The full-length PCR product was purified by electrophoresis on a 2% agarose gel and isolated using a QiAquick gel extraction kit (Qiagen Inc.). The purified PCR product
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was digested with EcoRI and BamHI and then inserted at the EcoRI and BamHI sites of pMAL-c2X (New England Biolabs, Beverly, MA). As a negative control, pMAL-c2X was digested with EcoRI and treated with Klenow fragment (Takara) to produce blunt ends and then self-ligated. The genes for the mutants of the wild-type fusion protein (MBP-EL5—(96–181)) were created by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene) using pMAL-c2X-EL5—(96–181) as the template. Mutagenic primers contained the following codon changes: V136A, GTC → GCG; L138A, CTC → GCC; R148A, AGG → GCG; C153A, TGC → GCC; E160A, GAG → GGC; V162A, GTC → GCC; V162W, GTC → TGG; D163A, GAC → GCC; M164A, ATG → GCC; W165A, TGG → GCC; W165I, TGG → ATC; L166A, CTC → GCC; T171A, ACC → GCC; L174A, CTC → GCC; R176A, CGC → GCC; R176D, CGC → GAC; and V162W/W165A, GTGGACATGTGG → TGGGACATGGCG. The sequences of all PCR constructs were verified by DNA sequencing. Escherichia coli BL21(DE3) cells that were first grown to OD600 nm < 0.5, then induced with isopropyl β-D-thiogalactopyranoside (1 mM final concentration), and finally cultured for an additional 3 h at 37 °C in M9 minimal medium plus 15N-labeled NH4Cl and 50 μg/ml ampicillin. Protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (final concentration), and 100 μM ZnSO4 (final concentration) was added at the same time. After 3 h, the cultures were centrifuged, and the pellets were frozen.

Cloning and Purification of Recombinant OsUBCSb—The recombinant OsUBCSb gene, prepared for this study, is a fusion construct with an upstream thioredoxin (Trx)-His6- tag sequence. The OsUBCSb nucleotide sequence was PCR-amplified using primers 5′-TTCCA-TGGGCTTACGGAGATCTCTTAAG-3′ and 5′-AACCTGAGCTAC-GCCCATAGCATATTCTTGGGT-3′. The purified PCR product was digested with NcoI and XhoI and then inserted into pET-32a (Novagen). BL21(DE3) cells were transformed with this expression vector. Bacteria were grown at 37 °C in 1 liter of LB medium containing 50 μg/ml ampicillin. Protein expression was induced with isopropyl β-D-thiogalactopyranoside (1 mM final concentration). After 3 h, the bacterial cultures were centrifuged, and the pellets were frozen.

To purify the OsUBCSb fusion construct, a frozen pellet was first thawed on ice, resuspended in 20 mM phosphate (pH 7.4) and 0.5 mM NaCl, and sonicated. Insoluble material was removed by centrifugation at 27,000 × g for 30 min. The supernatant was loaded onto a 5-ml HiTrap chelating HP column (Amersham Biosciences) to which nickel was bound (nickel-chelating column). After washing the column with 20 mM phosphate (pH 7.4) and 0.5 mM NaCl, protein was eluted with a linear gradient of 0–0.5 mM imidazole in 20 mM phosphate (pH 7.4) and 0.5 mM NaCl. Protein fractions were pooled, concentrated, and then applied to a HiLoad 26/60 Superdex 75 prep grade column (Amersham Biosciences) equilibrated with 20 mM phosphate (pH 7.4) and 0.1 mM NaCl. The Trx-His6 tag was removed from the Superdex-purified protein by thrombin (Novagen) proteolysis (28 units of thrombin/13 mg of protein) at 37 °C for 12 h, after which the solution was applied to a nickel-chelating column. To ensure that no residual tag remained, the protein in the flow-through fraction was further digested with enterokinase (130 units/5 mg of protein; Invitrogen) at 37 °C for 16 h. The solution was then applied to a HiLoad 26/60 Superdex 75 prep grade column. Purified OsUBCSb was dialyzed against 20 mM Tris-HCl (pH 7.0), 0.1 mM NaCl, and 5 mM dithiothreitol and concentrated using a Centricon spin dialysis tube (10-kDa cutoff; Millipore Corp.).

Cloning and Purification of Recombinant 15N-Labeled ELS—(129–181) Mutants CIS3A and W165A—The ELS—(129–181) mutants CIS3A and W165A were cloned as upstream Trx-His6-tagged nucleotide sequences into pET-32a. ELS—(129–181) mutant DNA fragments were amplified as described previously (8). The purified PCR products were digested with NcoI and BamHI and inserted into pET-32a, and the vector was introduced into E. coli BL21(DE3) cells. Bacteria were grown at 37 °C in M9 minimal medium plus 15N-labeled NH4Cl and 50 μg/ml ampicillin. Protein expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (final concentration), and 100 μM ZnSO4 (final concentration) was added at the same time. After 3 h, the cultures were centrifuged, and the pellets were frozen.

The Trx-His6-ELS—(129–181) mutants were purified using procedures similar to those used for 15N-labeled ELS—(129–181) (8). Frozen pellets were thawed on ice; suspended in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 μM ZnSO4, and 2.5 mM β-mercaptoethanol; and sonicated. The solution was centrifuged at 27,000 × g for 30 min, and the supernatant was loaded onto a 5-mL nickel-chelating column. The column was washed with 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 20 μM ZnSO4, 1 mM β-mercaptoethanol, and 15 mM imidazole, and bound protein was eluted in the same buffer containing 400 mM imidazole. Pooled protein fractions were concentrated and applied to a HiLoad 26/60 Superdex 75 prep grade column equilibrated with 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 20 μM ZnSO4, and 2.5 mM β-mercaptoethanol. To remove the Trx-His6 tag, proteins were incubated with enterokinase (75 units/31.5 mg of protein) at 37 °C for 16 h. Each solution was applied to a HiLoad 26/60 Superdex 75 prep grade column. The recovered protein solutions were diluted 5-fold with 50 mM Tris-HCl (pH 7.4), 20 μM ZnSO4, and 2.5 mM β-mercaptoethanol and then applied to a 5-ml HiTrap Q column (Amersham Biosciences). Purified proteins were dialyzed against 20 mM Tris-HCl (pH 7.0), 0.1 mM NaCl, 20 μM ZnSO4, and 1 mM dithiothreitol (which was the buffer used for NMR experiments) and concentrated using a Centricon spin dialysis tube (3-kDa cutoff).

Construction and Purification of Recombinant MBP Fusion Proteins Containing Various RING Finger Domains—cDNA fragments coding for a variety of RING finger domains were PCR-amplified with appropriate primers. PCR fragments were digested at EcoRI and BamHI sites and ligated to EcoRI/BamHI-digested pMAL-c2X vectors, which allowed for expression of upstream MBP fusion constructs. PCR products were verified by DNA sequencing. Plasmids were introduced into E. coli BL21(DE3) cells, and after culture, the recombinant proteins were purified by amyllose resin affinity column chromatography as described above for the MBP-EL5—(96–181) mutants.

In Vitro Ubiquitination Assay—Ubiquitination reactions were performed in 75-μl solutions containing 40 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 2 mM ATP, 2 mM dithiothreitol, 300 ng/μl bovine ubiquitin (Sigma), 50 ng of recombinant mouse E1, 100 ng of OsUBCSb, and 400 ng of one of the MBP-RING fingers. Reactions proceeded for 1 h at 30 °C and were stopped by addition of SDS-PAGE sample buffer. After boiling the samples for 5 min, proteins were separated on a 7.5% SDS-polyacrylamide gel and immunoblotted with anti-MBP antibody (New England Biolabs).

NMR Spectroscopy—All NMR spectra were recorded at 35 °C using a Bruker DMX750 or AV500 spectrometer equipped with a 5-mm inverse...
triple-resonance probe head with three-axis gradient coils. All spectra were processed using NMRPipe software (22). The $^1$H, $^{13}$C, and $^{15}$N chemical shifts were referenced to HDO (4.68 ppm at 35 °C), indirectly to sodium 3-(trimethylsilyl)-propionate ($^{13}$C) (23), and to liquid ammonia ($^{15}$N) (24), respectively.

RESULTS

Identification of EL5 RING-H2 Finger Residues That Are Critical for OsUBC5b Functional Interactions—EL5 RING-H2 finger residues were chosen for mutagenesis based on structural information obtained from
our previous EL5 RING-H2 finger/OsUBC5b NMR titration experiment (8) and an examination of the complex formed by the RING finger ubiquitin ligase c-Cbl and its cognate E2, UbcH7 (15). Previously, we found that the amide NMR signals of seven EL5-(121–181) residues (Val136, Cys137, Ala147, Arg148, Glu160, Thr171, and Leu174) were significantly perturbed when EL5 bound OsUBC5b. The amide signals of five other residues (Leu138, Val162, Asp163, Met164, and Trp165) were so broadened that they were not detectable. This phenomenon probably reflects an intermediary exchange rate for the free and protein-bound amide protons of those residues compared with the chemical shift time scale. The residues with chemical shift perturbations \( \pm 0.1 \) ppm (with perturbation defined as \( |\Delta \delta| = |\Delta \delta_{\text{HN}}| + 0.1|\Delta \delta_{\text{SN}}| \)) and those for which the resonances disappeared entirely (8) were mapped onto the EL5 RING finger surface (Fig. 1B). These residues are all located on the side of the molecule, which embodies the E2-binding surface. The location of this binding site parallels that of the c-Cbl RING finger-binding
site (15). Although the amide signal of EL5 Arg176 does not change position or broaden upon complex formation (8), we also prepared R176A and R176D mutants for the ubiquitination assay because a basic residue is often found in other E3 RING finger domains at the homologous position (Fig. 1A), and based on conformity with the crystal complex of c-Cbl/H18528 UbcH7 (15), Arg176 may interact with OsUBC5b. Thirteen mutants (V136A, L138A, R148A, E160A, V162A, D163A, M164A, W165A, L166A, T171A, L174A, R176A, and R176D) were prepared for the ubiquitination assay. The C153A mutant served as a negative control because it was not expected to chelate zinc, which should consequently disrupt the protein fold. The side chains of the mutated residues are displayed on an EL5 RING-H2 finger ribbon diagram (Fig. 1C).

The effects of the mutations were monitored by a ubiquitination assay using the recombinant MBP-EL5-(96–181) mutants, OsUBC5b, and mouse E1 (Fig. 2, A–C). We observed three distinguishable results. (i) Mutations V136A, W165A, L166A, R176A, R176D, and C153A completely abolished ubiquitination (Fig. 2A); (ii) Mutations L138A, R148A, V162A, D163A, and L174A significantly decreased ubiquitination (Fig. 2B); and (iii) mutations E160A, M164A, and T171A did not affect ubiquitination (Fig. 2C). Therefore, Val136, Cys153, Trp165, Leu166, and Arg176 participate in necessary functional interactions between the EL5 RING-H2 finger and OsUBC5b. C153A, which cannot chelate zinc, lost ubiquitination activity because it cannot fold properly, as will be discussed below. Three of
E2 Recognition Residues of the RING-H2 Finger Domain

FIGURE 5. Amino acid sequence alignments of rice RING-H2 and RING-HC finger domains. A, category I-a, RING-H2 fingers with the conserved Trp corresponding to residue 165 in EL5; B, category I-b, RING-HC fingers with the conserved Trp; C, category II-a, RING-H2 fingers without the conserved Trp; and D, category II-b, RING-HC fingers without the conserved Trp. The RING fingers used in the ubiquitination assays (Fig. 7) are identified by red asterisks. Metal-chelating residues are highlighted in yellow. Conserved Trp residues are highlighted in magenta. Residues in EL5 that abolished or decreased ubiquitination activity when mutated are highlighted in blue and green, respectively.
the five aforementioned residues (Val\textsuperscript{136}, Trp\textsuperscript{165}, and Leu\textsuperscript{166}) form the hydrophobic and shallow groove in EL5 (8), as was also found for c-Cbl (15). Of special note is Trp\textsuperscript{165}, which is at the center of the hydrophobic groove that defines the OsUBC5b-binding site. For c-Cbl, the homologous Trp is required for E3 activity (25). Additionally, because the R176A and R176D mutants are inactive, an electrostatic interaction must also be important for ubiquitination. Val\textsuperscript{136}, Leu\textsuperscript{138}, Val\textsuperscript{162}, Asp\textsuperscript{163}, and Leu\textsuperscript{174} are spatially near the residues that are essential for E3 activity. Val\textsuperscript{136} and Leu\textsuperscript{138} are located at and next to the N-loop, respectively; Val\textsuperscript{162}, Asp\textsuperscript{163}, Trp\textsuperscript{165}, and Leu\textsuperscript{166} are part of the central α-helix; and Leu\textsuperscript{174} and Arg\textsuperscript{176} are located in the C-loop. Therefore, these residues directly contact OsUBC5b.

Although the amide resonances of Glu\textsuperscript{160}, Met\textsuperscript{164}, and Thr\textsuperscript{171} shift significantly when the EL5 RING-H2 finger interacts with OsUBC5b (8), mutation of these residues did not affect ubiquitination. Therefore, the NMR titration experiment detected not only those residues in direct contact with OsUBC5b, but also nearby neighboring residues whose spatial orientations might have been altered upon binding.

Ubiquitination is therefore abolished by mutation of residues that contact OsUBC5b directly or when proper folding of the RING finger is prevented, as we demonstrated previously when zinc was removed from the EL5 RING-H2 finger (8). To establish whether the EL5 RING-H2 finger mutants C153A and W165A were inactive because they were not folded correctly, we recorded their \textsuperscript{1}H-\textsuperscript{15}N heteronuclear single quantum correlation (HSQC) NMR spectra and compared the spectra with that of the wild-type RING finger. The \textsuperscript{1}H-\textsuperscript{15}N HSQC NMR signals of the wild-type RING finger spectrum are well dispersed, which is a hallmark of a well defined molecular structure (Fig. 3A); but the \textsuperscript{1}H-\textsuperscript{15}N HSQC NMR spectrum of C153A does not have the characteristic chemical shift dispersion of a folded protein (Fig. 3B), which correlates with the loss of activity (Fig. 2A). Conversely, the \textsuperscript{1}H-\textsuperscript{15}N HSQC NMR signals of the W165A mutant are as dispersed as those of the wild-type RING finger (Fig. 3C). This mutant has a native fold, but is incapable of ubiquitination (Fig. 2A), which suggests that Trp\textsuperscript{165} interacts functionally with OsUBC5b.

Conserved Tryptophans That Are Part of RING-H2 Finger Central Helices Are Important for Ubiquitination—Our results support the hypothesis that the hydrophobic groove and the basic residue that is located at the tip of the groove functionally interact with OsUBC5b. Notably, Trp\textsuperscript{165} (located at the center of the hydrophobic groove) is well conserved in other E3 RING fingers, including those of c-Cbl, Rma1 (26), and Hrd1 (27). It was recently reported that CNOT4, a C4C4 type RING finger, is an E3 (28). The shallow hydrophobic groove of CNOT4, which interacts with UbcH5B, is reminiscent of the EL5 and c-Cbl grooves. However, although the Trp residues in the EL5 and c-Cbl grooves are at identical positions, for the CNOT4 RING finger, an Ile is found at the position homologous to EL5 Trp\textsuperscript{165}, and a tryptophan is found at the position homologous to EL5 Val\textsuperscript{162} (Fig. 4A). The EL5 Trp\textsuperscript{165} and Val\textsuperscript{162} side chains are displayed on a ribbon structure of the EL5 RING-H2 finger in Fig. 4B. Note that the side chains of both residues point in the same direction. To investigate the importance of Trp\textsuperscript{165} and its positional effect on ubiquitination, we prepared the EL5 RING-H2 finger mutants V162A, V162W, W165A, W165I, and V162W/W165A (Fig. 4C, lanes 9 and 10) and W165I (lanes 11 and 12), which lack a Trp. Inactive, the Trp\textsuperscript{165} mutants with a Trp in the central α-helix, such as V162A (Fig. 4C, lanes 5 and 6), V162W (lanes 7 and 8), and V162W/W165A (lanes 13 and 14), are active, although the V162W mutant is nearly inactive, perhaps because of a steric clash between the two Trp residues. Notably, V162W/W165A is active even though the Trp is displaced one turn along the central α-helix. The \textsuperscript{1}H-\textsuperscript{15}N HSQC NMR spectrum of the V162W/W165A RING-H2 finger indicates that the mutant folds correctly (data not shown). Therefore, it is probable that a Trp in the interior of the hydrophobic groove is necessary for ubiquitination activity.

Classification of Rice RING Finger Domains—The rice genome has been sequenced (O. sativa Genome Project), and as of 2003, 28,000 full-length cDNA clones had been prepared (21). Presently, there are 32,127 full-length cDNA clones, and their sequences are available at cDNA database. All rice RING finger sequences in the cDNA database can be listed in supplemental Fig. S1, with examples given in Fig. 5. About 0.7% (218/32,127) of the O. sativa proteins suggest the presence (category I) or absence (category II) of a conserved Trp at the position homologous to EL5 Trp\textsuperscript{165}. Each category was further subdivided into two: RING-H2 fingers (category a) and RING-HC fingers (category b). There are 121 category I-a, 26 category I-b, 21 category II-a, and 54 category II-b RING fingers; for which examples are given in Fig. 5. The lengths of the spacers, between the first and second pairs of metal-chelating residues (spacer I) and between the third and fourth pairs of metal-chelating residues (spacer II), are not conserved for pro-

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**FIGURE 6.** Number of residues in spacer I (A) or in spacer II (B) versus the percentage of proteins in each category with that number of residues in the spacer. Category I-a is colored red; category I-b is colored magenta; category II-a is colored blue; and category II-b is colored light blue.
TABLE ONE

Degree of conservation for E2-binding residues involved in OsUBC5b binding of category I-a (RING-H2 fingers with the conserved tryptophan), category I-b (RING-HC fingers with the conserved tryptophan), category II-a (RING-H2 fingers without the conserved tryptophan), and category II-b (RING-HC fingers without the conserved tryptophan).

There are 121 proteins in category I-a, 26 proteins in category I-b, 21 proteins in category II-a, and 54 proteins in category II-b. The number of RING fingers within a given category that have the designated residue is given in parentheses.

|       | Category I-a | Category I-b | Category II-a | Category II-b |
|-------|--------------|--------------|---------------|---------------|
|       | %            | %            | %             | %             |
| Val136| 95.9 (116)   | 96.2 (25)    | 85.7 (18)     | 90.7 (49)     |
| Leu138| 89.3 (88)    | 50.0 (13)    | 47.6 (10)     | 29.6 (16)     |
| Val162| 98.3 (119)   | 73.1 (19)    | 81.0 (17)     | 59.3 (32)     |
| Asp163| 55.4 (67)    | 3.8 (1)      | 11.5 (3)      | 5.5 (3)       |
| Leu174| 93.4 (113)   | 30.8 (8)     | 47.6 (10)     | 59.3 (32)     |
| Leu176| 79.3 (96)    | 65.4 (16)    | 76.2 (22)     | 40.7 (22)     |
| Arg177| 90.3 (108)   | 92.3 (24)    | 47.6 (10)     | 41.0 (22)     |
teins in any of the categories. To discern any pattern(s) associated with spacer lengths and type of RING finger, we plotted a bar graph of the spacer length versus the number of fingers (as a percentage) in a given category with the corresponding spacer length (Fig. 6).

To determine, in general, whether E3-type activity is dependent on the conserved Trp, we assayed randomly chosen RING fingers fused to an upstream MBP from each of the four categories in the presence of OsUBC5b (Fig. 7) or Ubch7 (data not shown). Only RING-H2 domains with the conserved Trp (category I-a) exhibit E3 activity and do so at levels comparable with MBP-EL5-96–181 (Fig. 7A, lanes 3–8). RING fingers of the other three categories are not active when OsUBC5b serves as E2 (Fig. 7A, lanes 9–14; and B, lanes 3–14). Therefore, probably the conserved Trp is required for the E3-type activity of RING-H2 fingers, at least when OsUBC5b is the E2. Additionally, none of the tested RING fingers are active in the presence of Ubch7 (data not shown).

DISCUSSION

A Consensus Structure for RING-H2 Finger E2-binding Sites—EL5 interacts with OsUBC5b, which is structurally related to the human E2 UbcH4/5 (20). The EL5 RING-H2 finger residues Val136, Leu138, Val162, Asp163, Trp165, Leu166, Leu174, and Arg176 participate in binding OsUBC5b (this work and Ref. 8). These residues delineate a shallow groove, which is the RING-H2 finger E2-binding site (Fig. 2, A and B).

The c-Cbl RING finger Ubch7-binding site includes the c-Cbl residues Ile192 in the N-loop; Cys404, Ser407, Trp408, and Ser411 in the central helix; Pro417 and Phe418 in the C-loop. These residues interact with the Ubch7 L1 and L2 loops (15). When c-Cbl Trp408, which is homologous to EL5 Trp165, is replaced with Ala, the c-Cbl RING finger binding affinity for the E2 UBC4/5 domain is reduced, and ubiquitination activity is lost (25). Zheng et al. (16) reported the structure of an SCF ubiquitin ligase complex, which includes the RING finger protein Rbx1 (but not an E2). These researchers inferred the crystallographic position of the loop-helix-loop Rbx1 E2-binding site by comparison with the c-Cbl-Ubch7 crystal structure (15). They substantiated their deduction using mutagenesis and found that mutants of Rbx1 containing an Ala substitution for Trp5, Lys6, Thr9, or Arg12 did not complement a yeast assay using yeast OsUBC5b and perhaps others of the UBC4/5 E2 subtype. A hydrophobic residue at the position corresponding to EL5 Val136 is found in most of the fingers, regardless of their assigned category (~90% homology). The high degree of conservation suggests that this position may also be required for folding/stabilization. Four other residues (Leu138, Val162, Leu174, and Leu176) are well conserved (~80–95%) in category I-a fingers, but are not in members of the other categories (<80%). The residue found at the position corresponding to EL5 Arg176 is almost always basic (~90% homology) in the category I-a and I-b fingers, whereas the residues at the corresponding positions in the category II-a and II-b proteins are much more diverse (~45% homology).

The sequence analysis of the rice RING-H2 fingers indicates that the category I-a proteins are probably E3-type proteins, which probably bind the E2 OsUBC5b and perhaps others of the UBC4/5 E2 subtype. This proposal is also supported by the fact that only category I-a RING fingers have E3-type activity in the presence of OsUBC5b (Fig. 7).

In summary, we identified EL5 RING-H2 finger residues that are necessary for ubiquitination. We also predict that the conserved Trp, found in all category I-a RING fingers and corresponding to EL5 Trp165, will be necessary for ubiquitination activity in conjunction with a UBC4/5 E2-type protein.

Role of the Trp for E3 Activity in the EL5 RING-H2 Finger Domain—The following results demonstrate that Trp165 is essential for EL5 RING-H2 finger/OsUBC5b functional interaction. (i) The Trp165 amide NMR signal broadens beyond detection when the RING finger is titrated with OsUBC5b (8). (ii) Trp165 is located at the center of the shallow groove, which is part of the E2-binding site (Fig. 3). (iii) Mutation of Trp165 to Ala or Ile completely abolishes E3 activity (Fig. 4). (iv) Mutants V162A and V162W and the double mutant V162W/W165A are active; therefore, a Trp at or near position 165 correlates with E3 activity (Fig. 4C). Additionally, other E3-type RING fingers, such as c-Cbl (25), Rma1 (26), and Hrd1 (27), have a Trp at the analogous position.

Characterization of Rice RING Finger Domains—There is intense interest in RING finger proteins because their occurrence in eukaryotes is ubiquitous. Within the Arabidopsis genome alone, there are 387 putative RING finger proteins (29). The biological functions of most of these proteins are not known. There are 218 cDNA clones of putative RING finger proteins, containing a total of 220 RING fingers, presently available in the rice cDNA library. Again, the functions of most of these proteins are not known. Supplemental Fig. S1 lists the sequences of all 220 RING finger domains according to one of our four categories, whereas Fig. 5 gives representative examples within each category. Two characteristics of these domains are worth examining. (i) The characteristic spacer length varies among the fingers of the different categories. Most of the category I-a fingers have spacer I lengths of 14 or 15 residues, whereas most of the category I-b fingers are 11 residues in length (Fig. 6A). The spacer I lengths for category II fingers, although centered on 11 residues, vary more than those for category I fingers (Fig. 6A). Most of the category I-a fingers have spacer II lengths of 10 residues (Fig. 6B). Unlike those for categories I-b and II-a, spacer II lengths for category II-b fingers vary significantly; some even have very long sequences. Spacer II lies between the central α-helix and the C-loop. (ii) In addition to Trp165, residues corresponding to Val136, Leu138, Val162, Asp163, Leu166, Leu174, and Arg176 are well conserved among category I-a RING fingers (Fig. 5, supplemental Fig. S1, and TABLE ONE). A hydrophobic residue at the position corresponding to EL5 Val136 is found in most of the fingers, regardless of their assigned category (~90% homology). The high degree of conservation suggests that this position may also be required for folding/stabilization. Four other residues (Leu138, Val162, Leu174, and Leu176) are well conserved (~80–95%) in category I-a fingers, but are not in members of the other categories (<80%). The residue found at the position corresponding to EL5 Arg176 is almost always basic (~90% homology) in the category I-a and I-b fingers, whereas the residues at the corresponding positions in the category II-a and II-b proteins are much more diverse (~45% homology).

The sequence analysis of the rice RING-H2 fingers indicates that the category I-a proteins are probably E3-type proteins, which probably bind the E2 OsUBC5b and perhaps others of the UBC4/5 E2 subtype. This proposal is also supported by the fact that only category I-a RING fingers have E3-type activity in the presence of OsUBC5b (Fig. 7).
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