High Precision NMR Structure and Function of the RING-H2 Finger Domain of EL5, a Rice Protein Whose Expression Is Increased upon Exposure to Pathogen-derived Oligosaccharides*

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EL5, a RING-H2 finger protein, is rapidly induced by N-acetylchitooligosaccharides in rice cell. We expressed the EL5 RING-H2 finger domain in *Escherichia coli* and determined its structure in solution by NMR spectroscopy. The EL5 RING-H2 finger domain consists of two-stranded β-sheets (β1, Ala147–Phe149, β2, Gly156–His158), one α-helix (Cys161–Leu166), and two large N- and C-terminal loops. It is stabilized by two tethered-prly coordinated zinc ions. This structure is similar to that of other RING finger domains of proteins of known function. From structural analogies, we inferred that the EL5 RING-H2 finger is a binding domain for ubiquitin-conjugating enzyme (E2). The binding site is probably formed by solvent-exposed hydrophobic residues of the N- and C-terminal loops and the α-helix. We demonstrated that the fusion protein with EL5-(96–181) and maltose-binding protein (MBP) was polyubiquitinated by incubation with ubiquitin, ubiquitin-activating enzyme (E1), and a rice E2 protein, *OsUBC5b*. This supported the idea that the EL5 RING finger domain is essential for ubiquitin-ligase activity of EL5. By NMR titration experiments, we identified residues that are critical for the interaction between the EL5 RING-H2 finger and *OsUBC5b*. We conclude that the RING-H2 finger domain of EL5 is the E2 binding site of EL5.

Upon sensing the invasion of microorganisms, plants evoke a variety of defense reactions, including the synthesis of antimicrobial compounds (phytoalexins) and proteins. Many of these biochemical reactions are based on the activation of defense-related genes. In some cases, the level of protein accumulation and the rapidity of gene induction in the host plant are correlated to the degree of its disease resistance. Therefore, it might be possible to control disease resistance by modifying the regulatory factors for the expression of defense-related genes.

Such regulatory factors could be elements of signal transduction pathways leading from the recognition of invading pathogens to the activation of defense-related genes. Most of the defense responses are reproducible in suspension-cultured cells treated with specific substances called elicitor (1). Chitin fragments (N-acetylchitooligosaccharides) can act as elicitors (2), which induce the transient expression of several "early responsive" genes, such as EL5 (3). EL5 is a RING finger protein, which is structurally related to proteins of the *Arabidopsis* family. These proteins are characterized by a transmembrane domain (domain I), basic domain (domain II), conserved domain (domain III), and RING-H2 finger domain (domain IV) followed by the C-terminal region with highly diverse amino acid sequences (4). Although some ATL family genes resemble EL5 in being induced in early stages of the defense responses (5), their biochemical function is obscure. Recently, it was shown that the fusion protein of EL5 with maltose-binding protein (MBP) was polyubiquitinated by incubation with ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2). Apparently, EL5 acted as a ubiquitin ligase (E3) and catalyzed the transfer of ubiquitin to the MBP moiety. Further, it was shown that a rice E2, *OsUBC5b* was induced by elicitor (6). Although these results strongly suggest that EL5 and *OsUBC5b* have roles in plant defense response through the turnover of protein(s) via the ubiquitin/proteasome system, their molecular function has not been clarified at the atomic level.

The RING finger motif, which is known from many functionally distinct proteins, was first identified in the product of the human gene RING1 (Really Interesting New Gene 1) that is located proximal to the major histocompatibility region on chromosome 6 (7). The RING finger motif is defined by the consensus sequence Cys2-X2-Cys-X29-35-Cys2-X3-His2-X3-Cys (Cys/His)-X-Cys-X48-Cys2-X2-Cys, in which X can be any amino acid. It binds two zinc atoms with its Cys and His residues in a unique “cross-brace” arrangement. The invariable spacing between the second and third pair of Cys/His residues indicates conservation of the distance between the two zinc-binding sites (8). The RING finger motif is widely distributed among proteins that play major roles in cell growth and differentiation (9). Certain types of RING finger domains seem to be required for multimerization, while others are elements of proteins involved in ubiquitination (10). Ubiquitination usually results in the formation of a bond between the C terminus of ubiquitin (Gly76) and the ε-amino group of a substrate Lys residue. This
reaction requires the sequential activation of three enzymes: (i) an activating enzyme (E1) that forms a thiol ester with the carboxyl group of Gli9 in ubiquitin, (ii) a conjugating enzyme (E2) that transiently carries the ubiquitin as a thiol ester, and (iii) a ligase (E3) that transfers the activated ubiquitin from the E2 to the substrate Lys residue. The efficiency and high selectivity of ubiquitination reactions depend on the accuracy of E3 action. All known E3s utilized either of catalytic domains, the RING finger domain or the HECT domain (11). The structures of RING finger domains have been determined by NMR (12, 13) and x-ray (14, 15). However, the relationship between molecular function and high order structure has not yet been established.

To elucidate the properties of EL5 at the atomic level, we determined the three-dimensional structure of its RING-H2 finger domain in solution by NMR spectroscopy. Furthermore, we characterized its functional properties by an ubiquitination assay in vitro and by NMR titration experiments. Our results illuminate the function of not only EL5 but also the ATL family proteins in general.

EXPERIMENTAL PROCEDURES

Cloning and Purification of Recombinant Proteins for NMR Experiments—EL5 RING-H2 finger domains and OsUCB5b were cloned as fusion proteins with thioredoxin (Trx) and His tag. The fusion proteins were constructed in the vector pET32a (Novagen). EL5(96–181) and EL5(96–181) DNA fragments from EL5 cDNA (AB045120) were amplified by polymerase chain reaction (PCR). pET32a was amplified by PCR from OsET32a cDNA (AB074412). Trx-EL5(129–181) was constructed using the primers 5'-GGATCCCATGCGAAGAGCGCGGTGAGT-3' and 5'-GCAATTCCTACAGCCAGCTAGTACG-3'. Trx-EL5(96–181) was constructed using the primers 5'-GGATCCATGGGGGTACGTCAGTGGAT-3' and 5'-GCGGATCTTCCTACAGCCAGCTAGTACG-3'. The purified PCR products were digested with NcoI and EcoRI and ligated into pET32a. Trx-UCB5b was constructed with the primers 5'-TCTTCCATGCGAAGAGCGCGGTGAGT-3' and 5'-AACCTCGGACTCCTAGGATCTCTTG-3'. The purified PCR product was digested with NcoI and XhoI and was ligated into pET32a.

All of the clones were transformed into Escherichia coli BL21(DE3) cells. The bacteria were grown at 37 °C in Luria Bertani for non-labeled protein and in M9 minimal medium for uniformly 15N- and 15N/13C-labeled protein, with 50 µg/ml ampicillin. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 1 mM. For EL5(129–181) and EL5(96–181), ZnSO4 (final concentration 100 µM) was added at the same time. After 3 h the bacteria were harvested by centrifugation, and the pellets were frozen. Trx-EL5(129–181) and Trx-EL5(96–181) were purified by a similar procedure. Frozen pellets were thawed on ice and resuspended in 50 mM Tris-HCl buffer (pH 7.4), 50 mM NaCl, 50 µM ZnSO4, and 2.5 mM β-mercaptoethanol, before lysis by sonication. Insoluble material remaining after lysis was removed by centrifugation at 27,000 × g for 30 min. The supernatant was loaded onto a 5-ml Ni-NTA column equilibrated in buffer (20 mM phosphate buffer, pH 7.4, 100 mM NaCl). Trx tag was removed by cleavage with 28 units of thrombin (Novagen) per about 13 mg of protein at 37 °C for 12 h. The solution was applied to a Ni-NTA column equilibrated with 28 units of thrombin (Novagen) per about 5 mg of protein at 37 °C for 16 h. Then the solution was applied to a HiLoad Superdex 75pg 26/60 column. Peak fractions were dialyzed against NMR buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.0, 5.0 m M dithiothreitol) and concentrated using a Centricon spin dialysis tube (3-kDa cutoff; Amicon, Inc.).

NMR Spectroscopy—All NMR spectra were recorded at 35 °C on a Bruker DRX750 spectrometer equipped with a 5-mm inverse triple resonance probe head with three-axis gradient coils. H1, 13C, and 15N resonance assignments were obtained using two-dimensional double resonance and three-dimensional double and triple resonance through-bond correlation experiments (16–18): two-dimensional H1/13C and 13C/15N-separated NOESY spectrum with mixing time of 100 ms, three-dimensional H1/13C/15N-separated NOESY spectrum with mixing time of 100 ms, and four-dimensional 13C/15N/13C-separated HMBC-NOESY-HMBC spectrum with mixing time of 100 ms. Amide-proton exchange rates were determined by recording a series of two-dimensional H1/15N NOESY spectra at different time points immediately after the D2O buffer was changed to H2O buffer. The cross-peak intensity with the amide proton was monitored over time using UPLC/HPLC technology (Bruker Biospin Diode Array). Our results were confirmed by monitoring the changes in the chemical shifts of the H1-15N cross-peaks of Ile119, Ile209, and Ile210.

Structure Calculations—NOE-derived interproton distance restraints were classified into four ranges: 1.8–2.7, 1.8–3.3, 1.8–4.3, and 1.8–5.0 Å, corresponding to strong, medium, weak, and very weak NOEs. The upper limit was corrected for constraints involving methyl protons and methylene protons that were not assigned spectroscopically (23). Hydrogen bond distance restraints were applied to N and O atoms (1.8–3.3 Å) and to HN and O atoms (1.8–2.3 Å), in regular secondary structures that had main chain amide hydrogen bond angle restraints on φ and ψ were derived from 1H/15N, coupling constants (24), short-range NOE contacts (δH to NH), and δH to NH contacts, and a data base analysis of backbone (15N, 13C), Hα, 13C=N chemical shifts using the program TALOS (25). Side-chain χ2 angle restraints were derived from HOHAA EAAb connectivities and the distances between δH and δH and between δH and δH, which were estimated from NOEs and ROEs. Values for δH and δH coupling constants were also taken into consideration. The structures of the RING-H2 finger domain of EL5 were calculated using the hybrid distance geometry-dynamical simulated annealing method (26), as contained in X-PLOR 3.1 (27). For structure calculations, we used 755 interproton distance restraints comprising 290 intraresidue, 185 medium range (1 < |i−j| < 5), and 207 long range (|i−j| ≥ 5) restraints obtained from heteronuclear three- and four-dimensional NOE spectra. In addition to the NOE-derived distance restraints, 18 distance restraints for 9 hydrogen bonds and 140 dihedral angle restraints (46 φ, 47 ψ, 38 χ1, and 9 χ2) were included in the calculation. A total of 15 lowest energy structures was selected from these calculations. None of them had NOE and dihedral angle violations of >0.5 Å and >5°, respectively. Structural statistics calculated for the final 20 structures are summarized in Table I. Statistics did not change significantly when the lowest energy structures were used for calculation. The average coordinate of the ensembles of the final 20 structures is summarized in Table I. Statistics did not change significantly when the lowest energy structures were used for calculation. The average coordinate of the ensembles of the final 20 structures is summarized in Table I. Statistics did not change significantly when the lowest energy structures were used for calculation.
the RING-H2 finger domain in 20 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl and 2 mM dithiothreitol, as a function of OsUBC5b concentration. Solutions containing 0.25 mM of either 15N-labeled RING-H2 finger domain or non-labeled OsUBC5b were mixed to yield molecular ratios (OsUBC5b : RING-H2 finger domain) of 0, 0.25, 0.5, 0.75, 1, 1.25, and 2. Assignments of the HSQC signals of EL5 RING finger domain bound to E2 were made by tracing the peaks during titration and were confirmed by analyzing three-dimensional triple resonance experiments carried out on the solution containing 0.6 mM 15N/13C-labeled EL5 RING finger domain and 0.72 mM OsUBC5b.

Cloning and Purification of Recombinant MBP Fusion Proteins—The EL5 fragments Gly96–Asn325 and Gly96–Val181 were amplified by PCR using 5′-H11032-GAATTCGGAGGAGGGGTCGACCCG-3′ and 5′-H11032-GGAATTCTCAATCCGGACATGCGC-3′ primers, respectively. The PCR products were digested with EcoRI and inserted into the EcoRI site of pMAL-p2 (New England BioLabs, Beverly, MA) to express MBP-EL5(96–325) and MBP-EL5(96–181). To yield MBP for negative control experiments, pMAL-p2 was digested with EcoRI, blunted, and self-ligated. All PCR products were verified by DNA sequencing. The plasmids were introduced into the E. coli strain JM109 to produce the recombinant proteins and were purified by amylose affinity chromatography according to the manufacturer’s instructions (New England BioLabs).

In Vitro Ubiquitination Experiments—The ubiquitination reaction was performed in 150 μl of ubiquitination buffer containing 300 ng/μl bovine ubiquitin (Sigma), 50 ng of mouse E1, 10 ng OsUBC5b as E2, and 250 ng of MBP fusion protein. The reaction buffer was incubated at 35 °C for 1 h, and the reaction was stopped by the addition of SDS sample buffer. After boiling for 5 min, the samples were separated by 7.5% SDS-PAGE and subjected to immunoblotting using anti-MBP antibody (New England BioLabs).

**RESULTS**

**Determination of NMR Experimental Conditions**—EL5 consists of a transmembrane domain (domain I), a basic domain (domain II), a conserved domain (domain III), and the RING-H2 finger domain (domain IV) followed by the C terminus (Fig. 1a). For NMR experiments, two protein fragments were prepared: EL5 (129–181, domain IV), containing the RING-H2 finger domain only, and EL5 (96–181, domain III + IV), which additionally contained the conserved domain. The 1H-15N HSQC spectrum of EL5(129–181) obtained at 35 °C showed dispersed, intense signals, indicating a well-defined molecular structure (Fig. 1b). In the spectrum of EL5(96–181), the strengths of peaks differed considerably (Fig. 1c). The weak signals corresponded to those of EL5(129–181), that is, to the RING-H2 finger domain. The intense signals were concentrated in the center of the spectrum, indicating that domain III is a highly flexible random coil structure.

To investigate the role of Zn2+ in the folding of the EL5 RING-H2 domain, Zn2+ was chelated by EDTA. Removal of zinc from the purified domain by addition of EDTA led to a loss of the chemical shift dispersion characteristics of the folded proteins (Fig. 1d). The protein was reversibly refolded upon addition of excess Zn2+, as indicated by the reappearance of a spectrum that was identical to that in Fig. 1b. Therefore we decided to carry out the purification of recombinant EL5(129–181) and its NMR analysis in the presence Zn2+.
Solution Structure and Function of EL5 RING Finger Domain

Table I

| Structural statistics for the RING-H2 finger domain of EL5 |
|-----------------------------------------------------------|
|             | SA= | SA= |
| **X-PLOR energies (kcal mol⁻¹)⁶**                           |     |
| E<sub>total</sub> | 182 ± 3.6 | 192 |
| E<sub>bound</sub> | 8.82 ± 0.4 | 9.702 |
| E<sub>angle</sub> | 107 ± 1.6 | 110.8 |
| E<sub>super</sub> | 14.1 ± 1.8 | 14.65 |
| E<sub>brw</sub> | 31.5 ± 1.8 | 16.48 |
| E<sub>inc</sub> | 4.5 ± 0.7 | 36.01 |
| E<sub>tab</sub> | 15.2 ± 0.4 | 4.591 |
| **RMSDs from idealized geometry**                          |     |
| Bond (Å)        | 0.0034 ± 0.0000 | 0.0036 |
| Angle (deg)     | 0.62 ± 0.005 | 0.70 |
| Improper (deg)  | 0.49 ± 0.01 | 0.48 |
| **RMSDs from experimental restraints**                    |     |
| Distances (Å)   | 0.029 ± 0.007 | 0.030 |
| Dihedral angles (deg) | 0.73 ± 0.07 | 0.73 |
| Ramachandran plot analysis (%)⁷ |     |     |
| Residues in most favored regions | 83.0 |     |
| Residues in additional allowed regions | 17.0 |     |
| Residues in generously allowed regions | 0.0 |     |
| Residues in disallowed regions | 0.0 |     |
| **RMSDs from mean structure (Å)⁸**                        |     |
| Backbone (N, Ca, Cα) atoms                             | 0.34 ± 0.003 | 0.47 |
| All non-hydrogen atoms                                 | 0.72 ± 0.005 | 0.88 |

¹H, ¹⁵N, and ¹³C Signal Assignments and Secondary Structure of the EL5 RING-H2 Finger Domain—The backbone amide resonances in the ¹H-¹⁵N HSQC spectrum were sequentially assigned to all non-proline residues based upon the analysis of correlations observed in CBCA(CO)/NH and HNCACB spectra, respectively. The former type of spectrum correlates each ¹⁵N, ¹H signal pair to the Ca and Cβ signals of the preceding residue, while the latter additionally provides the ¹⁵N, ¹H, Ca, and ¹⁵N, ¹H, Cβ intraresidue correlations. The assignments were checked and extended to backbone Hα and CO signals, and to ¹H and ¹³C side-chain signals by correlations observed in the three-dimensional spectra, ¹⁵N-separated HOHAHA-HSQC, HBHA(CO)/NH, HNCO, (CO)/HN, and HCCH-TOSCSY. Side-chain ¹H and ¹³C signals in aromatic regions were assigned to aromatic amino acids according to cross-peak patterns observed in ¹H-¹³C CT-HSQC and CT-HSQC-relay spectra that were optimized for aromatic side chains. The aromatic Hα and Cβ signals were then correlated to sequentially assigned backbone signals through cross-peaks observed in three-dimensional ¹⁵N-separated NOESY-HSQC and ¹⁵N/¹³C-separated NOESY-HSQC spectra and a four-dimensional ¹³C/¹⁵N-separated NOESY spectrum.

In order to obtain a high-resolution NMR structure, the resonance assignments were refined by stereospecific assignments of numerous methylene and methyl protons of Val and Leu residues. Stereospecific assignments of Gly α-protons were derived from ³J_{HNH} coupling constants (24), intrar residue NOEs between HN and α-protons, and sequential NOEs between Gly α-protons (i) and HN protons (i+1). β-protons were stereospecifically assigned using J couplings obtained from HNHB (29) and HOHAHA-HSQC spectra, and by intrar residue α-β distances estimated from NOE/ROE spectra, as described by Clore and Gronenborn (17). In some cases, information from local secondary structure and short-range NOEs was used to facilitate stereospecific assignments. Signals from all Val γ-methyl groups were stereospecifically assigned from estimates of ³J_{CCW}, ³J_{CHH}, ³J_{HH}, and ³J_{HHH} coupling constants and from NOEs between Val α-β and a-γ protons. Leu α-methyl group signals were stereospecifically assigned from NOE patterns observed in three-dimensional NOESY, ROESY, and four-dimensional NOESY spectra.

On the basis of essentially complete signal assignments, interproton distance constraints were derived from short range NOE connectives obtained from three-dimensional ¹⁵N-separated NOESY-HSQC, ¹³C/¹⁵N-separated NOE-HSQC, and four-dimensional ¹³C/¹⁵N-separated HSQC NOESY-HSQC spectra. Dihedral constraints for φ angles were determined from ³J_{HHH} coupling constants derived from Hα/HN intensity ratios measured in HNHA experiments. Slowly exchanging amide protons were assigned in ¹H-¹⁵N HSQC hydrogen exchange experiment and were identified as protons involved in interresidue hydrogen bonds. The β-strand and helical domains indicated by the NOE and J coupling data were corroborated by secondary structural elements predicted by the observed displacements of Ca and Cβ chemical shifts from their random-coil values (31). In summary, the analysis indicated that the RING-H2 finger domain of EL5 contained two β-strands and an α-helix in a ββα arrangement.

Tertiary Structure of RING-H2 Finger Domain of EL5—The three-dimensional structure of the RING-H2 finger domain of EL5 was determined by a hybrid distance geometry/dynamic-simulated annealing approach (26) based upon 913 experimental restraints derived from NMR spectroscopy. Structural statistics for the EL5 RING-H2 finger domain are shown in Table I. The superposed backbone N, Ca, and C’ coordinates of the final 15 structures (Fig. 2a) were well aligned, except for resi-
dues 129–131 at the N terminus and residues 179–181 at the C terminus. For the residues 132–178, the root mean square deviation (r.m.s.d.) for backbone heavy atoms was 0.34 Å, compared with 0.72 Å for all heavy atoms. A ribbon diagram (Fig. 2b) representing the backbone conformation of the restrained, energy-minimized mean structure of the RING-H2 finger domain illustrates its ββα fold (β1, Ala147–Phe149, β2, Gly156–His158, α1, Cys161–Leu166). There is a long flexible loop at each side of the βα structure (N-loop, Val133–Glu146, C-loop, Gly157–Val159).

The three-dimensional structures of a few RING finger domains have been determined. The molecular function of two of them, RAG1 and c-Cbl, is known. The V(D)J recombination-activating protein, RAG1, is a dimer. Its dimerization domain consists of a zinc finger and a RING finger (14). It also contains a unique binuclear zinc cluster instead of the mononuclear zinc site in the RING finger. The determination of the crystal structure of c-Cbl bound to a specific ubiquitin-conjugating enzyme (E2), UbcH7, has revealed that the RING finger domain of c-Cbl recruits the E2 (15). The RING finger domain of EL5 is structurally similar to those of RAG1 and c-Cbl (Fig. 3a). The backbones of the secondary structural elements of the proteins are almost superimposable. The backbone (N, Ca, C, O) atomic r.m.s.d. values for the β-strand- and α-helix-forming residues between the EL5 RING finger domain and RAG-1 or c-Cbl are 0.73 and 1.30 Å, respectively.

Although the overall three-dimensional structures of the RING finger domains of RAG1 and c-Cbl are similar, their molecular functions are different. To obtain information on the molecular function of the RING-H2 finger domain of EL5, we compared the RING finger domains of the three proteins at the atomic level. The RAG1 RING finger domain has one additional α-helix each at its N- and C-terminal residues (Fig. 3a). The RAG1 dimer interface is stabilized by an extensive hydrophobic core containing two clusters of three Phe residues in these additional α-helices (14). In accordance with the observation that c-Cbl is a monomer, its RING finger domain lacks the dimer-stabilizing N- and C-terminal α-helices. As the EL5 RING-H2 finger domain resembles c-Cbl in this respect, it is not expected to form dimers. The c-Cbl RING finger domain binds E2 along a hydrophobic groove (indicated by arrows in Fig. 3b) that is formed by the α-helix of the ββα structure and the two zinc-chelating N- and C-terminal loops (Ref. 15, compare electron potential map in Fig. 3b). On the contrary, the RING finger domain of RAG1, which is a DNA-binding protein without ubiquitin ligase (E3) activity (32), does not possess this groove. Its N- and C-terminal loops are closer together with the remaining space occupied by the side chain of Arg27 (Fig. 3b). Thus, the groove that enables the hydrophobic interaction between c-Cbl and E2 is occupied by a basic group (Arg27) in RAG1, preventing interactions between RAG1 and E2. The residues in c-Cbl that form the hydrophobic contact with E2 (His163, Cys164, Ser167, Trp169, Ser171, and Pro172) (15) are mostly conserved in the RING-H2 finger domain of EL5 (Val136, Cys137, Trp149, Ser161, and Pro162) as shown in Fig. 3b. The spatial arrangements of these hydrophobic residues is quite similar in the two molecules (Fig. 3b), suggesting that EL5 should bind E2 similarly as c-Cbl does.

The EL5 RING-H2 Finger Domain Catalyzes Auto-ubiquitination in Vitro—Takai et al. (6) showed that the fusion protein of EL5(96–325) and maltose-binding protein (MBP-EL5(96–325)) was polyubiquitinated by incubation with ubiquitin, ubiquitin-activating enzyme (E1), and UbcH5a or OsUBC5a/b, a rice E2. Thus, the EL5 domains III, IV, and the C-terminal region are sufficient to catalyze ubiquitin transfer to the MBP moiety in cooperation with E2. It was also demonstrated that replacement of Cys153 by Ser abolished the E3 activity. Our structural analysis suggested that the RING-H2 finger domain of EL5 binds E2. We expressed the EL5 RING-H2 finger domain as a fusion protein with MBP (MBP-EL5(96–181) and MBP-EL5(129–181), respectively) and determined its E3 activity. When MBP-EL5(129–181) was incubated with ubiquitin, E1 and OsUBC5a/b in an in vitro ubiquitination assay, only one ubiquitinated derivative of the fusion protein was detected. When MBP-EL5(96–181) was used in the same experiment, several ubiquitinated derivatives of the fusion protein were observed (Fig. 4, lanes 3 and 4). The latter result was similar to that obtained with MBP-EL5(96–325) (Fig. 4, lanes 1 and 2). These findings showed that only the RING-H2 finger domain was sufficient to bind E2, but that the polyubiquitination chain reaction was disturbed in the small construct, MBP-EL5(129–181), probably because the MBP moiety sterically hindered the development of a polyubiquitin chain. Therefore we decided to carry out the in vitro ubiquitination assay using MBP-EL5(96–181). Since Zn2+ is needed for the correct folding of the EL5 RING finger domain, we tested its effect in the ubiquitination assay. No ubiquitination was detectable in the presence of EDTA (Fig. 4, lanes 5 and 6), but the activity was restored by addition of excess ZnSO4 (Fig. 4, lanes 7 and 8). Thus, Zn2+ facilitates effective EL5-mediated ubiquitination by structurally stabilizing the EL5 RING-H2 finger domain.

Identification of Residues in the EL5 RING-H2 Finger Domain That Interact with OsUBC5—To identify EL5 residues that interact with E2, we recorded amide 15N and 1H chemical shifts of the EL5 RING-H2 finger domain as a function of the concentration of OsUBC5b. Residues located close to OsUBC5b in the E3/E2 complex were anticipated to exhibit large changes in their chemical shifts, because the shift depends on the residue’s magnetic environment. The HSQC signal of EL5(96–181) had indicated that the conserved domain remained unchanged (i.e. unstructured) in the presence of OsUBC5b, implying that it was not involved in the EL5/OsUBC5b interaction. Therefore we used EL5(129–181) in the NMR titration experiments.

In these experiments, the extreme broadening of the signals observed in several residues indicated intermediate exchange on the NMR time scale. From the titration curve that was plotted on the basis of the chemical shift variations observed in the RING-H2 finger domain, the stoichiometry between RING-H2 finger domain and OsUBC5b (1:1) and the dissociation constant (KD = 1 × 10−3 M) were calculated. Seven residues (Val136, Cys137, Ala147, Arg148, Glu160, Thr171, and Leu174) displayed significant chemical shift perturbations upon complex formation with OsUBC5b (Fig. 5a). The amide signals of 5 residues (Leu138, Val162, Asp163, Met164, and Trp169) were not detectable due to extreme broadening of the signal. We ascribe
this phenomenon to an exchange between the free and bound forms on the chemical shift time scale. The residues with the chemical shifts most sensitive (chemical shift perturbations, \(\Delta\delta > 0.15\) ppm) to complex formation were mapped on the free form structure of the EL5 RING-H2 finger domain (Fig. 5b). These residues are all localized on one side of the molecule where they form the E2 binding surface. The location of this binding site is in good agreement with that of the binding site of the c-Cbl RING finger domain for E2 (15).

**DISCUSSION**

Intense interest in RING finger proteins has arisen because of their role in human disease and their widespread occurrence.

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**Fig. 3.** a, comparison of the free RING finger domain of EL5 with the E2-bound form of c-Cbl and the dimerization domain of RAG1. The additional \(\alpha\)-helices at the N and C termini of RAG1 are shown in yellow. b, surface potential representations of these domains. Positively charged areas are blue, and negatively charged areas are red. The hydrophobic grooves in c-Cbl and EL5 are indicated by arrows. The atomic coordinates for c-Cbl and RAG1 were downloaded from the Protein Data Bank at Brookhaven National Laboratory (accession numbers: 1FBV and 1RMD, respectively).

**Fig. 4.** EL5 RING-H2 finger domain-catalyzed auto-ubiquitination in vitro. MBP-EL5(96–325) (lanes 1 and 2), MBP-EL5(96–181) (lanes 3 and 4) and MBP (lanes 9 and 10) were incubated at 35 °C with ATP, ubiquitin, E1, and OsUBC5b (E2) for 1 h, and were then subjected to SDS-PAGE followed by immunoblotting with an anti-MBP antibody. MBP-EL5(96–181) was incubated with EDTA before starting the assay (lanes 5–8), in the absence (lanes 5 and 6) or in the presence of an excess amount of ZnSO4 (lanes 7 and 8). Ladders of bands at higher molecular weights (lanes 2, 4, and 8) indicate the occurrence of ubiquitination.

**Fig. 5.** a, NMR chemical shift perturbation of the EL5 RING-H2 finger domain upon binding to OsUBC5b. Changes in the NMR chemical shifts of RING-H2 finger domain (\(\Delta\delta\)) as induced by complex formation with OsUBC5b, were calculated by the function \(\Delta\delta = \Delta\delta_{\text{HN}} \text{ (pink)} + 0.10 \Delta\delta_{\text{15N}} \text{ (magenta)}\). The light blue bars indicate resonances broadened beyond recognition. b, two views of the surface of the EL5 RING-H2 finger domain. Residues that showed highly sensitive backbone amide chemical shift (\(\Delta\delta > 0.15\) ppm) are colored magenta. Residues marked in red were not detectable due to extreme broadening of the signal after binding of OsUBC5b. The left view is in the same orientation as the models in Fig. 3, while the right view is from the opposite side.
In higher plants, numerous genes for RING finger proteins have been identified. For example, 387 RING finger proteins have been predicted from a search of the \textit{Arabidopsis} genome data base (33). Although RING finger proteins play important roles in plants, there is no information on their structure and/or the relationship between the structure and function.

The EL5 RING-H2 Finger Domain Interacts with UbcH4/5a-Type Ubiquitin-conjugating Enzymes—Our structural-based analysis suggested that the RING-H2 finger domain of EL5 could bind E2. NMR titration experiments and an \textit{in vitro} ubiquitination assay showed that the RING-H2 finger domain of EL5 could bind the E2, OsUBC5b, along a groove formed by a cluster of hydrophobic residues. The primary sequence of the EL5 RING-H2 finger domain shows high similarity with other RING finger domains that interact with UbcH4/5a (Fig. 6a). In fact, the fusion protein of EL5 with MBP was polyubiquitinated by incubation with E1 and UbcH5a. OsUBC5b, the E2 used in the present study, is highly similar to UbcH4/5a (6). Thus, available evidence suggests that the EL5 RING-H2 finger domain belongs to the group of UbcH4/5a-type E2 binding domains. Consequently, EL5 is a UbcH4/5a-type E2 binding E3.

Identification of Residues in the RING Finger Domain That Are Critical for E2 Interaction—The primary sequences of various RING finger domains are compared in Fig. 6a. Four groups can be distinguished on the basis of RING finger domain interaction with other proteins. The first group consists of ubiquitin ligases that cooperate with UbcH4/5a, but not with UbcH7/8; it includes EL5 and AO7 (34). Members of the second group, including HHAR1 (35), interact with UbcH7/8, but not with UbcH1 and UbcH5. c-Cbl forms a group on its own; it is E3-interacting both with UbcH7 (36) and UbcH4 (37). The fourth group includes KAP-1 (38) and RAG1 (14). Its members appear to function in the formation of macromolecular assemblages.

The result of the EL5-E2 NMR titration experiment detects an altered chemical environment for amide groups on EL5. The observed chemical shift changes reveal direct contacts as well
as dynamic conformational changes at a particular position or its close proximity. Our NMR titration experiments also shed some light on the structural basis of the functional classification of the four groups of RING finger domains. Seven residues of the EL5 RING-H2 finger domain (Val136, Cys137, Ala147, Arg148, Glu160, Thr171, Leu174) displayed significant perturbations of chemical shift. Five residues (Leu138, Val162, Asp163, Met164, Trp165) were undetectable in EL5/H9251/H9273.

The side-chain conformations (finger domain with the E2-bound form of c-Cbl suggested that CNOT4) of loop 1. A detailed comparison of the free EL5 RING domains (b) are well conserved in E2 binding RING finger regions is almost identical in c-Cbl and CNOT4 (Fig. 6b). The surface and chelating residues (positions 162 and 165 in EL5) is conserved to act as E3. Only in CNOT4 and HHARI, the position of the Trp residues in both E2 binding was completely abolished in a CNOT4 mutant fact, the Trp at this position is required for E3 activity, because these insights will facilitate the understanding of E2 recognition by ATL family RING finger proteins, which are widespread in higher plants.

**PDB and BMRB Accession Code**—The coordinates of the final structures and the structural constraints used for the calculations have been deposited in the RCSB Protein Data Bank (accession code 1IYM). The chemical shift values of the 

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High Precision NMR Structure and Function of the RING-H2 Finger Domain of EL5, a Rice Protein Whose Expression Is Increased upon Exposure to Pathogen-derived Oligosaccharides

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