39°C as described [R. B. Wickner, *Methods Mol. Genet.* **3**, 141 (1994)]. Translation extracts (cytoplasmic S30 extracts) were prepared exactly as described (*21*) and stored in liquid N_2. Capped and polyadenylated luciferase mRNA was prepared with the T7 luciferase (T7LUC) vector pRG166 (a gift of S. Green and Charles Moehle, RiboGene, Hayward, CA) and the Ampliscribe T7 transcription kit (Epicentre Technologies). mRNA was purified with the RNeasy total RNA kit (Qiagen). Translation assays were performed as described (*21*), except that the micrococcal nuclease treatment of the translation extracts was omitted. Luminescence was measured by adding 10 μl of translation mix to 100 μl of LUC assay reagent (Promega) and measuring the emission for 10 s on a Turner TD-20e luminometer.

15. To express the GST-yIF2 fusion protein in yeast under control of the yeast *GAL1* promoter, we inserted a portion of the *FUN12* cDNA from the plasmid fun12-1 [P. Sutrave, B. K. Shafer, J. N. Strathern, S. H. Hughes, *Gene* **146**, 209 (1994); a gift from P. Sutrave and S. Hughes, National Cancer Institute, Frederick, MD] into the yeast GST fusion expression vector pEGKT [D. A. Mitchell, T. K. Marshall, R. J. Deschenes, *Yeast* **9**, 715 (1993)], creating the plasmid pC485. GST and GST-yIF2 were purified from the *fun12*Δ strain J130 (*22*) transformed with the plasmids pEGKT and pC485, respectively. The GST-yIF2 fusion protein containing yIF2 amino acid residues 396 to 1002, starting 16 residues before the GTP binding domain and extending to the COOH-terminus of the protein, was able to fully complement the slow-growth phenotype of the *fun12*Δ strain, indicating that the fusion protein is functional in vivo. Crude protein extracts from transformants grown in synthetic minimal medium containing 10% galactose and 2% raffinose were incubated with glutathione Sepharose 4B and washed; the GST and GST-yIF2 proteins were eluted in buffer containing 10 mM reduced glutathione. The purified proteins were dialyzed against sample buffer [20 mM tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 10% (v/v) glycerol] and stored in liquid nitrogen. Purified yeast eIF2 [G. D. Pavitt, K. V. A. Ramaiah, S. R. Kimball, A. G. Hinnebusch, *Genes Dev.* **12**, 514 (1998)] was kindly provided by Graham Pavitt.
16. W. C. Merrick, *Methods Enzymol.* **60**, 108 (1979); S. L. Adams, B. Safer, W. F. Anderson, W. C. Merrick, *J. Biol. Chem.* **250**, 9083 (1975).
17. W. L. Zoll, S. K. Choi, T. E. Dever, W. C. Merrick, data not shown.
18. A. G. Hinnebusch, *J. Biol. Chem.* **272**, 21661 (1997).
19. N. C. Kyrpides and C. R. Woese, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 224 (1998).
20. W. C. Merrick and W. F. Anderson, *J. Biol. Chem.* **250**, 1107 (1975).
21. S. Z. Tarun and A. B. Sachs, *Genes Dev.* **9**, 2997 (1995).
22. The plasmid pC482 was generated by inserting 1.3 kb of *FUN12* 5′ flanking and 0.9 kb of *FUN12* 3′ flanking DNA into the *hisG::URA3::hisG* plasmid pNKY51 [E. Alani, L. Cao, N. Kleckner, *Genetics* **116**, 541 (1987)]. The *fun12*Δ strain J130 (*leu2-3 leu2-112 ura3-52 fun12::hisG*) is an ascospore product from the diploid strain H1056 that had been transformed with an Sph I–Mun I fragment from pC482.
23. R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989).
24. We inserted a 3.55-kb Sal I–Bam HI fragment from pC473 (*10*) into the corresponding sites of the low-copy-number *URA3* vector pRS316 (*23*) generating the plasmid pC476. We extended the *FUN12* insert in pC476 an additional 400 base pairs at the 5′ end by inserting a 1.56-kb PCR-generated Sal I–Eco 47III DNA fragment, creating the plasmid pC479. A Sal I–Bam HI fragment from pC479 containing the full-length *FUN12* DNA sequences was inserted between the same sites of the low-copy-number *LEU2* vector pRS315 (*23*), creating the plasmid pC480.
25. The *fun12::LEU2* allele in strain J135 (*10*) was disrupted by transforming to Ura^+ prototrophy with a Bgl II fragment containing the *leu2::hisG::URA3::hisG* cassette from the plasmid pNKY85 (Nancy Kleckner, Harvard University, Cambridge, MA). A Ura^+ Leu^- transformant was identified and then replica plated to 5-fluoroorotic acid (5-FOA) medium to select for a Ura^- segregant, generating the haploid strain J129 (*Matα leu2-3 leu2-112 ura3-52 fun12::leu2::hisG*).
26. S. U. Astrom, U. von Pawel-Rammingen, A. S. Bystrom, *J. Mol. Biol.* **233**, 43 (1993).
27. T. E. Dever, W. Yang, S. Astrom, A. S. Bystrom, A. G. Hinnebusch, *Mol. Cell Biol.* **15**, 6351 (1995).
28. C. M. Moehle and A. G. Hinnebusch, *ibid.*, **11**, 2723 (1991).
29. We thank G. Pavitt for purified yeast eIF2, M. Cigan for the *IMT4* plasmid, C. Moehle and S. Green for plasmid pRG166, P. Sutrave and S. Hughes for the *FUN12* cDNA clone, and A. Sachs for advice on the yeast in vitro translation assays; A. Hinnebusch, G. Pavitt, J. Anderson, and L. Phan and members of the Dever and Hinnebusch laboratories for helpful discussions; and A. Hinnebusch and R. Rolfes for comments on the manuscript. We thank T. Donahue for discussions and for communicating results before publication. Supported in part by National Institutes of Health grant 26796 (W.C.M.) and a training grant in cellular and molecular biology (GM08056 to W.L.Z.).

28 January 1998; accepted 13 April 1998

The Ubiquitin-Related Protein RUB1 and Auxin Response in *Arabidopsis*

J. C. del Pozo, C. Timpte,* S. Tan, J. Callis, M. Estelle†

The AXR1 (auxin-resistant) protein, which has features of the ubiquitin-activating enzyme E1, is required for normal response to the plant hormone auxin in *Arabidopsis thaliana*. ECR1 functions together with AXR1 to activate members of the RUB/NEDD8 family of ubiquitin-related proteins. Extracts from mutant seedlings lacking AXR1 did not promote formation of the RUB-ECR1 thiolester, indicating that AXR1 is the major activity in this tissue. AXR1 was localized primarily to the nucleus of dividing and elongating cells, suggesting that the targets of RUB modification are nuclear. These results indicate that auxin response depends on RUB modification of one or more nuclear proteins.

Plant growth and development depends on the coordinated action of several phytohormones. One of these hormones, indole-3-acetic acid (IAA or auxin), has been implicated in the control of diverse developmental processes (*1*). Auxin regulates these processes by promoting changes in cell division and cell elongation (*2*). Recessive mutations in the *AXR1* gene result in decreased auxin response, including reduced auxin-induced gene transcription (*3*). A screen for suppressors of the *axr1* phenotype resulted in identification of the *SAR1* gene. By genetic criteria, *SAR1* acts downstream of *AXR1* (*4*). Mutations in another gene, *TIR1*, also result in a defect in auxin response (*5*). Genetic studies indicate that *AXR1* and *TIR1* function in the same or overlapping pathways.

The molecular characterization of *AXR1* and *TIR1* implicates the ubiquitin pathway in auxin response. *AXR1* encodes a protein related to the NH_2-terminal half of the ubiquitin-activating enzyme, E1 (*6*), and proteins related to AXR1 can be found in yeast and mammals (*7*). Despite extensive similarity with E1, all lack the active-site cysteine required for thiolester bond formation with ubiquitin. *TIR1* encodes an F-box protein related to yeast Grr1p and human SKP2 (*5*). F-box proteins have been shown to function in a ubiquitin-ligase complex (E3) called the SCF (for Skp1 Cdc53 F-box). These results suggest that auxin response is mediated by ubiquitin or a ubiquitin-like protein modification.

Ubiquitin (UBQ) is one of the most conserved proteins among eukaryotes. Covalent attachment of UBQ to other proteins targets them for degradation by the 26*S* proteosome (*8*). UBQ is activated by E1 in an adenosine 5′-triphosphate (ATP)–dependent reaction in which a thiolester bond is formed between the COOH-terminus of UBQ and a cysteine within the E1 enzyme. The UBQ moiety is transferred to a cysteine residue on a UBQ-conjugating enzyme (E2). Finally, UBQ is covalently attached to a target protein by an isopeptide linkage directly from the E2 or by a UBQ-protein ligase such as the SCF (*9*).

Two conserved families of UBQ-related proteins (Smt3p/SUMO and RUB/NEDD8) have also been identified (*10*). In *Saccharomyces cerevisiae*, activation of Smt3p is performed by the E1-like heterodimer Aos1p/Uba2p rather than E1 (*11*). Aos1p is a member of the AXR1 family, while Uba2p is similar to the COOH-terminal half of E1 and contains the active-site cysteine. Smt3p is conjugated in vivo to other proteins, but these targets are yet to be identified (*11*). A protein related to Smt3p in

J. C. del Pozo, C. Timpte, M. Estelle, Department of Biology, Indiana University, Bloomington, IN 47405–6801, USA.
S. Tan and J. Callis, Section of Molecular and Cellular Biology, University of California–Davis, 1 Shields Avenue, Davis, CA 95616, USA.

*Present address: Department of Biological Sciences, University of New Orleans, New Orleans, LA 70148, USA.
†To whom correspondence should be addressed. E-mail: mestelle@bio.indiana.edu

humans, called SUMO-1, is covalently attached to the RanGAP1 (*12*) and PML proteins (*13*). This posttranslational modification affects the subcellular localization of these proteins rather than their metabolic stability.

Another member of the AXR1 family, yeast Enr2p, is required for Rub1p (for "related to ubiquitin") conjugation to the cell-cycle protein Cdc53p (*14*). Enr2p functions as a second bipartite activating enzyme, together with Uba3p (*14*)

Because several RUB genes have been identified in *Arabidopsis thaliana* (*15*), we investigated whether AXR1 was required for RUB activation. Through homology with Uba2p, we identified an expressed sequence tag (EST) from *Arabidopsis* that encodes a 55-kD protein (ECR1) related to the COOH-terminus of the E1 enzyme. Based on similarity to Uba2p and Uba3p, ECR1 contains a possible active-site cysteine at position 215 (Fig. 1A). The *ECR1* gene was mapped, using the recombinant inbred (RI) lines (*16*), to position 7.4 on chromosome 5, between CHS and g4560 (Fig. 1B). When recombinant AXR1 and ECR1 were mixed and incubated with radiolabeled RUB1 and ATP (*17*), a species of 65 kD size was observed (Fig. 2). This product was destroyed upon incubation with the reducing agent dithiothreitol (DTT) indicating that formation of the labeled complex involves a thiolester bond (Fig. 2). No thiolester products were generated when either AXR1 or ECR1 were omitted from the reaction. To investigate the specificity of the AXR1-ECR1 heterodimer, we determined if UBQ and NEDD8, a mouse protein with 84% identity to RUB1, could form a thiolester bond with ECR1. We found that AXR-ECR1 was capable of activating NEDD8 (Fig. 2), but not UBQ (*18*). To determine whether the thiolester bond is formed with AXR1 or ECR1, we used a glutathione S-transferase (GST)–ECR1 fusion protein in the thiolester assay. In this reaction, the DTT-sensitive product was detected at a size of ~85 kD (Fig. 2), indicating that the thiolester bond is formed between the COOH-terminus of RUB1 and ECR1. Furthermore, ECR1 mutants in which Cys^{215} was replaced with Ala were unable to activate RUB1 or NEDD8 (Fig. 2). This suggests that Cys^{215} forms the thiolester bond with the COOH-terminus of RUB1. On the other hand, the *axr1-3* mutation, which replaces the Cys^{154} with Ala, also prevents thiolester formation between ECR1 and the RUB/NEDD8 proteins (Fig. 2). This missense mutation in the *AXR1* gene results in reduced auxin responses in the *axr1-3* plants, confirming that the auxin defect is related to a decrease in RUB activation (6).

To investigate RUB activation in various auxin response mutants, we developed an in vitro RUB1 activation assay (*19*). When radiolabeled RUB1 was incubated with total protein extract from wild-type, *axr1-12* (*4*), *tir1-9* (6), *sar1-1* (*5*), or *axr1-12 sar1-1* double mutant plants, two DTT-sensitive products at ~50 and 65 kD were detected in wild-type, *tir1-9*, and *sar1-1* extracts, but not in *axr1-12* and *axr1-12 sar1-1* extracts (Fig. 3A). The 65-kD product is likely the ECR1 protein conjugated to one molecule of RUB1, and the 50-kD protein could be either an E2-RUB1 or E3-RUB1 thiolester. To confirm that these products were AXR1-dependent, recombinant AXR1 protein was added to *axr1-12* protein extract. This resulted in the reappearance of the DTT-sensitive 65- and 50-kD products (Fig. 3B). These results suggest that AXR1 is responsible for the majority of RUB1 activation, at least in seedlings. Further, our

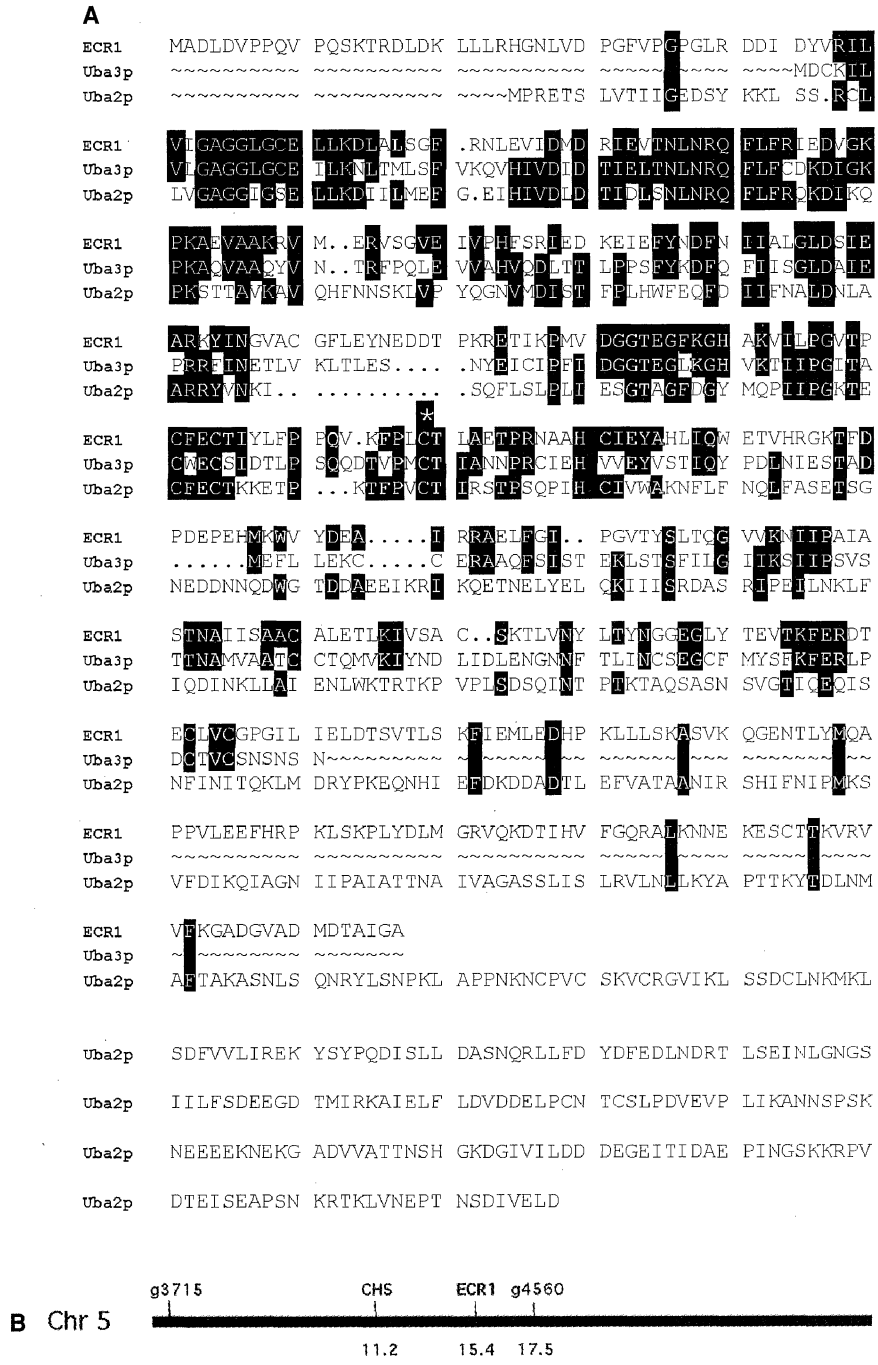

Fig. 1. ECR1 (GenBank AF051135) is related to yeast Uba2p (GenBank U32274) and Uba3p (EMBL Y16891) (*24*). (**A**) Alignment of ECR1 with Uba2p and YPR066W. Boxed residues are identical. The conserved active-site cysteine is indicated with an asterisk. (**B**) Map position of ECR1 on chromosome 5.

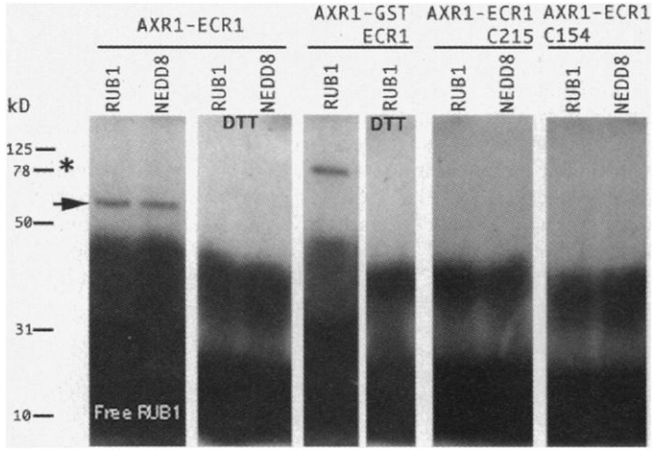

Fig. 2. Conjugation of RUB1 and NEDD8 with ECR1. Radiolabeled RUB1 or NEDD8 was incubated with recombinant AXR1 and ECR1 in a thiolester reaction (*17*). The reaction was terminated with SDS loading buffer in the absence or presence of DTT. The position of molecular size markers is indicated beside the gel. The arrow indicates the ECR1-RUB1 or ECR1-NEDD8 complex at ~65 kD. Radiolabeled RUB1 was incubated with the recombinant GST-ECR1 protein in a thiolester assay. The asterisk indicates the DTT-sensitive product GST-ECR1-RUB at 85 kD. Replacement of Cys^{215} in ECR1 or Cys^{154} in AXR1 with Ala were performed with the Stratagene direct mutagenesis kit. Neither AXR1-ECR1-C215 nor AXR1-C154-ECR1 heterodimers were able to form any DTT-sensitive product.

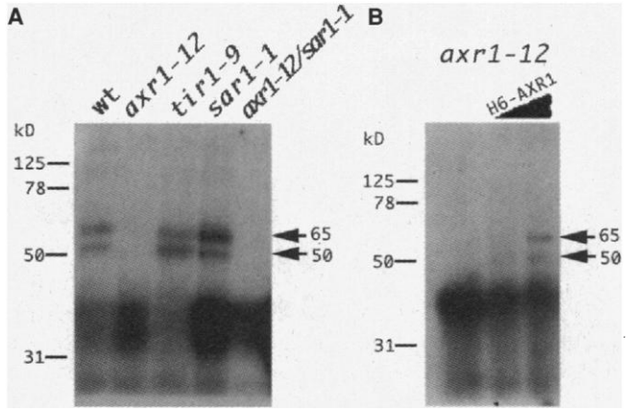

Fig. 3. RUB1-thiolester formation in protein extracts is dependent on AXR1. (**A**) Radiolabeled RUB1 was incubated with total plant protein from mutants as described (*19*). Arrows indicate two RUB1 thiolester adducts that were destroyed with DTT incubation (*18*). Free RUB1 protein ran off the gel. (**B**) Total protein extract from *axr1-12* mutant plants was incubated with RUB1. The biochemical phenotype (thiolester adducts at 50 and 65 kD) was restored when increasing amounts of recombinant AXR1 were supplied to the thiolester reaction.

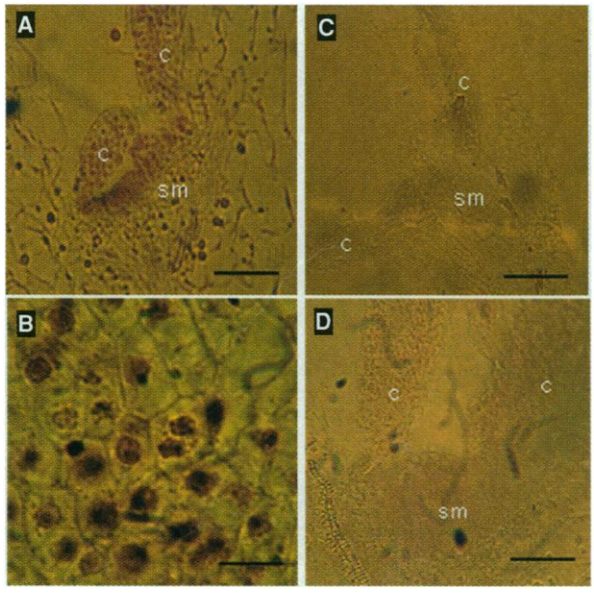

Fig. 4. AXR1 is located in the nucleus. Sections of 5-day-old *Arabidopsis* seedlings were used for immunolocalization of AXR1 protein by the method described (*20*). (**A**) Wild-type sections were labeled with AXR1 antiserum, which detected AXR1 protein in the nuclei of shoot meristem (sm) and expanding cotyledon (c) cells. (**B**) Higher magnification of expanding cotyledon cells showing the nuclear localization of AXR1. The identity of the nuclei in these sections was confirmed by staining with 4′,6′-diamidino-2-phenylindole (*18*). (**C**) No stain was observed with preimmune serum on wild-type sections. (**D**) Sections from an *axr1-12* plant were labeled with AXR1 antiserum. The *axr1-12* mutation introduces a stop codon in the exon 11 of the *AXR1* gene (*6*). AXR1 antiserum did not detect this truncated protein. Bars represent 100 μm in (A), (C), and (D), and 20 μm in (B).

results show that the *sar1-1* suppressor does not act by restoring RUB activation in *axr1* plants. This indicates that *sar1* acts to bypass the effects of the *axr1* mutation and is consistent with downstream function for *SAR1* (*4*).

In addition to the DTT-sensitive products described above, we also observed a broad DTT-resistant band of labeled protein migrating above the free RUB1 (Fig. 2 and Fig. 3, A and B). When the reaction was performed in the presence of [α-^{32}P]ATP and unlabeled RUB1, a labeled protein of this size was evident on the gel (*18*). This labeled protein was not present when the reaction was done with [γ-^{32}P]ATP. This result argues strongly that the smaller product is RUB1–adenosine 5′-monophosphate, an intermediate in RUB1 activation. Several other high molecular weight DTT-resistant products were also observed upon prolonged exposure. These proteins are likely to be RUB1 conjugates, indicating that the plant extracts are also capable of isopeptide bond formation between RUB1 and target proteins (*18*).

Analysis of *AXR1* genes expression by in situ hybridization indicate that *AXR1* expression is restricted to dividing and elongating cells (*18*). To determine the cellular location of AXR1, tissue sections of *Arabidopsis* seedlings were treated with AXR1 antiserum (*20*). Staining was associated primarily with the nuclei of dividing and expanding cells (Fig. 4, A and B). This staining was not detected in wild-type sections treated with preimmune serum or *axr1-12* mutant sections treated with AXR1 antiserum, which indicates that the AXR1 antiserum is specific (Fig. 4, C and D).

On the basis of these results, we propose that auxin response is mediated, at least in part, through modification of one or more proteins by RUB1 or a related protein. The nuclear localization of AXR1 suggests that these targets are probably nuclear proteins, which is consistent with studies in mammalian cells which show that most NEDD8-modified proteins are nuclear (*21*). Because the RUB/NEDD8 protein is conserved among the three eukaryotic kingdoms, it seems likely that RUB/NEDD8 modification will have an important regulatory function in all eukaryotes. In support of this, a human homolog of AXR1 called APP-BP1 interacts with the cytoplasmic domain of the amyloid precursor protein (APP), suggesting a role in APP function (*7*). In addition, a temperature-sensitive hamster cell line called ts41 carries a mutation in a hamster homolog of *AXR1* called *SMC1* (*22*). Mutations in *SMC1* result in a complex cell-cycle defect at the nonpermissive temperature (*22*). Further studies will be required to determine the biochemical

function of RUB/NEDD8 modification in both plant and animal systems.

In yeast, the most abundant Rub1p-modified protein is Cdc53p (*14*). Genetic evidence suggests that Rub1p modification regulates the activity of SCF^{Cdc4}, the E3 responsible for conjugation of UBQ to the CDK inhibitor Sic1p at the G_1-to-S phase transition. It is possible that RUB1 has a similar function in plant cells. For example, the *Arabidopsis* F-box protein TIR1 may be part of an SCF complex that is required for the degradation of negative regulators of auxin response. RUB1 may modify the activity of this SCF, perhaps in response to auxin (*23*). A homolog of *CDC53* exists in *Arabidopsis*, and it will be interesting to see if CDC53 is a target of RUB1 conjugation in plants.

REFERENCES AND NOTES

1. H. Klee and M. A. Estelle, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 1529 (1990).
2. M. L. Evans, in *Hormonal Regulation of Development II*, vol. 10 of *Encyclopedia of Plant Physiology*, T. K. Scott, Ed. (Springer-Verlag, Berlin, 1984), pp. 23–79.
3. L. Hobbie and M. A. Estelle, *Plant Cell Environ.* **17**, 525 (1994); C. Timpte, C. Lincoln, F. B. Pickett, J. Turner, M. Estelle, *Plant J.* **8**, 561 (1995).
4. A. Cernac, C. Lincoln, D. Lammer, M. Estelle, *Development* **124**, 1583 (1997).
5. M. Ruegger *et al.*, *Genes Dev.* **12**, 198 (1998).
6. H. M. O. Leyser *et al.*, *Nature* **364**, 161 (1993).
7. N. M. Chow, J. R. Korenberg, X. W. Chen, R. Neve, *J. Biol. Chem.* **271**, 11339 (1996); M. Shayeghi, C. L. Doe, M. Tavassoli, F. Z. Watts, *Nucleic Acids Res.* **25**, 1162 (1997).
8. A. L. Haas and T. J. Siepmann, *FASEB J.* **11**, 1257 (1997).
9. A. Ciechanover, S. Elias, H. Heller, A. J. Hershko, *J. Biol. Chem.* **257**, 2537 (1982).
10. M. Hochstrasser, *Annu. Rev. Genet.* **30**, 405 (1996).
11. E. Johnson, J. I. Schwienhors, R. J. Donmen, G. Blobel, *EMBO J.* **16**, 5509 (1997).
12. R. Mahajan, C. Delphin, T. Guan, L. Gerace, F. Melchior, *Cell* **88**, 197 (1997).
13. S. Muller, M. J. Matunis, A. Dejean, *EMBO J.* **17**, 61 (1998).
14. D. Lammer *et al.*, *Genes Dev.* **12**, 914 (1998); D. Liakopoulos, G. Doenges, K. Matuschewski, S. Jentsch, *EMBO J.* **17**, 2208 (1998).
15. J. Callis, T. Carpenter, C. Sun, R. D. Vierstra, *Genetics* **139**, 921 (1995); S. Tan and J. Callis, unpublished results.
16. C. Lister and C. Dean, *Plant J.* **4**, 745 (1993).
17. We diluted 20 ml of saturated culture of XL1-blue bearing His_6-tagged-AXR1 or ECR1 in 200 ml of LB medium containing ampicillin (75 μg/ml) and grew it at 30°C for 2 hours. Isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM, and the mixture was incubated for 4 hours. Cells were harvested, resuspended in 8 ml of TBS-T [20 mM Tris-Cl (pH 7.4), 150 mM NaCl, and 0.5% Tween-20], and sonicated to lyse the cells. Supernatant was portioned into aliquots and kept at −80°C. *Brassica napus* and mouse cDNAs encoding RUB1 (GenBank HO7679) and NEDD8 (GenBank D10918) were modified for insertion into pGEX-2TK, using polymerase chain reaction. *Arabidopsis thaliana* expresses a protein identical to *B. napus* RUB1 (J. Callis, unpublished data). The GST-RUB1 and GST-NEDD8 were expressed as described above. After purification of these recombinant proteins with the GST system (Pharmacia), they were labeled with ^{32}P using cyclic AMP kinase (Promega), and the fusion was cleaved with thrombin. The thiolester reactions contained AXR1 and ECR1 extracts, 1 mM ATP, 0.1 mM DTT, 10 mM $MgCl_2$ and ~200 cps of labeled protein. This mixture was incubated for 30 min at 23°C, and half of the reaction was stopped for 15 min with 4% SDS/10% glycerol and the other half with SDS/glycerol and 100 mM DTT.
18. J. C. del Pozo and M. Estelle, data not shown.
19. Five-day-old *Arabidopsis* seedlings were ground in liquid N_2, and proteins were extracted with 5 v/w in TBS-T. Total protein was precipitated with 100% saturated $(NH_4)_2SO_4$, resuspended in 1/10 volume of TBS, and dialyzed against TBS for 24 hours. The final volume was adjusted to 5% glycerol and kept at −80°C. Thiolester reactions contained approximately 30 μg/μl of plant protein, 2 mM ATP, 0.2 mM DTT, 10 mM $MgCl_2$, and ~400 cps of labeled RUB1. The reaction was performed at room temperature for 45 min and stopped as described (*13*).
20. AXR1 antiserum was prepared from rabbits by Pocono Rabbit Farm (Canadensis, PA), using recombinant AXR1 protein in an SDS gel slice [S. E. Perky, K. W. Nichols, D. E. Fernandez, *Plant Cell* **8**, 1977 (1996)].
21. T. Kamitani, K. Kito, H. P. Nguyen, E. T. H. Yeh, *J. Biol. Chem.* **272**, 28557 (1997).
22. S. Handeli and H. Weintraub, *Cell* **71**, 599 (1992); S. Handeli, personal communication.
23. W. M. Gray and M. Estelle, *Curr. Opin. Biotechnol.*, in press.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

25. We thank J. Turner (J.C.d.P., C.T., M.E.), B. McArdle, and J. Martin (S.T., J.C) for technical assistance. Supported by NSF grant 93-06759 (J.C.) and NIH grant GM43644 (M.E.). J.C.d.P. was supported by a long-term fellowship from the Spanish Government.

2 March 1998; accepted 12 May 1998

Close Contacts with the Endoplasmic Reticulum as Determinants of Mitochondrial Ca^{2+} Responses

Rosario Rizzuto,* Paolo Pinton, Walter Carrington, Frederic S. Fay,† Kevin E. Fogarty, Lawrence M. Lifshitz, Richard A. Tuft, Tullio Pozzan

The spatial relation between mitochondria and endoplasmic reticulum (ER) in living HeLa cells was analyzed at high resolution in three dimensions with two differently colored, specifically targeted green fluorescent proteins. Numerous close contacts were observed between these organelles, and mitochondria in situ formed a largely interconnected, dynamic network. A Ca^{2+}-sensitive photoprotein targeted to the outer face of the inner mitochondrial membrane showed that, upon opening of the inositol 1,4,5-triphosphate (IP_3)–gated channels of the ER, the mitochondrial surface was exposed to a higher concentration of Ca^{2+} than was the bulk cytosol. These results emphasize the importance of cell architecture and the distribution of organelles in regulation of Ca^{2+} signaling.

Upon physiological stimulation with IP_3-generating agonists, mitochondria undergo an increase in the concentration of Ca^{2+} in the matrix ($[Ca^{2+}]_m$) (*1*), well in the range of the Ca^{2+} sensitivity of the matrix dehydrogenases (*2*). This process, besides playing a direct role in the control of organelle function, may contribute to the modulation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), by buffering $[Ca^{2+}]_c$ (*3*) or influencing its spatiotemporal pattern (*4*). The accumulation of Ca^{2+} by mitochondria is rapid, despite the low affinity of their transport mechanisms (*5*). Because mitochondria might respond to microdomains of high $[Ca^{2+}]$ that were generated in their proximity by the opening of the IP_3-gated channels (*1*), we conducted high-resolution imaging of mitochondria and of their relation with the intracellular Ca^{2+} store (the ER). We directly monitored the $[Ca^{2+}]$ sensed by the mitochondrial Ca^{2+} uptake systems by using a targeted aequorin chimera.

The combined use of green fluorescent protein (GFP) chimeras with distinct spectral and targeting properties allows identification of two different subcellular structures in living cells (*6*). We expressed the S65T GFP mutant targeted to mitochondria [mtGFP(S65T)] (*6*) in HeLa cells (*7*) and used a high-speed imaging system that allows a three-dimensional (3D) fluorescence image of high resolution to be obtained from computationally deblurred optical sections (*8*). The 3D images, derived from image stacks taken at 30-s intervals with a 60× objective (pixel size 133 nm), revealed that mitochondria form a largely interconnected "tubular" network that undergoes

R. Rizzuto, P. Pinton, T. Pozzan, Department of Biomedical Sciences and the National Research Council Center for the Study of Biomembranes, University of Padova, Via Colombo 3, 35121 Padova, Italy.
W. Carrington, F. S. Fay, K. E. Fogarty, L. M. Lifshitz, R. A. Tuft, Biomedical Imaging Group, University of Massachusetts Medical Center, Worcester, MA 01605, USA.

*To whom correspondence should be addressed. E-mail: rizzuto@civ.bio.unipd.it
†Deceased.