videotapes for seal behavior. W. Evans and J. Thomas provided guidance on the analysis and identification of seal vocalizations. P. Canton assisted with manuscript preparation. We thank the many people with Antarctic Support Associates who provided logistical support in the field. This study was supported by the NSF Division of Polar Programs (grant OPP9614857) and the National Undersea Research Program (grant UAF98-0040). This study is dedicated to G. L. Kooyman on his 65th birthday.

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A Copper Cofactor for the Ethylene Receptor ETR1 from *Arabidopsis*

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The ETR1 receptor from *Arabidopsis* binds the gaseous hormone ethylene. A copper ion associated with the ethylene-binding domain is required for high-affinity ethylene-binding activity. A missense mutation in the domain that renders the plant insensitive to ethylene eliminates both ethylene binding and the interaction of copper with the receptor. A sequence from the genome of the cyanobacterium *Synechocystis* sp. strain 6803 that shows homology to the ethylene-binding domain of ETR1 encodes a functional ethylene-binding protein. On the basis of sequence conservation between the *Arabidopsis* and the cyanobacterial ethylene-binding domains and on in vitro mutagenesis of ETR1, a structural model for this copper-based ethylene sensor domain is presented.

Small gaseous molecules act as signals for a variety of organisms. In many cases, signal perception involves the use of a transition metal cofactor that mediates the interaction between the signal and its proteinaceous receptor. For example, sensors for NO in animal cells and O_2 in bacteria use a heme moiety to achieve high-affinity binding of the signal (*1*). The plant hormone ethylene is effective at nanomolar concentrations, reflecting the presence of high-affinity receptors (*2*). Theoretical considerations indicated Cu(I) as a possible receptor cofactor (*3*, *4*). The opportunity to directly investigate the role of a metal cofactor in ethylene sensing has been provided by the cloning and characterization of the *ETR1* gene from *Arabidopsis* (*5*). The ETR1 protein forms a membrane-associated disulfide-linked homodimer both in plant tissues and when heterologously expressed in yeast (*6*). Expression of truncated forms of ETR1 in yeast indicated that the first 165 amino acids of the protein contain the sequences that are necessary and sufficient to bind ethylene (*7*).

To further delineate the ethylene-binding domain of the ETR1 protein and to facilitate the purification of the binding activity, a chimeric gene consisting of the coding sequence for the first 128 amino acids of the ETR1 protein fused to the glutathione S-transferase (GST) coding sequence [ETR1(1-128)GST] was constructed (*8*). Yeast cells expressing the fusion protein showed ethylene-binding activity equivalent to that detected in cells expressing full-length ETR1 (Fig. 1A). Introduction of an amino acid substitution into the fusion protein, corresponding to the *etr1-1* mutant allele [$Cys^{65} \rightarrow Tyr^{65}$ (C65Y)], resulted in the complete loss of binding activity by the expressed protein, as was previously observed for the full-length etr1-1 protein (*7*). Both the mutant and the wild-type fusion proteins formed membrane-associated disulfide-linked homodimers and were expressed at equivalent levels as determined by immunoblot analysis using antibodies to GST (*9*).

We assessed binding in membrane extracts of yeast expressing both the full-length ETR1 protein and the ETR1(1-128)GST fusion protein (*10*, *11*). Addition of 300 μM $CuSO_4$ to the isolated membranes led to a 10- to 20-fold increase in ethylene-binding activity (Fig. 1B); up to twice that observed in intact cells. The addition of copper had no effect on ethylene binding in native yeast membranes nor in membranes expressing the mutant etr1-1(1-128)GST fusion protein. Of other transition metals tested (Fig. 1C), only silver ions stimulated ethylene-binding ac-

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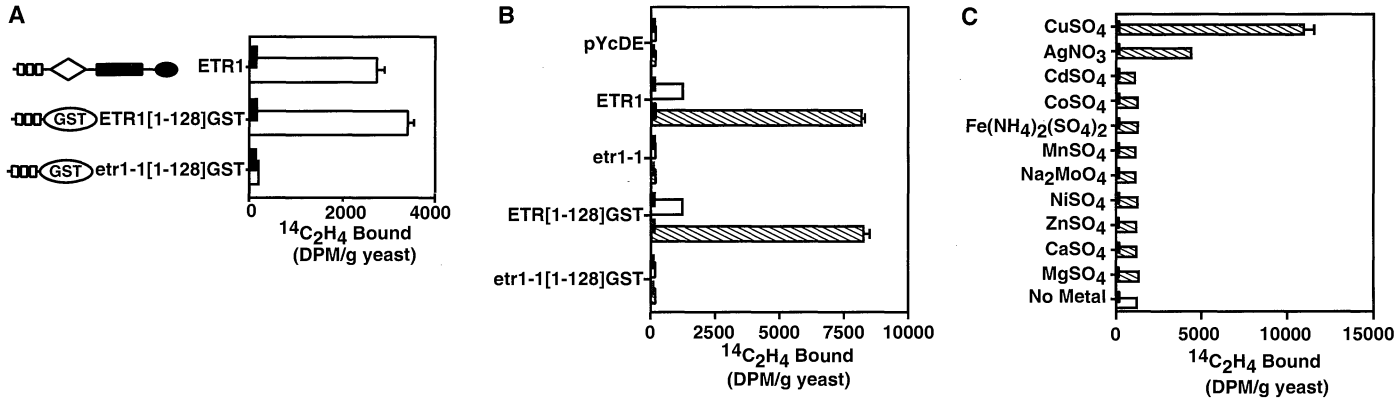

Fig. 1. Requirements for ethylene binding to the ETR1 protein expressed in yeast. Saturable ethylene binding is indicated as the difference in ^{14}C-ethylene between samples treated with ^{14}C-ethylene (0.1 μl/liter) (white or hatched bars) and identical samples treated with ^{14}C-ethylene (0.1 μl/liter) plus ^{12}C-ethylene (100 μl/liter) (overlapping black bars). DPM, disintegrations per minute. (**A**) Saturable ethylene-binding activity in yeast cells expressing different ETR1-derived constructs. Expressed proteins are depicted diagramatically, with the hydrophobic (small white squares), GAF (*19*) (white diamond), histidine kinase (black rectangle), receiver (black oval), and GST (white oval) domains indicated. (**B**) Effects of $CuSO_4$ addition to membranes extracted from yeast cells expressing the control vector (pYcDE) or the indicated ETR1-derived constructs. Ethylene-binding assays were performed with assay buffer alone (white bars) or with 300 μM $CuSO_4$ (hatched bars) (*11*). (**C**) Effects of $CuSO_4$ and other transition metals on ethylene-binding activity in yeast membranes expressing the ETR1 protein. Ethylene-binding assays were performed with assay buffer alone (white bar), or with 300 μM of the indicated metal salts (hatched bars).

tivity in membranes containing the ETR1 protein.

We were able to solubilize and purify the ETR1(1-128)GST fusion protein from yeast membranes in an active form (Fig. 2). The copper content of the purified protein was determined by graphite furnace atomic absorption spectroscopy. ETR1(1-128)GST preparations contained sixfold higher concentrations of copper (Fig. 2D) than did either native yeast (pYcDE) or mutant etr1-1(1-128)GST preparations. The net amount of copper (2.7 nmol) associated with the ETR1(1-128)GST sample was stoichiometrically similar to the amount of protein dimer (2.8 nmol) calculated from the difference in protein concentration between the pYcDE control and the affinity-purified ETR1(1-128)GST preparation. However, the estimated number of ethylene-binding sites in the purified preparation of ETR1(1-128)GST was only 0.9 nmol, based on its binding activity (Fig. 2B), which indicates that not all the purified fusion protein was biochemically active. Samples of purified mutant etr1-1(1-128)GST dimer (yield, 4.0 nmol) did not copurify with copper and lacked saturable binding activity, which indicates that Cys^{65} is an essential residue for both copper association and ethylene binding to the receptor.

In the development of a structural model for the ethylene-binding domain of ETR1, specific constraints are provided by amino acid residues that are conserved between functionally related proteins. These include the ERS1 protein from *Arabidopsis* and the *Nr* (*Never-ripe*) gene product from tomato (*12*), both of which showed ethylene-binding activity when expressed in yeast (*13*). Database searches also revealed that an open reading frame, designated slr1212, from the cyanobacterium *Synechocystis* strain 6803 (GenBank accession number D90905) showed sequence homology restricted to the ethylene sensor domain of ETR1. Ethylene-binding assays on cultured *Synechocystis* cell lines with wild-type and disrupted slr1212 (Fig. 3) showed that the wild-type gene encodes a functional ethylene-binding domain (*14*).

Alignment of *Synechocystis* and plant sequences indicate that conserved residues in the second and third hydrophobic subdomain align along a single face when these regions are modeled as α helices (Fig. 4). Conservation in the first helix does not fall along a single face, which may mean that an α-helical structure is incorrect for this region or that this helix may contact other protein regions along more than one face.

Evidence for a copper cofactor in ethylene binding to ETR1 places additional constraints on any structural model for the binding site. We hypothesized that particular amino acid residues, such as Cys and His, would serve as metal-coordinating ligands for the copper ion. As shown in Fig. 4, mutagenesis of Cys^{65} and His^{69} residues to Ser and Ala, respectively, resulted in complete loss of binding activity in the yeast-expressed ETR1 (Fig. 4B). These two residues align along a single face when the second hydrophobic subdomain is modeled as an α helix (Fig. 4A). Mutagenesis of other Cys, His, and Met residues in the ethylene-binding domain did not result in the loss of binding activity (Fig. 4B). These results, coupled with the lack of copper copurification with the C65Y mutant etr1-1(1-128)GST, prompt us to suggest that Cys^{65} and His^{69} may serve as ligands for a Cu(I) ion in the ETR1 binding site for ethylene.

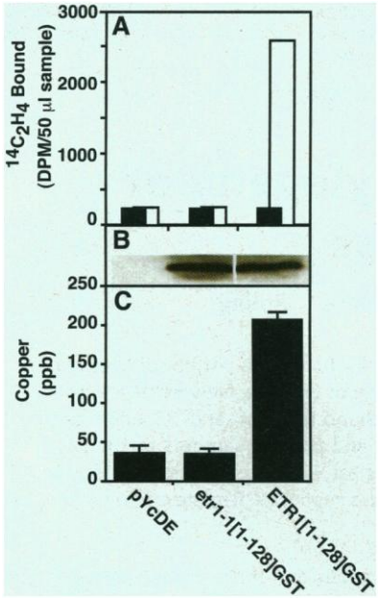

Fig. 2. Copurification of copper with the ETR1 ethylene-binding domain. (**A**) Saturable ethylene-binding activity was determined with 50-μl aliquots of the indicated affinity-purified preparations (1 ml total volume) (*20*) in the presence of ^{14}C-ethylene (white bars) or ^{14}C-ethylene plus ^{12}C-ethylene (overlapping black bars) (*11*). (**B**) Protein immunoblots of affinity-purified samples immunodecorated with antibodies to GST (*20*). (**C**) Copper contents of the purified samples determined by graphite furnace atomic absorption; ppb, parts per billion.

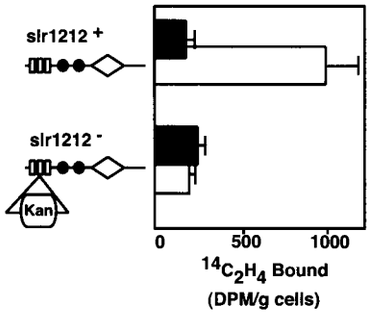

Fig. 3. Ethylene-binding activity in *Synechocystis* lines containing the intact (slr1212$^+$) or disrupted (slr1212$^-$) slr1212 ORF. The structural elements of the slr1212 coding sequence are indicated as follows: hydrophobic region related to ETR1 (small white rectangles), PAS (*22*) domains (black circles), and GAF motif (white diamond). The position of the insertion of the *Kan*^{r} gene is also shown. Ethylene-binding assays (*14*) were performed with ^{14}C-ethylene (white bars) or ^{14}C-ethylene plus ^{12}C-ethylene (overlapping black bars).

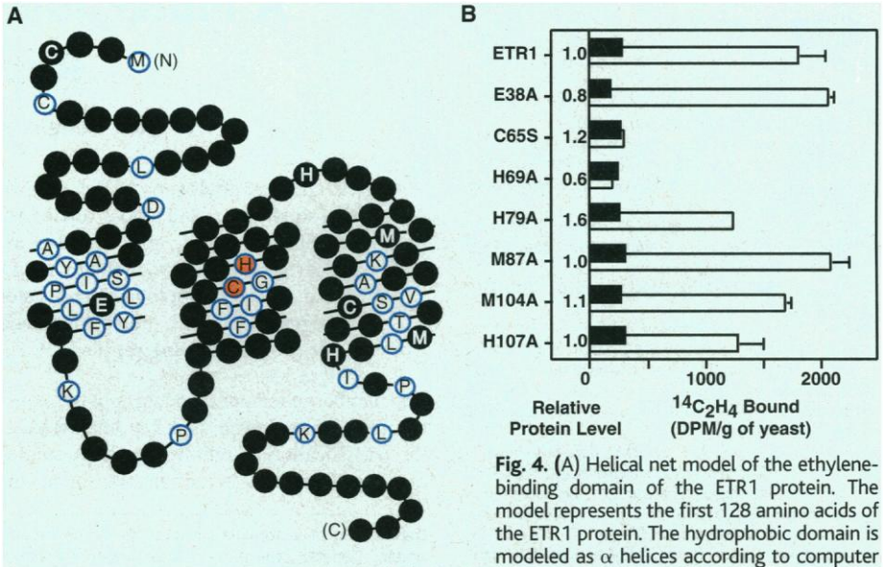

Fig. 4. (A) Helical net model of the ethylene-binding domain of the ETR1 protein. The model represents the first 128 amino acids of the ETR1 protein. The hydrophobic domain is modeled as α helices according to computer algorithms that predict membrane topology (*23*). Residues that are conserved between ETR1, ERS1, Nr, and slr1212 from *Synechocystis* are are outlined in blue (*24*). Nonconserved residues are represented as solid black circles. In vitro mutagenesis of potential metal-ligand residues either disrupted (red circles) or did not disrupt (white letters) ethylene binding in the yeast-expressed protein (*7*). (**B**) Ethylene binding by yeast expressing wild-type and mutant forms of ETR1. Ethylene-binding assays were performed as in Fig. 1A. Relative levels of expressed protein were determined as previously described (*7*).

The results presented here provide a mechanistic basis for how high-affinity ethylene binding to the sensor domain of ETR1 is achieved. We propose that ethylene interacts with a Cu(I) cofactor in an electron-rich hydrophobic pocket formed by membrane-spanning helices of the ETR1 dimer. The binding site must confer some unusual chemistry on the copper ion, because the stability of this ethylene/receptor complex (half-life for dissociation = 11 hours) (*7*) is much different from that observed for artificial copper complexes (*15*). The ability of Ag(I) ions to interact with the receptor and bind ethylene is of interest because silver ions can inhibit ethylene responses when applied to plant tissues (*4*). Silver ions may occupy the binding site and interact with ethylene but fail to induce the changes in the receptor that are needed to elicit downstream signaling.

The discovery of a functional ethylene-binding domain in the slr1212 coding sequence from *Synechocystis* raises interesting questions about the evolutionary origin of the higher plant receptors. *Synechocystis* is thought to share a common ancestor with the cyanobacterial lineage that evolved into the modern chloroplast of higher plants (*16*). The presence of both the ethylene-sensor domain and histidine-kinase transmitter domains in the cyanobacterial genome may have provided the raw materials for the evolution of the higher plant form of the ethylene receptors. Sequence homology to the ethylene-binding domain has not been identified in any other bacterial genomes sequenced to date, which supports a single origin for this functional domain in the evolution of photosynthetic organisms.

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8. The ETR1(1-128)GST fusion protein consists of the first 128 amino acids of ETR1, fused to the coding region of GST obtained from the bacterial expression vector pGEX4T1 (Pharmacia). The fusion protein was expressed in yeast with the use of the expression vector pYcDE-2 (*6*).
9. F. I. Rodriguez and A. B. Bleecker, data not shown.
10. Cultures of *Saccharomyces cerevisiae* strain LRB 520 (*6*) were grown to mid-log phase at 30°C and harvested by centrifugation at 1500*g* for 5 min. Cell pellets were washed and resuspended in 50 mM tris-HCl (pH 7.4), 10% glycerol, and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) at a concentration of 0.5 g of cells per milliliter. Cell suspensions were mixed with an equal volume of glass beads and disrupted in a bead beater with a cooling bath (BioSpec, Bartlesville, OK). Cell debris was separated from the homogenate by centrifugation at 10,000*g* and total cell membranes were harvested by centrifugation at 100,000*g* for 30 min and then resuspended in assay buffer [10 mM MES (pH = 5.5), 20% sucrose, 1% dimethyl sulfoxide, and 1 mM PMSF]. Membrane preparations obtained from 2 g of cells were diluted with assay buffer to 1 ml and tested for ethylene binding (*11*).
11. In vitro ethylene-binding assays were performed as described (*7*) with some modifications as follows: 500-μl aliquots of membrane suspensions representing 1 g of yeast cells were pipetted onto strips of Whatman paper 1M (2.5 cm by 20 cm) that were rolled and inserted in Eppendorf tubes. The filters were incubated for 4 hours in sealed glass chambers containing $^{14}C_2H_4$ (0.1 μl/liter) or $^{14}C_2H_4$ (0.1 μl/liter) plus $^{12}C_2H_4$ (100 μl/liter). The filters were aired for 10 min and transferred to individual sealed chambers containing 300 μl of 250 mM mercuric perchlorate in a scintillation vial; $^{14}C_2H_4$ trapped in the mercuric perchlorate was then determined as described (*7*).
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20. Membrane preparations (*10*) were made with 600 μM $CuSO_4$ in the extraction buffer. Ethylene-binding activity was solubilized as described by R. Serrano [*FEBS Lett.* **156**, 11 (1983)], using a solubilization buffer consisting of 50 mM tris (pH 8.0) and 0.5% (w/v) α-lysophosphatidylcholine (LPC). Solubilized samples were purified by affinity chromatography as described (*18*) using buffers including 0.5% LPC (w/v) previously purified in Chelex-100 columns. The purified extracts were desalted through Sephadex G-25 columns.
21. Samples were treated with 100 mM dithiothreitol and separated by SDS–polyacrylamide gel electrophoresis, and immunoblots were performed as described (*6*) using antibodies to GST (Sigma).
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A Molecular Phylogeny of Reptiles

S. Blair Hedges* and Laura L. Poling

The classical phylogeny of living reptiles pairs crocodilians with birds, tuataras with squamates, and places turtles at the base of the tree. New evidence from two nuclear genes, and analyses of mitochondrial DNA and 22 additional nuclear genes, join crocodilians with turtles and place squamates at the base of the tree. Morphological and paleontological evidence for this molecular phylogeny is unclear. Molecular time estimates support a Triassic origin for the major groups of living reptiles.

The number of temporal openings in the skull has long been viewed as a key character in the classification of reptiles (*1*, *2*). A single opening (synapsid condition) is found in mammals and their reptilian ancestors. Turtles and several late Paleozoic and early Mesozoic groups lack a temporal opening (anapsid condition). Most other living and fossil reptiles belong to a clade in which the ancestral condition was the presence of two temporal openings (diapsid condition). Among the living diapsid reptiles, the crocodilians and birds form one group, the Archosauria, and the tuataras and squamates (lizards, snakes, and amphisbaenians) form another group, the Lepidosauria. Other morphologi-

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