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ASSOCIATION OF DRG1 AND DRG2 WITH RIBOSOMES FROM PEA, *ARABIDOPSIS*, AND YEAST

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DRGs are highly conserved GTP binding proteins. All eukaryotes examined contain DRG1 and DRG2 orthologs. The first experimental evidence for GTP binding by a plant DRG1 protein and by DRG2 from any organism is presented. DRG1 antibodies recognized a single ~43-kDa band in plant tissues, whereas DRG2 antibodies recognized ~45-, 43-, and 30-kDa bands. An in vitro transcription and translation assay suggested that the 45-kDa band represents full-length DRG2 and that the smaller bands are specific proteolytic products. Homogenates from pea roots and root apices were used to produce fractions enriched in cytosolic and microsomal monosomes and polysomes. DRG1 and the 45- and 43-kDa DRG2 bands occurred in the cytosol and associated with cytosolic monosomes. In contrast, the 30-kDa form of DRG2 was strongly enriched in polysome fractions. Thus, DRG1 and the larger forms of DRG2 may be involved in translational initiation, and the 30-kDa form of DRG2 may be involved in translational elongation. DRG1 and the 45- and 43-kDa forms of DRG2 can reassociate with ribosomes in vitro, a process that is partially inhibited by GTP-γ-S. Cells expressing FLAG-tagged ribosomal proteins from transgenic lines of *Arabidopsis* and yeast also demonstrated DRG-ribosome interactions.

Keywords: DRG, GTP binding protein, *Pisum sativum*, proteolytic processing, ribosome, ribosome association.

Introduction

Well-studied GTP binding proteins (G proteins) are involved in essential physiological processes such as signal transduction (Ras and heterotrimeric G proteins), regulation of translational initiation and elongation (IF2, EF-G, EF-Tu, and their eukaryotic counterparts), targeting membrane vesicles to their proper subcellular compartment (Rab and Arf), and controlling passage through nuclear pores (Ran; Bourne et al. 1990, 1991; Leipe et al. 2002). The OBG/DRG subfamily of G proteins is ancient and highly conserved. OBGs occur in bacteria and eukaryotes. Although nuclear-encoded OBGs generally appear to be targeted to mitochondria and chloroplasts, the product of human ObgH2 localizes near nucleoli (Hirano et al. 2006). Bacterial *Obg* is an essential gene, which is needed for proper DNA replication and chromosome separation (Kobayashi et al. 2001; Morimoto et al. 2002; Slominska et al. 2002). OBG proteins from *Bacillus subtilis*, *Escherichia coli*, and *Caulibacter crescentus* cofractionate with ribosomes (Scott et al. 2000; Datta et al. 2004; Lin et al. 2004; Wout et al. 2004; Sato et al. 2005). The observed effects of *obg* mutants on DNA replication and chromosome separation may result from defects in ribosome biogenesis (Lin et al. 1999; Datta et al. 2004). Compared with other G proteins, bacterial OBGs are characterized by relatively low rates of GTP hydrolysis and high rates of GTP-GDP exchange (Lin et al. 1999; Wout et al. 2004). OBGs may regulate translation and stress responses through interactions with SpoT and RelA (Jiang et al. 2007). In this article, we will explore ribosome binding properties of DRGs from pea, *Arabidopsis*, and yeast.

Early studies identified DRGs in developing mouse brain (Sazuka et al. 1992) and human SV40-transformed fibroblasts (Schenker et al. 1994). These mouse and human genes became the archetypes for the *DRG1* and *DRG2* orthologous groups (Caldon et al. 2001; Leipe et al. 2002). All eukaryotes examined contain at least one member of each group (Li and Trueb 2000; Tatusov et al. 2003). Amino acid identity among plant, animal, and fungal representatives of each orthologous group is ~65%–70%, whereas paralogs from a single species share ~55%–60% identity. Both DRG1 and DRG2 from most organisms contain ~365–370 amino acid residues and have predicted molecular masses of ~41 kDa. DRG2 proteins from all plants examined (including the green alga *Chlamydomonas*) contain an ~32-amino extension at their C-termini that is not found in DRG2 proteins of other organisms. The predicted masses of plant DRG2 proteins is ~44.5 kDa. Although DRGs are presumed to be able to bind GTP, this property has been demonstrated experimentally only for DRG1 of two animals, mouse and *Drosophila* (Sazuka et al. 1992; Sommer et al. 1994).

Arabidopsis has three *DRG* genes. *AtDRG1* is a *DRG1* ortholog, and *AtDRG2* and *AtDRG3* are *DRG2* orthologs. *AtDRG1* and *AtDRG2* promoter-GUS fusions revealed similar but subtly different spatial expression patterns in seedlings and mature organs (Stafstrom 2008). For example, both promoters showed strong GUS expression in root apices, developing root primordia, the root stele, cotyledons, pollen, and stigmas. Neither promoter was active in shoot apices of young seedlings. The *AtDRG1* promoter was more active in leaf veins, and the *AtDRG2* promoter was more active in petals, siliques, and leaf trichomes. As assayed by quantitative RT-PCR, *AtDRG1* and

AtDRG2 mRNA levels were relatively high and similar to each other in every tissue examined (Stafstrom 2008). In contrast, *AtDRG3* expression was extremely low in the same tissues. However, *AtDRG3* expression increased ~1000-fold following 3 h of heat stress. Heat stress led to a modest increase in *AtDRG1* expression and a modest decrease in DRG2 expression. Microarray experiments also demonstrated that *AtDRG1* and *AtDRG2* expression is quite uniform under essentially all conditions tested and that *AtDRG3* is strongly stimulated by heat stress and stimulated ~10-fold by several other stresses and in pollen and developing seeds (Schmid et al. 2005). Whereas DRG1 protein levels were similar in all of these tissues, DRG2 protein levels were quite variable and notable low in older leaves (Stafstrom 2008). The basis for tissue-specific and developmental differences in relative mRNA and protein abundance is unknown. DRG1 and DRG2 protein levels were unaffected by most environmental and stress conditions tested. The sole exception was heat stress, which increased the accumulation of DRG2 slightly and also induced the appearance of an ~70-kDa band that was recognized by DRG2 antibodies. The identity of this band is unknown.

The DRG1 and DRG2 antibodies used in this study were described previously (Devitt et al. 1999; Stafstrom 2008). DRG1 antibodies recognized a single band with an apparent molecular mass of ~43 kDa protein (the deduced mass is 41.1 kDa); smaller bands (possible degradation products) were seen only occasionally. DRG2 antibodies recognized bands with apparent molecular masses of 30, 43, and 45 kDa, which varied in abundance in different tissues. The predicted mass of DRG2 from pea and *Arabidopsis* is ~44.5 kDa. As will be demonstrated here, we believe that the 43- and 30-kDa bands recognized by these antibodies are specific proteolytic products of the full-length DRG2 protein.

Relatively modest changes in *AtDRG* promoter activities and in mRNA and protein levels suggest that other types of regulation, such as altered subcellular localization, may be important for the cellular functions of DRGs. Many G proteins function through transient associations with various organelles. For example, Ras reversibly binds to plasma membranes, Ran helps to shuttle cargos between the cytoplasm and nucleoplasm, and Rab proteins provide specificity in targeting vesicles to membrane organelles (Kuersten et al. 2001; Pfeffer 2001; Pechlivanis and Kuhlman 2006). Using differential centrifugation, we found that pea DRG2 occurs predominantly in P150 and S150 fractions (Devitt et al. 1999). P150 is a 150,000 *g* pellet enriched in microsomal membranes and ribosomes, and S150 represents the postribosomal cytosolic fraction (Davies and Abe 1995). Etheridge et al. (1999) showed, using immunocytochemistry on sections prepared for light and electron microscopy, that *Arabidopsis* DRG2 (which they called DRG1) occurred in cytoplasmic granules or bodies. The organelle or structure responsible for DRG2 association with the P150 fraction or with these cytoplasmic granules is not known.

This report addressed three classes of questions. What are the identities of the 45-, 43-, and 30-kDa bands recognized by DRG2 antibodies? Can pea DRG1 and DRG2 bind GTP? Do DRGs bind to ribosomes? The first question was addressed by synthesizing ^{35}S-labeled DRG2 in vitro and observing its proteolytic processing in the presence of plant extracts. For the second question, we tested the ability of DRGs from tissue extracts to bind to GTP-agarose and determined whether binding was affected by various nucleotides. Ribosome binding properties of DRGs were the most intensively investigated aspect of this report. Homogenates from mature roots and root apices of pea were fractionated to yield four subpopulations of ribosomes: cytosolic monosomes, cytosolic polysomes, microsomal monosomes, and microsomal polysomes. Also, an assay was developed to examine whether DRGs could associate with ribosomes in vitro. Finally, yeast and *Arabidopsis* cells containing ribosomes incorporating a single FLAG-tagged ribosomal protein were used in affinity-binding assays to test for ribosome binding by DRGs in these organisms (Inada et al. 2002; Zanetti et al. 2005).

Material and Methods

Plants

Pea seeds (*Pisum sativum* L. cv. Alaska) were imbibed overnight in running tap water and germinated in the dark on moist paper towels for 2–3 d. Root apices were defined as the terminal ~2 mm, including the root cap. The next 1 cm of root tissue was discarded; the next 1 cm of root beyond that was considered to be fully elongated or mature root tissue. *Arabidopsis thaliana* seedlings were grown in a growth chamber for 7–10 d under a short-day photoperiod (10L : 14D) at 20°C. Growth was on vertically oriented sterile MS plates. Plant lines used were ecotype Columbia (Col-0) and *35S:HF-RPL18*, a genetically engineered line that expresses a fusion protein with the His-FLAG peptide at the N-terminus of ribosomal protein L18 (Zanetti et al. 2005). Tissues were frozen in liquid nitrogen and stored at −70°C until needed.

Western Blotting

The nucleotide sequence reported in this article for a *PsDRG1* cDNA has been submitted to GenBank under accession EU236700. DRG1 antibodies (antiserum 29) were raised against a His-tagged protein containing the entire 369 amino acid coding region of *Arabidopsis* DRG1. Pea and *Arabidopsis* DRG2 proteins contain 399 amino acid residues. DRG2 antibodies (antiserum 55) were raised against a His-tagged fusion protein containing the N-terminal 202 residues of pea DRG2 (Devitt et al. 1999). Despite the overall similarity of DRG1 and DRG2 proteins, there is little cross-reactivity between DRG1 and DRG2 antibodies under our experimental conditions. Western blots of *Arabidopsis drg1* or *drg2* T-DNA single gene knockout mutants demonstrate that DRG1 antibodies show no recognition for DRG2 and that DRG2 antibodies recognize DRG2 protein with at least a 10-fold greater specificity than they recognize DRG1 protein (J. D. Kubic and J. P. Stafstrom, in preparation). Both antisera were affinity purified on Affigel AG-10 columns (BioRad) to which the original antigen was covalently attached. Antibodies directed against maize RPS6 were obtained through the courtesy of J. Bailey-Serres (Williams et al. 2003). The predicted mass of RPS6 from several plants is ~28.1 kDa, but its apparent mass on SDS-PAGE is estimated to be 30–34 kDa (Williams et al. 2003; Chang et al. 2005). RPS6 is found only in 40S ribosome subunits and therefore is a marker for this subunit, 80S monosomes, and polysomes.

Methods for extracting and purifying proteins are described for each experiment.

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% or 12% acrylamide gels using standard techniques. Prestained molecular weight markers were included on all gels. For Western blots, proteins were electrophoretically transferred to nitrocellulose or polyvinylidene fluoride membrane blots using a semidry apparatus. Following transfer, the blots were rinsed briefly in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 500 mM NaCl) and then incubated for 1 h in blocking solution (TBS plus 5% instant dry milk). Blots were incubated overnight with affinity-purified primary antibodies at dilutions ranging from 1 : 100 to 1 : 1000 and then washed three times for a total of ~1 h in TBS-T (TBS plus 0.05% Tween-20). Blots were then incubated for 2 h with HRP-DAR secondary antibodies in TBS at a dilution of 1 : 5000 (donkey-antirabbit antibodies conjugated to horseradish peroxidase; Amersham), followed by a second series of washes in TBS-T. Following these washes, blots were incubated in Super-Signal West Pico chemiluminescence substrate (Pierce) and exposed to x-ray film.

Cell Fractionation

Constituents of pea root apices were fractionated by standard methods (Devitt et al. 1999). Frozen tissue was ground under liquid nitrogen, and soluble proteins were extracted with homogenization buffer (HB; 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 28 mM 2-mercaptoethanol, 400 mM sorbitol, and 2 mM PMSF). Samples were kept at 4°C during centrifugation and otherwise kept on ice. Crude homogenates were further disrupted by passing them through a 25-gauge needle five times and then cleared by filtration through Miracloth. The cleared homogenate was centrifuged at 20,000 g for 10 min. The pellet was discarded. The supernatant above this pellet was centrifuged at 150,000 g for 2 h. The resulting pellet, which was enriched in microsomal membranes and ribosomes, was resuspended in HB (P150). A postribosomal supernatant was also produced (S150). Three protein bands thought to be forms of DRG2 were quite abundant in P150. To analyze the nature of this association, resuspended P150 was separated into aliquots. The composition of these aliquots was adjusted to give a final pH of 5.5, 7.5, or 10 or a final NaCl concentration of 0.1, 0.25, 0.5, or 1.0 M (all at pH 7.5). These samples then were centrifuged at 150,000 g to obtain a second set of P150 fractions. These pellets were resuspended in SDS sample buffer, electrophoresed on 12% acrylamide gels, and analyzed by Western blotting with DRG2 antibodies.

Stability of ^{35}S-labeled DRG2 synthesized by coupled in vitro transcription and translation. Pea and *Arabidopsis DRG2* cDNA clones (Devitt et al. 1999) were used as templates for coupled in vitro transcription and translation using the TnT kit (Promega). A standard 25 μL TnT reaction contained the following: 0.5 μg of plasmid DNA; 0.5 U of T7 RNA polymerase, which produces sense RNA with these plasmids; an amino acid mixture depleted of methionine; and 1 μL of ^{35}S-Translabel, a mixture of labeled methionine and cysteine (Amersham). The reaction was for 90 min at 30°C. Samples were electrophoresed on 12% SDS-PAGE gels. For fluorography, gels were fixed in 10% acetic acid and 30%

methanol, rinsed three times for 15 min in water, soaked in Econo-Safe (Research Products) for 30 min, dried using a gel drier, and exposed to x-ray film. This procedure produced films showing a high level of background. For the experiments presented here, the products of the TnT reactions were purified further by immunoprecipitation. Ten microliters of DRG2 antibodies were combined with the products of a TnT reaction in a total volume of 100 μL in immunoprecipitation buffer (IB; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate). None of the buffers used for this experiment contained protease inhibitors. The reaction was gently agitated on ice for 1 h. A 15-μL aliquot of Protein A-Sepharose (Sigma P-3391) was then added and allowed to incubate under the same conditions for 12 h. Complexes were sedimented by centrifugation at 12,000 g for 20 s. Pellets were resuspended and washed three times in each of the following solutions: (1) IB; (2) 50 mM Tris, pH 7.5, 500 mM NaCl, 0.1% NP-40, and 0.05% sodium deoxycholate; and (3) 50 mM Tris, pH 7.5, 0.1% NP-40, and 0.05% sodium deoxycholate. Aliquots of a single immunoprecipitated DRG2 TnT reaction were incubated in the presence of homogenization buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 28 mM 2-mercaptoethanol) or cleared extracts from mature root tissue or root apices prepared in the same buffer. Equal amounts of total protein were present in each tissue extract. Incubations were for 30 min on ice or at 25°C. Samples were electrophoresed on 12% SDS-PAGE gels and subjected to fluorography.

GTP Binding Assays

Methods for GTP binding assays were based on published procedures (Pirovani et al. 2002). About 0.5 g of root apices were ground in liquid nitrogen and resuspended in 5 mL of ice-cold binding buffer (BB; 100 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1% Triton-X, 1 mM PMSF, 0.1 mM DTT, 5 mM MgCl$_2$). The homogenate was filtered through Miracloth and centrifuged at 20,000 g for 20 min at 4°C in an HB-6 rotor to produce a cleared homogenate (fig. 2, input). Before incubation with GTP-agarose (Sigma G-9768), 250 μL aliquots of the cleared homogenate were preincubated for 1 h on ice with no additions (control) or with various nucleotides (2 mM GTP, 10 mM GTP, 10 mM GDP, or 10 mM ATP). GTP-agarose was prepared by washing it three times in 50 mM Tris-HCl, pH 6.8. Fifty microliter aliquots of GTP-agarose were incubated with the homogenate samples for 5 h on ice with gentle shaking. After incubation, the resin was collected by centrifugation and then washed three times in BB. Bound proteins were released from the resin by heating at 95°C in SDS sample buffer. Samples were analyzed by SDS-PAGE and Western blotting using DRG1 and DRG2 antibodies.

Ribosome Fractionation

About 1 g of pea root or root apex tissue was ground in liquid nitrogen and immediately added to 10 mL protein isolation buffer (PIB; 200 mM Tris-HCl pH 8.5, 50 mM KCl, 25 mM MgCl$_2$; Davies and Abe 1995). Samples were kept at 4°C during centrifugation and otherwise kept on ice. Debris was removed by filtration through Miracloth to give a crude homogenate fraction. This homogenate was centrifuged at 3000 g

for 10 min to give a cleared homogenate, which was also designated as the total fraction (T) in figures 3 and 4. Samples containing ∼5 mg of protein were layered over a 3.5-mL 60% sucrose pad and centrifuged for 16 h at 170,000 g (Beckman L8–70M, rotor 70.1 TI). The resulting pellet was resuspended in 500 μL of PIB. This pellet, which contained ribosome subunits, monosomes, cytosolic polysomes, and membrane-associated polysomes, was designated as the total ribosome pellet (P). The postribosomal supernatant sample (S) also was analyzed.

The P fraction was fractionated further to produce four ribosome populations. First, the resuspended pellet was centrifuged for 20 min at 27,000 g using a Sorvall HB-6 rotor to separate microsomal ribosomes (pellet) and cytosolic ribosomes (supernatant). To release ribosomes from microsomal membranes, the pellet was resuspended in 500 μL of PIB containing 2 mM EGTA, 100 μg/mL heparin, 2% PTE, and 1% DOC (Davies and Abe 1995). Microsomal monosomes were separated from polysomes by centrifugation through a 60% sucrose pad (2 h at 210,000 g using a Beckman 70.1 TI rotor). The resulting supernatant contained microsomal monosomes (MM), whereas the pellet contained microsomal polysomes (MP). The total cytosolic ribosome sample (supernatant of the first centrifugation described in this paragraph) were fractionated as for microsomal ribosomes to produce cytosolic monosome (CM) and cytosolic polysome (CP) fractions. Ribosome-containing fractions were prepared on an equal-volume basis. Proteins were concentrated by acetone precipitation before analysis by Western blotting.

The following assay was used to determine whether cytosolic DRG proteins could associate with ribosomes in vitro. Cleared root apex homogenates (T) were prepared as described above. Aliquots were incubated on ice or at 25°C for 2 h in the presence or absence of added nucleotides (GTP, GDP, or GTP-γ-S, each at 0.5 mM). After incubation, total ribosome pellet (P) and postribosomal supernatant fractions (S) were prepared as described and analyzed by Western blotting.

Affinity Purification of Arabidopsis Ribosomes

Zanetti et al. (2005) generated a transgenic line of *Arabidopsis* (Col-0 background) that expressed ribosomal protein L18 (rpL18) with a dual His-FLAG peptide tag at its N-terminus. This protein was chosen because it was expected to be exposed on the solvent side of the 60S ribosome subunit. These authors demonstrated that ribosomes (60S subunits, 80S monosomes, and polysomes), together with associated mRNAs and proteins, could be purified by affinity methods using anti-FLAG M2-agarose. Transgenic line *35S:HF-RPL18* is referred to here simply as L18.

We used L18 and control Col-0 plant extracts to examine ribosome association with DRG proteins. Whole 7–10-d-old seedlings grown on MS plates were used. Approximately 0.25 g of tissue was ground under liquid nitrogen and immediately resuspended in 2 mL of polyribosome extraction buffer (PEB; 100 mM Tris-HCl, pH 9.0, 200 mM KCl, 25 mM EGTA, 36 mM $MgCl_2$, 5 mM DTT, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 1% PTE, 2% DOC). Homogenates were centrifuged at 16,000 g for 30 min, after which the cleared supernatant was removed as the ribosome-containing extract. Equal amounts of protein were added in each pull-down as-

say, and volumes were equalized by adding PEB to a total of 500 μL per assay. A 50-μL aliquot of anti-FLAG M2-agarose (Sigma A2220) was added to each assay. The reactions were incubated on ice for 1.5 h with gentle shaking. After incubation, resin was pelleted at 1000 g for 30 sec, and the supernatant was removed. The resin was washed three times for 15 min each with 500 μL of fresh PEB (without PTE or DOC). After the final wash, the resin was pelleted again. To elute bound proteins from the resin, SDS sample buffer was added directly to each reaction tube and heated at 95°C for 10 min. Heating released resin-bound polysomes and anything bound to them. Resin was removed by centrifugation at 14,000 g for 5 min. The resulting supernatant contained the eluted protein sample.

Affinity Purification of Yeast Ribosomes

Yeast (*Saccharomyces cerevisiae*) strain YIT613 contains an HF (His-FLAG) epitope tag fused to the C-terminus of ribosomal protein L25 (Inada et al. 2002). A functional rpL25 gene is essential for cell viability. The wild-type allele of the rpL25 gene was disrupted during the generation of YIT613, indicating that rpL25-HF is a functional component of translating ribosomes. As for *Arabidopsis* L18, 60S subunits, 80S monosomes and polysomes can be readily purified from YIT613 cells using anti-FLAG agarose.

Cultures of wild type and YIT613 were grown in 200 mL YPD at 30°C according to standard procedures (Guthrie and Fink 1991). Cells were collected by centrifugation in exponential phase (OD_{600} = 0.8) or in postdiauxic phase (∼18 h of additional growth; OD_{600} = 2.2; Fuge et al. 1994). Pellets were resuspended in 700 μL of binding buffer (100mM Tris-HCl, pH 7.5, 24 mM Mg(OAc)$_2$, 1mM DTT, 1mM PMSF, 50 U/ml RNAsin) containing a protease inhibitor cocktail (Complete Mini-EDTA Free, Roche) and lysed by vortexing in the presence of 700 μL of sterile glass beads. Homogenates were cleared by centrifugation in a microfuge at 10,000 rpm for 5 min, followed by a 20-min centrifugation at 10,000 rpm. The resulting supernatant was removed as the homogenate sample. For ribosome pull-down assays, 500 μL of homogenate in binding buffer was mixed with anti-FLAG M2-agarose and incubated for 2 h on ice with constant shaking. Resin was collected by centrifugation at 1000 rpm and washed five times with 1 mL of IXA-100 buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 12 mM Mg(OAc)$_2$, 1 mM DTT, 1 mM PMSF). Bound proteins were eluted with SDS sample buffer.

Results

In a previous report, we showed using differential centrifugation that DRG2 was located predominantly in P150 and S150 cell fractions of pea root apex. P150 was the 150,000 g pellet, which was prepared from a supernatant depleted of cell material that could be pelleted at 20,000 g. P150 was expected to be enriched in microsomal membranes and ribosomes, whereas the S150 supernatant contained the postribosomal cytosolic components of the cell. P150 contained protein bands recognized by DRG2 antibodies with apparent masses of ∼45, 43, and 30 kDa (fig. 1a). The nature of the interactions between these bands and other components of the
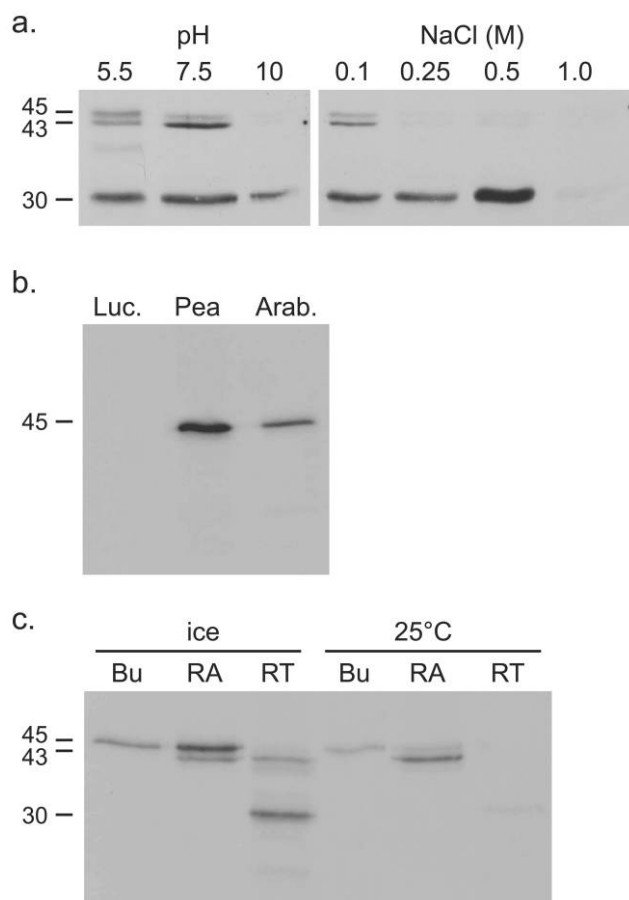
Fig. 1 Properties of protein bands recognized by DRG2 antibodies. *a*, A P150 fraction (150,000 *g* pellet) from pea root apices was resuspended in buffer at pH 7.5, incubated with various reagents for 30 min on ice, and then pelleted again at 150,000 *g*. This second P150 fraction was assayed for DRG2 bands by Western blotting. The 45- and 43-kDa bands were released in tandem (e.g., by pH 10 or by NaCl at 0.25 M higher concentrations). The 30-kDa band was released only by 1 M NaCl. *b*, *PsDRG2* (Pea) and *AtDRG2* (Arab.) cDNAs were used as templates for coupled in vitro transcription and translation in the presence of ^{35}S-labeled amino acids. The products were immunoprecipitated with DRG2 antibodies, separated by SDS-PAGE, and subjected to fluorography. Each cDNA yielded a single 45-kDa band. Luciferase cDNA (*Luc.*), a negative control, did not yield any precipitable bands. *c*, Aliquots of ^{35}S-labeled pea DRG2 were incubated in the presence of buffer (*Bu*), root apex extract (*RA*), or mature root extract (*RT*) for 30 min on ice or at 25°C. Protease inhibitors were not included in the incubation or when preparing the tissue extracts. DRG2 was stable in buffer at both temperatures. When incubated with a tissue extract, DRG2 appeared to be degraded first to a 43-kDa band and then to a 30-kDa band.

P150 fraction was investigated. To do so, the P150 pellet was resuspended, aliquots were incubated under various conditions, and the resuspended material was centrifuged again at 150,000 *g*. This second P150 pellet was analyzed by Western blotting using DRG2 antibodies (fig. 1*a*). The effect of pH was tested first (pH 7.5 was the control). All three bands could be repelleted at pH 5.5 or 7.5. At pH 10, the 45- and 43-kDa forms were released, whereas much of the 30-kDa band could be repelleted. NaCl at 0.25 M was sufficient to release the 45-

and 43-kDa bands. In contrast, the 30-kDa band was still bound at 0.5 M NaCl, but it could be released by 1 M NaCl. Thus, all bands could be released under some conditions, the 45- and 43-kDa bands were released in tandem, and the 30-kDa band was most firmly bound to other components of the P150 fraction.

The relationship between the three bands recognized by DRG2 antibodies was examined in an in vitro experiment. Complementary DNA clones corresponding to the complete coding regions of *PsDRG2* and *AtDRG2* were used as templates for coupled in vitro transcription and translation (TnT kit, Promega). These reactions were performed in the presence of ^{35}S-labeled methionine and cysteine. The products of these reactions were then analyzed by SDS-PAGE followed by fluorography. A very high level of background obscured the expected products at 45 kDa (data not shown). As a refinement, the products of the TnT reactions were further purified by immunoprecipitation with DRG2 antibodies. Following this procedure, *PsDRG2* and *AtDRG2* cDNAs each yielded a single 45 kDa band (fig. 1*b*). As a negative control, the luciferase cDNA provided with the TnT kit was used as a template. No precipitable bands were produced from this template.

Aliquots of the 45 kDa product of the *PsDRG2* TnT reaction were incubated with buffer, root apex extract, or mature root extract on ice or at 25°C for 30 min. The reactions were then subjected to SDS-PAGE followed by fluorography (fig. 1*c*). In the presence of buffer alone, the 45-kDa protein was relatively stable at both temperatures. In the presence of an extract from root apex or mature root, the 45-kDa protein appeared to undergo proteolytic processing, first to a 43-kDa protein, then to a 30-kDa protein, and finally to be fully degraded. This result suggests that the 43- and 30-kDa bands observed in tissue extracts are specific proteolytic products of the full-length DRG2 protein. Similar experiments were attempted with root apex proteins labeled in vivo. However, we were unsuccessful in immunoprecipitating DRGs in these experiments.

A pea *DRG1* gene has not been described previously. We isolated a full-length pea *DRG1* clone from an axillary cDNA bud library using *AtDRG1* as a probe. The *PsDRG1* cDNA (GenBank accession no. EU236700) contained an ORF that would encode a protein of 368 amino acid residues and a predicted mass of 41.2 kDa. On the basis of BlastP alignments (Schäffer et al. 2001), amino acid identity/similarity of *Ps*DRG1 with *Ps*DRG2 was 57%/74%, which is typical for DRG paralogs from a given species. Similar comparisons of *Ps*DRG1 with DRG1 orthologs from *Arabidopsis*, *Vitis*, *Oryza*, and *Chlamydomonas* indicated levels of identity/similarity of 91%/95%, 92%/97%, 91%/96%, and 74%/86% (AAK59539, CAO71761, BAC79856, and EDP08619, respectively). Similar to our previous work on *Arabidopsis* DRG1, our DRG1 antibodies specifically recognized a single band in pea tissues with an apparent mass of ~43 kDa band, with very small amounts of a possible degradation product seen only occasionally.

The ability of pea DRG1 and DRG2 from root apex extracts to bind to GTP was tested using GTP-agarose for pulldown assays (fig. 2). DRG1 and the 45- and 43-kDa forms of pea DRG2 bound to this resin, whereas the 30-kDa form of DRG2 did not (lane 2). High concentrations of free nucleotides in the incubation might inhibit DRG proteins from bind-
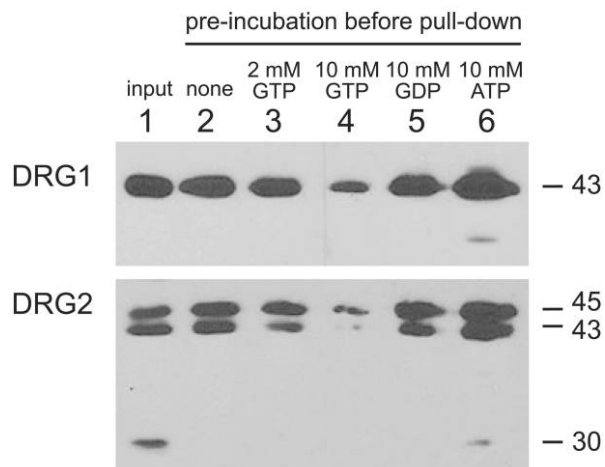
Fig. 2 GTP binding properties of pea DRG1 and DRG2. Input (cleared homogenate from root apices) contained DRG1 and three forms of DRG2. Pull-down samples contained proteins that became bound to GTP-agarose. Before incubation with GTP-agarose, the other aliquots were incubated on ice for 5 h with no additions (control), 2 mM GTP, 10 mM GTP, 10 mM GDP, or 10 mM ATP. Preincubation with 10 mM GTP reduced the ability of DRG1 and the 43- and 45-kDa forms of DRG2 to bind to GTP-agarose, but the other treatments did not. The 10 mM ATP lane is slightly overloaded, so a small amount of the 30-kDa band appears in this lane.

ing to GTP-agarose. This was tested by preincubating tissue extracts with nothing (control), GTP at 2 mM or 10 mM, or GDP or ATP at 10 mM. GTP at 10 mM significantly reduced the amount of binding of DRG1 and of the 45- and 43-kDa forms of DRG2 (lane 4). In contrast, preincubation with 2 mM GTP, 10 mM GDP, or 10 mM ATP did not reduce the amount of binding to GTP-agarose (lanes 3, 5, and 6, respectively). The ATP experiments (lane 6) were slightly overloaded, which may account for a small amount of the 30-kDa form of DRG2 appearing in that sample.

Ribosomes were thought to be a major component of the P150 fraction described above (fig. 1a). Experiments were conducted to assay for interactions between ribosomes and DRG proteins. Experiments were begun with equal amounts of protein in each sample. First, total cleared homogenates (T) were centrifuged through 60% sucrose pads to yield ribosome-enriched pellets (P) and postribosomal supernatants (S). Total ribosome pellets were subjected to differential centrifugation to produce fractions enriched in four ribosome subpopulations, namely, cytosolic monosomes (CM), cytosolic polysomes (CP), microsome-associated monosomes (MM), and microsome-associated polysomes (MP). Fractions were loaded on gels based on equivalent volumes, so the protein content of these fractions varied. A Coomassie stained gel of fractionated extracts from root apices showed many bands and relatively high protein content in the CP, MP, and T fractions, a moderate amount of protein in the CM and S fractions, and very little in the MM fraction (fig. 3a). RPS6 is a component of the 40S ribosome subunit and consequently occurs in this subunit, in 80S monosomes, and in polysomes. RPS6 is not known to be present elsewhere in the cell. A Western blot probed with RPS6 antibodies indicated a strong enrichment in the two
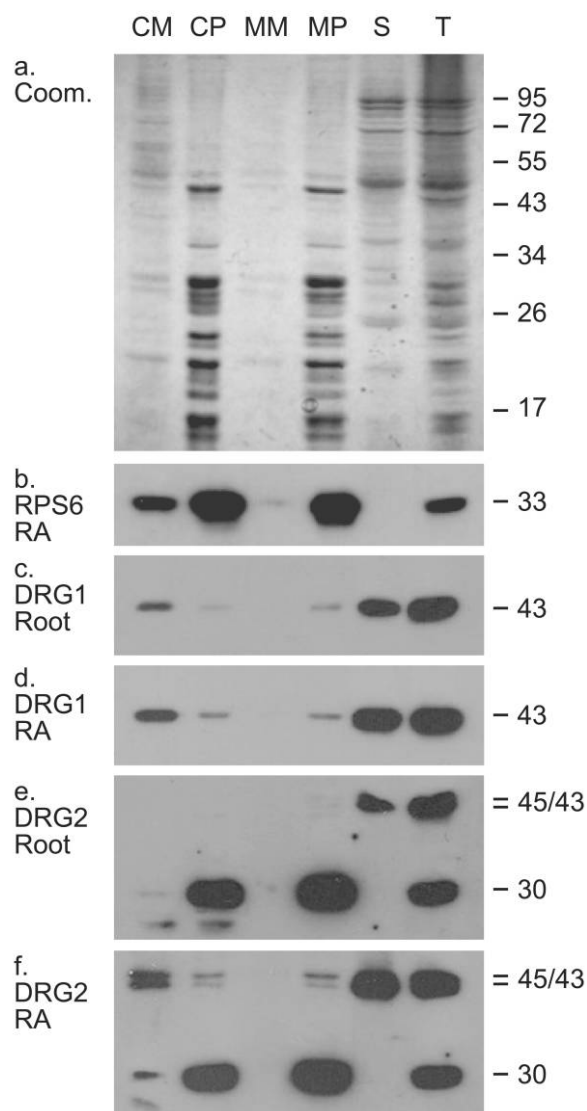

Fig. 3 Copurification of DRG1 and DRG2 with various ribosome types. A fraction enriched in all forms of ribosomes was subjected to differential centrifugation to yield fractions enriched in cytosolic monosomes (CM), cytosolic polysomes (CP), microsomal monosomes (MM), and microsomal polysomes (MP). The postribosomal supernatant (S) and total extract (T) were also examined. Equal volumes of each resulting fraction were assayed. a, A Coomassie-stained gel revealed total protein content of each fraction. b, A Western blot was probed for RPS6, which occurs in 40S subunits, 80S monosomes, and polysomes. Most of this protein occurred in polysomes (CP, MP), with a smaller amount in the cytosolic monosome/40S fraction. None was found in S. c, d, DRG1 was present in the cytosol of mature roots and root apices, as well as in the CM fraction. Relatively little was present in the polysome fractions. e, f, The fractionation patterns of the 43- and 45-kDa forms of DRG2 in fractions from root apices were similar to those of DRG1. In mature roots, however, these forms of DRG2 occurred in the cytosol but not in any of the ribosome-enriched fractions. The 30-kDa form of DRG2 was highly enriched in both types of polysomes from mature roots and root apices.

polysome fractions, CP and MP (fig. 3b). A moderate amount of RPS6 was present in the CM fraction, but very little occurred in the MM fraction. RPS6 was not detected in S, indicating that this fraction was depleted of ribosomes.

In addition to root apices, which contained actively dividing and elongating cells, mature roots also were examined. The patterns of DRG1 fractionation in roots and root apices were very similar (fig. 3c, 3d). A significant amount of DRG1 occurred in the S fraction and therefore was not associated with ribosomes. A small amount of DRG1 was associated with the two polysome fractions (CP and MP), but a greater amount was associated with the CM fraction, which contained 80S monosomes as well as 40S and 60S subunits. Given that the overall abundance of ribosomes is considerably lower in CM than in CP or MP, DRG1 appears to be considerably enriched in this fraction. DRG2 fractionation patterns were more complicated as a result of the presence of three forms of this protein (fig. 3e, 3f). The 45- and 43-kDa forms tended to cofractionate. In mature roots, these bands were detected only in S. In contrast, in root apices, these bands were enriched in the CM fraction and also were present in CP and MP; this fractionation pattern is very similar to that of DRG1. The 30-kDa form of DRG2 was strongly enriched in the two polysome fractions (CP and MP) of both roots and root apices. It was barely detectable in CM and was absent from S.

The previous experiment indicated that in root apices, some of the DRG1 and the 45- and 43-kDa forms of DRG2 associated with ribosomes (mostly cytosolic monosomes) but that much more remained in the postribosomal supernatant (fig. 3). An experiment was designed to test the ability of soluble DRGs to associate with ribosomes in vitro. Cleared homogenates were prepared from root apices and kept at 4°C during centrifugation steps and on ice at other times. Then, aliquots were incubated for 2 h either on ice or at 25°C. After incubation, a sample of total extract (T) was removed, and the remainder was used to isolate ribosomal pellet (P) and postribosomal supernatant (S) fractions. In incubations kept on ice, relatively small amounts of DRG1 and almost none of the 45- and 43-kDa forms of DRG2 occurred in the ribosome pellet (fig. 4, ice/cont.). Following incubation of the extracts at 25°C, much more DRG1 and the DRG2 45- and 43-kDa forms copurified with ribosomes (fig. 4, 25°C/cont.). At both temperatures, the 30-kDa form of DRG2 occurred exclusively in the ribosome pellet. Similar temperature shift incubations were performed in the presence of 0.5 mM GDP, GTP, or GTP-γ-S. Neither GDP nor GTP affected the ability of DRG1 or DRG2 45 and 43 kDa to bind to ribosomes during incubation at 25°C (data not shown). However, inclusion of GTP-γ-S in the reaction reduced the amount of both DRG1 and DRG2 that became bound to ribosomes (fig. 4; cf. P fractions in 25°C/cont. and 25°C/GTP-γ-S). The amounts of the 30-kDa form of DRG2 and RPS6 in the ribosome pellet were unaffected by any of these treatments.

Arabidopsis 35S:HF-RPL18 expresses a fusion between the HF (His-FLAG) peptide and ribosomal protein L18 (Zanetti et al. 2005). Ribosomes and anything bound to them can be easily purified from extracts of "L18" plants using agarose coupled to an anti-FLAG antibody for pull-down assays. We used this system to examine ribosome association of DRGs in whole *Arabidopsis* seedlings grown on MS plates (fig. 5a). In
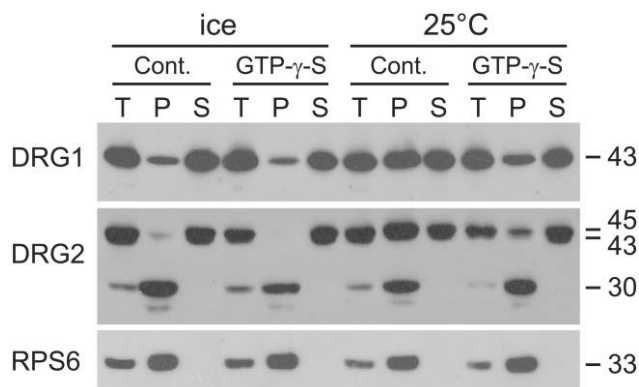
Fig. 4 Association of DRGs with ribosomes in vitro. Cleared homogenates were prepared from pea root apices and kept on ice. Aliquots were incubated for 2 h on ice or at 25°C, with or without 0.5 mM GTP-γ-S. The fractions analyzed were as follows: total cleared homogenates (*T*), total ribosomes (*P*), and postribosomal supernatants (*S*). Western blots were probed for the presence of DRG1, DRG2, and RPS6. In controls (no GTP-γ-S), a portion of DRG1 and DRG2 that had been in the soluble fraction became associated with ribosomes during incubation at 25°C. This association was partly inhibited by GTP-γ-S.

control experiments, cell extracts from wild type or L18 were used for pull-down assays and probed for RPS6. As expected, the pull-down fraction from L18 plants contained this protein, whereas that from wild-type plants did not. DRG1 also was detected in the pull-down fraction from L18 plants. A significant amount of the 30-kDa form of DRG2 also was present in the FLAG pull-down fraction. In contrast, only small amounts of the 45- and 43-kDa forms were present in this fraction.

Saccharomyces cerevisiae contains a DRG1 gene (YAL036c/FUN11/RBG1) and a DRG2 gene (YGR173w/GIR1/RBG2). The predicted molecular weight of each protein is ~41 kDa. Each of these proteins is recognized by antibodies raised against plant DRG1 or DRG2, and each has an apparent molecular weight of ~43 kDa. Yeast cell line YIT613 contains a C-terminal FLAG-His tag on ribosomal protein L25 (Inada et al. 2002). YIT613 does not contain a wild-type rpL25 gene, so translation depends on rpL25-HF. Similar procedures were used to affinity purify ribosomes from yeast cells as were used for *Arabidopsis*. Cells were harvested in late exponential phase ($OD_{600} = 0.8$) and ~18 h later when cells were in postdiauxic phase ($OD_{600} = 2.2$). Samples containing equal amounts of total protein from each strain and at each growth phase were analyzed. DRG1 antibodies recognized a single ~43-kDa band from yeast, which comigrated with *Arabidopsis* DRG1 (fig. 5b; *Arabidopsis* lanes are relatively overloaded). DRG2 antibodies also recognized a single band at ~43 kDa. The amount of yeast DRG1 and DRG2 in the total samples was similar in exponential and postdiauxic phase cells. However, slightly less DRG1 and considerably less DRG2 was in the ribosome pull-down fraction of postdiauxic phase cells compared with exponential phase cells.

Discussion

The very high level of sequence conservation among eukaryotic DRG1 and DRG2 orthologs implies that they play univer-
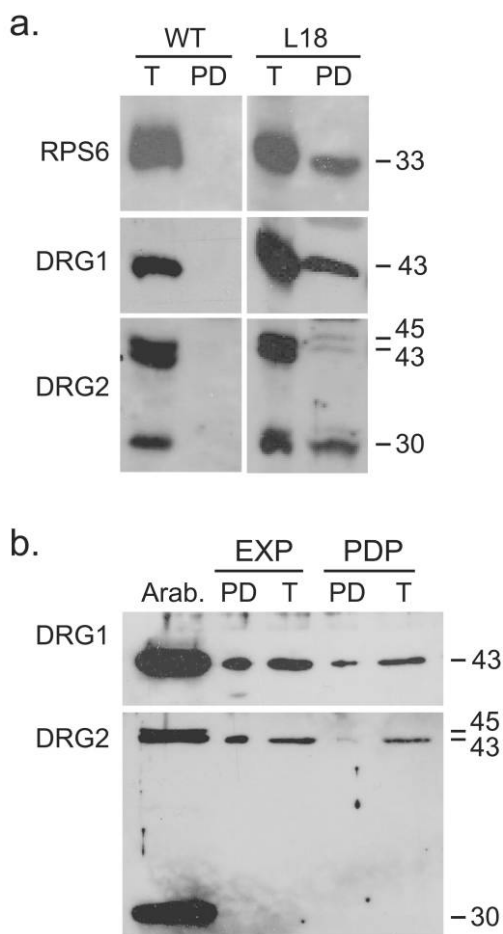
Fig. 5 Association of DRGs with FLAG-tagged ribosomes from *Arabidopsis* and yeast. *a*, Homogenates were prepared from *Arabidopsis* Col-0 wild-type (*WT*) and *35S:HF-RPL18* (*L18*) plants; the latter expressed FLAG-tagged ribosomal protein L18. Anti-FLAG M2-agarose was used to purify ribosomes and associated proteins. Total cleared homogenates (*T*) and pull-down (*PD*) fractions were probed for RPS6, DRG1, and DRG2 by Western blotting. None of these proteins were pulled down in extracts from wild-type plants. The PD fractions of L18 plants contained RPS6, DRG1, and the 30-kDa form of DRG2. Small amount of the 45- and 43-kDa forms of DRG2 also were in the PD fraction. *b*, Homogenates were prepared from *Saccharomyces cerevisiae* YIT613 cells, which expressed FLAG-tagged ribosomal protein L25. Cells were harvested in late exponential phase (*EXP*) or in postdiauxic phase (*PDP*). Total (*T*) and pull-down (*PD*) fractions were probed using DRG1 and DRG2 antisera. Each antiserum recognized a single ~43-kDa band. The amount of DRG1 and especially of DRG2 in PD fractions was reduced in postdiauxic cells relative to exponentially growing cells. Yeasts DRG1 and DRG2 are encoded by YAL036c and YGR173w, respectively.

sal and important roles in cell physiology. Nevertheless, an understanding of these functions remains elusive. Eukaryotic DRGs and bacterial OBGs are members of the obglike family of GTP binding proteins (conserved domain 01881; http://www.ncbi.nlm.nih.gov/). These proteins share considerable similarity in their GTP binding domains and also contain TGS domains near their C-termini, which may be important for RNA binding (Wolf et al. 1999). Interactions between ribo-

somes and OBG proteins are well documented. These interactions may be important for linking cell stress to the regulation of translation (Jiang et al. 2007). The primary goal of this report was to investigate ribosome binding properties of DRG1 and DRG2 proteins from pea, *Arabidopsis*, and yeast. Before discussing these results, we will address other aspects of this study.

DRG2 antibodies recognized protein bands with apparent masses of ~45, 43, and 30 kDa in tissues from pea (figs. 1–4) and *Arabidopsis* (fig. 5*a*; Stafstrom 2008). The actual sizes of the two larger bands may be closer to 44.5 and 41 kDa. In order to determine the identities of these bands, pea and *Arabidopsis DRG2* full-length cDNA clones were used as templates for in vitro transcription and translation reactions. Immunoprecipitation of the ^{35}S-labeled products indicated that full-length DRG2 from both plants has an apparent mass of ~45 kDa (fig. 1*b*). Labeled DRG2 synthesized in vitro was quite stable when incubated in buffer on ice or at 25°C (fig. 1*c*). However, incubation with cleared homogenates isolated from either mature roots or root apices led to specific proteolysis of the labeled 45-kDa band: an ~43-kDa band seemed to appear first, followed by a 30-kDa band. The rate or extent of proteolysis may have been greater at 25°C and in the presence of mature root extract. Most important, though, these results are consistent with the hypothesis that the 43- and 30-kDa bands isolated from plant tissues are specific proteolytic products of full-length DRG2.

Protease inhibitors were not used for some experiments involving ribosome purification (figs. 3, 4). Nevertheless, DRG proteins isolated from pea tissues appeared to be quite stable. For example, there were no marked differences between equivalent samples kept on ice at all times and ones incubated at 25°C for 2 h (fig. 4). Some recent proteomic studies on ribosomes and ribosome-associated proteins from *Arabidopsis* and *Chlamydomonas* also did not report using protease inhibitors during purification (Chang et al. 2005; Manuell et al. 2005; Carroll et al. 2008). Whereas DRG1 and DRG2 isolated from tissues appeared to be stable, DRG2 synthesized in vitro and combined with tissue extracts was processed to smaller forms (fig. 1*c*). What might account for this discrepancy? One possibility is that in cells, DRGs interact with stabilizing proteins. A class of such proteins, called DFRPs (DRG family regulatory proteins), has been described (Ishikawa et al. 2005). In *Xenopus*, DFRP1 interacts specifically with DRG1, and DFRP2 interacts specifically with DRG2. Physical interaction between a DRG and its DFRP partner inhibits polyubiquitination of DRG and its subsequent degradation. Engineered mutations in the chicken DFRP1 gene reduced the accumulation of DRG1 protein but not that of DRG2 (Ishikawa et al. 2005). DRG1 and DRG2 mRNA levels were unaffected in these mutants. DFRP gene homologs also occur in plants and fungi. We have found that a T-DNA knockout in the *Arabidopsis DFRP1* gene (At2g20280) reduces the accumulation of DRG1 and that a knockout in *DFRP2* (At1g51730) reduces the accumulation of DRG2 (J. D. Kubic and J. P. Stafstrom, in preparation). It is possible that DRG2 synthesized in vitro was not stable in the presence of cell extracts because it was not associated with a stabilizing partner, whereas DRGs isolated from cells were stabilized by such associations.

We demonstrated previously using differential fractionation techniques that pea DRG2 was localized predominantly in a

150,000 *g* pellet fraction (P150) and in the supernatant of this pellet (S150; Devitt et al. 1999). P150 is enriched in microsomal membranes and ribosomes, and S150 is the postribosomal cytosolic fraction. On the basis of several types of experiments presented here, DRG1 and DRG2 appear to copurify with ribosomes. Centrifugation of cell homogenates through a 60% sucrose pad yields a pellet enriched in ribosome subunits, monosomes, and polysomes (Davies and Abe 1995). DRG1 and DRG2 also occur in this pellet (fig. 4). This pellet was further fractionated to yield fractions enriched in cytosolic and microsomal monosomes and in cytosolic and microsomal polysomes (fig. 3). We examined root tissues at two developmental stages. Root apices contain actively dividing and elongating meristematic cells, whereas these processes have ceased in mature, fully elongated roots. RPS6 was used as a marker for 40S subunits, 80S monosomes, and polysomes (Williams et al. 2003). DRG1 and the three forms of DRG2 showed distinct patterns of ribosome cofractionation. In both tissues, DRG1 was present in the cytosolic fraction. A small amount of DRG1 cofractionated with the two polysome fractions, but the majority of DRG1 was associated with cytosolic monosomes (on the basis of RPS6 levels, membrane-associated monosomes were not abundant, so this fraction will not be discussed). The enrichment of DRG1 with cytosolic monosomes relative to polysomes is even more apparent when compared with the amount of RPS6 in these fractions. The 30-kDa form of DRG2 was highly enriched in cytosolic and microsomal polysomes of root apices and mature roots. Localization patterns of the 45- and 43-kDa forms of DRG2 isolated from root apices were similar to those of DRG1 in root apices and mature roots. In mature roots, however, these forms of DRG2 occurred in the cytosol but were not found in any of the ribosome-containing fractions. Developmental differences in DRG-ribosome association were also seen in yeast, most notably a considerable reduction in DRG2 in the ribosome pull-down fraction of postdiauxic phase cells (fig. 5*b*). Rates of protein synthesis in postdiauxic cells are no more than 10% of those of exponential cells (Fuge et al. 1994). Thus, yeast DRG2 appears to be associated primarily with translating ribosomes.

The 45- and 43-kDa forms of DRG2 occur in both the cytosol and the CM fraction (fig. 3), so initial attachment of DRG2 to monosomes or subunits may occur in one of these forms. Cleavage to produce the 30-kDa form might occur as monosomes are formed or after translational elongation begins to occur on polysomes. It is interesting that the 30-kDa form binds to the translation machinery much more tightly than the larger forms (fig. 1*a*). Since the 30-kDa form is essentially absent from the cytosol, its fate at the end of a translational cycle is unclear. Does it immediately interact with another polysome? Is it immediately degraded? It is also not known whether this form of DRG2 is necessary for some aspect of translational elongation. Further biochemical studies, together with analyses of mutants, should prove to be illuminating.

We are also interested in understanding how DRG1 and the three forms of DRG2 interact with ribosomes. These associations could involve protein-protein interactions with a ribosomal protein or with a ribosome-associated protein. Interactions also might result from rRNA-protein interactions mediated by the DRG TGS domain. Recent large-scale proteomic studies have examined the composition of ribosomes of *Arabidopsis* (Chang et al. 2005; Carroll et al. 2008), *Chlamydomonas* (Manuell et al. 2005), and other organisms. In addition to ribosomal proteins per se, each study identified a number of nonribosomal proteins that associate with ribosomes. None of these studies detected DRGs among the latter group. A study in yeast specifically sought to identify uncharacterized ribosome-associated proteins, which were referred to as translation machinery–associated proteins, or TMAs (Fleischer et al. 2006). TMA46 is the yeast homolog of *Xenopus* DFRP1, which, as described above, specifically interacts with DRG1 (Inada et al. 2002). TAP-affinity tags were fused to both TMA46 and DRG1, and each tagged protein was able to interact with the other protein in affinity assays (Fleischer et al. 2006). Thus, the association we observed between yeast FLAG-tagged ribosomes and DRG1 and DRG2 (fig. 5*b*) may be mediated by indirect interactions.

DRGs contain canonical G boxes and switch domains needed for binding guanine nucleotides (Leipe et al. 2002). However, experimental evidence for GTP binding previously was available only for DRG1 of mouse and *Drosophila* (Sazuka et al. 1992; Sommer et al. 1994). We demonstrated that pea DRG1 and DRG2 are capable of binding to GTP-agarose (fig. 2). This binding could be partly inhibited by 10 mM GTP but not by 2 mM GTP or 10 mM GTP or ATP. In contrast to these results, binding of soybean sucrose binding protein to GTP-agarose could be inhibited completely by either 2 mM GTP or GDP (Pirovani et al. 2002). Most G proteins exchange GDP for GTP in response to a specific guanine nucleotide exchange factor (GEF) and hydrolyze GTP to GDP in response to a specific GTPase activation protein (GAP; Bourne et al. 1990, 1991). Nothing is known about GEFs, GAPs, or guanidine dissociation inhibitors that might regulate GTP binding or hydrolysis by DRGs. Compared with most G proteins, OBGs exhibit relatively low rates of GTP hydrolysis and high rates of GTP/GDP exchange (Lin et al. 1999; Datta et al. 2004). The high concentration of GTP needed to partly inhibit DRG1 and DRG2 from binding to GTP-agarose may reflect relatively high rates of guanine nucleotide exchange by these proteins as well.

Unbound, cytosolic DRG1 and DRG2 appear to be capable of binding to ribosomes in vitro following incubation at 25°C for 2 h (fig. 4). This association could be partially inhibited by 0.5 mM GTP-γ-S (fig. 4) but not by GTP or GDP at the same concentration (not shown). Because GTP-γ-S cannot by hydrolyzed, it would tend to lock DRG in the GTP-bound state. As discussed above, though, it is not known whether DRGs, like OBGs, can release GTP without hydrolyzing it. More work needs to be done on the enzymology of GTP hydrolysis and GTP/GDP exchange and on the effects of GTP-γ-S and GDP-β-S at a range of concentrations. Significantly, the ribosome reassociation assay should be a useful tool.

Since the 45-, 43-, and 30-kDa forms of DRG2 showed distinctive patterns of ribosome association (fig. 3), it would be useful to know where full-length DRG2 is cleaved to produce the smaller forms. DRG2 has a GTP binding domain within the N-terminal ~30 kDa, which is followed by an ~10–12-kDa TGS domain. In addition, DRG2 proteins of plants and green algae contain an extension of ~32 residues at the C-terminus that is not found in DRG1 or DRG2 from other

organisms. The in vitro processing experiment suggested that the 43-kDa form appears before the 30-kDa form (fig. 1c). On the basis of Western blotting experiments of transgenic *Arabidopsis* plants expressing an N-terminal GFP-DRG2 fusion protein, we believe that this first cleavage occurs near the C-terminus (B. J. Nelson and J. P. Stafstrom, in preparation). If the second cleavage occurred nearer to the C-terminus, an ~30-kDa protein would be produced with an intact GTP binding domain. In this context, it is interesting to note that the 30-kDa form does not bind to GTP-agarose, whereas the larger forms do (fig. 2). This result might indicate that the cleavage that produces the 30-kDa form occurs within the GTP domain. To clarify this matter, we are attempting to purify the 30-kDa form of DRG2 in order to determine its N-terminal sequence.

In addition to showing interactions with pea ribosomes, we also have demonstrated interactions between DRG1 and DRG2 with ribosomes in *Arabidopsis* and yeast (fig. 5). Both of these efforts utilized copurification of DRGs with ribosomes containing a FLAG-tagged ribosomal protein (Inada et al. 2002; Zanetti et al. 2005). There is no published work that directly addresses DRG-ribosome interactions. However, the indirect ribosome-TMA46-DRG1 link in yeast is quite intriguing (Fleischer et al. 2006). Many additional suggestive interactions are listed in the Biological General Repository for Interaction Datasets (BioGRID; http://www.thebiogrid.org/), which contains nearly 200,000 interactions gleaned from many publications in the primary literature. A large number of interactions come from high-throughput studies of physical and genetic interactions in budding yeast. From this compilation, yeast DRG1 (YAL036c) was suggested to interact with translation initiation factors (TIF2, TIF4631), heat shock proteins and chaperones (HSP70- and HSP90-related genes), a component of the 26S proteasome protein degradation pathway (RPN1), and two DFRP domain-containing genes (TMA46, GIR2), among others. From the same data set, some of the genes/proteins suggested to interact with yeast DRG2 (YGR173w) are as follows: a translation elonga-tion factor (ELP2), a component of the amino acid starvation pathway (GCN1), a number of genes involved in rRNA processing and other aspects of ribosome biogenesis (POP7, POP8, RRP5), and a DFRP2 homolog (GIR2). With the exception of GIR2, there was no overlap between these lists of DRG1 and DRG2 interacters, suggesting that each plays a distinct role in ribosome assembly or activity or other cellular functions. For example, DRG1 interacted with two translation initiation factors, whereas DRG2 interacted with a translation elongation factor. Our ribosome fractionation experiments suggest similar associations (fig. 3). Specifically, we documented an enrichment of DRG1 in the CM ribosome fraction (which includes 40S and 60S subunits and 80S monosomes, which would be involved in translation initiation), and a highly specific association between the 30-kDa form of DRG2 and polysomes (where translation elongation factors would be abundant). Also of interest is the association of DRG1 with heat shock proteins and chaperones. OBG proteins, the bacterial cousins of DRGs, are associated with ribosomes and implicated in mediating stress responses (Jiang et al. 2007). In *Arabidopsis*, heat stress stimulates *AtDRG3* mRNA accumulation and also alters accumulation patterns of proteins recognized by DRG antibodies (Stafstrom 2008). We are continuing to study relationships between plant stress, ribosome activity, and DRG localization.

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