The KEEP ON GOING Protein of Arabidopsis Recruits the ENHANCED DISEASE RESISTANCE1 Protein to Trans-Golgi Network/Early Endosome Vesicles1[W][OA]

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Loss-of-function mutations in the Arabidopsis (Arabidopsis thaliana) ENHANCED DISEASE RESISTANCE1 (EDR1) gene confer enhanced resistance to powdery mildew infection, enhanced senescence, and enhanced programmed cell death under both abiotic and biotic stress conditions. All edr1-mediated phenotypes can be suppressed by a specific missense mutation (keg-4) in the KEEP ON GOING (KEG) gene, which encodes a multidomain protein that includes a RING E3 ligase domain, a kinase domain, ankyrin repeats, and HERC2-like (for HECT and RCC1-like) repeats. The molecular and cellular mechanisms underlying this suppression are poorly understood. Using confocal laser scanning microscopy and fluorescent protein fusions, we determined that KEG localizes to trans-Golgi network/early endosome (TGN/EE) vesicles. Both the keg-4 mutation, which is located in the carboxyl-terminal HERC2-like repeats, and deletion of the entire HERC2-like repeats reduced endosomal localization of KEG and increased localization to the endoplasmic reticulum and cytosol, indicating that the HERC2-like repeats facilitate the TGN/EE targeting of KEG. EDR1 colocalized with KEG to the TGN/EE when coexpressed but localized primarily to the endoplasmic reticulum when expressed alone. Yeast two-hybrid and coimmunoprecipitation analyses revealed that EDR1 and KEG physically interact. Deletion of the HERC2-like repeats abolished the interaction between KEG and EDR1 as well as the KEG-induced TGN/EE localization of EDR1, indicating that the recruitment of EDR1 to the TGN/EE is based on a direct interaction between EDR1 and KEG mediated by the HERC2-like repeats. Collectively, these data suggest that EDR1 and KEG function together to regulate endocytic trafficking and/or the formation of signaling complexes on TGN/EE vesicles during stress responses.

Programmed cell death (PCD) is a highly regulated process in multicellular organisms that is central to both normal development and coping with environmental challenges (Jacobson et al., 1997). PCD can be triggered by many stimuli and is often associated with misregulation of essential cellular processes such as DNA repair, cell cycle progression, and immune responses. In Arabidopsis (Arabidopsis thaliana), loss-of-function mutations in the ENHANCED DISEASE RESISTANCE1 (EDR1) gene confer enhanced PCD under both abiotic and biotic stress conditions. For example, PCD is induced in edr1 mutant leaves by infection with the powdery mildew fungus Golovinomyces cichoracearum (Frye and Innes, 1998) and by drought treatment (Tang et al., 2005). In addition, exogenous application of ethylene induces senescence of edr1 mutant leaves more rapidly than wild-type leaves (Frye et al., 2001). Significantly, the former two phenotypes require an intact salicylic acid (SA) signaling pathway, while the latter one does not (Frye et al., 2001; Tang et al., 2005), indicating that EDR1 functions upstream of, or in parallel to, SA signal transduction pathways.

EDR1 encodes a protein kinase similar to CONSTITUTIVE TRIPLE RESPONSE1 (CTR1; Frye et al., 2001), a negative regulator of the ethylene response pathway. Loss-of-function mutations in CTR1 confer a constitutive ethylene response phenotype (a short fat hypocotyl and an exaggerated apical hook when grown in the dark), constitutive expression of ethylene-inducible genes, and severe dwarfing (Kieber et al., 1993). However, the edr1 mutant does not show any of these phenotypes, indicating that these two kinases regulate separate pathways (Frye and Innes, 1998; Frye et al., 2001).

To identify components of signaling pathways required for edr1-mediated phenotypes, we mutagenized edr1 seeds and screened for second site mutations that suppressed the powdery mildew-induced lesion phenotype. From this screen, we identified a missense mutation in the KEEP ON GOING (KEG) gene that suppressed all known edr1 mutant phenotypes (Wawrzynska et al., 2008). This mutation, which we designated keg-4, is recessive but does not entirely eliminate KEG function, as T-DNA insertional knockouts of KEG (keg-1/2/3) are seedling lethal (Stone et al.,

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KEG is a multidomain protein that contains a functional RING finger E3 ubiquitin ligase domain, a kinase domain, nine ankyrin repeats, which have been implicated in mediating protein-protein interaction, and 12 HERC2-like (for HECT and RCC1-like) repeats of unknown function (Stone et al., 2006). The keg-4 mutation substitutes a conserved Gly to Ser in the fifth HERC2-like repeat. The KEG protein has previously been shown to regulate abscisic acid (ABA) signaling via regulating levels of the ABA-INSENSITIVE5 (ABI5) transcription factor through ubiquitin-mediated degradation (Stone et al., 2006). Recently, it has been further proposed that ABA promotes ABI5 accumulation by inducing self-ubiquitination and proteasomal degradation of KEG (Liu and Stone, 2010). Notably, mutation within the KEG kinase domain, or treatment with the general kinase inhibitor staurosporine, inhibits ABA-induced ubiquitination and degradation of KEG, indicating that phosphorylation is a key regulator of KEG activity (Liu and Stone, 2010). How ABA is perceived by or transduced to KEG is poorly understood.

Because all known edr1 mutant phenotypes can be suppressed by the keg-4 mutation, we hypothesize that KEG functions at the top of the EDRI-mediated signaling pathway. However, the molecular mechanisms underlying the genetic interaction between KEG and EDRI are not understood. Here, we show that KEG is predominantly localized to trans-Golgi network/early endosome (TGN/EE) vesicles and that this localization is dependent on the HERC2-like repeats of KEG. Furthermore, we show that KEG associates with EDRI in vivo and recruits EDRI to TGN/EE vesicles. In addition, yeast two-hybrid analysis indicates that KEG is likely a substrate of EDRI. Collectively, these data indicate that EDRI and KEG colocalize to TGN/EE vesicles and function together to regulate signaling and/or trafficking during stress responses.

RESULTS

KEG Is Localized to the TGN/EE

To determine the subcellular localization of KEG, super yellow fluorescent protein (sYFP; Kremers et al., 2006) was fused to the C terminus of the full-length KEG open reading frame. We showed that this fusion protein was functional by placing this construct under control of the native KEG promoter and transforming heterozygous keg-1 mutant plants. Phenotypically wild-type plants that were homozygous for the keg-1 insertion were recovered in the T1 generation, indicating that the transgene complemented the keg-1 mutation. This was confirmed in the T2 generation (data not shown). Analysis of transgenic plants by confocal laser scanning microscopy, however, failed to detect a fluorescent signal, indicating that KEG is expressed at very low levels under normal conditions.

To enable detection of the KEG-sYFP protein, we placed the KEG-sYFP construct under control of a steroid-inducible promoter (Aoyama and Chua, 1997) and transiently transformed it into Nicotiana benthamiana leaves using Agrobacterium tumefaciens infiltration. Twenty-four hours after treatment with 50 µM dexamethasone, epidermal cells were examined using confocal laser scanning microscopy. In all transformed cells, small fluorescent punctate structures were observed in the cortex of cells (Fig. 1A). In time-lapse imaging, these vesicles alternated between random saltatory and directional movements (Supplemental Movie S1), similar to the previously described movements of Golgi stacks (Boevink et al., 1998; Batoko et al., 2000; Brandizzi et al., 2002). To avoid possible mislocalization of KEG caused by the C-terminal fusion with sYFP, we also constructed a KEG N-terminal fusion with mCherry, and a similar localization pattern was observed (Fig. 1B). KEG-sYFP and mCherry-KEG completely overlapped with each other upon coexpression (Fig. 1C), suggesting that the fluorescent protein fusions did not impact the localization of KEG.

Since the KEG-sYFP fluorescence pattern resembled both the distribution and movement pattern of Golgi stacks, we performed colocalization experiments with the cis-Golgi cisternae marker GmMan49 (the first 49 amino acids of GmManI) fused with mCherry (Nelson et al., 2007) and with the trans-Golgi cisternae marker a-2,6-sialyltransferase (ST; Wee et al., 1998) fused with red fluorescent protein (RFP). Surprisingly, in both cases, very little overlap was detected. Instead, KEG-sYFP typically localized adjacent to Golgi stacks, revealing a twin-spot distribution pattern with Golgi stacks (Fig. 1, D–I). Time-lapse live imaging revealed that not all KEG-sYFP-containing vesicles associated with Golgi stacks. Instead, association with Golgi stacks appeared to be transient in nature (Supplemental Movie S1). We also performed analyses in Arabidopsis protoplasts using a native promoter-driven KEG-sYFP construct. The same localization pattern was observed as in N. benthamiana, although at a much lower level (Supplemental Fig. S1), indicating that the localization pattern is not an artifact of heterologous expression in N. benthamiana.

The localization pattern of KEG relative to Golgi stacks is similar to what has been previously reported for the TGN/EE (Uemura et al., 2004; Viotti et al., 2010). Therefore, we coexpressed KEG-sYFP with the TGN/EE marker protein SYP61 (Uemura et al., 2004) fused with mCherry at the N terminus. As a control, we also coexpressed KEG-sYFP with mCherry-SYP21, which localizes to the prevacuolar compartment/late endosomes (PVC/LE; Uemura et al., 2004). Upon coexpression, the fluorescence of KEG-sYFP colocalized with mCherry-SYP61, as shown in Figure 1, J to L, and their movement during time-lapse imaging was synchronous (Supplemental Movie S2). In contrast, KEG-sYFP conferred a distinct localization and movement pattern from mCherry-SYP21 (Fig. 1, M–O; Supplemental Movie S3). Furthermore, the movement of KEG-sYFP depended on...
the actin cytoskeleton, as treatment with latrunculin B, an actin-disrupting drug, blocked its directional movements (Supplemental Movie S4). We thus conclude that KEG-sYFP localizes to TGN/EE vesicles.

In plants, the TGN cannot be distinguished from EE (Dettmer et al., 2006), hence the dual name. Based on work in animal and yeast systems, the TGN is generally considered to play a role in sorting of newly synthesized proteins to the vacuole or plasma membrane, while EEs are formed by endocytosis of the plasma membrane. It appears, though, that in plant cells, EEs rapidly fuse with TGN vesicles (Dettmer et al., 2006); thus, plasma membrane-associated proteins can enter TGN/EE vesicles from both the Golgi-mediated secretory pathway and the endocytic pathway. Furthermore, specific isoforms of vacuolar ATPases that are localized to the TGN/EE are required for the proper function of both the endocytic and secretory pathways, indicating that the TGN/EE functions as a junction between these two pathways (Dettmer et al., 2006).

**Loss of KEG Function Does Not Block Endocytic Trafficking Pathways**

Ubiquitination plays an important role in controlling endocytosis and biogenesis of the endomembrane system in plants (Isono et al., 2010). Because KEG is an E3 ubiquitin ligase and is localized to the endomembrane system, we tested whether loss of KEG affected general endocytic trafficking pathways. To this end, the endocytic tracer dye FM4-64 was used to track the general endocytosis process from the plasma membrane to the central vacuole in root cells of both Columbia (Col-0) wild-type and keg-1 T-DNA insertion knockout lines. As shown in Figure 2, root cells of keg-1 mutants showed normal plasma membrane staining immediately after FM4-64 treatment (Fig. 2, A and E). No differences in FM4-64 staining between keg-1 and wild-type cells were observed at 20 min following FM4-64 application, by which time TGN/EE vesicles were visible (Fig. 2, B and F), at 100 min (Fig. 2, C and G; PVC/LE visible), or at 5 h (Fig. 2, D and H; central vacuole visible). The keg-1 mutant did not show any obvious morphological alterations in any endomembrane structures stained by FM4-64. Taken together, these data suggest that KEG does not control general endocytic trafficking routes from the plasma membrane to the central vacuole or the biogenesis of endomembrane vesicles.

**HERC2-Like Repeats Coordinate KEG Subcellular Targeting**

As described above, the keg-4 mutation, which is located in the HERC2-like repeat domain, suppresses the enhanced programmed cell death phenotype of edr1 mutant Arabidopsis (Wawrzynska et al., 2008). The finding that KEG-sYFP is primarily localized to the TGN/EE suggested that the enhanced programmed cell death phenotype of edr1 mutants may be related to endomembrane trafficking. Defects in endomembrane trafficking have previously been associated with triggering cell death in other systems (Surpin and Raikhel, 2004; Cheng and Lane, 2010). Therefore, we tested whether the edr1 mutation affected the localization of KEG. For this purpose, edr1 mutant protoplasts were transfected with KEG-sYFP and GmMan49-mCherry.
and their colocalization pattern was monitored. No change in KEG-sYFP localization was observed in the edr1 mutant background compared with wild-type protoplasts (Supplemental Fig. S2), indicating that loss of EDR1 does not disrupt the localization of KEG-sYFP or the formation of KEG-containing vesicles. To investigate whether the keg-4 mutation altered the normal subcellular localization of KEG-sYFP, we generated a keg-4-sYFP fusion and transiently expressed it in N. benthamiana. The vesicular signal of KEG-sYFP was reduced by the keg-4 mutation, whereas an increase in fluorescence signal was detected in both the cytosol and the endoplasmic reticulum (ER; Fig. 3, A–C). Wild-type KEG-sYFP was not detectable in the cytosol or ER under the same expression conditions (Fig. 3D).

The keg-4 mutation is located in the fifth HERC2-like repeat of KEG, changing a conserved Gly to Ser. This observation suggests that the HERC2-like repeats may regulate subcellular localization. To further test this hypothesis, a C-terminal deletion mutant lacking the entire HERC2-like repeat domain was made. This “RKA” construct retained the RING finger E3 ligase domain, the kinase domain, and the ankyrin repeat domain. Compared with KEG-sYFP, RKA-sYFP displayed much less localization to vesicles, instead localizing primarily to the cytoplasm and ER (Fig. 3, E–G). This change in localization was not due to the degradation of RKA-sYFP, as immunoblot analysis revealed RKA-sYFP to be intact (Fig. 3H). These results indicate that HERC2-like repeats play a central role in targeting KEG to the TGN/EE and that the keg-4 mutation partially abrogates this function.

Although deletion of the HERC2-like repeats substantially reduced the localization of KEG-sYFP to punctate structures, it did not entirely eliminate it (Fig. 3). To assess whether the remaining punctate structures represented TGN/EE vesicles, we coexpressed keg-4-sYFP and RKA-sYFP with wild-type KEG fused to mCherry (Supplemental Fig. S5). Both KEG derivatives colocalized with wild-type mCherry-KEG, indicating that the punctate structures corresponded to TGN/EE vesicles. Consistent with this conclusion, both keg-4-sYFP and RKA-sYFP showed a twin-spot pattern when coexpressed with the cis-Golgi marker GmMan-mCherry (Supplemental Fig. S5), as observed with wild-type KEG-sYFP (Fig. 1, D–F).

EDR1 Is Localized to the TGN/EE upon Coexpression with KEG

To better understand the molecular and cellular mechanisms behind the genetic interaction between EDR1 and KEG, we asked whether the subcellular localizations of EDR1 and KEG are interdependent. First, we transiently expressed a constitutive 35S promoter-driven EDR1-sYFP construct alone in N. benthamiana and observed a mostly cytosolic and ER distribution of the yellow fluorescent signal (Supplemental Movie S5). A similar localization pattern was also observed in Arabidopsis using protoplast transfection (Supplemental Fig. S4, A–D). Notably, neither system showed localization of EDR1 to TGN/EE-like vesicles, suggesting that EDR1 and KEG do not colocalize. However, when we transiently coexpressed EDR1-sYFP with KEG-mCherry in N. benthamiana, a major portion of EDR1-sYFP relocalized to KEG-containing vesicles (Fig. 4, A–C). To exclude that this localization change was an artifact caused by overexpression of KEG-mCherry, free sYFP was transiently coexpressed with KEG-mCherry in N. benthamiana. No translocation of free sYFP to KEG-containing vesicles was observed (Fig. 4, D–F), suggesting that KEG-induced relocalization to vesicles is specific to EDR1. Also, to demonstrate that the vesicle localization of EDR1-sYFP is specifically induced by KEG but not by other components involved in endocytic trafficking pathways, mCherry-SYP61 was coexpressed with EDR1-sYFP. No vesicular fluorescent signal was observed for EDR1-sYFP (Fig. 4, G–I). Lastly, to confirm that the EDR1-positive vesicles are TGN/EE vesicles, hemagglutinin (HA)-KEG, EDR1-sYFP, and mCherry-SYP61 were transiently coexpressed in N. benthamiana. EDR1-sYFP colocalized with mCherry-SYP61, showing that EDR1 localizes to TGN/EE vesicles when coexpressed with KEG (Fig. 4, J–L). We also observed that KEG-mCherry and EDR1-sYFP colocalized when coexpressed in...
Arabidopsis protoplasts (Fig. 5, A–E), indicating that this colocalization is not an artifact caused by expression in a heterologous system.

The HERC2-Like Repeats Are Essential for Recruitment of EDR1 to the TGN/EE

As demonstrated above, deletion of the HERC2-like repeats dramatically reduced KEG’s association with the TGN/EE but did not totally abolish it. To test whether the remaining TGN/EE-associated RKA protein can still mediate the relocalization of EDR1, we transiently coexpressed EDR1-sYFP and RKA-mCherry in Arabidopsis protoplasts. Deletion of the HERC2-like repeats abolished EDR1 localization to the TGN/EE (Fig. 5, F–J), indicating that the HERC2-like repeats are required for this process. However, the keg-4 mutation did not block EDR1-sYFP relocalization (Fig. 5, K–O), suggesting that the keg-4 mutation does not completely eliminate the function of the HERC2-like repeats. The HERC2-like repeats by themselves fused with mCherry did not display a clear vesicular localization pattern (data not shown), suggesting that targeting of KEG to the TGN/EE requires both the RKA domain and the HERC2-like repeats.

EDR1 Physically Interacts with KEG

The KEG-dependent recruitment of EDR1 to TGN/EE vesicles suggested that EDR1 and KEG might physically interact. We used yeast two-hybrid analysis to test this hypothesis. No direct interaction between wild-type EDR1 and KEG was detected (Fig. 6A). However, the interaction between a kinase and its substrate can sometimes be too transient to detect in yeast two-hybrid assays. In order to slow the process of EDR1 dissociation from its substrate, a “substrate trap” version of EDR1 (stEDR1) was generated. The kinase domain of EDR1 was mutated (D810A) to disrupt its phosphotransfer domain, which is necessary for substrate phosphorylation, thus stabilizing the potential interaction between EDR1 and its substrates (Gibbs and Zoller, 1991). Significantly, stEDR1 interacted with full-length KEG (Fig. 6A), suggesting that KEG may be a substrate of EDR1. The substrate trap mutation in stEDR1 did not affect its endosomal localization upon coexpression with KEG (Supplemental Fig. S3). Consistent with the finding that the HERC2-like repeats are required for EDR1 localization to the TGN/EE, deletion of the HERC2-like repeats abrogated stEDR1:KEG interaction in yeast (Fig. 6A), supporting the conclusion that the KEG-dependent EDR1 localization to the TGN/EE was based on a direct interaction between EDR1 and KEG mediated by the HERC2-like repeats.

To confirm the interaction between EDR1 and KEG in planta, we performed coimmunoprecipitation analyses using transient expression in N. benthamiana. Consistent with the yeast two-hybrid analysis, stEDR1-HA coimmunoprecipitated with KEG-Myc (Fig. 6B), demonstrat-
ing that the stEDR1:KEG interaction occurs in planta. In contrast to the yeast two-hybrid results, wild-type EDR1-HA also coimmunoprecipitated with KEG-Myc, although this interaction appeared to be slightly weaker than with stEDR1. Coimmunoprecipitation of RKA-Myc with either EDR1 or stEDR1 was not detectable, indicating that the HERC2-like repeats are required for EDR1:KEG interaction (Fig. 6B). To test whether immunoprecipitation of stEDR1-HA resulted in nonspecific precipitation of other TGN/EE-localized proteins, we included Myc-SYP61 as an additional control. Myc-SYP61 did not coimmunoprecipitate with stEDR1-HA, indicating that the EDR1:KEG interaction is specific and not the result of bulk precipitation of TGN/EE vesicles (Fig. 6B).

The HERC2-Like Repeats Mediate KEG:KEG Interactions

Because KEG appears to be endomembrane associated and many membrane-associated proteins are known to oligomerize (Engelman, 2005), we asked whether KEG interacts with itself and, if so, which domain is necessary and/or sufficient. Yeast two-hybrid analysis showed that full-length KEG can interact with itself (Fig. 7A). To confirm that KEG:KEG interaction occurs in planta, we performed coimmunoprecipitation analyses using transient expression in N. benthamiana. A full-length KEG-Myc construct was transiently expressed with KEG-sYFP, RKA-sYFP, or HERC2-sYFP. RPS5, a plant disease resistance protein, served as a negative control. Consistent with the yeast two-hybrid result, KEG-sYFP coimmunoprecipitated with KEG-Myc (Fig. 7B, left panel), demonstrating that KEG:KEG interaction occurs in planta. Coimmunoprecipitation of RKA-sYFP with full-length KEG-Myc was much weaker, whereas HERC2-sYFP coimmunoprecipitated strongly with full-length KEG-Myc (Fig. 7B, left panel). None of these three constructs coimmunoprecipitated with RPS5-Myc, demonstrating that the KEG:KEG interaction was specific (Fig. 7B, right panel).
Taken together, these data suggest that KEG can at least dimerize in vivo and that the HERC2-like repeats are required for this interaction.

**DISCUSSION**

Loss-of-function mutations in the *EDR1* gene confer enhanced resistance to powdery mildew infection, enhanced ethylene-induced senescence, and enhanced sensitivity to drought (Frye and Innes, 1998; Frye et al., 2001; Tang et al., 2005). All *edr1*-mediated phenotypes can be suppressed by the *keg-4* missense mutation (Wawrzynska et al., 2008). Because KEG is an E3 ubiquitin ligase, it suggests that KEG may function to regulate protein levels of specific substrates via ubiquitination followed by proteasome-mediated degradation. Indeed, Stone et al. (2006) have shown that KEG likely regulates the level of the ABI5 transcription factor, as steady-state levels of ABI5 increase in loss-of-function *keg-1* mutant seedlings. However, regulation of ABI5 levels cannot be the only function of KEG, as loss-of-function mutations in ABI5 only partially suppress *keg-1* mutant phenotypes (Stone et al., 2006).

Furthermore, transgenic plants expressing ABI5 protein at levels higher than that observed in *keg-1* seedlings do not undergo postgerminative growth arrest like that observed in *keg-1* mutants (Brocard et al., 2002; López-Molina et al., 2003). To gain more insight into the cellular processes controlled by KEG and the possible roles KEG plays in EDR1 signaling, we performed subcellular colocalization studies.

Given KEG’s putative role in regulating ABI5 levels through direct ubiquitination, we expected KEG to localize to the nucleus. To our surprise, KEG-sYFP was predominantly detected in intracellular vesicular structures that undergo dynamic association with and dissociation from Golgi stacks (Fig. 1; Supplemental Movie S1). These vesicles were subsequently identified as TGN/EE localization of KEG. However, a T-DNA insertion knockout of *KEG* did not disrupt general endocytic trafficking routes or the general morphology of endomembrane structures stained by the endocytic tracer dye FM4-64 (Fig. 2), suggesting...
that KEG may instead regulate other processes, such as the formation of signaling complexes on TGN/EE vesicles (discussed further below).

Targeting of KEG to the TGN/EE appears necessary for normal KEG function, as the $\text{keg-4}$ mutation dramatically reduced targeting of KEG to the TGN/EE while increasing the fraction of KEG associated with the ER and cytosol (Fig. 3). Deletion of the entire HERC2-like repeat domain, where the $\text{keg-4}$ mutation resides, resulted in even greater mislocalization of KEG (Fig. 3). Taken together, these data indicate that the HERC2-like repeats facilitate KEG endosomal targeting and suggest that KEG mislocalization may be causally related to the ability of $\text{keg-4}$ to suppress $\text{edr1}$ mutant phenotypes. KEG does not contain any obvious membrane-targeting motifs (e.g. palmitoylation or isoprenylation motifs); thus, we speculate that KEG is targeted to the TGN/EE at least in part by protein:protein interactions mediated by the HERC2-like repeats.

Full-length KEG does not appear to localize to the nucleus when KEG is overexpressed in either $\text{N. benthamiana}$ or Arabidopsis protoplasts (Fig. 3). This observation raises the question of how KEG may be regulating the levels of $\text{ABI5}$, a bZIP transcription factor, which is localized to the nucleus (López-Molina et al., 2003).

The plasma membrane has traditionally been viewed as the site of receptor-mediated signal transduction. However, recent work in animals and plants has revealed that signaling complexes often form after endocytosis of receptor-ligand complexes and that endocytosis is often required for proper signaling (Murphy et al., 2009). Activated receptors accumulate in TGN/EE vesicles, and certain signaling components exclusively reside in or undergo recruitment to the TGN/EE. Given that EDR1 and KEG both accumulate in the TGN/EE but do not appear to be required for endosome trafficking, we speculate that these proteins may function in regulating signaling by endosome-localized complexes. In the case of KEG, this could occur via ubiquitination of specific signaling complexes. E3 ligase-mediated ubiquitination has previously been shown to regulate endosomal signaling in animal cells (Burger et al., 2006). Ligand-induced endocytosis of the human EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) is one of the best-characterized examples of this. Upon ligand binding, internalization of EGFR is mediated by a RING finger E3 ligase, Casitas B lymphoma protein (Cbl), which binds to activated EGFR and ubiquitinates it, which in turn triggers EGFR endocytosis (de Melker et al., 2001; Haglund et al., 2003). The internalized EGFR associates with downstream signaling proteins that dock with the EEs. This protein complex then recruits mitogen-activated protein kinase cascade components (Di Guglielmo et al., 1994; Le Roy and Wrana, 2005). The ubiquitinated EGFR is then sorted toward multivesicular bodies for degradation to terminate signaling (Katzmann et al., 2002) or can be deubiquitinated by the deubiquitinating enzyme AMSH (for associated molecule with the SH3 domain of STAM) to be recycled back to the plasma membrane (Millard and Wood, 2006). EGFR levels in EEs (and, hence, signaling) are thus regulated in part by the counterbalancing ubiquitinating and deubiquitinating activities of Cbl and AMSH. The levels of Cbl and AMSH are themselves regulated by other E3 ubiquitin ligases (HECT E3 ligase, Ned4, RING finger E3 ligase, and RING FINGER PROTEIN11), which constitute a secondary regulatory framework for EGFR endoso-

**Figure 6.** KEG physically interacts with EDR1. A, Pairwise yeast two-hybrid assays between the indicated KEG and EDR1 constructs. B, Coimmunoprecipitation of KEG with EDR1. Wild-type EDR1-HA or the substrate trap form of EDR1-HA was transiently coexpressed with KEG-Myc, RKA-Myc, sYFP-Myc, or Myc-SYP61 in $\text{N. benthamiana}$. Proteins were immunoprecipitated with anti-HA affinity matrix (IP). A portion of each sample was taken prior to immunoprecipitation as the input control. Immunoblotting with the indicated antibodies was used to detect the epitope-tagged proteins.
mal signaling (Magnifico et al., 2003; Li and Seth, 2004). Therefore, ubiquitin E3 ligases play regulatory roles along the entire endosomal signaling process, including initiation, sustainment, and termination.

While receptor-based endosomal signaling in animals is well documented, the role of endocytosis in signaling in plants is poorly understood (Robatzek, 2007; Geldner and Robatzek, 2008). In Arabidopsis, the brassinosteroid receptor BRI1 localizes to both the plasma membrane and endosomes. The endosome-localized fraction can be increased by the addition of brefeldin A, which inhibits endosome trafficking from the Golgi to the plasma membrane and PVC. (Geldner et al., 2007). Significantly, brefeldin A application enhances brassinolide sensitivity in Arabidopsis seedlings, suggesting that the endosome-localized BRI1 protein is the functionally active form. In contrast to BRI1, endocytosis of the flagellin receptor FLS2 from Arabidopsis appears to terminate ligand-dependent signaling, as blockage of FLS2 internalization using cantharidin enhances ligand (flg22)-induced accumulation of reactive oxygen species (Serrano et al., 2007). However, FLS2 may also activate signaling pathways independent of reactive oxygen species, and these may be triggered from endosomes. For example, wortmanin, which impairs FLS2 trafficking, diminishes the activation of mitogen-activated protein kinases by flg22 while not affecting the oxidative burst (Chinchilla et al., 2007). As in animals, ligand-induced endocytosis of receptors in plants appears to be regulated in part by ubiquitination. For example, ligand-induced endocytosis of FLS2 in Arabidopsis is inhibited by depleting the levels of free ubiquitin moieties (Melikova et al., 2006; Robatzek et al., 2006), while in rice (Oryza sativa), the RING finger E3 ligase Xa21 binding protein 3 regulates levels of the receptor-like kinase Xa21 and Xa21-mediated immune responses (Wang et al., 2006).

As a RING E3 ligase associated with the TGN/EE, KEG could function in plant receptor endosomal signaling through mediating trafficking of receptor complexes, or degradation of other regulatory components, to fine-tune receptor signaling output. The physical association of EDR1 with KEG also implicates EDR1 in these processes. It is thus plausible that the enhanced PCD observed in edr1 mutants is caused by uncontrolled receptor signaling following activation, leading to elevated SA levels and overexpression of defense genes, as observed in the edr1 mutant (Christiansen et al., 2010). Strict control of the magnitude and timing of signal activation during endosomal signaling is critical for the regulation of cell survival and proliferation in animal systems. For instance, interfering with homeostasis by disrupting receptor turnover, and thus signal termination, is a key molecular pathology underlying many cancers (Le Roy and Wrana, 2005). Based on the genetic relation between EDR1 and KEG, we speculate that KEG may play a role in promoting stress-activated receptor signals. Furthermore, our data suggest that KEG might be controlled directly by EDR1 kinase activity and serve as a substrate of EDR1 (Fig. 6). Depending on the phosphorylation site on KEG, this modification could inhibit the E3 ligase activity of KEG and/or inhibit its association with specific substrates. Loss of EDR1 function would then lead to increased KEG-mediated activation of stress responses. Under this model, the keg-4 mutation would then reduce KEG activity, possibly due to mislocalization.

The keg-4 mutation causes a Gly-to-Ser substitution in the HERC2-like repeat domain of KEG, making the role of this domain in EDR1-KEG signal transduction of particular interest. The HERC2-like repeat domain is not found in any other known Arabidopsis protein but is conserved in KEG orthologs in other plant species (Stone et al., 2006). Each HERC2-like repeat is
defined as a 61-amino acid stretch conferring similarity to an interdomain region of the mammalian HERC2 protein, a HECT-type E3 ligase (Garcia-Gonzalo and Rosa, 2005; Stone et al., 2006). The structure of 12 HERC2-like repeats combined in a single protein is unique to KEG and its orthologs in plants. Here, we determined that the HERC2-like repeats are required for KEG-dependent recruitment of EDR1 to the TGN/EE and for physical association of KEG with EDR1 (Figs. 5 and 6), suggesting that the HERC2-like repeats mediate EDR1 association with KEG. In addition, we showed that the HERC2-like repeats also contribute to KEG:KEG interactions (Fig. 7). Taken together, these data demonstrate that the HERC2-like repeats mediate protein:protein interactions that are necessary for targeting of KEG to the TGN/EE and for interaction with EDR1. It will be of interest to determine whether the HERC2-like repeats regulate the E3 ligase activity of KEG and/or interaction with substrates.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (Arabidopsis thaliana) seeds were surface sterilized with 50% (v/v) bleach and 0.1% Triton X-100. Two days after stratification at 4°C, seeds were germinated and grown on half-strength Murashige and Skoog medium containing 0.8% agar and 1% Suc under a photoperiod of 9 h of light and 15 h of dark at 23°C. Seven-day-old seedlings were transferred from half-strength Murashige and Skoog plates to MetroMix 360 soil (Sun Gro Horticulture). Nicotiana benthamiana plants used for transient protein expression were grown under the same conditions, except that seeds were planted directly into MetroMix 360.

Plasmid Construction and Site-Directed Mutagenesis

Fusion of epitope tags and fluorescent proteins to KEG, EDR1, and various endomembrane marker proteins was accomplished using a multisite Gateway cloning strategy (Invitrogen) as follows. The full-length KEG open reading frame (At5g1330; Stone et al., 2006) was PCR amplified from a cDNA template and recombined into the multisite Gateway entry vectors pBSDONR P1-P4 (an ampicillin-resistant derivative of pDONR221 P1-P4) and pBSDONR P1r-P2 (an ampicillin-resistant derivative of pDONR221 P1r-P2) in which the attB3 sequence was replaced with attP2 by using Gateway BP Clonase II (Invitrogen). Similar “P1-P4” constructs were made for the RKA and HERC2-like repeats region of KEG, which span bases 1 to 2,547 and 2,548 to 4,875 of the KEG open reading frame, as well as for the full-length EDR1 open reading frame (At1g06720). “P4r-P2” constructs were made for full-length open reading frame clones of SYFP (At5g1330) and SYFP1 (At1g29490). Both P1-P4 and P4r-P2 constructs were made for SYFP (Kremers et al., 2006), mCherry, 3× epitope (HA), and a 5× c-Myc epitope. The keg-4 nonsense mutation (G1144S) was introduced into KEG full-length clones using the QuickChange site-directed mutagenesis kit (Stratagene). The substrate trap mutation in EDR1 (D810A) was also created using the QuickChange kit. To fuse proteins of interest with C-terminal epitope tags or fluorescent proteins, a P1-P4 clone (e.g. KEG) was mixed with a P4r-P2 tag (e.g. SYFP) and the desired destination vector containing attR1 and attR2 sites (e.g. pEarlyGate100; Earley et al., 2006) and recombined using Gateway LR Clonase II (Invitrogen). To construct N-terminal fusions, a P4r-P2 clone of the protein of interest was mixed with a P1-P4 clone of the tag and the desired destination vector. All clones were verified for correct construction using DNA sequencing. The primers used to create the above constructs are listed in Supplemental Table S1.

For stable transformation of N. benthamiana, the above pBSDONR constructs were recombined with the destination vector pEARLYGATE100 (Earley et al., 2006), which drives the expression of transgenes using a cauliflower mosaic virus 35S promoter, or with a modified version of the vector pMDC32 (Qi and Katagiri, 2009). To produce the latter vector, the 2x35S promoter of pMDC32 was replaced with the KEG native promoter (930 bp of DNA upstream of the KEG start codon) using Kpol and HindIII restriction sites flanking the 2x35S promoter region.

For transient transformation of N. benthamiana leaves and Arabidopsis protoplasts, the above pBSDONR constructs were recombined with the pTA7002-GW destination vector (Aoyama and Chua, 1997; McNellis et al., 1998) to generate dexamethasone-inducible protein constructs.

We also obtained previously constructed protein fusions for ER-localized mCherry and cis-Golgi-localized mcCherry from the Arabidopsis Biological Resource Center (stocks CD3-960 and CD3-968). The ST-RFP construct (trans-Golgi localized; Wee et al., 1998) was obtained from Dr. Jeanmarie Verchot-Lubicz at Oklahoma State University.

Arabidopsis Transformation

Plasmids were transformed into Agrobacterium tumefaciens strain GV3101 (pMP90) by electroporation with selection on Luria-Bertani plates containing 50 μg mL⁻¹ kanamycin sulfate (Sigma) and 20 μg mL⁻¹ gentamycin (Gibco). Arabidopsis plants were transformed using the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected either by growing on half-strength Murashige and Skoog medium with 0.8% agar and 30 μg mL⁻¹ hygromycin B (Sigma) or by spraying 1-week-old seedlings grown in MetroMix 360 with 300 μM BASTA (Finale) three times at 2-d intervals. Transformants selected on plates were transformed to pots and allowed to set seed. For complementation of the keg-1 mutant, heterozygous T-DNA insertion mutants (SALK_049542) were used for dipping, and homozygous insertion lines were confirmed by PCR in the T1 generation. Primers used for genotyping are listed in Supplemental Table S1.

Yeast Two-Hybrid Analyses

The full-length open reading frames of EDR1, EDR1 (D810A), and KEG were cloned into the DNA-binding domain vector pDEST32 (Invitrogen ProQuest two-hybrid system) and subsequently transformed into yeast strain AH109 by electroporation and selected on synthetic dextrose (SD)-Leu medium. The full-length open reading frame of KEG as well as the RKA and HERC2-like repeat domains were cloned into the activation domain vector pDEST22 and subsequently transformed into yeast strain Y187 by electroporation and selected on SD-Trp medium. Mating between the AH109 and Y187 strains carrying the relevant constructs was then performed in 2× yeast peptone dextrose A plus 0.003% adenine at 30°C for 20 h. Mating cultures were then diluted and plated on SD-.Tryp-Leu and SD-.Tryp-Leu-His.

Transient Protein Expression in N. benthamiana

Agrobacterium GV3101 (pMP90) strains transformed with the dexamethasone-inducible constructs described above were grown and prepared for transient expression as described previously (Wroblewski et al., 2005). Agrobacterium cultures were resuspended in water at an optical density at 600 nm of 0.5. For coexpression of multiple constructs, suspensions were mixed in equal ratios. Bacterial suspension mixtures were infiltrated using a needleless syringe into expanding leaves of 4-week-old N. benthamiana plants. Protein expression was induced by spraying the leaves with 50 μM dexamethasone (Sigma) 40 h after injection. Samples were collected for either protein extraction or microscopic imaging 24 h after hormone application.

Immunoprecipitations and Immunoblots

For total protein extraction, four leaves of infiltrated N. benthamiana were collected and ground in lysis buffer (50 μl Tris, pH 7.5, 150 μl NaCl, 0.1% Nonidet P40, and Plant Protein and Inhibitor Cocktail (Sigma)). Samples were centrifuged at 10,000 rpm at 4°C for 5 min, and supernatants were transferred to new tubes. Total proteins were mixed with 4× SDS loading buffer at a ratio of 3:1 and boiled for 5 min before loading. Immunoprecipitations were performed as described previously (Shao et al., 2003) using c-Myc Monoclonal Antibody-Agarose Beads (Clontech) or Anti-HA Affinity Matrix (Roche). The immunocomplexes were resuspended in 50 μl of 1× SDS loading buffer and boiled for 5 min. Total proteins and immunocomplexes were separated by electrophoresis on a 4% to 20% gradient Tris-HEPES-SDS polyacrylamide gel (Thermo Scientific). Proteins from duplicate gels and filters were transferred to a nitrocellulose membrane and probed with anti-c-Myc-peroxidase (Roche), anti-GFP-peroxidase (Thermo Scientific), or anti-HA-peroxidase (Sigma).
Isolation and Transient Transfection of Arabidopsis Protoplasts

The isolation and transient transfection of leaf mesophyll cell protoplasts from Arabidopsis plants (3 weeks old) was performed at room temperature following published procedures (Sheen et al., 1995). A total of 20 μg of plasmid DNA was used for each transfection experiment (plasmids were mixed in an equal ratio for cotransfections). For dexamethasone-inducible constructs, hormone was added immediately after transfection. Protoplasts were mixed in an equal ratio for cotransfections). For dexamethasone-inducible plasmid DNA was used for each transfection experiment (plasmids were mixed in an equal ratio for cotransfections). For dexamethasone-inducible constructs, hormone was added immediately after transfection.

Fluorescence Microscopy

To image fluorescent protein fusions in live cells, confocal laser scanning microscopy was performed using a Leica SP5 AOBS inverted confocal microscope (Leica Microsystems) equipped with a 63×, numerical aperture 1.2 water objective. syYFP (excited by the 514-nm argon laser) fluorescence was detected using a custom 522- to 545-nm band-pass emission filter, whereas mCherry fluorescence (excited using the 561-nm helium-neon laser) was detected using a custom 595- to 620-nm band-pass emission filter. To obtain three-dimensional images, a series of Z-stack images were collected and then combined and processed using the three-dimensional image-analysis software IMARIS 7.0 (Bitplane Scientific Software; http://www.bitplane.com).

To test whether vesicle movement was dependent on actin fibers, actin was depolymerized by infiltrating leaves with 25 μM latrunculin B (Calbiochem; stock solution, 10 mM in dimethyl sulfoxide) 1 h prior to imaging. To stain membranes with FM4-64 dye (Invitrogen), whole Arabidopsis seedlings were incubated in 1 μM FM4-64 for 2 min, transferred to liquid half-strength Murashige and Skoog medium, and incubated in the dark at room temperature for the indicated times.

Sequence data for the genes described in this study can be found in the GenBank/EMBL data libraries following the above accession numbers: KEG (At5g13530), EDR1 (At1g08720), SYP21 (At5g16830), and SYP61 (At1g28490).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Subcellular localization of native promoter-driven KEG-sYFP in Arabidopsis protoplasts.

Supplemental Figure S2. Localization of KEG-sYFP in Col-0 wild-type and edr1 mutant protoplasts.

Supplemental Figure S3. mdr1 colocalizes with KEG.

Supplemental Figure S4. Subcellular localization of 3SS:EDR1-sYFP in Arabidopsis protoplasts.

Supplemental Figure S5. Mutations in the HERC2-like repeats do not change the identity of KEG vesicular structures.

Supplemental Table S1. Primers used for this work.

Supplemental Movie S1. Time-lapse movie showing that KEG-sYFP-containing vesicles move independently from GmMan9-mCherry-labeled Golgi.

Supplemental Movie S2. Time-lapse movie showing colocalization of KEG-sYFP with mCherry-SYP61 during vesicle movement.

Supplemental Movie S3. Time-lapse movie showing independent movement of KEG-sYFP-containing vesicles and mCherry-SYP21-containing vesicles.

Supplemental Movie S4. Time-lapse movie showing that latrunculin B application inhibits the movement of KEG-sYFP-containing vesicles.

Supplemental Movie S5. Three-dimensional image of EDR1-sYFP localization in an N. benthamiana epidermal cell.

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