RESEARCH COMMUNICATION

Endosomal signaling of plant steroid receptor kinase BRI1

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The LRR receptor serine/threonine kinases are a major eukaryotic receptor family, for which the central regulatory mechanism of endosomal trafficking remains largely unaddressed. We show that the steroid receptor BRI1 localizes to both plasma membrane and early endosomal compartments, even when observed at low, endogenous expression levels, and that its localization and turnover are independent of ligand. However, increasing endosomal localization of BRI1 enhances activation of the pathway and genomic responses. Our data indicate distinct signaling and trafficking mechanisms within this receptor class and show that the use of endosomes as signaling compartments is an unexpectedly broad phenomenon in eukaryotes.

Receptor endocytosis has long been viewed as a mechanism to inactivate receptors and down-regulate signaling. This concept was challenged more than a decade ago, initially based on the observation that many activated receptors show considerable accumulation in endosomes [Baass et al. 1995]. It has now become apparent that key signaling components are localized exclusively to endosomes and that endocytosis is required to bring them into contact with their activated receptors, thereby allowing signaling to take place [Vieira et al. 1996; Wunderlich et al. 2001; Panopoulou et al. 2002]. Such a requirement for endosomal localization appears to be widespread in metazoans, as it has been observed in diverse receptor families, such as RTKs, TGF-β-Rs, and GPCRs [DeFea et al. 2000; Wunderlich et al. 2001; Panopoulou et al. 2002; Shenoy et al. 2006]. On the other hand, endocytosis of the Ste2p pheromone receptor in yeast appears only necessary for termination of the signal, but not for its transduction per se [Hicke et al. 1998]. Thus, it is unclear whether endosomes as signaling platforms are specific to animal cells or whether they repre-
It is possible that root meristem cells have close to maximal levels of activated receptors, precluding the detection of ligand-dependent localization changes when adding exogenous BL. Therefore, we depleted roots of endogenous BRs by growing them for 3 d on 5 µM brassinazole, a specific inhibitor of BR biosynthesis [Sekimata et al. 2001]. To our surprise, neither endosomal localization nor levels of BRI1-GFP were affected by this treatment [Fig. 2B, left]. Further, the levels and localization of BRI1-GFP remained unaltered even when high amounts of BL were added back to these ligand-devoid roots, which should induce a rapid shift toward completely ligand-occupied receptors [Fig. 2B, Supplementary Movie 3]. We conclude that BRI1 localizes to endosomes independently of its activation state.

Activation of signaling pathways can lead to a relevant increase in protein flux between compartments, without necessarily shifting their steady-state accumulation in one or the other direction [Ando et al. 2004]. In the case of BRI1, an increase in endocytosis and degradation could be offset by increased synthesis and secretion, without causing observable changes in overall localization or levels of BRI1-GFP. Therefore, we decided to directly measure BRI1 turnover rates in planta. To do so, we placed BRI1-YFP under the control of a heat-shock
promoter, generating lines that allow pulsed expression of the tagged BRI1 by a 20–30-min induction at 37°C (Supplementary Movie 4). In combination with quantitative confocal microscopy, this allowed for a pulse-chase analysis exclusively in intact root meristems [Fig. 2C; Supplementary Fig. 2; Supplementary Movie 5]. We determined the half-life of BRI1-YFP to be ~5 h. This relative stability suggests that BRI1 either recycles during its lifetime or that only a minor fraction of BRI1 engages in endocytic transport to the vacuole with a majority remaining immobile. Again, the measured degradation rate of BRI1 was unaffected by addition of BL either to untreated or ligand-depleted roots [Fig. 2C]. Based on these results, it is unlikely that BRs cause any difference in BRI1 intracellular transport, and we conclude that BRI1 trafficking is constitutive.

The activity status of BRI1 cannot be monitored directly at subcellular resolution. However, in untreated root cells a significant amount of plasma-membrane-localized BRI1 is apparently in an inactive state, as judged by the presence of the inhibitory BKI1 protein, which is released by BRI1-dependent phosphorylation after BR stimulation [Wang and Chory 2006]. We did not observe any BKI1 accumulation in endosomal structures, suggesting that the endosomal BRI1 may represent an active receptor pool [Fig. 2D]. To test this idea, we attempted to specifically manipulate the subcellular localization of BRI1 and to monitor the consequences on signaling activity. A number of known chemical inhibitors of trafficking were tested [data not shown], but only Brefeldin A (BFA) turned out to be sufficiently specific for our purpose. BFA is a widely used inhibitor of endosomal trafficking in plants [Geldner 2004]. BFA had the expected effects on BRI1 localization, although we observed a fairly strong persistence of BRI1 signals at the plasma membrane. Nonetheless, we quantified plasma membrane versus intracellular signal in time-lapse images and showed that BFA induced a shift of BRI1 localization into endosomal compartments [Fig. 3A,B; Supplementary Movie 6]. We also showed by pulse-chase analysis that BFA blocks translocation of BRI1 from earlier to late endosomal compartments and vacuoles, thereby interfering with BRI1-YFP degradation [Fig. 3C,D]. This block in vacuolar transport is visualized, because BFA-treated cells did not accumulate residual YFP signals in vacuoles some hours after the expression pulse [Fig. 3D]. Again, we did not observe any BKI1 accumulation in these BFA-induced endosomal aggregates, suggesting that the BRI1 therein could be part of active receptor complexes [Fig. 3E]. For summary cartoon of BFA effects on BRI1 trafficking, see Supplementary Figure 3.

We then investigated if increasing BRI1 endosomal accumulation could affect signaling activity. We first used a highly BR-sensitive cell suspension culture that shows BR-induced dephosphorylation of the BES1 transcription factor when treated with scant amounts of BL (1 nM vs. 10 nM in intact seedlings). Dephosphorylation of BES1 and its close family member, BZR1, leads to reduced expression of the BR early-response gene DWF4 [Fig. 4A]. Remarkably, BFA on its own was able to induce strong dephosphorylation of BES1 and to cause suppression of downstream genes over a similar time course and to a similar extent as exogenously applied BL [Fig. 4A]. Co-treatment of BFA together with exogenous BL further enhanced activation of the pathway [Fig. 4A]. The cell culture results were corroborated using intact seedlings overexpressing BRI1-GFP. We observed a small, but consistent, enhancement of BR signaling when seedlings exposed to low amounts of BL were cotreated with BFA [Fig. 4B].

The fairly mild enhancements of BR signaling in intact seedlings compared with cell cultures could be explained by problems of uptake and activity of BFA into the different BR-responsive organ and tissue types contained in whole seedlings. We therefore attempted to establish a more homogenous system where the same tissues could be simultaneously analyzed for the effect of BFA on BRI1 signaling and localization. We generated a bri1-null mutant line that contained heat-shock-inducible BRI1-YFP as the sole source of BRI1 activity. Since bri1-null mutants cannot be propagated as homozygous individuals, we established a cultured root system, where large amounts of root meristems were continuously formed from pre-existing roots. We then induced the expression of BRI1, which consequently led to induction of BRI1 signaling [Fig. 5]. BFA had the expected effect on BRI1 localization [Fig. 5, top panel], without increasing the actual amount of BRI1 over the time scale of the treatment [Fig. 5, middle panel]. Nonetheless, BFA was able to enhance BRI1-dependent signaling [Fig. 5, bottom panel] again suggesting that BRI1 signals preferentially from endosomes.

Our findings highlight the relevance of subcellular compartmentation of signaling components for the regu-
loration of plant receptor kinase pathways. We show that the endosomal and plasma membrane pool of BRI1 represent two distinct subpopulations, since it is only the latter that is complexed with an inhibitory scaffold protein. This subcellular partitioning appears to be functionally relevant, since increasing the ratio of endosomal to plasma membrane-localized BRI1 significantly enhances signaling activity. One prediction from our findings would be that the as yet unidentified direct downstream targets of the activated receptor complex may localize preferentially to endosomal compartments [Supplementary Fig. 4]. This is not unlike the situation for the unrelated TGF-β receptor in animals, which also has been shown to traffic in a ligand-independent fashion, but nonetheless transduces signals from endosomes [Panopoulou et al. 2002]. However, this contrasts to the recently demonstrated ligand-dependent trafficking of the related LRR-receptor kinase FLS2 [Robatzek et al. 2006].

Together, this now provides us with two plant receptor models that display opposite trafficking behavior. Interestingly, this difference in trafficking matches an underlying difference in the biology of these two receptors. Whereas FLS2 is a pathogen receptor evolved for rare but acute signaling, BRI1 is thought to perceive a more or less continuous signal, whose relevance lies in its modulation more than in acute presence or absence.

A number of interesting speculations have been put forward to explain why endosomal signaling might have evolved in animals. It was proposed that signaling from numerous motile endosomes might overcome problems of diffusion-based signaling cascades in large cells and that timing and specificity of signaling events can be better controlled in endosomes [Miaczynska et al. 2004]. Our data now indicate that endosomes in multicellular plants also act as signaling compartments, in spite of their independent sets of receptors and a differently organized endosomal system. We propose that a common, fundamental problem might have independently driven the development of signaling endosomes. Both multicellular plants and animals show vast increases of surface-localized receptor families. In such a situation, the available plasma membrane surface could become a limiting factor and trafficking of activated receptors to endosomes a means to increase the effective surface area available for signaling. This would restrict the dwelling time of receptors at the plasma membrane to ligand binding and activation, while compartmentalizing the longer-lasting downstream signaling events to the much less restricted inner surfaces of endosomal compartments.

Materials and methods

Plant material and growth conditions

Arabidopsis Columbia seedlings were grown vertically for 4–6 d with 24 h light at 22°C on 0.5× LS [Linsmayer-Skoog plant growth mixture] agar plates. Green cell suspension cultures were originally obtained from Ruishuang Geng (Ohio State University, Columbus, OH) and grown as described by Kim et al. [2003]. Suspension-grown root meristem cultures were obtained according to an online protocol at http://www.bio.net/bionet/mrn/arab-gen/1992/September/000708.html.

Constructs and generation of transgenic lines

A BRI1-GFP construct was cloned into a pGREEN II vector derivative containing nos-promoter-driven Basta resistance. It contains 1689 base

Figure 4. BRI1 signaling is induced by BFA treatment. [A] A highly sensitive Arabidopsis green cell suspension line shows dephosphorylation of BES1 [green, above] and suppression of early-response gene, DWF4 [green, below] already at 1 nM BL for 30 min. [Middle] BFA treatment alone leads to an efficient activation of BES1 [red] and suppression early-response genes. External BL enhances the BFA effect on BES1 [yellow, above] and DWF4 [yellow, below]. [B] Enhancement of BR signaling by BFA can also be observed in BRI1-overexpressing seedlings, when treating with low amounts of BL [10 nM] for 4 h, which leads to only half-maximal dephosphorylation of BES1 [above] and a twofold reduction in DWF4 levels [green, below]. Adding BFA [100 µM] after 30 min of BL pretreatment significantly enhances BES1 activation [above] and DWF4 suppression [red, below].

Figure 5. Induced BRI1-YFP expression in cultured roots. [A] BFA effects can also be observed in intact organs using a bri1-null mutant root culture containing heat-shock-inducible BRI1-YFP. In this system, the effects of BFA on localization and signaling can be simultaneously assessed. Top panel shows BRI-YFP signals, 8 h after a 30-min heat-shock or control treatment. BFA treatment [100 µM] started 4 h after heat shock. Middle panel shows BRI1-YFP levels as detected on immunoblot with α-BRI1 after same treatments as above. Bottom panel shows heat-shock-induced suppression of DWF4 [gray] and its further reduction by BFA treatment [red]. Bar, 10 µm.
pairs (bp) upstream of the BRI1 translational start codon and 629 bp downstream from the stop codon. The EGFP sequence was inserted at the C terminus as described by Friedrichsen et al. (2000). The contract was transformed into wild type, and a line with close to identical expression levels was selected. This line therefore still contains twice the amount of overall BRI1 product (endogenous BRI1 plus BRI1-GFP). The promoter of the HS–BRI1-YFP construct was described by Knox et al. (2003). YFP was fused to the C terminus as above.

Confocal microscopy and image quantification

Confocal microscopy was done with a Leica SP/2 inverted microscope. Live imaging of roots was done by placing root tips on an agar block that fit into silicon chambers with a coverslip bottom [Grace Biologs]. Specimens were either sealed with a slide (long-term observation) or overlaid with sufficient liquid media [short-term observations]. Image analysis was done with the Leica SP/2 software package and the ImageJ bundle provided by the Wright Cell Imaging facility.

Hormone and inhibitor treatments

BL was obtained from CIDTech Research, Inc., and Breidelin A was obtained from Sigma. For long-term treatments, hormones or drugs were dissolved into agar blocks onto which roots were placed [seen above]. Short-term observations were done by mixing the hormone/drug into a liquid drop overlaying the agar-embedded root tips [see above] while continuously scanning.

Heat-shock pulse-chase analysis

For quantification of the BR1 degradation rate, seedlings of homozygous HS–BRI1-YFP lines were transferred into in 200 µL of liquid 0.5x LS in a 2 mL cap and heat-shocked in a 37°C water bath for 20–30 min. Seedlings were then placed back onto an agar plate for 4.5 h, before being mounted into N.G., by the Howard Hughes Medical Institute, and by USDA and NSF–for maintenance of cell cultures; and F. Schaffl for the heat-shock protein.

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