Endosomes generate localized Rho–ROCK–MLC2–based contractile signals via Endo180 to promote adhesion disassembly

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The regulated assembly and disassembly of focal adhesions and adherens junctions contributes to cell motility and tumor invasion. Pivotal in this process is phosphorylation of myosin light chain-2 (MLC2) by Rho kinase (ROCK) downstream of Rho activation, which generates the contractile force necessary to drive disassembly of epithelial cell–cell junctions and cell–matrix adhesions at the rear of migrating cells. How Rho–ROCK–MLC2 activation occurs at these distinct cellular locations is not known, but the emerging concept that endocytic dynamics can coordinate key intracellular signaling events provides vital clues. We report that endosomes containing the promigratory receptor Endo180 (CD280) can generate Rho–ROCK–MLC2–based contractile signals. Moreover, we provide evidence for a cellular mechanism in which Endo180-containing endosomes are spatially localized to facilitate their contractile signals directly at sites of adhesion turnover. We propose migration driven by Endo180 as a model for the spatial regulation of contractility and adhesion dynamics by endosomes.

Introduction

Focal adhesions are sites of matrix engagement with cell surface integrin clusters that are linked to the actin cytoskeleton at stress fiber termini through interactions with multiple intracellular proteins, such as talin, vinculin, and paxillin (Webb et al., 2002; Carragher and Frame, 2004). The signaling and molecular mechanisms leading to focal adhesion assembly are well characterized and involve multiple Rho family GTPases, actin binding proteins, and integrin-matrix binding (Webb et al., 2002). In contrast, relatively little is known about the mechanisms involved in adhesion disassembly, but the involvement of Rho–Rho kinase (ROCK) signaling, calpains, and microtubules have been proposed (Carragher and Frame, 2004; Ezratty et al., 2005). In particular, Rho–ROCK promotes focal adhesion disassembly at the cell rear, and inhibition of this pathway produces a striking contractile and/or tail-retraction defect that is associated with decreased myosin light chain (MLC) 2 phosphorylation in various cell types (Itoh et al., 1999; Somlyo et al., 2000; Alblas et al., 2001; Worthylake et al., 2001; Riento and Ridley, 2003; Wilkinson et al., 2005).

ROCK-based contractility is not only involved in the disassembly of cell–matrix adhesions during tail retraction but can also disrupt the stability of cell–cell adhesions associated with adherens junctions (Sahai and Marshall, 2002). Adherens junctions occur at sites of cell–cell contact in organized epithelial cell monolayers and are formed via the homotypic interaction between E-cadherin on adjacent cells. The cytoplasmic tail of E-cadherin is linked to the actin cytoskeleton through interactions with catenin proteins (α, β, and p120) and actin binding proteins (vinculin). Adherens junctions can be regulated by translational events but are also subject to direct control by posttranslational cellular mechanisms, including their disassembly by the actin cytoskeleton and endocytosis (D’Souza-Schorey, 2005).

Endocytic dynamics have been shown to coordinate several key intracellular signaling events (Kermorgant et al., 2004; Polo et al., 2004; Le Roy and Wrana, 2005). In this study, we investigated whether endosomal signaling could represent an integral part of the deadhesion process, both in rear cell retraction and adherens junction breakdown. In particular, we have
investigated the role of the endocytic receptor Endo180 in these events. Endo180 (also known as CD280; uPARAP) is a 180-kD type I transmembrane receptor comprised of an N-terminal cysteine-rich domain followed by a fibronectin type II (FNII), 8 C-type lectin-like domains, a single transmembrane domain, and a short cytoplasmic domain (East and Isacke, 2002; Behrendt, 2004). Within this cytoplasmic domain, a critical dihydrophobic Leu\textsuperscript{1468}/Val\textsuperscript{1469} motif mediates the constitutive recruitment of Endo180 into clathrin-coated pits on the cell surface, which is followed by rapid internalization into intracellular endosomes and efficient recycling back to the cell surface (Isacke et al., 1990; Howard and Isacke, 2002). This trafficking of Endo180 is essential for its function as a collagen internalization receptor in which collagen bound to Endo180 is rapidly taken up into the endosomes and then dissociated from the receptor for delivery to, and degradation in, lysosomal compartments (Engelholm et al., 2003; Wienke et al., 2003; Kjoller et al., 2004; Curino et al., 2005). In addition to its role in ligand internalization, a promigratory function for Endo180 has also been demonstrated. Cells derived from mice with a targeted deletion in Endo180 and in which Endo180 expression is knocked down by siRNA oligonucleotides both display a reduced migratory capacity. Conversely, ectopic expression of Endo180 in Endo180-negative cell lines results in the acquisition of a polarized phenotype and enhanced cell migration (East et al., 2003; Engelholm et al., 2003; Sturge et al., 2003). Here, we have further investigated the promigratory function of this receptor and provide a mechanism by which intracellular Endo180 can spatially regulate cell contractility and adhesion dynamics.

**Results**

**Rear cell deadhesion and cell contractility are regulated by the endocytic receptor Endo180**

The potential involvement of endosomes in the spatial activation of ROCK during rear cell deadhesion was investigated by comparing the effects of ROCK inhibition with the down-regulation of constitutively recycling endocytic receptors. Treatment of MG63 osteosarcoma cells with ROCK inhibitor produced a tail-retraction defect that was associated with a decrease in MLC2 phosphorylation (Fig. 1, a and b; and Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200602125/DC1), consistent with its effects in other cell types (Itoh et al., 1999; Somlyo et al., 2000; Alblas et al., 2001; Worthylake et al., 2001; Riento and Ridley, 2003; Wilkinson et al., 2005). Three endocytic receptors, Endo180, transferrin receptor, and low-density lipoprotein (LDL) receptor, were targeted with siRNA oligonucleotides (Fig. S1 b). These receptors were chosen as Endo180 drives cell migration through an endocytosis-dependent mechanism (Sturge et al., 2003) and transferrin receptor enhances cell migration on a transferrin substrate (Bretscher, 1992). Because the LDL receptor (LDLR) has no reported role in cell migration, it was included as a negative control. Endo180 siRNA treatment

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Figure 1. Endo180 is required for contractile signals and rear cell deadhesion. (a–c) MG63 cells were plated onto uncoated glass coverslips or tissue culture plastic and treated with 0.3 μM ROCK inhibitor for 16 h or mock transfected, transfected with nontargeting scrambled Endo180 siRNA oligonucleotides (control), Endo180 single siRNA oligonucleotides (Endo180 siRNA), Endo180 SMARTPool siRNA oligonucleotides (Endo180 siRNA SP), or siRNA oligonucleotides against transferrin receptor (TIR), LDLR, or uPAR, and cultured for 72 h. (a) Cells were fixed, and the actin cytoskeleton was visualized by staining with Alexa 488 phalloidin (green). Cell nuclei were counterstained with TO-PRO-3 (blue). Bar, 50 μm. Images are representative of four separate experiments. (b) Cells in panel a were scored for tail formation by counting >100 cells in each of four separate experiments. Data are mean ± SEM. *, P < 0.0001, compared with untreated cells. (c) Cell lysates were resolved by SDS-PAGE and immunoblotted to detect diphospho-MLC2 and total MLC2. (top) A representative immunoblot of four separate experiments. (bottom) MLC2 phosphorylation levels quantified by densitometry. Data are mean ± SEM. *, P < 0.0001, compared with control. n = 4. (d) MDA-MB-231, H2:1080, BE, and MG63 cells were plated onto tissue culture plastic and treated with control or Endo180 siRNA oligonucleotides for 72 h, and levels of diphospho-MLC2 and total MLC2 were detected as described for panel c. Data are mean of four separate experiments ± SEM. *, P < 0.01, compared with control.
of MG63 cells resulted in a striking elongated phenotype, indicative of a tail-retraction defect, that was indistinguishable from that produced by ROCK inhibition (Fig. 1, a and b). This was not an off-target effect of Endo180 siRNA, as an identical phenotype was observed using alternate oligonucleotides (Fig. 1, a and b; and Fig. S1 c). In contrast, no defect in rear cell deadhesion was apparent in transferrin receptor or LDLR siRNA–treated cells (Fig. 1, a and b). Further, Endo180 was the only endocytic receptor required for MLC2 phosphorylation (Fig. 1 c and Fig. S1 c).

Defective cellular contractility and/or rear cell retraction resulting from the down-regulation of Endo180 was not restricted to MG63 osteosarcoma cells but was also confirmed in MDA-MB-231 breast carcinoma, HT-1080 fibrosarcoma, and BE colon carcinoma cells (Fig. 1 d and Fig. S2 a).

Endo180 is a coreceptor for the glycosphatidylinositol-anchored urokinase-type plasminogen activator (uPA)–uPA receptor (uPAR) complex (Behrendt et al., 2000) and is required for the activation of directional signaling pathways during sensing of a uPA gradient by migrating cells (Sturge et al., 2003). Because uPA–uPAR has been reported to regulate Rho–ROCK signaling and the phosphorylation of MLC2 (Nguyen et al., 1999; Jo et al., 2002), we considered this to be a potential regulatory component in rear cell deadhesion and contractility promoted by Endo180. To address this possibility, uPAR was targeted using siRNA oligonucleotides in MG63 and BE cells, which express low and high levels of uPAR, respectively (Fig. S1 b and Fig. S2 b). As previously reported (Vial et al., 2003), treatment with uPAR siRNA was effective at decreasing membrane ruffles in BE colon carcinoma cells (unpublished data). However, no tail-retraction defect or reduction in MLC2 phosphorylation was observed in either MG63 cells (Fig. 1, a–c) or BE cells (Fig. S2 c), indicating that uPA–uPAR does not promote cell contractility associated with rear cell tail retraction and is unlikely to have a role in this particular Endo180-mediated event during random cell migration.

**Endo180 does not require specific extracellular matrix components to regulate rear cell deadhesion and cell contractility**

Endo180 is a well-established collagen binding and internalization receptor (Engelholm et al., 2003; Wienke et al., 2003; Kjoller et al., 2004; Curino et al., 2005). To investigate whether the specific functions of Endo180 played a part in rear cell deadhesion and contractility, the behavior of cells targeted for knock down of Endo180 on both non–collagen- and collagen-based substrata was investigated. MG63 cells treated with Endo180 siRNA oligonucleotides for 72 h formed unretracted tails within 4 h of being seeded onto uncoated glass coverslips or glass coverslips coated with fibronectin, collagen I, or Matrigel (of which collagen IV is a major constituent), whereas on all substrata no tail-retraction defect was observed in control siRNA–treated cells (Fig. 2 a). Further, MG63 cells grown on uncoated tissue culture plastic or tissue culture plastic coated with fibronectin, collagen I, or Matrigel displayed similar decreases in MLC2 phosphorylation (Fig. 2 b). These findings are in keeping with a previous report (Sturge et al., 2003) and new data presented here (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200602125/DC1) that Endo180-mediated cell migration is not dependent on collagenous extracellular matrix substrata.

**Rho-ROCK signaling is a downstream target of Endo180**

To test the hypothesis that Endo180 regulates ROCK activity, a series of experiments were undertaken to assess whether Endo180 down-regulation could recapitulate the specific cellular and biochemical events associated with ROCK inhibition. First, a comparison using time-lapse microscopy of tail formation after global ROCK inhibition and Endo180 siRNA treatment revealed a similar sequence of cellular dynamics (Fig. 3 a and Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200602125/DC1). In both cases, cells exhibited a collapsed morphology, an increase in cell body movement (Fig. 3 b), and impaired translocation. This resulted in the formation of multiple and elongated tails, some of which then became protrusive and displayed localized membrane ruffles (Videos 1–3). In these assays, it was noted that the tail phenotype produced by ROCK inhibition was more extensive than that associated with Endo180 siRNA treatment. This most likely is a consequence of ROCK inhibition at multiple cellular locations, whereas tails

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**Figure 2. Endo180-generated contractile signals and rear cell deadhesion are not dependent on the extracellular matrix.** [a] MG63 cells were transfected with nontargeting [control] or targeting siRNA oligonucleotides against Endo180 for 72 h. Cells were seeded onto uncoated coverslips or coverslips coated with fibronectin, collagen type I, or Matrigel and allowed to adhere and spread for 4 h before fixation. The actin cytoskeleton was visualized by staining with Alexa 488 phalloidin (green), and cell nuclei were counterstained with TO-PRO-3 (blue). Images shown are representative of two separate experiments. Bar, 50 μm. [b] MG63 cells seeded onto uncoated tissue culture plastic or tissue culture plastic coated with fibronectin, collagen type I, or Matrigel were transfected with nontargeting [control] or targeting siRNA oligonucleotides against Endo180 for 72 h. Cell lysates were resolved by SDS-PAGE and immunoblotted to detect diphospho-MLC2 and total MLC2. (top) A representative immunoblot from a single experiment. (bottom) MLC2 phosphorylation levels quantified by densitometry. Data are mean of two separate experiments ± SEM.
resulting from Endo180 siRNA treatment were generally restricted to the rear of migrating cells and could result from the inhibition of spatially regulated ROCK activity. The global inhibition of ROCK also explains the more collapsed phenotype of cells treated with ROCK inhibitor that occurs before their formation of multiple tails (Fig. 3 a, t = 0).

Second, the signaling pathways downstream of ROCK were investigated. Activation of MLC2 by ROCK occurs through both diphosphorylation at threonine 20/serine 19 and a single phosphorylation event at serine 19. ROCK also phosphorylates LIM kinase (LIMK) 1/2 at threonine 508/505 and myosin phosphatase (MYPT) 1 at threonine 696 (Riento and Ridley, 2003; Croft et al., 2004). Treatment of MG63 cells with Endo180 siRNA or ROCK inhibitor not only reduced the diphosphorylation of MLC2 (Fig. 4 a, Fig. 1 c, and Fig. S1 a) but also reduced the monophosphorylation of MLC2 at serine 19 and decreased the phosphorylation of both MYPT1 and LIMK1/2 (Fig. 4 a). The reduction in phosphorylation attributed to Endo180 siRNA treatment was not due to an effect on kinase stability, as the levels of serine/threonine kinases involved in the phosphorylation of MLC2 remained unchanged (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200602125/DC1).

Finally, the involvement of the GTPase Rho, which functions as the key effector that directly binds and activates ROCK (Riento and Ridley, 2003), was investigated. Previous work established a role for Endo180 in the activation of the other two Rho family GTPases, Cdc42 and Rac (but not Rho), during uPA-mediated “directional” migration (chemotaxis; Sturge et al., 2003). However, the Endo180-dependent signaling pathways that promote “random” cell migration were not examined. To investigate whether ROCK activation by Endo180 occurs through the Rho pathway, levels of active Rho were measured in lysates of MG63 cells treated with Endo180 siRNA. The results of these experiments confirm that Rho activation is a downstream target of Endo180 (Fig. 4 b). Further, the inability of ROCK inhibitor to block Rho activity (Fig. 4 b) confirms previous reports that ROCK lies downstream of Rho. Interestingly,
the inhibition of Rho by TAT-C3 transferase in MG63 cells produced an unretracted tail phenotype similar to that observed in Endo180 siRNA– or ROCK inhibitor–treated cells (compare Fig. 4c and Fig. 1a). Collectively, these data suggest the existence of an Endo180–Rho–ROCK–MLC2 signaling pathway that is involved in rear cell retraction.

Spatial regulation of MLC2 phosphorylation in unretracted tails is associated with the accumulation of endosomal Endo180

Spatial regulation of MLC2 has been proposed to occur through localized signals that emanate from different upstream effectors (Totsukawa et al., 2004). It has also been hypothesized that endosomes have the capacity to perpetuate and/or amplify intracellular signaling pathways (Kermorgant et al., 2004; Polo et al., 2004; Le Roy and Wrana, 2005). Consequently, it was important to determine whether Endo180 could spatially localize to activate Rho–ROCK–MLC2 during rear cell deadhesion and tail retraction.

First, live cells were stained at 4°C with Endo180 antibody to assess the cell surface distribution of this receptor. Unlike integrins that cluster at the cell surface of unretracted tails after ROCK inhibition (Worthylake et al., 2001), no accumulation or clustering of plasma membrane Endo180 at the unretracted tails was observed in ROCK inhibitor–treated cells (Fig. 5a). Instead, the receptor remained uniformly distributed across the plasma membrane in punctate structures, consistent with previous reports that 10–30% of cellular Endo180 is localized to the plasma membrane in clathrin-coated pits (Isacke et al., 1990). In contrast, immunofluorescent staining and confocal microscopy of Endo180 in permeabilized ROCK inhibitor–treated cells revealed a dramatic accumulation of Endo180 in the majority of unretracted tails (Fig. 5b; 79 ± 4%; >50 cells scored in each of three separate experiments). High-magnification and multiple xz and yz confocal imaging of unretracted tails confirmed that this accumulated Endo180 was localized to intracellular endosomes (Fig. 5b). In contrast, in cells treated with either ROCK inhibitor or Endo180 siRNA, ROCK itself did not accumulate/relocalize to rear cell adhesion sites and remained diffusely cytosolic (unpublished data). Importantly, a small number (4 ± 1%; >50 cells scored in each of three separate experiments) of untreated migrating cells also displayed very strong localization of Endo180-containing endosomes to cell–matrix adhesion sites at the termini of stress fibers or unretracted tails (Fig. 5b, arrowhead). This confirms that accumulation of Endo180-containing endosomes occurs in normally migrating cells and suggests that the relatively small number of normal cells displaying localization of Endo180 to adhesion sites reflects the highly dynamic nature of endosomal trafficking during cell migration.

Second, the Endo180 endosomes were characterized to address two questions. Is the accumulation of Endo180 in the unretracted tails of ROCK inhibitor cells specific to this endocytic receptor, and does the accumulation of Endo180 reflect a change in endosomal trafficking caused by the inhibition of ROCK? For this purpose, the colocalization of Endo180 with transferrin receptor (a constitutively recycling receptor), early endosome antigen 1 (EEA1; a marker for sorting endosomes), and Rab11 (a marker for pericentriolar recycling endosomes; Zerial and McBride, 2001) was assessed. In untreated cells, Endo180 displayed near total colocalization with transferrin receptor and partial colocalization with both EEA1 and Rab11 (Fig. 6), demonstrating that Endo180, like the transferrin receptor (Sonnichsen et al., 2000), is localized to both rapidly recycling EEA1-positive sorting endosomes and slower recycling Rab11-positive pericentriolar endosomes. In ROCK inhibitor–treated cells, both transferrin receptor and EEA1 strongly accumulated with highest concentrations of these endosomal markers observed in the unretracted tails. A similar distribution of Rab11 was not evident in ROCK inhibitor–treated cells. As in untreated cells, Rab11 was uniformly distributed throughout ROCK inhibitor–treated cells, with the highest concentrations accumulated in pericentriolar recycling endosomes. The unretracted tails in ROCK inhibitor–treated cells were quantified by scoring for their accumulation of high concentrations of Endo180 together with high concentrations of transferrin receptor, EEA1, or Rab11. In this analysis, Endo180 displayed an almost total coaccumulation with transferrin receptor (98 ± 2%), a partial
Figure 6. **Endo180 in unretracted tails colocalizes with transferrin receptor and accumulates in early sorting but not recycling endosomes.** MG63 cells plated onto uncoated coverslips were left untreated or treated with 0.3 μM ROCK inhibitor for 4 h, fixed, and stained with anti-Endo180 mAb A5/158 (green) and antibodies against transferrin receptor (TfR), EEA1, or Rab11 as markers of different endosomal compartments (red). Nuclei were counterstained with TO-PRO-3 (blue). Images shown are representative of three separate experiments. Bar, 25 μm. Values represent the percentage of unretracted tails in ROCK inhibitor-treated cells that show coaccumulation of Endo180 with TfR, EEA1, or Rab11. Data represents >50 cells counted in each of three separate experiments ± SEM.

Figure 7. **Endo180-positive endosomes directly localize at sites of cell–matrix adhesion with increased contractile signals.** MG63 cells plated onto uncoated coverslips were fixed and stained with Alexa 488 phalloidin to visualize the actin cytoskeleton (green), anti-Endo180 mAb A5/158 (red), and anti-diphosphorylated MLC2 (blue). Two representative images are shown. Asterisks indicate regions shown in the right-hand images at higher (4×) magnification. Bar, 50 μm.

**Endo180 internalization is required for spatial adhesion assembly and disassembly and the generation of contractile signals**

The ability of Endo180 to regulate the adhesion/deadhesion process and generate contractile signals could emanate from the coaccumulation with EEA1 (24 ± 7%), and only minimal coaccumulation with Rab11 (4 ± 1%; Fig. 6), indicating that it is the redistribution of receptor-positive endosomes, rather than the altered internalization/export of receptors, that accounts for the tail localization and that accumulation of endocytic receptors in unretracted tails is not exclusive to Endo180. Despite this lack of exclusivity, it is notable that transferrin receptor also strongly accumulated in the unretracted tails of Endo180 siRNA–treated cells (unpublished data), supporting previous experimental findings (Fig. 1) that transferrin receptor has no functional role in the promotion of cell contractility during rear tail retraction. In contrast, further support for the mechanistic regulation of cell contractility by Endo180 was provided by the observation in untreated cells that Endo180-containing endosomes strongly accumulate at adhesion sites and unretracted tails that have a localized high level of MLC2 phosphorylation (Fig. 7).

**Endo180 internalization is required for spatial adhesion assembly and disassembly and the generation of contractile signals**

The ability of Endo180 to regulate the adhesion/deadhesion process and generate contractile signals could emanate from the plasma membrane or internalized endosomes. To address this, studies were undertaken using the Endo180(Ala1468/Ala1469) mutant, which is expressed at the cell surface but internalization defective (Howard and Isacke, 2002; Wienke et al., 2003). It has previously been demonstrated that the expression of wild-type Endo180 promotes MCF7 cell migration, whereas Endo180(Ala1468/Ala1469) does not (Sturge et al., 2003). For these experiments, stable transfectants with equal protein-expression levels of wild-type Endo180 and Endo180(Ala1468/Ala1469) were generated (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200602125/DC1). As expected from its promigratory function, expression of wild-type Endo180 increased cell spreading and the assembly of new focal adhesions (Fig. 8 a). In addition, these transfected cells showed enhanced adhesion to a Matrigel substratum (Fig. 8 b), consistent with a previous report that cells from mice with a targeted deletion in Endo180 show a defect in adhesion to a variety of collagen substrata (Engelholm et al., 2003). The Endo180(Ala1468/Ala1469) mutant contains an intact collagen binding domain and, as expected, supported a level of adhesion to Matrigel similar to that of wild-type Endo180 (Fig. 8 b). However, Endo180(Ala1468/Ala1469) did not promote cell spreading and spatial assembly of new focal adhesions (Fig. 8 a). Rather, both vector alone and Endo180(Ala1468/Ala1469) transfected cells developed an elongated phenotype upon plating (Fig. 8 a), with tail structures that were reminiscent of those observed in Endo180 siRNA– or ROCK inhibitor–treated cells (compare Figs. 1 a and 8 a). Moreover, in these cells, talin was seen to accumulate in the tail structures. These data indicate that an uncoupling of adhesion and deadhesion underlies the migration defect observed in cells expressing the internalization-defective Endo180(Ala1468/Ala1469) mutant and that although internalization of Endo180 is not necessary for the promotion of initial cell–matrix adhesions, it is required for the correct spatial formation and efficient turnover of cell–matrix adhesions during cell spreading and migration.

Next, several approaches were taken to confirm that Endo180 internalization is required for the generation of contractile signals.
First, it was demonstrated that MLC2 phosphorylation generated by serum stimulation of starved cells was significantly elevated in cells transfected with Endo180 compared with that generated by cells transfected with vector alone or Endo180(Ala1468/Ala1469) (Fig. 9 a and Fig. S5). Second, using a method that has been used to demonstrate the existence of intracellular endosomal signaling events (Kermorgant et al., 2004), cells were stimulated with serum for 10 min and the extracellular stimulus was withdrawn. Upon serum withdrawal, elevated MLC2 phosphorylation levels persisted for at least 2 h in Endo180-expressing cells but rapidly returned to basal levels within 10 min in vector alone transfected cells (Fig. 9 b). Finally, the recycling of endosomal components back to the plasma membrane was inhibited using primaquine (Woods et al., 2004) in serum-starved cells. As expected, primaquine resulted in a dramatic decrease in the localization of Endo180 at the cell surface (Fig. 9 c) but did not affect total cellular levels of Endo180 (Fig. 9 d), indicating that Endo180 had been internalized but not recycled back to the plasma membrane. The resultant fourfold increase in MLC2 phosphorylation in primaquine-treated cells was sufficient to stimulate a contractile response that was blocked by the presence of ROCK inhibitor (Fig. 9 e). The observation that MLC2 phosphorylation levels remain unchanged in primaquine-treated vector alone transfected cells (Fig. 9 e) provides independent evidence that other recycling receptors, such as transferrin receptor and β1 integrin (CD29), are not involved in the regulation of this signaling event. The results of these experimental approaches confirm that the internalization of Endo180 into endosomes can generate and sustain ROCK-dependent intracellular contractile signals.

Endo180–ROCK signaling disrupts adherens junctions

Because Endo180 can promote localized ROCK–MLC2 signaling during tail retraction, we further hypothesized that ectopic expression of this receptor in epithelial cells should be sufficient to promote disassembly of their adherens junctions, as ROCK-based contractility can disrupt cell–cell adhesions (Sahai and Marshall, 2002). The expression of Endo180 in MCF7 cells resulted in the loss of E-cadherin from cell–cell junctions, and this was reversed by treatment with ROCK inhibitor (Fig. 10, a and b). This effect was not specific to E-cadherin, as a similar redistribution was also observed with immunofluorescent staining of the junctional component α-catenin (unpublished data). Moreover, the decreased stability of MCF7 cell–cell junctions in cells ectopically expressing Endo180 was reversed by Endo180 siRNA treatment (Fig. 10, c and d). These findings suggest that in addition to regulating the disassembly of cell–matrix adhesions at the cell rear during cell migration, Endo180 has the capacity to activate ROCK and generate contractile signals that promote the disassembly of adherens junctions at epithelial cell–cell contacts.

Discussion

Our results point to a role for endosomes in the disassembly mechanism for cell–matrix and cell–cell adhesions. In particular, we have demonstrated that the endocytic localization of the Endo180 receptor activates mechanotransduction pathways that promote cell contractility and adhesion disassembly. A role for Endo180-containing endosomes in adhesion disassembly is complemented by a report that identified dynamin as a downstream target for microtubule-induced focal adhesion disassembly in fibroblasts (Ezratty et al., 2005). Dynamin provides a ubiquitous molecular mechanism for driving endocytosis via its ability to recruit actin monomers to the neck of clathrin-coated pits, where their polymerization is required to force endosome internalization and propulsion through the dense cytocortex into the cytosol (Orth and McNiven, 2003).

Because the expression of Endo180 is predominantly restricted to fibroblasts and other highly motile cells, including a range of highly invasive cancer cell types (Behrendt et al., 2000;
Figure 9. Internalization of Endo180 into endosomes promotes the generation of contractile signals. Cells were plated onto Matrigel-coated tissue culture plastic. (a) Serum-starved vector alone, Endo180, and Endo180(Ala1468/Ala1469) transfected MCF7 cells were treated with or without 10% FCS for 10 min. Lysates were resolved by SDS-PAGE and diphospho-MLC2 and total MLC2 detected by immunoblotting. (bottom) Representative immunoblots from 10 separate experiments. (top) MLC2 phosphorylation levels quantified by densitometry. Data are mean ± SEM. * P < 0.001, compared with FCS-stimulated vector alone and Endo180(Ala1468/Ala1469) transfected MCF7 cells. (b) Serum-starved vector alone and Endo180 transfected MCF7 cells were treated with or without 10% FCS for 10 min, washed twice in PBS, and incubated in serum-free medium for 10 min, 30 min, 1 h, or 2 h. Cells were lysed, and diphospho-MLC2 and total MLC2 were detected by immunoblotting. (top) Representative immunoblots from two separate experiments. (bottom) MLC2 phosphorylation levels quantified by densitometry. Data are mean ± SEM. n = 2. (c and d) Serum-starved vector alone or Endo180 transfected MCF7 cells were either untreated or treated with primaquine for 30 min. (c) The cell surface levels of Endo180, transferrin receptor (TfR), and β1 integrin (CD29) was assessed by flow cytometry. Data shown are mean relative fluorescent intensity normalized against isotype-matched IgG binding. (d) Cell lysates were resolved by SDS-PAGE, and Endo180 was detected by immunoblotting. (e) Serum-starved vector alone and Endo180 transfected MCF7 cells were untreated or treated with primaquine for 30 min. Where indicated, cells were incubated with 0.3 μM ROCK inhibitor for 1 h before primaquine stimulation. Lysates were resolved by SDS-PAGE, and diphospho-MLC2 and total MLC2 were detected by immunoblotting. (bottom) Representative immunoblots from four separate experiments. (top) MLC2 phosphorylation levels quantified by densitometry. Data are mean ± SEM. * P < 0.05, compared to all other treatments. n = 4.
a redistribution of Endo180/transferrin receptor–positive endosomes rather than from altered internalization/export kinetics of Endo180. However, we cannot rule out the possibility that export of Endo180 and transferrin receptor from sorting endosomes in the tails may also be reduced by ROCK inhibition. The colocalization of Endo180 and transferrin receptor in ROCK inhibitor–treated cells demonstrates that the accumulation of endocytic receptors is not exclusive to Endo180. However, the transferrin receptor also showed a strong accumulation in the unretracted tails of Endo180 siRNA–treated cells (unpublished data), and down-regulation of transferrin receptor by siRNA treatment had no effect on tail retraction or phosphorylation of MLC2. Moreover, primaquine treatment of Endo180-negative cells resulted in the intracellular accumulation of transferrin receptor and β1 integrin but did not enhance MLC2 phosphorylation. As a consequence, we conclude that, at least among the endocytic receptors examined, Endo180 has an exclusive functional role in promoting cell contractility during rear tail retraction. Finally, the observation that ROCK itself did not accumulate/relocalize to rear cell adhesion sites and remained diffusely cytosolic in either ROCK inhibitor– or Endo180 siRNA–treated cells indicates that Endo180 localization at these sites could be the rate-limiting step in the spatial activation of ROCK and has led us to propose that the localization of Endo180-containing endosomes results in the spatial activation of Rho–ROCK–MLC2 to promote adhesion disassembly.

In addition to regulating adhesion of cells to a substratum, ROCK can regulate the integrity of cell–cell adhesion complexes (Riento and Ridley, 2003). Although there are conflicting reports in the literature as to whether ROCK activity promotes an increased (Walsh et al., 2001; Eisen et al., 2004; Shewan et al., 2005) or decreased (Vaezi et al., 2002; Wojciak-Stothard and Ridley, 2002) integrity of intercellular junctions and permeability of cell monolayers, activation of ROCK has been shown to result in the disruption of E-cadherin–containing adherens junctions and the redistribution of junctional components in epithelial cells in culture (Sahai and Marshall, 2002; Croft et al., 2004). The demonstration here that ectopic expression of Endo180 results in a redistribution of E-cadherin in epithelial monolayers and that this redistribution can be reversed by treatment with ROCK inhibitor provides further support for a role of Endo180 as an upstream regulator of ROCK. These data additionally suggest that aberrant expression of Endo180 in epithelial cells may promote the acquisition of a more mesenchymal migratory phenotype, and in this respect it is of interest that the noninvasive MCF7 epithelial cell line is Endo180-negative, whereas the more aggressive BE and MDA-MB-231 tumor lines are Endo180-positive.

These studies, combined with previous work, have demonstrated that Endo180 has a dual function, acting both as a regulator of Rho–ROCK–MLC2 signaling and as a collagen internalization receptor. To date, the most striking in vivo phenotype resulting from manipulating Endo180 expression comes from crossing mice with a mammary tumor–predisposing transgene to mice with a targeted deletion in Endo180. These mice develop tumors at the same rate as those expressing wild-type Endo180 but show an increased collagen deposition in the tumor-associated stroma and a decreased tumor burden (Curino et al., 2005). Given the data presented here, we propose that this phenotype arises not only from the inability of Endo180-null fibroblasts to remodel the collagen-rich extracellular matrix but also because such cells will also have a mechanotransduction defect that will impair their motility within the tumor. Given that these stromal cells are major collagen producers, this impaired motility will contribute to the aberrant accumulation of extracellular collagen. Certainly it will be of interest to determine whether Endo180 plays a similar role in other pathological scenarios where altered fibroblast activity and matrix turnover are associated with disease progression.

Materials and methods

Antibodies and cells

Anti-Endo180 mAb A5/158 has been previously described (Sheikh et al., 2000). B3/25 anti-transferrin receptor mAb was a gift from C. Hopkins (Imperial College London, London, UK). Mouse anti-human uPAR was obtained from Fitzgerald. Mouse anti–human monophospho-MLC2 (clone MY21), MLCK, talin, and γ-tubulin were obtained from American Diagnostica, Inc. Rabbit anti–human monophospho-MLC2 (Ser19) and diphospho-MLC2 (Thr18/Ser19), UMK1, and phospho-UMK1 (Thr508)/UMK2 (Thr505) were obtained from Cell Signaling. Mouse anti-human MLC (clone MY21), MLCK, talin, and γ-tubulin were obtained from...
Sigma-Aldrich. Mouse anti-human MYPT1, ROCK1, ROCK2, ZIP kinase, EE1, and Rab11 were obtained from BD Biosciences. Rabbit anti-human phospho-MYPT1 (Thr696) was obtained from Upstate Biotechnology. Rabbit anti-human MRCKα and MRCKβ were a gift from S. Wilkinson (Institute of Cancer Research, London, UK; Wilkinson et al., 2005). Mouse anti-human CD29 was obtained from Serotec. Mouse anti-human E-cadherin (clone HECD-1) was obtained from Abcam; secondary antibodies Alexa Fluor 488/555, anti-rabbit Ig and Alexa Fluor 488/555 anti-mouse Ig, Alexa Fluor 488/555/633 phalloidin, and TO-PRO-3 were obtained from Invitrogen. HRP anti-rabbit Ig was obtained from Jackson ImmunoResearch Laboratories, and HRP anti-rabbit Ig was obtained from Santa Cruz Biotechnology, Inc. E-cadherin antibody was labeled using Zenon Alexa Fluor 488 (Invitrogen) and HRP anti-rabbit Ig was obtained from Santa Cruz Biotechnology, Inc. Alexa Fluor 488 was used to directly conjugate to Alexa Fluor 488 (Invitrogen) was used. MG63, MDA-MB-231, HT1080, and BE cells were maintained in DME + 10% FCS. MCF7 cells transfected with vector alone, wild-type Endo180, and Endo180(Ala1468/Ala1469) were cultured as previously described (Wienke et al., 2003).

Immunostaining and confocal imaging

Immunostaining and surface labeling of Endo180 were performed as described previously (Howard and Isacke, 2002; Sturge et al., 2003). For confocal imaging, cells were fixed, stained, and mounted in Vectashield H-1000 (Vector Laboratories) at room temperature. Images were captured at room temperature with a confocal microscope (TCS SP2; Leica) and Confocal Software (Leica) using 63× (1.40 NA; oil; Leica) or 40× (1.25 NA; oil; Leica) lenses and Immersol 518F oil (Carl Zeiss Microlumaging, Inc.). Images were imported into in Photoshop 8.0 (Adobe) for processing.

Immunoblotting and Rho activity assays

Immunoblotting was performed as described previously (Sturge et al., 2003). Rho activation assay kit was purchased from Upstate Biotechnology, and assays were performed according the manufacturer’s guidelines. The cell permeable TAT-C3 transferase toxin was a gift from G. Mavria (The Institute of Cancer Research, London, UK). Quantification of phosphorylation levels and Rho activation were measured using Image) densitometric software. Data is adjusted for loading and normalized to 100% for control levels ± SEM.

Cell treatments

Endo180, reversed Endo180 (control siRNA), and uPAR single siRNA oligonucleotides were as described previously (Sturge et al., 2003; Vial et al., 2003). Transferrin receptor, LDR, and Endo180 were targeted using SMARTpool siRNA oligonucleotides (Dharmacon). siRNA oligonucleotides (20 nmol/ml) were transfected into cells seeded on coverslips or culture dishes (30–50% confluent) with 100 μM Oligofectamine (Invitrogen) in Opti-MEM reduced-serum medium (Invitrogen). Knock down of targeted receptors was assessed by flow cytometry as previously described (Sturge et al., 2003; Wiencek et al., 2003). Optimal knockdown was obtained 72 h after transfection. The highly specific ROCK inhibitor (S)-1-2-Methyl-1-(4-methyl-5-isoxazoliny1)sulfonyl)homopiperazine, 2HCl (Calbiochem) was diluted in culture media from a stock solution of 10 mM in sterile water. TAT-C3 transferase toxin was diluted in culture media from a stock solution of 7.2 μM and used at a final concentration of 1 μM. To inhibit endocytic recycling, MCF7 cells were starved for 48 h in DME before incubation with 0.6 μM primacine (Sigma-Aldrich).

Video microscopy

MG63 cells were left untreated or treated with siRNA oligonucleotides for 72 h. Cells were seeded on coverslips and allowed to adhere for 1 h. The coverslips were then placed on to counting chambers (Hawksley Technology) and sealed with wax as previously described (Sturge et al., 2003). siRNA oligonucleotides were transfected into fresh growth medium, and ROCK inhibitor-treated cells were assessed in fresh growth medium containing 1 μM ROCK inhibitor, which was added to cells 30 min before the start of image collection. Images of cells were digitally recorded at a time-lapse interval of 1 min for 4 h using an microscope (IX70; Olympus) fitted with humidified 37°C incubation chamber, a 20× lens (0.4 NA; dry; Olympus), and Simple PCI acquisition software (Digital Pixel). Speed of cell body movement (mean ± SEM, n = 10 cells) was calculated as previously described for mean cell migratory speed (Sturge et al., 2003) using Motion Analysis software (Kinetix Imaging Ltd) and Mathematica software (Wolfram Research Ltd).

Flow cytometry

Flow cytometry was performed as previously described (Wiencek et al., 2003). Data is presented as relative fluorescent intensity (median fluorescent intensity of antibody binding/median fluorescent intensity of isotype-matched control IgG binding) in which the isotype-matched control IgG binding is set at zero.

Cell adhesion assay

To measure adhesion, 105 Calcein AM-labeled cells were added to each well of a Matrigel-coated 96-well plate in growth medium. Cells were left for 1 h at 37°C and washed two times, and adherent cells were counted using a fluorescence plate reader.

Online supplemental material

Fig. S1 shows a dose-dependent inhibition of MLC2 by ROCK inhibitor, flow cytometric analysis of receptor levels after siRNA treatment, and that the single Endo180 siRNA oligonucleotides and Endo180 SMARTpool oligonucleotides have similar effects on Endo180 knockdown and MLC2 phosphorylation. Fig. S2 shows that treatment of MDA-MB-231 breast cancer cells with Endo180 siRNA oligonucleotides results in a tail-retraction defect similar to that seen in MG63 cells, in BE cells there is efficient knock down of both Endo180 and uPAR after treatment with their respective siRNA oligonucleotides, and knock down of Endo180, but not uPAR, in BE cells results in a reduction of MLC2 phosphorylation. Fig. S3 shows that MCF7 cells expressing Endo180 plated on either Matrigel, collagen IV, or fibronectin show enhanced migration compared with vector alone transfected cells. Fig. S4 shows that treatment of MG63 cells with Endo180 siRNA oligonucleotides does not affect the expression levels of six MLCK regulatory kinases. Fig. S5 shows expression levels of Endo180 and Endo180(Ala1468/Ala1469) transfected into MCF7 cells. Videos 1–3 show time-lapse microscopy of control siRNA–, Endo180 siRNA–, and ROCK inhibitor-treated MG63 cells, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200602125/DC1.

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