Cortactin and dynamin are required for the clathrin-independent endocytosis of γc cytokine receptor

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Endocytosis is critical for many cellular functions. We show that endocytosis of the common γc cytokine receptor is clathrin independent by using a dominant-negative mutant of Eps15 or RNA interference to knock down clathrin heavy chain. This pathway is synaptojanin independent and requires the GTPase dynamin. In addition, this process requires actin polymerization. To further characterize the function of dynamin in clathrin-independent endocytosis, in particular its connection with the actin cytoskeleton, we focused on dynamin-binding proteins that interact with F-actin. We compared the involvement of these proteins in the clathrin-dependent and -independent pathways. Thus, we observed that intersectin, syndapin, and mAbp1, which are necessary for the uptake of transferrin (Tf), a marker of the clathrin route, are not required for γc receptor endocytosis. Strikingly, cortactin is needed for both γc and Tf internalizations. These results reveal the ubiquitous action of cortactin in internalization processes and suggest its role as a linker between actin dynamics and clathrin-dependent and -independent endocytosis.

Introduction

Receptor-mediated endocytosis is an essential function of eukaryotic cells. Although different routes of internalization have been described, the only pathway well characterized to date is the clathrin-dependent one. The current model is that clathrin is recruited to the plasma membrane to form clathrin-coated pits and vesicles. This recruitment is mediated by the adaptor protein AP-2 or an alternative adaptor (for reviews see Conner and Schmid, 2003; Traub, 2003). AP-2 interacts with clathrin and recognizes clathrin-coated pits' localization signals present in the cytosolic tail of receptors at the plasma membrane. In addition, AP-2 acts as a platform to provide sites of interaction for several proteins important for clathrin-dependent endocytosis, including amphiphysin, AP180, Epsin, and Eps15 (Marsh and McMahon, 1999; Traub, 2003). Eps15, via its Eps15 homology domain, also interacts with epsin, synaptojanin, and intersectin and thus plays a pivotal role in clathrin-coated pit formation. Altogether, clathrin, AP-2 (or possibly another adaptor), and their associated factors constitute the endocytic complex required for clathrin-dependent endocytosis (Traub, 2003). Finally, clathrin-coated vesicle fission is controlled by the large GTPase dynamin (Hinshaw, 2000). Although clathrin-dependent endocytosis is now well known, its links with actin dynamics have been studied only recently (Merrifield et al., 2002). These studies have pointed to the key role of dynamin in connecting endocytosis and the actin cytoskeleton (Orth and McNiven, 2003). Indeed, dynamin is a multidomain protein. Through its proline-rich domain (PRD), it binds Src homology 3 (SH3) domains present in proteins interacting directly or indirectly with F-actin, such as intersectin, syndapin, mammalian actin-binding protein (mAbp1), and cortactin (Simpson et al., 1999; Qualmann and Kelly, 2000; Kessels et al., 2001; Cao et al., 2003). These proteins were very recently reported to be involved in clathrin-dependent internalization, but their precise action during endocytosis is still unclear.

Glycosylphosphatidylinositol (GPI)-anchored proteins, the autocrine motility factor, the TGF-β receptor, the β chain of interleukin (IL)-2 receptor, toxins, but also viruses, are examples of the growing number of markers taken up by clathrin-independent endocytosis (for reviews see Gesbert et al., 2003; Nabi and Le, 2003; Nichols, 2003; Pelkmans and Helenius, 2003). These markers are not found in clathrin-coated pits; instead they are associated with membrane lipid microdomains enriched in cholesterol and glycosphingolipids, also called "rafts," caveolae being a subset of these domains that contain caveolin (Nabi and Le, 2003). The GTPase dynamin seems to play an essential role in most clathrin-independent internalization examples reported, but its function in these processes is unclear. However, dynamin is not required for all endocytic pathways, in particular pinocytosis of GPI-anchored proteins can be dynamin independent (Mayor and Riezman, 2004). Very little is known about the role of actin dynamics in

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Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; IL, interleukin; PI, phosphatidylinositol; PRD, proline-rich domain; SH3, Src homology 3; Tf, transferrin.

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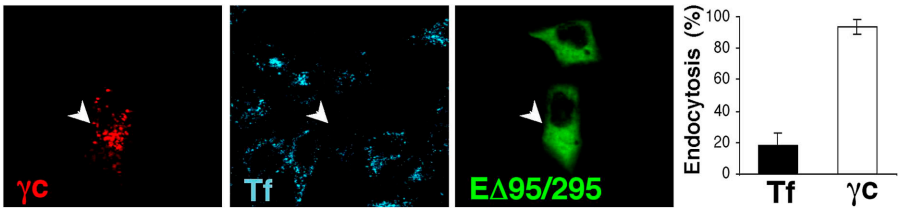

Figure 1. **Endocytosis of γc receptor is Eps15 independent.** Cells transfected with γc receptor and a GFP-tagged Eps15 dominant-negative mutant, EΔ95/295, were incubated for 15 min at 37°C with anti-γc receptor and Tf-Cy5 to follow endocytosis. The cells were fixed and permeabilized, and γc receptor was stained with labeled antibodies. The cells were analyzed by confocal microscopy and a medial optical cut is shown. The arrowhead indicates a doubly transfected cell expressing the Eps15 mutant and the γc receptor. The results were quantified by assessing the percentages of transfected cells having internalized Tf or γc receptor (right, mean of three experiments ± SD).

clathrin-independent endocytosis. It was shown, in the case of Simian Virus 40 uptake, that dynamin and actin were recruited to the virus-loaded caveolae (Pelkmans et al., 2002). In addition, the requirements for activated GTPases of the Rho family in the uptake of GPI-anchored protein and in the IL-2 receptor β chain entry are in favor of a role of the actin cytoskeleton in clathrin-independent mechanisms (Lamaze et al., 2001; Sabharanjak et al., 2002). More generally, due to the lack of molecular characterization of clathrin-independent endocytosis, these pathways are far from being understood.

The common cytokine receptor γc belongs to the type I cytokine receptor family that lack intrinsic kinase activity but recruit molecules that transduce the signal to the cell. The γc chain is shared by IL-2, -4, -7, -9, -15, and -21 receptors (Schluns and Lefrancois, 2003). Thus, the γc receptor plays a major role in lymphocyte proliferation and differentiation, leading when mutated to X-linked severe combined immunodeficiency (Schluns and Lefrancois, 2003). This receptor is rapidly internalized and reaches early endosomes (Hémar et al., 1995). Then, γc is targeted to late endosomes and lysosomes where it is degraded. Therefore, γc endocytosis leads to its down-modulation and thus is involved in the control of signaling pathways induced by the ILs that use it as a receptor component (Morelon et al., 1996). The γc receptor contains, in its cytosolic tail, sequences that enable its efficient and rapid endocytosis independently of the ligand or of other receptor chains (Morelon and Dautry-Varsat, 1998; Yu et al., 2000). However, the mechanism by which γc receptor is internalized is not known. The lack of a classic clathrin-coated pit localization signal in γc receptor and the clathrin-independent uptake of another cytokine receptor member, the β chain of IL-2 receptor, suggested that γc endocytosis might be clathrin independent. In this work, we show that indeed the γc receptor endocytosis is independent of AP-2 and clathrin and we analyzed the role of dynamin partners that also interact with F-actin.

Results

Endocytosis of the common cytokine receptor γc is clathrin independent

The common cytokine receptor γ (γc) is part of a subfamily of cytokine receptors and is shared by several members of the family, the receptors for IL-2, -4, -7, -9, -15, and -21 (Schluns and Lefrancois, 2003). When expressed by itself, the γc receptor is rapidly and efficiently endocytosed (Morelon and Dautry-Varsat, 1998). To characterize the endocytic pathway of the γc receptor, we made use of a dominant-negative mutant of Eps15 (EΔ95/295) that inhibits clathrin-coated pit formation when overexpressed (Benmerah et al., 1999). Endocytosis of γc receptor and transferrin (Tf), a marker of the classic clathrin-dependent pathway, were assessed at the same time in cells cotransfected with genes encoding the γc receptor and the GFP-tagged EΔ95/295. The cells were incubated for 15 min at 37°C with Cy5-labeled Tf and anti-γc monoclonal antibody to

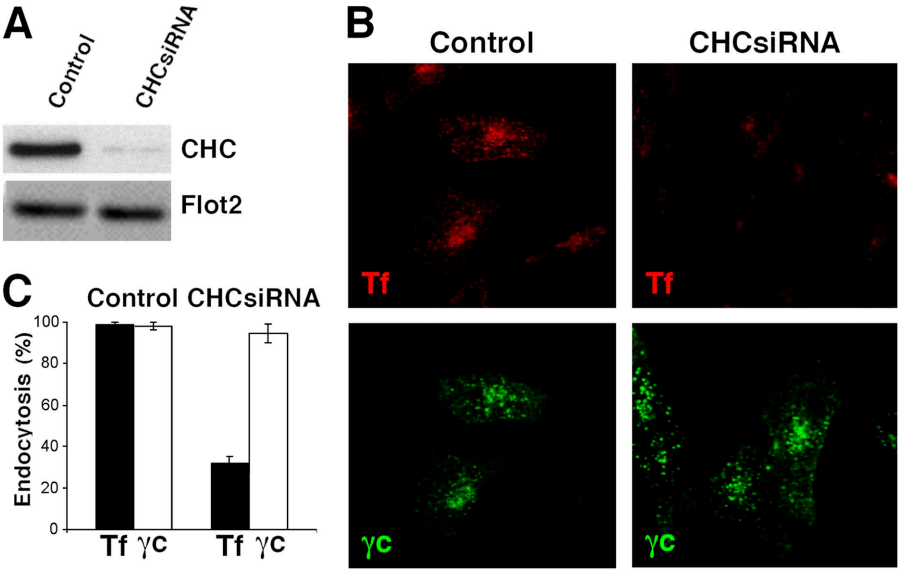

Figure 2. **Endocytosis of γc receptor is clathrin independent.** (A) Cells were transfected with the γc receptor and with siRNA against clathrin heavy chain (CHCsiRNA) or not (Control). Western blots analysis was performed on protein extracts from 10^5 cells with antibodies against clathrin heavy chain (CHC) and against Flotillin 2 (Flot2) as a control. (B) Cells transfected with γc receptor and with CHCsiRNA or not (Control) were incubated for 15 min at 37°C with anti-γc receptor antibody and Tf-Cy3 to follow endocytosis. The cells were fixed and permeabilized, γc receptor was stained with labeled antibodies, and the cells were analyzed by confocal microscopy. Confocal optical medial cuts are shown. The different images were taken with exactly the same settings. (C) The results were quantified by assessing the percentage of transfected cells having internalized Tf or γc receptor (mean of three experiments ± SD).

follow endocytosis. The cells were then fixed, permeabilized, stained with labeled second antibody, and observed by confocal microscopy. As shown in Fig. 1, in cells expressing EΔ95/295, endocytosis of γc receptor was not affected, whereas Tf uptake was inhibited in 84% of the transfected cells. This result shows that γc receptor internalization is Eps15 independent. Because overexpression of the Eps15 EΔ95/295 mutant prevents AP-2 recruitment to the plasma membrane (Benmerah et al., 1999), this result also shows that γc receptor endocytosis is AP-2 independent. It has been recently reported that some receptors, such as the epidermal growth factor receptor, could be internalized following a clathrin-dependent mechanism, but that AP-2 was not required for this process and could probably be replaced by other adaptors (Motley et al., 2003). Therefore, we assayed the role of clathrin in γc receptor uptake. For this assessment, we used a siRNA directed against clathrin heavy chain (CHCsiRNA) and transfected it twice into HeLa cells as described previously (Motley et al., 2003). The γc gene was introduced with the second round of CHCsiRNA transfection. Western blot analyses of total protein extracts showed that the introduction of CHCsiRNA in these cells resulted in an efficient reduction of the amount of clathrin heavy chain, without having a general effect on cellular proteins as seen by probing for a control protein, flotillin 2 (Fig. 2 A). Endocytosis of Tf and of γc receptor was assessed in these clathrin-depleted cells. As shown in Fig. 2 (B and C), clathrin depletion did not affect γc receptor endocytosis, whereas it inhibited Tf endocytosis (in ~70% of treated cells). Thus, clathrin depletion by siRNA treatment did not affect γc receptor uptake. Together, the results obtained with the Eps15 dominant-negative mutant and with clathrin siRNA show for the first time that γc receptor is endocytosed independently of clathrin-coated pits and vesicles.

γc receptor is associated with lipid rafts

So far, the receptors internalized by a clathrin-independent process are at least partially located into lipid rafts at the plasma membrane (Nabi and Le, 2003). Therefore, we asked if this was also the case for γc receptor. To test this we used the fact that raft-associated proteins are insoluble in Triton X-100 at 4°C and can be furthermore isolated by flotation on a sucrose gradient. Thus, cells constitutively expressing γc receptor were fractionated by this method, and we observed that γc receptor was associated with these membrane microdomains, as well as the raft resident protein flotillin 2 (Fig. 3). As other receptors internalized by clathrin-independent endocytosis, γc receptor is associated with lipid rafts.

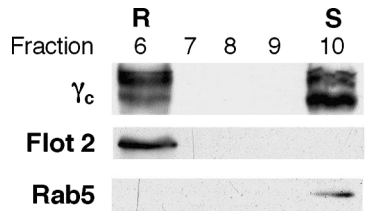

Figure 3. **The γc receptor is associated with lipid rafts.** Cells stably expressing γc receptor were extracted by 0.5% triton X-100 at 4°C and fractionated on a 36–5% sucrose density gradient and analyzed by Western blot. The low-density fraction (R) contained lipid raft-associated proteins such as flotillin 2 (Flot 2). The high-density fraction (S) contained the bulk-solubilized membrane-associated proteins such as Rab5.

Endocytosis of γc receptor is dynamin dependent

Next, we analyzed the involvement of the GTPase dynamin 2 in γc receptor internalization. The dynamin family contains three members, dynamin-1, the neuronal form, and dynamin-2 and -3 that share at least 70% homology, dynamin 2 being the ubiquitously expressed form (Cao et al., 1998). Dynamin is necessary for clathrin-dependent endocytosis, as well as for some, albeit not all, clathrin-independent pathways (Henley et al., 1998; Oh et al., 1998; Lamaze et al., 2001; Sabharanjak et al., 2002; Le and Nabi, 2003). To assay for dynamin involvement in γc receptor internalization, we made use of a dominant-negative mutant of dynamin 2 with an inactive GTPase, Dyn2K44A (Cao et al., 2000). Cells were cotransfected with γc gene and GFP-tagged Dyn2K44A or wild type. The cells were incubated at 37°C for 15 min with anti-γc receptor antibody to follow endocytosis. They were then fixed, permeabilized, and stained

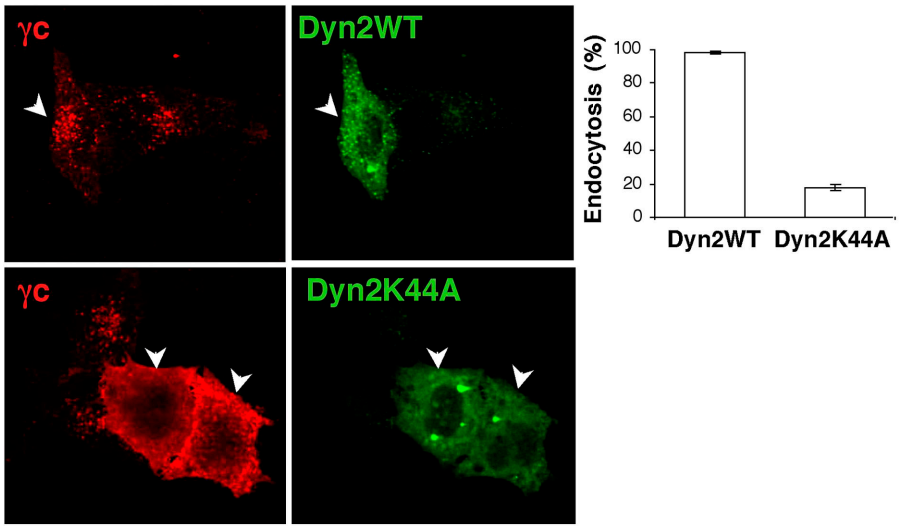

Figure 4. **Endocytosis of γc receptor is dynamin dependent.** Cells transfected with the γc receptor and GFP-tagged wild-type dynamin 2 (Dyn2WT) or GFP-tagged dominant-negative mutant (Dyn2K44A) were incubated for 15 min at 37°C with anti-γc receptor to follow endocytosis. They were then fixed, permeabilized, and labeled with second antibodies. Confocal medial optical cuts are shown. The arrowheads indicate doubly transfected cells. The results were quantified by assessing the percentage of transfected cells having internalized γc receptor (right, mean of three experiments ± SD).

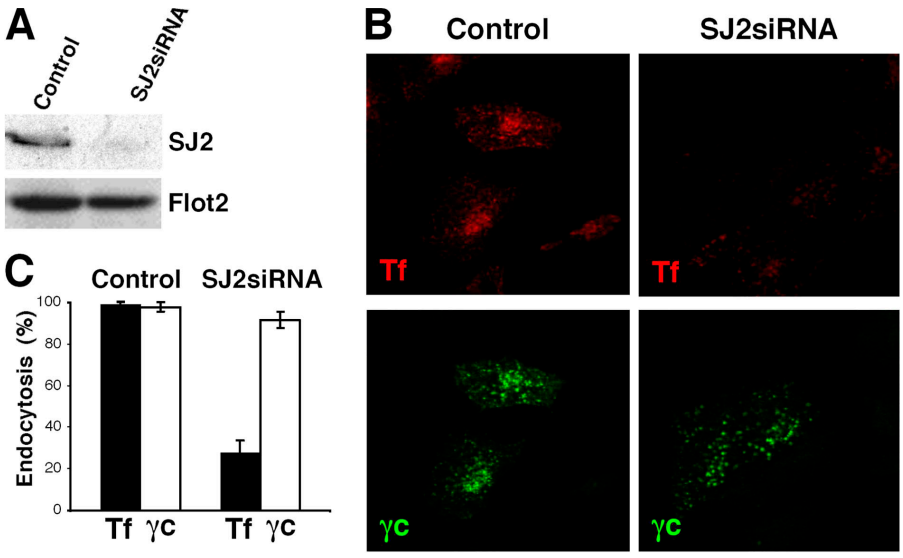

Figure 5. **Endocytosis of γc receptor does not require synaptojanin 2.** (A) Cells were transfected with the γc receptor and with siRNA against synaptojanin 2 (SJ2siRNA) or not (Control). Western blots analyses were done on protein extracts from 10^5 cells with antibodies against synaptojanin 2 (SJ2), and against flotillin 2 (Flot2) as a control. (B) Cells transfected with the γc receptor and with SJ2siRNA or not (control) were incubated for 15 min at 37°C with anti-γc receptor antibody and Tf-Cy3 to follow endocytosis. The cells were fixed and permeabilized, and γc receptor was stained with labeled second antibodies and the cells were analyzed by confocal microscopy. Confocal medial optical cuts are shown. The different images were taken with exactly the same settings. (C) The results were quantified by assessing the percentage of transfected cells having internalized Tf or γc receptor (mean of three experiments ± SD).

with labeled second antibody. In 85% of cells overexpressing Dyn2K44A, γc receptor mainly remained at the cell surface as observed by confocal microscopy (Fig. 4). As a control, overexpression of wild-type dynamin 2 did not affect γc receptor endocytosis (Fig. 4). This result shows that dynamin 2 is necessary for γc receptor internalization. Thus, γc receptor uptake represents another example of receptor-mediated endocytosis, which is clathrin independent and dynamin dependent.

Synaptojanin is not required for endocytosis of γc receptor

Dynamin has a pleckstrin homology domain that binds phosphatidylinositol (PI) lipids, thereby allowing for its association with membranes. The PI 5-phosphatase synaptojanin, which regulates PI(4,5)P_2 level in the cell, has recently been reported to be involved in the early steps of clathrin-dependent endocytosis (Malecz et al., 2000; Rusk et al., 2003). This protein appears to colocalize with dynamin on clathrin-coated pits and vesicles and can interact with various partners of dynamin and clathrin (Haffner et al., 2000; Rusk et al., 2003). Moreover, the fact that PI(4,5)P_2 is thought to transduce signals from the plasma membrane to the actin cytoskeleton suggests that synaptojanin could have a pleiotropic role in endocytic processes. Thus, we investigated the involvement of synaptojanin in γc receptor internalization. For this investigation, we engineered a siRNA directed against the ubiquitously expressed synaptojanin 2 and transfected it in cells expressing γc. In cells treated with synaptojanin 2 siRNA (SJ2siRNA), the amount of synaptojanin 2 was clearly reduced, as shown by Western blot, whereas a control protein, flotillin 2, was not affected (Fig. 5 A). Cells depleted of synaptojanin 2 with siRNA were allowed to internalize Tf and anti-γc receptor antibody for 15 min at 37°C. As shown in Fig. 5 (B and C), Tf endocytosis was deeply affected in more than 70% of siRNA-treated cells, in agreement with published data, whereas γc receptor endocytosis was not. Thus, the normal expression of synaptojanin is not necessary for the clathrin-independent pathway taken by γc receptor to function.

Actin polymerization is required for γc receptor endocytosis

In the past few years, it has been shown that actin polymerization was necessary for clathrin-dependent endocytosis (Lamaze et al., 1997; Merrifield et al., 2002). However, little is known in the case of clathrin-independent receptor-mediated internalization. We analyzed the effect on γc receptor entry of jasplakinolide, a drug-stabilizing actin filament, thereby preventing actin polymerization. Cells transfected with γc gene were pretreated with 1 μM jasplakinolide or DMSO, and γc receptor and Tf endocytosis were analyzed by confocal microscopy. Quantification of the results shows that γc receptor and Tf endocytosis were equally inhibited in ~65% of the jasplakinolide-treated cells (Fig. 6 A). Thus, actin polymerization is required for γc receptor uptake.

Role of proteins that link dynamin to the actin cytoskeleton in γc receptor internalization

Dynamin is a multidomain protein that is involved at different steps of clathrin-dependent endocytosis. Whereas its GTPase activity is responsible for vesicle scission, dynamin contains a carboxy-terminal PRD/arginine-rich domain that directly binds to SH3 domains of actin-associated proteins (Orth and McNiven, 2003). Recent reports have implicated several of these proteins in the classical clathrin-dependent pathway, showing links between this pathway and the actin cytoskeleton (Schafer, 2002). To further characterize the mechanism of γc receptor endocytosis, we focused on dynamin partners. Therefore, we investigated the potential implication of these actin- and dynamin-interacting proteins in γc receptor endocytosis.

Intersectin is a scaffolding protein belonging to this class of dynamin-binding proteins involved in clathrin-dependent endocytosis (Simpson et al., 1999). Intersectin has an Eps15 homology domain that recruits epsin. Epsin binds AP-2, thus linking intersectin to the clathrin-coated pathway (Yamabhai et al., 1998; Sengar et al., 1999). It also contains five SH3 domains binding to dynamin, synaptojanin, and N-WASP, the latter making a link with the actin cytoskeleton as it is a potent activator of

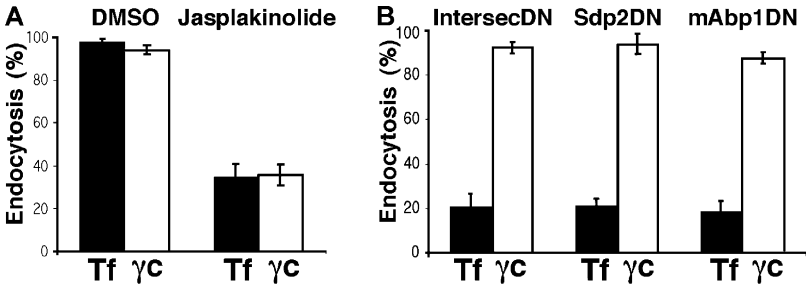

Figure 6. **Actin polymerization is required for γc receptor endocytosis, and intersectin, syndapin 2, and mAbp1 are not necessary.** (A) Cells transfected with the γc receptor were pretreated for 45 min at 37°C with DMSO or with jasplakinolide and incubated for 15 min at 37°C with anti-γc receptor antibody and Tf-Cy3 to follow endocytosis. (B) Cells were transfected with the γc receptor and either a GFP-tagged intersectin dominant-negative mutant (IntersecDN), the syndapin 2 dominant-negative mutant (Sdp2DN), or the mAbp1 dominant-negative mutant (mAbp1DN). The cells were incubated for 15 min at 37°C with anti-γc receptor antibody and Tf-Cy5 to follow endocytosis. In all experiments, the results analyzed by confocal microscopy were quantified by assessing the percentage of transfected cells having internalized Tf or γc receptor (mean of three experiments ± SD).

the Arp2/3 complex (Hussain et al., 2001). To test the involvement of intersectin in γc receptor internalization, cells were cotransfected with γc gene and with a dominant-negative mutant of intersectin composed of its five SH3 domains (IntersecDN; Simpson et al., 1999). Tf and γc receptor uptake were monitored in doubly transfected cells as described in the previous paragraph and their internalization was analyzed by confocal microscopy (Fig. 6 B). As expected, 85% of the transfected cells were deeply affected for Tf uptake. In contrast, no effect was observed for γc receptor endocytosis (Fig. 6 B). Thus, intersectin is a dynamin-binding protein that does not appear to be necessary for the clathrin-independent endocytosis of γc receptor.

The protein syndapin, which binds to the PRD domain of dynamin, has also been shown to regulate clathrin-dependent endocytosis (Qualmann and Kelly, 2000). In addition to dynamin, syndapin also interacts with N-WASP, providing a link with the actin cytoskeleton (Kessels and Qualmann, 2002). To assay for a putative function of syndapin in γc receptor uptake, cells were transfected with the dominant-negative mutant of syndapin 2 (Sdp2DN), composed of the SH3 domain of Sdp2 (Qualmann and Kelly, 2000), and endocytosis of the γc receptor was compared with that of Tf. In more than 80% of the cells overexpressing this dominant-negative mutant, Tf internalization was inhibited whereas endocytosis of the γc receptor was not affected (Fig. 6 B). Thus, syndapin is another example of a dynamin partner specifically involved in clathrin-mediated endocytosis.

Another protein, which links the actin cytoskeleton to endocytosis via dynamin, is mAbp1 (Schafer, 2002). This protein binds directly to the PRD domain of dynamin on one hand, and to F-actin on the other hand. mAbp1 is colocalized with dynamin in clathrin-coated pits and is involved in clathrin-mediated endocytosis (Kessels et al., 2001; Mise-Omata et al., 2003). Overexpression of the dominant-negative mutant of mAbp1 (mAbp1DN), composed of the SH3 domain of mAbp1, inhibits clathrin-mediated uptake (Kessels et al., 2001). Therefore, we transfected cells with this dominant-negative mutant and the γc gene, and assayed for γc receptor and Tf endocytosis. As previously reported, Tf internalization was inhibited in more than 80% of the cells expressing the mAbp1 mutant. However, endocytosis of γc receptor was not inhibited (Fig. 6 B). Once again, γc receptor endocytosis can be distinguished from clathrin-dependent internalization by the requirement for mAbp1.

Finally, the only other known F-actin binding protein that directly binds to the PRD domain of dynamin is cortactin (Orth and McNiven, 2003). As mAbp1, cortactin contains F-actin binding repeats and a SH3 domain at the COOH terminus that binds dynamin. In addition, it contains an NH_2-terminal motif (DDW) that binds directly to Arp3 to stimulate Arp2/3-dependent nucleation (Uruno et al., 2001; Weaver et al., 2002). Microinjection of anti-cortactin antibodies or transfection of the cortactin SH3 domain allowed

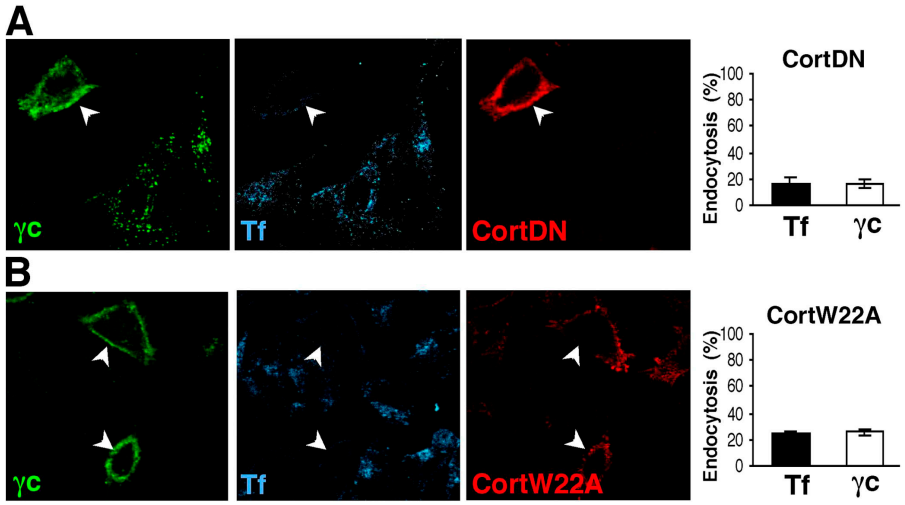

Figure 7. **Endocytosis of γc receptor is cortactin dependent.** Cells were transfected with γc receptor and with either the cortactin dominant-negative mutant (CortDN; A) or a mutant in the Arp3 binding site (CortW22A; B). The cells were incubated for 15 min at 37°C with anti-γc receptor antibody and Tf-Cy5 to follow endocytosis. They were then fixed, permeabilized, and stained with labeled antibodies and finally analyzed by confocal microscopy. A medial section is shown. The arrowheads indicate doubly transfected cells. The results were quantified by assessing the percentage of transfected cells having internalized Tf or γc receptor (right, mean of three experiments ± SD).

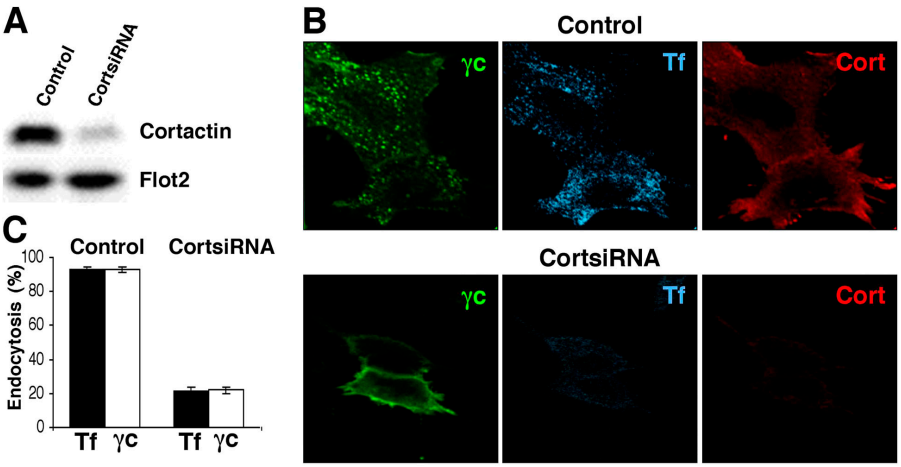

Figure 8. **Cortactin depletion affects clathrin-dependent and -independent endocytosis.** (A) Cells were transfected with the γc receptor and with siRNA targeting cortactin (CortsiRNA) or not (Control). Western blot analysis was performed on protein extracts from 10^5 cells with antibodies against cortactin and against flotillin 2 (Flot2) as a control. (B) Cells transfected with γc receptor and with cortsiRNA or not (Control) were incubated for 15 min at 37°C with anti-γc receptor antibody and Tf-Cy5 to follow endocytosis. The cells were fixed and permeabilized, γc receptor and cortactin were stained with labeled antibodies, and the cells were analyzed by confocal microscopy. Confocal optical medial cuts are shown. The different images were taken with exactly the same settings. (C) The results were quantified by assessing the percentage of transfected cells having internalized Tf or γc receptor (mean of three experiments ± SD). For CortsiRNA-treated cells, only cells depleted in cortactin were analyzed.

Cao et al. (2003) to demonstrate that cortactin is necessary for clathrin-mediated endocytosis. Therefore, we investigated its putative role in clathrin-independent uptake. Cells were cotransfected with γc gene and with the dominant-negative mutant of cortactin (CortDN), constituted by the SH3 domain of cortactin (Du et al., 1998). The distribution of Tf and of γc receptor were compared by confocal microscopy after allowing internalization of both markers simultaneously for 15 min at 37°C (Fig. 7 A). Tf internalization was inhibited in more than 80% of the cells expressing the cortactin dominant-negative mutant, in agreement with published data (Fig. 7 A). Strikingly, endocytosis of γc receptor was also inhibited in 85% of the cells expressing this mutant (Fig. 7 A). In addition, we used siRNA to decrease cortactin expression within the cells. In cells treated with cortactin siRNA (CortsiRNA), the amount of cortactin was clearly reduced, as shown by Western blot, whereas a control protein, flotillin 2, was not affected (Fig. 8 A). We analyzed the internalization of γc receptor and Tf in siRNA-treated cells by confocal microscopy (Fig. 8 B). As shown in Fig. 8 C, both Tf and γc receptor endocytosis were inhibited to the same extent (in ~80% of treated cells). Together, these experiments show that cortactin is essential for clathrin-dependent and -independent endocytosis. Cortactin and mAbp1, which have several common features, differ in their ability to bind directly to Arp3. We asked if the direct interaction of cortactin with Arp3 was important for endocytosis. Cells were cotransfected with γc gene and with the gene encoding cortactin mutated in the Arp3 binding site (CortW22A; Schafer et al., 2002). More than 80% of transfected cells were deficient in γc and in Tf internalization, as measured by confocal microscopy analysis and quantification (Fig. 7 B). Thus, cortactin needs to have its Arp3 binding site to participate correctly in these two endocytic pathways. In conclusion, cortactin is the only protein linking dynamin to the cytoskeleton known so far that is involved in both clathrin-dependent and -independent endocytosis.

Discussion

A growing number of proteins are internalized independently of clathrin-coated pits and vesicles, revealing the importance of these endocytic pathways. In this paper, we show that γc receptor is a new marker of clathrin-independent endocytosis. This receptor is associated with lipid rafts and its internalization requires dynamin and actin polymerization. In clathrin-dependent endocytosis, dynamin has been shown to play an important role in linking internalization and actin cytoskeleton. To further characterize the role of dynamin in clathrin-independent endocytosis, we examined the requirement of dynamin partners interacting with actin. We show that intersectin, syndapin, and mAbp1 are not required for γc receptor endocytosis, contrary to their participation in the clathrin-dependent way. Strikingly, the multidomain protein cortactin is necessary for both clathrin-dependent and -independent endocytosis. Cortactin is the first identified link between clathrin-independent endocytosis and actin dynamics.

We would like to discuss the specificity of action in internalization processes of each dynamin partner tested in this paper in light of what is currently known in the literature. The lipid $PI(4,5)P_2$ binds to several proteins such as epsin, AP-2, or dynamin and plays a central role in clathrin-mediated endocytosis (Jost et al., 1998; Ford et al., 2002; Rohde et al., 2002). Synaptojanin 2 is a phosphoinositide phosphatase that controls the amount of this lipid in the cell. In addition, synaptojanin 2, via its PRD domain, binds to clathrin-associated proteins endophilin and amphiphysin (Hill et al., 2001; Nemoto et al., 2001). Finally, synaptojanin 2 was shown to play an essential role in the early steps of clathrin-coated pits and vesicles formation (Rusk et al., 2003). Altogether, these findings suggest that synaptojanin is a component of the clathrin machinery. Therefore, our result showing that this phosphatase is not essential for γc receptor uptake is not surprising. Similar arguments can explain why intersectin is not necessary for the clathrin-independent endocytosis of γc receptor. Indeed, this protein contains

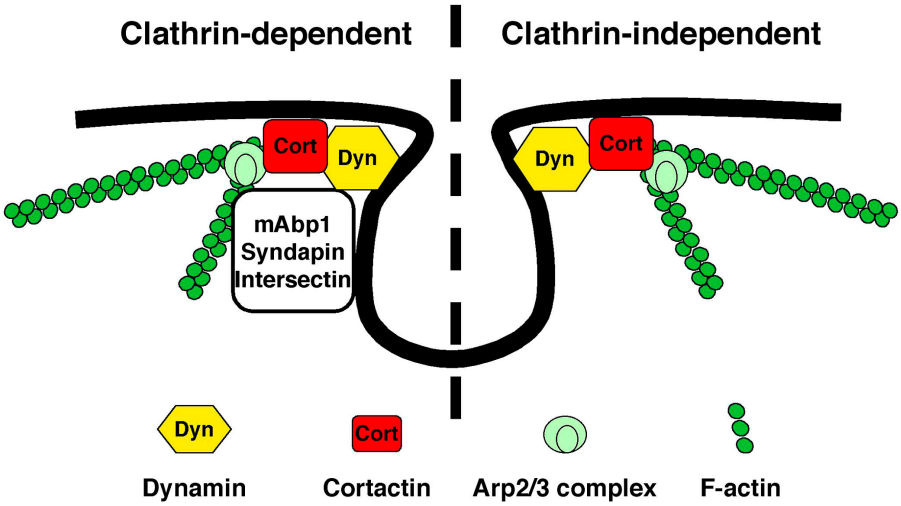

Figure 9. **Working model for the interface between endocytosis and actin cytoskeleton.** A core complex composed of dynamin, cortactin, Arp2/3 complex, and F-actin appears to be involved in both clathrin-dependent and -independent pathways. Cortactin is proposed to play a central role in connecting endocytosis to actin dynamics through dynamin. Syndapin, intersectin, and mAbp1 are actin-dynamin–interacting proteins specifically required in clathrin-coated pits and vesicles formation.

an Eps15 homology domain that interacts with epsin, a coiled-coil region binding to Eps15, and finally five SH3 domains interacting with several proteins including synaptojanin (Engqvist-Goldstein and Drubin, 2003). Thus, intersectin seems to be a specific player of clathrin-dependent endocytosis. However, a recent paper has shown that this protein was involved in caveolae fission in endothelial cells, suggesting that this protein may also be involved in caveolae-dependent internalization (Predescu et al., 2003). The abundance of caveolae in endothelial cells, and their crucial role in transcytosis, coupling endocytosis and exocytosis by rapid and efficient fusion-fission processes, suggested a particular function of caveolae in these cells. This finding might explain the special feature of intersectin in this case.

Syndapin has been involved in clathrin-dependent uptake (Qualmann and Kelly, 2000). In addition to dynamin, syndapin binds also to synaptojanin. Our results show that syndapin 2 is not required for the clathrin-independent endocytic pathway taken by γc receptor. Altogether, these findings suggest that syndapin may be a specific component of the clathrin-dependent endocytosis.

Cortactin and mAbp1 have been recently implicated in clathrin-mediated endocytosis (Kessels et al., 2001; Cao et al., 2003). Our study shows that whereas mAbp1 is not required for γc receptor uptake, cortactin is involved in this clathrin-independent endocytosis, as shown by using siRNA and cortactin mutants. These two proteins share a lot of common features. Indeed, both proteins bind directly to F-actin and dynamin (Orth and McNiven, 2003). In addition, cortactin and mAbp1 are Src kinase targets and are translocated to the cell periphery by the activation of Rac1 (Weed et al., 1998; Kessels et al., 2000). However, it is noteworthy that the two proteins have several specific partners. In particular, they differ by the presence of an acidic motif binding and activating Arp2/3 complex at the NH_2 terminus of cortactin (Weed et al., 2000). Interestingly, we found that a cortactin mutant (CortW22A), which no longer binds Arp3, inhibits clathrin-dependent and -independent endocytosis (Fig. 7 B). Thus, the differential effect of cortactin and mAbp1 on γc receptor endocytosis can be accounted for by their different properties in Arp3 binding. The results obtained with CortDN and CortW22A mutants suggest that both the SH3 domain and the Arp3 binding domain are important for cortactin function, as suggested by in vitro studies (Schafer et al., 2002). Cortactin is the only dynamin partner that has been shown to modulate actin dynamics. Indeed, mutations in cortactin avoiding its binding to Arp2/3 or dynamin decrease the actin dynamics of the cell (Schafer et al., 2002). Also, in vitro studies have shown that the complex formed by cortactin, Arp2/3, and dynamin promotes actin assembly (Schafer et al., 2002). Thus, cortactin constitutes the strongest link between endocytosis and the cytoskeleton. The fact that a cortactin mutant incapable of binding Arp3 affects both clathrin-dependent and -independent endocytosis suggests that the action of cortactin is linked to Arp2/3 complex and to actin polymerization.

Recently, clathrin-dependent endocytosis and caveolae-mediated uptake of Simian Virus 40 have been shown to involve actin polymerization at the site of entry (Merrifield et al., 2002; Pelkmans et al., 2002). We have shown here that actin polymerization is also necessary for clathrin-independent receptor-mediated endocytosis of γc receptor. Dynamin seems to play a crucial role in the actin cytoskeleton recruitment during endocytosis. The recent results concerning the clathrin-dependent uptake have allowed the identification of several dynamin-binding proteins that could act at the interface between endocytosis and actin dynamics (Orth and McNiven, 2003). In contrast, nothing was known for clathrin-independent endocytosis. However, the central role of dynamin in clathrin-dependent endocytic processes prompted us to test the role of actin-dynamin interacting partners in the clathrin-independent pathway used by the γc receptor. The majority of the known putative linker proteins seem to be solely involved in clathrin-mediated internalization. Cortactin is the only dynamin partner identified so far that is necessary for γc receptor internalization. Interestingly, the β chain of IL-2 receptor, another marker of the clathrin-independent pathway (Lamaze et al., 2001), also requires cortactin to be internalized (unpublished data), suggesting a general role of cortactin in clathrin-independent endocytosis. Thus, cortactin is involved in both clathrin-dependent

and -independent uptake. Our work points to an ubiquitous and pivotal role of cortactin in endocytosis. We propose a working hypothesis whereby dynamin, cortactin, Arp2/3, and F-actin constitute a core complex that would link endocytosis to actin dynamics (Fig. 9). The other dynamin partners, such as syndapin, intersectin, or mAbp1, specific to one endocytic pathway, could play a role in this dynamic process, in association to the core complex or not (Fig. 9). It is noteworthy that mAbp1, syndapin, and intersectin might be involved in some way in clathrin-independent uptake; if so, their role must be less critical than it is for the clathrin pathway. Future work will focus on the precise action of this complex during the different steps of internalization to characterize further the interface between endocytosis and the cytoskeleton.

Materials and methods

Cells and constructs

HeLa and Hep2 cells were maintained in DME containing 10% FCS. The T lymphocyte cell line Kit225, expressing constitutively γc receptor, was maintained in RPMI medium containing 10% FCS and 200 pM of IL-2 (a gift from A. Minty, Sanofi-Synthélabo, Labege, France). The plasmid pRCHγ encoding the γc receptor gene was described previously (Morelon and Dautry-Varsat, 1998). The GFP-tagged Eps15 dominant-negative mutant (EΔ95/295) was described previously (Benmerah et al., 1999). The GFP-tagged dynamin 2 aa wild type or mutant (K44A) were gifts from M.A. McNiven (Mayo Clinic, Rochester, MN; Cao et al., 1998, 2000). GFP-tagged SH3A-E of intersectin (IntersecDN) was a gift from P.S. McPherson (Montreal Neurological Institute, McGill University, Montreal, Canada; Simpson et al., 1999). The Xpress-tagged syndapin 2 SH3 (Sdp2DN) and the myc-tagged mAbp1 SH3 (mAbp1DN) were gifts of B. Qualmann and M.M. Kessels (Leibniz Institute for Neurobiology, Magdeburg, Germany; Qualmann and Kelly, 2000; Kessels et al., 2001). The myc-tagged cortactin SH3 (CortDN) and the flag-tagged cortactin with a point mutation in the Arp3 binding site (CortW22A) were gifts of J.T. Parsons (Health Sciences Center, University of Virginia, Charlottesville, VA; Du et al., 1998; Schafer et al., 2002).

Cells lysis and fractionation

Kit225 cells (5×10^7) were resuspended in 0.5 ml of ice-cold TBS (140 mM NaCl, 20 mM Tris, and 1 mM EDTA, pH 7.4), 0.5% Triton X-100, 1 mM phenylmethylsulfoxide, and 1% of inhibitor cocktail (Sigma-Aldrich). After 20-min incubation on ice, the lysate was passed 10 times through a 26G3/8 needle and centrifuged at 800 *g* for 10 min at 4°C. The supernatant mixed with an equal volume of 80% sucrose was fractionated on a 36–5% sucrose density gradient (Lamaze et al., 2001). Finally, proteins from the 10 fractions were precipitated by TCA and one third was loaded on SDS-PAGE and analyzed by Western blot with either goat anti-γc receptor (1:1,000, AF284; R&D Systems), mouse anti-rab5 (1:1,000; BD Biosciences), or mouse anti-flotillin 2 (1:2,000; BD Biosciences).

Transfection and siRNA experiments

HeLa cells (4×10^6 cells/point) were cotransfected by electroporation with 10 μg of pRCHγ and 20 μg of plasmid encoding the various mutants at 900 μF and 200 V (Easyject; Eurogentec). 24 h later, the cells were resuspended in DME containing 6 mM Na butyrate to enhance the expression of the mutants, and 24 h thereafter the cells were analyzed by immunofluorescence. siRNAs were synthesized by Dharmacon, and the siRNA duplexes were prepared according to the manufacturer's instructions to yield a 20-μM final concentration. The siRNA sequence targeting synaptojanin 2 was aacgugaacggaggaaagcagtt (Rusk et al., 2003), the one targeting clathrin heavy chain was taatccaattcgaagaccaat (Motley et al., 2003). Two siRNAs targeting cortactin were cotransfected in Hep2 cells, gacugagaagcaugccucctt (Bougneres et al., 2004) and ggagcauaucaacauacactt. We checked that an irrelevant siRNA had no effect on endocytosis. The cells were plated to 80% confluency in 6-well plates. 10 μl of duplexes siRNA or control (10 μl of TE 1/10: 1 mM Tris, pH 7.4, and 0.1 mM EDTA) were mixed in 42 μl of 250 mM $CaCl_2$ solution and added to the cells. Finally, 42 μl of HBS (50 mM Hepes, 280 mM NaCl, 10 mM KCl, and 1.5 mM Na_2HPO_4, pH 7.05) were added to the cells. 48 h later, 20 μg of pRCHγ DNA were introduced by electroporation in siRNA-transfected cells, together with 10 μl of CHCsiRNA or 10 μl of CortsiRNA duplexes, in experiments using these siRNAs. 24 h later, total protein extracts from 10^5 cells were loaded on SDS-PAGE and analyzed by Western blot with an anti-clathrin heavy chain mouse antibody (BD Biosciences; 1:1,000) or an anti-synaptojanin 2 rabbit antibody (1:2,000; a gift from M. Symons, Center for Oncology and Cell Biology, Institute for Medical Research at North Shore-LIJ, Manhasset, NY; Malecz et al., 2000) or an anti-cortactin mouse antibody (Upstate Biotechnology; 1:2,000) or an anti-flotillin 2 mouse antibody (BD Biosciences; 1:2,000). The secondary antibodies used were alkaline phosphatase-coupled anti–rabbit or anti–mouse antibodies (Pierce Chemical Co.). The Western blots were revealed by ECF (Amersham Biosciences) and quantified using a Storm FluoroImager (Molecular Dynamics).

Endocytosis, immunofluorescence, and confocal microscopy

Endocytosis of γc receptor and Tf were performed for 15 min at 37°C as described previously (Morelon and Dautry-Varsat, 1998) using anti-γc receptor Tugh4 (rat antibody; BD Biosciences; 1:50) or MAB284 (mouse antibody; R&D Systems; 1:50) and 50 nM of human iron-loaded Tf conjugated to Cy5 or Cy3 (Lamaze et al., 2001). In Fig. 6 A, HeLa cells were pretreated for 45 min at 37°C with either DMSO (1:1,000) or Jasplakinolide (1 μM; Molecular Probes). Immunofluorescence experiments were performed as described previously (Morelon and Dautry-Varsat, 1998) with anti-Xpress (mouse antibody; Invitrogen; 1:500), anti-myc (mouse antibody 9E10, ascites 1:500), anti-flagM5 (mouse antibody; Sigma-Aldrich; 1:100), or anti-cortactin mouse antibody (Upstate Biotechnology; 1:200). Secondary antibodies were Alexa Fluor 488–coupled anti–rat (Molecular Probes; 1:100), fluorescein (FITC)-coupled anti–mouse IgG (Southern Biotechnology Associates, Inc.; 1:100), or Texas red–coupled anti–mouse IgG_1 (Southern Biotechnology Associates, Inc.; 1:100). Confocal microscopy was performed on a microscope (model TCS4D; Leica) using a 63× objective. Z series of optical sections were acquired at 0.7 μm. Cy5, FITC, and Texas red emissions were collected separately to avoid fluorescence passage from one channel to another. Quantification of Tf and γc receptor uptakes were performed within the same cell and for at least 60 doubly transfected cells in at least three experiments.

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