Endosomal clathrin drives actin accumulation at the immunological synapse

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Summary
Antigen-specific cognate interaction of T lymphocytes with antigen-presenting cells (APCs) drives major morphological and functional changes in T cells, including actin rearrangements at the immune synapse (IS) formed at the cell–cell contact area. Here we show, using cell lines as well as primary cells, that clathrin, a protein involved in endocytic processes, drives actin accumulation at the IS. Clathrin is recruited towards the IS with parallel kinetics to that of actin. Knockdown of clathrin prevents accumulation of actin and proteins involved in actin polymerization, such as dynamin-2, the Arp2/3 complex and CD2AP at the IS. The clathrin pool involved in actin accumulation at the IS is linked to multivesicular bodies that polarize to the cell–cell contact zone, but not to plasma membrane or Golgi complex. These data underscore the role of clathrin as a platform for the recruitment of proteins that promote actin polymerization at the interface of T cells and APCs.

Key words: Actin, Clathrin, Cytoskeleton, Immunological synapse

Introduction
T cells have a central role in adaptive immunity, either by enhancing or suppressing immune responses through antigen-triggered cytokine secretion or by destroying antigen-bearing cells (Friedl et al., 2005). T cells are activated by the establishment of cell–cell contacts with antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages or B cells (Friedl et al., 2005; Reichardt et al., 2007). These contacts, which involve recognition of antigen-bearing MHC molecules on APCs by the T-cell receptor (TCR), are known as immunological synapses (ISs) (Billadeau et al., 2007; Friedl et al., 2005; Vicente-Manzanares and Sanchez-Madrid, 2004). The IS is highly dynamic and in its more structured form is organized into concentric rings of multimolecular assemblies. The central region, called the central supramolecular activation cluster (cSMAC), contains the clustered MHC-conjugated TCR complexes, including CD3, together with the co-stimulator CD28 and other signaling molecules; surrounding this structure is a ring of adhesion molecules called the peripheral supramolecular activation cluster (pSMAC), which includes the integrins LFA-1 (integrin αLβ2) and VLA-4 (integrin α4β1) (Dustin, 2009; Mittelbrunn et al., 2004). TCR stimulation during immune synapsing triggers major morphological and functional changes in T cells, including a large-scale accumulation of actin, which increases the area of T cell and APC interaction (Billadeau et al., 2007; Vicente-Manzanares and Sanchez-Madrid, 2004). Actin polymerization defects result in impaired T cell activation and deregulation of cytokine secretion (Nolz et al., 2007), demonstrating that these actin rearrangements are absolutely necessary for productive IS formation and lymphocyte activation.

Signaling initiated at the IS involves numerous TCR-proximal kinases and adaptor molecules whose actions culminate in the activation of the Arp2/3 complex, which promotes actin nucleation and polymerization, as well as branching of actin filaments (Billadeau et al., 2007; Vicente-Manzanares and Sanchez-Madrid, 2004). Arp2/3 activators identified at the IS include the Wiskott–Aldrich syndrome protein (WASP), Hs1 (cortactin homolog) and WAVE2 (Billadeau et al., 2007). Moreover, the large GTPase dynamin-2 has recently been shown to be recruited to the IS, where it regulates the reorganization of F-actin (Gomez et al., 2005). Nevertheless, the initial link between TCR activation and actin polymerization at the IS remains elusive.

In many aspects, the signaling and cytoskeletal rearrangements triggered by TCR activation at the IS resemble events that occur in other systems, such as lamellipodium formation at the advancing zone of migrating cells, or the contact zones formed between pathogens and host cells. For example, the receptor-activated signaling pathways that lead to actin polymerization during bacterial infection, similarly to those occurring at the IS, involve Arp2/3, N-WASP, WAVE, CD2AP (CD2-associated protein) and dynamin-2, among other proteins (Hamon et al., 2006; Veiga and Cossart, 2007).
2005). Interestingly, it has been recently demonstrated that clathrin, which is well known for its central role in endocytic processes, has a determining role in actin remodelling during bacterial and fungal infections (Veiga and Cossart, 2005; Veiga et al., 2007). These studies suggest that membranes coated with clathrin might serve as a molecular platform for recruitment of the protein machinery necessary to trigger localized actin polymerization during pathogen invasion. This hypothesis, together with the work of others (Yarar et al., 2005) challenged the classical view of clathrin-mediated endocytosis, which in mammalian cells was assumed to occur independently of actin (Conner and Schmid, 2003). Clathrin functions by binding to cell membranes through interaction with proteins called clathrin adaptors (Owen et al., 2004). The association of clathrin with the plasma membrane during endocytosis has been extensively studied (Conner and Schmid, 2003; Ungewickell and Hinrichsen, 2007). Clathrin is also detected in intracellular compartments, associated mainly with the Golgi complex (Jaiswal et al., 2009), where it is also involved in vesicle formation, and with late endosomes known as multivesicular bodies (MVBs) (Murk et al., 2003; Raiborg et al., 2002; Raiborg et al., 2006). MVBs form characteristic structures of vesicles within vesicles that can be detected by electron microscopy (Murk et al., 2003; Raiborg et al., 2002). Endocytic vesicles containing activated receptors are recognized by ESCRT complexes, four protein complexes (0, I, II, III, IV) that work in tandem to promote the internalization of the cargo-containing vesicles into the MVV (Slagsvold et al., 2006). These MVVs are able to fuse with lysosomes, thereby degrading the endocytic cargo (Hanson et al., 2009; Williams and Urbe, 2007). Recently, it has been described that TSG101, which forms part of ESCRT-I, regulates IS structure (Vardhana et al., 2010). Moreover, lyso-bis-phosphatidic acid, which is typically found in MVVs (Kobayashi et al., 1998), was detected at the cSMAC (Varma et al., 2006), suggesting that some interactions might occur between MVVs and the IS. However, whether MVVs polarize to the IS needs corroration, and the functional roles that MVVs have at the IS remain unknown. Here, we demonstrate that clathrin accumulates and regulates actin rearrangements at the IS in T cells. We additionally show that clathrin is required for the recruitment of proteins involved in actin polymerization such as dynamin-2, the Arp2/3 complex and CD2AP. We also show that the clathrin pool involved in actin accumulation at the IS is associated with MVVs, which polarize to the IS, and not to plasma membrane or Golgi complex.

**Results**

**Clathrin polarizes towards the immunological synapse**

We first examined clathrin localization in conjugates of T cells and APCs. Jurkat T cells bearing the TCR Vβ8 chain recognize the staphylococcal enterotoxin E (SEE) superfantigen bound to MHC class II in Raji APCs (Montoya et al., 2002). Upon stimulation of Jurkat T cells with SEE-loaded APCs, endogenous T cell clathrin relocalized and accumulated at the IS, as revealed by immunofluorescence using antibodies against the clathrin heavy chain (Fig. 1A). TCR stimulation by SEE-loaded APCs was confirmed by the clustering of CD3 at the cSMAC (Fig. 1A).

To quantify clathrin accumulation in individual immune synapses, we developed new algorithms and software, called Synapse Measures, which can be used as a plug-in for ImageJ (http://rsweb.nih.gov/ij/) and which will be made freely available. By analyzing and comparing fluorescence signals from several small regions in the T cell, the APC and in the IS, this program estimates the contribution of background fluorescence and the constitutive fluorescence of cell borders, thereby yielding more accurate measurements of localized immunofluorescence. A detailed description of the Synapse Measures program, including the algorithms used, is included in the Materials and Methods. Using this method, we were able to accurately quantify the ratio between the immunofluorescence intensity of T cell clathrin (and other proteins) at the IS with that remained in the rest of the T cell (Fig. 1B). The concentration of clathrin at the IS increased 2.6-fold when the TCR was stimulated with antigen (Fig. 1C; each dot in the figure corresponds to a single IS). A fluorescently tagged clathrin light chain fused to td-Tomato (td-Tomato–Lca) (Massol et al., 2006) also accumulated at the IS (Fig. 1D), validating its use for dynamics studies. The time courses of the recruitment of clathrin (td-Tomato–Lca) and actin (GFP–actin) (Boyer et al., 2006) to the IS were monitored by fast acquisition (~0.9 second intervals) of confocal stacks from live cells, using a Leica TCS-SP5 microscope equipped with a resonant scanner (Fig. 1E and supplementary material Movie 1). Over the period examined, the kinetics of clathrin accumulation at the IS mirrored that of actin (Fig. 1F).

To ascertain whether clathrin polarizes to the IS in a more physiological setting, we used conjugates formed by primary T cells obtained from peripheral blood lymphocytes (PBLs) of healthy human donors. To enrich the heterogeneous population of PBLs in T cells harbouring the TCR Vβ8 chain (which is able to recognize the SEE), the PBLs were treated with SEE, IL-2 and phytohaemagglutinin (PHA). This treatment selectively expanded the T cell population responding to SEE. We used Raji cells as APCs and primary DCs derived from monocytes obtained from healthy human donors. We examined clathrin localization in T-cell–APC conjugates. Clathrin from primary T cells bearing TCR Vβ8 chain relocalized and accumulated at the IS upon T cell stimulation with either Raji or primary DCs loaded with SEE, as revealed by immunofluorescence using antibodies against the clathrin heavy chain (Fig. 2A,B). TCR stimulation by SEE-loaded APCs was confirmed by the clustering of TCR at the IS (Fig. 2A,B). Quantification of clathrin accumulation using the Synapse Measures plug-in confirmed that the concentration of clathrin at the IS increased more than twofold when the TCR from primary T cells was antigen stimulated by both Raji and primary DCs (Fig. 2C).

**Clathrin is necessary for actin accumulation at the immunological synapse**

The role of clathrin in actin accumulation at the IS was examined by siRNA knockdown (KD) of clathrin heavy chain in Jurkat T cells. Reduced expression of clathrin was confirmed by fluorescence microscopy (Fig. 3A) and immunoblot (Fig. 3B). Note that clathrin reduction did not affect cell viability nor cell division rate (data not shown). In the presence of SEE-loaded APCs, clathrin-depleted T cells, which present the same levels of total actin that cells with normal amounts of clathrin (Fig. 3B), were unable to polymerize actin at the IS (Fig. 3A). However, CD3 accumulation at the T-cell–APC contact site appeared to be unaffected, suggesting that T cell activation is not impaired by partial depletion of clathrin (Fig. 3A, right). The frequency of T-cell–Raji conjugate formation measured by immunofluorescence (not shown), or by flow cytometry (looking for double positives: CD3+ for T cells and cellular marker CMTMR for Raji cells) was variable and dependent of the experiment, but showed no significant differences between control and clathrin-
knockdown (KD) Jurkat T cells (supplementary material Fig. S1A). Actin dynamics in clathrin KD T cells expressing GFP–actin was monitored by live-cell imaging using a Leica multidimensional microscope. Clathrin KD T cells were unable to accumulate GFP–actin to the IS (supplementary material Movie 2).

Quantification of actin accumulation with Synapse Measures confirmed that clathrin-depleted T cells were unable to accumulate GFP–actin to the IS (supplementary material Movie 2).

As the observed decrease in actin polymerization at the IS could be due to reduced levels of relevant receptors for TCR signaling and IS formation, as a control we further tested the effect of clathrin KD on T cell activation and surface expression of receptors implicated in IS signaling by flow cytometry. Surface levels of TCR, CD3, CD28, CD4, LFA-1 and VLA-4 were not reduced by clathrin depletion (Fig. 4A). CD3 surface expression was slightly increased within clathrin KD T cells, indicating that at least part of the CD3 population is constitutively removed from the plasma membrane by clathrin-mediated endocytosis. In addition, clathrin KD did not affect the increased surface expression of CD69, an early marker of T cell activation, observed upon stimulation of T cells with anti-CD3 and anti-CD28 antibodies (Fig. 4B). Likewise, clathrin depletion did not inhibit the rapid phosphorylation of PLCγ, Zap70 and ERK induced by antibody stimulation (Fig. 4C). Moreover, the levels of protein phosphorylation after antibody stimulation were slightly higher in clathrin KD T cells (Fig. 4D). Note that experiments shown in Fig. 3 were done in parallel (with the same samples) with those shown in Fig. 4.
To assess the functional consequences of the effect of clathrin silencing on the physiology of the T cells, we measured the secretion of IL-2. It has been shown that reducing actin polymerization at the IS, either by using drugs, or by depleting actin-binding proteins, alters the secretion of IL-2 (Nolz et al., 2007; Perez-Martinez et al., 2010). Clathrin reduction deregulates IL-2 secretion (supplementary material Fig. S1B), in a similar way to that observed by others using low doses of cytochalasin D (Nolz et al., 2007). Clathrin is necessary for recruitment of key adaptor and regulatory proteins involved in actin polymerization to the immunological synapse

To investigate the possibility that clathrin serves as a molecular platform for the recruitment of actin-polymerizing proteins, we assessed the role of clathrin in the recruitment of the large GTPase dynamin-2, which promotes actin polymerization at the IS (Gomez et al., 2005). Time-lapse confocal analysis of T-cell–APC conjugates showed that dynamin-2 and clathrin relocate to the IS at the same rate (Fig. 5A and supplementary material Movie 3); moreover, clathrin and dynamin-2 accumulated at the same areas of the IS (Fig. 5A,B). By contrast, clathrin-depleted T cells were unable to accumulate dynamin-2 when challenged with SEE-loaded APCs (Fig. 5B, bottom panels and Fig. 5C). Clathrin depletion also impaired the recruitment towards the IS of Arp2/3 complex (Fig. 5D). CD2AP, another important mediator of TCR-induced actin
Our fluorescence microscopy data suggest that clathrin at the IS associates with internal membranes and not with the plasma membrane (Fig. 1A and supplementary material Fig. S2). Clathrin has been detected in association with cellular organelles such as the Golgi complex and MVBs (Jaiswal et al., 2009; Murk et al., 2003; Raiborg et al., 2002), as well as at the plasma membrane. To identify the source of the clathrin pool that drives actin polymerization at the IS, we monitored the association of actin with markers of the Golgi and MVB during IS formation. Giantin, a Golgi marker (Linstedt and Hauri, 1993) did not colocalize with actin (Fig. 6A). The characteristic multivesicular appearance of MVBs on electron micrographs is driven by the ESCRT (endosomal sorting complex required for transport) machinery, which inserts vesicles into late endosomes (Linstedt and Hauri, 1993). In contrast to giantin, two ESCRT components, Hrs and Vps4 (Slagsvold et al., 2006; Williams and Urbe, 2007), localized with actin at the IS in SEE-stimulated T cells (Fig. 6A, middle and bottom panels), whereas in unstimulated T cells, these MVB markers were localized in vesicles all around the cell, and did not accumulate at cell–cell contacts (supplementary material Fig. S4A). These data confirm previous studies indicating that some interactions might occur between the IS and the MVBs. For example, lyso-bis-phosphatidic acid, a marker for MVBs, (Kobayashi et al., 1998), has been detected in association with cellular organelles such as the Golgi complex and MVBs (Jaiswal et al., 2009; Murk et al., 2003; Raiborg et al., 2002), as well as at the plasma membrane. To identify the source of the clathrin pool that drives actin polymerization at the IS, we monitored the association of actin with markers of the Golgi and MVB during IS formation. Giantin, a Golgi marker (Linstedt and Hauri, 1993) did not colocalize with actin (Fig. 6A). The characteristic multivesicular appearance of MVBs on electron micrographs is driven by the ESCRT (endosomal sorting complex required for transport) machinery, which inserts vesicles into late endosomes (Linstedt and Hauri, 1993). In contrast to giantin, two ESCRT components, Hrs and Vps4 (Slagsvold et al., 2006; Williams and Urbe, 2007), localized with actin at the IS in SEE-stimulated T cells (Fig. 6A, middle and bottom panels), whereas in unstimulated T cells, these MVB markers were localized in vesicles all around the cell, and did not accumulate at cell–cell contacts (supplementary material Fig. S4A). These data confirm previous studies indicating that some interactions might occur between the IS and the MVBs. For example, lyso-bis-phosphatidic acid, a marker for MVBs, (Kobayashi et al., 1998), has been recently detected in the IS (Varma et al., 2006).

We next studied the recruitment of clathrin to T cells depleted of AP-1, AP-2 or Hrs (Fig. 6B), which are adaptor molecules for the association of clathrin with the Golgi, plasma membrane and MVB, respectively (Owen et al., 2004). None of the siRNAs used affected surface expression of TCR, CD3, CD28, CD4, LFA-1 or VLA-4 (not shown). Knockdown of Hrs, and to a limited extent AP-1, impaired clathrin accumulation at the IS, whereas knockdown of the plasma membrane adaptor AP-2 had no effect (Fig. 6C). A similar dependence on Hrs was seen for actin accumulation at the IS.

**Actin rearrangements at the immunological synapse are driven by endosomal clathrin**

Our fluorescence microscopy data suggest that clathrin at the IS associates with internal membranes and not with the plasma membrane (Fig. 1A and supplementary material Fig. S2). Clathrin polymerization (Badour et al., 2003), also accumulated at the IS in non-silenced SEE-challenged T cells, and this accumulation was impaired in clathrin KD T cells (Fig. 5E). Remarkably, clathrin depletion also abolished IS-directed relocalization of Huntingtin-interacting protein-1-related protein (Hip1R), which regulates interactions between clathrin-coated membranes and the actin cytoskeleton (Engvigstad-Goldstein et al., 2004; Wilbur et al., 2008) (Fig. 5F). To verify that accumulation of clathrin, actin, dynamin-2, Arp2/3 complex, CD2AP and Hip1R at the IS was specific and to validate the Synapsis Measures software, we analyzed the cytoplasmic accumulation of GFP and CD45, a membrane tyrosine phosphatase, which after initial cell–cell contact does not accumulate at the IS (Blanchard et al., 2002; Freiberg et al., 2002; Varma et al., 2006), in T-cell–APC conjugates in the presence and absence of antigen. Endogenous CD45 and GFP did not accumulate at the IS after antigen stimulation in control or clathrin KD T cells, and there was no significant difference in TCR accumulation at the IS between control and clathrin KD Jurkat T cells. These data confirm the active and specific accumulation at the IS of clathrin, actin and actin-polymerization promoters. Nevertheless, a remaining question is the source of clathrin promoting such actin polymerization at the IS.
depletion of Hrs inhibited actin accumulation, whereas no major changes could be observed in cells depleted of AP-1 or AP-2 (Fig. 6D). The role of Hrs in actin accumulation at the IS was verified with an independent set of siRNAs targeting Hrs (supplementary material Fig. S4B). The mild alteration to actin polymerization in AP-1-depleted cells suggests a minor role of the Golgi in actin accumulation at the IS; however, these experiments indicate that clathrin associated with MVBs is the main source for the recruitment of proteins that promote actin polymerization during IS formation. Consistent with this view, typical MVB structures accumulated in T cells in close contact with SEE-loaded APCs (Fig. 6E).

To confirm the role of clathrin and MVBs in actin accumulation at the IS in primary T cells, clathrin heavy chain and Hrs were knocked down by siRNA (Fig. 7A). In the presence of SEE-loaded Raji APCs, actin clearly accumulated at the IS, whereas depletion of clathrin or Hrs in T cells impaired such actin accumulation at the IS (Fig. 7B,C). Depletion of clathrin or Hrs also impeded actin accumulation at the IS in conjugates formed by primary T lymphoblasts and DCs (Fig. 7D and supplementary material Fig. S5). Note that clathrin- and Hrs- depleted primary T cells present the same levels of total actin as seen in cells with normal amounts of clathrin and Hrs (Fig. 7A). TCR accumulation (visualized by immunofluorescence with anti-Vβ8 chain antibodies) at the T-cell–APC contact site appeared unaffected, suggesting that T cell activation is not impaired by partial depletion of clathrin or Hrs in primary T cells (Fig. 7B).

**Discussion**

The present study provides compelling evidence that endosomal clathrin is essential for massive actin polymerization at the IS formed between T cells and APCs. Clathrin is the major protein involved in clathrin-mediated endocytosis, where it forms a coat around the pinching membrane and enables multiple protein
interactions that are necessary for endocytosis to proceed (Ungewickell and Hinrichsen, 2007). It is well established that clathrin-mediated endocytosis in yeast requires actin rearrangement (Engqvist-Goldstein and Drubin, 2003). However, the situation in mammalian cells is less clear: whereas many authors show data involving actin in clathrin-dependent endocytosis (Chen and Brodsky, 2005; Engqvist-Goldstein et al., 2004; Ferguson et al., 2009; Toshima et al., 2005; Yarar et al., 2005), others show that actin depletion has no effect on the internalization dynamics of clathrin-coated vesicles (Boucrot et al., 2006). Studies with bacterial and fungal pathogens show that clathrin and actin both participate in pathogen internalization (Moreno-Ruiz et al., 2009; Veiga and Cossart, 2005; Veiga et al., 2007), and it has been proposed that mammalian cells support internalization of large membrane plaques in a process involving clathrin and actin that is very similar to ‘classical’ clathrin-mediated endocytosis (Saffarian et al., 2009).

There are two known connections between actin and clathrin assemblies. One involves dynamin 2 and cortactin, which activates the actin nucleation factor Arp2/3 (McNiven et al., 2000; Uruno et al., 2001; Weaver et al., 2001). The other is Hip1R, which contains binding sites for F-actin, cortactin and clathrin light chains (Engqvist-Goldstein et al., 2004; Wilbur et al., 2008). A recent study shows that Hip1R is necessary for actin recruitment to clathrin-coated pits during endocytosis in Dictyostelium discoideum (Brady et al., 2010). However, D. discoideum Hip1R does not have a cortactin-binding motif and therefore these results might not be applicable to mammalian cells. Our results not only confirm that clathrin participates in biological events requiring massive actin polymerization, but also demonstrate that clathrin serves as a molecular platform to recruit the necessary proteins able to polymerize actin (dynamin-2, CD2AP and Arp2/3) at the IS. We also show that clathrin is necessary to recruit Hip1R to the IS, but

![Fig. 6. Multivesicular bodies accumulate at the IS.](image-url)

(A) Confocal immunofluorescence images showing the cellular localization of giantin (Golgi marker) upper panels, HRS and VPS4 (MVB markers) middle lower panels, respectively (all in green) and actin (red) at the IS. APCs are identified in the merged image by the blue CMAC fluorescence. (B) Immunoblot showing suppression of Jurkat T cell protein expression of Hrs, AP-1 and AP-2 by siRNA knockdown (KD). (C, D) Control, Hrs KD, AP-1 KD and AP-2 KD T cells were conjugated with SEE-loaded (+) or unloaded (–) APCs, and Synapse Measures was used to analyze accumulation of immunostained clathrin (C) and actin (D) at the cell–cell contact area. Each dot corresponds to an individual T-cell–APC contact. More than 300 (C) or 400 (D) cellular contacts were analyzed in three independent experiments. ***P<0.005; **P<0.05 (Mann–Whitney test). Red lines indicate the median values. (E) Electron micrograph of the IS. The image on the right shows a high-magnification view of boxed region in the left image. Arrows indicate multivesicular bodies close to the cell–cell contact.
understanding the role played by Hip1R at the IS requires further investigation. Notably, clathrin depletion did not reduce the surface exposure of the main membrane receptors involved in IS formation, the number of T-APC conjugates or the TCR signalling. Note that CD3 surface expression appeared to be slightly increased in clathrin KD T cells, suggesting that a fraction of CD3 molecules is internalized by clathrin-mediated endocytosis in non-stimulated cells. In this regard, it has been shown that after CD3 stimulation, endocytosis of non-engaged (bystander) TCRs is clathrin dependent, in contrast to engaged TCRs where endocytosis was clathrin independent (Monjas et al., 2004). However, the complete view of TCR and CD3 recycling and endocytosis in resting and stimulated cells is far from understood and deserves further investigation.

IL-2 secretion appeared to be deregulated (increased) in clathrin KD cells, in a similar way to what has been shown in cells treated with low doses of cytochalasin D (Nolz et al., 2007), but the mechanisms linking the actin cytoskeleton, TCR-dependent signalling and cytokine secretion remain unclear. These data, together with the quantification of polymerized actin at the IS, show that actin polymerization occurs at low levels in clathrin and Hrs KD cells, in contrast to the extraordinary levels observed in control T cells expressing normal amounts of clathrin at MVBS. Partial inhibition of actin polymerization (using low doses of cytochalasin D) have long been known to enhance integrin function (Kucik et al., 1996), which, together with the slight increase of CD3 expression observed on the surface of clathrin KD cells, could partially explain the increase of IL-2 secretion. This increased IL-2 secretion, as well as the increased TCR-dependent signalling that occurs in clathrin KD T cells, could also be explained by recent data suggesting that the actin cytoskeleton somehow destabilizes TCR–MHC interactions, as shown by treatment with cytochalasin D and latrunculin A, which greatly prolonged TCR-MHC interactions at the IS (Huppa et al., 2010).

Our data with T cell lines and with primary T cells show that the clathrin adaptor for MVBS (Hrs) has a determinant role in actin polymerization at the IS. Notably, actin accumulation at the IS occurred independently of the main clathrin adaptors in the plasma membrane and Golgi complex. The dependence of clathrin and actin localization at the IS on Hrs indicates that the main platform for the recruitment of actin polymerization promoters during IS formation is clathrin associated with MVBS, which is consistent with the polar redistribution of MVBS observed by immunofluorescence and electron microscopy. These data are in agreement with recent reports suggesting MVBS recruitment to the IS. It has been shown that TSG101, an ubiquitin-binding protein that is involved in cargo sorting into MVBS, regulates IS structure (Vardhana et al., 2010). These authors also showed that lyso-bisphosphatidic acid, typically found in MVBS (Kobayashi et al., 1998), can be detected at the cSMAC (Varma et al., 2006), suggesting that some interactions might occur between MVBS and the IS. Our data confirm the proposed association of MVBS with the IS and furthermore, show a role for clathrin-rich MVBS as a platform to orchestrate massive actin polymerization. Interestingly, MVBS polarization to the plasma membrane also occurs during viral budding, another process in which clathrin has a major role (Morita and Sundquist, 2004). The recruitment dynamics of clathrin, actin and dynamin occur in parallel, suggesting that the machinery needed to initiate the actin polymerization process is preformed at the surface of the MVBS.

The findings presented here suggest a model for actin polymerization in which antigen stimulation induces rapid movements of clathrin-containing MVBS towards the cell–cell contact site to promote localized actin polymerization. Whether this MVBS-clathrin platform has a similar role in other cellular events (e.g. migration and adhesion) that require massive polarized actin polymerization remains to be determined.
Materials and Methods

Cells

The acute human leukaemia Jurkat J77/T20 Vβ1 T cell line and the Burkitt lymphoma Raji B cell line were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors by separation on a Lymphoprep gradient (Nycomed, Oslo, Norway) according to standard procedures. Monocytes were purified from PBMC by a 30 minute adherence step at 37°C in RPMI supplemented with 10% fetal calf serum. Nonadherent cells were washed off and the adhered monocytes were immediately subjected to the DC differentiation protocol, as described (Sallusto et al., 1995). Briefly, monocytes were cultured in RPMI, 10% FCS containing IL-4 (10 ng/ml), R&D Systems, Minneapolis, MN) and 1000 U/ml GM-CSF (Peprotech, London, UK). Cells were cultured for 6 days, with cytokine re-addition every 48 hours, to obtain a population of immature DCs. Phenotypic characteristics of these cells were assessed by flow cytometry on day 6 (HLA-DR, CD1a, CD209, CD14).

Human CD4+ T cells were purified from PBMCs, using MACS (magnetic-activated cell sorting) and anti-CD8 microbeads from Miltenyi (Bergisch Gladbach, Germany) and stained with FITC-conjugated F(ab')2 fragment of goat anti-mouse Ig. These cells were preincubated with 0.1 μg/ml of treatment, around 30% of the cells were positive for TCR-Vβ-3 days with 20 U/ml human recombinant IL-2 and 0.5% osmium tetroxide from Electron Microscopy Sciences.

purchased from Invitrogen, SEE from Toxin Technologies, cytochalasin D and mouse Ig was from DakoCytomation. Cell trackers CMAC and CMTMR were purchased from Molecular Probes. FITC-conjugated F(ab')2 fragment of goat anti-mouse Ig was from DakoCytomation. Cells were cultured for 6 days, with cytokine re-addition every 48 hours, to obtain a population of immature DCs. Phenotypic characteristics of these cells were assessed by flow cytometry on day 6 (HLA-DR, CD1a, CD209, CD14).

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AP-1 was knocked down with a single double-stranded RNA sequence (s 5'-H11032/Ctg-3'). All double-stranded RNAs were purchased from Ambion or Dharmacon. Typically 400 μl of 1 μM siRNA were electroporated into 2×106 cells. Experiments were performed 48 hours after RNA transfection.

Antibodies and reagents

Antibodies used were as follows: anti-β-actin mouse monoclonal antibody (mAb) (AC-15; Sigma); anti-AP-1 mAb (γ-adaptin, Sigma); anti-AP-2 mAb (AP-20, Benton Dickinson (BD)); anti-CDC2-AP rabbit polyclonal (rAb) (Abcam); anti-CDC3 mAb (BD); anti-CDC3 mAb (T3B; produced in the laboratory (PIL)); anti-CDC4 mAb (BD); anti-CDC5 mAb (BD); anti-CDC6 mAb (T3P-P5; PIL); anti-clathrin heavy chain mAb (X22; from Abcam and Pierce, normally used in western blots); anti-clathrin heavy chain mAb (Abcam), normally used in immunofluorescence assays. X22 was also used for immunofluorescence with the same results (not shown); anti-dynamin-2 rAb (Abcam), anti-Erk1/2 mAb (Millipore); anti-phosphorylated ERK1/2 mAb (Cell Signaling); anti-giantin rAb (Abcam); goat anti-guinea pig (Abcam); guinea pig anti-Hip1-R (gift from David G. Drubin, University of California at Berkeley, CA); anti-HRS rAb (HGS Abcam), anti-HRS mAb (HGS; Abnova); anti-LFA-1 pAb (Cell Signaling); anti-P-PLC-β1 rAb (Millipore); anti-Zap70 mAb (BD); anti-CD28 mAb (BD); anti-CD69 mAb (TP1-55; PiL); anti-clathrin heavy chain mAb (BD), anti-CD3 mAb (BD), anti-CD4 mAb (BD), anti-CD8 mAb (BD), anti-ERK1/2 mAb (BD), anti-p-guinea pig (Abcam); guinea pig anti-Hip1-R (gift from David G. Drubin, University of California at Berkeley, CA); anti-HRS rAb (HGS Abcam), anti-HRS mAb (HGS; Abnova); anti-LFA-1 pAb (Cell Signaling); anti-P-PLC-β1 rAb (Cell Signaling); anti-tubulin mAb (Sigma); anti-VP54 mAb (gift from Alberto Fraile, Centro Nacional de Biotecnologia (CSIC), Madrid, Spain); anti-α4-integrin mAb (PIL), anti-b1-integrin mAb (PIL); anti-Zap70 mAb (Abcam); anti-PLC-β1 mAb (BD); anti-Zap70 mAb (BD) (Y943; Abcam). Phalloidin and goat anti-rabbit and goat anti-mouse antibodies conjugated to Alexa Fluor 488, 546 and 647 were purchased from Molecular Probes. FITC-conjugated Fab'2 fragment of goat anti-mouse Ig was from DakoCytomation. Cell trackers CMAC and CMTMR were purchased from Invitrogen, SEE from Toxic Technologies, cytochalasin D and glutaraldehyde from Sigma, dicyclohexylcarbodiimide from Fluka and paraformaldehyde and osmium tetroxide from Electron Microscopy Sciences.

Plasmids

The plasmid encoding dynamin-2-GFP (the dynamin isoform aa) was a gift from Mark A. Mclnen (Mayo Clinic, Rochester, MN) (Cao et al., 1998). Plasmid encoding td-Tomato–LCA (Massol et al., 2006) was a gift from Tomás Kirchhausen (Harvard Medical School, Children’s Hospital and Immune Disease Institute, Boston, MA). The plasmid encoding GFP-actin (Boyer et al., 2006) was a gift from Emmanuel Lenèceix (Université de Nice, Nice, France).

Confluent microscopy

Confocal images were acquired with a Leica TCS-SP5 confocal microscope (63×) under the control of a Leica LAS AF. The images were processed with ImageJ (1.38; http://rsweb.nih.gov/ij/index.html).

Synapse measures

Quantification of the amount of clathrin (or any protein) at the T cell side of the IS (S) with respect to the amount in the rest of the T cell (T) presents a difficult problem. First, simply computing the ratio between the average pixel values at the synapse and at the T cell border does not account for the contribution to both averages of the background (Bg) fluorescence; and second, part of the fluorescence at the synapse is caused by the B cell (B) and the contribution of the constitutive fluorescence of the T cell border. To separate all these contributions, we measured the average intensity value of the image in small circles, as shown in Fig. 1B. For measurements covering more than one region (B, T, S), the circles were placed in such a way that each region (e.g. B cell and background in the case of the B measurements) occupied approximately one half of the circle area. Taking many measurements of each kind tends to compensate for the differences in area due to the manual placement of the circles. For each kind of measurement (Bg, B, T and S), we computed the pixel value average within the multiple circles associated with that region within the same image. As a result of the location of the circle, half of the fluorescence values from the B measurements are caused by the background fluorescence whereas the other half are caused by the constitutive fluorescence of the B cells plus the background fluorescence. The same idea applies to the T measurements. The S measurements have contributions from the background, half of the constitutive fluorescence of the B-cells, and half of the fluorescence of the extra amount of clathrin present at the synapse coming from the T cell. We can express this ideas algebraically as:

\[
\begin{align*}
\text{Bg} & = B + T \\
\text{B} & = B + T \\
\text{T} & = B + T \\
\text{S} & = B + T \\
\end{align*}
\]

where \( B, T, S \) are the average values of our measurements, \( B_g \) is an estimate of the expected background fluorescence, \( B \) is an estimate of the constitutive fluorescence of the B-cells, \( T \) is an estimate of the constitutive fluorescence of the T cells outside the synapse, and \( S \) is an estimate of the constitutive fluorescence of the T cells at the synapse. From the measurement model, the estimates can be easily derived as:

\[
\begin{align*}
\text{Bg} & = 1 - 0.5 + 0.5 \\
\text{B} & = 1 - 2 + 2 \\
\text{T} & = 1 - 0.5 + 0.5 \\
\text{S} & = 1 - 2 + 2 \\
\end{align*}
\]

From this we can estimate the ratio between the fluorescence at the T cell synapse and in T cell regions outside the synapse as \( Z = T / T \). This ratio is computed for each IS image.

Statistical analysis

Differences between groups were analyzed by the Mann–Whitney U-test (unless otherwise noted). Differences were considered significant at \( P<0.05 \). Unless otherwise stated, all experiments were performed at least three times, and data are presented as median values.

Cytochalasin treatment

J77 T cells were incubated with 10 μM cytochalasin in DMSO for 30 minutes at 37°C. J77 cells were then conjugated for 15 minutes with SEE-primed or unprimed Raji B cells at 37°C, and conjugates were fixed in 3% paraformaldehyde in PBS.
Clathrin promotes actin at the IS

II-6 secretion analysis

Conjugates were formed and cultured in flat-bottom, 96-well plates. Culture supernatant was harvested after culture for 16 hours and analyzed for II-6 concentration by ELISA (Diaclone, Gen-Probe-TDI, Stamford, CT).

Live-cell Imaging

Jurkat T cells expressing td-Tomato-LcA, GFP-actin or dynamin-2-GFP were reseeded in HBSS containing 2% FBS and seeded in a glass-bottomed dish. See-loaded CMAC-labelled Raji APCs were added, and conjugate formation was monitored for 15 minutes. Images were acquired with a Leica TCS-Sp5 confocal microscope equipped with a resonant scanner under the control of Leica LAS AF software. Three confocal planes were acquired approximately every second. Resulting images were processed with ImageJ 1.38 (http://rsbweb.nih.gov/ij/index.html). The movies presented showed the maximal projection of the three confocal planes.

Electron microscopy

T-cell-APC conjugates were fixed in 2.5% glutaraldehyde in PBS for 30 minutes, and the fixation buffer was then substituted with 1% osmium tetroxide for 45 minutes. Samples were dehydrated through a series of ethanol solutions (5%, 25%, 50%, 75%, 95% and 100%). After the last dehydration step, samples were embedded in DURCUPAN resin and stored overnight at room temperature. The resin column was then polymerized by baking at 60°C for 48 hours, after which sections were cut. Sections were warmed with a JEOL TEM1010 electron microscope (100 kV) equipped with a Bioscan digital camera (Gatan). Images were monitored with DigitalMicrograph 3.1 (Gatan).

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