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*J Immunol* 2004; 172:2030-2038; doi: 10.4049/jimmunol.172.4.2030
http://www.jimmunol.org/content/172/4/2030

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2004/02/06/172.4.2030.DC1

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Visualization of Antigen Presentation by Actin-Mediated Targeting of Glycolipid-Enriched Membrane Domains to the Immune Synapse of B Cell APCs

Claire Gordy, Sudha Mishra, and William Rodgers

Glycolipid-enriched membrane (GEM) domains, or lipid rafts, function in signaling in immune cells, but their properties during Ag presentation are less clear. To address this question, GEM domains were studied using fluorescence cell imaging of mouse CH27 B cells presenting Ag to D10 T cells. Our experiments showed that APCs were enriched with GEM domains in the immune synapse, and this occurred in an actin-dependent manner. This enrichment was specific to GEM domains, because a marker for non-GEM regions of the membrane was excluded from the immune synapse. Furthermore, fluorescence photobleaching experiments showed that protein in the immune synapse was dynamic and rapidly exchanged with that in other compartments of CH27 cells. To identify the signals for targeting GEM domains to the immune synapse in APCs, capping of the domains was measured in cells after cross-linking surface molecules. This showed that co-cross-linking CD48 with MHC class II was required for efficient capping and intracellular signaling. Capping of GEM domains by co-cross-linking CD48 and MHC class II occurred with corecapping of filamentous actin, and both domain capping and T cell-CH27 cell conjugation were inhibited by pretreating CH27 cells with latrunculin B. Furthermore, disruption of the actin cytoskeleton of the CH27 cells also inhibited formation of a mature immune synapse in those T cells that did conjugate to APCs. Thus, Ag presentation and efficient T cell stimulation occur by an actin-dependent targeting of GEM domains in the APC to the site of T cell engagement. The Journal of Immunology, 2004, 172: 2030–2038.
T cell, thus providing a mechanism for delivery of GEM-associated molecules to the immune synapse in the APC.

Materials and Methods

Cell culture and transfection
CH27 cells were grown in DMEM supplemented with antibiotics and 15% FBS. D10 cells were grown in complete medium containing RPMI 1640 supplemented with antibiotics, 10% FBS, glutamine, 10 μM ciprofloxacin hydrochloride, 2 U/ml IL-2, 200 U/ml IL-4, and 1 ng/ml IL-1β. All cells were maintained at 37°C in the presence of 5% CO2.

CH27 cells were transfected with vector encoding L10-GFP or S15-GFP (21) by electroporation (Gene Pulse II; Bio-Rad, Hercules, CA). For transfection, 107 cells were suspended in 50 μl of 150 mM NaCl, 20 mM sodium phosphate (pH 7.4; PBS) containing 25 μg of plasmid DNA. Settings of 330 V and 960 μF were used for electroporation. Forty-eight hours post-transfection, the cells were seeded at 104 cells/well in a 96-well plate in medium containing G418 (1.0 mg/ml). After drug selection, viable clones were selected and enriched for the expression of GFP using flow cytometry (Oklahoma Medical Research Foundation Flow Cytometry Core Facility).

Cell conjugation
D10 cells recognize conalbumin in the context of I-Ak (22). CH27 cells were therefore pulsed with conalbumin as previously described (1) before adding them to the D10 cells. In brief, CH27 cells were incubated overnight with 500 μg/ml conalbumin at a cell density of 5 × 105/ml in DMEM containing antibodies, 15% FBS, and 50 mM HEPES (pH 7.4). For cell conjugation, an equal number of D10 and Ag-pulsed CH27 cells were mixed and sedimented by brief centrifugation (Eppendorf model 5417C; Brinkmann Instruments, Westbury, NY). The sample was then transferred to 37°C and incubated for 15 min before seeding onto coverslips coated with polylysine (Sigma-Aldrich, St. Louis, MO). In experiments measuring the frequency of cell conjugation by flow cytometry, D10 cells were labeled with a 20 μg/ml solution of Dil (Molecular Probes, Eugene, OR) in RPMI 1640 before adding them to Ag-pulsed CH27 cells. After labeling, the D10 cells were washed and added to CH27 cells at a ratio of 5:1. Conjugation was performed as described above, except conjugate formation was measured by flow cytometry immediately after incubation of the samples at 37°C. In experiments in which cells were treated before conjugation, 107/ml CH27 cells were incubated for the indicated times in DMEM plus 50 mM HEPES (pH 7.4) containing either 10 mM methyl-β-cyclodextrin (MβCD; Sigma-Aldrich), 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine (PP2; Calbiochem, La Jolla, CA), or 5 μM latrunculin B (Calbiochem). Additions were made from 100X stock solutions in DMSO (Sigma-Aldrich), and the samples were repeatedly washed with DMEM containing 50 mM HEPES (pH 7.4) before addition of D10 cells.

Conjugation of CH27 cells to Ab-coated beads was performed using 6-μm latex beads (Polysciences, Warrington, PA) coated with Ab to I-Ak (clone 11-5-2; BD Pharmingen, San Diego, CA), CD48 (clone HM48-1; BD Pharmingen), or I-Ak and CD48. The beads were prepared as previously described (23). For conjugation, beads at a density of 107/ml were added to an equal number of CH27 cells. The samples were briefly centrifuged (Eppendorf; Brinkmann Instruments) and transferred to 37°C for 10 min before seeding onto polylysine-coated coverslips.

Fluorescence labeling of cells
Samples were fixed with a 2% paraformaldehyde solution in PBS after seeding onto coverslips. For experiments including staining of intracellular proteins, the sample was permeabilized after fixation using 0.2% Triton X-100 in PBS. Filamentous actin was stained using a 0.1 μg/ml solution of Texas Red-conjugated phallloidin (Molecular Probes). I-Ak was stained using clone 11-5-2 in the first step and Texas Red-conjugated Fc-specific anti-Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) in the second step. Phosphotyrosine was stained using biotinylated clone 4G10 (Upstate Cell Signaling Solutions, Charlottesville, VA) and streptavidin-conjugated Texas Red (Molecular Probes). Protein kinase C-θ (PKC-θ) was stained using primary Ab from rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) and Texas Red-conjugated anti-rabbit Ig.

Fluorescence microscopy and image analysis
Confocal imaging was performed using a Zeiss LSM510 laser scanning confocal microscope (Oberkochen, Germany; Cell Imaging Core Facility, Oklahoma Medical Research Foundation). GFP imaging was performed by exciting at 488 nm and collecting emission wavelengths between 530 and 560 nm. For imaging of Texas Red fluorescence, the samples were excited at 543 nm, and emission wavelengths 600–660 nm were collected. In double-labeling experiments, GFP and Texas Red fluorescence were collected simultaneously in separate channels. Size calibrations were performed using calibrated fluorescent beads (Polysciences). During live cell imaging, the samples were maintained at 37°C in complete medium plus 50 mM HEPES (pH 7.4).

Image processing was performed using IP Lab Spectrum software (Signal Analytics, Vienna, VA). Protein enrichment in immune synapses was quantitated as previously described (16) based on the average fluorescence intensity of the synapse and nonsynapse regions of the outer membrane. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) measurements were performed as previously described (24), except that in the FRAP experiments the regions were photobleached using a 2-s pulse of laser illumination, and recovery of fluorescence was monitored by collecting frames at a frequency of every 7 s. The mobile fraction of protein and time constant for its recovery were quantitated by fitting the recovery to the function F(t) = F∞ + F0 × e−t/τ, where F∞ represents the fraction of recovery at infinite time and indicates the mobile fraction of the molecule in the bleached region or, inversely, its immobile fraction, and τ is the time constant for recovery and is inversely proportional to the diffusion coefficient. The recovery values were corrected for loss in signal during the experiment as previously described (24, 25). The diffusion coefficient (D) of each sample was calculated by fitting the bleaching profile to a Gaussian function (24, 26, 27).

Results

GEM domains are quickly and specifically targeted to the immune synapse in APCs
APCs are enriched with GEM domains in the immune synapse (10, 28). However, the kinetics of targeting GEM domains in the APC to the cell interface is unknown. To address this question, live cell imaging was performed with conalbumin-pulsed CH27 cells expressing L10-GFP and presenting Ag to D10 T cells (Fig. 1A and supplemental Fig. 1). The top row of Fig. 1A consists of confocal images of a CH27 cell as it formed an immune synapse with a D10 T cell. The positions of the CH27 and T cells are visible in the differential interference contrast (DIC) images in the bottom row. Contact between the T cell and the B cell began at 0:00 h, and enrichment of L10-GFP in the region of cell-cell adhesion was visible 2 min after the initial contact. An even greater enrichment occurred 4 min after initial cell-cell contact, and this was maintained through the rest of the experiment for the following 20 min. Thus, enrichment of L10-GFP in the immune synapse occurred within minutes of initial cell conjugation, and it was sustained for a prolonged period of time.

To determine whether the enrichment of L10-GFP in the APC represented a specific enrichment of GEM domains, the localization of L10-GFP in CH27 cells conjugated to D10 cells was compared with that of S15-GFP, which contains the first 15 aa of p60(CD79a or CD79b) fused to the N terminus of GFP and labels the non-GEM regions of the plasma membrane (21). In this experiment, Ag-pulsed CH27 cells that expressed either L10-GFP or S15-GFP were mixed with the D10 cells and incubated at 37°C for 15 min before fixing and measuring by confocal microscopy. An example of the localization of L10-GFP and S15-GFP in the plasma membrane of an APC relative to an immune synapse is shown in the confocal images in Fig. 1B and is quantitated in the accompanying fluorescence intensity profiles. The profiles were generated by measuring the intensity of the plasma membrane of the CH27 cells around the circumference of the cell. The vertical lines in the plots mark the boundaries of the immune synapse. These data show that the L10-GFP in Fig. 1B exhibited a 2- to 2.5-fold enrichment in the immune synapse over the nonsynapse regions of the plasma membrane. Furthermore, the region of the immune synapse enriched with L10-GFP was coenriched with I-Ak (Fig. 1C). Conversely,
S₁₅-GFP was depleted from the immune synapse relative to the remaining plasma membrane. The data in Fig. 1B are representative of many additional images that were acquired in the same manner. For example, the relative enrichment of L₁₀-GFP and S₁₅-GFP in immune synapses was measured for populations of B cell-T cell conjugates by dividing the average fluorescence intensity of the plasma membrane of each CH27 cell where it contacted a T cell by the average intensity of the remaining plasma membrane. The measured values are plotted in the histograms in Fig. 1D, and these show that S₁₅-GFP was most frequently depleted from the immune synapse, but L₁₀-GFP was often enriched by >1.6-fold. Thus, the plasma membrane of CH27 cells was specifically enriched with GEM domains where it formed an immune synapse with T cells.

**Targeting of MHC II to the cell interface requires GEM domains**

Consistent with earlier findings (10), we found that B cell-T cell conjugation was sensitive to treatment of CH27 cells with MβCD. For example, measurement of cell conjugation by flow cytometry showed that an ∼50% reduction in conjugate formation occurred when CH27 cells were pretreated with MβCD (Fig. 2A). However, the effect of MβCD on cell conjugation was reversible, because CH27 cells that were returned to complete medium for several hours after extraction exhibited similar levels of conjugation to D10 cells as untreated samples (data not shown). Thus, CH27 cells remained viable after treatment with MβCD.

**FIGURE 1.** L₁₀-GFP is quickly targeted to the immune synapse in CH27 cells, but S₁₅-GFP is excluded. A. Live cell imaging of a CH27 cell expressing L₁₀-GFP as it forms an immune synapse with a D10 T cell. The top and bottom rows contain confocal and DIC images of the sample, respectively. 0:00 represents the time when the B cell and the T cell first make contact. B. Top, Confocal (top) and DIC (bottom) images of D10-conjugated CH27 cells expressing either L₁₀-GFP (left) or the non-GEM marker S₁₅-GFP (right). The arrowheads indicate immune synapses that formed by conjugation of the CH27 cells with the D10 T cells. The white bars represent 5 μm. B. Bottom, The fluorescence intensity of the plasma membrane was measured around the perimeter of each of the CH27 cells in the top panels and is plotted in the corresponding graphs. The y-axis of the plots represents the relative fluorescence intensity of the pixels and has arbitrary units that range in value from 0–255. The x-axis represents the distance around the periphery of the cell. The region between the vertical lines corresponds to the immune synapse. C. Left, Confocal images of a CH27 cell expressing L₁₀-GFP and stained with an mAb specific to I-A^k and a Texas Red-conjugated secondary Ab. The white arrow indicates an immune synapse that has formed where the CH27 cell has conjugated to a D10 cell. The position of each cell is visible in the DIC image. C. Right, Fluorescence intensity profiles of the cell measured in the GFP (green) and Texas Red (red) channels. The bars indicate the boundaries of the immune synapse. D. Histograms of enrichment of L₁₀-GFP (top) and S₁₅-GFP (bottom) in immune synapses. CH27 cells expressing L₁₀-GFP or S₁₅-GFP were conjugated to D10 cells as described in B. The relative enrichment of GFP fluorescence in the immune synapse was calculated by dividing the average intensity of the plasma membrane in the synapse by the average fluorescence intensity of the remaining outer membrane. The sample sizes were 35 and 38 for the L₁₀-GFP and S₁₅-GFP samples, respectively.
As shown in Fig. 2A, a measurable fraction of CH27 cells continued to conjugate to D10 cells after cholesterol extraction. However, these cells showed an inhibited targeting of I-Ak to the cell interface (Fig. 2B). For example, the fraction of D10-conjugated CH27 cells that had a 1.5-fold or greater enrichment of I-Ak at the B cell-T cell interface decreased from 50% in the untreated sample to 20% in that extracted with MβCD. Thus, intact GEM domains are necessary for efficient targeting of MHC II complexes to the B cell-T cell interface.

One explanation for the observed effect of MβCD on cell conjugation and targeting of MHC II complexes to the cell interface is that extraction of cholesterol causes protein aggregation (8, 29), which then inhibits protein mobility for diffusion to the site of Ag presentation. To test this hypothesis, FRAP measurements were performed using MβCD-treated CH27 cells expressing L10-GFP. In FRAP experiments, a region of the outer membrane is photobleached by a brief pulse of laser illumination, and recovery of fluorescence in the bleached region is indicative of mobile protein. One such FRAP experiment of an MβCD-treated CH27 cell is shown in Fig. 2C and supplemental Fig. 2, and the confocal images (top panel) show most of the fluorescence recovered after photobleaching. The recovery of L10-GFP was quantitated by fitting the data to a monoexponential function (30), and from this it was determined 78% of the L10-GFP was mobile (Fig. 2C, bottom panel). We therefore conclude that GEM-associated proteins such as L10-GFP remain mobile after treatment with MβCD for targeting to the cell interface to form an immune synapse.

GEM-associated proteins are mobile in the immune synapse, and they exchange quickly with nonsynapse protein

To measure the mobility of L10-GFP in the immune synapse of APCs, FRAP measurements were performed using L10-GFP-expressing CH27 cells conjugated to D10 cells. One example of a FRAP experiment measuring the diffusion of L10-GFP in the immune synapse is shown in Fig. 3A and supplemental Fig. 3. In this experiment and others, a large majority of the L10-GFP was mobile, such that the average mobile fraction and time constant of L10-GFP measured in six separate CH27 cells were 77% (SD = 18%) and 31 s (SD = 6 s), respectively. Furthermore, the recovery of L10-GFP corresponded to a diffusion coefficient (D) of ~0.02

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μm²/s, similar to that measured for other GEM-associated molecules in cell membranes (31). Conversely, negligible recovery occurred if the cells were fixed before the FRAP measurements (not shown).

To further characterize the dynamics of GEM-associated proteins present in immune synapses, FLIP experiments were performed to measure the exchange of L₁₀-GFP between the immune synapse and the remaining plasma membrane (30). In the FLIP experiments, a region of the immune synapse was repeatedly bleached with prolonged pulses of laser illumination (5 s), and the decay of the fluorescence signal of the cell was measured by acquiring an image of the sample after each photobleaching pulse. Thus, FLIP experiments measure the diffusion of fluorophore from all regions of the cell into the path of the laser illumination. An example of a FLIP experiment with photobleaching of L₁₀-GFP within an immune synapse is shown in Fig. 3B and supplemental Fig. 4, during which most of the plasma membrane fluorescence was photobleached within ~1 min. Interestingly, intracellular membranes that contained L₁₀-GFP were also photobleached during the experiment, and this could occur by trafficking of protein between the plasma membrane and intracellular compartments. To quantitate the rate of photobleaching in Fig. 3, the fluorescence intensity of the entire cell was measured at each time point, and these values were plotted vs time (Fig. 3B, bottom panel). The fluorescence decay was best fit to a double-exponential function with time constants of 40 and 3 s. This result is consistent with the exchange of protein between the immune synapse and the remaining cell occurring by multiple processes, such as diffusion within the plasma membrane and intracellular trafficking. Together, the results in Fig. 3 show that L₁₀-GFP diffuses quickly in the immune synapse and between the immune synapse and nonsynapse regions of the cell.

Specific capping of GEM domains occurs by co-cross-linking I-A<sup>K</sup> and CD48

To identify the signals in APCs for assembling GEM domains in immune synapses, CH27 cells expressing L₁₀-GFP were incubated with 6-μm polystyrene beads that were coated with Ab to I-A<sup>K</sup>, CD48, or both I-A<sup>K</sup> and CD48. CD48 is a GPI-anchored protein that is a ligand for CD2, and it has been demonstrated in T cells to provide signals for membrane capping (32). Confocal microscopy showed that only those cells that were incubated with the cocated beads exhibited capping of GEM domains at the bead-cell interface (Fig. 4A, panel 3). Furthermore, the capping induced by the cocated beads represented a specific clustering of GEM domains, as S₁₅-GFP was not enriched in the membrane caps (Fig. 4B). Capping of either L₁₀-GFP or S₁₅-GFP by the Ab-coated beads was quantitated for many cells by measuring the relative enrichment of GFP fluorescence at the cell-bead interface. Again, those cells containing a 1.5-fold or greater enrichment of fluorescence were scored as exhibiting capping. The results are plotted in Fig. 4C, and they are consistent with those in Fig. 4, A and B, showing that efficient capping of GFP fluorescence occurred only in CH27 cells that expressed L₁₀-GFP and were incubated with cocated beads. Thus, cross-linking I-A<sup>K</sup> alone is not sufficient for inducing capping of GEM domains, but, rather, requires additional signals that are provided by co-cross-linking CD48 with I-A<sup>K</sup>.

Signals for capping GEM domains in APCs are GEM dependent and downstream of Src kinases

To further characterize the signals for capping GEM domains, protein tyrosine phosphorylation was measured in CH27 cells after cross-linking either I-A<sup>K</sup> or CD48 or co-cross-linking both molecules together. Consistent with the data in Fig. 4, the most robust signaling occurred in the sample with co-cross-linking (Fig. 5A). Furthermore, by separating the Triton X-100-soluble and insoluble membrane fractions after stimulation, it was determined that phosphotyrosine signals occurred in the detergent-insoluble GEM fraction (data not shown). Similarly, phosphotyrosine signals colocalized with L₁₀-GFP where beads cocated with Abs to I-A<sup>K</sup> and CD48 contacted the cell surface (Fig. 5B, arrowhead). Extensive capping of L₁₀-GFP is not evident in Fig. 5B, most likely because the beads were incubated in the sample for only 5 min before fixing and staining, rather than for 15 min as in the experiments shown in Fig. 4.

The results in Fig. 5, A and B, suggests that the signals for capping GEM domains in CH27 cells include protein tyrosine kinase activity that is enriched in GEM domains. To further test this hypothesis, CH27 cells were pretreated with either MβCD or the Src kinase inhibitor PP2 before stimulation by co-cross-linking I-A<sup>K</sup> and CD48. As shown in Fig. 5C, both PP2 and MβCD significantly inhibited protein tyrosine phosphorylation after cell

![FIGURE 4. GEM domains in CH27 cells are assembled into membrane caps by co-cross-linking CD48 and I-A<sup>K</sup>.](http://www.jimmunol.org/Downloadedfrom HTTP://www.jimmunol.org)
stimulation. In addition, measurement of confocal images of PP2- and MβCD-treated CH27 cells expressing L10-GFP demonstrated that these treatments also inhibited capping of GEM domains (Fig. 5D). These results therefore show that capping of GEM domains in CH27 cells requires Src kinase activity and GEM-associated signaling molecules.

The actin cytoskeleton of the APC is essential for both capping GEM domains and T cell-APC conjugation

Our earlier studies with T cells and adherent cells showed that GEM domains are assembled into membrane patches and caps by associating with the actin cytoskeleton (7, 16). Similarly, we determined that the GEM-enriched caps of CH27 cells stimulated with Ab-coated beads were coenriched with filamentous actin (Fig. 6A). These caps occurred at the site of cell-bead contact, including regions around the bead as the APC membrane began to surround the bead. To determine whether the actin cytoskeleton functioned in assembling GEM domains in immune synapses in CH27 cells, cells expressing L10-GFP were treated with latrunculin B prior to addition of cocated beads. As shown in Fig. 6B, latrunculin B caused an ~75% reduction in the frequency of capping of GEM domains by co-cross-linking CD48 and I-Ak.

To further determine the role of the actin cytoskeleton in Ag presentation, cell conjugation was measured in samples in which CH27 cells were pretreated with latrunculin B. CH27 cells were untreated or treated with PP2 or MβCD before stimulating by co-cross-linking CD48 and I-Ak. Control samples received carrier alone. A. Both CD48/I-Ak-mediated capping of L10-GFP in CH27 cells and T cell-APC conjugation are actin dependent. A, Top, Confocal images of a CH27 cell expressing L10-GFP and stimulated with beads coated with anti-CD48 and anti-I-Ak. The sample was immunostained with Ab to phosphotyrosine. The arrowheads indicate an L10-GFP-enriched patch at the bead-cell interface that is coenriched with phosphotyrosine. The position of the bead is indicated by the asterisk. B, Bottom, Fluorescence intensity profiles of the cell that were measured in the GFP (green) and Texas Red (red) channels. The vertical lines indicate the boundaries where the bead contacts the cell surface. C, Antiphosphotyrosine immunoblots of CH27 cells treated with PP2 or MβCD before stimulating by co-cross-linking CD48 and I-Ak. Control samples received carrier alone. D, Inhibition of capping of GEM domains by PP2 and MβCD. CH27 cells were prepared and stimulated as described in C. The plot represents the fraction of those cells in each sample that had a 1.5-fold or greater enrichment of L10-GFP at the bead-cell interface. The sample sizes were 40, 40, and 33 for the untreated, PP2-treated, and MβCD-treated samples, respectively. ***, p < 0.001, determined using the untreated sample as the reference.

FIGURE 6. Both CD48/I-Ak-mediated capping of L10-GFP in CH27 cells and T cell-APC conjugation are actin dependent. A, Top, Confocal images of a CH27 cell expressing L10-GFP and stimulated with beads coated with anti-CD48 and anti-I-Ak. The asterisks indicate the positions of two separate beads. The sample was double-labeled with Texas Red-conjugated phalloidin (Phal-TR). A, Bottom, Fluorescence intensity profiles of the cell that were measured in the GFP (green) and Texas Red (red) channels are also shown. The vertical lines indicate the boundaries where the beads contact the cell surface. B, Measurement of the effect of latrunculin B on capping of GEM domains. CH27 cells expressing L10-GFP were pretreated with latrunculin B before incubation with cocated beads. Capping of GEM domains was scored based on the enrichment L10-GFP at the bead-cell interface as described in Fig. 4. The sample sizes were 69 and 66 for the untreated and treated samples, respectively (p < 0.01). C, Measurement of B cell-T cell conjugates using either untreated (control) or latrunculin B-treated CH27 cells. Eighty-one and 28% of the untreated and latrunculin B-treated CH27 cells, respectively, bound D10 cells.
washed before adding them to D10 cells to avoid affecting the cytoskeleton of the T cells, and cell conjugation was measured by flow cytometry. This experiment showed that latrunculin B caused a significant reduction in the formation of B cell-T cell conjugates (Fig. 6C). For example, 81% of the untreated CH27 cells formed conjugates with D10 cells, but only 28% of the latrunculin B-treated cells did so. Furthermore, CH27 cells that did conjugate to D10 cells failed to exhibit enrichment of either Lel-GFP or I-A\(^{b}\) at the site of cell contact (data not shown). Together, the results in Fig. 6 show that the actin cytoskeleton of APCs functions to assemble GEM domains for immune synapses and in the conjugation of APCs to T cells.

The actin cytoskeleton of the APC is necessary for forming an immune synapse in T cells

T cells form a distinct signaling complex when stimulated by p-MHC II complexes, and this consists of a central supramolecular activation complex (c-SMAC) that occurs in the center of the immune synapse (3). The c-SMAC contains TCR and other signaling proteins, including PKC-\(\theta\) (1, 3, 33). To determine the effect of disrupting the actin cytoskeleton of APCs on the formation of c-SMACs in T cells, D10 cells were fixed and stained with Ab to PKC-\(\theta\) and Texas Red-labeled secondary Ab (anti-PKC-\(\theta\)). Fig. 7A shows confocal images of D10 cells conjugated to either an untreated or a latrunculin B-treated CH27 cell. Enrichment of PKC-\(\theta\) at the cell interface occurred only in the T cell that was conjugated to the untreated CH27 cell. To quantitate the effect of latrunculin B on targeting of PKC-\(\theta\) to the c-SMAC, the enrichment of PKC-\(\theta\) at the cell interface was measured in ~40 B cell-T cell conjugates. These measurements showed that enrichment of PKC-\(\theta\) decreased from an average of ~4.5 for D10 cells conjugated to untreated B cells to 1.0 or no enrichment in cells conjugated to latrunculin B-treated CH27 cells (Fig. 7B). We conclude from these results that an intact cytoskeleton in the APC is necessary for generation of a mature immune synapse in T cells.

![Confocal and DIC images of D10 T cells conjugated to either an untreated or latrunculin B-treated CH27 cell. The cells were fluorescently labeled with anti-PKC-\(\theta\) and Texas Red-labeled secondary Ab.](http://www.jimmunol.org/)

**FIGURE 7.** The actin cytoskeleton of APCs is necessary for generation of a mature immune synapse in conjugated T cells. A, Confocal and DIC images of D10 T cells conjugated to either an untreated or latrunculin B-treated (right) CH27 cell. The cells were fluorescently labeled with anti-PKC-\(\theta\) and Texas Red-labeled secondary Ab. The arrowhead indicates enrichment of PKC-\(\theta\) in an immune synapse. The untreated cell was incubated with carrier alone. B, Quantification of the enrichment of PKC-\(\theta\) in immune synapses in D10 cells conjugated to either untreated or latrunculin B-treated CH27 cells. The samples were prepared as described in A. Enrichment of PKC-\(\theta\) in immune synapses was quantitated by dividing the average intensity of Texas Red-labeled PKC-\(\theta\) at the T cell-APC interface by the average intensity of the remaining cell. The sample sizes were 38 and 30 for the untreated and latrunculin B-treated cells, respectively (\(p < 0.001\)).

**Discussion**

We report in this study results showing that Ag presentation occurs by an actin-dependent assembly of GEM domains at the site of presentation. Consequently, disruption of the actin cytoskeleton of the APC inhibits both formation of an immune synapse and conjugation of the APC to T cells. Our data are therefore consistent with the model in which the immune synapse forms by the APC actively targeting molecules to the cell interface (34) rather than by a passive rearrangement of molecules in the APC that is driven by migration of associated proteins in T cell (2, 3, 18).

We have previously demonstrated a role for GEM domains in T cells for targeting molecules to the immune synapse (16). Similarly, MHC II is constitutively associated with GEM domains (14), and disruption of GEM domains with MβCD inhibited targeting of MHC II to the cell interface (Fig. 2B). These findings suggest that GEM domains serve as a vehicle for targeting p-MHC II complexes to the site of Ag presentation as well. Consistent with this hypothesis is the observed recruitment to immune synapses of both irrelevant and Ag-loaded MHC class II molecules (10) as well as MHC II molecules loaded with a null peptide (18). Thus, colocalization of multiple species of p-MHC II complexes in the same GEM domains would result in their corecruitment to the immune synapse.

One recent study showed that inhibition of B cell-T cell conjugation by MβCD was most effective at low Ag concentrations (10). This finding suggests that GEM domains can function to increase the concentration of p-MHC II complexes within discrete regions of the plasma membrane of the APC for binding to the TCR. Accordingly, we propose that GEM domains function in Ag presentation by a two-step process. First, GEM domains cause a localized increase in the effective concentration of p-MHC II complexes. Once MHC II-dependent signals for actin polymerization have occurred, GEM domains function as a vehicle for targeting associated p-MHC II complexes to the cell interface to form an immune synapse.

In contrast to our finding showing the important role of the actin cytoskeleton of the APC in forming immune synapses, an earlier study has reported that accumulation of MHC II at the B cell-T cell interface occurred after pretreatment of B cells with cytochalasin D (18). One interpretation of the latter finding is that a threshold amount of Ag presentation can occur in the absence of an actin-mediated clustering of GEM domains. We predict that Ag presentation in these conditions is weaker than that where a robust targeting of p-MHC II and GEM domains to the cell interface occurs, and this is evidenced by the less efficient Ag presentation by p-MHC II complexes in planar membranes rather than in plasma membrane (14).

We have previously shown in T cells and adherent cells that the actin cytoskeleton assembles GEM domains into membrane patches and immune synapses by directly associating with GEM domains (7, 16). Such an association could occur through actin-binding proteins that reside in GEM domains (35) and by GEM domains hosting signals for actin polymerization (36). Another mechanism by which actin filaments could assemble GEM domains is by a passive clustering with actin filaments (37–39). However, our data showing a specific enrichment of GEM domains in the immune synapse are more consistent with domain clustering occurring by a direct association between the actin cytoskeleton and GEM domains rather than a passive entrapment of molecules. Actin-mediated assembly of GEM domains is essential for T cell stimulation (16), and as we have shown in this study, Ag presentation, and the mechanism underlying this phenomenon represents an important question that deserves further study. Importantly,
other classes of APCs, such as dendritic cells and macrophages, may have separate or additional methods for assembling MHC II-peptide complexes at the immune synapse. The role of GEM domains in Ag presentation in APCs other than B cells therefore warrants further study as well.

Photobleaching experiments showed that the majority of synapse-associated L10-GFP is mobile and exchanges rapidly with nonsynapse protein (Fig. 3). This contrasts with measurements of planar membranes showing that diffusion of p-MHC II in the immune synapse is slow and restricted to a small fraction of the protein (2). One interpretation of the latter result is that diffusion of p-MHC II complexes is inhibited due to engagement with T cell proteins and does not reflect the properties of other proteins in the immune synapse. We have also shown that the immune synapse undergoes an active and continuous exchange of protein with non-synapse regions of the cell. Accordingly, the immune synapse undergoes a high flux of molecular components, similar to that shown for other cell structures (40).

Interestingly, assembly of GEM domains into an immune synapse was mimicked only by co-cross-linking MHC II and CD48. Thus, cross-linking MHC II alone is not sufficient to cause capping of GEM domains, but requires additional signals that can be provided by CD48. One explanation for the role of CD48 in initiating capping of GEM domains is suggested by the documented heterogeneity of GEM domains (41). Specifically, signaling molecules that are necessary for actin polymerization and capping may be distributed between separate species of domains (42). Hence, co-cross-linking CD48 and I-A<sup>B</sup> may allow signaling molecules that reside in distinct GEM domains to mix and interact to initiate signaling events for actin polymerization and domain clustering.

Signaling after co-cross-linking I-A<sup>B</sup> and CD48 was inhibited by M6ICD (Fig. 5), and this is consistent with the idea that GEM domains are discrete signaling compartments in cell membranes. Furthermore, signaling from MHC II and CD48 required Src kinase activity. As with MHC II, many Src kinases are constitutively associated with GEM domains (43–45). Thus, signaling from MHC II may be initiated in GEM domains through interaction of MHC II-associated molecules and Src kinases. Interestingly, Lang and co-workers (46) showed that the Igα and Igβ (CD79a/CD79b) heterodimer of the B cell Ag receptor associates with MHC II in Ag-primed B cells and functions in transducing signals from MHC II after cross-linking MHC II. The B cell receptor is recruited to GEM domains after its engagement (47), and this may serve to colocalize CD79a and CD79b in GEM domains with MHC II for cell signaling. Future studies will show the degree to which signaling downstream of MHC II is compartmentalized to GEM domains, and whether this occurs by partitioning of signaling molecules between GEM and non-GEM fractions, as has been shown for other immune cells (47–49).

In summary, our data show that the APC actively targets GEM domains to the site of engagement with the TCR. As MHC II is constitutively associated with GEM domains, our results suggest a mechanism by which p-MHC II complexes are delivered to the site of Ag presentation. Importantly, our findings contrast with the idea that the APC serves only as a passive membrane platform for presenting Ag to the TCR. Furthermore, we have shown that targeting of GEM domains to immune synapses occurs by signals downstream of the MHC II and as a consequence of actin remodeling. Thus, similar to events in the plasma membrane of T cells, our data support the idea that GEM domains serve as an actin-driven vehicle for delivery of molecules to the immune synapse and generation of an immune response.

Acknowledgments
We thank J. Byrum and S. Jordan for technical assistance, and A. Bothwell and D. Pflugh for supplying D10 and CH27 cell lines and their helpful discussions.

References