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A Novel Adaptor Protein Orchestrates Receptor Patterning and Cytoskeletal Polarity in T-Cell Contacts

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Summary

Recognition of antigen by T cells requires the formation of a specialized junction between the T cell and the antigen-presenting cell. This junction is generated by the recruitment and the exclusion of specific proteins from the contact area. The mechanisms that regulate these events are unknown. Here we demonstrate that ligand engagement of the adhesion molecule, CD2, initiates a process of protein segregation, CD2 clustering, and cytoskeletal polarization. Although protein segregation was not dependent on the cytoplasmic domain of CD2, CD2 clustering and cytoskeletal polarization required an interaction of the CD2 cytoplasmic domain with a novel SH3-containing protein. This novel protein, called CD2AP, is likely to facilitate receptor patterning in the contact area by linking specific adhesion receptors to the cytoskeleton.

Introduction

T-cell activation requires T-cell antigen receptor (TCR) recognition of peptides bound to MHC molecules (antigen). The physical dimensions of the TCR interacting with antigen suggest that TCR recognition will require the formation of a narrow 15 nm gap between the T cell and antigen-presenting cell membranes (Garboczi et al., 1996; Garcia et al., 1996). Because the affinity of the TCR for antigen is very low, on the order of 10^{-4}–10^{-6} M (Corr et al., 1994; Matsui et al., 1994), and the number of ligands is likely to be limited, the interaction of the TCR with antigen seems unlikely to be sufficient to drive formation of this tight contact (Davis and van der Merwe, 1996; Shaw and Dustin, 1997). Other mechanisms must therefore exist to initiate and stabilize the cell–cell contact.

One of these mechanisms is the use of adhesive molecules to form a specialized junction between the T cell and the antigen-presenting cell (APC). An important feature of this junction is a specific pattern of receptors with an outer ring of LFA-1 surrounding an inner circle containing TCR (Monks et al., 1998). The exact function is unclear, but several features are consistent with receptor patterning playing a role in facilitating TCR engagement. First, including the TCR in a central cluster allows cosegregation with receptors like CD4, CD28, and CD2 that have similar physical dimensions (Shaw and Dustin, 1997) Thus, the engagement of these molecules with their ligands helps to promote a tight, homogeneous interaction between the membranes of the T cell and the APC of about 15 nm. Second, generation of a 15 nm gap will require the exclusion of larger molecules such as LFA-1 and the tyrosine phosphatase CD45 from the TCR contact area. Third, concentrating many TCRs in the contact area may be important to enhance the ability of TCRs to engage rare ligand molecules. Lastly, formation of a suitable contact area is likely to be critical for cytoskeletal polarity. Cell polarity allows cytotoxic agents and cytokines to be focused directly at the cell in contact.

Surprisingly, the mechanism of molecular patterning and cytoskeletal polarization in the junction between T cells and antigen-presenting cells is unknown. We have begun to analyze this process by studying the T-cell membrane protein CD2, a 50 kDa protein expressed on the surface of T lymphocytes and natural killer (NK) cells. Although heavily studied in the last 15 years, the exact function of CD2 is still unclear. But its expression on T and NK cells suggests that it plays an important role in the biology of these cells.

Because it is an adhesion molecule that binds specific ligands expressed on a wide range of APCs, CD2 is well-positioned to participate in contact area formation. In humans, the principle ligand for CD2 is CD58 (Shaw et al., 1986). In rodents, the related molecule CD48 is the ligand for CD2 (Davis and van der Merwe, 1996). Receptor–ligand interactions in this system are species specific since human CD2 does not bind rodent CD48 and rat CD2 does not bind human CD58. The complex of CD2 bound to its ligand spans a gap of 15 nm (van der Merwe et al., 1995), suggesting that CD2 adhesion might serve to solve a topological problem inherent in TCR recognition of ligand. The formation of a junction using adhesion molecules that are similar in size to the TCR would clearly facilitate engagement of MHC by the TCR.

When plated on its ligand, CD2 concentrates and forms a small junction in which thousands of CD2–CD58 interactions cooperate to closely align the T cell and APC membranes (Dustin et al., 1997b). Because it is associated with the TCR, CD2 clustering could also serve to recruit the TCR to the contact surface (Bockenstedt et al., 1988; Beyers et al., 1992). Concentrating the TCR along with associated molecules like CD2, CD4, CD8, and CD28 in two-dimensional space could generate enough attractive force to stabilize the interaction between the two cells as well as to force larger proteins to the periphery of the contact.

In this study, we investigated the process of CD2 clustering stimulated by ligand binding and T-cell activation. We found that CD2 binding to ligand stimulates

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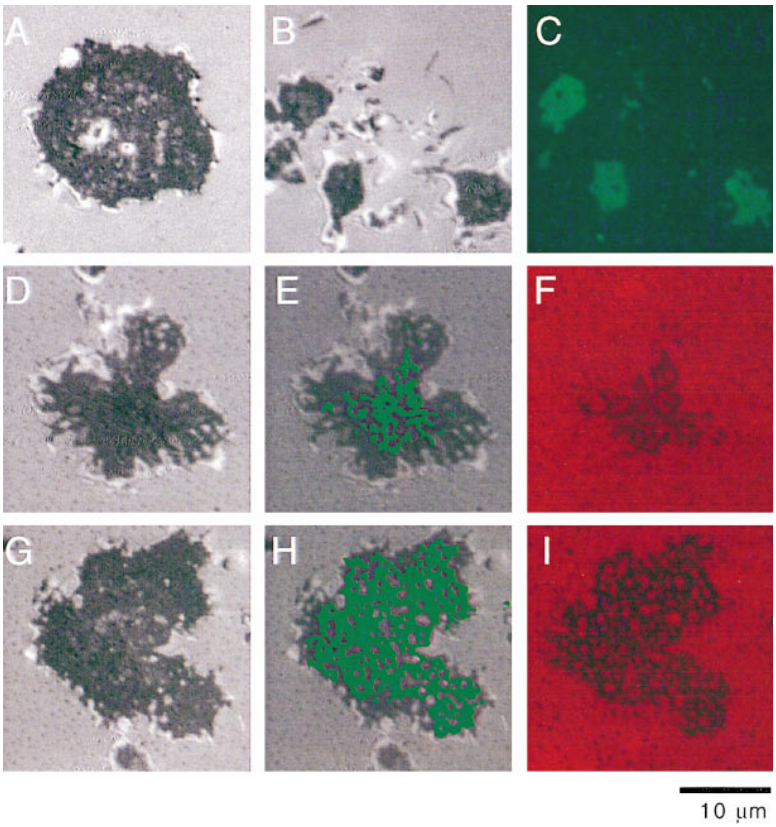

Figure 1. Comparison of Integrin Spreading and CD2 Concentration in T-Cell Bilayer Junctions

Jurkat T cells expressing wild-type rat CD2 were treated with 10 ng/ml PMA and 1 μM ionomycin and were plated on planar bilayers with ICAM-1 (A) or FITC-CD48 (B and C). In (A) and (B) the junctions, as visualized by IRM, are dark. (C) shows FITC-CD48 concentration in the junctions. Next, the rat CD2 Jurkat cells were activated as above and plated on bilayers containing TRITC-ICAM-1 and FITC-CD48. (D) shows the junction, (E) shows the superposition of FITC-CD48 concentration on the junction, and (F) shows the exclusion of ICAM-1 from the areas of FITC-CD48 accumulation. ICAM-1 monomers do not bind strongly enough to LFA-1 to be concentrated in the contact areas. (G), (H), and (I) are the same as (D), (E), and (F), respectively, except that the C-terminal 20 amino acids are deleted from the rat CD2 (CY97).

clustering of CD2 and also polarization of the T cell. Clustering and polarization required the CD2 cytoplasmic domain as cells expressing forms of CD2 that lack the cytoplasmic domain were unable to cluster CD2 or to polarize. These processes are mediated by the binding of a novel SH3-containing protein that binds to the cytoplasmic domain of CD2. Binding of this novel protein, called CD2-associated protein (CD2AP), to CD2 is induced by T-cell activation and is required for CD2 clustering and T-cell polarization. Thus, CD2AP seems likely to function as a molecular scaffold for receptor patterning and cytoskeletal polarization. Both of these events are critical to the formation of an effective T-cell–antigen-presenting cell junction.

Results

CD2 and LFA-1 Have Distinct Roles in Contact Formation

To study the mechanisms that regulate formation of the junction between a T cell and an antigen-presenting cell, we began by determining whether we could simulate antigen-specific junction formation using purified ligands for CD2 and LFA-1 embedded into lipid bilayers. Because the two major adhesive proteins on the T-cell surface are LFA-1 and CD2 (Shaw et al., 1986), we reasoned that they are likely to be involved in generating the junction between T cells and APCs. T cells were treated with phorbol ester and calcium ionophore before plating on the substrate to simulate the effects of antigen receptor engagement on LFA-1 and CD2 avidity (Dustin and Springer, 1989; Hahn et al., 1992). Previously, it had been shown that engagement of LFA-1 or CD2 elicit distinct T-cell behaviors (Dustin and Springer, 1988). When T cells are plated on lipid bilayers containing ICAM-1, the ligand for LFA-1, the cells spread, forming a large broad junction as visualized by interference reflection microscopy (IRM, Figure 1A). In contrast, when T cells are plated on lipid bilayers containing CD58, the ligand for human CD2, cells round up and CD2 clusters, forming a small, discrete junction (Figures 1B and 1C).

We next tested the behavior of Jurkat T cells on lipid bilayers containing fluorescently labeled CD48 (green) and ICAM-1 (red). Areas of CD48 concentration within the contact mark the sites of CD2 engagement. Activated Jurkat T cells expressing full-length rat CD2 were plated on the lipid bilayer. IRM demonstrated that T cells plated on a bilayer containing both ligands form a broad contact typical of cells plated on ICAM-1 (Figure 1D). Visualization of CD48 accumulation demonstrated that CD2 engagement is biased to the center of the contact (Figure 1E). Furthermore, ICAM-1 molecules are excluded from the sites of CD2 engagement (Figure 1F). Thus, key elements of receptor patterning observed in T cell–antigen-presenting cell contacts can be simulated using artificial lipid bilayers containing the ligands for CD2 and LFA-1. Receptor patterning was dependent on T-cell activation as only broad CD2-mediated contacts were formed when cells were plated without prior stimulation with PMA and ionomycin.

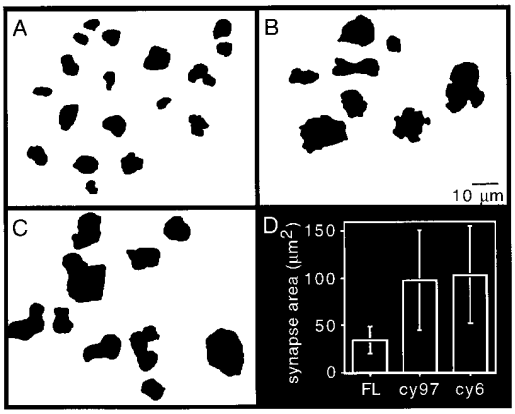

Figure 2. CD2 Concentration Requires the Last 20 Amino Acids of the Cytoplasmic Domain

Jurkat cells transfected with full-length rat CD2 (FL, panel A), rat CD2 with a 97–amino acid cytoplasmic domain (CY97, panel B), or rat CD2 with a 6–amino acid cytoplasmic domain (CY6, panel C) were treated with PMA and ionomycin and plated on 600 molecules/μm^2 of CD48 embedded in glass-supported planar bilayers. After 60 min at 37°C the cells were fixed and observed by IRM. The gray IRM images were used to generate the black segments shown. The average areas of greater than 100 contacts for each cell type are summarized in (D). These data are representative of three experiments.

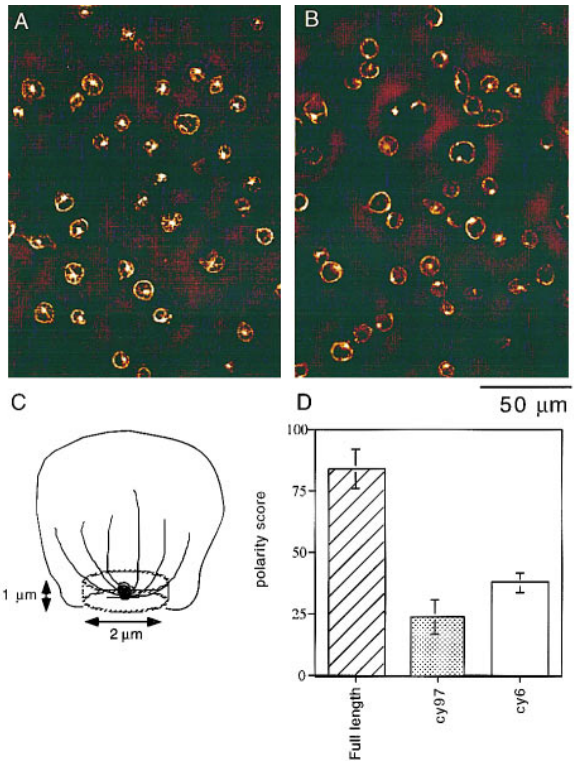

Figure 3. The Cytoplasmic Domain of CD2 Regulates Cytoskeletal Polarity

Jurkat T cells expressing wild-type rat CD2 (FL, panel A), rat CD2 with a 97–amino acid cytoplasmic tail (CY97, panel B), or rat CD2 with a 6–amino acid cytoplasmic tail (CY6, not shown) were treated with PMA and ionomycin and incubated on planar bilayers containing 1000 molec/μm^2 of mouse or rat CD48. Cells were treated with 10 ng/ml PMA and 1 μM ionomycin. After 60 min the cells were fixed and the position of the MTOC determined by fluorescence microscopy. (C) indicates the criteria for scoring a cell as polarized. This cylindrical volume represents approximately 1% of the cytoplasmic volume such that this positioning of the MTOC is unlikely to occur at random. The data in (D) are from two experiments.

Clustering and Segregation of CD2 Requires Its Cytoplasmic Domain

To determine the molecular basis for CD2 clustering after contact formation, we began by testing whether the cytoplasmic domain of CD2 was required. Jurkat cells stably transfected with either full-length rat CD2 or forms of CD2 lacking either the complete cytoplasmic domain (CY6) or the 20 C-terminal residues (CY97) were activated and tested for their ability to cluster CD2 by measuring contact size (He et al., 1988; Figure 2). The use of rat CD2 allowed us to distinguish mutated CD2 molecules from the wild-type human CD2 present in Jurkat cells. Unlike cells expressing wild-type CD2, T cells expressing either of the two truncated forms of CD2 formed larger, more heterogeneous junctions (Figure 2). This suggested that the last 20 residues of the cytoplasmic domain of CD2 were required for the normal regulation of CD2 junction size.

We next tested the ability of mutated forms of CD2 to segregate in the center of the junction when plated on lipid bilayers containing both CD48 and ICAM-1. CD2 molecules lacking the complete cytoplasmic domain (data not shown) or the C-terminal 20 residues did not concentrate at the center of the contact, but rather were distributed throughout the entire contact (Figure 1 H). Visualization of ICAM-1 demonstrated that it was still excluded from contact areas containing engaged CD2 (Figure 1I). This suggests that extracellular CD2 engagement is primarily responsible for ICAM-1 exclusion and, consistent with our topological model, that CD2/CD48 and LFA-1/ICAM interactions are mutually exclusive (Shaw and Dustin, 1997). Our data therefore suggest that initial segregation of adhesion molecules in contact areas is mediated by size incompatibility, but the central localization of CD2 requires the C-terminal 20 residues of CD2.

The Cytoplasmic Domain of CD2 Can Mediate T-Cell Polarization

Because contact formation is the first step in cell polarization, we tested whether T-cell activation and CD2 clustering might be sufficient to induce T-cell polarization. Polarization of T cells is characterized by the movement of the Golgi complex and the microtubule-organizing center (MTOC) to a region of the cytoplasm just adjacent to the area of contact (Kupfer et al., 1986). T cells expressing wild-type or truncated forms of CD2 were plated on CD48, fixed, and then stained with anti-tubulin to mark the position of the MTOC. The MTOC was then visualized by optical sectioning microscopy (Figures 3A and 3B), and cells were scored positive for polarization if the MTOC was visible within 1 μm of the planar bilayer and 1 μm of the contact center (Figure 3C). Greater than 80% of the cells expressing wild-type

CD2 were polarized, but significantly fewer cells expressing either of the cytoplasmic truncations of CD2 were polarized (Figure 3D). This establishes that the last 20 residues of the CD2 cytoplasmic domain are required for CD2 clustering and T-cell polarization and suggests that both processes may be linked.

Identification of a Novel SH3-Containing Protein that Interacts with the Cytoplasmic Tail of CD2

Examination of the last 20 residues of CD2 demonstrates the presence of multiple proline residues that could serve as ligands for conserved protein binding modules such as SH3 domains. SH3 domains are found in a wide variety of signaling and cytoskeletal proteins and bind proline-rich sequences. We used the yeast two-hybrid screen to identify CD2-interacting proteins (Fields and Song, 1989; Vojtek et al., 1993). From a mouse embryo library, a partial cDNA encoding a novel SH3 domain was identified. The protein bound specifically to CD2 as expression of the DNA-binding domain of LexA alone or a control fusion protein, LexA-lamin, did not transactivate the reporter construct (data not shown).

A full-length cDNA was obtained by screening a cDNA library from mouse thymus. The deduced amino acid sequence predicts a 641 amino acid protein with a molecular weight of approximately 70 kDa (Figure 4A) containing three SH3 domains in the amino-terminal half of the protein. The SH3 domain cloned using the yeast two-hybrid screen represented the most amino-terminal of the three SH3 domains. The sequence of the latter half of the protein is proline-rich and contains some sequence similarity to neurofilament proteins as well as a recently cloned myosin I-binding protein, Acan125, from *Acanthamoeba* (Xu et al., 1995). A possible role for CD2AP in binding actin is suggested by the presence at the C terminus of a sequence similar to the monomeric actin–binding protein, thymosin-β4, (Van Troys et al., 1996). Because of its identification as a CD2-binding protein, the protein is named *CD*2-*a*ssociated *p*rotein or CD2AP.

Northern blotting studies performed to analyze tissue distribution (Figure 4B) detected message in all tissues tested except brain. The pattern of protein expression was verified using a rabbit polyclonal antiserum. Immunoblotting of multiple tissues demonstrated that the antiserum recognized an approximately 80 kDa protein from liver, thymus, and spleen (Figure 4C). No protein was detected in brain, kidney, or lung. It is not clear what explains the discrepancy between the Northern and Western blotting. Although the protein mobility is slower than predicted based on the protein sequence, it is similar to the mobility of protein expressed from the full-length cDNA (Figure 5). Immunoblotting of lysates from cell lines confirmed that the protein is expressed abundantly in cells known to express CD2 such as T cells and NK cells (Figure 4C). It was also expressed in fibroblast cell lines.

The First SH3 Domain of CD2AP Binds to a Proline Sequence at the C Terminus of CD2

To define the features of CD2AP and CD2 that mediate their interaction, we first tested whether the interaction

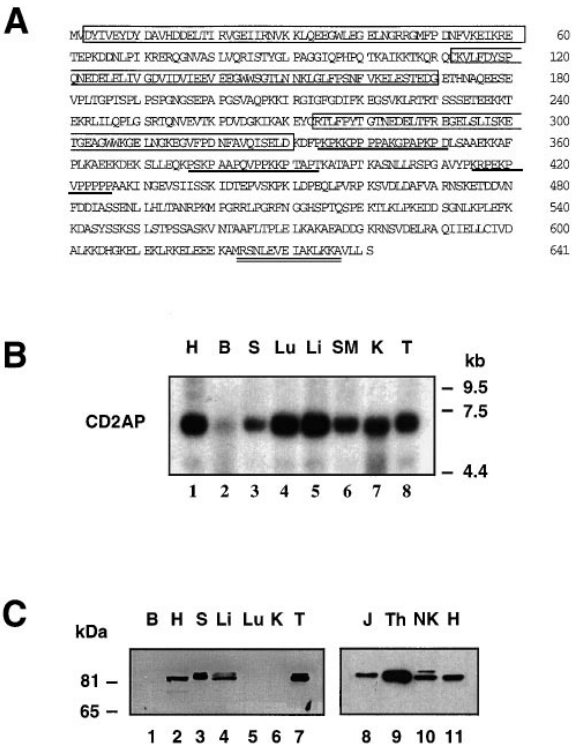

Figure 4. A Novel SH3 Domain-Containing Protein Identified in the Yeast Two-Hybrid Screen using the Cytoplasmic Domain of CD2

(A) Sequence of a novel triple SH3 domain–containing protein (CD2AP) identified using the two-hybrid screen using the one-letter amino acid code. SH3 domains are boxed. Proline-rich stretches are underlined. A putative C-terminal monomeric β-Thymosin-like actin binding domain is doubly underlined. Psi-Blast analysis (Altschul et al., 1997) of the second half of this sequence demonstrated similarities with intermediate filaments and with a myosin I–binding protein (Xu et al., 1995).

(B) Multiple tissue Northern blotting analysis of CD2AP. A commercial membrane was hybridized with a labeled CD2AP DNA probe. Tissue types: H, heart; B, brain; S, spleen; Lu, lung; Li, liver; SM, smooth muscle; K, kidney; T, thymus; J, jurkat; Th, T helper clone; NK, natural killer cell line; H, HeLa.

(C) Immunoblotting analysis of CD2AP protein expression in various tissues and cell lines. Mouse tissues (lanes 1–7) and cell lysates (lanes 8–11) were probed with a rabbit polyclonal anti- CD2AP sera, followed by a secondary HRP-conjugated antibody and developed using chemiluminescence. Tissue and cell types are indicated above each lane.

between CD2 and the CD2AP could be reconstituted in HeLa cells. The CD2AP cDNA was tagged with a myc epitope (Evan and Bishop, 1985) while CD2 was expressed as a chimera with the extracellular and transmembrane domains of the viral glycoprotein VSV-G. The proteins were coexpressed in HeLa cells and complex formation was tested by analyzing VSV G/CD2 immunoprecipitates for coprecipitating CD2AP (Figure 5B). Both full-length CD2AP, which has a molecular mobility of approximately 80 kD, and the first CD2AP SH3 domain alone were coprecipitated efficiently with the G/CD2 chimeric protein (Figures 5B and 5C, lane 2). This was specific because no association was detected when CD2AP was coexpressed with a construct that contains only the extracellular and transmembrane domains of VSV G, G T− (Figures 5B and 5C, lane 1).

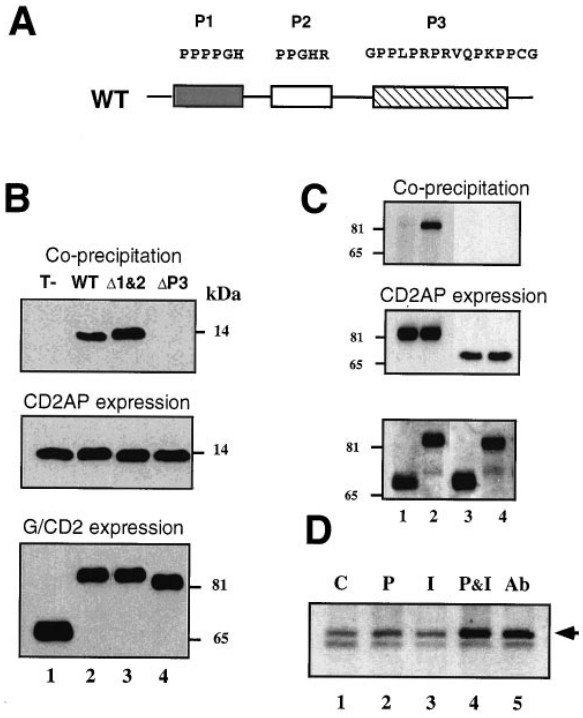

Figure 5. Mapping the Interaction between CD2AP and CD2

(A) Schematic diagram of VSV-G-CD2 constructs used to map the interaction between SH3–1 of CD2AP with murine CD2. The proline-rich sequences are represented by the gray and open boxes.

(B) CD2AP binds to a proline-rich region at the C terminus of the CD2 cytoplasmic domain. The first CD2AP SH3 domain was appended with the myc epitope and coexpressed with VSV-G/CD2 constructs lacking either P1 and P2 or the P3 proline sequences using the vaccinia-T7 expression system in HeLa cells. VSV-G constructs were immunoprecipitated, separated by SDS-PAGE, transferred, and blotted with anti-myc followed by a secondary HRP-conjugated antibody, and developed using chemiluminescence. Immunoblotting of cell lysates to control for expression of myc-CD2AP (middle panel) and VSV G (lower panel) are shown.

(C) Only the first SH3 domain of CD2AP binds to CD2. The VSV G/CD2 chimera (lanes 2 and 4) was coexpressed with either full-length myc-CD2AP (lanes 1 and 2) or with a myc-CD2AP construct lacking the first SH3 domain (lanes 3 and 4). A VSV-G tail minus construct, T− (lanes 1 and 3), was used as a control. The VSV-G immunoprecipitates were separated by SDS-PAGE and immunoblotted following transfer to nitrocellulose using a monoclonal antibody to the myc epitope tag. The proteins were visualized using a secondary HRP-conjugated antibody and chemiluminescence. Expression controls for myc-CD2AP (middle panel) and for VSV G (lower panel) are shown.

(D) Activation-dependent association between CD2 and CD2AP in Jurkat T cells. Jurkat T cells were treated with PMA (P), Ionomycin (I), PMA + Ionomycin (P&I), with an activating pair of CD2 antibodies (Ab), or left untreated (C). Cells were lysed and immunoprecipitates prepared with antibodies to CD2, separated by SDS-PAGE, transferred to nitrocellulose, and developed with polyclonal antibodies to CD2AP.

To define the segment of CD2 that interacts with CD2AP, we focused on proline-rich sequences because they are known to bind to SH3 domains (Ren et al., 1993). We focused on three proline-rich segments designated in Figure 5A as P1, P2, and P3. G/CD2 constructs that lacked each of the proline sequences were coexpressed with the first SH3 domain of CD2AP and tested for complex formation. Deletion of P1 or P2 alone or together had no effect on the ability of CD2 to coprecipitate with CD2AP (Figure 5B, lane 3 and data not shown). Deletion of P3, however, completely abrogated the ability of CD2 to coprecipitate CD2AP (Figure 5B, lane 4). Therefore, CD2AP interacts with a proline-rich sequence contained within the last 30 residues of the cytoplasmic domain of CD2.

Three overlapping 20-mer peptides based on the last 30 residues of CD2 were generated and tested for binding using surface plasmon resonance (SPR). Only one peptide containing the amino-terminal 20 of the last 27 residues of the CD2 cytoplasmic domain was sufficient for binding (see below and data not shown). Mutagenesis data demonstrated that the primary binding site is a Type II SH3 ligand (PPLPRPR) and five to seven C-terminal flanking residues to the motif are required for binding (data not shown). Thus, CD2AP binding to CD2 requires sequences that are lacking in the CY97 form of CD2.

Cloning of the first SH3 domain of CD2AP in the original yeast screen demonstrated that it was sufficient by itself to mediate the interaction between CD2 and CD2AP. However, the presence of multiple proline-rich segments in CD2 and multiple SH3 domains in CD2AP suggested that other interactions between CD2 and CD2AP might also occur. We tested this by generating a construct that lacks the first SH3 domain of CD2AP, ΔSH3#1, and expressing it with G/CD2 (Figure 5C, lanes 3 and 4). No ΔSH3#1 could be detected in the G/CD2 immunoprecipitates. The interaction between CD2 and CD2AP is therefore mediated solely by the first SH3 domain of CD2AP.

The Association of CD2AP with CD2 in T Cells Is Activation Dependent

To determine whether CD2AP associates with CD2 in T cells, CD2 immunoprecipitates from Jurkat T cells were immunoblotted with antibodies against CD2AP. From unstimulated T cells, CD2 immunoprecipitates contained a small amount of CD2AP (Figure 5D, lane 1). However, several different methods of activating T cells strongly induced the association of CD2AP with CD2. Phorbol ester and ionophore treatment, which can together activate T cells, strongly enhanced CD2/CD2AP association (Figure 5D, lane 4). But treatment with either agent alone had only a small effect on the association of CD2 with CD2AP (Figure 5D, lanes 2 and 3). T cells can also be activated by ligation of CD2 with specific pairs of CD2 monoclonal antibodies (Olive et al., 1986). Treatment of T cells with such a pair of CD2 monoclonal antibodies (CD2.1 and TS2/18) significantly augmented the amount of CD2AP associated with CD2 (Figure 5D, lane 5). As expected, treatment with either of the anti-CD2 antibodies alone had no effect on the association (data not shown). The association of CD2AP with CD2 is therefore regulated by T-cell activation.

The CD2AP SH3 Domain Binds to CD2 with High Affinity and Specificity

Using surface plasmon resonance, the affinity of the first CD2AP SH3 domain for CD2 was measured. A GST

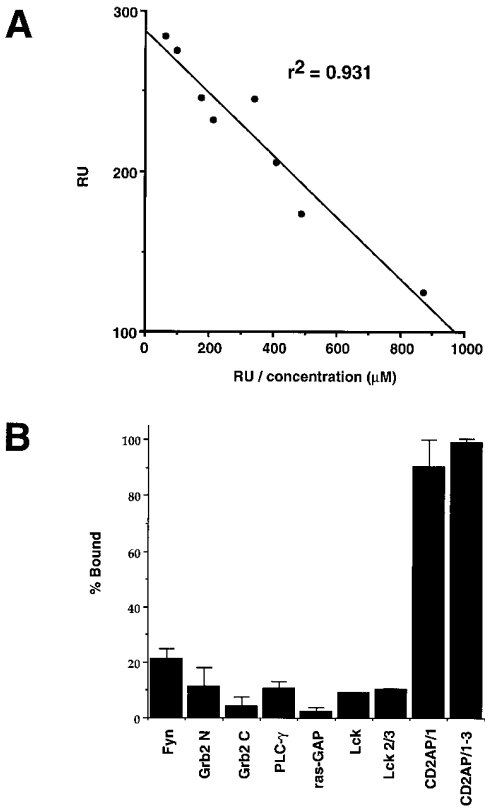

Figure 6. CD2AP Binds to CD2 with High Specificity and High Affinity

(A) Scatchard analysis for the binding of the first SH3 domain of CD2AP to a CD2 peptide measured by surface plasmon resonance. A peptide corresponding to CD2 residues 302–322 was biotinylated and bound to a streptavidin sensor chip. CD2AP SH3–1-GST was flowed over the chip at various concentrations and the change in surface plasmon resonance measured. Response units (RU) were measured after equilibrium binding and after correction for bulk effects of background. A representative experiment is shown of five trials. The calculated K_d of the above experiment is 194 nM with an r^2 of 0.931. Values obtained in other experiments ranged from 150 to 194 nM.

(B) Various SH3-GST fusion proteins (1 μM) were flowed over a sensor chip bound with a CD2 peptide corresponding to residues 317–337. Binding was measured using surface plasmon resonance. The data are an average of three experiments and are presented as percent of binding compared to the binding of a GST fusion protein containing either the single SH3 domain or full-length CD2AP.

fusion protein containing the first SH3 domain was purified and its affinity towards a CD2 peptide (residues 317–337) was determined by measuring the kinetics of binding as well as by Scatchard analysis. The k_{on} and k_{off} rates were determined to be 3.5×10^3 s/M and 3.8×10^{-4}/s, giving a K_d of 130 nM for the interaction between CD2 and CD2AP. Scatchard analysis gave comparable values for the K_d of between 150 and 190 nM (Figure 6A). This is an extremely high affinity for an SH3 domain as most SH3 interactions are in the 10–20 μM range. But it is comparable to an affinity recently reported for the SH3 domain of a Rho GTP exchange factor, PIX, to PAK kinase (Manser et al., 1998).

To determine whether this high affinity was intrinsic to either the peptide or the SH3 domain, we compared the ability of a wide variety of SH3 domains to bind to the CD2 peptide (Figure 6B). Each of the SH3 proteins was purified as a GST fusion protein and binding was compared at a concentration of 1 μM by SPR. At this concentration, only the CD2AP SH3 domain exhibited significant binding. Direct affinity measurements of the Fyn and Lck SH3 domains for the CD2 peptide generated affinity values of 15–20 μM, consistent with those previously reported for SH3 domains from Src kinases (Rickles et al., 1995).

A Dominant-Negative Form of CD2AP Disrupts Receptor Patterning and Cell Polarization

To confirm the involvement of CD2AP, we tested whether a truncated form of CD2AP could block receptor patterning by inhibiting wild-type CD2AP binding to CD2. A chimeric protein was generated consisting of the first two SH3 domains of CD2AP fused to green fluorescent protein (GFP). We reasoned that this protein should bind constitutively to CD2, but might inhibit CD2AP function because it lacks most of the other protein-binding motifs of wild-type CD2AP. The chimera, CD2AP SH3-GFP, was transiently expressed in Jurkat T cells. T cells were plated on lipid bilayers containing ICAM-1 and CD58 24–30 hr after transfection. As GFP fluorescence is similar to fluorescein, CD58 was labeled with TRITC (yellow in Figure 7) and ICAM-1 was labeled with Cy5 (red in Figure 7). Visualization of LFA-1 engagement was enhanced by using ICAM-1 dimers with higher affinity for LFA-1 (Miller et al., 1995). A construct containing the SH3 domain of Fyn fused to GFP was used as a control (Figures 7G-7I).

Cells expressing the CD2AP SH3-GFP chimera disrupted CD2 recruitment to the center of the contact (Figure 7). In most of the cells, junctions were disorganized with no clear central patterning of CD2 (Figure 7C). Interestingly, a significant proportion of junctions from CD2AP SH3-GFP–expressing cells showed central clusters of LFA-1 surrounded by a ring of CD2 engagement (Figures 7D–7F).

We next tested whether CD2AP-GFP protein could inhibit cytoskeletal polarization. Expression of the CD2AP SH3-GFP chimera strongly inhibited the ability of CD2 to stimulate T-cell polarization as compared to untransfected T cells or cells transfected with the Fyn-GFP chimera (Figures 7J–7L). These data support the idea that CD2AP binding to CD2 mediates the ability of CD2 to induce T-cell polarization and receptor patterning.

Antigen Receptor Engagement Triggers CD2 Clustering

In the foregoing experiments we utilized phorbol esters and calcium ionophore to simulate antigen receptor engagement. Without prior activation, receptor patterning and polarity was not detected using Jurkat T cells. To confirm that receptor patterning and CD2 clustering were dependent on antigen receptor engagement, we used T cells from 3A9 TCR transgenic mice that recognize the MHC molecule I-A^k complexed with peptide 48–62 from hen egg lysozyme (I-A^k∼HEL48–62) presented by supported planar bilayers (Dustin et al.,

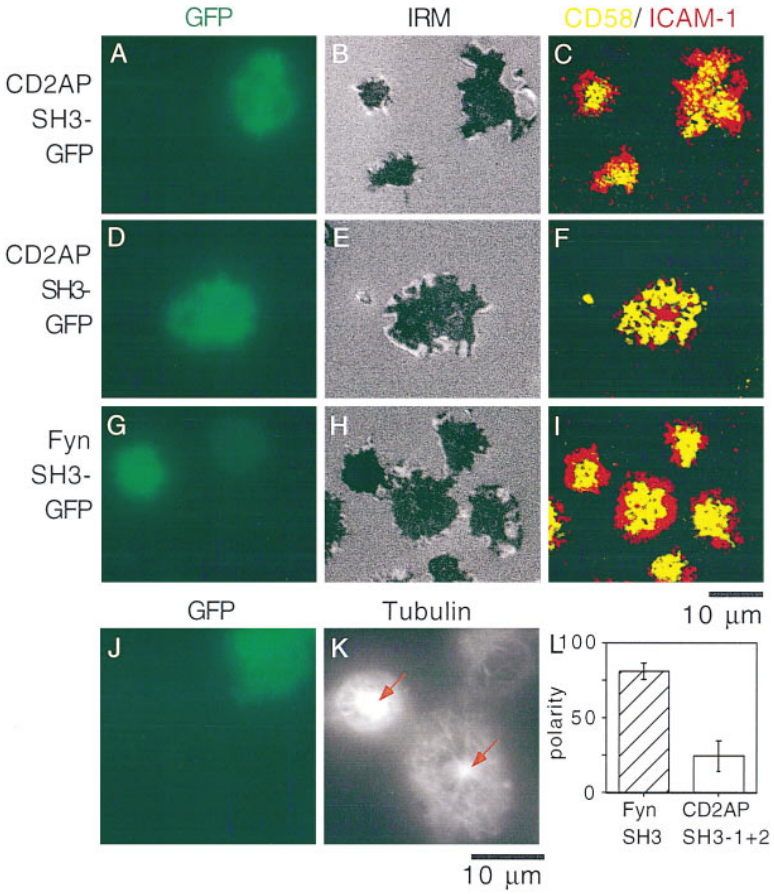

Figure 7. Dominant Negative CD2AP Inhibits Receptor Patterning and Cytoskeletal Polarity

Jurkat T cells transiently expressing the two amino-terminal SH3 domains of CD2AP fused to GFP (CD2AP SH3-GFP) (A-F, J, and K) or with GFP fused to the SH3 domain of Fyn (Fyn SH3-GFP) (G-I) were treated with PMA and ionomycin and plated on planar bilayers with TRITC-CD58 (yellow) and Cy5 ICAM-1 dimers (red) (A-I) or on unlabeled CD58. After 1 hr at 37°C the cells were fixed and stained for tubulin (J and K). The GFP-expressing cells were identified by green fluorescence (A, D, G, and J) and junctions were defined by IRM (B, E, and H). In (C), (F), and (I), receptor patterning is visualized as areas of discrete LFA-1/ICAM-1 engagement (red) and CD2/CD58 engagement (yellow). The pattern of CD2 engagement is disorganized (C) or completely inverted (F) in the CD2AP-GFP-positive cells, but is not affected in Fyn SH3-GFP cells (I). In (K) the positions of the MTOC within 1 μm of the substrate are indicated with arrows. Polarity data from 3 experiments with at least 30 GFP-positive cells are summarized in (L).

1997a). The 3A9 T cells were plated on bilayers with ICAM-1 and FITC-CD48, with and without I-A^k∼HEL48–62. In the absence of antigen the T cells crawled on the substrate and relatively little CD2 engagement was detected based on imaging CD48 redistribution (Figure 8A). The presence of a low amount of I-A^k∼HEL48–62 in the bilayer resulted in a 13- to 14-fold increase in CD2 engagement and formation of pronounced CD2 clusters in the central region of >80% of junctions (Figure 8B). In these junctions the CD2 clusters are surrounded by areas of LFA-1/ICAM-1–mediated contact as indicated by IRM images. Thus, antigen receptor engagement is required for physiological formation of CD2 clusters and for receptor patterning.

Discussion

The ability of antigen-specific T cells to locate and grasp target cells that express specific peptide/MHC complexes involves an ordered and complex series of events that is required for T-cell activation. The first step requires the adhesion molecules LFA-1 and CD2 to mediate an initial transient interaction (Shaw et al., 1986). This initial adhesive interaction facilitates engagement

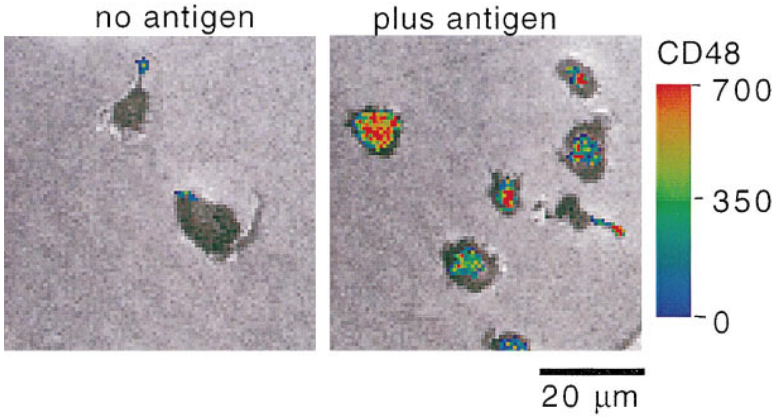

Figure 8. Requirement of Antigen Receptor Engagement for Receptor Patterning

T cells from 3A9 TCR transgenic mice were incubated with bilayers containing 500 molec/μm² ICAM-1 and 300 molec/μm² FITC-CD48 without or with antigen I-A^k∼HEL48–62 at 50 molec/μm². The accumulation of CD48 was determined by fluorescence microscopy and represented with the indicated color scale. In the absence of antigen, many fewer cells were able to adhere to the bilayer. In the figure, CD48 accumulation is superimposed on the grayscale IRM. The dark gray areas in IRM represent regions of LFA-1/ICAM-1 adhesion. The average accumulation of CD48 in the absence and presence of antigen is 1160 and 15,600 molec/junction, respectively.

of the TCR by peptides bound to MHC. TCR engagement leads to suppression of T-cell locomotion, formation of a specialized junction, and T-cell polarization (Kupfer and Singer, 1989; Dustin et al., 1997a). This combination of a specialized junction, cell polarization, and positional stability bears a striking similarity to the classical synapse of the nervous system (Paul and Seder, 1994). The "immunological synapse" is characterized by a specific pattern of molecules in the contact; LFA-1 is localized to the periphery of the contact while the TCR and accessory molecules like CD2, CD4, and CD28 are localized to the center of the contact.

The orchestrated receptor movements that characterize formation of the immunological synapse have two key features: (1) segregation of surface molecules into a least two domains and (2) the localization of one group of molecules in a central cluster. Here we have identified some of the mechanisms for molecular segregation and formation of the central cluster.

Recently, we proposed that the forces that govern contact cap formation are likely to include the size of the molecules involved (Shaw and Dustin, 1997). This was based on the fact that the molecules that occupy the central region of the immunological synapse share a common topology. Structural data indicate that the interaction of TCR with MHC, CD2 with CD48, CD28 with CD80, and CD4 or CD8 with MHC all span a gap of 15 nm. In contrast, the LFA-1/ICAM-1 interaction is predicted to span ~30–40 nm (Staunton et al., 1990). In fact, ICAM-1 alone is ~20 nm long, too large to be easily accommodated in the 15 nm gap. These theoretical arguments, however, had yet to be tested experimentally.

Here, we demonstrated that ICAM-1 is strongly excluded from CD2/CD48-mediated contact areas. This exclusion is probably related to the difference in molecular size between CD2/CD48 and ICAM/LFA-1, supporting a simple biophysical mechanism for initial protein segregation and for the exclusion of large molecules like CD45 and CD43 from contact areas. Because the ability of the CD2/CD48 interaction to exclude ICAM-1 was not dependent on the cytoplasmic tail of CD2, exclusion and segregation are mainly the result of ectodomain interactions and steric considerations.

Here we demonstrated that the central clustering of CD2 was dependent on sequences contained in the last 20 amino acids of the CD2 cytoplasmic domain. We then identified a protein, CD2AP, which interacts with this region. CD2AP has three SH3 domains and multiple polyproline motifs in a tandem array suggesting that it functions as an adaptor protein. Support for a role for CD2AP in cytoskeletal rearrangement and CD2 clustering was obtained by overexpressing a dominant-negative form of CD2AP, which blocked both CD2-triggered cytoskeletal polarization and immunological synapse formation. Central clustering of CD2 may have an important role in maintaining a stable domain for sustained antigen receptor engagement and signaling.

Antigen receptor engagement is the central event in the formation of the immunological synapse (Paul and Seder, 1994). Therefore, it is significant that we found that CD2 engagement and clustering is dependent on antigen receptor engagement. Antigen receptor engagement induces CD2AP binding to CD2 resulting in CD2 clustering in the central region of the junction. These data are consistent with earlier reports that antigen receptor engagement regulates CD2 avidity (Hahn et al., 1992) and with studies demonstrating that T-cell polarization is mediated primarily by TCR engagement (Kupfer et al., 1986; Lowin-Kropf et al., 1998). Our results directly demonstrate antigen-regulated CD2/CD48 interactions. We suspect that the small amounts of CD2 engagement detected in the absence of antigen allow the TCR to scan for MHC-peptide complexes in the small 15 nm contact areas. Recognition of the correct peptide/MHC complex by a TCR would then result in a chain of events leading to T-cell activation. Increased LFA-1 avidity and CD2 clustering would result in immunological synapse formation. Synapse formation would allow the rare specific peptide/MHC complex to serially trigger multiple TCRs (Valitutti et al., 1995).

The structure of CD2AP supports an important role for CD2AP in contact formation and polarization. In yeast, the protein BEM1, which contains two amino-terminal SH3 domains, is required for cell polarization (Chenevert et al., 1992). It functions by organizing the cytoskeleton around the polarized site where budding will take place (Peterson et al., 1994). Thus, BEM1 is thought to link membrane proteins that mark the site of polarization with the cytoskeleton. The binding of CD2AP to CD2 in the contact area may play an analogous role.

Although T cells from mice lacking CD2 do not exhibit a significant phenotype (Kileen et al., 1992), the simplest explanation is that another molecule can compensate for the loss of CD2. We would favor that other molecules that are topologically similar in size to CD2, like CD28, could share some functions with CD2. In addition, the powerful role of thymus selection would allow the generation of T cells that are adapted to depend on other molecules that share function of CD2.

Our model suggests that T-cell engagement of the APC is first mediated by LFA-1 and CD2 in distinct domains of non-antigen-specific contact areas. Engagement of TCR stimulates the binding of CD2AP to CD2 and enhances the avidity of LFA-1 leading to formation of a well-organized contact. CD2AP functions by clustering CD2 and possibly by marking the polarized surface. While interaction of CD2AP is not required to exclude large glycoproteins from sites of CD2 engagement, CD2 clustering by CD2AP will consolidate exclusion of larger molecules like CD45, CD43, and LFA-1 by driving these molecules to the periphery of the contact and may also play a role in TCR recruitment to the contact. Thus, the biophysical forces that result in the organization of a T-cell contact are related to protein size and the ability of the cytoskeleton to facilitate protein clustering and concentration. The end result of this activity is that TCR engagement is protected in a stable central portion of the contact area. Understanding these forces in greater detail will lead to a more profound understanding of the process of T-cell activation.

Experimental Procedures

Antibodies

The MAbs, OX34 (CD2; Jefferies et al., 1985), 9E10 (Evan and Bishop, 1985), OX78 (Kato et al., 1992), YN1/1 (Takei, 1985), TS2/9 (Shaw et

al., 1986), RR1/1 (Dustin and Springer, 1988), CL203 (Temponi et al., 1988), CD2.1 (Olive et al., 1986), and TS2/18 (Shaw et al., 1986), were used after purification from ascites or culture supernatants. The rabbit anti-CD2AP sera was generated against full-length CD2AP expressed and purified from bacteria.

Adhesion Molecules

Human CD58, mouse CD48, and human ICAM-1-GPI were affinity purified from human erythrocytes using TS2/9 MAb, C3F6 cells using OX78 MAb, and CHO cells using RR1/1 MAb, respectively. Each protein was labeled while attached to the respective MAb to protect the active site. Dyes were used at 0.05 mg/ml of MAb-Sepharose. The labeled proteins were then eluted, free dye was removed by ultrafiltration, and the protein was analyzed by SDS-PAGE. Labeled molecules retained full activity and mediated adhesion in the physiological density range. The activity of labeled and unlabeled molecules was identical. I-A^kHEL48–62 was purified and reconstituted as described (Dustin et al., 1997a).

Preparation of Glass-Supported Planar Bilayers

Glass-supported planar bilayers were prepared by the method of McConnell et al. (1986). Briefly, adhesion molecules were combined with 0.4 mM egg phosphatidylcholine (egg PC) in PBS with 1% octylglucoside and were dialyzed against PBS to form liposomes with incorporated adhesion molecules. The liposome suspensions were incubated on a clean glass coverslip in a parallel plate flow cell to form bilayers (Bioptechs, Butler, PA). The surface was treated with 5% non-fat dry milk (Carnation). Adhesion experiments were performed in HBS with 1 mM Mg and $CaCl_2$ and 5% serum at 37°C. All adhesion was inhibited by antibodies to LFA-1 and CD2. Adhesion molecule density was determined by binding of iodinated Fab fragments of MAb. ICAM-1 was reconstituted at 500 molec/μm^2, human CD58 at 200 molec/μm^2, mouse CD48 at 300–1000 molec/μm^2, and I-A^kHEL48–62 at 50 molec/μm^2.

Microscopy

Images were acquired using an inverted microscope (Yona Microscopes, Silver Spring, MD and Carl Zeiss, Thornwood, NY) (Dustin et al., 1997b). Filters: dichroic mirror XF93; emission filter XF93; excitation filters XEXM2, XEXM3, XEXM4 (Omega Optical, Brattleboro, VT). IRM images were obtained excitation filter XF32. Objective: 100× Neofluar 1.3 N.A. Camera: PXL1400 with resolution of 650 × 508 pixels = 108 × 85 μm (Photometrics, Tuscon, AZ). Images were processed using IP-lab software (Scanalytics, Vienna, VA).

Determination of Jurkat Cell Polarity

Jurkat cells interacting with ligand-containing planar bilayers were fixed, permeabilized, and stained for tubulin (Stowers et al., 1995). The samples were imaged by IRM to identify the junction and were optically sectioned by fluorescence microscopy from 12.8 μm below the junction to 12.8 μm above the junction at 0.2 μm steps. An experimental point spread function was used for image restoration using the linear least squares method with the program XCOSM (Preza et al., 1992; URL at http://ibc.wustl.edu/). The restored images, in which out-of-focus fluorescence is reassigned to the correct position, were used to score the position of the MTOC with respect to the junction in *x*, *y*, and *z* axis.

Yeast Two-Hybrid Screen and Cloning

The yeast two-hybrid system was used as described by Fields and Song (1989) and as modified by Vojtek et al. (1993). Briefly, a construct encoding the DNA-binding domain of the LexA fused to the cytoplasmic domain of mouse CD2 (Clayton et al., 1987) was produced by PCR and cloned into pBTM116. A murine embryonic cDNA library (Vojtek et al., 1993) was transfected into yeast and approximately 1 × 10^6 colonies were screened. Seventeen colonies expressed LacZ only when coexpressed with the LexA-CD2 construct and were sequenced. A murine, 16-day, embryonic, thymic λt11 library was screened using a random primed cDNA probe representing the full sequence of the clone identified in the two hybrid screen essentially as described in Sambrook et al., 1989. The longer of two clones isolated was approximately 2.1 kb in length. The nitrocellulose membrane containing the RNA from various murine tissues was obtained from Clontech (Palo Alto, CA).

Cell Lysates

Total protein lysates from murine brain, heart, spleen, liver, lung, kidney, and thymus were prepared by mincing the tissues in an ice-cold hypotonic buffer and then homogenized using a polytron. Cellular debris was removed by centrifugation, and cellular protein was normalized before immunoblotting.

DNA Constructs, Mutagenesis and Transfections

The cDNA constructs for mapping the interaction of CD2AP with CD2 were constructed using PCR-based mutagenesis. Transient protein expression for mapping studies was performed using the vaccinia-T7 expression system as previously described (Richard et al., 1995). For immunoprecipitations, HeLa cells were lysed in a lysis buffer containing 1% digitonin in 150 mM NaCl, 25 mM Tris-HCl (pH 7.4). Immunoprecipitations were performed on cleared lysates using 2 μl of antisera. The beads were then washed with lysis buffer and then analyzed by SDS-PAGE. Whole cell lysates were prepared by lysing transfected cells directly with Laemmli sample buffer. For immunoblotting, proteins were separated on 8% SDS-PAGE gels, transferred to nitrocellulose, and visualized using chemiluminescence.

Binding Studies and Scatchard Analysis

Binding and affinity studies were conducted using surface plasmon resonance on a BIAcore 2000 (Pharmacia). A peptide corresponding to residues 317–337 (QKGPPLPRPRVQPKPPCG) of the CD2 tail was synthesized as previously described (Muslin et al., 1996), biotinylated at the N terminus, and linked to a streptavidin-coated sensor chip. SH3-GST fusion proteins at a concentration of 1 μM were passed over the chip at a flow rate of 5 μl/min at 15°C in 50 mM HEPES (pH. 7.4), 150 mM NaCl, 0.001% Tween 20, 5 mM βME. Peptide competition studies were performed to determine the exact binding site of CD2AP on CD2. CD2AP SH3I-GST (1 μM) was incubated with 10 to 500 mM of competing peptides (QKG**A**PL**A**RP RVQPKPPCG, QKGPPLPRP**A**VQPKPPCG, QKGPPLPRPRVQ**A**KP **A**CG) and analyzed as above. Kinetic studies were analyzed using BIAevaluation 2.1 (Biacore AB). Scatchard analysis was performed using CD2AP SH3-GST concentrations from 0.070 to 70 μM. Fyn and Lck SH3-GST Scatchard analyses were performed similarly.

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GenBank Accession Number

The nucleotide sequence corresponding to the amino acid sequence shown in Figure 4 has been deposited in GenBank (AF077003).