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This information is current as of December 8, 2021.

J Immunol 2001; 167:641-645; ;
doi: 10.4049/jimmunol.167.2.641
<http://www.jimmunol.org/content/167/2/641>

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Cutting Edge: Integration of Human T Lymphocyte Cytoskeleton by the Cytolinker Plectin

Martin J. Brown,^{1*} John A. Hallam,* Yin Liu,* Kenneth M. Yamada,[†] and Stephen Shaw*

Chemokine-induced polarization of lymphocytes involves the rapid collapse of vimentin intermediate filaments (IFs) into an aggregate within the uropod. Little is known about the interactions of lymphocyte vimentin with other cytoskeletal elements. We demonstrate that human peripheral blood T lymphocytes express plectin, an IF-binding, cytoskeletal cross-linking protein. Plectin associates with a complex of structural proteins including vimentin, actin, fodrin, moesin, and lamin B in resting peripheral blood T lymphocytes. During chemokine-induced polarization, plectin redistributes to the uropod associated with vimentin and fodrin; their spatial distribution indicates that this vimentin-plectin-fodrin complex provides a continuous linkage from the nucleus (lamin B) to the cortical cytoskeleton. Overexpression of the plectin IF-binding domain in the T cell line Jurkat induces the perinuclear aggregation of vimentin IFs. Plectin is therefore likely to serve as an important organizer of the lymphocyte cytoskeleton and may regulate changes of lymphocyte cytoarchitecture during polarization and extravasation. *The Journal of Immunology*, 2001, 167: 641–645.

Circulating lymphocytes are spherical but assume a polarized morphology as they emigrate from circulation to tissue (1). Chemokines play a central role in lymphocyte recruitment in vivo (2) and are sufficient to trigger the polarization of peripheral blood T lymphocytes (PBT)² and other leukocytes in vitro. Chemokine-induced PBT polarization involves the rapid and synchronized reorganization of many cytoskeletal proteins (reviewed in Ref. 3). A fundamental gap in our understanding of the mechanisms of lymphocyte polarization is how the various components of the lymphocyte cytoskeleton are structurally and func-

tionally interconnected. This gap is particularly apparent for vimentin intermediate filaments (IFs), which are arguably the least understood component of the lymphocyte cytoskeleton. During the first minutes of chemokine-stimulated polarization, vimentin IFs undergo a pronounced transformation from an array of cytoplasmic filaments to a perinuclear aggregate localized to the uropod, or tail end, of the polarized PBT (4). The collapse of vimentin IFs requires actin and myosin contractility, is influenced by the orientation of microtubules (4, 5), and coincides with the redistribution of cytoskeletally associated proteins such as fodrin (6). These relationships clearly suggest physical connectivity between lymphocyte IFs and other cytoskeletal elements. However, the molecular interactions controlling the organization of IFs in circulating PBT and their reorganization during polarization remain largely undefined.

Cytolinkers are multifunctional proteins connecting different cytoskeletal filaments and other intracellular components, and they are important to the assembly and dynamics of cytoplasmic structural networks (7). Plectin, a member of the plakin/cytolinker family of proteins, was originally identified as an abundant IF-associated protein in several cultured cell lines (8) and has subsequently been characterized as a widely expressed cytoskeletal cross-linker (reviewed in Ref. 9). Plectin binds to IFs (10, 11), actin microfilaments (11, 12), microtubules (11, 13, 14), fodrin (14), integrins (15, 16), and itself (9) and thus has the capacity to bridge many cellular structures (9). Overexpression of the plectin IF-binding domain (IFBD) in fibroblasts and epithelial cells induces the collapse of the IF cytoskeleton into a condensed, juxtannuclear aggregate (12, 15–20). This process, although distinct from the PBT response to chemokines, resembles the collapse of the IF network during lymphocyte polarization. We therefore examined human PBT for plectin expression. We find that plectin is expressed in PBT and associates with a complex of structural and cytoskeletal proteins including vimentin and fodrin. Our results indicate that plectin is an integral component of the lymphocyte cytoskeleton and suggest that it plays a role in the polarization process.

Materials and Methods

Cells and Abs

Resting human PBT (>95% purity) were isolated from PBMC by immunomagnetic negative selection as described previously (4). SV40 large T-Ag transfected Jurkat (Jurkat-TAg) and HeLa cells were grown in RPMI 1640 or DMEM (Life Technologies, Rockville, MD), respectively, with 10% FCS. The following Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): goat anti-plectin, anti-lamin B, anti-vimentin, anti-ezrin, and anti-fodrin (spectrin β 1); mouse anti-HA (F-7); rabbit anti-HA (Y-11); and anti-vimentin mAb V9. Other primary Abs included: guinea pig anti-plectin (Research Diagnostics, Flanders, NJ); anti- α -fodrin mAb AA6 (ICN Pharmaceuticals, Costa Mesa, CA); and anti- α -tubulin mAb

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Received for publication April 12, 2001. Accepted for publication May 23, 2001.

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² Abbreviations used in this paper: PBT, peripheral blood T lymphocyte; ABD, actin-binding domain; CA, calyculin A; F-actin, filamentous actin; IF, intermediate filament; IFBD, intermediate filament binding domain; SDF-1 α , stromal cell derived factor-1 α ; VPF, vimentin-plectin-fodrin; HA, hemagglutinin.

B-5-1-2 (Sigma, St. Louis, MO). Normal goat IgG, normal mouse IgG, and preadsorbed secondary Abs conjugated with FITC, rhodamine, Cy5, and HRP were obtained from Jackson ImmunoResearch (West Grove, PA).

RT-PCR

Total RNA was extracted from 2×10^7 PBT and 1×10^7 HeLa cells using Tri Reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed using SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies). Primers for human plectin were designed based on the nucleotide sequence of human plectin cDNA (gi|1477645). The 3' primer was selected in the 3' region of exon 32 (Plect3'-1, nucleotides 8453–8473). 5' primers were selected toward the 3' end of exon 30 (Plect5'-1, 3925–3945) and toward the 5' end of exon 32 (Plect5'-2, 7738–7754). Primer pair 5'-1 and 3'-1 (1-1) detects plectin message without exon 31. Primer pair 5'-2 and 3'-1 (2-1) detects plectin message regardless of exon 31 splicing.

Immunomagnetic isolations and Western blot

Tosyl-activated, 2.8- μ m paramagnetic beads were conjugated with donkey anti-goat or donkey anti-mouse IgG following the manufacturer's protocol (DynaL, Lake Success, NY). Conjugated beads were coated with goat anti-plectin, goat anti- β -fodrin, anti-vimentin V9, normal goat IgG, or normal mouse IgG. PBT homogenates for immunomagnetic isolations were prepared by lysing 2×10^7 PBT in a cytoskeleton-stabilizing extraction buffer (0.1 M sodium-PIPES, 2 M glycerol, 1 mM EGTA, 1 mM MgSO₄, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and Complete protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN; pH 6.9). Lysates were sonicated and precleared for 1 h at 4°C with 200 μ g control IgG-coated beads. Cleared lysates were transferred to tubes containing 100 μ g washed, Ab-coated beads for 1 h at 4°C. Beads were washed 5 \times in lysis buffer and eluates resolved by SDS-PAGE. Whole cell lysates were prepared by lysing 1×10^7 PBT or 1×10^6 HeLa in modified radioimmunoprecipitation assay buffer. Lysates were diluted 1/1 with 2 \times reducing sample buffer, boiled, sonicated, and resolved by SDS-PAGE. Western blots were performed using ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Transfections

Constructs encoding N-terminally HA-tagged plectin actin-binding domain (ABD) and plectin IFBD (provided by Dr. A. Sonnenberg, The Netherlands Cancer Institute, Amsterdam, The Netherlands) have been described previously (15). Jurkat-TAG cells (1×10^7) in RPMI 1640 with 20 mM HEPES were transfected by electroporation using 310 V, 950 μ F, and 10 μ g DNA per sample. After 16 h, cells were adhered to poly-L-lysine-coated coverslips at 37°C for 5 min, fixed with 2% paraformaldehyde, and processed for indirect immunofluorescent staining.

Confocal microscopy

Suspended PBT were stimulated with 100 ng/ml SDF-1 α (PeproTech, Rocky Hill, NJ), fixed, and processed for indirect immunofluorescence as described previously (4). Cells were stained with various combinations of primary Abs followed by fluorophore-conjugated secondary Abs. Alexa 568 phalloidin was used to stain filamentous actin (F-actin; Molecular Probes, Eugene, OR). Samples were examined on a Zeiss LSM410 laser scanning confocal microscope with a $\times 100$ (1.4 numerical aperture) objective. Images were acquired as single optical sections or as serial sections and converted to two-dimensional projection images using Zeiss software (Zeiss, Thornwood, NY).

Results and Discussion

Expression of plectin splice variants in human PBT

The cytolinker plectin is one of the largest proteins expressed in mammalian cells. Plectin exhibits a broad, complex expression pattern with numerous forms resulting from variable splicing of at least 32 exons (21, 22). Although plectin message has been detected in many organs including thymus and lymph node (21), expression in hemopoietic cells has not been characterized. We examined human PBT for plectin expression by Western blot and RT-PCR. Western blot analysis of PBT whole cell lysates with two anti-plectin Abs consistently shows the presence of a dominant high molecular mass form of plectin ($M_r > 300$ kDa), as well as variable amounts of a reactive lower molecular mass species ($M_r \sim 180$ kDa) (Fig. 1A). The high molecular mass band is consistent

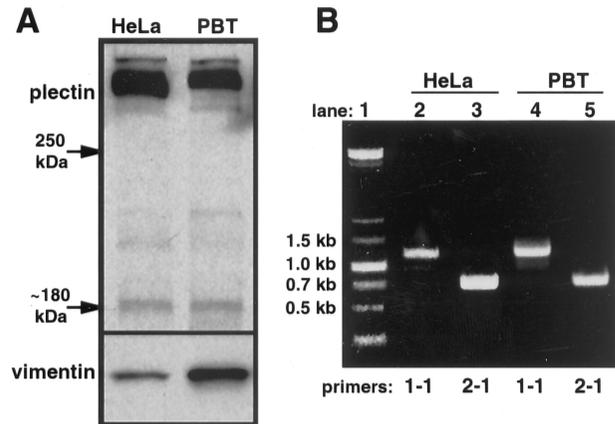


FIGURE 1. PBT express alternate forms of plectin with and without exon 31. *A*, Western blots comparing plectin and vimentin expression in human PBT and HeLa whole cell lysates; *B*, RT-PCR products from HeLa (lanes 2 and 3) and PBT total RNA (lanes 4 and 5) amplified with two combinations of PCR primers: primer pair 1-1 (lanes 2 and 4): predicted 1168-bp product from message lacking exon 31; primer pair 2-1 (lanes 3 and 5): predicted 736-bp product from all plectin transcripts having exon 32.

with full-length forms of plectin (532 kDa; $M_r > 300$ kDa) (22). Plectin has been described as dumbbell-like in shape, with a long central rod region and globular N- and C-terminal domains (9). The lower molecular mass form detected is consistent with smaller rodless variants of plectin, produced by alternative splicing in which the large exon 31 is removed (21). The presence in PBT of message for rodless plectin (exon 31 spliced out) was confirmed by RT-PCR (Fig. 1B).

Plectin redistributes to the uropod with vimentin and fodrin during PBT polarization

Immunofluorescent staining of resting PBT demonstrates extensive colocalization of plectin and vimentin IFs, consistent with the original characterization of plectin as an IF-associated protein (8). Vimentin and plectin are detected throughout the PBT cytoplasm, and both closely follow the contours of nuclear invaginations visible in single optical sections (Fig. 2, column I). Stimulation of PBT with the chemokine stromal cell-derived factor 1- α (SDF-1 α) causes the majority of PBT plectin to rapidly redistribute with vimentin to a focal accumulation within the uropod (Fig. 2, column II). In addition to vimentin, a variety of other proteins including fodrin (6), ezrin-radixin-moesin (23), CD43, ICAMs (24), and integrins (25) have been previously shown to localize to the uropod of polarized lymphocytes (reviewed in Refs. 3 and 26). Of these, we observe only fodrin acutely redistributing in SDF-1 α -stimulated PBT, accumulating at the distal tip of the uropod concomitant with the redistribution of vimentin and plectin (Fig. 2, columns III and IV). Plectin shows a consistent spatial relationship to the other molecular components of the uropod, spanning the juxtanuclear vimentin aggregate and the more peripheral fodrin accumulation. This staggered, overlapping distribution suggests that vimentin associates with the nucleus whereas plectin links vimentin to fodrin and the cortical actin cytoskeleton.

PBT plectin associates with a stable complex of structural proteins

Plectin binds to lamin B, fodrin, actin, and vimentin *in vitro* (12, 14, 27), and has been shown to form complexes with these proteins in epithelial cells (28). To examine the physical associations between plectin and other structural components in lymphocytes, we

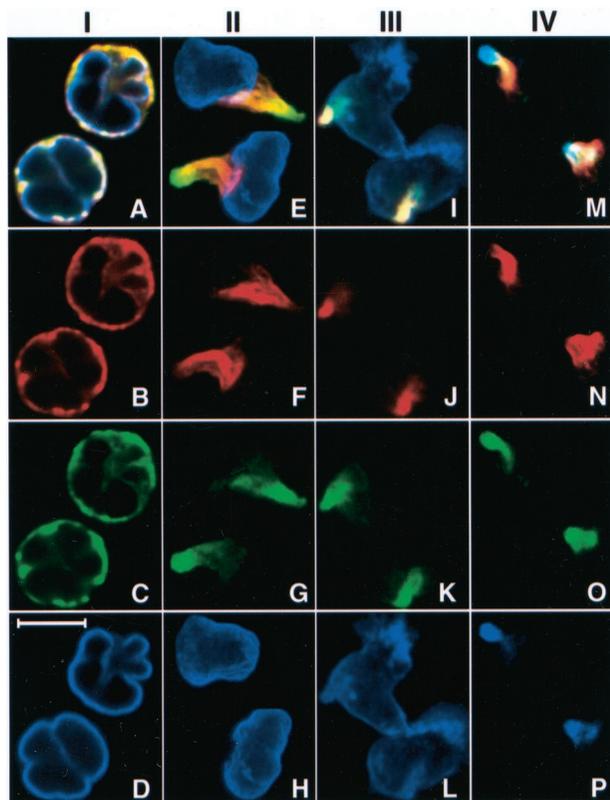


FIGURE 2. Plectin colocalizes with vimentin in resting PBT and redistributes to the uropod during polarization. Column I, Relative distribution in spherical PBT of vimentin IFs (red, *B*), plectin (green, *C*), and nuclear lamin B (blue, *D*); *A*, overlay. Column II, Redistribution of vimentin (red, *F*) and plectin (green, *G*) to the uropod following 3 min of stimulation with 100 ng/ml SDF-1 α ; *H*, lamin B (blue); *E*, overlay. Column III, Coaccumulation of fodrin (red, *J*) and plectin (green, *K*) within the uropod of SDF-1 α polarized PBT; *L*, F-actin (blue); *I*, overlay. Column IV, Overlapping distributions of vimentin (red, *N*), plectin (green, *O*), and fodrin (blue, *P*) in the uropod; *M*, overlaid. *A–D* are single optical sections; *E–P* are projection images produced from three-dimensional confocal serial sections. Bar = 5 μ m.

isolated cytoskeletal complexes from PBT whole cell lysates using an immunomagnetic separation technique (28). Coisolation of proteins from resting PBT homogenates using anti-vimentin or anti-fodrin beads demonstrated the existence of protein complexes containing vimentin, plectin, fodrin, lamin B, actin, and moesin (Fig. 3). Similar results were obtained using anti-plectin beads (not shown). Tubulin was not detected, suggesting either that microtubules do not associate with the complex or more likely that these interactions are not preserved during the isolation procedure. Tubulin is an abundant protein in PBT; its absence in the immunomagnetic isolates, as well as the lack of detectable proteins in control bead isolates, supports the specificity of the isolation procedure.

The molecular assembly of plectin, fodrin, vimentin, lamin B, and actin is detected both before and after chemokine stimulation (Fig. 3A), indicating that these components remain associated during polarization. In contrast, moesin is lost from anti-fodrin isolates within 1 min of SDF-1 α stimulation (Fig. 3B). These results show that vimentin IFs are physically connected to a variety of structural proteins in both resting and polarized PBT. With the exception of moesin, this cytoskeletal complex appears to redistribute to the uropod with no gross change in its composition. It is plausible that the complex is reorganized during polarization with-

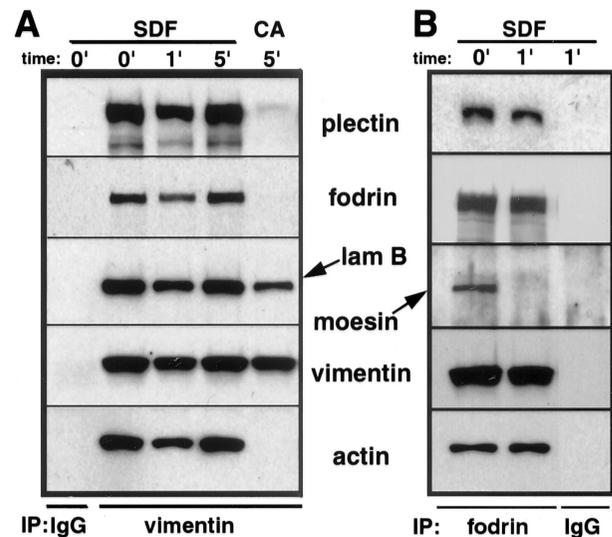


FIGURE 3. Plectin associates with multiple structural proteins in PBT. *A*, Western blots demonstrating that anti-vimentin magnetic beads coisolate (IP) vimentin, plectin, actin, fodrin, and lamin B (lam B) from PBT homogenates. Stimulation of PBT with 100 ng/ml SDF-1 α does not consistently change the yield of any of the shown proteins during the polarization. CA (25 nM for 5 min) treatment eliminates plectin, fodrin, and actin, but not lamin B, from anti-vimentin isolates. *B*, Western blots of anti-fodrin isolates (IP) confirm the association of plectin, fodrin, vimentin, actin, and also moesin. Moesin dissociates within 1 min of stimulation with SDF-1 α . Control isolations used magnetic beads coated with normal mouse IgG (*A*) or normal goat IgG (*B*).

out a detectable net change in its core components, because all of the coisolated proteins are capable of self-association as well as interactions with multiple binding partners. Redistribution during polarization may also involve the association or dissociation of as yet unidentified components. We view vimentin, plectin, and fodrin as the core cytoplasmic components of this assembly (a VPF complex) based on their simultaneous redistribution to the uropod, and also because a large fraction of the total cellular content of each of these proteins is isolated with the complex (data not shown). The cross-linking capacity of plectin makes it a likely candidate for an integrator of the PBT cytoskeleton in general and of the PBT VPF complex in particular.

The VPF complex associates with lamin B before and after SDF-1 α stimulation, indicating that a physical connection between the cytoplasmic cytoskeleton and the nucleus is maintained during polarization. Both vimentin (29) and plectin (27) have been reported to directly associate with lamin B in vitro. The spatial separation of plectin from the nucleus of polarized cells (Fig. 2) suggests that plectin is not the primary attachment of the VPF assembly to the nucleus. The interactions of plectin with lamin B and vimentin are modulated in vitro by phosphorylation (27). We therefore treated PBT with the phosphatase inhibitor calyculin A (CA) to attempt to disrupt or otherwise modify the assembly of proteins in the complex. Treatment of PBT with CA displaces plectin, fodrin, and actin from anti-vimentin isolates, whereas a significant amount of lamin B remains associated (Fig. 3A), indicating that plectin-independent interactions exist between vimentin and the nucleus. Actin, fodrin, and moesin remain associated in anti-fodrin isolations of CA-treated cells (not shown). The concurrent loss of fodrin, actin, and plectin supports a model in which plectin provides the bridge linking the nucleus and vimentin IFs to fodrin and the cortical cytoskeleton.

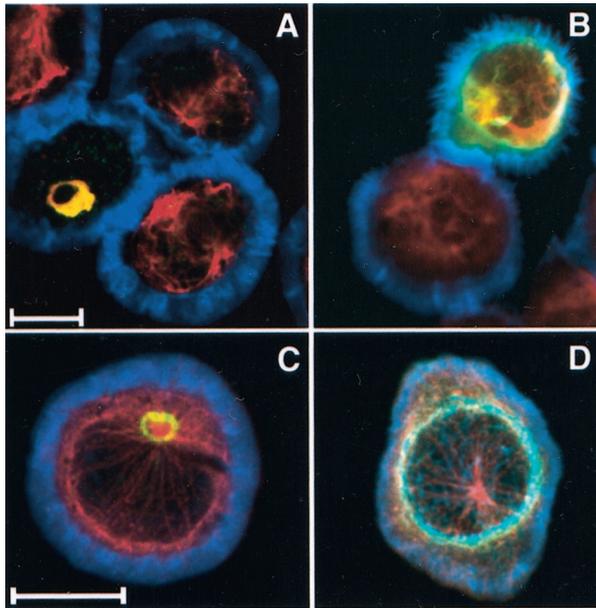


FIGURE 4. Overexpression of plectin-IFBD in Jurkat causes IF collapse. Jurkat were transfected with HA-tagged plectin-IFBD (A and C) or HA-tagged plectin-ABD (B and D) and triple-labeled with anti-HA (green, all panels), fluorescently conjugated phalloidin to stain F-actin (blue, all panels), and either anti-vimentin (red, A and B), or anti-tubulin (red, C and D). IFBD expression (A and C) causes the collapse of vimentin filaments. Collapsed IFs colocalize with HA-IFBD (A, yellow) and are found adjacent to the microtubule organizing center (C). Plectin-ABD colocalizes with F-actin but does not alter the distribution of IFs (B and D). Images are single optical sections. Bars = 5 μ m.

Transfection with plectin-IFBD induces IF collapse in Jurkat

Plectin has a well-defined intermediate filament-binding site in its C-terminal region (20). Transfection of the T cell line Jurkat with the plectin-IFBD induces complete condensation of vimentin into a single dense juxtannuclear aggregate (Fig. 4A). As with chemokine-induced uropod formation, the IFBD-induced vimentin aggregate always occurs at a site adjacent to the microtubule organizing center (Fig. 4C). The immediate proximity of the IF aggregate to the nucleus and the lack of fodrin coaccumulation (not shown) is consistent with our hypothesis that plectin forms a bridge connecting vimentin and the nucleus to fodrin and the cortical cytoskeleton. Among possible mechanisms to explain the IF collapse (15, 17, 18), we favor the model in which plectin-IFBD competes with endogenous plectin for plectin-vimentin binding sites and thereby: 1) disrupts the proposed function of plectin as a strut that prevents condensation of adjacent vimentin filaments; and 2) releases the major connection of vimentin to the membrane via actin and fodrin. Using as a control the N-terminal plectin ABD, we find no IF collapse, but rather plectin association with and modification of actin filaments (Fig. 4, B and D). Thus, in addition to physically linking vimentin to the actin cytoskeleton in PBT, plectin may also regulate the dynamic distribution and organization of both IFs and microfilaments, as described in other cell types (12, 17). Transfection with the plectin-IFBD does not induce uropod formation in Jurkat. If plectin actively regulates uropod formation, it is likely that both the IF- and actin-binding regions would be required. However, we have been unable to obtain a functional construct encoding both regions to test this prediction.

The tensegrity model (30) of cytoskeletal organization emphasizes the physical and mechanical continuity that exists within eu-

karyotic cells. We have demonstrated a new level of structural continuity within human lymphocytes and propose that plectin is an integrator of a contiguous web of proteins reaching from the nucleus to the plasma membrane in PBT. Plectin is essential to the maintenance of normal cytoarchitecture in cells exposed to mechanical stress (31). The human disorder epidermolysis bullosa simplex, involving skin blistering and muscular dystrophy, has been linked to mutations resulting in the functional loss of plectin (32). Targeted inactivation of plectin in mice results in skin blistering, degenerative abnormalities of heart and skeletal muscle, and death within days of birth (31). The increased fragility of plectin-deficient cells may be due to a loss of anchorage, stability, and/or organization of IFs (31), which themselves are major contributors to the structural integrity of cells (33). We recently reported that vimentin IFs limit the mechanical deformation of spherical human PBT and proposed that the compaction of vimentin IFs into the uropod would facilitate transendothelial migration by allowing increased cell deformation (4). Whether serving as a passive structural cross-linker or as an active regulator of lymphocyte cytoarchitecture, plectin is likely to be important to the regulated shape changes occurring during lymphocyte recruitment in vivo.

Acknowledgments

We thank Arnoud Sonnenberg for generously sharing reagents and Tilmann Brotz for use of the EIB Microscopy and Digital Imaging Facility.

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