The microtubule cytoskeleton participates in control of $eta_2$ integrin avidity

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This study was supported by NIH grant GM54715 to Jianxun Li and by a Career Development Award from the Dept. of Veterans Affairs to Dennis Kucik.
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Summary

Leukocyte avidity is regulated by cytoskeletal constraints, which keep $\beta_2$ integrins in an inactive mode. Releasing these constraints results in increased lateral mobility and clustering of integrins, effectively activating adhesion. At least part of the constraint on $\beta_2$ integrins is due to actin; whether other cytoskeletal components are involved has not previously been investigated. Microtubules are a candidate for control of integrin rearrangement, since they modulate focal adhesions, which are sites of interaction between integrins and the cytoskeleton. Here we report that both depolymerization of microtubules by colchicine or nocodazole and stabilization of microtubules by taxol increased the lateral mobility of $\beta_2$ integrins, activating adhesion.

Increased integrin mobility was accompanied by an increase in tyrosine phosphorylation of paxillin, a biochemical event associated with activation of $\beta_2$ integrins. Further, C3 exoenzyme, an inhibitor of Rho, blocked induction of integrin mobility by nocodazole, but not by taxol, suggesting that there are multiple microtubule-dependent pathways to integrin rearrangement, only some of which require Rho activity. Taken together, our data suggest that a dynamic microtubule system is required to regulate integrin-cytoskeleton interactions. Further, these data demonstrate that microtubules participate in control of integrin rearrangement, one of the earliest steps in activation of integrin-mediated adhesion.
Introduction

The β₂ integrin family of adhesion molecules plays an important role in cell adhesion and cell spreading in the immune system. An early event of cell adhesion is integrin activation (1). Following adhesion, the assembly of a focal adhesion complex, consisting of many cytoskeletal proteins, is required for cells to spread (2). Many intracellular and extracellular signals regulate integrin activation (3). In the case of β₂ integrins, activation is tightly regulated by a number of intracellular signals, often originating from other receptors. For example, on T cells, adhesion is activated by crosslinking of T cell receptors (4). Activation of integrins by signals originating within the cell is termed “inside-out” signaling (5). Although the mechanism of inside-out signal transduction is not clear, both protein kinase C (PKC) and the cytoskeleton are involved (for review see (3;6;7).

Protein kinase C-mediated effects on the cytoskeleton play a key role in β₂-integrin activation. In unactivated leukocytes, β₂ integrins are relatively immobile in the membrane because of cytoskeletal constraints. Activation of PKC with phorbol ester releases these constraints, allowing the integrins to diffuse and increasing their probability of encountering ligand (8). Although the exact mechanism of PKC’s involvement is not clear, its kinase activity causes a relaxation of cytoskeleton. It has been shown by direct measurement of integrin mobility that not only phorbol esters, but also PKC-activating cytokines (9) stimulate the mobility of β₂ integrin molecules on the membrane. The PKC signal is most likely transduced by MacMARCKS protein (10;11), because either lack of this protein, or a mutation that prevents phosphorylation, prevents both PKC-stimulated molecular mobility of integrin (9) and β₂-integrin-mediated cell adhesion (12-14).
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The actin cytoskeleton is important to activation of adhesion in at least three ways. First, the restriction of mobility of integrins before activation is mediated by the cytoskeleton. The role of the cytoskeleton in this process was confirmed previously by treating cells with low-dose (0.1-0.3 µg/ml) cytochalasin D to partially depolymerize the actin cytoskeleton. This not only stimulated β2 integrin mobility, but also activated β2-mediated cell adhesion (8). Second, cytoskeletal relaxation results in integrin clustering (15;16). Both a conformational (affinity) change (17;18) and an avidity change (8;16;19;20) occur, and may be complementary in some cases (21;22). β2 integrin clustering seems to be automatic once the cytoskeletal constraint is removed. Multivalent or clustered ligand may then promote further formation of integrin clusters. Integrin clustering results in “outside-in” signaling, activating signaling pathways within the cell. Third, cytoskeletal proteins participate in activation of adhesion by their involvement in outside-in signaling. In response to integrin clustering, paxillin (23;24) and other proteins (25) are tyrosine-phosphorylated. Subsequently, cytoskeletal proteins are recruited to link the cytoskeleton to integrins, forming focal adhesions. This stabilizes the clusters (26), enhancing cell adhesion and spreading. At this stage, an intact actin cytoskeleton is required for the assembly of focal adhesions, because at high concentrations of cytochalasin D, cells fail to spread. It is interesting that the actin filaments play opposite roles in cell adhesion and cell spreading. While partial disruption of actin results in activation of the β2 integrin, an intact actin cytoskeleton is needed to establish adhesions for cell spreading.

It has been demonstrated that microtubules are also involved in cell spreading (27-29). For example, depolymerization of microtubules with nocodazole causes spreading macrophages to retract quickly into a spherical shape (30). Similarly, in fibroblasts, cell spreading is impaired when microtubules are disassembled (27;28). Indeed, cell spreading closely correlates with
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Microtubule integrity, which can influence both the growth of actin stress fibers and the assembly of focal adhesions (32;33). Growing microtubule filaments are targeted toward the focal adhesion site (33), and such microtubule targeting seems to influence the turnover rate of focal adhesions (35). In addition to microtubules themselves, microtubule-associated proteins are also involved in cell spreading. For example, kinesin, the plus-end directed microtubule-dependent motor protein, is involved, because anti-kinesin antibody decreases cell spreading when injected into cells (29). Dynamitin, a component of dynein/dynactin motor complex, is also involved in cell spreading (31) via its P62 subunit, which localizes to focal contacts (34). It has not yet been established, however, whether the microtubule cytoskeleton also plays a dual role, as actin does, affecting activation of adhesion and cell spreading in different ways. Such a dual role in leukocytes would be consistent with a recent study that showed differential effects of microtubule disrupting agents on adhesion and spreading in an adenocarcinoma cell line (36).

In this report, we investigated the roles of both microtubule disruption and stabilization on β2 integrin mobility and its consequences for leukocyte adhesion. We used Single Particle Tracking, in which receptor movement was visualized by conjugating small particles (40-200 nm) to the integrins (8;37-41) and positions of single molecules were determined with nanometer-level spatial resolution and 30-ms time resolution (42;43). Restricted thermal motion of the integrins is then used as a readout for cytoskeletal connections (8,9,38). We showed that depolymerization of microtubules enhanced mobility of the β2 integrin molecules, a very early step in β2 integrin activation. Our data clearly indicate that although depolymerization of microtubules inhibits cell spreading, it releases β2 integrins to diffuse freely, resulting in activation of cell adhesion. This is associated with increased tyrosine phosphorylation of paxillin, a biochemical marker of outside-in signaling (14;23). Further, similar effects of
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stabilization of microtubules by taxol indicate that it is the dynamics of the microtubule system, not their presence or absence, that is required for regulation of $\beta_2$ integrin dependent adhesion. These results provide new insights into the mechanism of regulation of integrin mobility, and define an unforeseen role for microtubules.
Experimental Procedures

Materials

J774 and Wehi 274.1 cells were purchased from ATCC. MP EBV-transformed B cells were a gift from Eric Brown, University of California at San Francisco. Hybridoma HB 226, which produces hamster anti-mouse β2 integrin antibody (2E6)(44), was purchased from ATCC and was grown in serum-free media from Gibco (Grand Island, NY). After removing the hybridoma, we collected the antibody-containing supernatant and concentrated it for later use. The carboxylated fluorescent latex beads were purchased from Molecular Probes (Cat No. F-8811, Oregon, 200 nm in size). Colloidal gold beads were from EY Laboratories, San Mateo, CA. DMEM and other cell culture media were purchased from Gibco. PMA (phorbol 12-myristate 13-acetate), cytochalasin D, colchicine, nucodazole, taxol, neuraminidase X, and other routine chemicals were purchased from Sigma (St. Louis, MO).

Cell preparation

J774 cells, Wehi 274.1.1 cells, and mutant cells were cultured in DMEM with 10% fetal bovine serum (FBS). MP EBV-transformed B cells were cultured in RPMI with 10% FBS. Before the experiment, 5 x 10^6 cells were washed with phosphate-buffered saline (PBS) once and then treated with neuraminidase X (1 mU in 3 ml buffer containing 0.13 M NaCl, 0.05 M NaAC, pH 6.5) for 30 min. Then 0.5 ml of the cell suspension was added to 2.5 ml of Hanks’ solution and plated on an acid-washed coverslip coated with poly-L-lysine (8;45).

Conjugation of antibodies to the beads

For the MP cell experiments, colloidal gold particles were coated with antibody IB4, a monoclonal specific for β2 integrins (46). For the EBV-transformed B cells experiments, 100 nm. colloidal beads were coated by adsorption as described previously, to be consistent with Kucik et
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al., 1996 (8). For J774 and Wehi experiments, Fab2 fragments of anti-β2 integrin antibody (2E6) at a concentration of 10 mg/ml were conjugated to the carboxylated beads according to the manufacturer’s instructions (Molecular Probes, Oregon). In a glass tube, 100 µl of antibody or BSA were added to the reaction mixture containing 50 µl MES (200 mM), 50 µl H₂O, and 200 µl of carboxylated latex beads and gently shaken at room temperature for 15 min. Then 2 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) were added to the tube, which was shaken for 2 h at room temperature. At the end of 2 h, 450 µl of glycine (1 M, pH = 6.25) were added, and the tube was shaken for an additional 30 min to terminate the reaction. The unconjugated antibodies were removed by dialyzing the reaction mixture in a dialyzing bag (M.W. cutoff 300,000) against 1 liter of 50 mM MES (pH 6.0) overnight. The buffer was then changed to PBS by dialyzing the beads against 1 liter of PBS for another 4 h. The beads were stored at 4°C in 1.5 ml PBS containing 1% BSA and 0.02% azide.

Because the latex microbeads used here have autofluorescence, at the end of the experiments the beads were illuminated for their fluorescence so that they would not be confused with the subcellular organelles (9).

Video microscopy

The detailed microscopic procedure was previous described (9). Briefly, cells were plated on poly-L-lysine-coated coverslips. In some experiments, cells were incubated in 15 µg/ml C3 exoenzyme for 24 hours as previously described (53). Coverslips were then mounted in a steel chamber and placed on a water-jacketed heating stage on a Zeiss Axiovert 100 microscope. After the cells were spread on the poly-L-lysine-coated surface and their lamellipodia clearly defined, the anti-β2 integrin antibody conjugated beads were added at a concentration of 4-5 beads per cell. PMA (100 nM), colchicine (10 µM), nocodazole, and taxol at indicated concentrations were
added at the same time as the beads. Recording started as soon as the antibody-conjugated beads fell onto and bound to the cells and continued for 30 s. Although the larger latex beads may be phagocytosed by macrophages, based on vertical imaging on the confocal microscope the beads used here were not phagocytosed during the observation time, because either the beads were too small to cause phagocytic receptor aggregation or the 30-s observation time was too short. Beads coated with BSA or antibody against MHC II were used as controls as described previously (9). Bead motion was observed using video-enhanced differential interference contrast microscopy. A CCD camera system (Optronic DE750) with digital contrast enhancement and a Pentium II 400-MHz computer with 384-MB RAM recorded the beads’ motion directly into RAM at 30 frames/s. For each track, a 36-s segment was recorded using PIXCI software (Epix Inc, IL) and transferred later to the hard drive. Location of the beads was determined manually in each video frame for 15 s, i.e., 450 frames.

Data analysis

The $xy$ coordinates of a particle at any given time point were automatically recorded from the digitized image using a in-house written software created with Visual Basic on Window 95 platform. For each track of a bead, the mean square displacement (MSD) for each time interval was calculated from the $xy$ coordinates of the particles according to the formulas [1], [2] and [3] (for review see (43)) using the same software:

$$\rho_X(n\Delta T) = \sum_{i=0}^{N} (x_{i+n} - x_i)^2/(N + 1)$$  \[1\]

$$\rho_Y(n\Delta T) = \sum_{i=0}^{N} (y_{i+n} - y_i)^2/(N + 1)$$  \[2\]

$$\text{MSD}_{\text{total}} = \rho_n = \rho(n\Delta T) = \rho_X(n\Delta T) + \rho_Y(n\Delta T)$$  \[3\]
The obtained MSD is the sum of the random and directed motion:

\[ \text{MSD}_{\text{total}} = \text{MSD}_{\text{random}} + \text{MSD}_{\text{directed}} \]  \[4\]

because

\[ \text{MSD}_{\text{random}} = 4Dt \]  \[5\]
\[ \text{MSD}_{\text{directed}} = (vt)^2 \]  \[6\]

thus,

\[ \text{MSD}_{\text{total}} = 4Dt + (vt)^2 \]  \[7\]

By fitting the MSD calculated from the experimental data to the quadratic equation [7], the diffusion coefficient \( D \) can be extracted.

Tyrosine phosphorylation of paxillin:

Cells in 100 mm dishes were treated with either PMA (100 nM), taxol (20 \( \mu \)M) or nocodazole (5 \( \mu \)M) for 20 min. The cells were then lysed in 500 \( \mu \)l of ice-cold Tris-HCl lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8, 0.5% Triton X-100 and a cocktail of protease inhibitors). Paxillin was immunoprecipitated with 2.5 \( \mu \)g of anti-paxillin antibody (BD Biochemicals, San Diego, CA) for 1 h at 4 °C, followed by a 1:1 mixture of 50% slurry of Protein A- and Protein G-Sepharose for 30 min at 4 °C. After SDS PAGE, proteins were transferred to Immobilon
membrane and probed with 4G10, an anti-phosphotyrosine (Upstate Biochemicals, NY) followed with peroxidase-conjugated secondary antibody. Later, the membrane was stripped and probed again for paxillin to show the amount of paxillin in each lane.

*Cell aggregation assay*

J774 or Wehi 274.1.7 cells were cultured overnight in DMEM containing 10% FBS in Teflon beakers at a concentration of 2x10⁶ cells/ml. The cells were then treated with either nocodazole, taxol, or PMA at the indicated concentrations. The aggregation of cells in the Teflon beakers was quantified as follows: After suspending cells with a gentle swirling, an aliquot of cell suspension was transferred to a hemacytometer, and unaggregated cells were counted. The cells were then subjected to vigorous pipeting to break all aggregates. An aliquot was transferred to the hemacytometer and total cells were counted. The difference was the number of cells in the aggregates. Percentage aggregation was obtained by dividing the number of aggregated cells by the number of total cells.

To inhibit the aggregation with antibodies, the formed aggregates were dispersed by repeated pipetting, and the cells were resuspended in the 35-mm dishes in 1 ml of RPMI 1640 containing 10% FBS. Fab fragments of antibodies were then added to the dishes.
Results

Colchicine stimulates $\beta_2$ integrin mobility on MP EBV-transformed B cells

The actin cytoskeleton is required for the cytoskeletal constraint on the molecular mobility of $\beta_2$ integrin, and, thus, regulation of $\beta_2$ integrin mediated adhesion (8;9). Here we tested whether the microtubule cytoskeleton also participates in this cytoskeletal constraint on integrin mobility. Using the MP EBV-transformed B cell line, in which it was first demonstrated that both PMA and low-dose (0.1-0.3 $\mu$g/ml) cytochalasin D increase integrin mobility and activate adhesion (8), we tested the effect of microtubule depolymerization on $\beta_2$ integrin mobility. SPT was used to measure diffusion of $\beta_2$ integrins on MP cells before and after treatment with 10 $\mu$M colchicine. Colchicine increased the lateral mobility of $\beta_2$ integrins in a manner similar to previous results with cytochalasin D. That is, mobility was increased, without a statistically significant component of directed motion ($p < 0.01$). This increase in random motion, illustrated in the particle tracks of figure 1, A and B, is consistent with a release of the $\beta_2$ integrins from cytoskeletal constraints. The motion of the $\beta_2$ integrins was quantified by fitting the MSD plot data (fig. 1C; see experimental procedures section) and expressed as a diffusion coefficient ($D$). As can be seen in figure 1D, colchicine induced a significant increase in $\beta_2$ integrin mobility ($p < 0.01$). This confirms that, in the MP cell system, microtubules also play a role in control of integrin mobility in these cells.

Nocodazole and taxol stimulate the lateral mobility of $\beta_2$ integrin on macrophage cell lines in a concentration-dependent manner

The molecular basis of the cytoskeletal constraints on $\beta_2$ lateral mobility has been shown recently to involve MacMARCKS (9). This was demonstrated in both Wehi and J774
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macrophage cell lines. Since MacMARCKS binds to dynamin, a microtubule-associated protein (31), we asked whether microtubules were also necessary for restriction of lateral mobility in the macrophage system. We measured the effect of microtubule depolymerization on \( \beta_2 \) integrins in J774 cells. Further, to confirm that the effects on integrin mobility (as shown in figure 1) were due to microtubule disruption, rather than a nonspecific effect of colchicine, we used nocodazole, another microtubule-depolymerizing reagent (47), for these experiments. When nocodazole was added to J774 macrophage, we observed a concentration-dependent increase in mobility of \( \beta_2 \) integrin (Fig. 2). The effect of nocodazole on integrin mobility reached a plateau at a concentration of 12.5 \( \mu \)M. At this concentration, nocodazole caused an 5.8-fold increase in integrin mobility, which was close to the previously reported effect of depolymerization of actin by cytochalasin D (8;9). As with the colchicine-treated lymphocytes, the increase in \( \beta_2 \) integrin mobility was due to an increase in random motion. Thus, we conclude that microtubule cytoskeleton is involved in constraining integrin molecules.

Because the microtubule cytoskeleton is a dynamic system, constantly undergoing polymerization and depolymerization in living cells, we also tested taxol, a reagent that keeps microtubules in the polymerized state (48). We observed a concentration-dependent increase in mobility of \( \beta_2 \) integrin in J774 cells treated with taxol, similar to that caused by nocodazole (Fig. 3). This shows that, while taxol stabilizes microtubules, it also removes the cytoskeletal constraint on \( \beta_2 \) integrin mobility. Therefore, the dynamics of the microtubule system, and not merely the state of polymerization, are important for constraints on integrin mobility.

*Microtubule-regulated integrin mobility is independent of PKC.*
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We showed previously that the PKC signal is upstream of the actin cytoskeleton in regulating integrin mobility (8). Further, the effect of PKC is mediated by a PKC substrate MacMARCKS (9). Expression of a dominant negative mutant of MacMARCKS (J774-ED, effector domain deletion (14)) in J774 macrophages or a lack of MacMARCKS expression in Wehi 274.1.7 cells (13) both blocked PMA-stimulated integrin mobility (9). On the other hand, actin filaments depolymerized with cytochalasin D bypassed MacMARCKS mutation (9). To determine the relationship between the microtubule system and PKC and MacMARCKS, we tested the effects of both taxol and nocodazole on β2 integrin mobility in J774-ED and Wehi 274.1.7 cells, two types of cells that do not respond to PMA. Nocodazole (20 µM) and taxol (20 µM) stimulated 11.3-fold and 14.5-fold increases of β2 integrin mobility in J774 ED cells, respectively (Fig. 4A). In Wehi cells, nocodazole and taxol also stimulated 8.3-fold and 9.1-fold increases, respectively (Fig. 4B). This was similar to the effect of cytochalasin D (9). Lower concentrations of nocodazole and taxol stimulated lesser increases in integrin mobility, consistent with figure 2 (data not shown). Therefore, disruption of the microtubule cytoskeleton bypassed the defect in PKC/MacMARCKS signal transduction pathways, which demonstrates that microtubule cytoskeleton is independent of the PKC/MacMARCKS signals.

Microtubule depolymerization induced integrin-mediated signal transduction and cell-cell adhesion of J774 cells.

To determine whether the enhanced β2 integrin mobility correlated with integrin-dependent signal transduction, we examined whether inhibition of microtubule dynamics would also affect tyrosine phosphorylation of paxillin, shown earlier to be a marker of outside-in signaling through β2 integrins (14;23;24). After treatment of cells with taxol or nocodazole,
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paxillin was immunoprecipitated from cell lysates, and its tyrosine phosphorylation was probed with the anti-phosphotyrosine antibody 4G10 (Fig. 5). Both taxol (5 μM) and nocodazole (5 μM) treatments induced increased tyrosine phosphorylation of paxillin compared to the untreated control cells (although to a lesser extent than PMA, a strong inducer of paxillin phosphorylation (23;24) and used here as a positive control). Thus, at drug concentrations at which integrins are beginning to be released from the constraints on their diffusion (c.f. figures 2 and 3), both disruption and stabilization of microtubules have a clear (but not maximal) effect on signaling pathways associated with β2 integrin activation.

To determine whether the increased integrin mobility induced by microtubule disruption was sufficient to activate integrin-mediated functions, we tested integrin-mediated cell-cell adhesion and cell spreading. Cell-cell adhesion assays were done with J774 macrophages in Teflon beakers, where the cells do not adhere to the culture surface. Before the addition of PMA or nocodazole, these cells did not aggregate (Fig. 6A). After adding nocodazole at 25 μM, a concentration that induces maximal integrin mobility (c.f. fig. 2) or PMA (100 ng./ml.) for 6 hours, J774 cells formed large aggregates (Fig. 6A). To confirm that this was β2 integrin-mediated cell aggregation, the aggregates were dispersed by repeated pipeting and the cells were recultured in the presence or absence of the Fab fragment of anti-β2 antibody (2E6). Within 6 hours, cells cultured without anti-β2 reaggregated, whereas those with antibody remained dispersed (Fig. 6B). Thus, we conclude that depolymerization of microtubules is sufficient to activate β2-integrin-mediated cell adhesion.

This β2 integrin-mediated aggregation was also examined in J774-ED cells. Because of the MacMARCKS mutation, J774-ED cells did not aggregate in response to the addition of PMA (Fig. 6C). However, 25 μM nocodazole induced aggregation in these mutant cells (Fig. 6C).
This further supports the contention that the microtubule-mediated constraint on integrin mobility is independent of PKC and MacMARCKS.

**Intact microtubules are required for cell spreading.** In cell culture plates in media containing 10% FBS, J774 macrophages adhere to the culture dish surface but do not spread extensively. Treating these cells with PMA is sufficient to induce $\beta_2$ integrin-dependent spreading (Fig. 7). Although depolymerization of microtubules has the same effect as PMA in activating integrin-mediated adhesion, it does not stimulate cell spreading (Fig. 7). In fact, adding nocodazole (5 $\mu$M) inhibits macrophage spreading. This is a minimal nocodazole concentration, which only partially mobilizes integrins (c.f. figure 2), suggesting that cell spreading is exquisitely sensitive to microtubule disruption.

**C3 exoenzyme, an inhibitor of Rho, prevents nocodazole-, but not taxol-induced increases in integrin mobility.** Rho regulates cytoskeletal rearrangement, and has effects on cell spreading and focal contact formation (32;55;58;59). Since microtubule inhibitors have been shown to affect Rho function, it is reasonable that Rho might be involved in microtubule-mediated regulation of integrin mobility. To explore the molecular basis of regulation of integrin mobility, we tested the involvement of Rho in nocodazole-, taxol- and PMA-stimulated integrin diffusion. We found that while the C3 strongly inhibits the nocodazole-stimulated increase in integrin diffusion, it has little effect on the taxol- or PMA-mediated increase (fig. 8). This observation suggests that while both taxol and nocodazole treatment result in enhanced integrin mobility, their mechanisms of action may be different, since the nocodazole effect is Rho-dependent, while taxol and PMA can exert their effects independently of Rho.
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Discussion

In this report, we demonstrated by direct diffusion measurements that the microtubule cytoskeleton participates in control of $\beta_2$ integrin avidity. This is the first report implicating microtubules in integrin rearrangement, and provides new insights into the regulation of activation of leukocyte adhesion. We demonstrated that disruption of the microtubule cytoskeleton causes an increase in the mobility of $\beta_2$ integrins. Further, not only the integrity, but also dynamics of microtubules are important for their role in controlling integrin mobility, since taxol, which stabilizes microtubules, also releases the integrins to diffuse.

It has been known for some time that microtubule integrity is necessary for cell spreading (54). It is generally believed that integrin activation precedes cell spreading. Thus, one might assume that microtubule integrity would be required for integrin activation. Interestingly, our data show that the opposite is the case. While we confirm that intact microtubules are required for cell spreading, it is interference with microtubule dynamics, either by depolymerization or stabilization, that results in integrin activation. This is true for both increased integrin mobility and enhanced cell-cell adhesion. Thus, microtubule dynamics are necessary to maintain $\beta_2$ integrins in the immobile, inactive state. Together with the result that partial disruption of the actin cytoskeleton is sufficient to mobilize $\beta_2$ integrins and activate adhesion (8), this supports the diffusion-limited model of $\beta_2$ integrin activation (8), demonstrating a link between integrin mobility and activation of integrin function.

Once the integrins have been mobilized, high affinity binding to ligand through conformational change (17;18), or high avidity ligand binding through molecular clustering (8;16;19;20), can occur. The fact that, although cytoskeletal disruption activates adhesion, it inhibits cell spreading indicates either an unknown control mechanism for cell spreading.
downstream of the cytoskeleton, or that an intact cytoskeleton is needed to facilitate spreading. We prefer the latter, since a large body of evidence indicates that an intact cytoskeleton is an integral part of the spreading mechanism.

Regulation of both actin and tubulin polymerization in cells is complex, and the molecular details remain to be worked out. Clearly, the PKC pathway is involved, because activation of PKC achieves the same effect on integrin mobility as dissociating the cytoskeleton ((8) and this report). The PKC substrate MacMARCKS is also clearly involved, since mutation of MacMARCKS blocks increased $\beta_2$ integrin mobility (9). Microtubule involvement, however, is independent of PKC and MacMARCKS signals, because disruption of microtubules bypasses the mutation of MacMARCKS. This could indicate that the microtubule effect is downstream of PKC and MacMARCKS, or that the pathways are separate.

Our data also show that once the constraint on microtubule cytoskeleton is released, the $\beta_2$ integrin is sufficiently activated to both initiate signal transduction (as indicated by paxillin phosphorylation) and to mediate cell-cell adhesion. This effect is similar to that of partial disruption of the actin cytoskeleton (8). Thus, once the $\beta_2$ integrins are released to diffuse, both adhesion and signal transduction are activated.

Rho family GTPases are intimately involved in cytoskeletal rearrangement, and, in particular, in regulation of stress fibers and focal contacts (32;55;58;59). It has long been observed that microtubule disrupting agents promote the formation of stress fibers and focal adhesions (54;56). However, the molecular mechanisms underlying the microtubule effects are not worked out in detail. Recently, it was demonstrated that microtubule-disrupting agents affect the activity of one of the Rho family GTPases, Rac1 (57). For that reason, we tested whether
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Microtubule-dependent mobilization of $\beta_2$ integrins might be linked to Rho family effects on cytoskeletal rearrangement.

We found that the microtubule-dependent regulation of integrin diffusion does involve the Rho family, but in a complex manner. That is, inhibition of Rho with the C3 exoenzyme blocked the effect of microtubule disruption by nocodazole on $\beta_2$ integrin mobility. However, although stabilization of microtubules with taxol also led to increased integrin mobility (and activation of integrin-mediated adhesion), this could not be blocked by C3. Therefore, the relationship between microtubule dynamics and the Rho family GTPases is not simple and direct. This is not surprising, since regulation of integrin mobility and of cytoskeleton rearrangement are both complex processes. Regulation of microtubule dynamics necessarily involves regulation of both polymerization and depolymerization. It is possible that taxol-mediated stabilization of microtubules affects regulation of integrin dynamics in a way that is independent of the Rho family. Alternatively, other Rho family GTPases, such as Rac1 and/or Cdc42, might be involved. Based on the recent report that microtubule growth promotes Rac1 activation and lamellipodia protrusion (57), it possible that taxol stimulated integrin mobility involves Rac1.

It is also interesting that talin has been shown to bind to the cytoplasmic tail of unactivated $\beta_2$ integrins (49), as well as to the $\beta_1$ and $\beta_3$ cytoplasmic domains (50;51). PKC activation results in dissociation of the talin-integrin link by promoting the proteolytic degradation of talin (49). Whether talin is involved in microtubule-mediated regulation of $\beta_2$ integrin function, and how it may be involved with Rho family GTPases, remains to be investigated. It will be important to work out in detail the molecular mechanism of the
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microtubule system’s role in control of leukocyte adhesive function. This will be the focus of future work.

The finding that the microtubule cytoskeleton is involved in control of integrin mobility provides important new information about regulation of integrin activation. This report demonstrates that the microtubule cytoskeleton has dual effects: while disruption of the microtubule cytoskeleton results in integrin activation, its integrity is required for cell spreading. Our data, demonstrating that it is the dynamics, not the integrity of microtubules that maintains integrins in the cytoskeleton-restricted state indicates that microtubules probably regulate, rather than physically restrict, integrin motion. Evidence is accumulating that microtubules play unexpected roles in several adhesion-mediated cell functions, including adhesion, spreading and migration. However, the role of microtubules is often assumed to involve either structural effects or transport of intracellular vesicles. Our finding that microtubules are involved activation of integrins will have implications for understanding integrin function in general and its regulation in cell functions.
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Footnote

All correspondence should be addressed to DFK or JL.

$ The computer software created in JL's laboratory for Single Particle Tracking is available to
anyone upon request. Please send request to JL.

Abbreviations

DMEM: Dulbecco's modified Eagle's medium. ED: Effector domain deletion mutant of
MacMARCKS. EDAC: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. FAK: Focal Adhesion
Kinase. ICAM-1: Intercellular Adhesion Molecule 1. MARCKS: Myristoylated Alanine-rich C
Kinase Substrate. MacMARCKS: Macrophage-enriched Myristoylated Alanine-rich C Kinase
Substrate. MCP-1: (Macrophage chemoattractant protein-1). MES: 2-(N-
morpholino)ethanesulfonic acid. MSD: Mean square displacement. PBS: Phosphate-buffered
saline. PKC: Protein kinase C. PMA: Phorbol 12-Myristate 13-Acetate. SPT: Single Particle
Tracking.
Figure legends

Figure 1. Depolymerization of microtubules releases cytoskeletal constraints on β2 integrin diffusion in EBV-transformed B cells. Fig. 1 A and B are examples of particle tracks before and after release by colchicine of cytoskeletal constraints on motion. Random integrin mobility is increased by colchicine, with no significant component of directed motion, as calculated by a statistical method based on that of Berg (52) and described earlier (8); thus, the major effect of microtubule depolymerization is an increase in random motion of the integrin. C) Representative MSD plots of β2 integrin motion before and after release from cytoskeletal constraints on mobility. Triangles = before colchicine treatment (10 µM); squares = after colchicine. These examples are chosen to be near the mean D for each group. From these plots, a diffusion coefficient can be extracted to quantify random motion of the integrins, and this can be used as a readout of constraints on mobility by the cytoskeleton. D) Comparison of mean D ± SEM demonstrates the increase in mobility of β2 integrins due to microtubule depolymerization. (D = 0.24 ± 0.05 x 10^{-10} cm^2/s before treatment and 3.9 ± 1.2 x 10^{-10} cm^2/s after 10 mM colchicine.)

Fig. 2. Microtubule depolymerization induces an increase in lateral mobility of β2 integrins. J774 macrophages were incubated with the indicated concentration of nocodazole. Integrin mobility was assayed using SPT methods as described in Experimental Procedures. Diffusion coefficients (D) obtained at different concentrations of nocodazole were as follows: 0.74±0.19x10^{-10} cm^2/sec at 0 µM, 1.14±0.50x10^{-10} cm^2/sec at 1 µM, 1.79±0.34x10^{-10} cm^2/sec at
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5 µM, 2.71±0.79x10⁻¹⁰ cm²/sec at 10 µM, 4.27±0.95x10⁻¹⁰ cm²/sec at 12.5 µM, 4.38±0.97x10⁻¹⁰ cm²/sec at 30 µM.

Fig. 3. Taxol induces an increase in lateral mobility of β₂ integrin. J774 macrophages were treated with the indicated concentrations of taxol, and integrin mobility was measured using SPT methods as described in the Experimental Procedures. The following diffusion coefficients (D) at different concentrations of taxol were obtained: 0.74±0.19x10⁻¹⁰ cm²/sec at 0 µM, 1.25±0.52x10⁻¹⁰ cm²/sec at 0.1 µM, 1.41±0.68x10⁻¹⁰ cm²/sec at 1 µM, 4.50±1.33x10⁻¹⁰ cm²/sec at 5 µM, 5.15±1.52x10⁻¹⁰ cm²/sec at 10 µM.

Fig. 4. Nocodazole and taxol bypass the defect in PKC/MacMARCKS signal transduction pathways. (A) J774 cells with a MacMARCKS effector domain deletion mutant (J774-ED). (B) Wehi 274.1.7 cells, which lack MacMARCKS expression. In both cell types, the β₂ integrins have been shown earlier to be unable to respond to PMA stimulation (13,14). However, in both cell types, 20 µM taxol and 20 µM Nocodazole each induced a significant increase in the diffusion coefficient of β₂ integrins (p < 0.01).

Fig. 5. Both depolymerization and stabilization of microtubules induce tyrosine phosphorylation of paxillin. J774 cells were treated with PMA (100 nM), taxol (5 µM) or nocodazole (5 µM) for 20 min. Paxillin was immunoprecipitated with anti-paxillin antibody followed by SDS PAGE. After transfer to an Immobilon membrane, paxillin phosphorylation was probed with anti-phosphotyrosine antibody 4G10. The ratio of phosphorylated paxillin is:
control: PMA: Nocodazole: Taxol = 1.0:4.4:2.6:2.5. The same membrane was then stripped and reblotted with anti-paxillin.

Fig. 6. Nocodazole promotes β₂ integrin-mediated cell aggregation of J774 macrophages. A) J774 macrophages were treated with PMA or nocodazole at the indicated concentrations for 6 h in Teflon beakers and photographed. The percentage of cells aggregated was: no addition: 1.2 + 0.1%; PMA: 79.5 + 4.3%; Nocodazole: 79.5 + 9.3%. Both PMA- and nocodazole-induced aggregation was significantly greater than control (p < 0.01 by T test). B) Aggregated cells were dispersed by repeated pipetting and then allowed to re-aggregate in the presence or absence of antibody against β₂ integrin (2E6). The percentage aggregated was: Dispersed: 1.2 + 0.3%; reaggregated: 73.7 + 8.0%; Reaggregated with antibody: 16.7 + 5.9%. Anti-β₂ antibody caused a statistically significant decrease in aggregation (p < 0.01). C. The MacMARCKS mutation in J774-ED cells resulted in an absence of PMA-stimulated aggregation. However, the defect was bypassed with 25 µM nocodazole. The percentage aggregated was: No addition: 1.0 + 0.2%; PMA: 7.8 + 1.5%; Nocodazole: 88.6 + 3.4%. Nocodazole resulted in a statistically significant increase in aggregation (p < 0.01).

Fig. 7. PMA-stimulated cell spreading is inhibited by nocodazole. Untreated J774 macrophages did not spread extensively. PMA (100 nM) induced these cells to spread in 15 min. Adding nocodazole (5 µM) together with PMA abolished PMA-stimulated cell spreading.

Fig. 8. Effect of C3 exoenzyme on microtubule-dependent integrin mobility. J774 cells were incubated with 15 µg/ml of C3 in DMEM for 24 hours. The cells were then treated with taxol,
nocodazole or PMA as described in experimental procedures. The mobility of β2 integrin molecules was recorded and expressed as a diffusion coefficient. Statistical analysis showed that only the differences between nocodazole samples are significant.
Fig 2

J774_WT + Nocodazole

Diffusion Coefficient (x 10^-10 cm^2/sec) of J774_WT

0  5  10  15  20  25  30  

μM
Fig 3

J774_WT + Taxol

Diffusion Coefficient ($10^{-10} \text{ cm}^2/\text{sec}$) of J774_WT

- 0 µM: 1
- 2 µM: 2
- 4 µM: 3
- 6 µM: 4
- 8 µM: 5
- 10 µM: 6

Error bars indicate variability.
Fig 4

A. J774_ED + Nocodazole/Taxol

Diffusion Coefficient (x 10^-10 cm^2/sec) of J774_ED

- Nothing Add
- + Noc 20μM
- + Taxol 20μM

B. Wehi + Nocodazole/Taxol

Diffusion Coefficient (x 10^-10 cm^2/sec) of J774_ED

- Nothing Add
- + Noc 20μM
- + Taxol 20μM
Fig 5

Control | PMA | Nocodazole | Taxol

Blot Phosphotyrosine

Blot Paxillin
Fig 7

No Add  + PMA (100ng/ml)  + PMA & Nocodazol

[Images of cells under different conditions]
Fig. 8
The microtubule cytoskeleton participates in control of beta2 integrin avidity
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J. Biol. Chem. published online September 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104029200

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