The GTPase Rap1 Regulates Phorbol 12-Myristate 13-Acetate-stimulated but Not Ligand-induced β1 Integrin-dependent Leukocyte Adhesion*

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Leukocyte migration from bloodstream to tissue requires rapid, coordinated regulation of integrin-dependent adhesion and de-adhesion. In a previous study we demonstrated that inhibition of protein geranylgeranylation inhibited phorbol ester-stimulated avidity modulation of β1 integrin in several leukocyte cell lines. Both RhoA and Rap1 require post-translational modification by geranylgeranylation for full function. In this report we identify Rap1, not RhoA, as a critical geranylgeranylated protein mediating phorbol ester-stimulated β1 integrin and β2 integrin-dependent adhesion of Jurkat cells. Overexpression of the Rap1-specific GTPase-activating protein, SPA-1, or inactivated form of Rap1 (N17Rap1) blocked phorbol ester-stimulated adhesion of Jurkat cells to fibronectin (αβ1) and ICAM-1 (αβ2). With high concentrations of fibronectin as ligand, Jurkat cells adhered spontaneously without phorbol ester stimulation. Unlike the phorbol ester-stimulated adhesion, adhesion induced by high density ligand was not dependent upon Rap1 activation or actin cytoskeleton reorganization. Thus, the “inside-out” adhesion signal induced by phorbol ester and the “outside-in” signal induced by high density ligand involve different pathways.

The essential role of leukocyte integrin receptors in cell-cell and cell-substrate adhesion in the inflammatory and immune systems is well established. The adhesive capacity of leukocyte integrins is highly regulated. Integrin receptors in a low adhesive state do not mediate strong adhesion to other cells or ligands. However, when leukocytes are appropriately activated, there is often a detectable increase in integrin adhesive-ness within a few seconds to minutes. Some activation stimuli induce a measurable change in integrin receptor affinity, whereas others mediate their effects without altering affinity but instead utilize post-receptor events involving cytoskeleton-dependent clustering of receptors that serve to increase overall adhesivity. Increases in integrin adhesivity produced by post-receptor events without changes in receptor affinity have been defined as increased avidity. Post-receptor events also regulate “outside-in” signaling in which integrins transduce information from the exterior to the interior of the cell, engaging classic signaling pathways that control growth, differentiation, apoptosis, and cytokine expression.

Whether leukocyte integrin adhesivity is regulated primarily by affinity or avidity modulation is still somewhat controversial (1). For a given leukocyte cell type, different activation stimuli may modulate integrin adhesivity by one or the other mechanism. For example, the functional activity of β1 integrins on human T-cells can be regulated by treatment with certain divalent cations or activating monoclonal antibodies (mAbs),1 which directly increase the affinity of β1 integrins for their ligands, presumably by altering receptor conformation (2). In contrast, the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) generally promotes β1-dependent leukocyte adhesion by targeting events that occur following receptor occupancy without significantly affecting the affinity of the receptor for the ligand (3). Moreover, for the same stimulus, integrin receptors of one subfamily may respond differently from those of another. For example, Weber et al. (4) noted that there was differential regulation of αβ1 and αβ2 integrin adhesiveness in eosinophils stimulated by the same chemoattractant. β2 integrin-mediated adhesion was dependent upon affinity modulation, whereas αβ1 integrin-mediated adhesion involved post-receptor events. For leukocyte β1 integrins, the regulation of adhesivity may be mediated predominantly by post-receptor events such as diffusion/clustering in the membrane and subsequent cytoskeletal interactions rather than by affinity modulation (3). Activation of leukocyte β1 integrins also involves avidity modulation, although affinity mechanisms clearly apply (5). Although a number of cytoplasmic protein regulators of integrin affinity have been identified and characterized in a variety of cell types (1), the signal transduction pathways involved in avidity modulation in leukocytes have not been fully elucidated. One current model of avidity modulation proposes that leukocyte integrins are loosely restrained in the plasma membrane in an inactive, non-clustered state by interaction of the β-subunit cytoplasmic tail with cortical actin cytoskeleton (1). Regulatory proteins such as MacMARCKS (6) and L-plastin (7) maintain the cortical cytoskeleton. Diverse stimuli trigger activation of the integrin receptors by "inside-out" signaling, resulting in activation of PKC, phosphorylation of L-plastin and MacMARCKS, increases in intracellular Ca2+, and activation of calpain. These

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1 The abbreviations used are: mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; FN, fibronectin; ΔGRD, GAP-related domain; PKC, protein kinase C; MacMARCKS, macrophage-enriched myristoylated alanine-rich C kinase substrate; PGGT, protein geranylgeranyl transferase; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); GAP, GTPase-activating protein; PBS, phosphate-buffered saline.
 signals promote the release of integrin receptors from the cytoskeletal constraints, allowing diffusion of the receptors within the membrane. With increased diffusion there is clustering of receptors upon contact with immobilized ligand, augmenting cellular avidity for the ligand-coated surface. Subsequently, other signaling components such as cytohesin-1 (8), Rac1 (9), and GTPases are recruited to the clusters, leading to adhesion strengthening, actin stress fiber formation, spreading, actin deposition "activation" epitopes, and assembly of signaling molecules. All of these events may take place without significant changes in receptor affinity.

Several Ras/Rho small GTPases have also been reported to regulate leukocyte integrin avidity. H-Ras and Rac were shown to be involved in stimulated β2 integrin-dependent leukocyte adhesion (10). Previous studies (11) have established a critical role for RhoA in integrin clustering, adhesion, and spreading in a wide variety of adherent cell types. However, studies of leukocyte adhesion using C3 exoenzyme to inactive RhoA specifically have yielded conflicting results. Blockade of RhoA function by the C3 exoenzyme was reported to inhibit β2 integrin-dependent stimulated neutrophil adhesion (12). However, in other studies treatment with C3 exoenzyme did not affect β2 integrin-dependent adhesion of neutrophils (13) or JY lymphocytic cells (14) or β1 integrin-dependent adhesion of peripheral blood T-cells (15) or U937 monocytic cells (16). Finally, several recent studies have implicated Rap1, a small GTPase with 53% amino acid sequence homology to K-Ras, in stimulated β1 (17–19) and β2 (10, 19–21) integrin-dependent leukocyte adhesion, as well as β2 integrin-dependent platelet adhesion (22).

In a previous study (23) we demonstrated that inhibition of protein geranylgeranylated inhibited phorbol ester-stimulated avidity modulation of β2 integrin in several leukocyte cell lines. Both RhoA and Rap1 require post-translational modification by geranylgeranylation for full function. In this report we identify Rap1, not RhoA, as a critical geranylgeranylated protein mediating phorbol ester-stimulated β2-integrin-dependent adhesion of Jurkat T-cells. We show further that adhesion to fibronectin (FN) stimulated by PMA and "spontaneous" adhesion induced by binding to a high concentration of FN involve different mechanisms. Unlike PMA-stimulated adhesion, adhesion induced by binding to high density ligand is not dependent upon Rap1 activation, actin cytoskeleton reorganization, PKC activation, or tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat cells (American Type Culture Collection, Manassas, VA) and NIT1/Rap1-transfected Jurkat cells (10) a gift from Dr. T. Kinashi, Kyoto University, Kyoto, Japan) were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and non-essential amino acids (Invitrogen).

Reagents—The blocking anti-β2 integrin subunit (CD29) monoclonal antibody (mAb) 5D1I was generated in our laboratory (24). Monoclonal antibody 5A2 is an anti-actin activator antibody directed to β1 integrin subunit (25). Phorbol myristate acetate (Sigma) was used at 100 ng/ml in Me2SO, Molecular Probes, Eugene, OR) for 30 min at room temperature in the dark. The cells were then washed with PBS. After centrifugation for 7 min at 300 g, the infected Jurkat cells were resuspended in 1 ml of phenol red-free medium and then labeled by incubation with 5 µl of the fluorescent dye calcein-AM (1 mg/ml in Me2SO, Molecular Probes, Eugene, OR) for 30 min at room temperature in the dark. The cells were then washed with phenol red-free medium. After incubation with PMA or the β2 integrin-activating mAb 5A2 (2 µg/ml) in 30 min in control medium at room temperature, cells (~1 × 10^6/well) were added to triplicate wells. After incubation for 30 min at 37 °C, the total population of cells in the well was analyzed using a fluorescence plate reader (Perspective Biosystems, Framingham, MA). Unbound cells were removed by washing the plate three times with phenol red-free medium, and the plate was then reanalyzed to determine the fluorescence of bound cells. After subtraction of background, the percent adherence was calculated as the emission at 530 nm of bound cells divided by the emission of total cells.

Detection of Small G-Protein Activation—Small G-protein activation was detected with an activation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, cells were lysed with Mg2+/lysine/wash buffer (MBL) containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% CA-630, 10 mM MgCl2, 1 mM EDTA, 10 mM glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, and 25 mM sodium fluoride. The lysates were incubated at 4 °C for 30 min with Raf-1-PBD-agarose to detect Rap1 or Ras activation or with PAK-1-PBD-agarose for Rac. After washing three times with MB1, the pellet was subjected to SDS-PAGE. The gels were then analyzed by immunoblotting with an anti-Rap1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ras antibody (Upstate Biotechnology, Inc.), or anti-Rac antibody (Upstate Biotechnology, Inc.). Because Raf-1-PBD has a much higher binding affinity for Rap1 and Rac (30), only Rap1 was detected by western blotting. In the experiment, the cell lysate was pre-cleared by immunoprecipitation of Ras with the anti-Ras antibody.

β2 Integrin Receptor Clustering—An assay to detect stimulated β2 integrin diffusion/clustering was developed based upon the report from Rehemtulla et al. (31). Briefly, control or treated cells were incubated in medium (2 mM sodium butyrate: syndecan sulfate: fibronectin) (DTSSP) (Pierce), which covalently links molecules within 12 Å. Following cross-linking, cells were lysed, and limiting anti-β2 mAb was added. More integrin receptor was immunoprecipitated if β2 integrin receptors were either freely diffusible, allowing "capture" by the cross-linker, or clustered. In contrast, with excess anti-β2 mAbs equivalent amounts of integrin receptor were immunoprecipitated, independent of receptor mobility or clustering. Jurkat cells (~5 × 10^6) were untreated or treated with 100 ng/ml PMA or with cytochalasin D (1 µg/ml) for 30 min at room temperature before adding 1.25 mg/ml of DTSSP. After cross-linking for 3 h at room temperature, DTSSP was removed by
waxing the cells three times with PBS. Immunoprecipitation
was performed with the anti-β1 mAb 8A2 at a concentration of 1 µg/ml. Immunoprecipitates were subjected to SDS-PAGE, and immunoblot-
ing was then performed with an anti-

Staining was then performed with an anti-

The geranylgeranylation of Rap1 was also inhibited by GTGT-298 at the same time course and dose as GTGT-298-inhibited cell adhesion (23). We investigated the role of Rap1 by transducing cells with the Rap1-specific GTPase-activating protein (GAP), SPA-1 (27). Tsukamoto et al. (27) demonstrated previously that overexpression of SPA-1 blocked Rap1 activation through its GAP activity in 293T cells and that a mutant SPA-1 without a GAP-related domain, ΔGRD, was without effect. As shown in Fig. 2, PMA activated Rap1 from

the GDP- to GTP-binding form. Compared with the vector control, overexpression of SPA-1 suppressed Rap1 activation in both resting and PMA-stimulated cells. However, the mutant SPA-1-ΔGRD enhanced production of Rap1-GTP. SPA-1 did not inhibit the GTP loading of Ras or Rac GTPases (Fig. 2).

Overexpression of SPA-1 significantly reduced PMA-stimulated Jurkat cell adhesion to FN (Figs. 1 and 3A). There was some increased adhesion with PMA compared with basal adhesion in the SPA-1-overexpressing cells, but maximal adhesion was much lower than that of vector control or that of SPA-1-ΔGRD (Fig. 3B). The SPA-1-ΔGRD mutant moderately increased cell adhesion compared with vector control (Fig. 3B), consistent with its effect on Rap1-GTP binding in vivo (Fig. 2).

Cells overexpressing an inactivated form of Rap1, N17Rap1, evidenced reduced adhesion in response to PMA (Fig. 3C). In contrast to its effect on PMA stimulation, overexpression of SPA-1 had no effect on adhesion induced by the activating mAb 8A2 (Fig. 3A).

Overexpression of SPA-1 Does Not Inhibit Cytochalasin D-Induced Adhesion or β1 Integrin Diffusion/Clustering—Previous reports (33) showed that low doses of cytochalasin D induced cell adhesion, whereas higher doses inhibited it. We found that overexpression of SPA-1 did not block cytochalasin D-induced adhesion (Fig. 4). As shown in Fig. 5, PMA treatment of Jurkat cells markedly increased the amount of β1 integrin immunoprecipitated by limiting amounts of anti-β1 mAb in the presence of cross-linker, indicating an increase in diffusion, clustering, or both. Cytochalasin D treatment also markedly increased β1 integrin immunoprecipitated in resting cells, consistent with dissolution of basal cytoskeletal restraints, allowing increased diffusion and capture by cross-linker. SPA-1 did not block cytochalasin D- or PMA-induced integrin diffusion/clustering as detected by this technique (Fig. 5).

Overexpression of SPA-1 Also Inhibits PMA-Stimulated β2 Integrin-Dependent Adhesion—As shown in Fig. 6, overexpression of SPA-1 reduced α1β2 integrin-dependent Jurkat cell adhesion to ICAM-1 as well as α1β1-dependent adhesion to FN. Thus, the effect of SPA-1, and hence the role of Rap1, involves β2 as well as β1 integrin receptor activation.

The Inhibitory Effect of SPA-1 Is Dependent Upon the Density of Ligand—As shown in Fig. 7A, the adhesive behavior of Jurkat cells differed dramatically with the concentration of FN coating the wells. PMA stimulation markedly increased adhesion of vector control and SPA-1-ΔGRD cells, but not of SPA-1 cells, to wells coated with 1 µg/ml FN. When the concentration

\[ \text{Fig. 1. Blockade of RhoA or RhoA kinase activity does not inhibit phorbol ester-stimulated leukocyte adhesion. SPA-1-overexpressing Jurkat cells and Jurkat cells treated overnight with medium (control), 10 µg/ml C3 exoenzyme (C3) or for 1 h with 30 µM Y-27632 (Y-27632) were stimulated with phorbol ester to adhere to 1 µg/ml FN for 30 min. Values are means ± S.D. of four replicates in a representative experiment.} \]

\[ \text{Fig. 2. SPA-1 down-regulates Rap1 activation. 2.0 × 10^6 Jurkat cells were starved for 5 h in serum-free medium. Cells were lysed in 1 ml of MLB. The activated GTP-bound form of Rap1 or Ras was bound to Rap-1-RBD-agarose, and the immunoblot was detected with anti-Rap1 antibody or anti-Ras antibody. The activated GTP-bound form of Rac was bound to PAK-PBD-agarose, and the immunoblot was performed with anti-Rac antibody.} \]
of FN used to coat the wells was increased from 1 ("low") to 2 ("high") μg/ml, the adhesion of unstimulated vector control cells increased from 5 to 65% and unstimulated SPA-1-ΔGRD cells from 16 to 79% and even that of the SPA-1 cells increased from 1 to 51%. Although overexpression of SPA-1 reduced both unstimulated and PMA-stimulated adhesion to low FN, SPA-1 had little effect on the adhesion of SPA-1 cells to high FN. At 1 μg/ml FN the PMA-stimulated adhesion of SPA-1 cells was only 22%, and those of the vector control and SPA-1-ΔGRD were 74 and 86%, respectively. For PMA-stimulated SPA-1 cells, adhesion increased to 88% at 2 μg/ml FN, which was
PMA did induce Rap1 activation in control and SPA-1-FN, based on the GTP-loading assay. In the same experiment Rap1 was not activated in any of the cells adherent to high (93%) cells.

B

but not in SPA-1 cells (Fig. 7).

Replicates (*, p < 0.05 versus PMA stimulation by 100 ng/ml PMA). Values represent means ± S.D. of three replicates corresponding SPA-1-infected cells). B, Rap1 activation assays were performed with 2 × 10^6 cells as in Fig. 1. Vector control cells (control), SPA-1-infected cells (SPA-1), and SPA-1-ΔGRD-infected cells (ΔGRD) were treated with or without 100 ng/ml PMA in suspension or adhered to wells treated with 4 μg/ml FN without PMA (+FN). Rap1-GTP was detected by pull-down assay with Raf-1-RBD and anti-Rap1. Because Raf-1-RBD has higher affinity for Ras than for Rap1, cell lysate was pre-cleared with anti-Ras antibody before the pull-down assay was performed.

A

The Outside-in Signal Is Distinct from the Inside-out Signal—PMA-stimulated cell adhesion was dependent upon the activation of Rap1 (Fig. 3A), whereas adhesion induced by high FN was independent of Rap1 activation (Fig. 7A). The inactivated form of Rap1, N17Rap1, also failed to inhibit unstimulated adhesion to high density of FN (5 μg/ml). Unstimulated adhesion of control cells was 12% to low FN and 30% to high FN. Adhesion of N17Rap1 cells to low FN was 4.5 versus 28% to high FN (means of eight replicates). These results suggested that PMA-stimulated and high density ligand-induced adhesion involved distinct mechanisms. This was supported by the observation that PMA-stimulated adhesion was inhibited by cytochalasin D, whereas adhesion to high density FN was not affected (Fig. 8A).

Additional studies were performed to determine the signaling pathways involved in Jurkat cell adhesion at high density FN, which was not dependent upon Rap1 or cytoskeletal reorganization. Both PMA-stimulated adhesion to low FN and unstimulated adhesion to high FN were blocked by deoxyglucose and azide (Fig. 8B), indicating that both required active cell metabolism. PMA-stimulated adhesion to low FN was reduced by treatment with the tyrosine kinase inhibitor, genistein, and the PKC inhibitor, staurosporine, whereas neither agent affected unstimulated adhesion to high FN (Fig. 8B).

DISCUSSION

We had determined previously (23) that a 20–30-kDa protein(s) whose geranylgeranylation was catalyzed by PGGT-1 was a candidate to regulate PMA-stimulated, β1 integrin-dependent Jurkat cell adhesion. Geranylgeranlyation of both RhoA and Rap1 proteins was inhibited when PMA-stimulated cell adhesion was blocked by lovastatin or the PGGT-1 inhibitor GGTI-298 (23). However, treatment of cells with C3 exoenzyme, which ADP-ribosylates RhoA protein and prevents RhoA interaction with its downstream protein, did not inhibit PMA-stimulated adhesion to FN (Fig. 1). This suggested that RhoA activation was not required for PMA-stimulated, β1 integrin-dependent leukocyte adhesion. That conclusion was further supported by the observation that inhibition of RhoA kinase,
the downstream effector of RhoA, with the specific inhibitor Y-27632 did not inhibit PMA-stimulated α5β1 integrin-mediated adhesion (Fig. 1). In addition, we have shown that RhoA activation is not required for stimulated α5β2 integrin-dependent neutrophil adhesion but is instead involved in the process of de-adhesion (34). With RhoA excluded as the geranylgeranylated protein mediating PMA-stimulated Jurkat adhesion, we focused on Rap1. Tsukamoto et al. (27) first implicated Rap1 in cell adhesion with the observation that overexpression of SPA-1, a Rap1-specific GAP, blocked granulocyte-colony stimulating factor-induced promyelocytic 32D cell adhesion to culture dishes. Reedquist et al. (18) reported that Rap1 was involved in CD31-induced, β1 integrin-dependent adhesion of Jurkat cells. Rap1 was also shown to be the activation signal for PMA-stimulated, LFA-1-dependent adhesion of Jurkat cells to ICAM-1 (10), for lipopolysaccharide-induced, β2 integrin-dependent adhesion and spreading of macrophages (21), and for erythropoietin- or interleukin-3-induced, β1 integrin-dependent adhesion of 32D cells (17). Constitutively activated Rap1, V12Rap1, was recently shown to increase β1 and β2 integrin-dependent adhesion of lymphocytes (19). Consistent with these studies, we found that overexpression of the Rap1-specific GAP, SPA-1, in Jurkat cells markedly reduced PMA-stimulated, α5β1-mediated adhesion to FN, a process dependent upon avidity modulation. The effect of SPA-1 on cell adhesion was further confirmed with the inactivated form of Rap1, N17Rap1 (Fig. 3C) (10). Both N17Rap1- and SPA-1-transfected Jurkat cells exhibited significantly lower adhesion when stimulated with PMA (Fig. 3, A and C). The results with N17Rap1 confirm that SPA-1 is involved in cell adhesion by regulating Rap1 activation. In contrast, the transfection of SPA-1 did not inhibit Jurkat cell adhesion to FN by the β1 integrin-activating mAb 8A2, which directly modulates β1 integrin affinity (Fig. 3A). Because the overexpression of SPA-1 also reduced β2 integrin-dependent Jurkat cell adhesion to ICAM-1, we conclude that Rap1 activation is involved in inside-out activation of both β1 and β2 integrins by PMA in Jurkat cells.

Previous studies (35) have shown that low dose cytochalasin D stimulates α5β1 and α5β2 integrin-dependent leukocyte adhesion, presumably by releasing cytoskeletal restraints and allowing diffusion of integrin receptors in the membrane with subsequent ligand-induced clustering. We found that overexpression of SPA-1 did not inhibit cytochalasin D-induced adhesion (Fig. 4). Like PMA, cytochalasin D also induced β1 integrin receptor mobility in the plasma membrane, and overexpression of SPA-1 did not block this effect induced by either PMA or cytochalasin D (Fig. 5). Thus, blockade of Rap1 by overexpression of SPA-1 did not interfere with the process of integrin release from the cytoskeleton. PMA stimulated a dose-dependent adhesion of both the vector control and the SPA-1ΔGRD-infected cells to FN (Fig. 3B). Interestingly, SPA-1ΔGRD-infected cells exhibited greater Rap1 activation and increased adhesion when com-
pared with vector control (Figs. 3 and 7), consistent with overexpressed SPA-1-GRD functioning as a dominant-negative mutant of endogenous SPA-1. However, with overexpression of SPA-1 there was an initial stimulation at the very low dose of 1 ng/ml PMA but no further stimulation at concentrations up to 100 ng/ml PMA. Taken together, these results support the suggestion by van Kooyk and Figdor (1) that PMA modulates leukocyte integrin-dependent adhesion by a multistep process. In the first step, similar to cytochalasin D, PMA activation loosens actin cytoskeletal restraints, thereby allowing diffusion of integrin receptors. This suggestion is supported by the cross-linking experiment. Both PMA and cytochalasin D increased capture of β1 integrin by a cross-linker, as assessed by immuno-precipitation with a limiting amount of anti-β1 integrin mAb, consistent with greater diffusion. Notably, overexpression of SPA-1 did not block this effect of cytochalasin D or PMA. Zhou and Li (6) reported that PMA induced integrin receptor diffusion through activation of PKC and phosphorylation of MacMARCKS. Because SPA-1 overexpression has no effect on the initial diffusion, we speculate that PMA-stimulated phosphorylation of MacMARCKS and Rap1 activation are either two independent signals or that Rap1 activation is downstream of MacMARCKS phosphorylation. The second step in the process of PMA stimulation of integrin-dependent adhesion involves integrin clustering, cytoskeletal rearrangement, and a complex assembly to promote efficient binding to ligand. Because overexpression of SPA-1 blocked PMA-stimulated Rap1 activation and stable adhesion, but did not inhibit β1 integrin diffusion/clustering, we propose that activation of Rap1 plays a role in this second step.

After cells bind to ligand(s) via integrin receptors, there is outside-in signaling, which engages classic signaling pathways controlling growth, differentiation, apoptosis, and cytokine expression (36). Previous studies (27, 37) have also noted spontaneous αβ1-dependent adhesion to high density ligand. Our results suggest that adhesion to wells coated with the high concentration of FN involved a mechanism distinct from PMA-stimulated inside-out signaling. With increasing concentrations of FN, spontaneous, unstimulated adhesion of Jurkat cells increased markedly for cells overexpressing SPA-1 as well as for control and SPA-1-GRD-infected cells (Fig. 7A). Although overexpression of SPA-1 blocked PMA-stimulated adhesion to wells coated with lower concentrations of FN, it had little effect at the higher concentrations of FN (Figs. 3B and 7A). Notably, Tsukamoto et al. (27) reported that adhesion of HeLa cells to high FN induced Rap1 activation, despite overexpression of SPA-1. Although their studies did not specifically address the integrin dependence of the adhesion process, it is likely that these cells utilized β1 integrin to adhere to FN. In our studies, however, overexpression of SPA-1 did not block Jurkat cell adhesion to high density FN, and adhesion to high density FN alone did not induce Rap1 activation. These results suggest that leukocyte adhesion induced by high density ligand in the absent of exogenous stimulation does not involve Rap1. In addition, the outside-in signal leading to spontaneous adhesion was different from the inside-out signaling triggered by PMA in that it was not blocked by cytochalasin D (Fig. 8A). Furthermore, the tyrosine kinase inhibitor, genistein, and the PKC inhibitor, staurosporine, significantly inhibited PMA-stimulated adhesion but had much less or no effect on high FN-induced adhesion (Fig. 8B). Because mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and Rho kinase have been reported to modulate integrin-dependent adhesion, we tested the effect of mitogen-activated protein kinase inhibitor, PD 98050, the phosphatidylinositol 3-kinase inhibitor, LY294002, and the RhoA kinase inhibitor, Y-27632, on both PMA-stimulated and FN-induced adhesion. None of these inhibitors inhibited either of these two pathways (data not shown). Hence, it is unlikely the activation of these kinases is essential for cell adhesion in this model system.

Fig. 9 depicts a model for these two adhesive mechanisms. With PMA-stimulated adhesion (Fig. 9A), the integrins are first released from the cytoskeletal restraints, and their diffusion rate in the plasma membrane is increased. The increased diffusion accounts for the initial increase in cell adhesion. PMA may activate this process through phosphorylation of MacMARCKS (6), whereas low dose cytochalasin D directly releases integrins from the cytoskeleton by inhibiting actin polymerization. In this initial step, the adhesion complex is not supported by a reorganized actin cytoskeleton, so the cells are not stably adhered. In the second step, integrin receptors are clustered; the phosphorylated MacMARCKS is dephosphorylated, and the adhesion complex is supported by a reorganized actin cytoskeleton, leading to stable strong adhesion. Thus, when actin cytoskeleton-dependent re-structuring was disrupted by the high concentration of cytochalasin D, cell adhesion was inhibited. Our studies indicate that Rap1 activation is required for this second step, perhaps in part modulating receptor affinity as suggested by Guerrero et al. (22).

Adhesion induced by outside-in signaling with high density ligand is clearly distinct from PMA-stimulated, inside-out signaling. In contrast to PMA-stimulated adhesion, it is not dependent upon Rap1 activation or reorganization of actin cytoskeleton. It was also not blocked by treatment with inhibitors of tyrosine kinase (genistein), phosphatidylinositol 3-kinase (LY-294002), PKC (staurosporine), mitogen-activated protein kinase (PD-98059) or Rho kinase (Y-27632).

It is surprising that such relatively small changes in ligand density in vitro (i.e. from 1 to 2 µg/ml FN in this study or from 200 to 700 sites/µm2 of VCAM-1 in the study by Grabovsky et al. (37), so dramatically affect integrin-dependent adhesion. Unstimulated adhesion induced by high ligand density was nearly comparable with PMA-stimulated adhesion at low ligand density and was independent of Rap1 activation or cytoskeletal rearrangement. Because leukocytes might reasonably encounter high density ligand on the surface of endothelial cells or in tissue, further characterization of this novel pathway of adhesion is warranted.

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