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Affinity Modulation of Very Late Antigen-5 Through Phosphatidylinositol 3-Kinase in Mast Cells

Tatsuo Kinashi, Tetsuo Asaoka, Ruri Setoguchi, and Kiyoshi Takatsu

Adhesiveness of integrins is up-regulated rapidly by a number of molecules, including growth factors, cytokines, chemokines, and other cell surface receptors, through a mechanism termed inside-out signaling. The inside-out signaling pathways are thought to alter integrin affinity for ligand, or cell surface distribution of integrin by diffusion/clustering. However, it remains to be clarified whether any physiologically relevant agonists induce a rapid change in the affinity of β1 integrins and how ligand-binding affinity is modulated upon stimulation. In this study, we reported that affinity of β1 integrin very late Ag-5 (VLA-5) for fibronectin was rapidly increased in bone marrow-derived mast cells by Ag cross-linking of FcεRI. Ligand-binding affinity of VLA-5 was also augmented by receptor tyrosine kinases when the phospholipase Cγ-1/protein kinase C pathway was inhibited. Wortmannin suppressed induction of the high affinity state VLA-5 in either case. Conversely, introduction of a constitutively active p110 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) increased the binding affinity for fibronectin. Failure of a constitutively active Akt to stimulate adhesion suggested that the affinity modulation mechanisms mediated by PI 3-kinase are distinct from the mechanisms to control growth and apoptosis by PI 3-kinase. Taken together, our findings demonstrated that the increase of affinity of VLA-5 was induced by physiologically relevant stimuli and PI 3-kinase was a critical affinity modifier of VLA-5. The Journal of Immunology, 1999, 162: 2850–2857.

Cell-cell and cell-matrix adhesion mediated by integrins influences cell migration and localization (1, 2). To accomplish this process, the adhesiveness as well as expressions of specific integrins must be regulated cooperatively. While regulations of expressions of adhesion molecule usually take several hours to days, a transient change of integrin adhesiveness (avidity modulation) is known to occur in a short time period (3). The importance of both types of regulations is exemplified in physiologic processes, such as endothelial transmigration, in which leukocytes and lymphocytes migrate to inflamed tissues through the cascade of adhesion mediated by selectin and integrin as well as their counter-receptors (4–6). Several external stimuli were reported to modulate avidity of integrins without changes of integrin expressions, such as Ag, chemokines, and cytokines (4–8). These stimuli were thought to exert their effects on integrin adhesiveness through intracellular molecules, and this process is referred to as inside-out signaling (3).

Avidity modulation of integrins is thought to be regulated by the spatial distribution or ligand-binding affinity of the integrin (9–12). Avidity modulation in integrins detected with soluble ligands or Abs recognizing the high affinity state was reported for α5β1, α8β1, α1β2, and αimβ3 integrins in cells stimulated with activating Abs, manganese ions, or cross-linking of the TCR (13–19). Cross-linking of the TCR increased the affinity of VLA-4 for fibronectin (FN) in a T lymphoid cell line (13), but it was reported that affinity of VLA-4 for FN and VCAM-1 did not change in primary T cells (18, 20). Several studies showed that PMA enhanced adhesion without detectable change in ligand-binding affinity of integrins (13, 17, 21), suggesting that affinity change does not account for all avidity modulation processes. In fact, the physiologic relevance of affinity modulation of integrins has been questioned, since artificial agonists are often used and it has not been clearly demonstrated that a natural agonist can modulate the affinity of β1 integrins (22).

To gain a clearer understanding of avidity regulations of integrins, we have examined adhesion mediated by VLA-5 in bone marrow-derived mast cells. Mast cells are distributed exclusively in peripheral tissues and play critical roles in allergy and inflammation. The tissue distribution of mast cells, therefore, influences the magnitude of these inflammatory events. We and others previously reported that steel factor (SLF) transiently stimulated mast cells to adhere to FN via VLA-5 at concentrations 100-fold lower than those required for growth stimulation (23, 24). Furthermore, avidity of VLA-5 was regulated independently by the PI 3-kinase and PLCγ-1/protein kinase C (PKC) pathways of c-kit and the PDGF receptor (25–27). Cross-linking of FcεRI also stimulated mast cells to bind to FN (28). However, it remains unclear whether these agonists affect ligand-binding affinity of VLA-5 and how ligand-binding affinity is regulated upon stimulation. In this study, we reported that the affinity modulation of VLA-5 by physiologically relevant agonists caused adhesion of mast cells to FN. We further showed that the ligand-binding affinity of VLA-5 was increased by PI 3-kinase stimulated by receptor tyrosine kinases and FcεRI.

Abbreviations used in this paper: VLA, very late Ag; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; FN, fibronectin; HA, hemagglutinin; HRP, horseradish peroxidase; PDGF, platelet-derived growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PY, phosphotyrosine; SLF, steel factor.
Materials and Methods

Cell lines, Abs, and chemicals

Primary bone marrow–derived mast cell cultures were conducted as described (23). Primary mast cells were used from 4–10 wk of culture after establishment. Retroviruses–mediated transfection was employed to introduce the wild-type, mutant PDGF receptors (25), or constitutively active PI 3-kinase, p110-CAAX (29) into mast cells, as described (25).

The anti-mouse VLA-5 mAb MFR-5 (5H10) (rat IgG2a) (30) was purified by affinity chromatography on protein G-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Anti-DNP IgE was a culture supernatant of a hybridoma, IGE.1.2 (American Type Culture Collection, Manassas, VA). Recombinant murine SLF (Genzyme, Boston, MA), PMA (Sigma, St. Louis, MO), and manganese chloride tetrahydrate (Sigma) were purchased.

Purification of FN and production of the 80-kDa fragment

FN was purified as described (31) from frozen human plasma obtained from the Japanese Red Cross Central Blood Center. The 80-kDa tryptic fragment that contains the RGDF-binding motif for VLA-5, but lacks VLA-4-binding domains, was produced by a modified procedure (32). Briefly, purified FN was digested with 1 or 2 μg/ml of trypsin (Wako Pure Chemical Industries, Osaka, Japan) for 90 min at 37°C in digestion buffer containing 25 mM Tris-Cl buffer (pH 7.6), 50 mM NaCl, and 0.5 mM EDTA. The 80-kDa fragment was purified by affinity chromatography on heparin-Sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden). After extensive washing with digestion buffer, a fraction containing a 80-kDa FN fragment was obtained with elution buffer containing 25 mM Tris-Cl buffer, pH 7.6, 100 mM NaCl, and 0.5 mM EDTA, and further purified by HPLC on Superdex 200 HR 10/30 (Pharmacia Biotech). The purity of the 80-kDa fragment was more than 95%, as assessed by SDS-PAGE, and its function was confirmed by adhesion assays.

Adhesion assays

Assays of adhesion to FN were performed as described (23). Briefly, mast cells labeled with 2′,7′-bis-(2-carboxyethyl)-5′-(and -6)-carboxyfluorescein (BCECF) in 96-well plates precoated with FN (1 μg/well) were incubated in triplicate at 37°C for 30 min in the presence of PMA (10 ng/ml Sigma), SLF (1 U/ml), DNP (1 μg/ml), or medium alone (RPMI 1640 supplemented with 0.02% BSA and 10 mM HEPES, pH 7.4), as indicated. For manganese stimulation, HBSS (Life Technologies, Grand Island, NY) was used instead of RPMI 1640, and BSA was dialyzed against PBS. The amounts of these factors were chosen to give the maximum response. For cross-linking of FcεRI, labeled mast cells were sensitized with a culture supernatant containing anti-DNP IgE for 30 min at room temperature before stimulation with DNP-conjugated BSA (1 μg/ml). After washing the plate four times, bound fluorescence was measured with a fluorescence concentration analyzer (IDEXX Laboratories, Westbrook, ME). The level of adhesion was calculated by dividing bound fluorescence by input fluorescence. Mast cells did not adhere to wells precoated with BSA with or without stimulation (data not shown). For the assay with Abs, labeled mast cells were preincubated at room temperature with 20 μg/ml of Abs, as indicated, and the adhesion assay was performed in the presence of the Abs. For pretreatment with PMA, mast cells were incubated with 100 ng/ml of PMA for 40 h. The pretreatment had no effect on viability of mast cells. PMA was washed away before the adhesion assay. For the experiment with wortmannin (Wako Pure Chemical, Tokyo, Japan), cells were treated with wortmannin at room temperature for 10 min, as described (33).

For the experiment with the soluble 80-kDa FN fragment, the plates were coated with the 80-kDa FN fragment (1 μg/well), then blocked with 3% BSA for 1 h at 37°C. Mast cells labeled with BCECF mixed with varying amounts of the soluble 80-kDa FN fragment were subjected to the adhesion assay described above.

125I labeling of the 80-kDa FN fragment

The 80-kDa FN fragment was radioiodinated with a modified method using chloramine T (34). Briefly, carrier-free Na125I (1 mCi) (iodine-125, NEZ-033A; DuPont NEN, Boston, MA) was mixed with 250 μg of the 80-kDa FN fragment in labeling buffer containing 10 mM sodium phosphate, pH 7, 150 mM NaCl, and 30 μg/ml of chloramine T (Katayama Chemical, Osaka, Japan). After incubation for 4 min at room temperature, the iodination was stopped by adding sodium pyrosulfite (Na2S2O5; Katayama Chemical) at 60 μg/ml and 0.1% NaI (Wako Pure Chemical Industries). The labeled protein was collected with the PD-10 column (Pharmacia Biotech, Piscataway, NJ). Radioactivity of the protein was measured with a gamma counter. The typical sp. of the labeled 80-kDa fragment used in our experiments was about 3.7 × 106 cpm/μmol.

Ligand-binding assay

The ligand-binding assay was performed basically as previously described (31). Mast cells were washed once with binding buffer containing RPMI 1640 (Sigma), 0.1% BSA (Life Technologies), and 10 mM HEPES, pH 7.4 (Sigma), and suspended with the same buffer at 1 × 107 cells/ml. In a typical binding assay, performed in a 1.5-ml microcentrifuge tube, 100 μl of cells (1 × 106 cells/tube) was mixed with 50 μl of the radiolabeled 80-kDa fragment and 50 μl of stimulant (e.g., 1 mM manganese, 10 ng/ml PMA, 10 U/ml SLF, or 1 μg/ml DNP), with or without inhibitors (unlabeled 80-kDa FN fragment, wortmannin). For inhibition with Abs (20 μg/ml), mast cells were preincubated with Abs for 20 min at 25°C. In the case of manganese stimulation, HBSS was used instead of RPMI 1640 and BSA was dialyzed against PBS. After incubation for 30 min at 37°C, samples were layered onto 100 μl of separation oil (80% Di-n-butyl phthalate (Wako Pure Chemical Industries) and 20% olive oil (Katayama Chemical)) in 0.5-ml tubes, and centrifuged at 8000 rpm for 1 min. The tip of tubes was anapunted from the body with a blade and applied to a gamma counter to measure radioactivity of the bound (the tip) and the unbound (the body). The nonspecific binding was determined at each data point in the presence of a 50-fold excess of the unlabeled 80-kDa fragment. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

In vitro PI 3-kinase assay

PI 3-kinase assays were performed basically as described (35). Mast cells (107 cells) were stimulated for 5 min at 37°C with PMA (100 ng/ml) and SLF (50 U/ml). For stimulation with DPGF (50 ng/ml), mast cells expressing the PDGF receptor were used. Cell lysates prepared as described above were immunoprecipitated for 2 h at 4°C with an anti-phosphotyrosine (PY) mAb (4G10; Upstate Biotechnology, Lake Placid, NY), followed by incubation for 40 min at 4°C with protein G-Sepharose (Pharmacia Biotech) to collect the immune complex. The beads were washed three times with PI 3-kinase buffer, and three times with PI 3-kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, and 200 μM adenosine). PI 3-kinase activity was measured in a volume of 100 μl of PI 3-kinase buffer. The kinase reaction was initiated by adding 10 μg sonicated phosphatidylinositol (Sigma), 10 μg ATP, and 10 μg [γ-32P]ATP (Amersham, Arlington Heights, IL). Reactions are conducted for 15 min at room temperature and stopped by the addition of 20 μl of 35% trichloroacetic acid. Lysates of the radiolabeled kinase were dialyzed against chloroform:methanol (1:1, v/v) and were separated on oxalate-treated TLC plates using a solvent of chloroform:methanol:water:28% ammonia (45:35:7.5:2.5, v/v). Labeled phosphatidylinositol was detected and quantified by a phosphor imager (BAS1000; Fujifilm, Tokyo, Japan).

Analysis of expression and phosphorylation of Akt and cell survival

Mast cells were prepared for cell lysates as described (25). Equal amounts of protein were subjected to SDS-PAGE. Following SDS-PAGE, the separated proteins were electrophoretically transferred to a PVDF membrane. After blocking with 5% BSA, the membrane was incubated with a 1/1000 dilution of anti-phospho-Akt Ab (New England Biolabs, Beverly, MA) and then with a 1/2000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit Ab. The bands were visualized using enhanced chemoluminescence (ECL; Amersham). The same membrane was stripped and reprobed with a 1/1000 dilution of anti-Akt Ab (New England Biolabs) and a 1/2000 HRP-conjugated anti-rabbit Ab.

To detect HA-tagged c-Akt and gsk-Akt in transfected mast cells, cell lysates (5 × 106 cells) were immunoprecipitated with 5 μg of an anti-HA mAb (12CA5; Boehringer Mannheim, Indianapolis, IN) and protein G-Sepharose (Pharmacia Biotech). After blocking with 5% BSA, the membrane was incubated with a 1/1000 dilution of anti-phospho-Akt Ab (New England Biolabs, Beverly, MA) and then with a 1/2000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit Ab. The bands were visualized using enhanced chemoluminescence (ECL; Amersham). The same membrane was stripped and reprobed with a 1/10000 dilution of anti-Akt Ab (New England Biolabs) and a 1/2000 HRP-conjugated anti-rabbit Ab.

Results

Adhesion to FN and ligand-binding affinity of VLA-5

Mouse bone marrow–derived mast cells did not adhere to FN without stimulation. Upon stimulation with PMA, SLF, and cross-linking of FcεRI for 30 min, mast cells adhere to FN at comparable levels (Fig. 1A). In all cases, adhesion to FN was blocked by an
FIGURE 1. Adhesion and ligand-binding affinity of mast cells for FN. A, Adhesion of mast cells to FN. Mast cells were unstimulated (−) or stimulated with PMA (10 ng/ml), SLF (10 U/ml), or cross-linking of FcεRI (1 μg/ml of DNP-BSA) for 30 min. Closed bars represent untreated mast cells, and hatched and open bars represent mast cells treated with the control Ab and anti-VLA-5 Ab, respectively. Adhesion assays were performed in triplicate, as described in Materials and Methods. The average and SE are shown. B, The specific binding of 125I-labeled FN80 fragment. The ligand-binding assay was performed in triplicate using 125I-labeled FN80 (0.2 μg). Mast cells were unstimulated (−), or stimulated with PMA (10 ng/ml), SLF (10 U/ml), cross-linking of FcεRI (1 μg/ml of DNP-BSA), or manganese (1 mM Mn2+), as indicated. The specific bindings of mast cells treated with the anti-VLA-5 Ab are indicated (anti-VLA-5). The data shown are representative of several experiments with similar results, and the average and SEs are shown. C, The saturation curve and Scatchard plot (inset) analysis of the 125I FN80 fragment bindings to mast cells stimulated with cross-linking of FcεRI. The total (open square) and specific binding (filled square) were measured as described in Materials and Methods. The data are representative of several independent experiments. D, Inhibition of adhesion with the soluble FN80 fragment. Various amounts of soluble FN80 were included in adhesion assays with untreated mast cells stimulated with PMA, SLF, and FcεRI cross-linking (open circle), as indicated. The specific bindings were measured in the presence of an excess amount of the cold fragment, as described in Materials and Methods. The total (open square) and specific binding (filled square) were measured as described in Materials and Methods. Inhibition of adhesion with soluble FN. Various amounts of soluble FN80 were included in adhesion assays with untreated mast cells stimulated with PMA (square), SLF (filled circle), FcεRI cross-linking (triangle), Mn2+ (open circle), as indicated. The average and SE for results of triplicate experiments are shown.

Affinity modulation of VLA-5 through PI 3-kinase

To examine the effect of these stimuli on ligand-binding affinity of VLA-5, the soluble 80-kDa FN (FN80) fragment, which has a single binding site of VLA-5 containing the classical RGD motif (1), was radiolabeled and used for ligand-binding assays. Unstimulated and stimulated mast cells were incubated with 125I-labeled FN80 fragment, and the specific bindings were measured in the presence of an excess amount of the cold fragment, as described in Materials and Methods (Fig. 1B). Stimulation with PMA or SLF did not augment the binding of the FN80 fragment, although they had a potent effect on adhesion (Fig. 1A). In contrast, FcεRI-stimulated mast cells showed significant increase of specific ligand binding. The increased binding was mediated by VLA-5, because the binding was inhibited with the anti-VLA-5 Ab. The effect of manganese on the binding was also measured, as manganese is known to act directly on integrins and activate them (36–38). As expected, manganese also increased the specific binding, which was also inhibited with the anti-VLA-5 Ab.

High affinity ligand binding of VLA-5 by FcεRI cross-linking

Although unstimulated, PMA-, or SLF-stimulated mast cells showed low binding affinity for the FN80 fragment as above, we were unable to obtain the specific saturation curve that was reproducible (data not shown). In contrast, mast cells stimulated with cross-linking of FcεRI showed specific saturation curves with increased amounts of labeled FN80 fragment, and Scatchard analysis indicated a $K_d$ of 37.7 ± 3.8 nM ($n = 3$) (Fig. 1C). The total number of VLA-5 was calculated as 3.85 ± 0.20 × 10^6 molecules/cell, which was similar to the number obtained with 125I-labeled anti-VLA-5 Ab (data not shown). As a comparison, we also measured ligand-binding affinity of VLA-5 in mast cells stimulated with manganese. Manganese also induced dose-dependent bindings of the FN80 fragment, and Scatchard analysis showed a $K_d$ of about 10 nM (data not shown). The affinity of VLA-5 obtained with FcεRI cross-linking and manganese was in agreement with result of a previous report using β1 integrin-activating Abs (15).

Inhibition of adhesion with soluble FN

To examine whether the increase in ligand-binding affinity of VLA-5 accounts for adhesion to immobilized FN, various doses of soluble FN80 fragment were added in adhesion assays. While there
Association of PI 3-kinase activity with anti-PY immunoprecipitates in stimulated mast cells

Previously, we showed that avidity of VLA-5 was up-regulated by PI 3-kinase of c-kit and the PDGF receptor (25). To assess the relative ability of stimuli used in adhesion assays to activate PI 3-kinase in mast cells, we measured activities of PI 3-kinase that were immunoprecipitated with an anti-PY Ab in mast cells expressing the wild-type receptor, and was markedly increased in cells expressing the mutant receptor that lacks the PLCg-1 supported PDGF-induced adhesion, whereas the mutants that were defective in both binding sites eliminated PDGF-induced adhesion (25). Upon stimulation with PDGF, ligand-binding affinity for FN was modestly increased in mast cells expressing the wild-type receptor, and was markedly increased in cells expressing the mutant receptor that lacks the PLCg-1 binding site (Fig. 4). In contrast, there was little change in ligand-binding affinity for FN of receptor tyrosine kinases

Inhibition of the high ligand-binding affinity of VLA-5 by wortmannin

To explore the possibility that PI 3-kinase is involved in the affinity modulation of VLA-5, we used wortmannin, a specific PI 3-kinase inhibitor (40). Wortmannin reduced FcεRI-induced ligand-binding affinity for FN by about half at as low as 10 nM (Fig. 3). Further reduction was barely observed even at 500 nM. On the other hand, manganese-induced ligand-binding affinity was not affected at all by wortmannin (Fig. 3), demonstrating the specific inhibition of FcεRI-induced ligand-binding affinity of VLA-5 by wortmannin. These results suggest that PI 3-kinase is involved at least in part in the affinity modulation of VLA-5 by FcεRI cross-linking.
affinity for FN in mast cells expressing the mutant receptor that was defective in binding to PI 3-kinase, although they adhered to FN at levels comparable with that of cells expressing the wild-type and PLCγ-1-defective mutant receptors (data not shown). The mutation of both the PI 3-kinase and PLCγ-1 binding sites abolished the ligand bindings. These results suggest a critical role for PI 3-kinase in ligand-binding affinity for FN.

The fact that the mutant receptor that lacks the PLCγ-1 binding site induced higher ligand-binding affinity than wild-type suggests that activation of the PLCγ-1/PKC pathway inhibits affinity modulation for FN. To examine the possibility that the PLCγ-1/PKC pathway inhibits affinity modulation of VLA-5 by SLF/c-kit, mast cells were pretreated with PMA to down-regulate PKC. Although PMA-pretreated mast cells did not respond to PMA for adhesion, they still adhered to FN when stimulated with SLF (Fig. 5A). The adhesion of PMA-pretreated mast cells was almost completely inhibited with wortmannin, showing the PI 3-kinase-dependent adhesion to FN (Fig. 5A). In contrast to untreated mast cells (Fig. 1B), PMA-pretreated mast cells showed increased ligand bindings to FN upon SLF stimulation (Fig. 5B). The increase in ligand-binding affinity for FN was completely abolished by wortmannin. Taken together, these results support the notion that the PLCγ/PKC pathway counteracts the affinity modulation of PI 3-kinase through receptor tyrosine kinases.

To further confirm modulation of ligand-binding affinity by PI 3-kinase, we introduced a constitutively active p110 subunit of PI 3-kinase (p110-CAAX) (29, 41) into mast cells. Two independent mast cell lines expressing p110-CAAX adhered to FN without stimulation (Fig. 6A). Moreover, the ligand-binding affinity for FN was also increased (Fig. 6B), indicating that PI 3-kinase can modulate ligand-binding affinity for FN. Taken together with the inhibitory effect of wortmannin on ligand binding to FN, these results indicate that PI 3-kinase modulates ligand-binding affinity for FN.

PI 3-kinase is known to activate several signaling molecules including protein kinases, such as Akt, which play an important role in growth and apoptosis (42). Stimulation with SLF and FceRI cross-linking induced phosphorylation of Akt, which was abolished by the treatment of wortmannin, indicating the PI 3-kinase-dependent activation of Akt (Fig. 7A). The degree of phosphorylation of Akt by SLF was higher than that by FceRI cross-linking, consistent with the relative ability of activation of PI 3-kinase (Fig. 2). To examine whether Akt is capable of stimulating adhesion, a constitutively active Akt, gag-Akt, or c-Akt (43) was introduced into mast cells. Mast cells expressing gag-Akt or c-Akt failed to stimulate adhesion to FN (Fig. 7B). However, gag-Akt, but not

**FIGURE 5.** Effects of down-regulation of PKC on adhesion and ligand-binding affinity for FN. A, Adhesion of mast cells to FN following pretreatment with PMA. Mast cells pretreated with PMA (100 ng/ml) for 48 h were unstimulated (open bar), or stimulated with PMA (hatched bar) or SLF (closed bar) for adhesion to FN, as described in Fig. 1. Mast cells were incubated with DMSO (1, 10, 100, 500 nM) for 10 min at 25°C before assays. B, Specific bindings of 125I-labeled FN80 fragment of PMA-pretreated mast cells unstimulated (open bar), or stimulated with SLF (closed bar). Pretreatment with DMSO (D) or wortmannin was performed as in A before ligand-binding assays. The average and SE for triplicate experiments are shown.

**FIGURE 6.** p110-CAAX induced FN adhesion (A) and ligand-binding affinity (B). Two independent mast cell lines expressing p110-CAAX (p110-CAAX-1, p110-CAAX-2), or the neomycin-resistance gene alone (neo) were established after retrovirus-mediated transfection. A, Adhesion to FN with (open bars) or without (closed bars) the anti-VLA-5 Ab. B, Specific bindings of 125I-labeled FN80. The specific ligand bindings of 125I-labeled FN80 and adhesion to FN were measured as in Fig. 1. The average and SE for triplicate experiments are shown.
results suggest that the affinity modulation mechanism by PI 3-kinase is distinct from the mechanisms to control growth and apoptosis by PI 3-kinase.

Discussion

Bone marrow-derived mast cells adhered to FN via VLA-5 when stimulated with PMA, SLF, cross-linking of FceRI with Ag and IgE. Through our study of adhesiveness and ligand-binding affinity for FN, we demonstrated that cross-linking FceRI induced high affinity state VLA-5 that mediated adhesion to FN, indicating that a distinct physiologic agonist can increase ligand-binding affinity of β1 integrin. We also showed that PI 3-kinase was a critical affinity modulator of VLA-5 by using the specific PI 3-kinase inhibitor, the mutant PDGF receptor that lacked the binding site of PI 3-kinase, and the constitutively active PI 3-kinase. The affinity modulation of PI 3-kinase through receptor tyrosine kinase was augmented when the PLCγ-1/PKC pathway was inhibited, suggesting a fine tuning of a balance between these two pathways determines the ligand-binding affinity of VLA-5.

Cross-linking of FceRI stimulated mast cells to adhere to FN as reported (28), through VLA-5 in a rapid and transient fashion as SLF (unpublished data). The different effects of FceRI cross-linking and SLF on ligand-binding affinity of VLA-5 could result in distinct adhesion behaviors under physiologic and pathologic circumstances. Since FceRI cross-linking enhanced mast cell chemotaxis though FN-coated matrix by chemokines (45), induction of the high affinity state VLA-5 by FceRI could facilitate migration and accumulation toward Ag. Our result that soluble FN competitively inhibited the FceRI-induced adhesive interaction suggests that the high affinity state of VLA-5 rather weakens firm attachment when soluble FN concentrations are augmented due to plasma protein extravasation at allergic and inflammatory sites and influences tissue localization of mast cells. Further studies are required to establish the specific role of the high affinity state VLA-5 in mast cells.

Although integrin adhesiveness is thought to be regulated through alterations of integrin affinity for ligand or cell surface distribution by diffusion/clustering (9–12), the physiologic relevance of affinity modulation of integrins, in particular β1 integrin, has been questioned (11), because artificial agonists were used to induce the high affinity state integrins. Our study clearly showed affinity modulation of β1 integrin in a physiologically relevant system. PI 3-kinase was shown to be an important intracellular mediator in the inside-out signaling pathway by using mutant receptors that were defective in the binding sites of PI 3-kinase or by using a dominant-negative form of PI 3-kinase (25, 46, 47). Our study using a constitutively active PI 3-kinase ruled out the possibility that other molecules that bind to the same sites of receptors stimulate integrin adhesiveness, and confirmed PI 3-kinase as a critical affinity modulator of VLA-5 and probably other integrin subfamilies as well.

It is unknown how PI 3-kinase modulates the affinity of integrins. Our result with a constitutively active Akt suggests distinct mechanisms to control ligand-binding affinity of integrins from those to regulate growth and apoptosis. We also confirmed that Btk, another downstream target of PI 3-kinase, which played a critical role in Ca²⁺ influx, was not involved in FceRI-induced adhesion (48). Although downstream targets of PI 3-kinase are unknown at present, affinity modulation of integrins probably occurs through the cytoplasmic region of integrin α- and/ or β-chains since mutations or deletions of the cytoplasmic regions resulted in
high affinity state integrins (49, 50). Interactions of integrin cytoplasmic regions with other molecules such as cytoskeletal proteins may control the ligand-binding affinity of integrins.

Apparently, other adhesive mechanisms exist besides affinity modulation of integrins, because ligand-binding affinity for FN was not changed significantly by PMA and SLF in mast cells. Recent studies have shown that an increase in lateral diffusion of integrins by PMA or cytochalasin D at low doses facilitates adhesion, suggesting that release of integrins from cytoskeletal constraints is an important step in activation of adhesion (12, 51). We observed that PMA or SLF, but not cross-linking of FceRI, induced surface redistribution of VLA-5 in mast cells (unpublished data). Therefore, regulation of spatial distribution of integrin molecules by clustering and diffusion may be an alternative regulatory mechanism to control integrin adhesiveness probably through PKC, because PMA is a direct activator of PKC (52). Our result that suppression of the PLCγ-1/PKC pathway either by mutation of binding sites of PLCγ-1 in the PDGF receptor or down-regulation of PKC augmented ligand-binding affinity upon stimulation suggests that a balance between the PI 3-kinase and PLCγ-1/PKC pathways determines diffusibility and ligand-binding affinity of integrins and controls cell adhesion behavior.

Interestingly, down-regulation of PKC did not enhance ligand-binding affinity of VLA-5 by FcεRII (unpublished data), although cross-linking of FceRI leads to Ca2+ influx and PKC activation. This result implies differences in activation patterns of PKC among FcεRI and receptor tyrosine kinases. It is currently under investigation which isotypes of PKC are activated and responsible for stimulation of adhesion and inhibition in affinity modulation of integrins in mast cells.

Regulation of the adhesive interactions with extracellular matrix undergoes complex mechanisms regulating activation and inactivation of integrin adhesiveness. Elucidation of the mechanistic basis to the control of these processes requires a better understanding of the specific signaling pathways. Our study has yielded important insights into, as well as a useful experimental system with which to examine, inside-out signaling in physiologic conditions.

Acknowledgments
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References
10. Stewart, M., and N. Hogg. 1996. Regulation of leukocyte integrin function: affinity modulation of integrins, because ligand-binding affinity for FN was not changed significantly by PMA and SLF in mast cells. Recent studies have shown that an increase in lateral diffusion of integrins by PMA or cytochalasin D at low doses facilitates adhesion, suggesting that release of integrins from cytoskeletal constraints is an important step in activation of adhesion (12, 51). We observed that PMA or SLF, but not cross-linking of FceRI, induced surface redistribution of VLA-5 in mast cells (unpublished data). Therefore, regulation of spatial distribution of integrin molecules by clustering and diffusion may be an alternative regulatory mechanism to control integrin adhesiveness probably through PKC, because PMA is a direct activator of PKC (52). Our result that suppression of the PLCγ-1/PKC pathway either by mutation of binding sites of PLCγ-1 in the PDGF receptor or down-regulation of PKC augmented ligand-binding affinity upon stimulation suggests that a balance between the PI 3-kinase and PLCγ-1/PKC pathways determines diffusibility and ligand-binding affinity of integrins and controls cell adhesion behavior.

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References
10. Stewart, M., and N. Hogg. 1996. Regulation of leukocyte integrin function: affinity modulation of integrins, because ligand-binding affinity for FN was not changed significantly by PMA and SLF in mast cells. Recent studies have shown that an increase in lateral diffusion of integrins by PMA or cytochalasin D at low doses facilitates adhesion, suggesting that release of integrins from cytoskeletal constraints is an important step in activation of adhesion (12, 51). We observed that PMA or SLF, but not cross-linking of FceRI, induced surface redistribution of VLA-5 in mast cells (unpublished data). Therefore, regulation of spatial distribution of integrin molecules by clustering and diffusion may be an alternative regulatory mechanism to control integrin adhesiveness probably through PKC, because PMA is a direct activator of PKC (52). Our result that suppression of the PLCγ-1/PKC pathway either by mutation of binding sites of PLCγ-1 in the PDGF receptor or down-regulation of PKC augmented ligand-binding affinity upon stimulation suggests that a balance between the PI 3-kinase and PLCγ-1/PKC pathways determines diffusibility and ligand-binding affinity of integrins and controls cell adhesion behavior.

Interestingly, down-regulation of PKC did not enhance ligand-binding affinity of VLA-5 by FcεRII (unpublished data), although cross-linking of FceRI leads to Ca²⁺ influx and PKC activation. This result implies differences in activation patterns of PKC among FcεRI and receptor tyrosine kinases. It is currently under investigation which isotypes of PKC are activated and responsible for stimulation of adhesion and inhibition in affinity modulation of integrins in mast cells.

Regulation of the adhesive interactions with extracellular matrix undergoes complex mechanisms regulating activation and inactivation of integrin adhesiveness. Elucidation of the mechanistic basis to the control of these processes requires a better understanding of the specific signaling pathways. Our study has yielded important insights into, as well as a useful experimental system with which to examine, inside-out signaling in physiologic conditions.

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