Distinct Mechanisms of $\alpha_5\beta_1$ Integrin Activation by Ha-Ras and R-Ras*

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To investigate the possible roles of the Ras/Rho family members in the inside-out signals to activate integrins, we examined the ability of Ras/Rho small GTPases to stimulate avidity of $\alpha_5\beta_1$ (VLA-5) to fibronectin in bone marrow-derived mast cells. We found that both Ha-Ras\textsuperscript{Val-12} and R-Ras\textsuperscript{Val-38} had strong stimulatory effects on adhesion and ligand binding activity of VLA-5 to fibronectin. However, only Ha-Ras\textsuperscript{Val-12}, but not R-Ras\textsuperscript{Val-38}-induced adhesion was inhibited by wortmannin, which suggests that Ha-Ras\textsuperscript{Val-12} is dependent on phosphatidylinositol (PI) 3-kinase on adhesion whereas R-Ras\textsuperscript{Val-38} has another PI 3-kinase independent pathway to induce adhesion. The effector loop mutant Ha-Ras\textsuperscript{Val-12}E37G, but not Y40C retained the ability to stimulate adhesion of mast cells to fibronectin. Consistently, PI 3-kinase p110$\delta$, predominantly expressed in mast cells, interacted with Ha-Ras\textsuperscript{Val-12}E37G, but not Y40C, which was also correlated with the levels of Akt phosphorylation in mast cells. Furthermore, marked adhesion was induced by a membrane-targeted version of p110$\delta$. These results indicate that Ha-Ras\textsuperscript{Val-12} activated VLA-5 through PI 3-kinase p110$\delta$. The mutational effects of the R-Ras effector loop region on adhesion were not correlated with PI 3-kinase activities, consistent with our contention that R-Ras has a distinct pathway to modulate avidity of VLA-5.

Adhesion mediated by integrins controls cell migration and localization. Adhesive interactions through integrins are modulated by adhesiveness (avidity) as well as expressions of integrins (1, 2). Although both types of regulations are important, avidity modulation of integrins in particular plays a critical role in leukocyte migration and localization during inflammatory responses (3). Several external stimuli were reported to modulate avidity of integrins without changes of integrin expressions, such as antigen, chemokines, and cytokines (3–5). A rapid change of avidity of integrins occurs within minutes and is triggered by intracellular signaling pathways, which are referred to as inside-out signals (6).

Avidity modulation of integrins is regulated by increasing affinity to ligands, or by spatial redistribution of integrins on cell surface, which increases the number of integrins on the contact site (7–10). Which types of avidity regulations are utilized largely depends on stimuli that induce adhesion. PMA$^1$ enhanced adhesion without detectable change in ligand-binding affinity of integrins (11–13). Recent studies have shown that an increase in lateral diffusion and clustering of integrins by PMA or cytochalasin D at low doses facilitates adhesion (10, 14), suggesting that the adhesion is mediated by low affinity, but multivalent bindings of integrins. On the other hand, affinity modulation in integrins detected with soluble ligands or antibodies recognizing the high affinity state was reported for $\alpha_5\beta_1$, $\alpha_5\beta_2$, $\alpha_1\beta_3$, and $\alpha_{IIb}\beta_3$ integrins in cells stimulated with activating antibodies, manganese ions, or cross-linking of the T cell receptor (12, 13, 15–19). We previously demonstrated that PMA-stimulated mast cells adhered to fibronectin without accompanying affinity modulation of VLA-5, while FcRI cross-linked mast cells adhered to fibronectin by the high affinity state of VLA-5. Steel factor-induced adhesion was considered to be brought by both mechanisms (20). Changes in the affinity state of integrins influenced cell migratory speeds on substrates (21). However, the physiological significance of two modes of avidity modulation has not yet been demonstrated clearly.

The Ras/Rho family of small GTPases regulates the actin cytoskeleton and contributes to the formation of membrane ruffling and focal adhesion (22, 23). Cytoskeletal reorganization subsequent to attachment to substrate leads to marked cell shape changes and strengthens adhesive interactions. Several members of the Ras/Rho family have been reported to influence integrin-mediated adhesion. Ha-Ras was shown to suppress the active form of $\alpha_{IIb}\beta_3$ chimeras through the MAP kinase pathway (24). A constitutively active R-Ras was found to enhance cellular adhesion to fibronectin by enhancing $\beta_3$-integrin ligand-binding affinity (25). We have recently shown that Rap1 has a unique property that causes an increase of ligand binding affinity of the $\beta_2$ integrin LFA-1, and that Rap1 was critically involved in T-cell receptor-mediated LFA-1/ICAM-1 adhesion (26). However, there are few comprehensive studies that examine whether or not the Ras/Rho family of small GTPases can modulate avidity of $\beta_1$ integrins directly.

To gain a clearer understanding of avidity regulations of $\beta_1$

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** The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; MAP, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
integrins by the Ras/Rho family of small GTPases, we employed bone marrow-derived mast cells as a model system to analyze their ability to modulate avidity of VLA-5, because mast cells have been shown to adhere to fibronectin through VLA-5 upon physiologically relevant stimulation such as steel factor (27), or antigen cross-linking of FcεRI (20) and are considered to be suitable for activation signal-dependent adhesion. With mast cells, one can also examine the affinity state of VLA-5 as we demonstrated that with antigen cross-linking of FcεRI (20). Here we report that the active mutants of Ha-Ras and R-Ras among the Ras/Rho family member lead to strong adhesion to fibronectin with the high affinity state of VLA-5. Furthermore, our study reveals distinct mechanisms of Ha-Ras and R-Ras in regulation of avidity of VLA-5 through analyses of effector mutants and constitutively active downstream signaling molecules: PI 3-kinase p110δ is critical to avidity modulation by Ha-Ras, while R-Ras has other mechanisms to regulate avidity of VLA-5.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Chemicals—Primary bone marrow-derived mast cell cultures were carried out as described (27). Primary mast cells expanded from 4 to 10 weeks of culture after establishment. Retrovirus-mediated transfection was employed to introduce cDNAs into mast cells as using GP+ E86 packaging cells (28). The anti-mouse VLA-5 monoclonal antibody M510 (5H10–27) (29) was purified by affinity chromatography on protein G-Sepharose (Amersham Pharmacia Biotech). Monoclonal anti-Myc epitope (9E10) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-7 ε epitope (Novagen, Madison, WI), rabbit polyclonal anti-Ha-Ras antibody and anti-RalA antibody (Transduction Laboratory, Lexington, KY), anti-HA 12CA5 (Roche Molecular Biochemicals, Indianapolis, IN) antibody, anti-PI 3-kinase p110α, p110β (Santa Cruz Biotechnology), p110δ (30), anti-PI 3-kinase p85 subunit (Upstate Biotechnology, Inc., Lake Placid, NY), anti-phospho-Akt antibody (Ser473) (New England Biolabs Inc., Beverly, MA), and peroxidase-linked anti-mouse or rabbit antibody (Amersham Pharmacia Biotech) were used for immunoprecipitation and immunoblotting as described below. Wortmannin (Wako Pure Chemicals, Tokyo, Japan), phorbol 12-myristate 13-acetate (PMA), recombinant murine steel factor (Genzyme, Boston, MA), and β-estradiol (Sigma) were purchased. Plasmids—Constitutively active Ha-Ras, R-Ras, Rap1A, Ra1A, Rac, and Rho mutants were produced from their cDNAs by a single point mutation; substitution of glutamine with valine at position 12 (Ha-RasVal12, RacVal12, and Rap1Val12) was a gift from Drs. M. Hattori and N. Minato, Kyoto University, substitution of glutamic acid for leucine at position 72 (Raf1E72L, a gift from Dr. H. Koide, Tokyo Institute of Technology). Epitope tags were attached at the amino-terminal end of the mutant small GTPases (31): Myc tag for Raf1 and Rap1Val12; HA (32) to the 3′ end of p110δ. Epitope tag for RacVal12 and Rap1 (Santa Cruz Biotechnology, Inc.) served as an internal control for the expression level. Epitope tag for Rap1. Effector loop mutants of Ha-RasVal12 (T55S, E57G, D85E, and Y40C) were described (31). To make effector loop mutants of R-RasVal38, polymerase chain reaction was used with R-RasVal38 as a template to introduce a point mutation; substitution of threonine with serine at position 61 (E61S), glutamic acid with glycine at position 63 (E63G), aspartic acid with glutamic acid at position 64 (D64E), and tyrosine with cysteine at position 66 (Y66C), p110α-CAAX was made by attaching the 20-carboxy-terminal amino acids (KMSKDGKKKSKSKKKKC) of K-Ras as described for Raf1-CAAX (32) to the 3′ end of p110δ by polymerase chain reaction. All cDNAs were verified by sequencing both strands, and subcloned into a retrovirus vector pMX-neo to introduce into mast cells, or pSG5 (Strategene, La Jolla, CA) for COS7 cells. cDNAs encoding for Raf1ER (33), Rap1-CAAX (34), and Raf1-CAAX (32) were also subcloned into pMX-neo.

Preparation of Fibronectin and the 80-kDa Fragment, and Integrin Affinity Measurement—Fibronectin and its 80-kDa tryptic fragment that contains the RGD binding motif for VLA-5 were produced as described (35, 36). The 80-kDa fibronectin fragment was radioiodinated with a modified method using chloramine T (37). The typical specific activity of the labeled 80-kDa fragment used in our experiments was about 3.5 × 10⁶ dpm/nmol. Its binding to cells was measured as described (20). Briefly, mast cells were washed once with binding buffer containing RPMI 1640 (Sigma), 0.1% BSA (Life Technologies, Inc.), and 10 mM HEPES, pH 7.4 (Sigma), and suspended with the same buffer at 1 × 10⁶ cells/ml. In a typical binding assay, performed in a 1.5-ml microcentrifuge tube, 100 μl of cells (1 × 10⁶ cells per tube) were mixed with 100 μl of the radiolabeled 80-kDa fragment. For inhibition with antibodies or Wortmannin, mast cells were preincubated with antibodies (20 μg/ml), or Wortmannin for 15 min at 37 °C before assays. After incubation for 30 min at 37 °C, samples were oil-separated by centrifugation at 8000 rpm for 1 min. The tip of tubes was amputated from the blade and applied to a blade 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.

Flow Cytometric Analysis—Cells (1 × 10⁶) were incubated on ice for 30 min with 50 μl of staining buffer (phosphate-buffered saline, 0.1% BSA, 0.05% sodium azide). The cells were stained with fluorescein isothiocyanate-labeled anti-Rat IgG as above. The stained cells were analyzed by FACSCan (Becton Dickinson, San Jose, CA).

Adhesion Assays—Assays of adhesion to fibronectin were performed as described (27). Briefly, mast cells labeled with 2′,7′-dihydro-2-carboxyethyl-5-(and-6)-carboxyfluorescein (BCECF) in 96-well plates precoated with fibronectin (1 μg/well) or 1% bovine serum albumin (BSA) were incubated in triplicate at 37 °C for 30 min, or in the presence of PMA as indicated. 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 subtracting the bound antibodies or Wortmannin, labeled mast cells were preincubated at room temperature for 15 min with 20 μg/ml antibodies or Wortmannin as indicated before assays.

Western Blot—Mast cells were prepared for cell lysates as described (28). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, the separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane. After blocking with 5% BSA, the membrane was incubated with antibodies as indicated and detected with the appropriate secondary antibody conjugated with horseradish peroxidase. The bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Stripping and reprobing were performed according to the manufacturer’s instructions.

For detection of Myc-tagged Raf-CAAX or, HA-tagged RalA-CAAX, cell lysates (5 × 10⁶ cells) were immunoprecipitated with anti-Myc (9E10) or anti-HA (12CA5) antibodies and subjected to Western blotting detected with anti-Raf (Upstate Biotechnology, Inc.) or anti-HA antibodies. For detection of p110α-CAAX, cells (5 × 10⁶ cells) were suspended with SDS sample buffer, and briefly sonicated and boiled before SDS-PAGE and Western blotting.

Interactions of Ha-Ras Mutants and p110δ—Cos7 cells were transfected with Ha-Ras mutants (5 μg) and Myc-tagged p110δ (5 μg). After 48 h, cells were harvested and lysed with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM Tris, pH 7.4, 10 mM MgCl₂, 150 mM Glycine, 1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin). Immunoprecipitation was performed using anti-Myc epitope antibody (9E10) or anti-Ras antibody (Y13-238, Oncogene Science, Uniondale NY). Immunocomplexes were collected with protein G-Sepharose (Amersham Pharmacia Biotech) and washed with lysis buffer three times. SDS-PAGE and Western blotting were performed as above. Blots were incubated with anti-p110δ antibody (30) or anti-Ras antibody (Transduction Laboratories).

Assay for MAP Kinase Activity—Mast cells (5 × 10⁶ cells) that introduced RafER were stimulated with estradiol (1 μM) or steel factor (10 μM) for 15 min at 37 °C for 30 min. Cells were lysed with 1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.6, 1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin). Cell lysates were incubated with 1 μg of anti-ERK2 antibody (Santa Cruz). Immunocomplexes were collected with protein G-Sepharose. Immune complex kinase assays were performed with myelin basic protein (Sigma) as a substrate (33). The levels of phosphorylation of myelin basic protein were quantitated with a PhosphorImager (BAS1000, Fujifilm, Tokyo, Japan).

RESULTS

The Effects of the Ras/Rho Family of Small GTPases on Adhesion to Fibronectin through VLA-5—To examine the ability of the Ras/Rho small GTPases to modulate avidity of VLA-5 to fibronectin, we established bone marrow-derived mast cells by cultivating bone marrow cells with interleukin-3 for 4 weeks, and then introduced active forms of Ha-Ras (Ha-RasVal12), Rap1 (T7-tagged Rap1Val12), Ra1A (Ra1A-Leu72), R-Ras (Myc-
Tagged R-RasVal-38, Rac (Myc-tagged RacVal-12), Rho (Myc-tagged RhoVal-14), or neomycin only by retrovirus. Following drug selection, we examined adhesion of these transfectants to fibronectin. Control mast cells (neo) and uninfected mast cells did not adhere to fibronectin significantly when compared with BSA (Fig. 1). However, cells expressing Ha-RasVal-12 and R-RasVal-38 adhered strongly to fibronectin without stimulation. In both cases, adhesion to fibronectin was blocked by an anti-VLA-5 antibody, 5H10, indicating that adhesion was mediated by VLA-5 (Fig. 1). Adhesion to fibronectin was also induced slightly in Rap1Val-12 or RacVal-12 expressing cells, which was inhibited with anti-VLA-5 antibody, but RalALeu-72 or RhoVal-14 transfectants did not change the level of adhesion significantly. Expressions of VLA-5 of transfectants were similar to control cells (Neo) (Fig. 2A), or uninfected cells (not shown). We demonstrated expression of introduced active forms of the Ras/Rho small GTPases in mast cells by Western blot analysis (Fig. 2B). These results indicated that adhesiveness of VLA-5 was increased in cells expressing Ha-RasVal-12 and R-RasVal-38. Transfectants expressing Rap1Val-12, RalALeu-72, RacVal-12, or RhoVal-14 adhered to fibronectin when stimulated with steel factor or PMA, indicating that these active forms of small GTPases did not exert inhibitory effects on activation-dependent adhesion of mast cells (data not shown).

Ha-RasVal-12 and R-RasVal-38 Increase Ligand Binding Activity of VLA-5—R-RasVal-38 was previously shown to augment ligand binding activity to fibronectin (25). To examine whether ligand binding activities of VLA-5 are augmented in cells expressing Ha-RasVal-12 and R-RasVal-38, we measured ligand-binding affinity using a soluble 80-kDa fibronectin fragment (FN80) containing the RGD motif that was recognized by VLA-5. We previously demonstrated that ligand bindings of unstimulated mast cells was low, but increased by FceRI cross-linking, but not PMA stimulation (20). In mast cells expressing either Ha-RasVal-12 or R-RasVal-38, ligand bindings were augmented compared with those of control cells (Fig. 3). The level of ligand bindings of cells expressing Ha-RasVal-12 was higher than that in R-RasVal-38 expressing cells. The increased ligand bindings were inhibited by anti-VLA-5 antibody, indicating that ligand binding activity of VLA-5 was increased in these cells.

To explore whether PI 3-kinase is involved in affinity modulation of VLA-5 by Ha-RasVal-12 and R-RasVal-38, as is the case in mast cells stimulated with FceRI cross-linking (20), ligand binding assays were performed in the presence of wortmannin. Bindings to FN80 in both Ha-RasVal-12 and R-RasVal-38 expressing cells were completely inhibited with low doses of wortmannin (Fig. 3) or LY294002 (data not shown), suggesting that PI 3-kinase is involved in affinity modulation by Ha-RasVal-12 and R-RasVal-38.

Differential Effects of Wortmannin on Adhesion to Fibronectin of Cells Expressing Ha-RasVal-12 and R-RasVal-38—Wortmannin also inhibited adhesion of Ha-RasVal-12 expressing cells to fibronectin at a concentration similar to those abolished bindings to FN80 (Fig. 4). On the other hand, adhesion of R-RasVal-38 expressing cells was resistant to treatment of wortmannin even at 100 nM, the dose of which completely blocked the bindings to FN80 (Fig. 3). LY294002 also failed to inhibit adhesion to fibronectin (data not shown). Anti-VLA-5 inhibited adhesion of R-RasVal-38 expressing cells to fibronectin in the presence of wortmannin (Fig. 4). These results indicate that the high affinity state of VLA-5 by PI 3-kinase likely accounted for adhesion induced by Ha-RasVal-12, while R-RasVal-38-induced adhesions were mostly independent from PI 3-kinase activities, and did not require the high affinity state of VLA-5 for adhesion.

The Effects of Mutations in the Effector Loop Region of Ha-RasVal-12 on Adhesion and Akt Phosphorylation—Point mutations in the effector loop region of the active form of Ha-Ras was reported to selectively inhibit the interaction and activation of downstream signal molecules such as Raf, RaIGDS, and PI 3-kinase (38). Raf was shown to interact only with Ha-RasVal-12.
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FIG. 3. The specific bindings of the 125I-labeled fibronectin 80 fragment. The ligand binding assay was performed in triplicate using 125I-labeled fibronectin 80 (0.2 μg) and mast cells expressing the neomycin gene (open bar), Ha-Ras<sup>Val-12</sup> (closed bars), or R-Ras<sup>Val-38</sup> (hatched bars). The specific bindings of mast cells pretreated with the anti-VLA-5 antibody (anti-α5, 5H10), or wortmannin at concentrations indicated. The data shown are representative of several experiments with similar results, and the average and standard errors are shown.

FIG. 4. Effects of wortmannin on adhesion to fibronectin of mast cells expressing Ha-Ras<sup>Val-12</sup> (closed bars) or R-Ras<sup>Val-38</sup> (hatched bars). Left panel, mast cells were pretreated with dimethyl sulfoxide (0.1%) (DMSO) or indicated amounts of wortmannin (10, 50, and 100 nM) for 15 min before adhesion assays. Right panel, adhesion to fibronectin of R-Ras<sup>Val-38</sup> expressing mast cells with/without the anti-VLA-5 antibody (anti-α5, 5H10). The data are shown as in Fig. 1.

T35S and Ha-Ras<sup>Val-12</sup> D38E, and RalGDS and PI 3-kinase p110α interacted only with Ha-Ras<sup>Val-12</sup> E37G and Y40C, respectively. To confirm PI 3-kinase dependence of Ha-Ras for adhesion, mast cells expressing Ha-Ras<sup>Val-12</sup>, T35S (Ser<sup>35</sup>), E37G (Gly<sup>37</sup>), D38E (Glu<sup>38</sup>), or Y40C (Cys<sup>40</sup>) were established (Fig. 5B). There were no significant changes in surface levels of VLA-5 in these transfectants (data not shown). Contrary to our expectation, cells expressing the Gly<sup>37</sup>, but not Cys<sup>40</sup> mutant showed the levels of adhesion equivalent to, or more than, cells expressing Ha-Ras<sup>Val-12</sup>, whereas those of adhesion of mast cells expressing the Ser<sup>35</sup>, Gly<sup>37</sup>, or Cys<sup>40</sup> mutants were reduced considerably (Fig. 5A). We also examined the phosphorylation of Akt in effector mutant expressing cells as its phosphorylation is dependent on activities of PI 3-kinase (39). The level of Akt phosphorylation was augmented only in mast cells expressing the Gly<sup>37</sup> mutant, the level of which was more than that of the Val<sup>12</sup> mutant (Fig. 5C). The Ser<sup>35</sup> slightly increased Akt phosphorylation compared with control (Fig. 5C). The phosphorylation of Akt was completely abolished with treatment of wortmannin, confirming the requirement of PI 3-kinase activity for Akt phosphorylation (data not shown). Thus the levels of Akt phosphorylation were in good correlation with those of adhesion to fibronectin (Fig. 5C), which is consistent with the notion that PI 3-kinase is critically involved downstream of Ha-Ras for adhesion, and suggest that the interaction of Ha-Ras and PI 3-kinase occurs in the Gly<sup>37</sup>, but not Cys<sup>40</sup> mutants in mast cells.

 Associations of PI 3-Kinase p110δ with Ha-Ras Effector Mutants—To confirm the possibility that the Gly<sup>37</sup>, but not Cys<sup>40</sup> mutant associates with PI 3-kinase in mast cells, we examined the isoforms of the p110 catalytic subunit that were expressed in mast cells. Mast cells expressed predominantly p110δ, while p110α and β were barely detected (Fig. 6A). The interactions of Ha-Ras effector mutants and Myc-tagged p110δ were examined by co-transfection into COS cells and immunoprecipitation with either anti-Myc antibody (Fig. 6B) or anti-Ha-Ras antibody (Fig. 6C) for associations with Ha-Ras mutants or p110δ, respectively. In both cases, p110δ was co-immunoprecipitated with the Gly<sup>37</sup> as efficiently as Ha-Ras<sup>Val-12</sup>. This result is consistent with strong Akt phosphorylation in the Gly<sup>37</sup> expressing mast cells (Fig. 5C).

FIG. 5. Adhesion to fibronectin of mast cells expressing effector loop mutants of Ha-Ras<sup>Val-12</sup>. A, adhesion to fibronectin. Mast cells transfected with the neomycin gene (Neo), Ha-Ras<sup>Val-12</sup> (V12), or effector loop mutants (Ser<sup>35</sup>, Gly<sup>37</sup>, Glu<sup>38</sup>, and Cys<sup>40</sup>) in the background of the Val-12 mutation were subjected to adhesion assays without (closed bars) or with 10 ng/ml PMA (hatched bars). The data are shown as in Fig. 1. B, expressions of Ha-Ras mutants in mast cells transfected with the neomycin gene, or Ha-Ras mutants as indicated. C, phosphorylation of Akt. Cell lysates of mast cells expressing the neomycin gene (Neo) or Ha-Ras mutants were analyzed by Western blotting for phosphorylation of Akt by the antibody specific for phosphorylation of serine 473 of Akt (upper panel). The membrane was stripped and reprobed with anti-Akt antibody recognizing both phosphorylated and unphosphorylated Akt (lower panel).

p110δ-CAAX Induces Adhesion to Fibronectin—To directly demonstrate that p110δ itself is sufficient to induce adhesion to fibronectin, we introduced an activated membrane-targeted version of p110δ, p110δ-CAAX, into mast cells. We also tested membrane-targeted versions of two known effector molecules that bind to Ha-Ras, Raf-CAAX and Rlfs-CAAX (Fig. 7A). Mast cells expressing p110δ-CAAX strongly adhere to fibronectin without stimulation. In contrast, cells expressing Raf-CAAX or Rlfs-CAAX failed to adhere to fibronectin while they responded well to PMA to adhere to fibronectin. As shown in Fig. 7B, mast cells expressing p110δ-CAAX showed marked cell attachment.

Adhesion to fibronectin of mast cells expressing effector loop mutants of Ha-Ras<sup>Val-12</sup>.
and spreading on fibronectin compared with control cells (neo). Cell attachment and spreading of p110δ-CAA-expressing mast cells were comparable to those of Ha-RasVal-12, while R-RasVal-38 expressing cells tended to spread more on fibronectin (Fig. 7B).

We further examined the effect of activation of the Raf-MAP kinase pathway on adhesion by introducing a chimera of the Raf kinase domain and the hormone-binding domain of the estrogen receptor (rafER) (33). A conditional activation of the Raf kinase activity by estradiol increased the kinase activity of ERK2 more than that by steel factor (Fig. 8B). Stimulation with estradiol did not affect adhesion to fibronectin. We introduced R-RasVal-38 effector mutants that carried the same replacement mutations as Ha-Ras at the corresponding sites in the background of the Val38 mutation. Established mast cells expressed comparable amounts of PI 3-kinase in mast cells. We found that wortmannin had the marginal effect on adhesion induced by Myc-tagged Raf-CAAX, HA-tagged Rlf-CAAX, or p110δ-CAAX were analyzed for adhesion to fibronectin. Adhesion assays were performed without stimulation (closed bars) or with PMA (hatched bar). The results are shown as in Fig. 1. Lower panel, expressions of Raf-CAAX, Rlf-CAAX, and p110δ-CAAX (arrow). Lanes 1, 3, and 5, mast cells transfected with the neomycin gene. Lane 2, Raf-CAAX (72 kDa); lane 4, Rlf-CAAX (60 kDa); lane 6, p110δ-CAAX (110 kDa). B, appearance of adhesion of control mast cells (neo), or mast cells expressing p1006-CAAX, Ha-RasVal-12, or R-RasVal-38. The original magnification is 100-fold.

Akt phosphorylation to the comparable degrees in all effecter mutants (Fig. 9C). This result was in contrast to that of Ha-RasVal-12, in which the levels of adhesion paralleled with PI 3-kinase activities, and supports PI 3-kinase independent mechanisms of R-RasVal-38 in stimulating adhesion to fibronectin.

DISCUSSION

In this study, we examined the ability of a series of the Ras/Rho family of small GTPases to activate VLA-5 to adhere to fibronectin in bone marrow-derived mast cells. We found that the active forms of Ha-Ras (Ha-RasVal-12) and R-Ras (R-RasVal-38) were most potent in stimulating adhesion to fibronectin. Both Ha-RasVal-12 and R-RasVal-38 induced the high affinity state of VLA-5. However, PI 3-kinase inhibitors abrogated adhesion by Ha-RasVal-12, but not R-RasVal-38. Among effector loop mutants of Ha-RasVal-12, only the Gly37 mutant retained the ability to stimulate adhesion and also the ability to associate with p110δ, which is consistent with strong phosphorylation of Akt in the Gly37 mutant expressing cells. The membrane-targeted version of p110δ was sufficient to stimulate adhesion to fibronectin. These results indicate that Ha-RasVal-12 depends on PI 3-kinase δ to activate VLA-5 in mast cells.

We showed that wortmannin had the marginal effect on adhesion induced by R-RasVal-38, whereas it abolished the increase in ligand binding activity of VLA-5. This result suggests that R-Ras depends on PI 3-kinase on induction of the high
affinity state of VLA-5, which only made a minor contribution to R-Ras Val-38-induced adhesion. The effects on adhesion of mutations in the effector loop region of R-Ras Val-38 were distinct from those of Ha-Ras Val-12 and the decrease of phosphorylation of Akt did not result in loss of adhesion, which further support the PI 3-kinase independent mechanism of R-Ras Val-38 to activate VLA-5. Although it is currently unclear about adhesion mechanisms of R-Ras, it is conceivable that R-Ras Val-38 has other mechanisms that induce adhesion mediated through the low affinity state of VLA-5, possibly by modulating lateral diffusion/clustering on VLA-5 on cell surface.

We have recently shown that PI 3-kinase is an affinity modulator of VLA-5, which is critically involved in adhesion induced by FcεRI (20). Our results that PI 3-kinase was involved in Ha-Ras Val-12 and R-Ras Val-38 induced the high affinity state of VLA-5 are in line with previous reports (38, 41) on activation of PI 3-kinase by these small GTPases and further implicate physiological roles in Ha-Ras and R-Ras in FcεRI-induced adhesion. In fact, activation of Ha-Ras, but not R-Ras was most seen in mast cells when stimulated with cross-linking of FcεRI. The kinetics of Ha-Ras activation paralleled with that of adhesiveness of VLA-5 increased by FcεRI.2 Thus, Ha-Ras could contribute to the high affinity state of VLA-5 by FcεRI. However, we could not demonstrate that Ha-Ras was responsible for the high affinity state of VLA-5 by FcεRI, because a dominant negative Ha-Ras was not expressed in mast cells, possibly due to the inhibition of their growth.

Ha-Ras was reported to suppress the activation of integrins through the Raf/MAP kinase pathway. In this study, chimeras of the extracellular regions of αIIbβ3 and intracellular regions of α5β1 or α6β1 integrins were used in adherent Chinese hamster ovary cells (24). In the present study, we showed that Raf-CAAX did not affect adhesion induced by PMA (Fig. 7A). We also showed that a conditional activation of the Raf/MAP kinase pathway did not increase adhesion (Fig. 8), and had no effect on steel factor- and FcεRI-induced adhesion (data not shown). In fact, it has not been demonstrated directly whether Ha-Ras plays an inhibitory role in integrin activation by inside-out signals. The reason of the discrepancy is not known at present, but it is likely due to the differences in experimental systems including integrin adhesive property and structure. The other investigators reported that Ha-Ras was involved in adhesion induced by interleukin-3 and the activated form of Ha-Ras induced adhesion to fibronectin in an interleukin-3-dependent cell line Ba/f3, which was blocked by an inhibitor of phospholipase C, U-73122, but not inhibitors for PI 3-kinase (42). At present, we cannot explain this discrepancy. It could be due to the difference in cell context, or the other effects of

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2 T. Kinashi, unpublished data.
The activated Ha-Ras interacts with and activates the PI 3-kinase catalytic subunit, p110α (38). The association of Ha-Ras and p110α was further characterized with the effector loop mutations of Ha-Ras (31). Notably the Cys40 mutant was shown to interact with p110α. p110α belongs to class IA of PI-3 kinase (45, 46). It is specifically expressed in leukocytes (30). Ha-Ras was also shown to interact with p110α. p110α has biochemical properties similar to p110α, including the sensitivity of PI 3-kinase inhibitors (30). However, p110β was co-immunoprecipitated with the Gly37 mutant, but poorly with the Cys40 mutant, as we showed in this study. The amino acid sequence of p110β in the Ras-binding domain is considerably diverged from that of p110α and p110β (30, 47). The sequence divergence in this region among p110 subunits likely contributes to the difference in the specific interaction sites of the effector loop region of Ha-Ras. The Gly37 mutant was previously shown to interact with RalGDS and Rif, GTP exchange factors for Ral (31, 34). However, the experiments with active forms of Ral, Rif, or RalGDS (data not shown) rule out their critical roles in activation of integrins. Instead, the fact that Akt phosphorylation was augmented in the Gly37, but not Cys40 mutant expressing mast cells indicates that the association and activation of p110β with the Gly37 mutant occur in mast cells. Taken together, our results demonstrate that p110β is a critical effector molecule of Ha-Ras in activating integrins in mast cells.

R-RasVal-38 was reported to increase ligand-binding affinity to αβ6 in an interleukin-3 dependent myeloid cell line, 32D cells (25). We showed here that both R-RasVal-38 and Ha-RasVal-12 induced the high affinity state of VLA-5 in mast cells. The ligand binding activity was higher in Ha-RasVal-12 transfectants than in R-RasVal-38 transfectants. Our preliminary experiments showed that the dissociation constant of VLA-5 in Ha-RasVal-12 transfectants was between 20 and 50 nM, which was higher than that reported in R-RasVal-38 expressing 32D cells (250 nM) and equivalent to that induced by FezF1 cross-linking (20). Importantly, our study revealed that the high affinity state of VLA-5 in R-RasVal-38 expressing cells was dispensable for adhesion to fibronectin, since the treatment of Wortmannin abolished the high affinity state with a small inhibitory effect on adhesion. This is in contrast with Ha-RasVal-12 expressing cells, in which Wortmannin abolished both ligand binding activity and adhesion to fibronectin at the similar doses. The PI-3 kinase independent adhesion by R-Ras was also supported by the analysis using the effector loop mutants. All of the effector loop mutations at the homologous sites of Ha-Ras resulted in decrease of Akt phosphorylation at the similar degree, but did not parallel levels of adhesion. Recently it has been reported that the R-Ras Gly65 mutants among other mutants interacted more with the Ras-binding domain of PI 3-kinase p110α, and that adhesion by the Gly65 mutant was partially inhibited by a dominant negative Rac or Ral (48). We failed to detect distinct sites of the R-Ras effector loop region to interact with p110β by co-immunoprecipitation. The difference could be due to low homologies in the Ras-binding domain between p110α and p110β as discussed above. In addition, RacVal-12 or RalaGlu-72 by itself failed to induce adhesion in our study, ruling out their critical roles downstream of R-Ras in activating integrins in our case, although they might promote adhesion by modulating cytoskeletal organization such as cell spreading.

Adhesion through integrins is mediated through multiple steps initiated by integrin activation by inside-out signals, leading to cytoskeletal reorganization and firm attachment by outside-in signals upon adhesion. Here in this study, we examined the ability of a series of Ras/Rho family of small GTPases to activate integrins in search for the possible inside-out signals. Our study clearly demonstrated that distinct members of small GTPases had the ability to regulate adhesiveness of integrins, which gives important clues to dissect regulatory processes of adhesion through integrins.

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