Steel Factor and c-*kit* Regulate Cell-Matrix Adhesion

By Tatsuo Kinashi and Timothy A. Springer

***Steel (Sl)* and *white spotting (W)* loci encode steel factor (c-*kit* ligand) and the c-*kit* tyrosine kinase receptor, respectively. Mutations at these loci affect migration and differentiation of primordial germ cells, neural crest-derived melanoblasts, and hematopoietic cells. In these processes, cell adhesion molecules are hypothesized to be crucial. We have examined the role of steel factor and c-*kit* in cell-extracellular matrix adhesion using bone marrow-derived mast cells as a model system. Steel factor stimulates mast cells to bind to fibronectin and, to a lesser extent, to vitronectin, whereas interleukin-3 and interleukin-4, which are also mast cell growth factors, do not. Activation of adhesiveness is transient, occurs at concentrations of steel factor 100-fold lower than required for growth stimulation, and requires the integrin VLA-5. Mast cells from c-*kit* mutant mice adhere to fibronectin on stimulation with phorbol 12-myristate 13-acetate (PMA), but not on stimulation with steel factor, indicating that stimulation of integrin adhesiveness requires activation of the c-*kit* protein tyrosine kinase. By contrast, c-*kit* mutant and wild-type mast cells adhere equally well to COS cells expressing membrane-anchored steel factor, showing that the kinase activity of c-*kit* is not required for adhesion directly mediated by c-*kit*. Our findings suggest that regulation of adhesion is an important biologic function of steel factor.**

MUTATIONS IN THE MOUSE at the *steel (Sl)* or *white spotting (W)* loci affect the development of several migratory cell lineages, including primordial germ cells, neural crest-derived melanocytes, hematopoietic stem cells, and mast cells.[1,2] Mice bearing either mutation have similar phenotypes, including sterility, severe anemia, white coat color, and mast cell deficiency. Grafting experiments indicate that the *Sl* gene affects the microenvironment that supports the cells mentioned above, whereas the *W* gene acts cell-autonomously.[3-8] The *W* locus encodes the proto-oncogene c-*kit*, which is a member of the transmembrane tyrosine kinase family that has homology to colony-stimulating factor 1 and platelet-derived growth factor receptors.[9-11] The *Sl* locus encodes a ligand for c-*kit*[12-14] that has been designated stem cell factor,[14-16] mast cell growth factor,[12,17,18] and the *kit* ligand (KL)[13,19]; herein, we refer to the c-*kit* ligand as steel factor.[20] Alternative splicing gives rise to both cell surface and soluble forms of steel factor with indistinguishable mast cell growth activity in vitro.[17,21]

Because the above studies have suggested that steel factor and c-*kit* regulate cell migration, and cell migration is dependent on adhesive interactions with the extracellular matrix,[22] we have investigated how the steel factor/c-*kit* system regulates cell-extracellular matrix adhesion using mast cells as a model system. Mast cells are relatives of granulocytes that are found in connective tissues near blood vessels and mucosa. They are centrally important in regulating local inflammatory reactions. Mast cells are easily obtained in a large quantity compared with other cells affected by the *Sl* and *W* mutations. Mice bearing the *Sl* or *W* mutation lack mast cells in peripheral tissues, but mast cell precursors exist in the bone marrow and can be expanded with interleukin-3 (IL-3) as in normal mice.[8,23]

We show here that soluble steel factor is a much more potent regulator of cell adhesion than cell proliferation. Activation of adhesiveness to fibronectin (FN) is rapid, transient, and requires steel factor concentrations 100-fold lower than those required for stimulation of cell growth. The mast cell growth factors IL-3 and IL-4 have no effect on adhesion. Activation of the FN receptor requires the tyrosine kinase activity of c-*kit*, whereas cellular adhesion through steel factor and c-*kit* does not.

MATERIALS AND METHODS

Cell lines. The MC/9 mast cell line[24] was maintained in RPMI 1640 containing 10% fetal calf serum, 50 μmol/L β-mercaptoethanol, and 50 μg/mL gentamycin (complete medium) supplemented with 10% WEHI-3B conditioned medium as a source of IL-3. Homogeneous cultured mast cells were established by maintaining nonadherent cells of bone marrow derived from Balb/c mice with the same medium as above, and were used from 4 to 10 weeks after establishment. WBB6 W/Wv and control +/+ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mast cells were established from bone marrow as described above. Mast cells cultured in vitro for less than 6 to 7 weeks bind better than those cultured for more than 8 weeks.

Antibodies, growth factors, and chemicals. The MFR-5 or 5H10 monoclonal antibody (MoAb) (rat γ2a/κ) was produced as described[25] by immunizing with MC/9 and screening for inhibition of adhesion to fibronectin. The characterization of MFR-5 will be described elsewhere. MFR-5 was purified from culture supernatants by ammonium sulfate precipitation, followed by the affinity chromatography on protein G-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).

Ascites of ACK2, an MoAb against murine c-*kit*,[26,27] was generously provided by Dr S-I. Nishikawa (Kumamoto University, Kumamoto, Japan) and murine steel factor (10^5 U/mg) was the kind gift of Dr S. Gillis (Immunex Corp, Seattle, WA). Purified murine IL-3 and IL-4 were purchased from Genzyme (Boston, MA). Human fibronectin, vitronectin, RGDS, and RGES peptides were from Telios Pharmaceuticals (San Diego, CA). Mouse laminin and collagen type IV were from Collaborative Research (Bedford, MA). Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem (San Diego, CA).

Adhesion assays of extracellular matrix proteins. Adhesion assays were performed in a 96-well polystyrene plate (Linbro-Titertek; Flow Laboratories, McLean, VA) coated with extracellular matrix proteins. FN, laminin, collagen IV, and vitronectin (100 mL of 100 mg/mL in RPMI 1640 supplemented with 10 mmol/L HEPES, pH 7.4) was added to each well and incubated at 37°C for

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2 hours or 4°C overnight. Subsequent to experiments in Fig 1, FN was used at 20 μg/mL. RPMI 1640 with 3% bovine serum albumin (BSA fraction V; Sigma Chemical Co, St Louis, MO) was added for 1 hour at 37°C to block nonspecific binding sites. The wells were then washed four times with RPMI 1640 containing 10 mmol/L HEPES and 0.03% BSA (the binding medium). MC/9 cells and cultured mast cells were labeled with 2',7'-bis-(2-carboxyethyl)-5 (and -6)carboxyfluorescein (BCECF; Molecular Probes, Inc, Eugene, OR) as described,[28] suspended in the binding medium, and transferred into coated wells (5×10^4 cells in 100 μL) with or without growth factors as indicated. The plates were incubated at 37°C for 30 minutes and the unbound cells were then washed away by vacuum pump aspiration of the medium through a pipette tip (multifit nonbeveled tip; Marsh, Rochester, NY). Binding medium was added (150 μL) and washing was repeated four times. Input and bound fluorescence were directly quantitated from the 96-well plates using a Pandex fluorescence concentration analyzer (Baxter Healthcare Corp, Mundelein, IL). The level of adhesion was calculated by dividing bound fluorescence by input fluorescence.

For kinetic studies of adhesion to FN, BCECF-labeled mast cells were treated at 37°C with steel factor or PMA for varying times in suspension before transfer into wells and centrifugation of the plate at 400 rpm for 1 minute. The plate was then incubated for 6 minutes at 37°C and washed four times by flicking media from the plate with 150 μL of the binding medium added between each wash. The time indicated in Fig 4 includes acceleration, 1 minute at speed, deceleration, handling time (4 minutes), the 6 minutes of incubation in the plates, and varying times in suspension.

For inhibition of the adhesion assay with antibodies, BCECF-labeled cells were pretreated at room temperature with a saturating amount of antibody (1 μg of MFR-5 for 5×10^4 cells) for 30 minutes. The treated cells were then transferred to 96-well plates together with the antibody.

Growth assay of mast cells. Mast cells were cultured in 96-well plates (5×10^4 cells in 0.2 mL/well) for 48 hours in complete medium supplemented with various amounts of steel factor as indicated. Methyl-^{3}H-thymidine (6.7 Ci/mmol; NEN, Boston, MA) was added into wells (1 μCi/well) 6 hours before cells were harvested. Incorporated ^{3}H-thymidine was collected onto glass filters and counted by liquid scintillation.

Flow cytomeric analysis. Cells (5×10^5 cells) were incubated with 50 μL of a saturating amount of antibody (1 μg for MFR-5) at 4°C for 40 minutes and then washed twice with cold phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS). Cells were further incubated with 50 μL of a 1/40 dilution of fluorescein isothiocyanate (FITC)-labeled goat antirat IgG (Zymed Immunochemicals, San Francisco, CA) and washed under the same conditions as above. Cells were analyzed using FACScan (Becton Dickinson, Lincoln Park, NJ). For kinetic studies, cells were immediately fixed with 2% paraformaldehyde, stained with antibodies, and analyzed as above.

Adhesion assays of transfected COS cells. COS cells, 1 day after plating at 1.5×10^6 cells per 150-mm culture dish, were transfected with murine full-length steel factor cDNA (KL-M1) in the transient expression vector pcDNA I[21] or vector alone (mock) using diethylaminoethyl (DEAE) dextran as described.[29] Twenty-four hours after transfection, COS cells were replated into 6-well dishes (6×10^4 cells/well) and incubated for an additional 48 hours before use in adhesion assays. The experiment was performed using COS cells from the same 150-mm dish. Mast cells (5×10^5 cells/well), with or without preincubation for 30 minutes with ACK-2 (1/1,000 dilution), were incubated in complete medium with transfected COS cells at 37°C for 2 hours with occasional swirling. Unbound cells were washed away by aspirating and adding the medium five times. Remaining mast cells were detached with trypsin/EDTA and counted with a hemocytometer.

RESULTS

Adhesion of mast cells to extracellular matrix proteins. We tested the IL-3–dependent mast cell line MC/9 and bone marrow-derived mast cells for adhesion to extracellular matrix proteins. MC/9 cells adhered well to FN and laminin, and to a lesser extent to vitronectin (Fig 1A). Unstimulated bone marrow-derived mast cells did not bind significantly to any of the tested extracellular matrix proteins; however, bone marrow-derived mast cells that were stimulated with steel factor, but not those stimulated with IL-3 or IL-4, bound to FN and less markedly to vitronectin (Fig 1B). We confirmed that both IL-3 and IL-4 stimulated growth of our cultured mast cell cells (Fig 1C), as previously reported,[30-32] showing that their lack of effect on adhesion was not caused by receptor modulation. In contrast to their synergistic effect with steel factor on growth, we did not find any synergistic effect of IL-3 and IL-4 in combination with steel factor on FN binding (data not shown). Mast cells did not bind laminin and collagen type IV, even at a higher concentration of steel factor (200 U/mL; data not shown). PMA also stimulated mast cells to bind to FN and vitronectin. In contrast to mast cells cultured from bone marrow, the established mast cell line MC/9 showed only slightly augmented binding to FN and laminin in the presence of steel factor (data not shown). It appears that adhesiveness of MC/9 may be constitutively activated.

Adhesion to FN requires a much lower concentration of steel factor than does cell growth. Stimulation of adhesiveness of cultured bone marrow mast cells was exquisitely sensitive to steel factor. Half-maximal binding of mast cells to fibronectin was achieved with only 0.03 U/mL, whereas 10 U/mL was required to stimulate half-maximal growth of mast cells (Fig 2). These results show that the effects of steel factor on mast cell adhesiveness for FN and growth can be dissociated.

Steel factor-stimulated adhesion to FN is mediated through the murine homologue of VLA-5. A 120-kD fragment of FN containing the classical RGD tripeptide cell attachment site[33] was able to substitute for native FN in mediating steel factor-stimulated adhesion of mast cells (Fig 3). MFR-5, a rat MoAb that recognizes murine VLA-5, blocked adhesion to the 120-kD fragment and to intact FN. The RGDS peptide, but not the control RGES peptide, completely inhibited the binding to the 120-kD fragment, indicating that the RGD cell attachment site is responsible for adhesion.

Transient adhesiveness to FN. To investigate the kinetics of adhesiveness, mast cells were stimulated for varying time periods in suspension, centrifuged onto the fibronectin substrate for 1 minute, and incubated on fibronectin for 6 minutes at 37°C before washing. The adhesiveness stimulated by both steel factor and PMA was transient, and maximal adhesiveness was seen at about 10 minutes (Fig 4), the earliest time point measurable after stimulation. Binding declined to background levels after 90 minutes. At 120 minutes, mast cells were restimulated with either 100 U/mL steel factor or 10 ng/mL PMA; however, neither heterologous nor homologous restimulation had any further effect on adhesiveness. Cell viability after restimulation was more

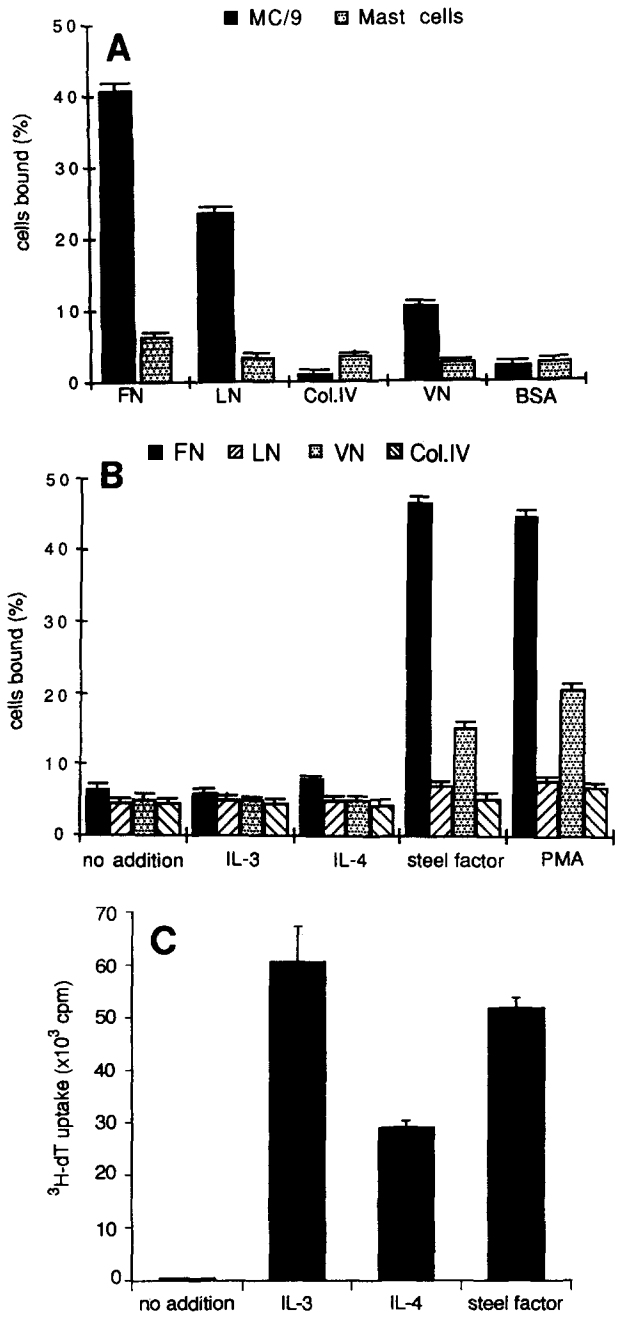

Fig 1. Adhesion of MC/9 and mast cells to extracellular matrix proteins. (A) Cell binding was measured to FN, laminin (LN), collagen type IV (Col.IV), vitronectin (VN), and albumin (BSA) coated on polystyrene culture plates and used for the binding assay. (B) Adhesion of mast cells stimulated with IL-3, IL-4, and steel factor (100 U/mL each) or PMA (10 ng/mL) to extracellular matrix proteins. (C) ^{3}H-thymidine uptake of mast cells in response to IL-3, IL-4, and steel factor (100 U/mL each). The average and SE of triplicate determination are shown.

than 95% as judged by trypan blue exclusion. Transient adhesiveness was unrelated to the level of expression of the FN receptor on mast cells, as shown by staining with MFR-5 before and at several time points after stimulation with steel factor or PMA (Fig 5).

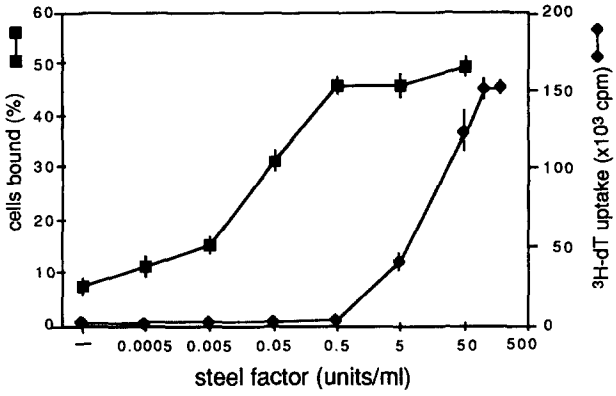

Fig 2. Comparison of adhesion-stimulating and growth-stimulating activities of steel factor. Stimulation of binding to fibronectin and ^{3}H-thymidine uptake of mast cells were assayed in triplicate. Bars show the SE.

W/W*v* *mast cells are defective in steel factor-stimulated adhesion but not in adhesion to steel factor.* To investigate how *W* mutations affect adhesion, we characterized *W/Wv* mast cells. The *W* mutation results in a null phenotype, whereas the *Wv* mutation is a single amino acid substitution in the kinase domain of c-*kit* that is expressed comparably to the wild-type,[34] (data not shown). Binding to FN of *W/Wv* mast cells stimulated with steel factor was dramatically reduced compared with wild-type, whereas the responsiveness to PMA remained intact (Fig 6A). This result shows that the tyrosine kinase activity of c-*kit* is crucial to induce adhesiveness to FN. The importance of the tyrosine kinase activity is also supported by experiments with the tyrosine-specific protein kinase inhibitors, genestein and herbimycin A, in which these inhibitors abolished the steel factor-induced FN binding, whereas PMA-induced binding remained relatively intact (data not shown). By contrast to adhesion stimulated by c-*kit*, direct adhesion through c-*kit* was unaffected by the W^v mutation. Mast cells from +/+ and *W/Wv* mice adhered equally well to COS cells transfected with full-length steel factor (Fig 6B). This cellular adhesion was dependent on c-*kit*, because the blocking antibody to c-*kit*, ACK-2, abolished adhesion.

DISCUSSION

We have investigated regulation of cell-matrix adhesion by steel factor and c-*kit*. We found that steel factor specifi-

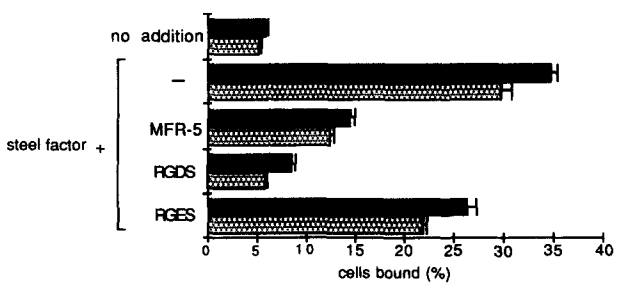

Fig 3. Adhesion to (■) intact fibronectin or (□) its 120-kD fragment was stimulated with steel factor in the presence of MFR-5 or of 1.5 mmol/L of RGDS or RGES peptide.

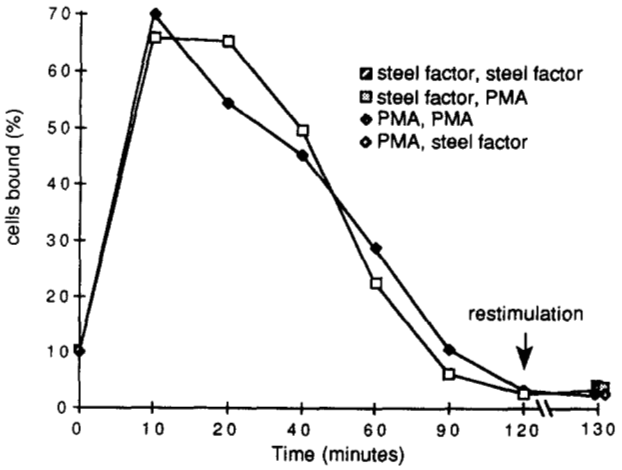

Fig 4. Kinetics of adhesion of mast cells stimulated with steel factor or PMA. Mast cells were stimulated with steel factor (10 U/mL) or PMA (10 ng/mL) in suspension for varying times before transfer into wells and centrifugation. The plate was then incubated for 6 minutes at 37°C before washing. Zero time indicates adhesion without stimulation. The time indicated includes all steps before washing. At 120 minutes, cells were restimulated with 100 U/mL steel factor or 10 ng/mL PMA in all four possible combinations, as indicated in the figure. The average of triplicates is shown; the SE was less than 3%.

cally stimulated mast cells to bind FN and, to a lesser extent, vitronectin. PMA also stimulated mast cells to bind FN, as previously reported.[35] Adhesiveness of mast cells to FN was stimulated transiently by both PMA and steel factor. Adhesion reached its peak within 10 minutes and declined thereafter. This transient adhesion of FN was mediated at least in part by the classical FN receptor, VLA-5, as shown by inhibition with MoAb to VLA-5 and RGD peptide. Transient adhesiveness of mast cells to FN was not accompanied by any change in the density on the mast cell surface of the VLA-5 receptor. These findings suggest that the affinity or some other qualitative change in the FN receptor is stimulated by steel factor and PMA. This "inside-out" signalling is reminiscent of the increased adhesiveness in LFA-1 stimulated by T-cell receptor cross-linking.[36] T-cell receptor cross-linking was shown to result in transient adhesiveness of LFA-1, whereas adhesiveness stimulated by PMA was stable, showing both similarities and differences between the two systems.

Steel factor was much more efficient for stimulation of FN binding than for stimulation of DNA synthesis. Although IL-3 and IL-4 are known to have mast cell growth activities,[30-32] neither of them stimulated mast cells to bind FN. Signals activating the FN receptor thus can be dissociated from growth signals. Because steel factor is 100-fold more efficient in stimulating adhesiveness than growth, regulation of adhesiveness may be more important than regulation of cell growth by steel factor in many contexts in vivo. Meininger et al[37] reported that steel factor acted as a mast cell chemoattractant at concentrations comparable to those that induced adhesion in our study. IL-3 was also potent as a mast cell chemoattractant.[38] The effects of steel factor are thus more selective in stimulating adhesion to extracellular matrix than in the chemotaxis assay.

c-kit is a member of the receptor tyrosine kinase family with five Ig domains in the extracellular region. All loss-of-function *W* mutations impair tyrosine kinase activity, suggesting that the tyrosine kinase activity of c-*kit* is crucial in the phenotype of *W* mutants.[34,39] The *Wv* allele encodes a c-*kit* protein with a single point mutation in the kinase domain. We found that mast cells from *W/Wv* mice failed to bind to FN on stimulation with steel factor, but remained responsive to PMA stimulation. However, there was no significant difference between +/+ and *W/Wv* mast cells in ability to bind COS cells transfected with full-length cDNA of steel factor.[21] These findings emphasize the importance of the tyrosine kinase domain in initiating the signal for increased integrin adhesiveness.

Steel factor binding to c-*kit* can both directly mediate adhesion and stimulate integrin adhesiveness to FN that is transient. These signals may differ, because they differ in the concentration of steel factor that is required. Steel factor

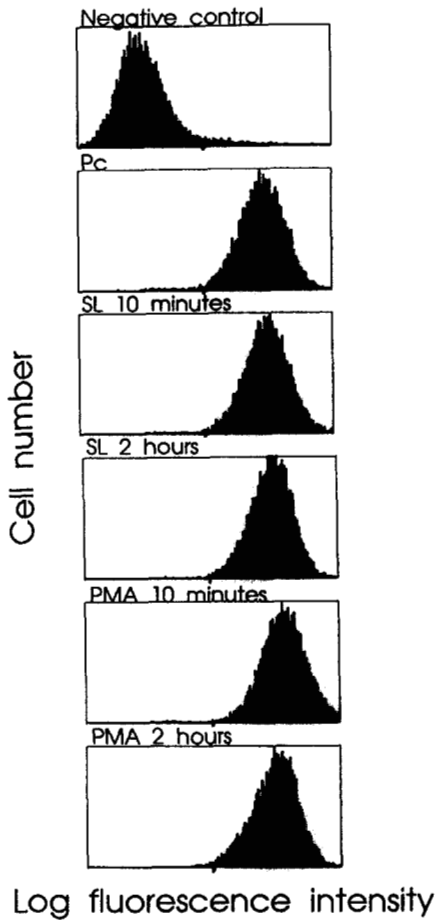

Fig 5. Expression of the FN receptor of mast cells before and after stimulation with steel factor or PMA. Mast cells were stained with MFR-5 MoAb followed by FITC-labeled antirat IgG before (0 minutes) or at 10 minutes and/or 2 hours after stimulation with steel factor (10 U/mL) (SL) or PMA (10 ng/mL), or cells were stained only with FITC-labeled antirat IgG (negative control) and subjected to fluorescence flow cytometry.

stimulation of integrin adhesiveness appears particularly relevant to the effect of the *W* and *Sl* mutations on migration of several cell lineages in vivo; adhesiveness is required for cell migration and this regulation of adhesiveness is thought to be closely coordinated with cell migration.[40,41] It was suggested that cell-cell adhesion through membrane-anchored steel factor and c-*kit* could be important for cell migration.[21] However, the crucial requirement of the tyrosine kinase activity of c-*kit* for the stimulation of integrin adhesiveness is consistent with the strong effect of kinase-defective *W* mutations on cell migration. By contrast, we found that *W/Wv* mast cells were fully competent in direct binding of c-*kit* to steel factor, showing that this does not require the kinase activity. Although we have used soluble steel factor in our experiments, both soluble and membrane-bound steel factors may be important in stimulating integrin adhesiveness in vivo. In situ analysis of steel factor mRNA[42] shows that steel factor is expressed in tissues associated with the migratory pathways and homing sites of melanoblasts, germ cells, and hematopoietic stem cells. FN is essential in migration of cell types, including those derived from the neural crest[22]; however, FN is much more widely distributed than steel factor. The localized distribution of steel factor may therefore be important in guidance of cells along the migratory pathway.

Our studies have shown that a receptor tyrosine kinase can regulate adhesion at concentrations of ligand lower than those required for regulation of growth control. Whereas receptor tyrosine kinases are generally thought to be important in development for regulation of cell growth or differentiation, our study on c-*kit* suggests an alternative mechanism of regulation of cell adhesion.

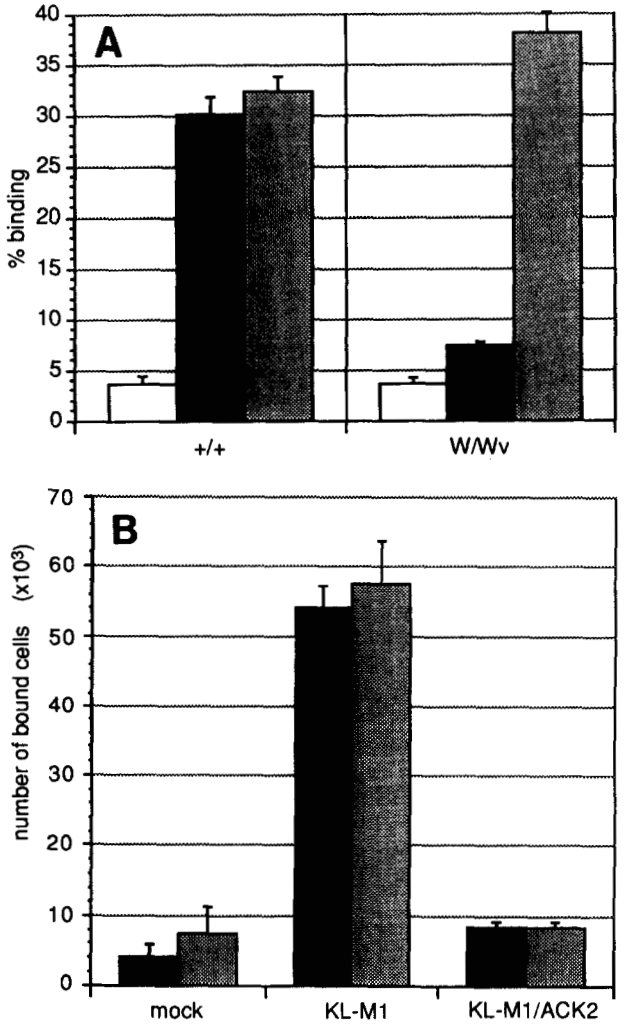

Fig 6. Effect of c-*kit* tyrosine kinase mutation on adhesion to FN (A) or steel factor on the cell surface (B). (A) Mast cells from +/+ and *W/Wv* mice were incubated with no addition (□), steel factor (■), or PMA (▨), and assay for adhesion to FN was performed as described in Fig 2. (B) Adhesion of +/+ and *W/Wv* mast cells was assayed to COS transfected with a vector alone (mock), full-length steel factor cDNA (KL-M1), or full-length steel factor cDNA in the presence of anti–c-*kit* antibody, ACK2 (KL-M1/ACK2).

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