Genetic Analysis Reveals Cell Type-specific Regulation of Receptor Tyrosine Kinase c-Kit by the Protein Tyrosine Phosphatase SHP1

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Summary

Receptor protein tyrosine kinases (RTKs) transmit downstream signals via interactions with secondary signaling molecules containing SH2 domains. Although many SH2-phosphotyrosyl interactions have been defined in vitro, little is known about the physiological significance of specific RTK/SH2 interactions in vivo. Also, little is known about the mechanisms by which specific RTKs interact with and/or are regulated by specific protein tyrosine phosphatases (PTPs).

To address such issues, we carried out a genetic analysis of the previously reported biochemical interaction between the RTK c-Kit, encoded at the *W* locus, and the SH2-containing non-transmembrane PTP SHP1, encoded at the *motheaten* (*me*) locus (1). Mice carrying a kinase-defective allele of c-Kit ($W^v/+$) were crossed with *me*/+ mice, which carry one effectively null allele of SHP1, and then backcrossed to generate all possible allelic combinations.

Our results indicate strong intergenic complementation between these loci in hematopoietic progenitor cells. Compared to progenitors purified from normal mice, bone marrow progenitor cells (lin^-) from *me/me* mice markedly hyper-proliferated in response to Kit ligand (KL) stimulation. Superimposition of the *me/me* genotype increased the number of bone marrow–derived CFU-E from $W^v/+$ mice. Conversely, the presence of one or two copies of W^v decreased the number of macrophages and granulocytes in *me/me* lung, skin, peripheral blood and bone marrow, thereby decreasing the severity of the *me/me* phenotype. The decrease in dermal mast cells in W^v/W^v mice was rescued to levels found in $W^v/+$ mice by superimposition of the *me/me* genotype. Surprisingly, however, the presence or absence of SHP1 had no effect on the proliferative response of bone marrow-derived cultured mast cells to KL or IL3 ex vivo. Nevertheless, the immediate-early response to KL stimulation, as measured by KL-induced tyrosyl phosphorylation, was substantially increased in mast cells from $W^v/+$:*me/me* compared to $W^v/+$:+/+ mice, strongly suggesting that SHP1 directly dephosphorylates and regulates c-Kit. Taken together, our results establish that SHP1 negatively regulates signaling from c-Kit in vivo, but in a cell type-specific manner.

The phosphorylation of proteins on tyrosyl residues is a key mechanism for regulating proliferation and differentiation of hematopoietic cells. The steady state level of phosphotyrosine on any protein is determined by the opposing actions of protein tyrosine kinases (PTKs)[1] and protein tyrosine phosphatases (PTPs). Two types of receptors evoke tyrosyl phosphorylation events to transduce signals generated by polypeptide growth and differentiation factors. Many growth factors signal via receptor tyrosine kinases (RTKs)(2, 3), which possess intrinsic tyrosine kinase activity. In contrast, most cytokine receptors, such as the IL3 receptor, lack intrinsic enzymatic activity but associate with non-transmembrane (non-TM) PTKs (4, 5).

Upon ligand binding, RTKs oligomerize, become acti-

[1] *Abbreviations used in this paper:* BMCMC, bone marrow-derived cultured mast cells; KL, Kit ligand; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatases; RTK, receptor protein tyrosine kinase; WBC, white blood count.

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vated and trans-phosphorylate on multiple tyrosyl residues. Analogously, cytokine receptor stimulation leads to the activation of the associated non-TM PTK(s), which phosphorylate themselves and the cognate receptor. Downstream signals from RTKs and cytokine receptors are transmitted via interactions of receptor phosphotyrosyl residues with secondary signaling molecules containing SH2 domains (6–8).

Although many SH2-phosphotyrosyl interactions have been defined in vitro and in tissue culture cells (i.e., ex vivo), little is known about the biological significance of these interactions in vivo. Experiments in lower eukaryotes provide the only clear examples in which the physiological importance of an interaction between a specific SH2-containing protein and a putative phosphotyrosyl target has been established. Molecular genetic analysis of the *let*-23 pathway in *C. elegans* indicated that *sem*-5, a Grb2 homologue, is required for activation of the *ras* homologue *let*-60 (9). Analogous experiments in *Drosophila* established the importance of the *sem*-5/Grb2 homologue *drk* (10), as well as the guanine nucleotide exchange factor *sos*, in *ras* activation by the *sevenless* (11) and *torso* (12) RTKs. Similar protein–protein interactions exist in mammalian cells, but the function(s) and importance of these complexes in specific cell types in vivo have not been established.

Even less is known about the mechanism(s) by which signals induced by growth and differentiation factors are terminated. Dephosphorylation of phosphotyrosyl protein targets, as well as other mechanisms such as receptor endocytosis, could play important roles in signal termination. However, the relative importance of such processes in terminating specific signaling pathways is largely undefined. Likewise, for those pathways in which tyrosyl dephosphorylation is believed to be important for negative regulation, the specific PTP(s) that control inactivation remain largely unidentified.

We have evaluated the biological significance of the interaction between a specific RTK, c-Kit, and a specific PTP, SHP1, by adopting a molecular genetic approach. The c-*kit* proto-oncogene encodes a classical RTK, which serves as the receptor for Kit ligand (KL) (also known as Steel factor, stem cell factor, or mast cell growth factor) (13–18). c-Kit function has been implicated in the proliferation, survival, migration, and differentiation of germ cells, neural crest-derived cells (e.g., melanocytes), and hematopoietic stem cells, as well as in cell type-specific functions (e.g., chemotaxis, secretion) in more differentiated cells (19). SHP1 (20), formerly known as SHPTP1 (21), PTP1C (22), HCP (23), or SHP (24), is a non-transmembrane SH2 domain-containing PTP expressed primarily in hematopoietic cells of all lineages and all stages of maturation. Previous biochemical studies established that, after KL stimulation of factor-dependent Mo7e cells, SHP1 binds to tyrosyl phosphorylated c-Kit and itself becomes tyrosyl phosphorylated (1). Since SHP1 has been found to negatively regulate several other receptors of the hematopoietic system (EPOR [25], IFNα [26], TCR [27, 28], CSF1R [29]), it seemed likely that it might be important for c-Kit inactivation. However, the functional significance of the c-Kit/SHP1 interaction has not yet been established, either in cell lines or in whole animals.

To assess the relevance of c-Kit/SHP1 interactions, we took advantage of mouse models of c-Kit and SHP1 deficiency. In mice, c-Kit is encoded at the dominant *White Spotting* (*W*) locus. Several different mutations affecting the c-*kit* gene have been described (30). Depending on the severity of c-Kit impairment caused by these mutations, *W* mutant mice show varying degrees of several phenotypes: (*a*) change of black coat color to white because of deficiency of neural crest cell migration, which results in impaired melanocyte development; (*b*) sterility; (*c*) macrocytic anemia resulting from decreased number of erythroid progenitor cells and impaired erythroid differentiation; and (*d*) deficient mast cell production in vivo and decreased or absent proliferation of bone marrow-derived cultured mast cells in response to KL ex vivo.

Mutations at the SHP1 locus cause the *motheaten* (*me*) phenotype (31, 32). The *me* allele encodes a frameshift mutation near the 5′-end of the SHP1 coding sequence, which results in the absence of detectable SHP1 protein. Thus, *me/me* mice are effectively SHP1 nulls. These mice display a panoply of disorders affecting virtually all hematopoietic lineages and die within two to three weeks after birth (33). Prominent amongst these defects are myeloid hyper-proliferation and inappropriate activation, which leads to the characteristic "motheaten" skin lesions and the interstitial pneumonia that causes their early demise. Although their erythroid progenitor cells (CFU-E) are intrinsically hyper-responsive to EPO stimulation, *motheaten* mice are anemic. Anemia in *me/me* mice probably results from a combination of factors, including the displacement of erythroid progenitor cells from the bone marrow (to the spleen) as a consequence of the markedly expanded population of immature and mature myeloid cells (myelopthisis), as well as hypersplenism. Coat color is normal in *me/me* mice, whereas the status of mast cells has not been studied rigorously.

We examined the effect of the *me/me* background on c-Kit-responsive cell types in mice bearing normal and *White viable* (W^v) c-Kit alleles, as well as the effects of the W^v mutation on several aspects of the *me/me* phenotype. To accomplish this, we crossed mice heterozygotic for *me* with mice heterozygotic for W^v. We chose the W^v allele because, owing to a point mutation within its kinase domain, W^v has markedly diminished, but importantly, still detectable c-Kit kinase activity (30, 34). We reasoned that if there were a biologically important interaction between c-Kit and SHP1, mice carrying mutations at both loci should manifest genetic interaction; i.e., W^v and *me* might exhibit intergenic complementation. We found that c-Kit and SHP1 do show genetic interaction, but in a tissue-restricted manner. There was strong intergenic complementation in the hematopoietic progenitor cells of $W^v/+$:*me/me* and W^v/W^v:*me/me* mice. The presence of one and especially two copies of W^v decreases the severity of the *me/me* phenotype, as manifested by fewer skin lesions and delayed on-

set and decreased severity of interstitial pneumonia. Lack of SHP1 improves *ex vivo* erythropoiesis in the $W^v/+$ background, enhances the proliferative response of bone marrow-derived progenitor cells to KL, and increases ("rescues") the numbers of dermal mast cells in the ears of W^v/W^v mice. However, we detected no genetic interaction between W^v and *me* in the melanocyte lineage in vivo or in bone marrow-derived cultured mast cells (BMCMC) ex vivo. Nevertheless, biochemical analysis of KL-induced signaling events in BMCMC from mice bearing different combinations of c-Kit and SHP1 alleles provides direct evidence for negative regulation of the c-Kit pathway by SHP1. Our results are consistent with a model in which SHP1 is a critical negative regulator of c-Kit signaling in some, but not all, cell types.

Materials and Methods

Mice. F1 (combined heterozygotic $W^v/+$:*me*/+) mice were generated by mating heterozygotic $W^v/+$ (C57BL/6J-lx W^v; Jackson Labs, Bar Harbor, ME) with heterozygotic *me*/+ (C3HeB/FeJle-a/a-*me*; Jackson Labs, Bar Harbor, ME) mice. F2 mice, carrying all possible combinations of mutations at the W^v and *me* loci, were generated by mating of combined heterozygotic mice. Genotypes at the *W* locus were identified by coat color, whereas genotypes at the *me* locus were determined by polymerase chain reaction (PCR), using primers described previously (31). Based on initial studies, which showed no differences between +/+ and me/+ mice, both were used as "normal" controls, indicated as +/+(*me*). 14–18-d-old mice were used throughout the study.

Primary BMCMC. Mice were euthanized by inhalation of CO_2 before femurs and tibias were flushed with PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl; pH 7.4). Bone marrow cells were washed twice with PBS and then resuspended at 5×10^5–5×10^6/ml in BMCMC medium (RPMI-1640, 10% bovine calf serum, 15% WEHI 3-conditioned medium (source of IL3), 0.1 mM MEM non-essential amino acid solution (GIBCO BRL, Gaithersburg, MD), 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, and antibiotics (Penicillin plus Streptomycin; GIBCO BRL). BMCMC were isolated by continuous transfer of cells growing in suspension to new flasks for 4–6 wk. Homogeneity of BMCMC was confirmed by May-Gruenwald/Giemsa staining (35).

Histology and Morphometry. Tissues from euthanized mice were fixed in 10% neutral-buffered formalin. Cardiac blood was taken immediately after euthanasia for hematological analysis. Tissue samples were embedded in paraffin, cut at 5 μm, and stained with hematoxylin-eosin for histological evaluation. Samples of ear skin and back skin were obtained and processed for 1-μm Epon-embedded, Giemsa-stained sections (36, 37). Sections were coded and then were examined by observers who were unaware of their identities. The number of mast cells per square millimeter of dermis in each specimen was determined as described previously (38, 39).

Colony Forming Assays. Femurs were flushed with RPMI-1640, 10% fetal bovine serum (FBS)(RPMI, 10%). Bone marrow cells were washed, resuspended in 10 ml RPMI, 10% per mouse, and twice plated for 1 h at 37°C onto 100 mm plates to deplete adherent macrophages. 10^5 non-adherent cells were resuspended in 3 ml colony assay medium (IMDM [GIBCO BRL], 50% methyl cellulose, 25% FBS, 10% bovine serum albumin [BSA], 5×10^{-5} M β-mercaptoethanol, 2 U/ml erythropoietin [Amgen, Thousand Oaks, CA]), supplemented with the indicated concentrations of KL (recombinant mouse KL; Genzyme, Boston, MA) or IL3 (BioSource International, Camarillo, CA). The cell mixture was plated in three wells, containing 1 ml each. Colonies were scored after 6–7 d. Experiments shown represent one of at least three independent experiments.

Purification of lin⁻ Bone Marrow Progenitor Cells and Flow Cytometric Analysis. Bone marrow cells were purified and depleted of adherent macrophages as described above (see colony forming assays). Red blood cells were lysed for 5 min on ice in 0.15 M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$. The remaining cells were washed and resuspended in PBS, 2% FBS (1 ml/mouse) and 0.2 μg/10^6 cells of the following antibodies (all purchased from PharMingen, San Diego, CA) were added for 20 min on ice: Ter119, CD4, CD8, B220, GR1, Mac1. Cells were washed and 1 ml/ 2.5×10^7 cells of sheep anti–rat IgG coupled to Dynabeads M450 (Dynal, Lake Success, NY) was added for 20 min at 4°C. After two rounds of exposure to a magnetic field, lin⁻ cells were isolated. Lin⁻ bone marrow progenitor cells (2×10^5) were stained with anti-c-Kit antibody (CD117), precoupled to FITC (purchased from PharMingen), in PBS, 3% BSA and 0.1% sodium azide. Stained progenitor cells (5,000) were analyzed by flow cytometry using a FACScan® instrument (Becton Dickinson, Bedford, MA).

Proliferation Assays. BMCMC (2×10^4/well/200 μl medium) or lin⁻ bone marrow progenitor cells (1.4×10^4/well/200 μl medium) were plated in triplicate in BMCMC medium without WEHI 3-conditioned medium, supplemented with the indicated amount of KL or IL3 and incubated for 60 h. They were then pulsed with 1 μCi of [^{3}H]thymidine for 4 h, harvested, and [^{3}H]thymidine incorporation was measured using a cell-harvester (Skatron Instruments, Sterling, VA).

Immunoprecipitations and Immunoblotting. Cells lysates were prepared from primary human melanocytes (Clonetics) and from primary BMCMC. Some BMCMC cultures were stimulated with KL (500 ng /2 × 10^7 cells/ml) for the indicated times before lysis. Cells (4×10^7/ml) were lysed in NP-40 buffer (0.5% Nonidet P-40 [NP-40], 150 mM NaCl, 50 mM Tris-HCl [pH 7.6], 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 mM NaF, leupeptin [10 μg/ml], aprotinin [1 μg/ml], pepstatin A [1 μg/ml], antipain [1 μg/ml], and phenylmethylsulfonyl fluoride [20 μg/ml]). Lysates were clarified by centrifugation for 20 min at 100,000 *g* at 4°C, and immunoprecipitations with anti-phosphotyrosine agarose (30 μl of 50% suspension PT66 coupled to agarose; Sigma Chem. Co., St. Louis, MO) or with anti-SHP1 antibodies (40) were performed on cleared lysates. Immune complexes were washed five times in NP-40 buffer. Total cell lysates and immune complexes were resolved by SDS-PAGE and electroblotted onto Immobilon P. Immunoblotting was performed as described previously (40), using the following antibodies (with concentrations recommended by the manufacturers): monoclonal anti-SHP1 (Transduction Laboratories, Lexington, KY), monoclonal anti-phosphotyrosine 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.), polyclonal anti-MAPK (generous gift of Dr. J. Blenis) and anti-phospho-specific MAPK (New England Biolabs; Beverly, MA). Immunoblots were developed using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Results

To examine the potential role of SHP1 in c-Kit signaling, we adopted a genetic approach. We reasoned that if

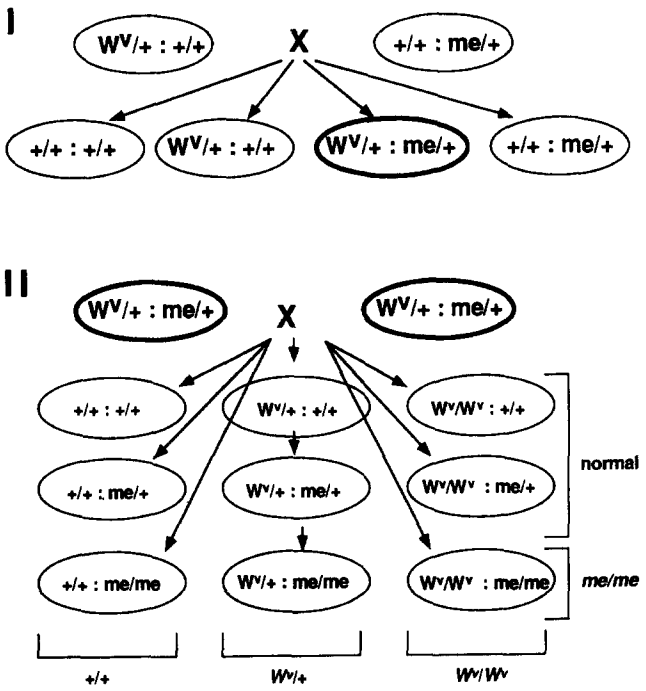


Figure 1. Schematic representation of strategy used to generate F1 (*I*) and F2 (*II*) mice with different combinations of mutations at *W* and *me* loci. The first ten litters showed the following distribution of F2 genotypes: 23 +/+:+/+(*me*); 9 +/+:*me/me*; 40 +/W^v:+/+(*me*); 13 +/W^v:*me/me*; 15 W^v/W^v:+/+(*me*); 7 W^v/W^v:*me/me*.

SHP1 negatively regulated c-Kit in vivo, loss of SHP1 activity (in *me/me* mice) might lead to hyper-responsiveness of KL-sensitive cell types. Moreover, since W^v retains some kinase activity, superimposing partial or complete loss of SHP1 (on W^v/+ or W^v/W^v mice) might to some extent rescue defective c-Kit function. Conversely, impaired c-Kit function might "normalize" certain aspects of the *me/me* phenotype. An *in vivo* approach also might have the advantage of revealing whether any of the observed effects were cell type/tissue-specific. Therefore, we analyzed the progeny of crosses between *me*/+ and W^v/+ mice. F1 double heterozygotic mice (W^v/+:*me*/+) were obtained and crossed with each other to generate an F2 population with all nine possible genotypes (Fig. 1), which resulted in six different phenotypes (see Materials and Methods).

W^v Reduces the Severity of the me/me Phenotype, but me/me Does Not Alter the Effect of W^v on Melanocyte Development. All combinations of the two mutations were viable, and the F2 generation showed the expected distribution of the different genotypes (Fig. 1). The presence or absence of the *me* allele had no obvious consequence on the effects of the *c-kit* locus on coat color phenotype (Fig. 2, *A–C*). Earlier studies had not established whether SHP1 is expressed in normal neural crest-derived cell types, although it has been reported that SHP1 is expressed in PC12 cells, a rat pheochromocytoma-derived cell line (41). Consistent with the lack of genetic interaction between *me* and W^v for coat color determination, immunoblotting revealed no detectable expression of SHP1 in primary melanocytes (Fig. 2 *D*). These results suggest that SHP1 is not a limiting negative regulator of c-Kit in melanocytes nor, most likely, in less differentiated neural crest cells. W^v/W^v and *me/me* mice are sterile. We did not address the question of whether W^v/W^v:*me/me* mice were sterile because most of these mice died prior to, or just at the time of, reaching sexual maturity.

White (i.e., W^v/W^v) *me/me* mice appeared to have markedly fewer skin lesions than their black (i.e., c-Kit +/+) *me/me* littermates. Moreover, white *me/me* mice appeared healthier at younger ages (2–3 wk) and tended to live longer than their black *me/me* littermates. Although we did not maintain large numbers of W^v/W^v:*me/me* or +/+:*me/me* mice until their natural deaths, at least 7 W^v/W^v:*me/me* lived 6–7 wk, whereas none of the +/+:*me/me* mice obtained in this cross or in our much larger *me*/+ breeding stock ever reached this age. These clinical/morphological observations were confirmed by histological analysis. At two to three weeks of age, *me/me* mice exhibited the characteristic extensive infiltrates of granulocytes and macrophages in their skin, whereas double homozygotic (W^v/W^v:*me/me*) mice had both markedly fewer and less severe dermal lesions (Fig. 3, *A–H*). Similarly, age-matched W^v/W^v:*me/me* mice had minimal or absent myeloid cell infiltration in their lungs, resulting in less severe interstitial pneumonia than was exhibited by their littermate +/+:*me/me* mice (Fig. 3, *G–H*).

SHP1 Negatively Regulates c-Kit in Hematopoietic Progenitor Cells. The results described above suggested that a marked reduction of c-Kit activity can reduce the severity of the *me/me* phenotype in vivo. Further support for this conclusion was obtained by analysis of peripheral blood counts (Table 1). The dramatically elevated white blood count (WBC) in *me/me* mice was reduced to approximately normal levels in W^v/W^v:*me/me* or W^v/+:*me/me* mice. However, the macrocytic anemia caused by the W^v allele was not improved by loss of SHP1. There are several possible reasons why loss of SHP1 might not improve the anemia of *White viable* mice: (*a*) SHP1 might not regulate c-Kit signaling in erythroid progenitor cells; (*b*) SHP1 might regulate c-Kit in erythroid progenitor cells, but loss of SHP1 might not be sufficient to "rescue" the W^v mutation; or (*c*) SHP1 might regulate c-Kit in erythroid progenitor cells, but since *me/me* mice are themselves anemic, the combination of the W^v and *me/me* mutations might not result in restoration of a normal hemoglobin/hematocrit (33, 42).

To distinguish between these possibilities, we examined the differentiation/proliferation capacity of bone marrow-derived hematopoietic progenitor cells in a more controlled environment by using ex vivo colony forming assays. Macrophage-depleted bone marrow cells from normal and *me/me* mice were tested for their abilities to form erythroid (BFU-E) and myeloid (CFU-GM) colonies in response to increasing amounts of KL (Fig. 4). Compared to normal littermates, bone marrow from *me/me* mice gave rise to increased numbers of BFU-E at all doses of KL tested (Fig. 4 *A*). KL is not typically viewed as a growth

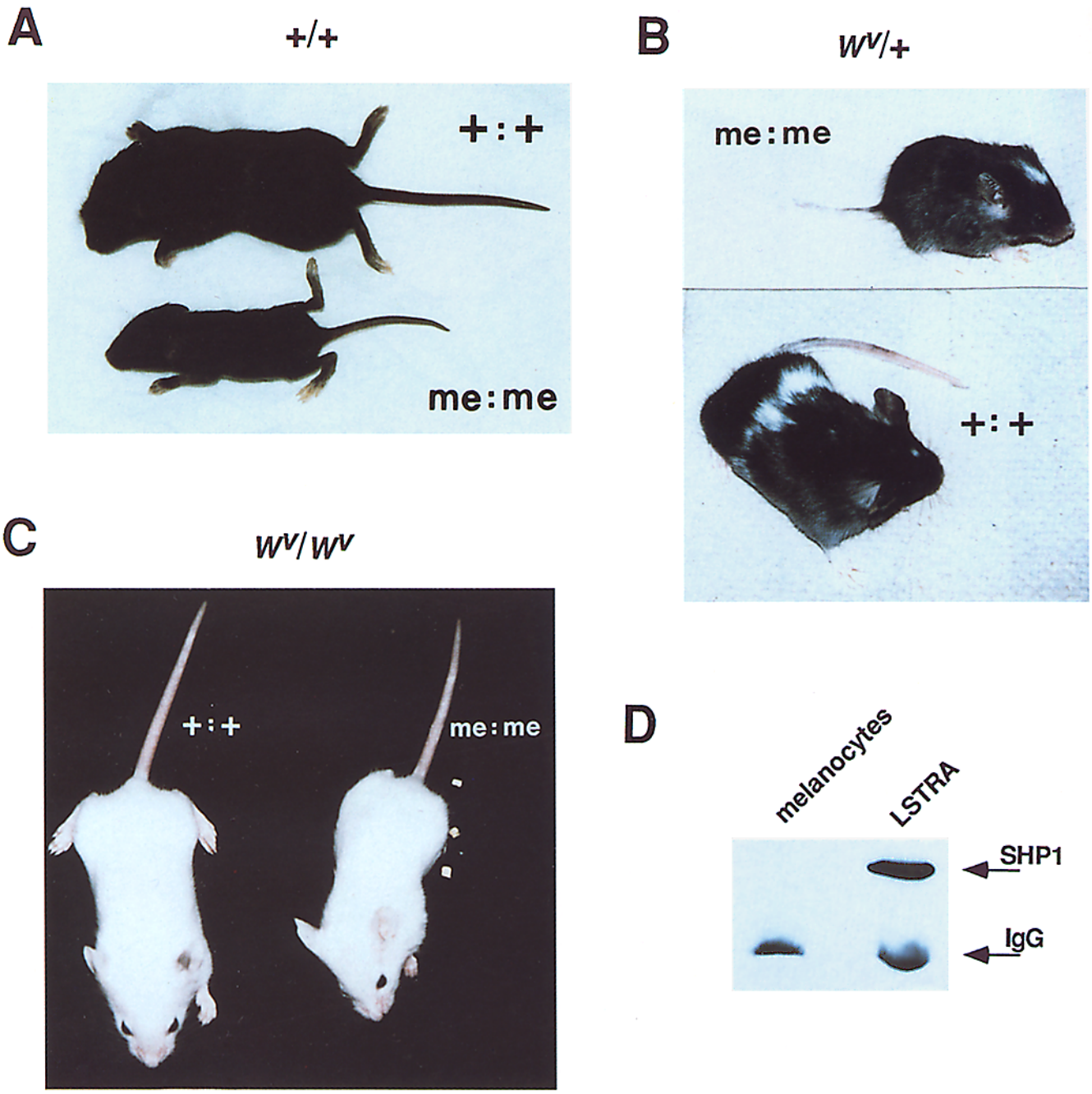
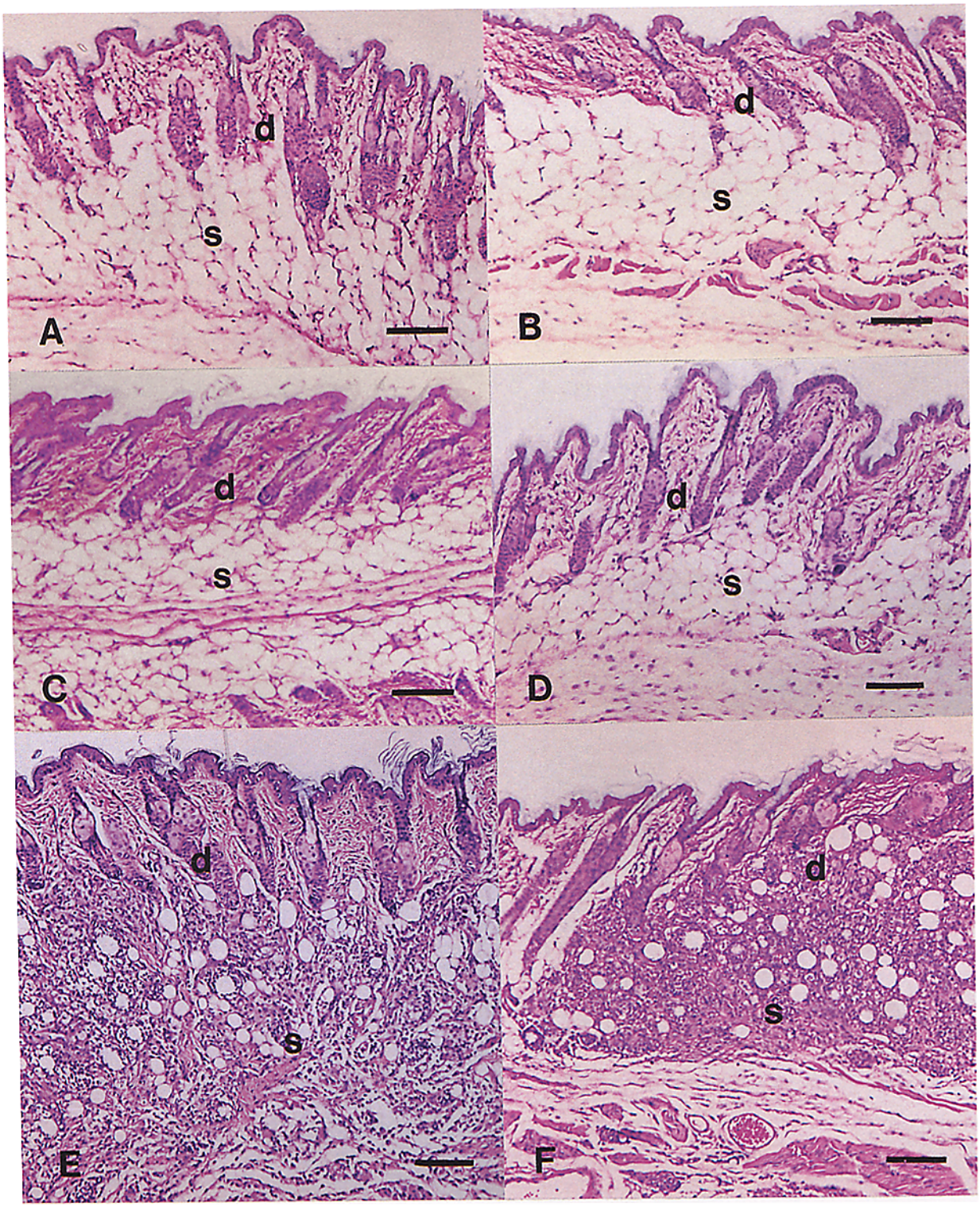


Figure 2. (*A–C*). Photographs of F2 generation mice depicting the six skin and coat color phenotypes. (*A*) +/+:+/+ and +/+:*me/me*, (*B*) W^v/+:+/+ and W^v/+:*me/me*, (*C*) W^v/W^v:+/+ and W^v/W^v:*me/me*. (*D*) SHP1 is not expressed in melanocytes. SHP1 was immunoprecipitated from primary cultures of human dermal melanocytes (10^7)(Clonetics, San Francisco, CA) or LSTRA cells (10^7), which express abundant amounts of SHP1 (40). After SDS-PAGE (8% gel), SHP1 was detected by immunoblotting with anti-SHP1 antibodies.

factor that directs myeloid cell differentiation. However, at high doses or in synergy with granulocyte macrophage-colony stimulating factor, KL can stimulate low levels of CFU-GM production (43). In *me/me* mice, KL was more effective at promoting CFU-GM (Fig. 4 *B*). Notably, if we extensively depleted the large excess of macrophages and other adherent cells from the bone marrow of *me/me* mice, we did not observe significant numbers of either BFU-E or CFU-GM in the absence of added KL (or other growth factors; data not shown), arguing that hematopoietic progenitor cells from *me/me* mice, like those from normal mice, are not capable of autocrine colony formation.

Although other explanations are not formally excluded (see below), these data are consistent with the hypothesis that SHP1 negatively regulates c-Kit signal transduction pathway(s) that control erythropoiesis and that may poten-



tiate myelopoiesis. Further support for this notion was provided by analysis of compound mutants. Bone marrow cells from W^v/W^v mice could not be stimulated by KL to produce BFU-E or CFU-GM regardless of the alleles at the SHP1 locus. However, a significantly increased number of KL-evoked BFU-E was obtained from $W^v/+$:*me*/*me*, compared with $W^v/+$:+/+(*me*) mice (Fig. 4 *C*). These results suggest that loss of SHP1 is not sufficient to rescue the virtually absent kinase activity of c-Kit in W^v/W^v mice, but can increase significantly the effect of the residual c-Kit activity present in $W^v/+$ mice.

Taken together with previous biochemical studies show-

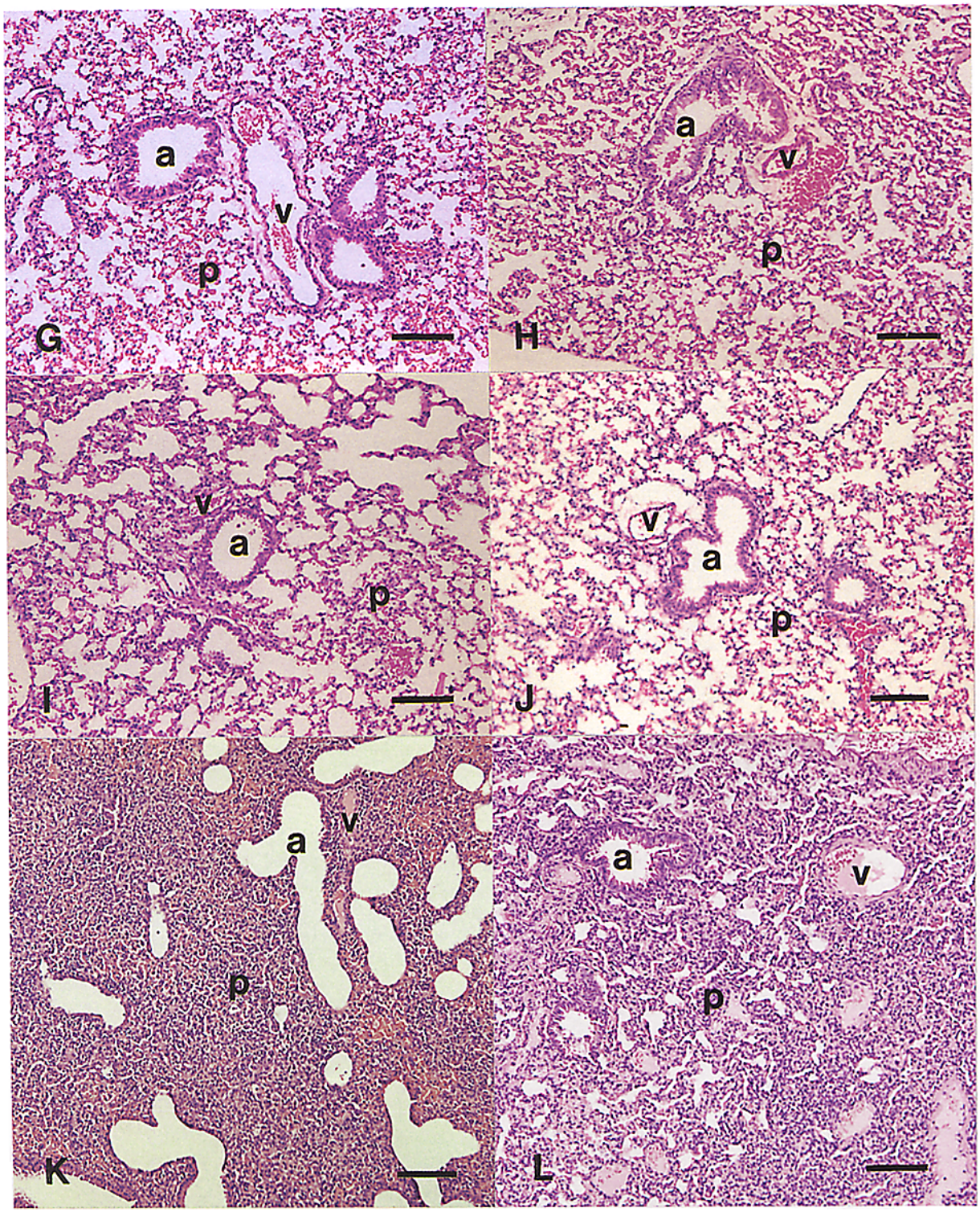


Figure 3. Sections of lung and skin of mice with different combinations of mutations at *W* and *me* loci. *A-F* show sections of skin, *G–L* show sections of lung , stained with hematoxylin-eosin; bars = 115 μm. (*A, G*) W^v/W^v:+/+(*me*), (*B, H*) W^v/+:+/+(*me*), (*C, I*) W^v/W^v:*me/me*, (*D, J*) +/+:+/+(me), (*E, K*) +/+:*me/me*, (*F, L*) W^v/+:*me/me*. (*A–F*) Skins from the W^v/W^v:+/+(*me*)(*A*), W^v/+:+/+(*me*)(*B*), W^v/W^v:*me/me* (*C*), and +/+:+/+(*me*)(*D*) were normal. No histological alterations could be seen in the dermis (*d*) or subcutis (*s*), whereas the skins from the +/+:*me/me* (*E*), and W^v/+:*me/me* (*F*) mice had a diffuse and localized inflammatory cell infiltration in the dermis and subcutis with a marked effacement of the dermal and subcutaneous architecture. (*G–L*) Lungs from W^v/W^v:+/+(*me*)(*G*), W^v/+:+/+(*me*)(*H*), W^v/W^v:*me/me* (*I*), and +/+:+/+(*me*)(*J*) were normal. No histological alterations could be seen in the alveolar parenchyma (*p*), around airways (*a*), or in blood vessels (*v*), whereas lungs from the +/+:*me/me* (*K*), and W^v/+:*me/me* (*L*) mice had a diffuse inflammatory cell infiltration in the alveolar parenchyma, around airways, and in blood vessels, in particular in the +/+:*me/me* (*K*) mice.

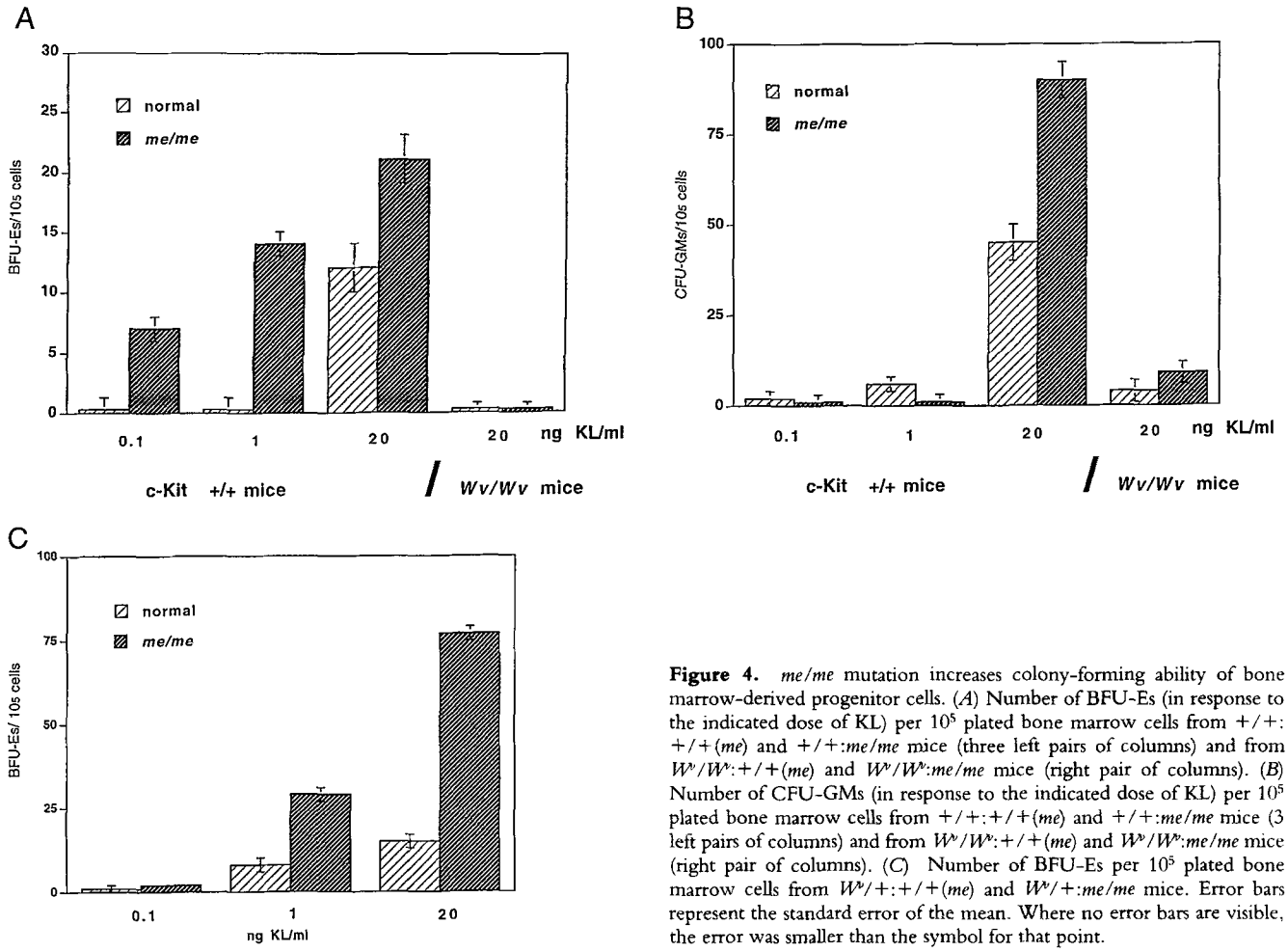


Figure 4. *me/me* mutation increases colony-forming ability of bone marrow-derived progenitor cells. (*A*) Number of BFU-Es (in response to the indicated dose of KL) per 10^5 plated bone marrow cells from +/+:+/+(*me*) and +/+:*me/me* mice (three left pairs of columns) and from W^v/W^v:+/+(*me*) and W^v/W^v:*me/me* mice (right pair of columns). (*B*) Number of CFU-GMs (in response to the indicated dose of KL) per 10^5 plated bone marrow cells from +/+:+/+(*me*) and +/+:*me/me* mice (3 left pairs of columns) and from W^v/W^v:+/+(*me*) and W^v/W^v:*me/me* mice (right pair of columns). (*C*) Number of BFU-Es per 10^5 plated bone marrow cells from W^v/+:+/+(*me*) and W^v/+:*me/me* mice. Error bars represent the standard error of the mean. Where no error bars are visible, the error was smaller than the symbol for that point.

ing that SHP1 binds directly to c-Kit, these genetic data are consistent with the hypothesis that SHP1 is a critical negative regulator of c-Kit signaling in hematopoeitic progenitor cells. However, there are other possibilities. The response to KL is dependent on the level of c-Kit surface expression as well as its enzymatic activity. Therefore, we investigated whether the *me* mutation affects c-Kit expression levels. Early (lineage-negative, lin^-) hematopoietic progenitor cells from normal and *me/me* littermates were isolated by negative selection (see Materials and Methods) and analyzed for c-Kit surface expression by flow cytometry. Three sub-populations could be identified based on their relative c-Kit expression levels: c-$Kit^{neg.}$, c-Kit^{low}, and c-Kit^{high} (Fig. 5). Although there were no major differences in the level of c-Kit expression between normal and *me/me* bone marrow progenitor cells (Table 2), there was a slight

Table 1. *Hematological Parameters of Mice with Different Allelic Combinations at W and me loci*

Genotype: c-kit: *me*	+:+ +:+(*me*)	+:+ *me:me*	W^v:+ +:+(*me*)	W^v:+ *me:me*	W^v:W^v +:+(*me*)	W^v:W^v *me:me*
WBC ($\times$ mm^{-3})	10.7 ± 3.8	32.3 ± 13.6	7.2 ± 2.8	15.6 ± 2.3	8.8 ± 3.6	12.0 ± 6.3
RBC ($\times$ 10^{-6} mm^{-3})	5.8 ± 0.4	3.9 ± 0.4	4.3 ± 0.2	3.6 ± 0.3	3.3 ± 0.3	2.8 ± 0.3
Hgb (g/dl)	12.0 ± 0.3	9.2 ± 1.3	9.5 ± 0.4	8.5 ± 0.4	8.6 ± 0.6	7.6 ± 0.6
MCV	58.3 ± 3.3	66.8 ± 3.9	63.7 ± 2.0	69.4 ± 3.1	76.3 ± 2.6	74.3 ± 6.6

WBC, white blood count; RBC, red blood count; Hgb, hemoglobin concentration; MCV, mean corpuscular volume.

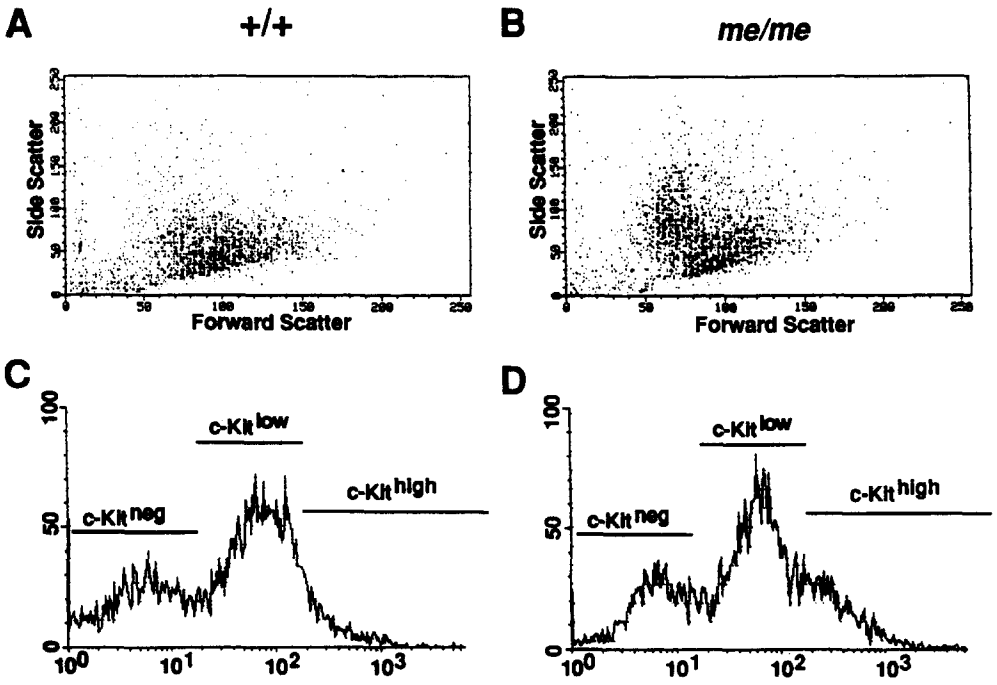


Figure 5. Flow cytometric analysis of normal and *me/me* lin$^-$ bone marrow progenitor cells. (*A, B*) Dot plots displaying forward versus side scatter of lin$^-$ bone marrow progenitor cells from normal (*A*) and *me/me* (*B*) mice. (*C, D*) Histograms of c-Kit surface expression of lin$^-$ bone marrow progenitor cells from normal (*C*) and *me/me* (i) mice. Thresholds used to characterize c-Kit^{neg}, c-Kit^{low}, and c-Kit^{high} are indicated. Percentages of respective subpopulations are listed in Table 2.

increase in the relative number of c-Kit^{high} cells (14–21%) in *me/me* mice.

It has been reported that lin$^-$ c-Kit^{high} cells are enriched for erythroid progenitor cells (44). Although the increased percentage of such cells in *me/me* mice could in part account for the observed increase in BFU-Es, it is unlikely to account for the nearly ten-fold increase in BFU-E production in response to sub-saturating KL concentrations (Fig. 4 *A*; see Discussion). The increased number of BFU-E in the presence of the *me/me* mutation also may be due to the hyper-responsiveness of one or more PTK pathways that act within early hematopoeitic progenitor cells to give rise to increased numbers of c-Kit-responsive cells. Although flow cytometric analysis of lin$^-$ cells allowed us to determine reliably the relative proportion of c-Kit-positive and -negative cells (which is essentially the same in normal and *me/me* mice), this analysis does not allow accurate determination of the absolute number of c-Kit-positive cells in total bone marrow. Because lin$^-$ cells represent such a small percentage of total bone marrow cells, such an estimate would be prone to large errors.

Therefore, we evaluated whether a given number of lin$^-$ cells from *me/me* mice displayed increased sensitivity to KL compared with their normal littermates (shown by flow cytometry to contain comparable fractions of c-Kit-positive cells) by measuring their proliferative response to KL stimulation (Fig. 6). Progenitor cells from *me/me* mice showed dramatic hyper-proliferation (50–60×) in response to KL stimulation. Again, this substantially increased response cannot easily be explained solely by the ~50% increase in c-Kit^{high} cells (see above, Table 2, and Discussion). Instead, it supports the hypothesis that SHP1 is a direct negative regulator of c-Kit in hematopoietic progenitor cells.

W^v/W^v Affects Myelopoiesis in me/me Mice. We observed that *me/me* mice had a less severe "motheaten" phenotype in the presence of the W^v/W^v genotype (Figs. 2 and 3). Hyper-proliferation of the myeloid lineage is thought to be one of the major problems in *me/me* mice that results in their early death. We reported in Table 1 that W^v/W^v reduced the peripheral blood WBC of *me/me* mice to values even lower than those in normal (at the *me* locus) mice. We also assessed whether the presence of W^v/W^v could suppress increased myelopoiesis of *me/me* bone marrow progenitor cells ex vivo. We observed a dramatic increase in the number of CFU-GM in +/+:*me/me* and $W^v/+$:*me/me*, compared with +/+:+/+(*me*) and $W^v/+$:+/+(*me*) mice, respectively (Fig. 7). However, consistent with their improved phenotype, the number of CFU-GMs was reduced to almost normal levels in W^v/W^v:*me/me* mice. This result shows that c-Kit indeed contributes to the control of myeloid progenitor cell numbers. In addition, further supporting the hypothesis that SHP1 negatively regulates c-Kit function, these data suggest that deregulated (excessive) c-Kit

Table 2. *Surface Expression of c-Kit on lin$^-$ Bone Marrow Progenitor Cells from Normal [+/+(me)] and me/me Mice (two Individuals Each) as Determined by Flow Cytometry*

	c-Kit^{low}*	c-Kit^{high}*
normal 1	54%	14%
normal 2	55%	14%
me/me 1	52%	21%
me/me 2	43%	21%

*Thresholds for c-Kit^{low} and c-Kit^{high} are as shown in Fig. 5.

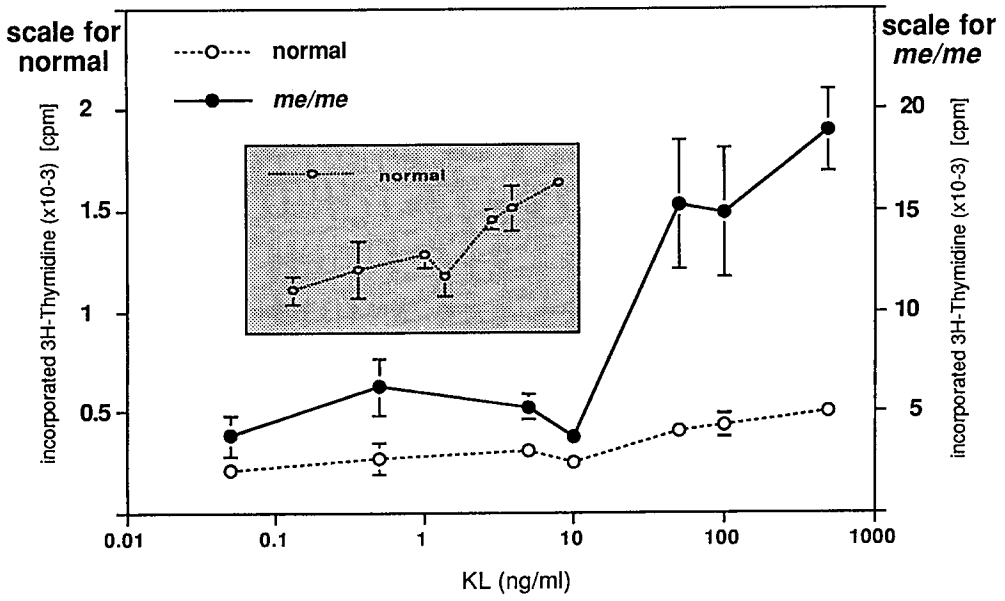


Figure 6. Hyper-proliferation of *me/me* bone marrow-derived progenitor cells in response to KL. Proliferative responses to increasing amounts of KL as measured by [^{3}H]thymidine incorporation of lin$^-$ bone marrow progenitor cells from normal (*n*) and *me/me* (*l*) mice. Note that scales are different for normal and *me/me* proliferative responses. Proliferative response of normal progenitor cells is shown in the insert to elucidate their responsiveness to KL stimulation. Error bars represent the standard error of the mean. Where no error bars are visible, the error was smaller than the symbol for that point.

activity contributes significantly to the generation of the *me/me* phenotype (see Discussion).

SHP1 Regulates Tyrosine Phosphorylation of c-Kit and Numbers of Dermal Mast Cells in W^v/W^v Mice In Vivo, but not KL-induced BMCMC Proliferation Ex Vivo. Mice carrying one or two W^v alleles display defective mast cell production (19). This is reflected by diminished numbers of dermal mast cells in vivo and by severely impaired proliferation of BMCMC in response to KL ex vivo (35). Measurements of dermal mast cell density in the back skins and ear skins of mice showed that, in the absence of SHP1, the number of dermal mast cells, particularly in the ears of W^v/W^v mice, increased (Table 3). These data are consistent with the hypothesis that loss of the negative regulator SHP1 permits the residual kinase activity of the kinase-defective c-Kit encoded by the W^v allele to rescue, at least partially, the mast cell-deficiency of W^v/W^v mice. However, it remained possible that other abnormalities present in *me/me* mice, but not intrinsic to mast cells (e.g., over-production or inappropriate production of other cytokines in the absence of SHP1) might have compensated for the deficient c-Kit activity in W^v/W^v mice.

To assess c-Kit signaling intrinsic to mast cells, we investigated whether the presence or absence of SHP1 affected the ability of BMCMC to proliferate in response to KL. In-

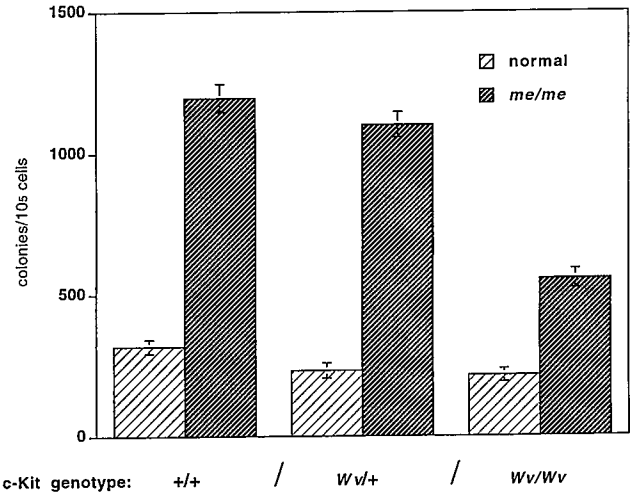


Figure 7. W^v mutation attenuates increased granulopoiesis observed in *me/me* mice. Number of CFU-GMs per 10^5 plated bone marrow cells in response to saturating amounts of IL3 (10 ng/ml) from +/+ (two left columns), $W^v/+$ (two middle columns), and W^v/W^v (two right columns) mice. Error bars represent the standard error of the mean. Where no error bars are visible, the error was smaller than the symbol for that point.

Table 3. *Mast Cell Density (Number of Mast Cells Per mm^2) of Mice Carrying Various Genotypes*

Tissue	Genotype c-Kit: SHP1	Mean ± SE	Statistics
Back Skin	+/+:++(*me*)	48.40 ± 11.05	
	+/+:*me/me*	35.61 ± 6.11	$P = 0.350$
	$W^v/+$:+/+(*me*)	44.94 ± 7.34	
	$W^v/+$:*me/me*	28.32 ± 4.12	$P = 0.096$
	W^v/W^v:+/+(*me*)	16.04 ± 3.61	
	W^v/W^v:*me/me*	21.50 ± 2.67	$P = 0.258$
Ear Skin	+/+:+/+(*me*)	77.14 ± 5.97	
	+/+:*me/me*	50.24 ± 8.68	$P = 0.063$
	$W^v/+$:+/+(*me*)	51.62 ± 10.77	
	$W^v/+$:*me/me*	53.01 ± 7.83	$P = 0.920$
	W^v/W^v:+/+(*me*)	8.86 ± 1.02	
	W^v/W^v:*me/me*	44.31 ± 13.89	$P = 0.044$

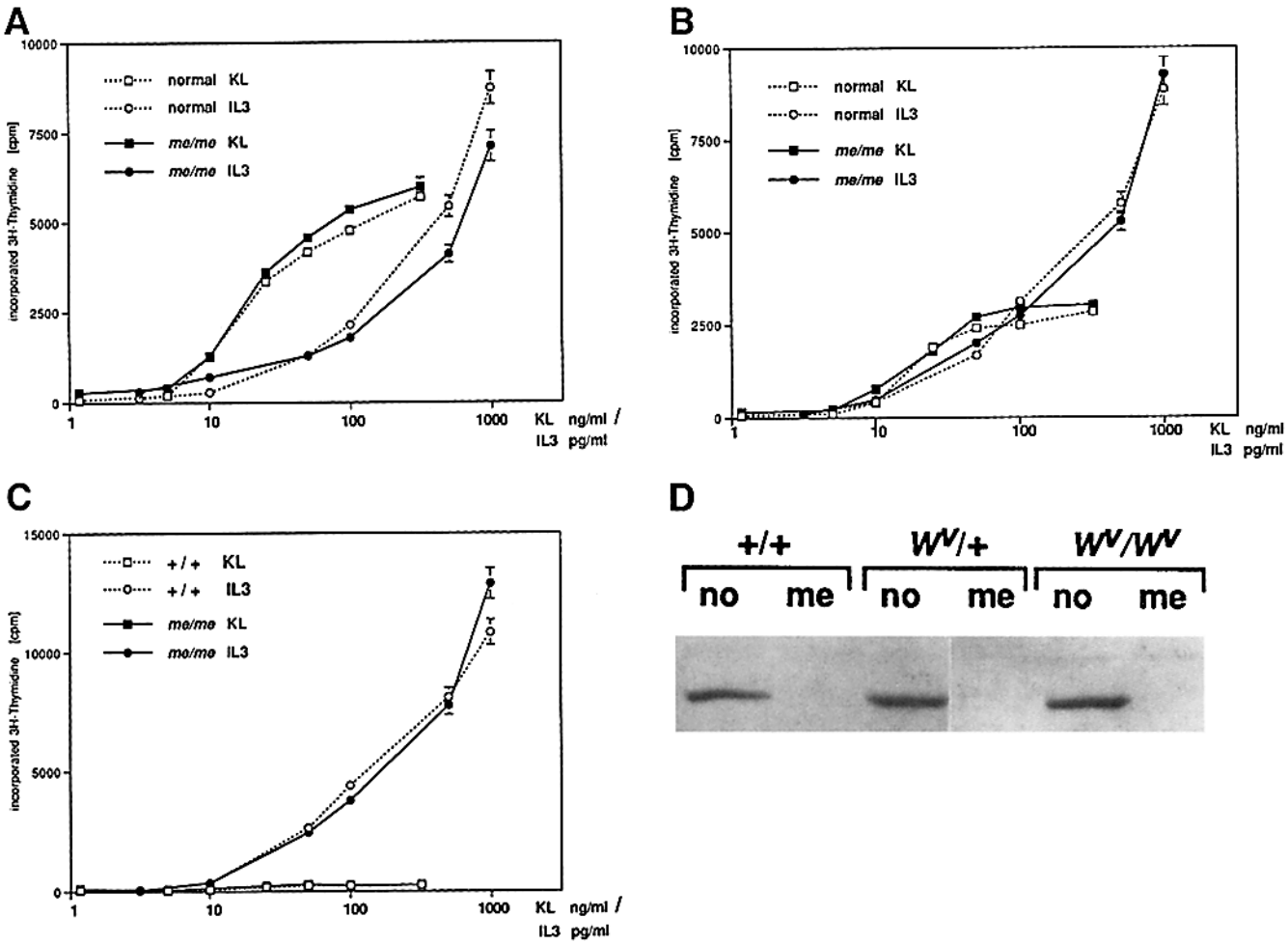


Figure 8. (*A–C*) Proliferative responses of normal (*m* and *q*) or *me/me* (*l* and *n*) BMCMC to IL3 (*m* and *l*) or KL (*q* and *n*) stimulation. BMCMC from (*A*) +/+:+/+(*me*) and +/+:*me/me* mice; (*B*) W^v/+:+/+(*me*) and W^v/+:*me/me* mice; and (*C*) W^v/W^v:+/+(*me*) and W^v/W^v:*me/me* mice. Error bars represent the standard error of the mean. Where no error bars are visible, the error was smaller than the symbol for that point. (*D*) SHP1 is expressed in BMCMC. Total cellular lysates of BMCMC (2×10^6) were analyzed for SHP1 expression by SDS-PAGE (8% gel) followed by immunoblotting with anti-SHP1.

terestingly, in contrast to the morphometric analyses of dermal mast cell production in vivo (see above and Table 3) and to the results of our assays of hematopoietic progenitor cells, there was no detectable difference in KL-driven proliferation (as measured by [^{3}H]thymidine incorporation) between normal and *me/me* BMCMC (Fig. 8, *A–C*). As expected, W^v/+-derived BMCMC displayed a decreased proliferative response to KL compared with BMCMC derived from littermates which expressed normal c-Kit, whereas W^v/W^v BMCMC were completely unresponsive to KL. Again, however, in five out of six mice, complete loss of SHP1 expression had no demonstrable effect on the proliferative response to KL. There also was no difference in proliferation between normal and *me/me* mast cells in response to IL3 stimulation, even though SHP1 has been reported to regulate IL3-induced proliferation in the DA-3 myeloid cell line (45). Intriguingly, mast cells grown from one W^v/W^v:*me/me* mouse responded to a limited extent to KL (to a level comparable to the typical response of W^v/+ mice), whereas the IL3 response of these cells was unaltered (data not shown). The reason for this discrepancy is not clear, but these findings raise the possibility that a third locus may influence the ability of SHP1 to regulate c-Kit in mast cells (see Discussion).

Our results suggest that SHP1, at least with respect to the effects of KL or IL3 on proliferation, is not a limiting negative regulator (for c-Kit or the IL3 receptor) in BMCMC. However, unlike in melanocytes, SHP1 is expressed in such cells (Fig. 8 *D*). Even though SHP1 did not detectably influence KL-induced proliferation in BMCMC, we examined whether immediate-early signaling events stimulated by KL binding, such as tyrosyl phosphorylation and MAP kinase (MAPK) activation (46), still might be influenced by SHP1. BMCMC were stimulated with KL, and total tyrosyl phosphorylation was analyzed by immunoprecipitation with anti-phosphotyrosine antibodies followed by anti-phosphotyrosyl immunoblotting (Fig. 9 *A*). KL-stimulated BMCMC showed a rapid increase in protein ty-

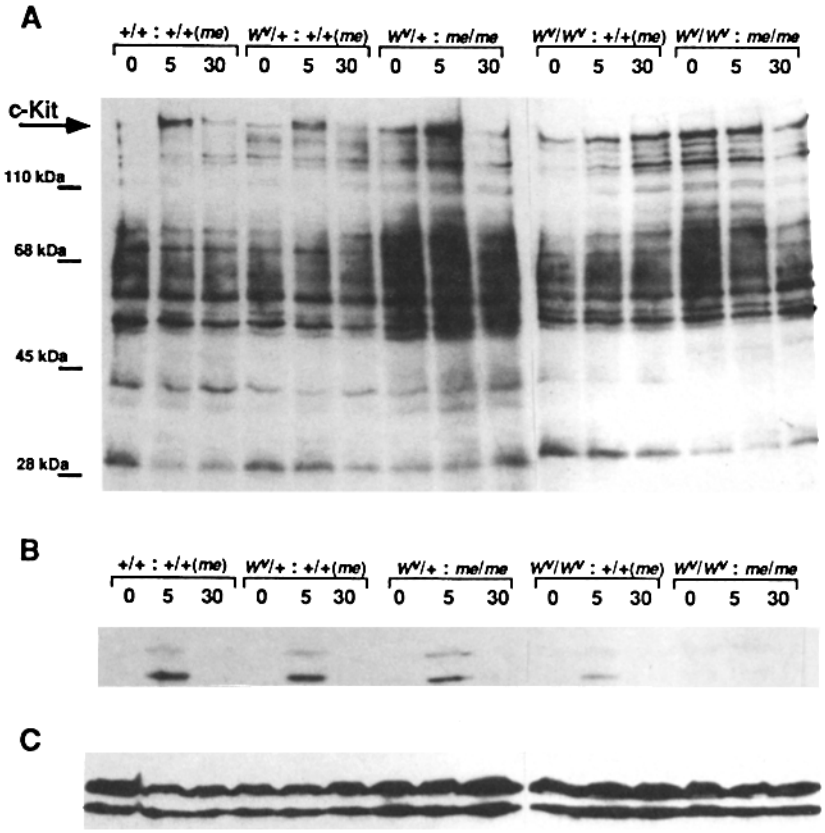


Figure 9. *me/me* mutation improves the immediate-early KL response of BMCMC from $W^v/+$ mice, as measured by KL-induced tyrosyl phosphorylation, but does not affect MAP-kinase activation. BMCMC from mice of the indicated phenotypes were stimulated for 0, 5, and 30 min with KL. (*A*) Lysates from KL-stimulated BMCMC (2×10^7) were immunoprecipitated with anti-phosphotyrosine antibodies and, after separation by SDS-PAGE (8%), immunoblotted with anti-phosphotyrosine. Note that the top tyrosyl phosphorylated band in W^v/W^v BMCMC is a background, nonspecific band, not c-Kit, which is not tyrosyl phosphorylated in such cells. (*B* and *C*) Total cellular lysates (2×10^6) of the same KL-stimulated BMCMC as used in *A* were analyzed by SDS-PAGE (8% gel) and immunoblotting with anti-phospho-MAP-kinase (*B*) or anti-MAP-kinase (*C*).

rosyl phosphorylation. Most prominent amongst these phosphotyrosyl proteins was an ~150-kD species, which is c-Kit itself. As expected, $W^v/+$ BMCMC also responded to KL, although the magnitude of the response was significantly reduced, whereas W^v/W^v BMCMC were unresponsive. We were unable to obtain sufficient quantities of BMCMC from +/+:*me/me* mice to allow biochemical analysis. The non-responsiveness of W^v/W^v BMCMC could not be rescued by *me/me*, but in $W^v/+$ BMCMC, increased tyrosyl phosphorylation in the presence of the *me/me* mutation was observed repeatedly. Notably, c-Kit phosphorylation was increased significantly in the *me/me* background, showing levels of phosphorylation equal to or higher than c-Kit in normal BMCMC. The increased tyrosyl phosphorylation of $W^v/+$:*me/me* compared with $W^v/+$:+/+(*me*) mice provides direct biochemical evidence that SHP1 regulates c-Kit tyrosyl phosphorylation ex vivo. However, SHP1 regulation of c-Kit clearly is not limiting for the control of KL-driven proliferation under these conditions. (see Discussion).

By assaying MAPK activation using activation-specific antibodies, we then determined whether the differences in KL-induced tyrosyl phosphorylation translated into alteration of a downstream component of the c-Kit signaling pathway (Fig. 9 *B*). Interestingly, there was no significant difference in MAPK activation between stimulated normal and *me/me* mast cells. These results are consistent with SHP1 not being a limiting negative regulator of KL-induced BMCMC proliferation ex vivo.

Discussion

Our combined results reveal two types of genetic interaction between the RTK c-Kit and the PTP SHP1: (*a*) improvement of the *motheaten* phenotype upon reduction of c-Kit activity, and (*b*) cell-type specific regulation of c-Kit activity by SHP1. Together with previous biochemical analyses (1), as well as our biochemical studies of the immediate-early responses to KL in BMCMC (Fig. 9), these results strongly suggest that SHP1 is a critical negative regulator of c-Kit signaling in some, but not all, KL-responsive cell types. Moreover, our data suggest that dysregulation of c-Kit signaling pathways in hematopoietic progenitor cells may play an important role in the development of the *me/me* phenotype. These results constitute the first genetic demonstrations in a mammalian system of the biological relevance of a biochemical interaction between a RTK and a given SH2-containing protein and between an RTK and its specific regulatory PTP.

F2 double homozygotic mice (W^v/W^v:*me/me*) appeared healthier than "c-Kit normal" *motheaten* mice (+/+:*me/me*). This was confirmed histologically by the finding of decreased numbers of myeloid cells infiltrating the lungs and skin, by the restoration of almost normal numbers of WBC

in the blood, and by ex vivo colony forming assays, which revealed a decreased number of bone marrow-derived CFU-GM. We interpret these results to mean that impaired c-Kit activity decreases the generation of hematopoietic progenitor cells, which limits the myeloid expansion usually observed in *me/me* mice. Although KL is not generally viewed as a myeloid growth factor, high levels of KL can support CFU-GM generation (Fig. 4 *B* and reference 47). In the absence of SHP1 (i.e., in *me/me* mice), hyper-activation of c-Kit may contribute directly to the generation of the *me/me* phenotype by increasing the number of myeloid progenitor cells. These results raise the interesting possibility that pharmacological inhibition of SHP1 activity ex vivo (for example, in human bone marrow stem cell cultures) might help to promote progenitor cell expansion.

Genetic interaction between c-Kit and SHP1 could occur in two general ways. c-Kit could act primarily on an early progenitor cell(s), in which SHP1 does not act, whereas SHP1 controls signaling pathways in more differentiated cells. With substantially reduced c-Kit signaling (i.e., in W^v/W^v mice), far fewer early progenitors would be produced, thus limiting the myeloid expansion caused by the loss of SHP1. Alternatively, SHP1 could regulate c-Kit in the same (early progenitor) cell, as well as possibly controlling several signaling pathways in later cell populations. We favor the latter explanation, in view of: (*a*) the biochemical data demonstrating a physical interaction between SHP1 and c-Kit in Mo-7e (1), a cell line derived from a relatively undifferentiated cell (48); (*b*) our direct demonstration that lin^- $c\text{-}Kit^+$ progenitor cells are dramatically hyper-responsive to KL stimulation ex vivo (Fig. 6), which argues that absence of SHP1 leads to hyper-sensitivity of c-Kit; and (*c*) our demonstration that the absence of SHP1 in BMCMC leads to enhanced c-Kit and total cellular tyrosyl phosphorylation (Fig. 9 *A*).

However, the influence of SHP1 on c-Kit is complex and cell type-specific. This is perhaps not totally surprising, since the quantitative requirements for various cellular responses to KL (e.g., proliferation, suppression of apoptosis, etc.) are different. Nevertheless, our results emphasize the importance of the specific cellular context in which a given SH2/phosphotyrosyl protein interaction occurs in determining the biological relevance of that interaction. For example, coat color, which is controlled by neural crest-derived cells, is not detectably affected by SHP1 status (Fig. 2). SHP1 is not expressed detectably in cultured melanocytes (Fig. 2 *D*). We did not assess SHP1 expression in neural crest primordia, but we suspect that SHP1 is not expressed in these cells either. Most likely c-Kit signaling is regulated by other PTPs and/or by other mechanisms (e.g., receptor modulation) in neural crest cells.

The macrocytic anemia observed in mice expressing (the) W^v allele(s) also is unaffected by loss of SHP1. Nevertheless, ex vivo analysis of cells derived from single or double mutant mice indicates a critical role for SHP1 in regulating c-Kit function in erythroid progenitor cells. In the absence of SHP1 the number of KL-induced BFU-E increased in cells derived from +/+ or $W^v/+$ mice. However, no response could be evoked in W^v/W^v cells, indicating that absence of SHP1 can up-regulate a KL-driven response but requires a minimal amount of c-Kit activity for such enhancement to be physiologically significant. This rather complicated result could reflect a number of possibilities: (*a*) the level of c-Kit surface expression could be dependent on SHP1 expression and higher levels of expression could result in an increased response to KL; (*b*) there could be an increased number of erythroid progenitor cells because loss of SHP1 affects some other pathway(s) earlier in development (this could be a pathway involving c-Kit or a completely different pathway); and/or (*c*) a higher percentage of progenitor cells responds to a given amount of KL due to the loss of SHP1, which normally negatively regulates c-Kit activity.

More than one of these explanations may apply. A comparable percentage of cells expressing low to medium levels of c-Kit was observed in lin^- cells isolated from both types of mice; however *me/me* progenitor cells displayed an ~50% increase in the proportion of cells expressing high levels of c-Kit (Table 2). Given the report that Kit^{high} cells are enriched for erythroid progenitors (44), the increased percentage of such cells could account, at least in part, for the increased number of BFU-Es observed in *me/me* mice (42). This 50% increase in the percentage of $c\text{-}Kit^{high}$ progenitor cells also could account for much (if not all) of the ~1.5-fold increase in BFU-E observed at saturating levels of KL. However, it is highly unlikely that this relatively small increase in the percentage of $c\text{-}Kit^{high}$ progenitor cells could account for the 10–15-fold increase in BFU-Es in *me/me* mice observed at sub-saturating levels of KL (Fig. 4 *A*). Flow cytometric analysis only indicates the relative levels of c-Kit expressing cells. Due to the very small percentage of lin^- cells in the bone marrow (<1%), such analyses cannot yield accurate estimates of the absolute numbers of progenitor cells. Therefore, we measured directly the proliferative response of purified lin^- bone marrow cells to KL. Under these conditions, in which we knew, on the basis of flow cytometric analysis, that approximately equal numbers of c-Kit-expressing cells were being compared, lin^- cells from *me/me* mice proliferated 50–60-fold more than did their normal counterparts. Even though there probably are more KL-responsive progenitor cells in *me/me* mice than in normal mice, it is clear that *me/me* cells also are intrinsically more responsive to KL stimulation. These results strongly support the hypothesis that SHP1 acts directly on c-Kit (within the same cell), thereby controlling c-Kit activity and/or the phosphorylation of c-Kit targets. Indeed, the increase in $c\text{-}Kit^{high}$ cells observed in *me/me* mice most likely is a consequence of loss of SHP1 activity in the c-Kit pathway in vivo. However, we cannot exclude formally the unlikely possibility that, due to the absence of SHP1, progenitor cells from *me/me* mice are predisposed to hyper-react to KL stimulation by a mechanism independent of the direct interaction between c-Kit and SHP1.

Although SHP1 regulation of c-Kit appears to be critical in KL-responsive progenitor cells, it is not limiting in the

more differentiated mast cell population. $W^v/+$ and especially W^v/W^v mice exhibit a diminished number of tissue mast cells in vivo. We had anticipated that the absence of SHP1 might result in an effective increase in c-Kit-derived signaling in $W^v/+$ and/or W^v/W^v mice, with a corresponding increase in mast cell number. Indeed, morphometric analysis of the number of dermal mast cells revealed that in the ear skin, loss of SHP1 did "rescue" the number of dermal mast cells of W^v/W^v mice to levels similar to those observed in $W^v/+$ mice (Table 3). In contrast, we did not detect any influence of loss of SHP1 on dermal mast cell number in the back skin. This could be due to differences in the properties of the mast cells (e.g., their sensitivity to changes in c-Kit activity) or differences in their origin and/or differentiation pathway. Loss of SHP1 could influence the number of dermal mast cells in the ear by effects on cells early in the mast cell differentiation pathway, e.g., by generating more mast cell progenitors, and/or by affecting later stages of differentiation and influencing viability and thereby life span of mature mast cells. Therefore, we analyzed the effects of W^v and *me* mutations on the proliferative capacity of BMCMC, a lineage-committed population of immature mast cells (49, 50), in response to KL. In all $W^v/+$:*me/me* and nearly all (5/6) W^v/W^v:*me/me* BMCMC cultures tested, the absence of SHP1 did not affect KL-induced proliferation detectably. These results suggest that SHP1 regulates dermal mast cell generation at an earlier stage of differentiation or by effects on processes other than proliferation, or that SHP1 is limiting for c-Kit regulation only in selected mast cell subpopulations (e.g., dermal mast cells, but not BMCMC). Interestingly, however, a BMCMC culture derived from one W^v/W^v:*me/me* mouse did proliferate significantly in response to KL, whereas all of the W^v/W^v:+/+(*me*) BMCMC cultures tested were wholly non-responsive. Since the parental $W^v/+$ and *me*/+ mice in our cross were of different genetic backgrounds (see Materials and Methods), a third, stochastically segregating locus could play a role in negative regulation of c-Kit.

IL3-directed proliferation of BMCMC also is independent of SHP1 genotype (Fig. 8, *A–C*). Previous studies, in which antisense SHP1 RNA was expressed in DA-3 cells, revealed an inverse correlation between lack of SHP1 expression and IL3-driven proliferative response (45). Likewise, our results (Fig. 7 and data not shown) suggest that *me/me* bone marrow progenitor cells, as well as bone marrow-derived macrophages (Chen, H., and B.G. Neel, unpublished observations) are hyper-sensitive to growth factors (IL3, GM-CSF) that signal through the common IL3 receptor beta chain. Together with our KL/c-Kit results, these data suggest that hematopoietic progenitor cells and BMCMC may have distinctly different mechanisms of terminating RTK and cytokine receptor-generated signals. Loss of SHP1 also might influence KL- or IL3-induced pathways other than proliferation (e.g., potentiation of degranulation, chemotaxis) in BMCMC.

Although SHP1 is not limiting for KL-driven proliferation in BMCMC, biochemical analysis of these cells indicates clearly that SHP1 contributes to the inactivation of KL-initiated signals. Superimposition of the *me/me* genotype resulted in substantial recovery of KL-induced tyrosyl phosphorylation in the $W^v/+$ background, although W^v/W^v BMCMC could not be "rescued" (Fig. 9 *A*). These results provide direct biochemical validation of the genetic interaction between c-Kit and SHP1. Since the tyrosyl phosphorylation of c-Kit is modulated by SHP1 expression, and SHP1 binds directly to c-Kit (1), our data suggest that c-Kit may be a direct target of SHP1. Despite the virtually complete recovery of KL-induced tyrosyl phosphorylation in $W^v/+$:*me/me* BMCMC, normal KL-induced MAPK activation was not restored. This result parallels the lack of effect of SHP1 loss on KL-induced proliferation of $W^v/+$ BMCMC. There are several possible explanations for this apparent discrepancy: (*a*) In addition to its negative regulatory role in the c-Kit pathway, SHP1 may also have a positive signaling function upstream of MAPK, such that loss of SHP1 restores immediate-early tyrosyl phosphorylation events, but not MAPK activation; (*b*) although total tyrosyl phosphorylation is restored to apparently normal levels in $W^v/+$:*me/me* mice, subtle differences in either specific c-Kit targets and/or in the kinetics of c-Kit tyrosyl phosphorylation may have been missed by our assays; (*c*) the W^v mutation may be impaired in another c-Kit function (e.g., endocytosis) important for downstream signaling and independent of its impaired PTK activity; and/or (iv) SHP1 may only dephosphorylate certain sites on c-Kit and/or certain downstream targets of c-Kit, whose phosphorylation cannot be restored to normal simply by the loss of SHP1. We suspect that SHP1 exerts similar biochemical effects on c-Kit-induced tyrosyl phosphorylation in hematopoietic progenitor cells, but that either the absence of secondary pathways of c-Kit inactivation and/or differences in the details of c-Kit-induced proliferative pathways in such cells results in a significant increase in KL-induced effects in the absence of SHP1. The difficulty in obtaining sufficient numbers of lin^- Kit^+ cells for biochemical analysis limits our ability easily to resolve these issues. Further studies will be required to elucidate the molecular explanation for tissue specific regulation of c-Kit by SHP1.

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