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5. Carp were maintained in 2000-liter aquaria at 30°C for at least 3 months under a 12-hour day length and fed with a commercial trout diet (Mainstream expanded pellets; B.P. Nutrition, Northwich, Cheshire, UK). They were gradually cooled over a 3-day period from 30°C to 23°C on day 1, 17°C on day 2, and 10°C on day 3 (*3*) and were maintained at 10°C for the remainder of the experiment. Cooling was at 1°C per hour to the desired temperature and was then held constant for the remainder of the day. At intervals, the hepatic postmitochondrial microsomal membrane fraction was isolated by differential centrifugation and recovered by centrifugation at 100,000*g* for 60 min. Lipids were isolated and phospholipid fractions were separated by thin-layer chromatography and transmethylated as described [A. R. Cossins and C. L. Prosser, *Biochim. Biophys. Acta* **687**, 303 (1982)]. The resulting methyl esters were analyzed by capillary gas chromatography (25 m × 0.25 mm; FFAP, Phase Separations, Queensferry, Clwyd, UK). Molecular species were analyzed by high-performance liquid chromatography (HPLC) separation of dinitrobenzene derivatives as described [H. Takamura, H. Narita, R. Urade, M. Kito, *Lipids* **21**, 356 (1986)].
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8. A commercial carp liver cDNA library (Stratagene) was screened with the Bam HI–Sac I fragment from pDs3-358 (*7*) labeled with [α-^{32}P]deoxycytidine 5′-triphosphate (dCTP) by random priming (Stratagene Prime-It II kit). Hybridization was done in 50% formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution, 1% SDS, and calf thymus DNA (100 μg/ml) at 30°C. Posthybridization washes were done twice for 20 min in 2× SSC and 0.1% SDS at 21°C and 30°C, respectively, and in 0.2× SSC and 0.1% SDS at 30°C. Plasmid pcDsL7 was excised from purified positive lambda plaque CL7 according to manufacturer's (Stratagene) instructions. The full cDNA insert was removed by Kpn I–Sac I digestion and labeled with [α-^{32}P]dCTP as above. RNA was extracted from carp liver [P. Chomzynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)] polyadenylated [poly(A)$^+$] RNA selected and separated by electrophoresis in 1% agarose-formaldehyde followed by capillary transfer to polyvinylidene difluoride (PVDF) membrane [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, vol. 1)]. Blots were hybridized to the full-length cDNA insert from pcDsL7 at 42°C with the same medium as detailed above with highly stringent posthybridization washes according to manufacturer's (Millipore) instructions. The hybridization product was 2.7 kb (P. E. Tiku, data not shown). Unidirectional deletion subclones of pcDsL7 were generated by exonuclease III digestion (Erase-a-base; Promega) and autosequenced (Applied Biosystems model 373A) with universal pUC/M13 primers. The full sequence was confirmed by autosequencing of the anticoding strand as having 2652 bp with an ORF of 879 bp, with a 520-bp 5′ untranslated region (UTR) and a 1253-bp 3′ UTR (GenBank, accession number CCU31864). Translation of this ORF indicated a polypeptide of 292 amino acid residues and a calculated molecular mass of 33.65 kD. For the clustal alignment (Fig. 2B), we used an identity residue weight table and DNAStar Lasergene software. Amino acid sequences were obtained from cDNA sequences from GenBank and EMBL: rat (accession number J02585), tick (U03281), and yeast (J05676).
9. Total RNA (2.5 μg) isolated from carp livers was hybridized at 45°C simultaneously to antisense probes for carp desaturase, carp β-actin, and human 18*S* rRNA (Ambion, Austin, TX). Ribonuclease protection assay was performed with the RPA-II kit from Ambion. Probes were synthesized by in vitro transcription with T7 RNA polymerase (MAXIscript; Ambion) and α-^{32}P uridine 5′-triphosphate. The β-actin template was derived by subcloning the Sac I–Xba I fragment from CA16–Sal I [Z. Liu *et al.*, *DNA Sequence–J. DNA Sequencing and Mapping* **1**, 125 (1990)] into the Promega vector pGEM7Zf(+). The recombinant clone pG7ccactb was digested with Hpa I to generate a template from which a probe of ~700 nucleotides (nt) was synthesized. Unidirectionally deleted pcDsL7 resulted in subclone pcDsL7D*, which had all of the 5′ UTR deleted. This was digested with Xho I and protected with α-phosphorothioate deoxynucleotide triphosphates. Unidirectional deletion of Hind III–digested and protected pcDsL7D* resulted in subclone pcDsL7d4, which was digested with Nae I to give an 830-bp fragment, which was then used as a template for producing the 300-nt desaturase antisense probe. The 109-nt anti–18*S* rRNA probe was synthesized from pT7 18*S* RNA (Ambion). Nuclei were isolated from carp liver, RNA synthesized with unlabeled nucleotide triphosphates [W. F. Marzluff and R. C. C. Huang, in *Transcription and Translation: A Practical Approach*, B. D. Hames and S. J. Higgins, Eds. (IRL Press, Oxford, 1984), pp. 89–130] and 10 μg of RNA analyzed by RPA with the antisense desaturase probe.
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12. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
13. Equal quantities of microsomal protein were subjected to 12% SDS–polyacrylamide gel electrophoresis, wet-electroblotted onto PVDF membrane, and probed with polyclonal antibody to rat desaturase (*7*). The use of this antibody against a 33-kD carp protein has been described (*10*). In Fig. 4A, the secondary antibody was goat antibody to rat immunoglobulin G conjugated to alkaline phosphatase, and the substrate was 5-bromo-4-chloro-3-indolyl phosphate (BCIP). All bands shown in Fig. 4A were run on the same gel. For the assays shown in Fig. 4B, the chemiluminescent substrate Lumiphos 530 (Boehringer-Mannheim) was used. Exposed x-ray films were analyzed with a Molecular Dynamics (Sunnyvale, CA) laser densitometer, correcting for variations in background and normalizing against a rat desaturase standard for variations between blots in band density. Band density was proportional to protein loading ($r^2 > 0.95$). Δ^9-Desaturase activity was assayed at 30°C by measuring the rate of conversion of palmitoyl-CoA (final concentration, 12.5 μmol/liter) to palmitoleoyl-CoA with trace quantities of ^{14}C-labeled substrate [J. B. Leifkowith, *Biochim. Biophys. Acta* **1044**, 13 (1990)].
14. We thank E. Fodor for HPLC analyses of molecular species; P. Strittmatter for providing the antibody to rat desaturase and the cloned rat cDNA, pDs3-358; P. B. Hackett for the carp β-actin clone CA16–Sal I, and M. Caddick, B. Maresca, and J. Crampton for helpful discussions. Supported by a grant and postgraduate support from the Natural Environmental Research Council (UK).

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Identification of a Committed Precursor for the Mast Cell Lineage

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Mast cells originate from hematopoietic stem cells, but the mast cell–committed precursor has not been identified. In the study presented here, a cell population in murine fetal blood that fulfills the criteria of progenitor mastocytes was identified. It is defined by the phenotype Thy-1^{lo}c-Kit^{hi}, contains cytoplasmic granules, and expresses RNAs encoding mast cell–associated proteases but lacks expression of the high-affinity immunoglobulin E receptor. Thy-1^{lo}c-Kit^{hi} cells generated functionally competent mast cells at high frequencies in vitro but lacked developmental potential for other hematopoietic lineages. When transferred intraperitoneally, this population reconstituted the peritoneal mast cell compartment of genetically mast cell–deficient *W/W*^{v} mice to wild-type levels.

Mature mast cells reside in mucosal and connective tissues where they can act as key mediators in immunoglobulin E (IgE)–dependent allergic reactions. In addition, through the release of cytokines as well as proteases and other mediators, mast cells can participate in a wide array of immunological and inflammatory responses in which they function in the recruitment of leukocytes into sites of inflammation and in the local regulation of vascular or epithelial permeability (*1*). Mast cells originate from hematopoietic stem cells (HSCs) (*2*), and in vitro assays of colony formation indicate that mast cell precursor activity occurs at low frequency in the bone marrow, peripheral blood, and mesenteric lymph nodes of murine rodents (*3*, *4*). It has been proposed that mast cell precursors leave the bone marrow, migrate in the peripheral blood, and invade mucosal and connective tissues where they undergo differentiation into morphologically characteristic mature mast cells. However, a mast cell–committed precursor cell, that is, a cell type distinguished by morphology and developmental potential from a multipotent HSC, has not been purified from bone marrow or blood (*3*, *4*). Here, we report the identification of a cell population purified from murine fetal blood that satisfies the criteria of a mast cell–committed precursor at a stage before tissue

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invasion. This progenitor mastocyte (promastocyte) is both morphologically and functionally distinct from HSCs.

The leukocyte fraction in fetal blood (FB) from day 15.5 of gestation contains several distinct cell populations that can be resolved on the basis of expression of Thy-1 and the receptor tyrosine kinase c-Kit (Fig. 1A) (*5*). Fetal blood Thy-1$^-$c-Kit$^+$ cells (Fig. 1B) and FB Thy-1$^+$c-Kit^{lo} cells were recently defined as populations containing multipotent hematopoietic stem cells and pro-thymocytes, respectively (*5*, *6*). Beginning on day 14.5 of gestation, a third population, defined by the phenotype Thy-1^{lo}c-Kit^{hi}, can also be isolated from FB (Fig. 1C). Both FB Thy-1$^-$c-Kit$^+$ and FB Thy-1^{lo}c-Kit^{hi} cell types displayed large nucleus-to-cytoplasm ratios and strongly (Thy-1$^-$c-Kit$^+$) or weakly (Thy-1^{lo}c-Kit^{hi}) basophilic cytoplasm, features that are consistent with those of immature hematopoietic precursors. However, FB Thy-1^{lo}c-Kit^{hi} cells (Fig. 1E), but not FB Thy-1$^-$c-Kit$^+$ cells (Fig. 1D), exhibited variable numbers of prominent basophilic cytoplasmic granules.

To assess their hematopoietic potential, we cultured FB Thy-1$^-$c-Kit$^+$ and FB Thy-1^{lo}c-Kit^{hi} cells with various hematopoietic cytokines in methylcellulose assays in vitro (*7*). Fetal blood Thy-1$^-$c-Kit$^+$ cells formed colonies in response to interleukin-3 (IL-3) alone, a response augmented by stem cell factor (SCF). In contrast, FB Thy-1^{lo}c-Kit^{hi} cells were unresponsive to either IL-3 or SCF alone, but did generate colonies in IL-3 and SCF at frequencies higher than those of FB Thy-1$^-$c-Kit$^+$ cells (Fig. 2). In numerous assays (*8*), FB Thy-1$^-$c-Kit$^+$ cells, but not FB Thy-1^{lo}c-Kit^{hi} cells, proved capable of multilineage development. Fetal blood Thy-1^{lo}c-Kit^{hi} cells, but not FB Thy-1$^-$c-Kit$^+$ cells, failed to generate colonies in response to macrophage–colony-stimulating factor (M-CSF), granulocyte-macrophage (GM)–CSF, and granulocyte (G)–CSF. The expression of RNA encoding β^{major} globin, which is indicative of erythroid lineage commitment, was inducible with IL-3, SCF, and erythropoietin in FB Thy-1$^-$c-Kit$^+$ but not in FB Thy-1^{lo}c-Kit^{hi} cells (*8*). As was in agreement with these in vitro findings, in vivo myeloid-erythroid precursor activity, as revealed by day 8 and day 12 spleen colony-forming units (*9*), was contained in FB Thy-1$^-$c-Kit$^+$ cells but not in up to 5×10^3 FB Thy-1^{lo}c-Kit^{hi} cells (the highest analyzed cell concentration). Pre–B cell colonies arose from FB Thy-1$^-$c-Kit$^+$ cells, but not from FB Thy-1^{lo}c-Kit^{hi} cells, when both types of progenitors were analyzed in a sensitive culture system supporting B cell development on stromal cells (PA-6) with IL-7 (*10*). Finally, FB Thy-1^{lo}c-Kit^{hi} cells, but not FB Thy-1$^-$c-Kit$^+$ cells, lacked T cell precursor potential on adoptive intrathymic transfer (*6*). Thus, FB Thy-1^{lo}c-Kit^{hi} cells, but not FB Thy-1$^-$c-Kit$^+$ cells, lack developmental potential for macrophages, granulocytes, erythrocytes, and B and T lymphocytes.

Interleukin-3 and SCF can support mast cell colony formation from bone marrow cells (*3*, *4*, *11*) and from mature mast cells in vitro (*4*, *11*, *12*). Ex vivo isolated FB Thy-1^{lo}c-Kit^{hi} cells contained cytoplasmic granules that stained weakly with toluidine blue but not with berberine sulfate or safranin, dyes that stain granules of mature connective tissue–type mast cells (CTMCs) (*13*). After culture in IL-3 and SCF (*8*), FB Thy-1^{lo}c-Kit^{hi} cells displayed an immature mast cell morphology with small nucleus-to-cytoplasm ratios and more abundant toluidine blue–positive granules. When observed by transmission electron microscopy (*14*–*18*), freshly sorted FB Thy-1^{lo}c-Kit^{hi} cells exhibited criteria characteristic of very immature mast cells (Fig. 3A) and differed from immature basophils according to the ultrastructural characteristics of their nuclei, immature cytoplasmic granules, Golgi apparatus, and endoplasmic reticulum (*15*, *17*, *18*). When FB Thy-1^{lo}c-Kit^{hi} cells were cultured for 13 days with IL-3 and SCF, ultrastructural examination revealed a more abundant cytoplasm and more numerous granules, which are indicative of further maturation (Fig. 3B). Ultrastructural analysis of sorted FB Thy-1$^-$c-Kit$^+$ and FB Thy-1$^+$c-Kit^{lo} cells (Fig. 1), representing multipotent and pro-thymocyte populations (*5*), respectively, revealed no evidence for cytoplasmic granule development.

Mast cells are frequently characterized by expression of their specific secretory granule proteases (*19*). Therefore, FB Thy-1$^-$c-Kit$^+$ and FB Thy-1^{lo}c-Kit^{hi} cells were analyzed by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) for expression of RNAs encoding

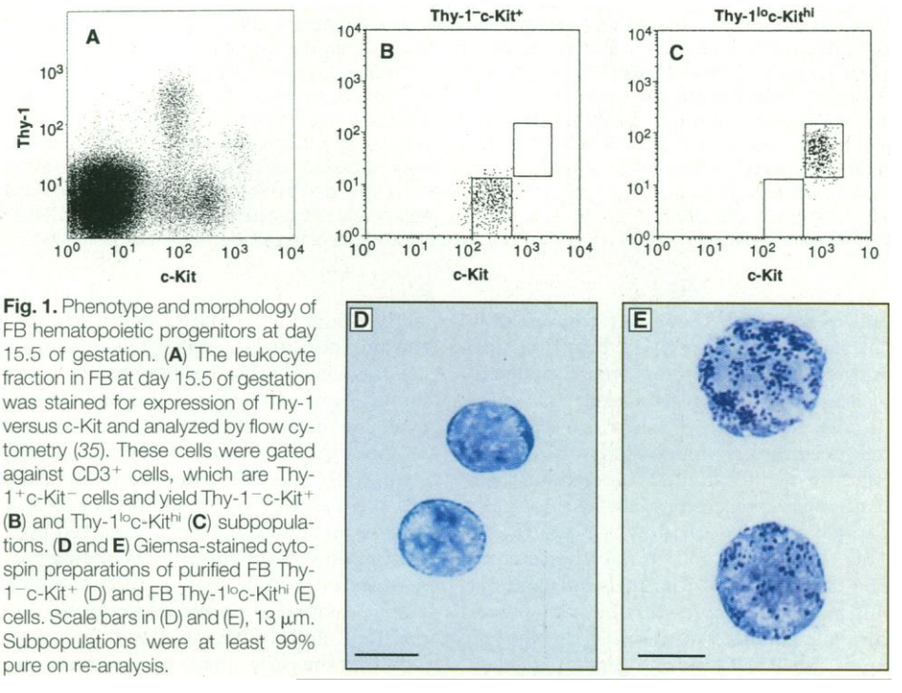

Fig. 1. Phenotype and morphology of FB hematopoietic progenitors at day 15.5 of gestation. (**A**) The leukocyte fraction in FB at day 15.5 of gestation was stained for expression of Thy-1 versus c-Kit and analyzed by flow cytometry (*35*). These cells were gated against CD3$^+$ cells, which are Thy-1$^+$c-Kit$^-$ cells and yield Thy-1$^-$c-Kit$^+$ (**B**) and Thy-1^{lo}c-Kit^{hi} (**C**) subpopulations. (**D** and **E**) Giemsa-stained cytospin preparations of purified FB Thy-1$^-$c-Kit$^+$ (D) and FB Thy-1^{lo}c-Kit^{hi} (E) cells. Scale bars in (D) and (E), 13 μm. Subpopulations were at least 99% pure on re-analysis.

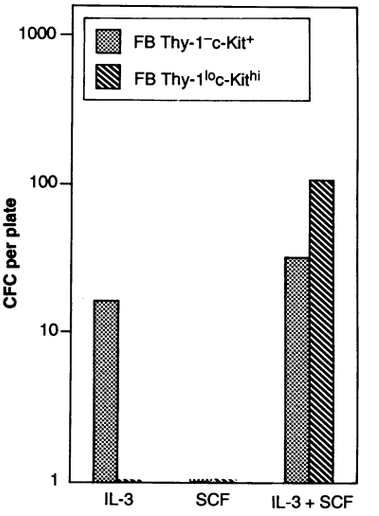

Fig. 2. In vitro colony formation of FB-derived Thy-1$^-$c-Kit$^+$ and Thy-1^{lo}c-Kit^{hi} populations. Purified Thy-1^{lo}c-Kit^{hi} and Thy-1$^-$c-Kit$^+$ cells (isolated as in Fig. 1) were plated into methylcellulose medium in the presence of various cytokines, and colony-forming cells were determined after 10 days in vitro (*7*, *8*). Data are representative of at least three independent experiments per analyzed condition.

three mast cell–specific proteases (MC-CPA, MMCP-2, and MMCP-4) (*19–21*). All three were expressed in ex vivo isolated FB Thy-1^{lo}c-Kit^{hi} cells (in the quantitative order MC-CPA > MMCP-4 > MMCP-2), but were expressed in FB Thy-1^{-}c-Kit^{+} cells only after culture under conditions that promote mast cell development (IL-3 plus SCF).

Because mature mast cells express high-affinity IgE receptors (FcεRI) (*22*), we analyzed FB Thy-1^{-}c-Kit^{+} and FB Thy-1^{lo}c-Kit^{hi} cells for expression of RNA encoding the FcεRI α chain (*23*). FcεRIα RNA expression was undetectable in both ex vivo purified populations (Fig. 3C). However, after culture in IL-3 and SCF (*8*), FcεRIα RNA was expressed in progeny from both FB Thy-1^{-}c-Kit^{+} and FB Thy-1^{lo}c-Kit^{hi} cells (Fig. 3C). FcεRIα RNA was ~10 to 100 times more abundant in progeny derived from FB Thy-1^{lo}c-Kit^{hi} cells as compared with progeny from FB Thy-1^{-}c-Kit^{+} cells, which suggests a greater frequency or maturity of mast cell lineage precursors in the FB Thy-1^{lo}c-Kit^{hi} population. Expression of FcεRIα RNA paralleled the capacity to bind IgE to the cell surface, as determined by flow cytometry. Fetal blood Thy-1^{lo}c-Kit^{hi} cell–derived mast cells were functionally competent, as shown by their capacity to incorporate serotonin and to release this mediator (*24*) after antigen [2,4-dinitrophenyl (DNP)–carrier] or antibody [antibody to IgE (anti-IgE)] stimulation of IgE antibody to DNP (anti–DNP-IgE)–occupied FcεRI (Fig. 3D).

To assess their developmental potential for the mast cell lineage in vivo, we transferred FB Thy-1^{lo}c-Kit^{hi} or FB Thy-1^{-}c-Kit^{+} cells into mast cell–deficient *W/W^{v}* mice (*25, 26*). Typical CTMCs in the peritoneal cavity stain strongly with berberine sulfate (Fig. 4, A and B) and safranin (Fig. 4C) (*13, 26*). *W/W^{v}* mice deficient in c-Kit completely lack CTMCs in the peritoneal cavity (Fig. 4G) (*25, 26*). On intraperitoneal transfer of only 5 × 10^{3} FB Thy-1^{lo}c-Kit^{hi} progenitors, the CTMC compartment in the peritoneal cavity of *W/W^{v}* mice was reconstituted to wild-type levels (Fig. 4D), and the cytoplasmic granules of the reconstituted mast cells showed the typical CTMC staining pattern (Fig. 4, E and F). Intravenous, but not intraperitoneal, injection of multipotent FB Thy-1^{-}c-Kit^{+} cells into *W/W^{v}* mice also reconstituted the CTMC compartment (Fig. 4H). However, as verified by donor-globin analysis, intravenous injection of FB Thy-1^{-}c-Kit^{+} cells also produced HSC engraftment in *W/W^{v}* recipients. No HSC engraftment was detectable after intravenous injection of FB Thy-1^{lo}c-Kit^{hi} cells into *W/W^{v}* recipients.

Mast cell precursor activity has been demonstrated in the murine day 9.5 embryonic yolk sac (*27*), in adult bone marrow (*2*), and in adult peripheral blood (*3, 28*), as well as in mesenteric lymph nodes of *Nippostrongylus brasiliensis*–infected mice (*4, 29*). Nevertheless, a mast cell–committed precursor cell, functionally or morphologically distinct from HSCs or from other multipotent progenitor cells, had not been purified from bone marrow or blood (*3, 4*). The purification and initial characterization of FB Thy-1^{lo}c-Kit^{hi} cells indicate that this population represents the earliest precursors committed to the mast cell lineage to be identified in ontogeny. Our data strongly suggest that: (i) Commitment into the mast cell lineage and expression of mast cell–associated proteases can precede tissue immigration. (ii) Circulating pro-mastocytes are indeed granulated cells that are morphologically distinct from multipotent stem cells. (iii) Formation of cytoplasmic granules can precede expression of FcεRI in vivo [as in certain immature mast cell populations in vitro (*30*)]. (iv) Circulating pro-mastocytes are selective in their cytokine requirement (IL-3 plus SCF) for further expansion and differentiation in vitro. Whether the early appearance of mast cell–

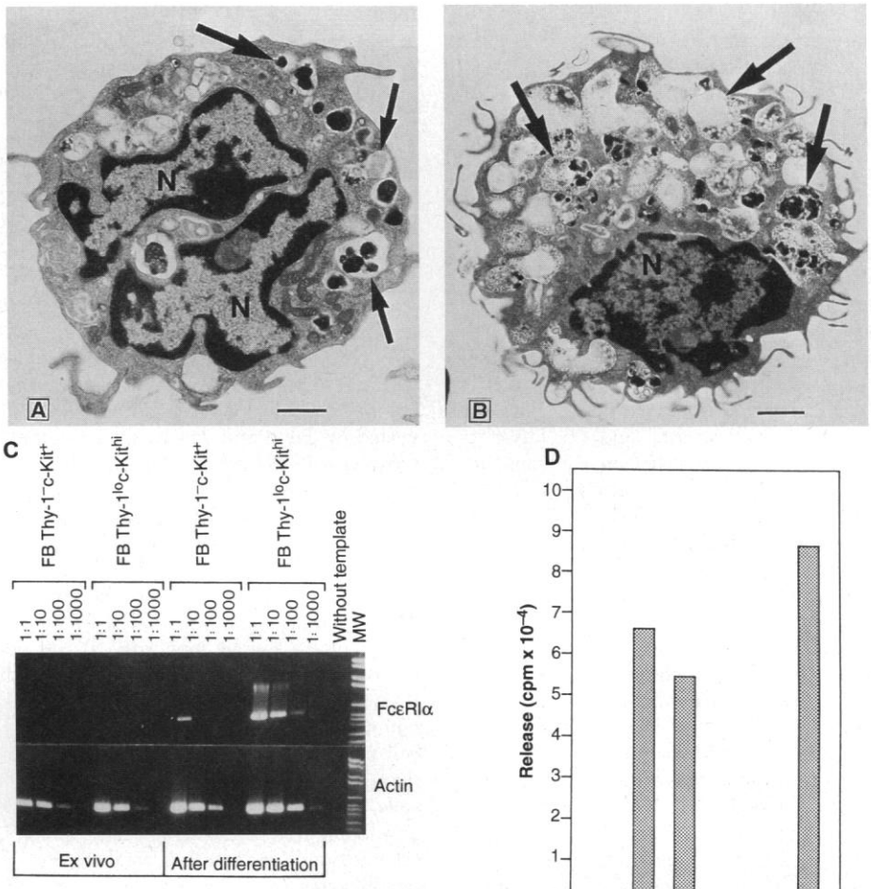

Fig. 3. Fetal blood Thy-1^{lo}c-Kit^{hi} cells give rise to functional mast cells in vitro. Purified FB precursors isolated ex vivo (**A** and **C**) or cultured in methylcellulose medium with IL-3 and SCF (*8*) (**B** to **D**) were examined by transmission electron microscopy (*18*) [(A) and (B)], analyzed by semiquantitative RT-PCR for expression of RNAs encoding FcεRIα [(C), top panel] or actin [(C), bottom panel] (*23, 33*), or analyzed for ^{3}H-serotonin release after stimulation through FcεRIα (*24, 36*) (D). The representative ex vivo isolated mast cell precursor (A) has a lobular nucleus (N) and several immature cytoplasmic granules (arrows) containing mixtures of vesicles, electron-dense progranules, lightly dense matrix material, and electron-lucent areas. In (B), the cultured FB Thy-1^{lo}c-Kit^{hi} mast cell is also immature by morphological criteria. However, compared with the cell in (A), the cytoplasm is more abundant and the immature granules (arrows) are more numerous. Scale bars in (A) and (B), 1 μm. In (D), ^{3}H-serotonin–labeled FB Thy-1^{lo}c-Kit^{hi} cells cultured for 14 days in IL-3 and SCF (*8*) were incubated with monoclonal mouse anti–DNP-IgE (lanes 1 through 3) or medium alone (lanes 4 through 6) and subsequently stimulated with medium alone (lane 1), with anti-IgE (lanes 2 and 4) or DNP-HSA (lanes 3 and 5), or with ionomycin (*36*). Release in lanes 1 through 6 corresponds to ~2, 56, 41, 1, 1, and 72%, respectively, of total release after lysis in Triton X-100 (0.1%) (100% release = 1.19 × 10^{5} cpm). Data are representative of three independent experiments.

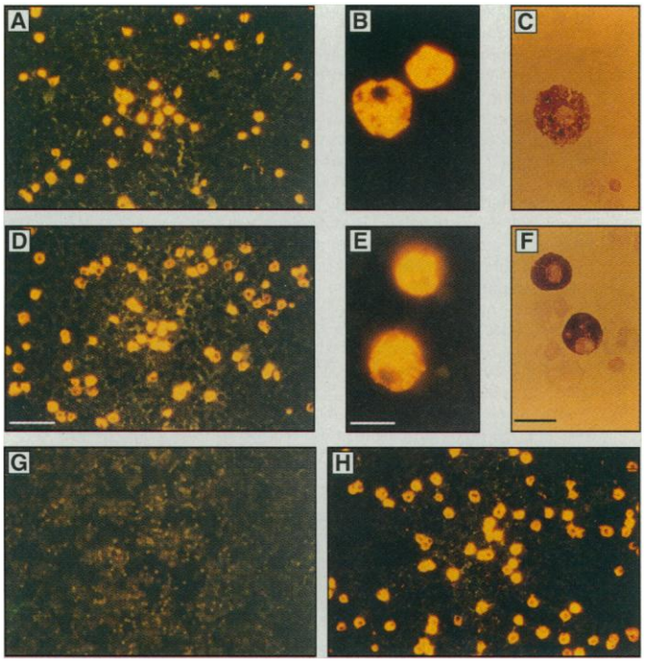

Fig. 4. Fetal blood Thy-1^{lo}c-Kit^{hi} cells reconstitute the peritoneal mast cell compartment to wild-type levels in mast cell–deficient *W/W^v* mice. Fetal blood Thy-1^{lo}c-Kit^{hi} cells or Thy-1$^-$c-Kit$^+$ cells purified by fluorescence activated cell sorting were injected (5 × 10^3 cells per mouse) intravenously (i.v.) or intraperitoneally (i.p.) into 250 rad–irradiated (i.v. transfer) or unirradiated (i.p. transfer) mast cell–deficient *W/W^v* recipient mice (*25, 26, 37*). Three months after cell transfers, peritoneal exudate cells (PECs) were harvested from wild-type C57BL/6 mice (**A** through **C**) or from *W/W^v* mice that were injected i.p. with FB Thy-1^{lo}c-Kit^{hi} cells (**D** through **F**) or uninjected (**G**) or injected i.v. with FB Thy-1$^-$c-Kit$^+$ cells (**H**). PECs (2 × 10^5) were cytospun onto glass slides and stained with berberine sulfate [(A), (B), (D), (E), (G), and (H)] or alcian blue–safranin [(C) and (F)] (*13, 26*). Cells with weakly berberine-positive nuclei are lymphocytes and monocytes, and large cells with strong cytoplasmic staining are peritoneal CTMCs, which are completely absent in *W/W^v* mice [compare (A) and (G)] (*25*). Higher magnification reveals granular staining with berberine [(B) and (E)] and safranin [(C) and (F)]. The recipient mouse displaying CTMC reconstitution after i.v. transfer of multipotent FB Thy-1$^-$c-Kit$^+$ cells (H) also showed HSC engraftment by donor-globin analysis. Identical results were obtained in three independent experiments. Scale bars in (A), (D), (G), and (H), 100 μm; in (B), (C), (E) and (F), 17 μm.

associated proteases plays any role in tissue invasion from the blood remains to be assessed. Also, the origin of this pro-mastocyte population in fetal development has yet to be identified; we could not identify a corresponding population in fetal liver in mid or late gestation. The number of FB Thy-1^{lo}c-Kit^{hi} cells in the blood (~1/40 of the CD45$^+$ leukocyte fraction on day 15.5) declines from day 15.5 up to birth.

The identification of this most immature mast cell precursor may help to elucidate the earliest molecular events underlying mast cell lineage commitment and differentiation into mature mast cells. Our findings may also prove useful for the further classification of mast cell malignancies; indeed, an immature human mast cell line derived from a patient with mast cell leukemia expresses a mast cell granule–associated protease but not FcεRI (*31*).

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8. Subpopulations purified by fluorescence-activated cell sorting were plated at 1500 cells per plate into 1 ml of methylcellulose medium with IL-3, or SCF, or IL-3 and SCF, or M-CSF with and without IL-3, or GM-CSF with or without IL-3, or G-CSF, or IL-3 with SCF and erythropoietin. After 10 days, colonies were counted. After culture in IL-3 with SCF and erythropoietin, colonies were harvested and β^{major} globin and actin RNA expression were analyzed by RT-PCR (*32, 33*). Expression of β^{major} globin was detectable only in colonies derived from Thy-1$^-$c-Kit$^+$ and not from Thy-1^{lo}c-Kit^{hi} precursors. Cytokines used were IL-3 [always 350 U/ml, corresponding to 0.1% conditioned medium from cells transfected with an IL-3–expressing vector (*34*)], SCF (10 ng/ml) (R&D Systems, Abingdon, U.K.), M-CSF (25 U/ml) (Genzyme, Cambridge, MA), GM-CSF (1 ng/ml) (Gibco-BRL, Gaithersburg, MD), G-CSF (25 U/ml), or erythropoietin (2 U/ml) (both from Boehringer Mannheim, Germany).
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36. Cells were labeled with 5-hydroxy[G-^{3}H]tryptamine creatinine (^{3}H-serotonin) (Amersham, Buckinghamshire, U.K.) at 1 μCi/ml for 8 hours, washed, and incubated with monoclonal mouse anti–DNP-IgE (SPE-7; Sigma, St. Louis, MO) (10 μg/ml) or medium alone for 1 hour on ice. Cells were subsequently stimulated for 10 min at 37°C with medium alone, with monoclonal rat antibody to mouse IgE (R35-72; PharMingen, San Diego, CA) (2 μg/ml) or DNP–human serum albumin (HSA) (1 μg/ml; Sigma), or with ionomycin (1 μM; Sigma).
37. *W/W^v* recipient mice were F_1 animals derived from crosses of C57BL/6-*W^v*/+ × WB-*W*/+ parents (Japan-SLC, Japan).
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Activation of BTK by a Phosphorylation Mechanism Initiated by SRC Family Kinases

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Bruton's tyrosine kinase (BTK) is pivotal in B cell activation and development through its participation in the signaling pathways of multiple hematopoietic receptors. The mechanisms controlling BTK activation were studied here by examination of the biochemical consequences of an interaction between BTK and SRC family kinases. This interaction of BTK with SRC kinases transphosphorylated BTK on tyrosine at residue 551, which led to BTK activation. BTK then autophosphorylated at a second site. The same two sites were phosphorylated upon B cell antigen receptor cross-linking. The activated BTK was predominantly membrane-associated, which suggests that BTK integrates distinct receptor signals resulting in SRC kinase activation and BTK membrane targeting.

BTK is a member of the BTK/TEC family of nonreceptor tyrosine kinases (NRTKs) (*1*–*3*). These proteins are distinct among NRTKs in containing conserved NH_2-terminal regions consisting of a pleckstrin homology (PH) domain and a proline-rich sequence, in addition to their conserved SRC homology 2 (SH2), SH3, and kinase domains. Deficient function of BTK is responsible for both human X-linked agammaglobulinemia (XLA) and murine X-linked B cell immunodeficiency (XID) (*1*, *4*–*6*). The sequelae of deficient BTK function suggest that a BTK-dependent signal is required for the expansion, functional maturation, or both of B cell progenitors (pro-B) (*7*). A diverse group of receptors is capable of activating BTK (*8*–*10*). Knowledge of how these receptors control BTK activity is necessary for understanding BTK-dependent signaling and how specific BTK mutations can interrupt these events.

Several lines of evidence implicate SRC family kinases in receptor-mediated activation of BTK, including activation kinetics (*8*), studies with LYN$^{-/-}$ mice (*11*), and in vitro interaction studies (*12*). In an effort to synthesize these data, we studied the consequences of coexpression of BTK and LYN. We used vaccinia-driven expression of BTK, LYN, or both in a B cell line transformed with Epstein-Barr virus (EBV) and deficient in BTK mRNA and protein production (Fig. 1A). Coexpression of LYN and BTK increased the tyrosine phosphorylation of BTK five times relative to that present after expression of BTK alone (Fig. 1A). These results were comparable to the 5 to 10 times increase in BTK tyrosine phosphorylation that follows cross-linking of the high-affinity immunoglobulin E (IgE) receptor (FcεRI) on mast cells, membrane IgM on B cells, and the interleukin-5 (IL-5) receptor on pro-B cells (*8*, *9*). To eliminate the potential contribution of other B cell–specific signaling pathways, endogenous LYN, or regulation through hematopoietic phosphatases, we coexpressed BTK and LYN in a nonhematopoietic cell line. Coexpression increased both tyrosine phosphorylation of BTK and BTK enzymatic activity 5 to 10 times in multiple experiments (Fig. 1B), a significantly greater magnitude than that seen after receptor-mediated activation of BTK (*8*, *9*). Coexpression of the SRC family kinase, FYN, with BTK resulted in similar increases in both BTK tyrosine phosphorylation and enzymatic activity (*13*). In contrast, coexpression of the SYK tyrosine kinase and BTK resulted in no significant increase in BTK tyrosine phosphorylation or activity (*13*, *14*). Coexpression of BTK and LYN therefore reproduces the changes in BTK tyrosine phosphorylation and activation that follow hematopoietic receptor stimulation and provides a simplified system for the study of BTK activation.

We used this system to determine whether the tyrosine kinase activities of LYN or BTK were necessary for the LYN-dependent phosphorylation of BTK. Coex-

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