

Expression of Functional TrkA Receptor Tyrosine Kinase in the HMC-1 Human Mast Cell Line and in Human Mast Cells

By See-Ying Tam, Mindy Tsai, Masao Yamaguchi, Koji Yano, Joseph H. Butterfield, and Stephen J. Galli

Nerve growth factor (NGF) can influence mast cell development and function in murine rodents by interacting with its receptors on mast cells. We now report the identification of mRNA transcripts of full-length tyrosine kinase-containing *trkA*, *trkB*, and *trkC* neurotrophin receptor genes in HMC-1 human mast cell leukemia cells. Although HMC-1 cells lacked p75 mRNA, they expressed transcripts for the exon-lacking splice variant of *trkA* (*trkAI*), truncated *trkB* (*trkB.T1*), and truncated *trkC*. By flow cytometry, HMC-1 cells exhibited expression of TrkA, TrkB, and TrkC receptor proteins containing full-length tyrosine kinase domains. NGF stimulation of HMC-1 cells induced tyrosine phosphorylation of TrkA protein, increased expression of the early response genes *c-fos* and NGF1-A, and activation of ERK-mitogen-activated protein (MAP) kinase, results which indicate that TrkA receptors in HMC-1 cells are fully functional. Highly purified populations of human lung mast cells expressed mRNAs for *trkA*, *trkB* and *trkC*, whereas preparations of human umbilical cord

blood-derived mast cells expressed mRNAs for *trkA* and *trkC*, but not *trkB*. Moreover, preparations of human umbilical cord blood-derived immature mast cells not only expressed mRNA transcript and protein for TrkA, but exhibited significantly higher numbers of chymase-positive cells after the addition of NGF to their culture medium for 3 weeks. In addition, HMC-1 cells expressed mRNAs for NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), the cognate ligands for TrkA, TrkB, and TrkC, whereas NGF and BDNF transcripts were detectable in human umbilical cord blood mast cell preparations. Taken together, our findings show that human mast cells express a functional TrkA receptor tyrosine kinase and indicate that NGF may be able to promote certain aspects of mast cell development and/or maturation in humans. Our studies also raise the possibility that human mast cells may represent a potential source for neurotrophins.

© 1997 by The American Society of Hematology.

NERVE GROWTH FACTOR (NGF) represents the prototype for a family of related neurotrophic factors known as the neurotrophins, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5).¹ The neurotrophins are essential for the growth, differentiation, and survival of many neurons in the developing nervous system and for the maintenance of some neurons in the mature nervous system.² Although neurotrophins are thought to exert their actions predominantly on neurons, some neurotrophins, particularly NGF, can also influence the development and activation of many hematopoietic cell types, including basophils and mast cells.³⁻⁶

Mast cells are derived from hematopoietic precursors and represent critical effector cells in allergic diseases and other IgE-dependent responses.⁷⁻⁹ Several lines of evidence indicate that NGF can influence rat and mouse mast cell development and function. In neonatal rats, subcutaneous injection of NGF can induce mast cell hyperplasia and hypertrophy in many tissues and organs,^{6,10,11} and mast cell numbers can be greatly decreased in rats by antibodies to NGF.¹¹ In the presence of IL-3, NGF can promote colony formation and induce maturation of mouse bone marrow-derived cultured mast cells *in vitro*.¹² NGF can also promote the survival of rat peritoneal mast cells (PMCs) by suppressing apoptosis.^{13,14} Moreover, *in vitro* studies have indicated that NGF can induce degranulation and mediator release from rat PMCs.¹⁵⁻¹⁸ Finally, a recent report has demonstrated that rat PMCs can synthesize, store, and spontaneously release biologically active NGF.¹⁹ Taken together, these findings indicate that rat mast cells represent a potential source of NGF, and that neurotrophins may mediate autocrine and/or paracrine effects on rat and mouse mast cell development and function.

All neurotrophins can interact with two distinct types of cell surface receptors to exert their biologic effects. The low-affinity neurotrophin receptor, known as p75, binds all neurotrophins and appears to mediate a number of diverse functions in neurons, such as increasing the high-affinity binding of NGF, promoting the retrograde transport of neuro-

trophins, increasing the discrimination of ligand, and regulating NGF-dependent apoptosis.²⁰ By contrast, the high-affinity neurotrophin receptors have been identified as the Trk family of receptor tyrosine kinases. NGF, BDNF, and NT-3 are primary ligands for TrkA, TrkB, and TrkC, respectively, whereas TrkA and TrkB also serve as receptors for NT-3 and NT-4/5.²¹

Mature rat mast cells, such as PMCs, express TrkA, but not p75.^{14,18} The TrkA detected in these cells is functional and appears to interact specifically with NGF to initiate relevant signaling responses, such as tyrosine autophosphorylation and the induction of expression of early response genes.^{13,14,18} On the other hand, cultured mouse mast cells maintained in interleukin-3 (IL-3) express gene transcripts for both TrkA and p75,²² suggesting that the pattern of mRNA expression of these two types of NGF receptors can differ among distinct mast cell preparations, or according to species.

To begin to characterize the pattern of expression of neurotrophin receptors in human mast cells, we analyzed the human mast cell line, HMC-1. HMC-1 cells, which are de-

From the Departments of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA; and the Division of Allergic Diseases, Department of Internal Medicine, Mayo Clinic, Rochester, MN.

Submitted November 15, 1996; accepted April 22, 1997.

Supported in part by United States Public Health Service Grants No. CA/AI-72074, AI/CA-23990, and HL-56383 (Project 4) (to S.J.G.) and by the Beth Israel Hospital Pathology Foundation (Boston, MA).

Address reprint requests to See-Ying Tam, PhD, Department of Pathology, Research North, Beth Israel Deaconess Medical Center-East, 330 Brookline Ave, Boston, MA 02215.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1997 by The American Society of Hematology.
0006-4971/97/9005-0017\$3.00/0

rived from a patient with mast cell leukemia,²³ express many of the features of immature mast cells, and in some respects, resemble the mouse "pro-mastocyte."²⁴ We found that HMC-1 mast cells expressed the gene transcripts and the proteins of TrkA, TrkB, and TrkC receptors, but lacked expression of p75 mRNA. Moreover, the TrkA receptors present in HMC-1 cells could function to initiate signaling responses. In addition, we detected mRNA transcripts for *trkA*, *trkB*, and *trkC* in highly purified human lung mast cells. We also demonstrated that human umbilical cord blood-derived mast cells can express the transcript and protein for TrkA receptor, and showed that treatment with NGF *in vitro* can significantly increase the number of chymase-positive cells in such mast cell population. Finally, we found that HMC-1 cells expressed mRNAs for NGF, BDNF, and NT-3, and that human umbilical cord blood-derived mast cell preparations expressed mRNAs for NGF and BDNF. Some of our results have been previously reported in abstract form.²⁵

MATERIALS AND METHODS

Culture of human cell lines and sources of animal tissues. The human mast cell line HMC-1 was originally established from the peripheral blood of a patient with mast cell leukemia.²³ HMC-1 cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and L-glutamine. K-562, a human chronic myelogenous leukemia cell line, and Hs294T, a human melanoma cell line, were obtained from American Type Culture Collection (ATCC) (Rockville, MD). K-562 cells were grown in RPMI medium supplemented with 10% FCS, whereas Hs294T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose supplemented with 10% FCS. The fetal monkey mesencephalon was dissected from coronal sections of a brain removed from an African green monkey fetus (St Kitts Biochemical Research Foundation, St Kitts) at 150 days of gestation.²⁶

Culture of human umbilical cord blood-derived mast cells. Normal human mast cells were generated as previously described by Saito et al²⁷ with slight modifications. Briefly, mononucleated cells were isolated from human umbilical cord blood (Advanced Biotechnologies, Columbia, MD) by centrifugation over Histopaque-1077 (Sigma Chemical, St Louis, MO), and were then cultured in Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), *E coli*-derived recombinant human stem cell factor (SCF, at 80 ng/mL, generously provided by Dr Keith E. Langley, AMGEN Inc, Thousand Oaks, CA), recombinant human IL-6 (50 ng/mL, AMGEN), and prostaglandin E₂ (PGE₂) (1 μmol/L; Cayman Chemical, Ann Arbor, MI). The culture medium was changed weekly, and the cells were maintained for 12 to 23 weeks. For sensitizing cells with IgE, cells were cultured in 24-well culture plate with human myeloma IgE (Biodesign International, Kennebunk, ME) at a cell density of 2 × 10⁵/mL.

Preparation of 99% pure human lung mast cells. Lung mast cell purification was performed using gradient centrifugation and magnetic cell separation systems.²⁸ Briefly, lung mast cells were dispersed from ~12.0 g of macroscopically normal human lung tissue obtained from patients undergoing partial pneumonectomy for lung cancer. The lung tissue was cut into strips with scissors, washed extensively in calcium- and magnesium-free Tyrode's buffer, and then incubated in 0.5 mg/mL collagenase (type I, Sigma) in the same buffer for 45 minutes at 37°C. Cells dispersed by this procedure were separated from undissociated tissue by subsequent filtration through 800 μm and 80 μm pore-sized nylon gauze and washed twice with the buffer by centrifugation at 500g for 10 minutes at room temperature. The numbers and percentage of mast cells were

assessed by counting in a hemocytometer after metachromatic staining with Kimura stain.²⁹ In a typical experiment, mast cells obtained at this stage were ~1.58 × 10⁶/g of wet lung tissue and the purity was 9.7%. Mast cells were enriched by centrifugation through a Percoll gradient. The dispersed lung cell suspension was overlaid onto 30%/80% isotonic Percoll layers and was centrifuged at 500g, 4°C for 20 minutes. The interface was harvested and washed with four to five times volume excess of calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) supplemented with 2% FCS. Mast cells at this step represented a purity of 30.9% and 1.08 × 10⁶ cells/g. Cells were cultured overnight in RPMI 1640 containing 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

The next day, cells were washed twice and were resuspended in 2 mL of phosphate-buffered saline (PBS) containing 5 mmol/L EDTA and 0.5% bovine serum albumin (BSA) (PBS/BSA), 8% rat serum, 50 μg/mL human IgG for 15 minutes at 4°C (to help reduce any nonspecific binding of antibodies). The cells were then pelleted and resuspended in 15 μg/mL SR-1 monoclonal mouse IgG_{2a} antibody against human c-kit (provided by Dr Keith Langley) for 15 minutes at 4°C. After being washed, cells were incubated with 15 μg/mL of rat-antimouse-IgG_{2a+b} antibody conjugated with colloidal microbeads (Miltenyi Biotec, Sunnyvale, CA) in PBS/BSA for 30 minutes at 4°C. The c-kit-positive cells were recovered using MACS cell separation system, according to the manufacturer's specifications (Miltenyi Biotec), yielding preparations of >99% mast cells according to staining with Kimura stain or with a mouse monoclonal antibody against human mast cell tryptase (see below).

Stimulation of human mast cells with anti-IgE. Cells were sensitized overnight with 1 μg/mL of human myeloma IgE (Biodesign International). They were then washed twice and stimulated with goat antihuman IgE (1 μg/mL, Sigma) or with vehicle alone for 2 hours at 37°C (1.5 × 10⁵ cells in 500 μL) in HBSS supplemented with 0.4 mmol/L MgCl₂, 1.0 mmol/L CaCl₂, and 0.1% BSA. The specimens were washed with ice-cold HBSS/0.1% BSA and resuspended in an adequate amount of buffer. Cells were pelleted again and stored at -80°C until RNA was extracted.

Immunocytochemical analysis of human cord blood-derived mast cells. Immunocytochemical identification of human mast cell tryptase and chymase was performed as reported by Saito et al with slight modifications.²⁷ Briefly, cytospin preparations were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature, and washed in Tris-buffered saline (TBS). After incubation with 5% rabbit serum in TBS for 20 minutes, cells were incubated with a mouse monoclonal IgG_{1κ} antibody against either human mast cell tryptase or human mast cell chymase (Chemicon International, Temecula, CA), or a control mouse IgG_{1κ} antibody (MOPC21, Sigma) at 2 μg/mL for 1 hour at room temperature. Slides were then washed and incubated with rabbit antimouse IgG (APAAP kit, Dako, Carpinteria, CA) for 1 hour. After washing, slides were incubated with alkaline phosphatase-antimouse alkaline phosphatase complex (APAAP) (Dako) for 1 hour. Cells were then reacted with red substrate (Fast Red TR/Naphthol AS-MX with 1 mmol/L levamisole; Sigma) for 5 minutes, counterstained with hematoxylin, and covered with CrystalMount (Biomed, Foster City, CA). From each slide, 500 cells were analyzed for chymase using light microscopy by an observer who was unaware of the identity of the individual slides, and the strength of chymase positivity was graded from negative (-) to strongly positive (+++). The distribution of mast cells exhibiting various intensities of chymase positivity (- to +++) in individual specimens was compared using the χ² test.

Cytokines and antibodies. Purified mouse 2.5S nerve growth factor was purchased from Upstate Biotechnology (Lake Placid, NY). For flow cytometry and Western blotting experiments, specific affinity-purified rabbit anti-TrkA (Product No. 763), anti-TrkB (794),

and anti-TrkC (798) polyclonal antibodies and their respective control peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These three Trk antibodies were raised against three different polypeptides, each comprised of 15 nonhomologous amino acids present in the tyrosine kinase domain of each of the three Trk receptors. These antibodies recently have been used to characterize Trk receptors in human transformed B lymphocytes by flow cytometry³⁰; this study also showed that these antibodies could identify TrkA, TrkB, and TrkC proteins in PC12 cells and rat embryonic dorsal root ganglia by Western blotting and in human dorsal root ganglia and sural nerve biopsy specimens using immunohistochemistry.³⁰

Reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern analysis. Total RNA was extracted from cultured cells and from fetal monkey midbrain using the RNazolB method according to the manufacturer's specifications (Biotech Laboratories, Houston, TX). Human substantia nigra poly A+ mRNA was purchased from Clontech Laboratories (Palo Alto, CA). Approximately 5 μ g of total RNA, or \sim 0.1-0.5 μ g of heparinase-treated total RNA (for cultured and purified human mast cells),³¹ or 0.5 μ g of poly A+ mRNA, was reversed transcribed with random hexamers using the StrataScript RNaseH⁻ reverse transcriptase (Stratagene, La Jolla, CA) at 37°C for 60 minutes. The cDNA synthesized was subsequently amplified in a 100- μ L volume reaction mixture by a DNA thermal cycler for 30 seconds at 96°C, 2 minutes at 55°C, and 3 minutes at 72°C for 38 thermocycles followed by an additional 7 minutes at 72°C using 2.5 U of *Ampli*Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR products were analyzed on 1.5% agarose gels, and then transferred onto Zetabind nylon membrane (Cuno, Meriden, CT). Blots were hybridized at 42°C and washed at 58°C to a final stringency of 0.1 \times SSC.

Full-length isoforms of *trk* transcripts. To amplify simultaneously *trkA*, *trkB*, and *trkC* cDNAs that contain the full-length tyrosine kinase domains, we used two degenerate oligo primers (A and B) corresponding to the highly conserved regions of the extracellular domain [PTH(Y,M,V)NNG(N,D)YTL] and of the tyrosine kinase domain (EGAFGKVFLEAEC) shared among human TrkA, mouse TrkB, and porcine TrkC receptor proteins.³²⁻³⁴ The sequences of the primers are: Primer A, 5'-ATGGATCCAC(CT)CA(CT)(TGA)(AT)(CG)AA(CT)AA(CT)GG(CA)(AG)ACTACAC(GAC)CT-3' (sense); Primer B, 5'-TCTCGAGCACTC(GCA)GC(CTA)AGGAA(GA)AC(CT)TTCCCAAAGGC(TG)CC(CT)TC-3' (antisense). To characterize the different splice variants of *trkA* gene transcripts, PCR was performed using a pair of primers (C and D) flanking the putative alternative splicing region of human *trkA* mRNA.¹⁸ Primer C: 5'-ATGGACAACCCTTCGAGTTCAAC-3' (5' corresponding to residue 1219); Primer D: 5'-GACCCCAAAGGTGTTTCGTCC-3' (3' corresponding to residue 1299).

Truncated isoforms of *trkB* and *trkC* transcripts. To amplify cDNAs encoding human truncated TrkB (TrkB.T1) by PCR, primers E and F, which are specific for human truncated *trkB*.T1 sequence, were used.³⁵ Primer E: 5'-ATGGGCTGGCCTGGAATTGACGAT-3' (5' corresponding to residue 1249); Primer F: 5'-CATCAACCAACAAGCACCACAGCC-3' (3' corresponding to residue 1567 of human *trkB*.T1). To amplify cDNAs encoding human truncated TrkC by PCR, specific human *trkC* primers G and H were used.³⁵ Primer G: 5'-AGATGCCATGGTTAAGAGGCTTGG-3' (5' corresponding to residue 1162); Primer H: 5'-CCACTGGGCACAGCC-AACCAGACC-3' (3' corresponding to residue 1751 of human truncated *trkC*).

p75. To detect human p75 mRNA transcripts by PCR, primers R and S, which are specific for human p75 cDNA sequence, were used.³⁶ Primer R: 5'-ACAGGCCTGTACACACACAGC-3' (5' corresponding to residue 213); Primer S: 5'-GCCAGGGATCTCCTCGACTC-3' (3' corresponding to residue 672).

NGF, BDNF, and NT-3. Human NGF, BDNF, and NT-3 genes lack any introns.³⁷ Accordingly, it was crucial for valid RT-PCR analysis of the corresponding transcripts that the total RNA isolated from the HMC-1 cells and human umbilical cord blood-derived mast cells be treated with DNase I (RQ1 RNase-free DNase) (Promega, Madison, WI) at 37°C for 30 minutes, at 1 U/10 μ g total RNA, to eliminate any contamination by genomic DNA. The DNase I-treated RNA was subsequently reverse transcribed as described above (human umbilical cord blood-derived mast cell RNA was subjected to an additional treatment with heparinase I before the reverse transcription procedure), and the resulting cDNA was amplified by PCR using native *Pfu* DNA polymerase (Stratagene) with three different sets of gene-specific primers as listed in the following³⁷⁻³⁹: NGF, Primer T: 5'-TCATCATCCCATCCCATCTTCCAC-3' (5' corresponding to residue 1825); Primer U: 5'-CACAGCCTTCCTGCTGAGCACACA-3' (3' corresponding to residue 2144). BDNF, Primer V: 5'-GACCCTGCCGCCGAGGGGAG-3' (5' corresponding to residue 466); Primer W: 5'-AGTGTCTATCCTTATGAA-TCGCC-3' (3' corresponding to residue 764). NT-3, Primer X: 5'-CGGTACGCGGAGCATAAGAGTCAC-3' (5' corresponding to residue 487); Primer Y: 5'-TCCGATTTTTCTCGACAAGGCACA-3' (3' corresponding to residue 817).

cDNA probes. The full-length human *trkA* (ATCC# 41055) and mouse *zif/268* cDNA (ATCC# 63027) clones were obtained from ATCC. The *c-fos* probe is a 1.2-kb *HindIII/EcoRI* mouse cDNA fragment kindly provided by Dr Brent Cochran (Massachusetts Institute of Technology, Cambridge). The partial cDNA probes for monkey *trkB*, *trkC*, and p75 were generated by RT-PCR using fetal monkey midbrain total RNA and primers A and B (*trkB* and *trkC*) or primers R and S (p75) as previously described.²⁶ The authenticity of the PCR-amplified monkey cDNAs was verified by subcloning the DNA fragments into pGEM-T Vector (Promega) and subsequent DNA sequencing analysis using Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH).²⁶ Similarly, the partial cDNA probes for human NGF and NT-3 were generated by RT-PCR of human brain poly A+ mRNA using *Pfu* DNA polymerase (Stratagene) and primers T and U (NGF) or primers X and Y (NT-3). The human BDNF probe was generated by RT-PCR of DNase I-treated RNA from HMC-1 cells using *Pfu* DNA polymerase and primers V and W. The amplified DNA fragments of the predicted sizes were subcloned into pCR-Script SK(+) vector (Stratagene), and subsequent DNA sequencing showed that they contained authentic human cDNA sequences for NGF, BDNF, and NT-3. The cDNA probes were labeled with [α -³²P] deoxycytidine triphosphate (New England Nuclear, Boston, MA) using the *exo(-)* Klenow fragment of DNA polymerase I with random nonamer primers (Stratagene).

Northern analysis. Total RNA (20 μ g/lane) was isolated by the RNazolB method, electrophoresed in 1% agarose-formaldehyde denaturing gel, and transferred onto Zetabind nylon membrane. RNA blots were hybridized at 42°C, and washed at 42°C to a final stringency of 0.2 \times SSC.

Flow cytometry. Approximately 1.0 \times 10⁶ HMC-1 cells, or human umbilical cord blood-derived mast cells, were fixed with a 1:1 mixture of methanol/ethanol for 30 minutes at 4°C and subsequently incubated with the primary anti-TrkA, anti-TrkB, or anti-TrkC antibody at 10 μ g/mL for 30 minutes at 4°C. After three consecutive washes with PBS containing 1% BSA (pH 7.3) and 15% goat serum, cells were incubated with a fluorescein-conjugated goat F(ab')₂ anti-rabbit IgG (10 μ g/mL) (Santa Cruz Biotechnology). Cells were resuspended in PBS and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Negative controls were cells incubated with a rabbit IgG preparation (10 μ g/mL) (Sigma) instead of a specific primary antibody. We also assessed the specificity of staining by preincubation each anti-Trk antibody for 10 minutes before using it for flow cytometry analysis, with 0.02 to 20 μ g/mL

of either a peptide representing the appropriate Trk receptor or a peptide representing an inappropriate Trk receptor (Santa Cruz Biotechnology).

TrkA immunoprecipitation and Western blotting. Approximately 2.0×10^7 cells were lysed in 1.0 mL of ice cold RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.1 mg/mL phenylmethylsulfonyl fluoride, 63 μ g/mL aprotinin, 1 mmol/L sodium orthovanadate) by passage through 21-gauge needles. The lysate was cleared by centrifugation, and incubated with 2 μ g of anti-TrkA antibody (763) for 3 hours at 4°C, and subsequently incubated with 20 μ L of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) with gentle mixing overnight at 4°C. Immunoprecipitates were washed four times with RIPA buffer, subjected to 6% SDS-polyacrylamide gel electrophoresis, and then electroblotted onto a Hybond-ECL nitrocellulose membrane (Amersham, Arlington Heights, IL). Membranes were incubated in blocking buffer (0.01 mol/L Tris, pH 7.5, 0.1 mol/L NaCl, 0.1% Tween 20, 1% BSA) at 37°C for 20 minutes and then immunoblotted with a horseradish peroxidase-conjugated recombinant antiphosphotyrosine antibody RC20 (Transduction Laboratories, Lexington, KY) at 1:2,500 dilution at 37°C for 20 minutes. The phosphorylated proteins were detected using an enhanced chemiluminescent detection method (Amersham).

Mitogen-activated protein (MAP) kinase activity. Previous studies from our laboratory have shown that ERK1-pp44 and ERK2-pp42 MAP kinases are coordinately regulated in mouse mast cells that are stimulated with SCF or IgE and antigen.⁴⁰ Therefore, only pp44 MAP kinase activity was measured in this study. ERK1-MAP kinase pp44 was partially purified from ERK1 ImmunoCruz System (Santa Cruz Biotechnology), which consists of an immunoaffinity column containing ERK1 rabbit polyclonal IgG antibody coupled to an agarose matrix, as described in the instruction manual supplied by the manufacturer. The eluted antigen was assayed for phosphorylation of myelin basic protein (Santa Cruz Biotechnology) using a substrate peptide kinase assay as previously described.⁴¹ Briefly, 30 μ L of the eluted antigen was incubated with 25 mmol/L Tris-HCl (pH 7.5), 1 mmol/L MgCl₂, 50 μ mol/L adenosine triphosphate (ATP), 2.5 μ g of myelin basic protein, and 1 μ Ci of [γ -³²P]ATP in a reaction volume of 50 μ L. After incubation at 30°C for 15 minutes, 25 μ L of the reaction was spotted onto a 1.5 \times 1.5 cm P-81 cellulose phosphate paper (Life Technologies, Gaithersburg, MD) and washed twice with 1% phosphoric acid and then water before quantitation of radioactivity by liquid scintillation counting. The protein concentration for each eluted antigen sample was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), and used to calculate specific activities (cpm/ μ g protein).

RESULTS

HMC-1 cells express mRNA transcripts for *trkA*, *trkB*, and *trkC* that contain the full-length tyrosine kinase domains. Recent studies have indicated that mRNA for *trkA*, but not *trkB* or *trkC*, is detectable in rat and mouse mast cells.^{18,22} To assess the pattern of expression of *trk* mRNAs in human mast cells, we first searched for the presence of mRNA transcripts encoding the tyrosine kinase-containing isoforms of different members of the Trk receptor family in HMC-1 mast cells.

RT-PCR was performed on HMC-1 RNA with two degenerate oligo primers, A and B, which correspond to highly conserved regions of the extracellular domain and of the tyrosine kinase domain shared among human TrkA, mouse TrkB, and porcine TrkC receptor polypeptides.³²⁻³⁴ These two primers have previously been shown to be effective

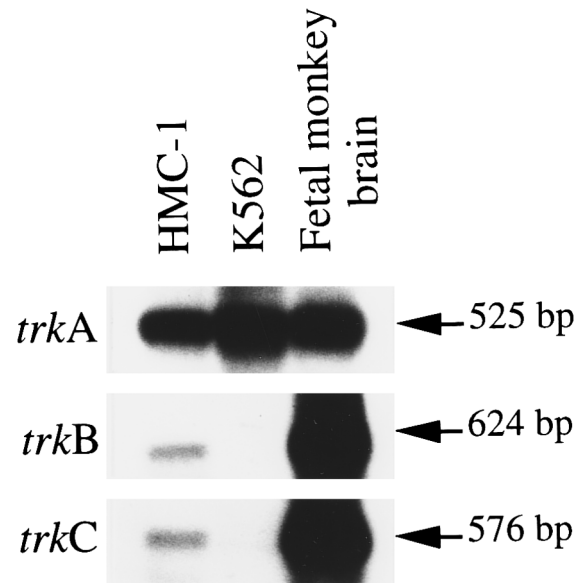


Fig 1. Expression of mRNA transcripts encoding *trkA*, *trkB*, and *trkC* genes in the HMC-1 mast cell line, the K-562 chronic myelogenous leukemia cell line, and fetal monkey brain tissue. The cDNA was synthesized from total RNA by reverse transcriptase and amplified by PCR using two degenerate oligonucleotide primers A and B. The identity of the PCR products was confirmed by Southern blot analysis using ³²P-labeled human *trkA*, monkey *trkB*, and monkey *trkC* probes.

in simultaneously amplifying partial *trkA*, *trkB*, and *trkC* sequences by PCR with fetal monkey brain cDNAs.²⁶ The partial monkey *trkB* and *trkC* sequences amplified are highly homologous to their human counterparts,^{26,35} and thus were used as cDNA probes in our subsequent Southern hybridization studies. Southern analysis of the PCR products using human *trkA* and monkey *trkB* and *trkC* cDNAs as probes detected strong signals for the mRNA expression of *trkA* (predicted size 525 bp) in HMC-1 cells and in fetal monkey brain (Fig 1). Moreover, we could detect weaker, but consistently reproducible, hybridization signals indicating the expression of mRNAs for *trkB* (predicted size 624 bp) and *trkC* (predicted size 576 bp) in HMC-1 cells. These hybridization signals did not appear to result from nonspecific cross-hybridization of different *trk* cDNA probes with the HMC-1 *trk* gene messages, as illustrated by the observation that the human K-562 cell line, which is known to express exclusively the TrkA receptor,³² exhibited no detectable expression of *trkB* and *trkC* mRNAs (Fig 1). Moreover, the *trkB* and *trkC* signals detected on the Southern blot exhibited the expected difference in their molecular weights (*trkB* > *trkC*) (Fig 1). The results shown in Fig 1 were obtained in at least four separate experiments.

It has been reported that two different splice variants of *trkA* mRNA transcripts are expressed in PC12 cells and rat PMC (*trkAI* and *trkAII*).^{18,42} Moreover, transfection studies in PC12^{nmr5} cells show that, in contrast to TrkAI receptors, TrkAII receptors exhibit enhanced functional responses to stimulation by the presumably noncognate ligand, NT-3.⁴³ To assess which of the two *trkA* transcripts was expressed in HMC-1 cells, PCR was performed using a pair of primers

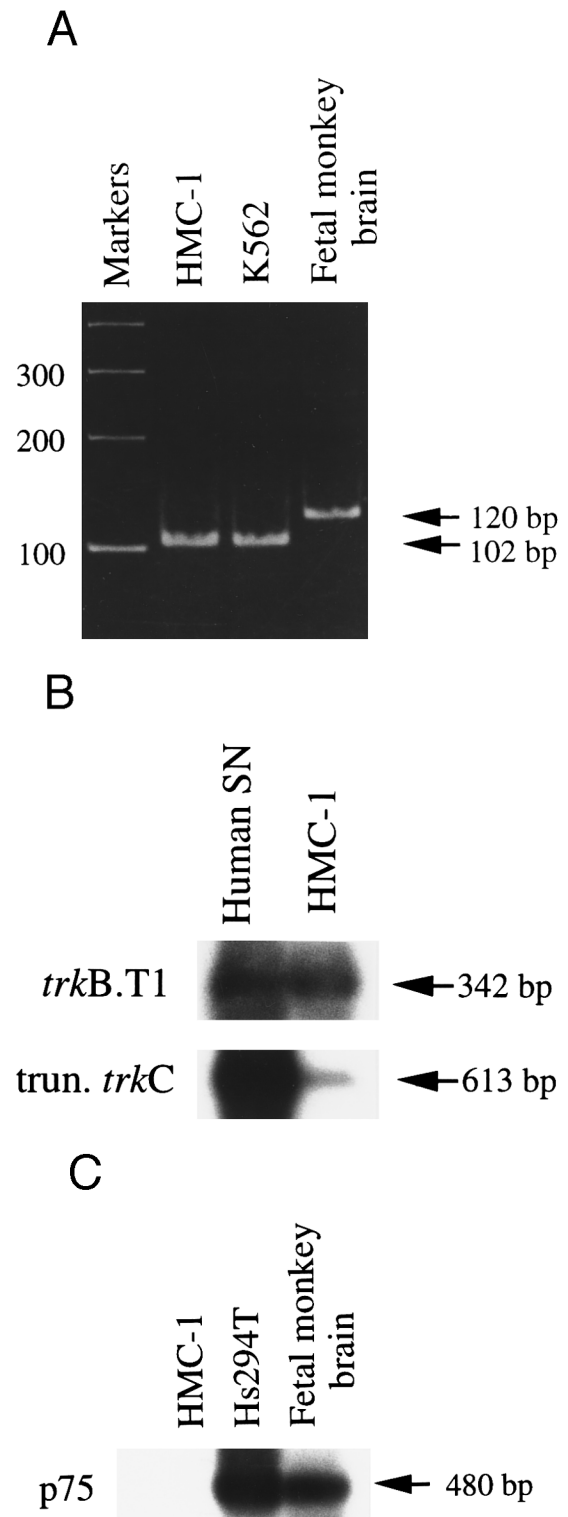
Fig 2. RT-PCR analysis to detect: (A) alternatively spliced *trkA* mRNA isoforms in HMC-1 cells, K-562 cells, and fetal monkey brain tissue, (B) the expression of truncated isoforms of *trkB* and *trkC* in human substantia nigra and HMC-1 cells, and (C) the expression of p75 mRNA transcripts in HMC-1 cells, Hs294T cells, and fetal monkey brain tissue. The cDNAs were synthesized from total RNA or poly A+ mRNA from each of the indicated cells or tissues by reverse transcriptase and amplified by PCR using primers C and D for *trkA* in (A), and primers E and F for *trkB.T1* in (B), and primers G and H for truncated *trkC* in (B), and primers R and S for p75 in (C). The PCR products were analyzed on a 5% nondenaturing polyacrylamide gel in (A). The identity of PCR products in (B) and (C) was confirmed by Southern blot analysis using ³²P-labeled monkey *trkB* and *trkC* cDNA probes in (B), and a monkey p75 cDNA probe in (C).

that flank the putative alternative splicing region of *trkA* mRNA.¹⁸ As shown in Fig 2A, the 120-bp band amplified from the monkey brain represents the exon-containing form (*trkAII*), whereas the HMC-1 cells and the K-562 cells appeared to express exclusively the exon-lacking form (*trkAI*), as represented by the 102-bp amplified fragment. To confirm the identities of these DNA fragments, DNA gel bands amplified from HMC-1 cells and fetal monkey brain were purified and subcloned into pGEM-T Vector. Our DNA sequencing analysis confirmed that the HMC-1 cells expressed the *trkAI* isoform, whereas the *trkAII* isoform was found in the specimen from fetal monkey brain.

HMC-1 cells express truncated isoforms of trkB and trkC transcripts. Isoforms of *trkB* and *trkC* transcripts encoding truncated tyrosine kinase domains have been identified in rats and in humans.^{35,44,45} To assess if these isoforms were expressed in HMC-1 cells, RT-PCR was performed with PCR primers specific for the previously described human truncated *trkB* (*trkB.T1*) and *trkC* cDNAs.³⁵ As shown in Fig 2B, which shows human substantia nigra mRNA as a positive control, Southern analysis using partially overlapping monkey *trkB* and *trkC* probes indicated that both truncated *trkB* (predicted size 342 bp) and *trkC* (predicted size 613 bp) transcripts were detectable in the HMC-1 cell line. These results were obtained in two separate experiments.

p75. It has been reported that rat PMCs lack expression of both mRNA and protein of the low-affinity neurotrophin receptor, p75,¹⁸ whereas p75 mRNA is detectable in mouse mast cells that have been derived from the spleen and maintained in IL-3 in vitro.²² To assess whether HMC-1 cells expressed the p75 gene transcript, RT-PCR was performed with two oligo primers specific for the human p75 cDNA sequence.³⁶ As shown in Fig 2C, Southern analysis of the PCR products using monkey p75 cDNA as a probe showed no detectable signal of p75 message in HMC-1 cells. In contrast, positive controls such as the Hs294T cells and the fetal monkey brain exhibited strong signals of hybridization for p75 mRNA (predicted size 480 bp).

Expression of Trk proteins in HMC-1 cells. To assess whether the expression of *trk* gene transcripts detected in HMC-1 cells could be correlated with the expression of Trk receptor proteins, the cells were incubated with specific antibodies against various Trk peptides, and analyzed by flow cytometry. As assessed in six experiments, we found that the



HMC-1 cells exhibited positive labeling with TrkA, TrkB, or TrkC antibodies, indicating that the HMC-1 cells express proteins for all three types of Trk receptors (Fig 3). The staining obtained with each antibody appeared to be specific, as it could be competitively blocked in a concentration-de-

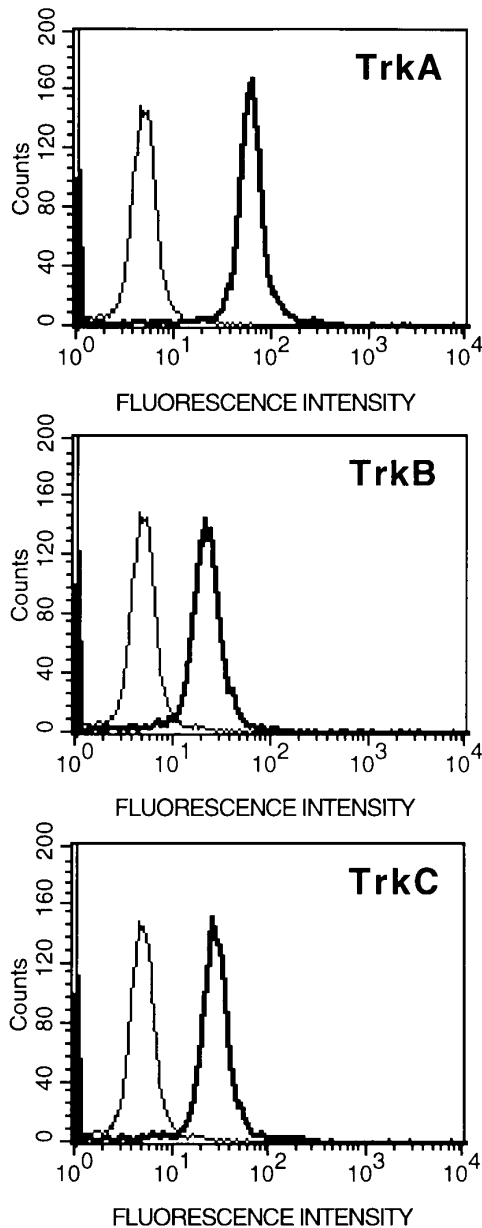


Fig 3. Flow cytometric analysis (relative log fluorescence) of expression of Trk proteins in HMC-1 mast cells. HMC-1 cells were incubated with an anti-TrkA, anti-TrkB, or anti-TrkC antibody (as indicated in the figures), or with rabbit IgG (negative control, the thin line in the figures), and subsequently stained with fluorescein-conjugated goat antirabbit IgG and analyzed by flow cytometry. Data are representative of six separate experiments.

pendent manner by preincubation with the appropriate Trk receptor control peptides, but not with the irrelevant peptides (data not shown). Furthermore, because these antibodies were raised to recognize the respective tyrosine kinase domains in the three Trk receptor proteins, these results also support the conclusion that the Trk receptors (A, B, and C) expressed in HMC-1 cells are of the full-length isoforms.

Induction of signaling responses by NGF in HMC-1 cells. The predominant expression of *trkA* (as opposed to *trkB* or

trkC) gene message and protein in HMC-1 cells is consistent with the possibility that the TrkA receptor tyrosine kinase may play a more significant role in mast cell development/function in humans than either the TrkB or TrkC receptors. However, normal human mast cells are usually available only in very small numbers, the amount of which would not be sufficient for functional analysis of TrkA at the cellular level. We, therefore, used HMC-1 cells to assess whether the TrkA receptors expressed by these cells are functional. Specifically, we examined the ability of NGF, the cognate ligand for TrkA, to initiate relevant intracellular signaling responses in these cells.

NGF stimulation of HMC-1 cells induces tyrosine phosphorylation of TrkA. In many different cell types that express the TrkA protein, including rat PMCs, exposure to NGF induces phosphorylation of the tyrosine residue on the TrkA receptor protein.^{14,18,21} To assess the phosphorylation of TrkA in response to NGF stimulation, HMC-1 cells were treated with NGF (200 ng/mL) for 5 or 10 minutes, and the cell lysates were immunoprecipitated with a specific anti-TrkA antibody. Immunoblot analysis of TrkA with a recombinant antiphosphotyrosine antibody showed that tyrosine phosphorylation of a protein of approximately 140 kD occurred 5 minutes after NGF treatment and that levels of the phosphorylated species decreased by 10 minutes after stimulation (Fig 4). The results shown in Fig 4 are representative of those obtained in four separate experiments. Similar results were also obtained for NGF-stimulated PC12 cells (50 ng/mL) using the same anti-TrkA antibody and identical immunoblotting procedures and reagents (data not shown).

Induction of expression of early response genes in HMC-1 cells by NGF. The induction of expression of early response genes such as *c-fos* and NGF1-A (*zif/268*) represents a marker for NGF signal transduction via the TrkA receptor tyrosine kinase in many cell types, including rat PMCs.^{13,46} To determine whether the TrkA receptor expressed in HMC-1 cells could interact with NGF to initiate a similar response, we examined the effects of NGF on the expression of early response genes by Northern analysis. HMC-1 cells were starved in RPMI medium with 0.1% FCS for 16 hours before NGF stimulation. Figure 5, which is representative of three

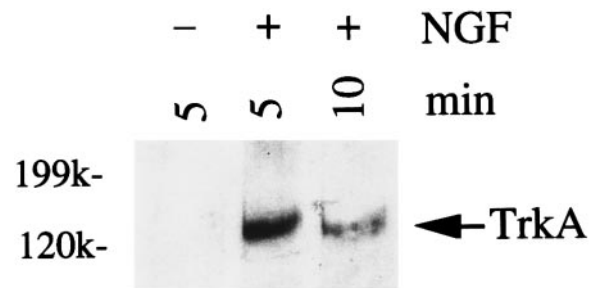


Fig 4. Western analysis of tyrosine phosphorylation of TrkA in HMC-1 mast cells stimulated with NGF. HMC-1 cells were stimulated with NGF (200 ng/mL) for 5 or 10 minutes. TrkA was immunoprecipitated with specific anti-TrkA antibody from lysates of NGF-treated (+) or untreated (-) cells, and the immunoprecipitates were probed with the RC20 antiphosphotyrosine antibody.

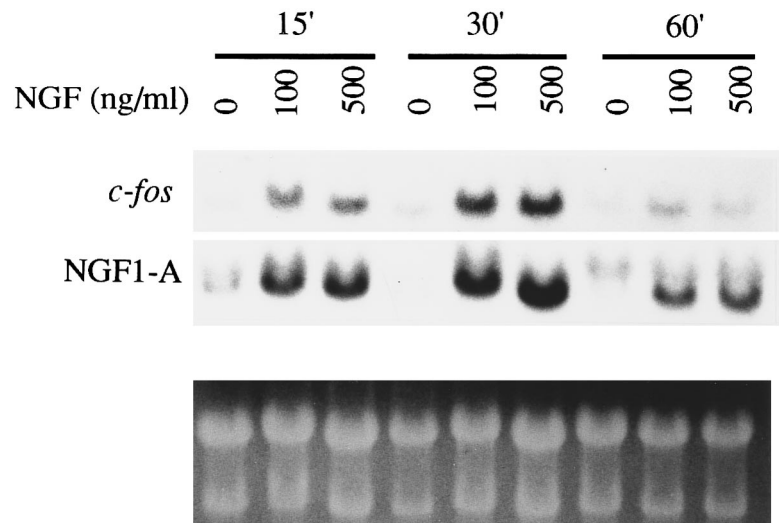


Fig 5. Induction of expression of early response genes by NGF in HMC-1 mast cells. HMC-1 cells were stimulated with NGF, at the concentrations indicated, for 15, 30, or 60 minutes. Total RNA was extracted and Northern blots were hybridized sequentially to mouse cDNAs of *c-fos* and *zif/268* (NGF1-A). Ethidium bromide staining was used to assess loading of RNA.

experiments that gave similar results, shows that NGF at 100 or 500 ng/mL induced increased expression of mRNAs for *c-fos* and NGF1-A at 15 and 30 minutes after stimulation, but the enhancement effects had diminished by 60 minutes after stimulation. The time course of this NGF effect in HMC-1 cells is reminiscent of that observed in rat PMCs stimulated with NGF.¹³ Lower doses of NGF (≤ 25 ng/mL) were found to be ineffective in eliciting a detectable early gene response in these HMC-1 cells (data not shown).

Activation of MAP kinase activity in HMC-1 cells by NGF. It has been shown in many cell types that the Ras/Raf/MAP kinase signaling pathway is activated in response to stimulation by many different growth factors, including NGF.⁴⁷⁻⁴⁹ To assess whether the activation of MAP kinases might represent part of the cascade of signal transduction initiated by NGF in mast cells, we examined the effects of NGF stimulation on MAP kinase activity in HMC-1 cells. The pp44 ERK-1 MAP kinase was partially purified from control and NGF-stimulated HMC-1 cells, and MAP kinase activity was quantified by a substrate peptide kinase assay using myelin basic protein as the substrate. Figure 6 shows the time course of NGF effects on MAP kinase activation in HMC-1 cells; the data shown were derived from four different experiments, all of which gave similar results. Stimulation of HMC-1 cells with NGF (100 ng/mL) significantly elevated pp44 MAP kinase activity (by threefold to sevenfold), as assessed at 5, 15, and 30 minutes after stimulation. However, by 60 minutes, the MAP kinase activity in HMC-1 cells had returned to basal levels.

Expression of different *trk* mRNA transcripts in human cord blood-derived mast cells and in mast cells purified from human lungs. To assess the physiological relevance of Trk receptor expression in the HMC-1 mast cell line, we performed RT-PCR using primers A and B, followed by Southern blot analysis, to examine the patterns of mRNA expression of different *trk* genes in human cord blood-derived mast cells that had been cultured in SCF, IL-6, and PGE₂ for 14 weeks (purity of 93% according to metachromatic staining with Kimura stain), and in human mast cells that had been

purified from human lungs (purity of 99% according to metachromatic staining with Kimura stain or immunocytochemistry for tryptase).

In our initial studies, two rounds of PCR with nested primers were necessary to yield a detectable *trkA* signal on a Southern blot of unstimulated human umbilical cord blood-derived mast cells (data not shown). However, when these cells were sensitized and stimulated with anti-IgE, weak signals for *trkA* and *trkC*, but not *trkB*, could be detected in these cells after one round of PCR amplification (data not

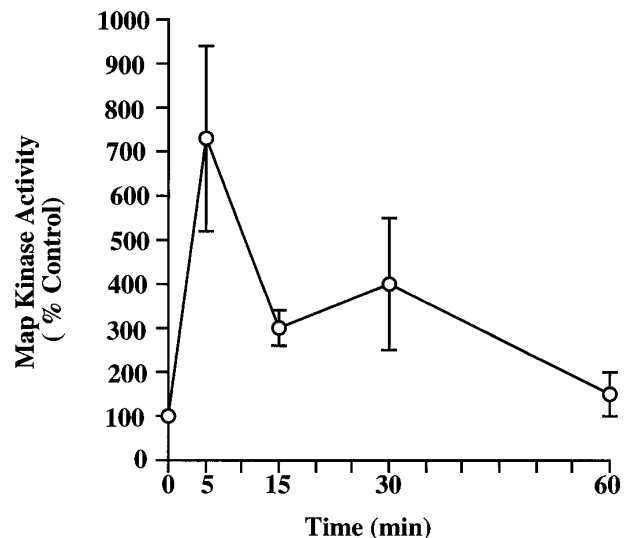


Fig 6. Kinetics of induction of MAP kinase activity by NGF in HMC-1 mast cells. HMC-1 cells were stimulated with NGF (100 ng/mL) for 5, 15, 30, or 60 minutes. pp44ERK-1 MAP kinase was partially purified from HMC-1 cells using an immunoaffinity column. MAP kinase activity of the eluted antigen was quantified by a substrate peptide kinase assay using myelin basic protein as the substrate, and the specific activity was expressed as cpm/ μ g eluted protein. The results shown are the mean \pm standard error of mean (SEM) of values obtained from four separate experiments.

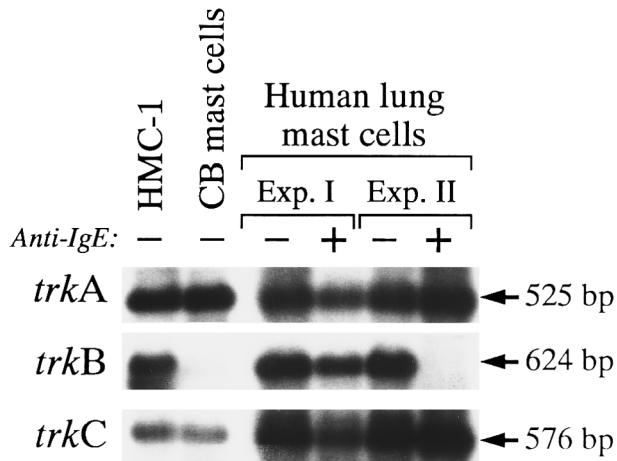


Fig 7. Expression of mRNA transcripts encoding *trkA*, *trkB*, and *trkC* in HMC-1 cells, human umbilical cord blood-derived mast cells, and mast cells purified from human lungs. The cDNA was synthesized from total RNA (HMC-1) or heparinase-treated total RNA (human umbilical cord blood-derived or lung mast cells) by reverse transcriptase and amplified by PCR using degenerate oligonucleotide primers A and B. The identity of the PCR products was confirmed by Southern blot analysis using ^{32}P -labeled human *trkA*, monkey *trkB*, and monkey *trkC* cDNA probes. (+) denotes specimens from cells that were sensitized with human IgE and subsequently stimulated with antihuman IgE, whereas (-) denotes specimens from cells not stimulated with antihuman IgE (see Materials and Methods for details).

shown). We speculated that the weak signals detected by RT-PCR for *trk* mRNA expression in these populations of mast cells might have reflected the low amounts of RNA that were extracted from these samples. In a subsequent study, better RNA yield was obtained from a population of unstimulated human umbilical cord blood-derived mast cells (23 weeks, 99% purity). RT-PCR and Southern analysis showed strong signals for the expression of *trkA* and *trkC*, but not *trkB*, in these mast cells (Fig 7).

On the other hand, consistently good RNA yields were obtained from samples of unstimulated mast cells purified from human lungs. When such samples were analyzed by RT-PCR, we found that robust hybridization signals were readily detected for *trkA*, *trkB*, and *trkC* messages (Fig 7). The intensities of the hybridization signals were comparable to those observed with HMC-1 cells (the two Southern blots illustrated in Fig 7 showing *trkB* and *trkC* expression had been highly overexposed as compared with those shown in Fig 1, which used fetal monkey brain as the positive controls). Interestingly, in one of two experiments (Experiment II), anti-IgE stimulation appeared to downregulate the expression of mRNA transcript for *trkB*, but not *trkA* or *trkC*, in purified human mast cells (Fig 7). Indeed, prolonged exposure of the Southern blot did not show any detectable signal for *trkB* message (the lack of *trkB* signal did not appear to occur as a result of poor PCR amplification, as *trkA* and *trkC* mRNAs could be detected using the same Southern blot with the same PCR amplification reaction mixture) (Fig 7).

These experiments were performed primarily to determine whether transcripts for TrkA, TrkB, or TrkC receptor were

detectable in purified preparations of normal human mast cells. However, our results also raise the possibility that anti-IgE stimulation of human lung mast cells can downregulate the cells' expression of *trkB* mRNA. Thus, the extent of specific histamine release (% release from anti-IgE-stimulated cells minus % release from cells without anti-IgE) detected in response to anti-IgE challenge was higher in Experiment II (~18% specific release) than in Experiment I (no specific release). This result suggests that the level of mast cell activation achieved in the second experiment was substantially greater than that in Experiment I.

Human umbilical cord blood-derived mast cells express functional TrkA receptor protein and respond to NGF stimulation by exhibiting increased expression of chymase. To assess the physiologic relevance of the expression of functional TrkA receptor protein in HMC-1 cells, we examined the expression of TrkA protein in human umbilical cord blood-derived mast cells (of 90% purity by Kimura stain or immunocytochemistry for tryptase) using flow cytometry. As shown in Fig 8, specific staining of these human mast cells with the TrkA antibody could be detected, indicating that these cells indeed express TrkA protein.

To assess further whether these TrkA receptors were functional, we examined the ability of NGF, the cognate ligand of TrkA, to influence the expression of chymase in these mast cells. We performed this study because of two preliminary reports: one of them indicated that the tryptase activity in HMC-1 cells was significantly increased after these cells had been cultured in the presence of NGF,⁵⁰ while the other report indicated that populations of human umbilical cord blood-derived mast cells, which had been cultured with NGF, contained more mast cells that expressed chymase, as well as tryptase, than did populations of control mast cells,

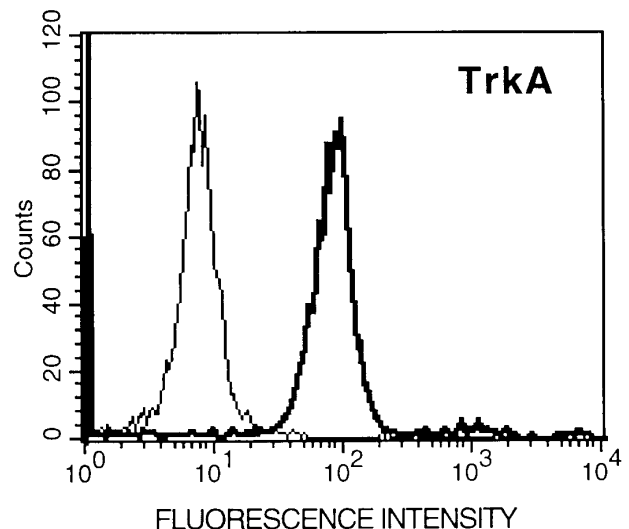


Fig 8. Flow cytometric analysis (relative log fluorescence) of expression of TrkA protein in human umbilical cord blood-derived mast cells. Human umbilical cord blood-derived mast cells were incubated with an anti-TrkA and subsequently stained with fluorescein-conjugated goat antirabbit IgG and analyzed by flow cytometry. Data are representative of two experiments.

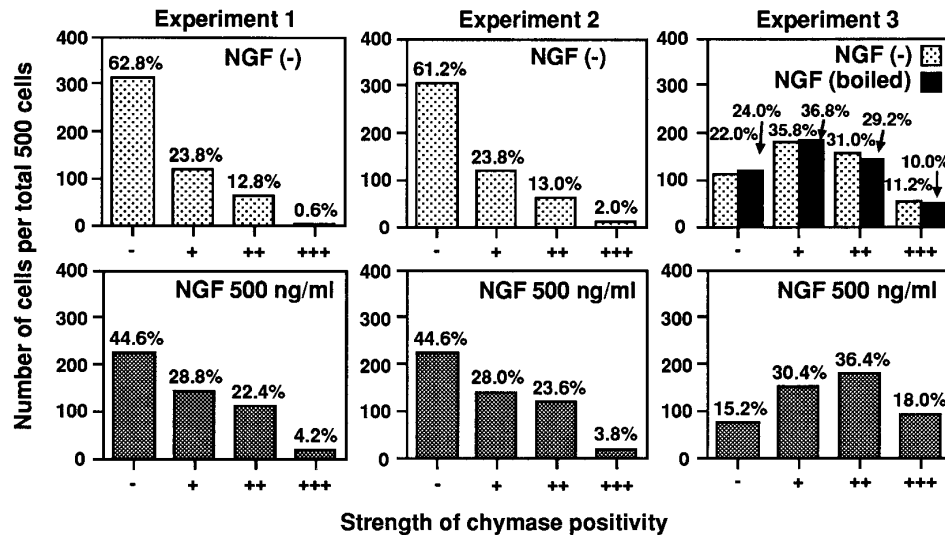


Fig 9. Human umbilical cord blood-derived mast cells cultured with NGF exhibit increased expression of chymase. Human umbilical cord blood-derived mast cells were cultured in SCF, IL-6, and PGE₂ for 19 or 20 weeks and were maintained for an additional 3 weeks in medium with or without NGF at 500 ng/mL (see text). Immunocytochemical analysis of the expression of chymase in these mast cells was performed as described in the Materials and Methods. The strength of chymase positivity was graded from negative (-) to strongly positive (+++) (as illustrated in Fig 10), and the distribution of mast cells exhibiting various intensities of chymase positivity in individual specimen was compared using the χ^2 test. In each of the three independent experiments shown, the difference between the distribution of cells exhibiting various degrees of chymase positivity in cultures with or without exogenous NGF was significant ($P < .0001$, $P = .0014$, and $P = .046$ in Experiments 1, 2, and 3, respectively). In Experiment 3, we found that NGF which had been boiled (black bars in Experiment 3), when used at 500 ng/mL instead of native NGF in medium containing SCF and PGE₂, had no detectable effect on chymase immunoreactivity ($P = .93$ v values for cells cultured without NGF in medium containing SCF, IL-6 and PGE₂, $P < .01$ v values for cells cultured with native NGF).

which had been cultured without NGF.⁵¹ To evaluate whether NGF might affect chymase expression by human mast cells, we maintained human cord blood-derived mast cells that had been cultured in SCF, IL-6, and PGE₂ for 19 to 20 weeks for an additional 3 weeks in medium containing SCF (80 ng/mL), IL-6 (50 ng/mL), and PGE₂ (1 μ mol/L) or SCF (80 ng/mL), PGE₂ (1 μ mol/L) and native or (as a control) boiled NGF at 500 ng/mL.

Figure 9 shows the data from three different experiments, all of which gave similar results. Photomicrographs depicting the immunohistochemistry results obtained in one of these experiments (Experiment 1 in Fig 9) are presented in Fig 10. As illustrated by data obtained from Experiment 1, NGF did not affect the total number of cells (1.2×10^6 cells in NGF (-) culture versus 1.1×10^6 cells in the NGF (+) culture) or the proportion of mast cells in the culture (% of tryptase-positive cells (ie, mast cells) in the NGF (-) culture was 99.8% versus 99.8% in the NGF (+) culture). However, immunocytochemical staining showed that the number of chymase-positive mast cells was increased by the NGF treatment (% of chymase-positive cells in the NGF (-) culture was 37.2% versus 55.4% in the NGF (+) culture) (Fig 9, Experiment 1). Moreover, in each of the three experiments, a significantly higher level of chymase expression was detected in the mast cells cultured with NGF, as opposed to those maintained in the absence of NGF ($P < .05$ –.0001, χ^2 test). However, NGF treatment did not appear to alter significantly the histamine content in these populations of human umbilical cord blood-derived mast cells [NGF(-)

cultures: 12.2 ± 1.1 pg/cell, $n = 3$; NGF (+) cultures: 11.6 ± 3.3 pg/cell, $n = 3$].

According to the manufacturer, the purified NGF used in our studies contained <0.0002 ng of lipopolysaccharide (LPS)/mg of NGF (Dr Lúsa Eisenlohr, Upstate Biotechnology, personal communication, January 1997), and our FCS preparations contained <0.005 ng of LPS/mL, according to a *Limulus* amoebocyte assay (LAL assay; Whitaker Bioproducts, Inc, Walkersville, MD). Thus, when used at 500 ng/mL, these NGF preparations would have contributed little, if any, LPS to the culture medium. Accordingly, it is very unlikely that our results were affected by whatever small amounts of LPS might have been present in the NGF preparations. Nevertheless, in two experiments, we compared the expression of chymase immunoreactivity in mast cells cultured for 3 weeks in SCF, PGE₂, and either native NGF or NGF, which had been boiled for 1 hour (each at 500 ng/mL). We found that boiled NGF, in contrast to native NGF, had no detectable effect on the overall number of chymase-positive mast cells in cultures containing SCF and PGE₂ or on the intensity of chymase expression in the mast cell population (see black bars in Experiment 3 in Fig 9). This experiment showed that essentially identical levels of chymase immunoreactivity were expressed by mast cells cultured for 3 weeks in SCF, IL-6, and PGE₂ or SCF, PGE₂, and boiled NGF ($P = .93$). Similar results were obtained in the second experiment, in which mast cells that had been cultured for 3 weeks in SCF, PGE₂, and native NGF expressed more chymase immunoreactivity than did aliquots

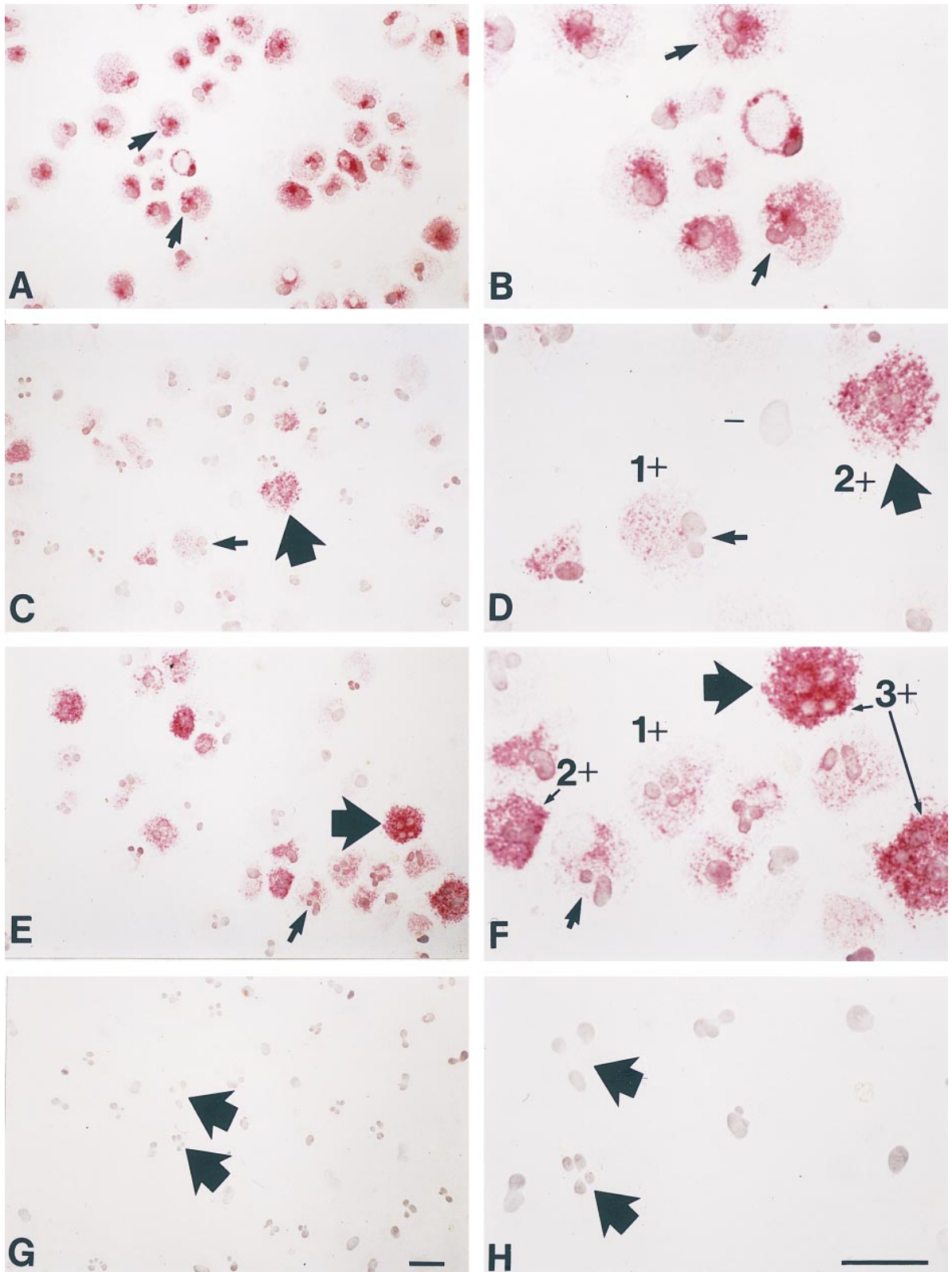


Fig 10.

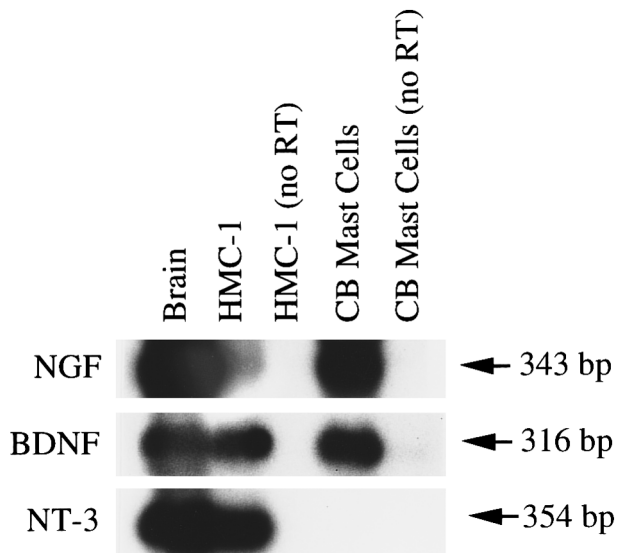


Fig 11. Expression of mRNA transcripts for NGF, BDNF, and NT-3 in human brain, HMC-1 cells, and human umbilical cord blood-derived mast cells (99% purity according to staining for tryptase). Poly A+ mRNA (human brain) or total RNA treated with DNase I (for both HMC-1 cells and human umbilical cord blood-derived mast cells) and heparinase I (for human umbilical cord blood-derived mast cells only) was reverse transcribed. The cDNAs were amplified using specific human NGF primers (T and U), BDNF primers (V and W), and NT-3 primers (X and Y). As a negative control for genomic DNA contamination, DNase I-treated RNAs from HMC-1 cells and from human umbilical cord blood-derived mast cells were amplified by PCR without the reverse transcription procedure (as shown in lanes labeled as "no RT"). The resulting PCR products were analyzed by Southern hybridization using ^{32}P -labeled cDNA probes.

of the same mast cells that had been maintained for 3 weeks in SCF, PGE₂, and boiled NGF ($P < .05$ by χ^2 test, data not shown).

HMC-1 cells and human umbilical cord blood-derived mast cells express mRNA transcripts for NGF and other neurotrophins. As shown in Fig 9, different preparations of umbilical blood-derived human mast cells expressed, in the absence of treatment with exogenous NGF, variable por-

portions of cells that exhibited detectable immunoreactivity for chymase. A previous report showed the expression of mRNA for NGF in rat PMCs,¹⁹ raising the possibility that mast cells themselves might represent a potential source of NGF. To assess whether human mast cells could also express NGF mRNA and to determine whether these cells might express mRNAs for other members of the neurotrophin family, we performed RT-PCR and Southern analysis of DNase I-treated RNAs derived from HMC-1 cells and human cord blood-derived mast cells.

As shown in Fig 11 (representative of two experiments that gave similar results), positive hybridization signals of the predicted sizes for the mRNA expression of NGF (343 bp), BDNF (316 bp), and NT-3 (354 bp) could be detected in HMC-1 cells and in specimens of human brain. By contrast, human umbilical cord blood-derived mast cells appeared to express only the transcripts of NGF and BDNF, but not NT-3. However, when the DNase I-treated RNAs from HMC-1 cells and from human umbilical cord blood-derived mast cells were amplified by PCR without the reverse transcription step (as a negative control for genomic DNA contamination), no detectable hybridization signals could be detected for the three neurotrophins (Fig 11). Furthermore, the DNA fragment that was amplified by PCR using the HMC-1 RNA and the BDNF specific primers was subcloned and sequenced and shown to contain sequence identical to that reported for human BDNF cDNA.³⁹

DISCUSSION

The HMC-1 cell line, which possesses many properties of immature mast cells,^{23,24} has proven to be a useful model for studying the molecular events underlying many cellular responses in human mast cells.⁵²⁻⁵⁴ In this study, we have characterized in detail, at the molecular level, the various types and isoforms of neurotrophin receptors expressed by HMC-1 cells. Our data show that HMC-1 cells express both the gene transcripts and the proteins for the TrkA, TrkB, and TrkC receptor tyrosine kinases, but lack detectable mRNA expression for the p75 low-affinity neurotrophin receptor. The TrkA receptors detected in HMC-1 cells are functional, as demonstrated by the ability of NGF, the cognate ligand

Fig 10. Long-term incubation with NGF enhances chymase immunoreactivity in human umbilical cord blood-derived mast cells. Human umbilical cord blood-derived mast cells (19 weeks after start of culture; > 99% purity by tryptase immunostaining) were maintained for an additional 3 weeks in culture with or without purified NGF at 500 ng/mL (see text). At the start of culture, tryptase immunoreactivity (red reaction product) is detectable in essentially all of the mast cells (A, B). (B) is a higher magnification view of some of the same cells shown in (A); the arrows in (A and B) depict the same two cells, both of which appear to have bilobed nuclei (a common finding in such *in vitro*-derived mast cells). (C and D) Mast cells that were maintained for an additional 3 weeks without NGF exhibit a range of chymase immunoreactivity (red reaction product), from none (eg, "-" in D), to 1+ or 2+ in intensity (examples depicted in D). (D) is a higher magnification view of some of the same cells shown in (C), some of which have bilobed (small arrow) or multilobed (thick arrow) configurations. (E and F) Mast cells that were maintained for an additional 3 weeks with NGF exhibit increased chymase immunoreactivity (red reaction product), with some cells of 3+ staining intensity, as well as those of 1+ or 2+ intensity (examples indicated in F). (F) is a higher magnification view of some of the same cells as in (E), some of which have bilobed (small arrow) or multilobed (thick arrow) nuclear configurations. (G and H) Negative control (a mouse monoclonal IgG_{1k} antibody of irrelevant antigen specificity, rather than either the mouse monoclonal IgG_{1k} antitryptase or antichymase antibody, was used as the first antibody) showing lack of nonspecific immunoreactivity in these mast cells (these were aliquots of the same cell population depicted in C and D). (H) is a higher magnification view of some of the same cells shown in (G), some of which have multilobed nuclei (thick arrows), a common finding in such *in vitro*-derived mast cells. These cells are from Experiment 1 in Fig 9. Scale bars in G (for A, C, E and G) and H (for B, D, F, and H) represent 10 μm .

of TrkA, to initiate appropriate cellular signaling responses in these cells. Thus, NGF stimulation of HMC-1 cells was shown to induce tyrosine autophosphorylation of TrkA, increased expression of early response genes, and activation of MAP kinase activity.

It is currently quite difficult to obtain normal human mast cells in large numbers. Accordingly, the full extent to which our observations with HMC-1 cells are relevant to normal human mast cells can only be established by further studies. However, we found that highly purified populations of both cultured umbilical cord blood-derived human mast cells and mast cells isolated from adult human lung tissue predominantly express *trkA* mRNA, and that human umbilical cord blood-derived mast cells also express TrkA protein. Moreover, the TrkA receptors expressed in human umbilical cord blood-derived mast cells appear to mediate at least one potentially physiological response to NGF stimulation, the increased expression of chymase.

In contrast to mRNA for *trkA*, the expression of *trkB* and *trkC* mRNAs in HMC-1 cells (and in populations of purified human lung mast cells) was unexpected, particularly in light of recent studies indicating a lack of expression of these *trk* mRNAs in either rat PMCs or mouse mast cells derived in IL-3-containing media.^{13,22} Indeed, of all of the hematopoietic cells examined to date, human mast cells, including the HMC-1 cell line and populations of mature lung mast cells, appear to be the only cell type that can express all three *trk* gene transcripts. However, unlike preparations of human lung mast cells, populations of human umbilical cord blood-derived mast cells, which have features of immature mast cells, express transcripts for *trkA* and *trkC*, but not *trkB*. These findings indicate that populations of human mast cells can express all three Trk receptors, at least at the level of mRNA, but that the patterns of expression of the three receptors in individual human mast cell populations (eg, lung *in vitro*-derived cord blood mast cells) may vary.

Furthermore, we found that HMC-1 cells express mRNAs encoding the truncated forms of human TrkB (TrkB.T1) and TrkC receptors, as well as mRNAs for the full-length, tyrosine kinase-containing, forms.³⁵ While the exact function(s) of these truncated TrkB and TrkC receptors have not been fully determined, there is speculation that they may act as "scavenger receptors" to maintain high levels of neurotrophins at the cell surface, or may initiate distinct cellular responses through ligand-dependent interactions with downstream signaling elements.⁵⁵

Our findings indicate that there may be significant differences in the expression of neurotrophin receptors by mast cells of different species, as well as by different mast cell populations in the same species. Thus, recent studies show that rat PMCs can express transcripts for both the TrkAI and TrkAII isoforms,¹⁸ whereas we report here that the HMC-1 cell line expresses solely transcripts for the shorter TrkAI isoform that lack the 18 nucleotide insert.^{42,43} In addition, the p75 transcript is detectable in cultured mouse mast cells,²² but in neither rat PMCs^{14,18} nor HMC-1 cells (this study). Taken together, these findings and all of our other data are consistent with the suggestion of Jippo et al²² that the patterns of expression of neurotrophin receptors by mast

cells can be quite heterogeneous, depending both on the cells' stage of development and species of origin.

Despite the apparent heterogeneity of neurotrophin receptor expression in mouse and human mast cells, the interaction of NGF with its receptors on different populations of mast cells appears to be consistently of the low-affinity type. An earlier report indicated that NGF binds to its receptor on rat PMCs with a K_D that is consistent with low-affinity binding (4.0×10^{-9} mol/L).⁵⁶ Similarly, a recent study shows that the NGF binding detected in cultured mouse mast cells also exhibits a low-affinity (2.1×10^{-9} mol/L).²² The dose response curves for NGF effects on serotonin release and on cell survival in rat PMCs are also consistent with a low-affinity interaction,^{13,18} although one recent apparently conflicting report has indicated that NGF can promote survival of rat PMCs via a high-affinity NGF receptor.¹⁴ In accord with most of these observations, we found that the doses of NGF required to induce the expression of early response genes in HMC-1 cells are consistent with a low-affinity type of interaction between NGF and the NGF receptors expressed in these cells.

The TrkA receptor tyrosine kinase expressed by HMC-1 cells appears to be functional, as NGF stimulation of these cells elicited signaling responses that are characteristic of NGF/TrkA interaction in other NGF-responsive cell types. These responses include tyrosine phosphorylation of TrkA, the induction of early response gene expression, and the activation of MAP kinase activity. However, we found that the kinetics of MAP kinase activation and expression of early response genes in HMC-1 cells stimulated with NGF were more transient than those observed with other cell types. In HMC-1 cells, these two NGF-induced signaling responses appeared to dissipate completely by 1 hour after stimulation (Figs 5 and 6), whereas both MAP kinase activity and the expression of *c-fos* remained elevated 1 hour after NGF-stimulation in PC12 cells and human B lymphocytes.^{57,58} Very similar transient kinetics of MAP kinase activation and early response gene expression were exhibited in rat PMCs that were treated with NGF,¹³ as well as in mouse bone marrow-derived cultured mast cells, which had been activated via *c-kit* (with SCF) or Fc ϵ RI (with IgE and specific antigen).^{40,59} These findings are consistent with the widely held view that distinct patterns or kinetics of activation of common signaling elements may be used by different cell types to achieve specificity in their cellular responses to ligand-dependent stimulation of Trk receptors.⁴⁶ In particular, it has recently been suggested that variation in the duration of MAP kinase activation may represent a mechanism that can elicit differential cellular responses to stimulation via receptor tyrosine kinases.⁶⁰

Previous studies have indicated that NGF can promote the maturation of mouse bone marrow-derived cultured mast cells *in vitro* in the presence of IL-3.¹² We show in this study, in confirmation of results reported by Igarashi et al⁵¹ in abstract form that NGF can enhance the expression of the cytoplasmic granule-associated protease, chymase, in human umbilical cord blood-derived mast cells cultured in the presence of SCF. Previously, Furitsu et al⁶¹ reported that factors derived from mouse 3T3 fibroblasts can induce the mast cell

precursors that are present in the human umbilical cord blood mononuclear cell preparations to develop predominantly into mast cells that contain both tryptase and chymase.⁶¹ Based on this characteristic, such *in vitro*-derived mast cells resemble the mature mast cells that are present in human dermis and certain other sites.⁶² On the other hand, the human cord blood-derived mast cells, which are generated *in vitro* in the absence of 3T3 cells, but in the presence of SCF, exhibit many phenotypic characteristics of immature mast cells, including a predominantly tryptase⁺ chymase⁻ or low phenotype.²⁷ The ability of NGF to enhance the expression of chymase in umbilical cord blood-derived human mast cells thus provides evidence that NGF may represent one of the microenvironmental factors, which can synergize with SCF in promoting at least one of the phenotypic changes (ie, increased chymase expression) that is associated with the maturation/differentiation of certain populations of human mast cells *in vivo*. On the other hand, treatment with NGF did not significantly increase the cells' histamine content. This result suggests that NGF may have different effects on different aspects of human mast cell phenotype.

It has been proposed that there are complex and bidirectional, developmental, and functional interactions between mast cells and the nervous system.⁴⁻⁶ Our finding that various populations of human mast cells can express TrkA and TrkB, or Trk A, TrkB and TrkC, offers further support for this hypothesis. Moreover, a recent report showed that rat PMCs, which express TrkA receptors, can synthesize, store, and spontaneously release biologically active NGF.¹⁹ In the present study, we found that HMC-1 mast cells and cultured human umbilical cord blood-derived mast cells can express the gene transcript for BDNF, as well as NGF mRNA. Moreover, HMC-1 cells also express mRNA for NT-3. While the extent to which these findings pertain to other types of normal human mast cells and the extent to which human mast cells can produce and secrete neurotrophins, remain to be determined, our results raise the possibility that mast cell-derived neurotrophins in addition to NGF may mediate autocrine and/or paracrine effects on mast cell development and function.

ACKNOWLEDGMENT

We thank Drs Claudia Cabral and Chris S. Lantz for assistance with the flow cytometry, Dr Jürg Boesiger for analysis of the histamine content of human umbilical cord blood-derived mast cells, and Dr Keith Langley of AMGEN Inc for recombinant human SCF and IL-6 and the SR-1 antibody.

REFERENCES

- Bothwell M: Functional interactions of neurotrophins and neurotrophin receptors. *Ann Rev Neurosci* 18:223, 1995
- Snider WD: Functions of the neurotrophins during nervous system development: What the knockouts are teaching us. *Cell* 77:627, 1994
- Tsuda T, Wong D, Dolovich J, Bienenstock J, Marshall J, Denburg JA: Synergistic effects of nerve growth factor and granulocyte-macrophage colony-stimulating factor on human basophilic cell differentiation. *Blood* 77:971, 1991
- Bienenstock J, Stead RH, Marshall JS: Mast cells and the nervous system, in Kaliner MA, Metcalfe DD (eds): *The Mast Cell in Health and Disease*. New York, NY, Dekker, 1993, p 687
- Stanisz AM: Neuronal factors modulating immunity. *Neuroimmunomodulation* 1:217, 1994
- Levi-Montalcini R, Skaper SD, Dal Toso R, Petrelli L, Leon A: Nerve growth factor: From neurotrophin to neurokinin. *Trends Neurosci* 19:514, 1996
- Bochner BS, Lichtenstein LM: Anaphylaxis. *N Engl J Med* 324:1785, 1991
- Paul WE, Seder RA, Plaut M: Lymphokine and cytokine production by FcεRI⁺ cells. *Adv Immunol* 53:1, 1993
- Galli SJ: New concepts about the mast cell. *N Engl J Med* 328:257, 1993
- Aloe L, Levi-Montalcini R: Mast cells increase in tissues of neonatal rats injected with the nerve growth factor. *Brain Res* 133:358, 1977
- Aloe L: The effects of nerve growth factor and its antibody on mast cells *in vivo*. *J Neuroimmunol* 18:1, 1988
- Matsuda H, Kannan Y, Ushio H, Kiso Y, Kanemoto T, Suzuki H, Kitamura Y: Nerve growth factor induces development of connective tissue-type mast cells *in vitro* from murine bone marrow cells. *J Exp Med* 174:7, 1991
- Horigome K, Bullock ED, Johnson EM: Effects of nerve growth factor on rat peritoneal mast cells: Survival promotion and immediate-early gene induction. *J Biol Chem* 269:2695, 1994
- Kawamoto K, Okada T, Kannan Y, Ushio H, Matsumoto M, Matsuda H: Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the *trk* proto-oncogene receptor. *Blood* 86:4638, 1995
- Bruni A, Bigon E, Boarato E, Mietto L, Leon A, Toffano G: Interaction between nerve growth factor and lysophosphatidylserine on rat peritoneal mast cells. *FEBS Lett* 138:190, 1982
- Pearce FL, Thompson HL: Some characteristics of histamine secretion from rat peritoneal mast cells stimulated with nerve growth factor. *J Physiol* 372:379, 1986
- Marshall JS, Stead RH, McSharry C, Nielsen L, Bienenstock J: The role of mast cell degranulation products in mast cell hyperplasia. I. Mechanism of action of nerve growth factor. *J Immunol* 144:1886, 1990
- Horigome K, Pryor JC, Bullock ED, Johnson EM: Mediator release from mast cells by nerve growth factor: Neurotrophin specificity and receptor mediation. *J Biol Chem* 268:14881, 1993
- Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, Levi-Montalcini R: Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci USA* 91:3739, 1994
- Chao MV, Hempstead BL: p75 and Trk: A two-receptor system. *Trends Neurosci* 18:321, 1995
- Barbacid M: The Trk family of neurotrophin receptors. *J Neurobiol* 25:1386, 1994
- Jippo T, Ushio H, Hirota S, Mizuno H, Yamatodani A, Nomura S, Matsuda H, Kitamura Y: Poor response of cultured mast cells derived from *mi/mi* mutant mice to nerve growth factor. *Blood* 84:2977, 1994
- Butterfield JH, Weiler D, Dewald G, Gleich GJ: Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res* 12:345, 1988
- Rodewald H-R, Dessing M, Dvorak AM, Galli SJ: Identification of a committed precursor for the mast cell lineage. *Science* 271:818, 1996
- Tam S-Y, Tsai M, Butterfield JH, Galli SJ: Expression of functional TrkA receptor tyrosine kinase in human mast cell line HMC-1. *Soc Neurosci Abstr* 20:34, 1994 (abstr)
- Tam S-Y, Elsworth JD, Sladek JR, Redmond DE, Roth RH: Identification of novel variants of *trkC* mRNA transcripts in brain of African green monkeys. *Exp Neurol* 143:172, 1997
- Saito H, Ebisawa M, Sakaguchi N, Onda T, Iikura Y, Yanagida M, Uzumaki H, Nakahata T: Characterization of cord-blood-

derived human mast cells cultured in the presence of steel factor and interleukin-6. *Int Arch Allergy Immunol* 107:63, 1995

28. Okayama Y, Hunt TC, Kassel O, Ashman LK, Church MK: Assessment of the anti-c-kit monoclonal antibody YB5.B8 in affinity magnetic enrichment of human lung mast cells. *J Immunol Methods* 169:153, 1994

29. Kimura I, Moritani Y, Tanizaki Y: Basophils in bronchial asthma with reference to reagin-type allergy. *Clin Allergy* 3:195, 1973

30. Schenone A, Gill JS, Zacharias DA, Windebank AJ: Expression of high- and low-affinity neurotrophin receptors on human transformed B lymphocytes. *J Neuroimmunol* 64:141, 1996

31. Tsai M, Miyamoto M, Tam S-Y, Wang Z-s, Galli SJ: Detection of mouse mast cell-associated protease mRNA: Heparinase treatment greatly improves RT-PCR of tissues containing mast cell heparin. *Am J Pathol* 146:335, 1995

32. Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M: Molecular and biochemical characterization of the human *trk* protooncogene. *Mol Cell Biol* 9:24, 1989

33. Middlemas DS, Lindberg RA, Hunter T: *trkB*, a neural receptor protein-tyrosine kinase: Evidence for a full-length and two truncated receptors. *Mol Cell Biol* 11:143, 1991

34. Lamballe F, Klein R, Barbacid M: *trkC*, a new member of the *trk* family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* 66:967, 1991

35. Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, Carroll K, Spencer SD, Levinson AD: Human *trks*: Molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesions. *J Neurosci* 15:477, 1995

36. Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M: Expression and structure of the human NGF receptor. *Cell* 47:545, 1986

37. Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramie GR, Nikolics K, Winslow JW: Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* 4:767, 1990

38. Ullrich A, Gray A, Berman C, Dull TJ: Human β -nerve growth factor gene sequence highly homologous to that of mouse. *Nature* 303:821, 1983

39. Jones KR, Reichardt LF: Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc Natl Acad Sci USA* 87:8060, 1990

40. Tsai M, Chen R-H, Tam S-Y, Blenis J, Galli SJ: Activation of MAP kinases, pp90^{sk} and pp70-S6 kinases in mouse mast cells by signaling through the c-kit receptor tyrosine kinase or Fc γ RI. Rapamycin inhibits activation of pp70-S6 kinase and proliferation in mouse mast cells. *Eur J Immunol* 23:3286, 1993

41. Kharbanda S, Saleem A, Emoto Y, Stone R, Rapp U, Kufe D: Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. *J Biol Chem* 269:872, 1994

42. Barker PA, Lomen-Hoerth C, Gensch EM, Meakin SO, Glass DJ, Shooter EM: Tissue-specific alternative splicing generates two isoforms of the *trkA* receptor. *J Biol Chem* 268:15150, 1993

43. Clary DO, Reichardt LF: An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin-3. *Proc Natl Acad Sci USA* 91:11133, 1994

44. Valenzuela DM, Maisonnier PC, Glass DJ, Rojas E, Nunez L, Kong Y, Gies DR, Stitt TN, Ip NY, Yancopoulos GD: Alternative forms of rat TrkC with different functional capabilities. *Neuron* 10:963, 1993

45. Tsoulfas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez JL, Rosenthal A, Nikolics K, Parada LF: The rat *trkC*

locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. *Neuron* 10:975, 1993

46. Arenander AT, Herschman HR: Primary response gene expression in the nervous system, in Loughlin SE, Fallon JH (eds): *Neurotrophic Factors*. San Diego, CA, Academic, 1993, p 89

47. Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD: ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65:663, 1991

48. Thomas SM, DeMarco M, D'Arcangelo G, Halegoua S, Brugge JS: Ras is essential for nerve-growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* 68:1031, 1992

49. Wood KW, Sarnecki C, Roberts TM, Blenis J: ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* 68:1041, 1992

50. Welker P, Grabbe J, Grutzkau A, Henz BM: Influence of the growth factors NGF and GM-CSF on the expression of mast cell characteristics. *J Allergy Clin Immunol* 266:334a, 1996 (abstr)

51. Igarashi Y, Kando N, Amano H, Kurosawa M, Miyachi Y, Saito H, Ikura Y, Nakahata T, Matsuda H: Effect of nerve growth factor on subtypes of cultured human mast cells. *Jpn J Allergology* 44:1059, 1995 (abstr)

52. Sillaber C, Bevec D, Butterfield JH, Heppner C, Valenta R, Scheiner O, Kraft D, Lechner K, Bettelheim P, Valent P: Tumor necrosis factor α and interleukin- 1β mRNA expression in HMC-1 cells: Differential regulation of gene product expression by recombinant interleukin-4. *Exp Hematol* 21:1271, 1993

53. Furitsu T, Tsujimura T, Tono T, Ikeda H, Kitayama H, Koshimizu U, Sugahara H, Butterfield JH, Ashman LK, Kanayama Y, Matsuzawa Y, Kitamura Y, Kanakura Y: Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest* 92:1736, 1993

54. Selvan RS, Butterfield JH, Krangel MS: Expression of multiple chemokine genes by a human mast cell leukemia. *J Biol Chem* 269:13893, 1994

55. Eide FF, Lowenstein DH, Reichardt LF: Neurotrophins and their receptors—Current concepts and implications for neurologic disease. *Exp Neurol* 121:200, 1993

56. Cattaneo A: Thymocytes as potential target cells for nerve growth factor, in Levi-Montalcini R, Calissano P, Kandel ER, Maggi A (eds): *Molecular Aspects of Neurobiology*. New York, NY, Springer-Verlag, 1986, p 31

57. Kruijer W, Schubert D, Verma IM: Induction of the proto-oncogene *fos* by nerve growth factor. *Proc Natl Acad Sci USA* 82:7330, 1985

58. Franklin RA, Brodie C, Melamed I, Terada N, Lucas JJ, Gelfand EW: Nerve growth factor induces activation of MAP-kinase and p90^{sk} in human B lymphocytes. *J Immunol* 154:4965, 1995

59. Tsai M, Tam S-Y, Galli SJ: Distinct patterns of early response gene expression and proliferation in mouse mast cells stimulated by stem cell factor, interleukin-3, or IgE and antigen. *Eur J Immunol* 23:867, 1993

60. Marshall CJ: Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80:179, 1995

61. Furitsu T, Saito H, Dvorak A, Schwartz LB, Irani AA, Burdick JF, Ishizaka K, Ishizaka T: Development of human mast cells in vitro. *Proc Natl Acad Sci USA* 86:10039, 1989

62. Schwartz LB: Heterogeneity of human mast cells, in Kaliner MA, Metcalfe DD (eds): *The Mast Cell in Health and Disease*. New York, NY, Dekker, 1993, p 219