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Monokine Induced by Interferon-γ Acts as a Neurotrophic Factor on PC12 Cells and Rat Primary Sympathetic Neurons*[S]

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We found that a monokine induced by interferon-γ (Mig, CXCL9), which belongs to the CXC chemokine subfamily, acts as a neurotrophic factor on PC12 cells and rat primary sympathetic neurons. PC12 cells were shown to express a single class of high affinity binding sites for Mig (670 receptors/cell, K_d = 2.9 nM). Mig induced neurite outgrowth in PC12 cells in a dose-dependent manner. Comparison of extracellular signal-regulated kinase signaling pathways between Mig and nerve growth factor (NGF) revealed that these pathways are crucial for Mig action as well as NGF. K252a, an inhibitor of tyrosine autophosphorylation of tyrosine kinase receptors (Trks) did not inhibit the action of Mig, suggesting that Mig action occurs via a different receptor from that of NGF. Furthermore, Mig as well as NGF promoted PC12 survival under serum-free conditions and activated Akt/protein kinase B downstream from phosphatidylinositol 3-kinase (PI3K). Because the PI3K inhibitor LY294002 prevented the Mig- and NGF-induced survival effect, this effect is probably mediated by the PI3K signaling pathway. Mig also promoted survival of rat primary sympathetic neurons that die when deprived of NGF. These results suggest that chemokines, including Mig (CXCL9) have neurotrophic effects on the nervous system.

Chemokines are small secreted proteins (8–10 kDa) that were initially recognized as playing roles in leukocyte communication and migration in both physiological and pathological contexts in the immune system (1–3). They are classified into four classes based on the positions of key N-terminal cysteine residues: C, CC, CXC, and CX3C (1–3). The two major families are the CXC and CC chemokines, which interact with seven-transmembrane G-protein-linked CXCR and CCR receptors, respectively (1–3). Besides their well established roles in the immune system, several recent reports have demonstrated that these proteins also have roles in the CNS. The best known major action is their ability to act as immunoinflammatory mediators. These proteins regulate leukocyte infiltration in the brain during neuroinflammatory conditions and diseases, which range from bacterial and viral meningoencephalitis (4, 5) to multiple sclerosis (6, 7), human immunodeficiency virus encephalopathy (8–10), and cerebral ischemia (11, 12). Aside from their function as immunoinflammatory mediators, recent studies have led to the realization that some members of the chemokine family and their cognate receptors have an important role in cellular communication in developing and normal adult CNS. As support for this concept, chemokines and their receptors have been observed in both developing and adult brains by *in situ* studies (13–17). In addition, studies with knock-out mice have shown that the stromal-cell-derived factor 1/SDF-1/CXCL12, which is synthesized constitutively in the developing brain, plays an essential role in neuron migration during formation of the granule-cell layer of the cerebellum (18, 19). The chemokines are a large and ever-expanding family of proteins with more than 50 members. However, these known chemokine functions in the CNS represent only the tip of the iceberg.

Nerve growth factor (NGF),[2] a prominent neurotrophic factor, plays a crucial role in promoting growth, differentiation, and survival of sympathetic nerve cells (20). The PC12 cell line was derived from a pheochromocytoma tumor of the rat adrenal medulla (21), and it has been used as a model system for neuronal differentiation and death. PC12 cells respond to NGF by extension of neurites and the acquisition of sympathetic neuronal phenotype (21). PC12 cells undergo apoptosis when cultured in serum-free medium without NGF. However, supplementation of serum-free medium with NGF promotes the survival of PC12 cells (22). To obtain novel neurotrophic factors like NGF, we investigated the effect of over 200 small secreted proteins, such as cytokines, chemokines, and bioactive peptides on PC12 cells. As a result, we found that one of the CXC chemokines, monokine induced by interferon-γ (Mig, CXCL9) promotes the neuronal differentiation and survival of PC12 cells. Mig also promoted the survival of rat primary sympathetic neurons that die when deprived of NGF.

EXPERIMENTAL PROCEDURES

Materials—PC12 cell line was purchased from ATCC. Antibodies for phospho-ERK1/2, ERK1/2, phospho-Akt, and Akt were from Santa Cruz Biotechnology. Anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase were purchased from Amersham Biosciences; all human chemokines (Mig, IP-10, and I-TAC), PD98059, LY294002, K252a, pertussis toxin, anti-GAP-43 antibody were from Sigma; anti-human Mig antibody was from Chemicon; and mouse NGF (2.5S) was from Promega.

Western Blot Analysis—Cells were lysed in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM NaF, 1 mM vanadate, 1% Nonidet P-40, EDTA-free Complete™ protease inhibitor mixture (Roche Molecular Biochemicals)). Protein extracts transferred to nitrocellulose membranes were incubated with primary antibodies followed by horseradish peroxidase-coupled secondary antibodies. Signals were detected by the ECL Plus system (Amersham Biosciences) using Hyperfilm (Amersham Biosciences) for exposure.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: NGF, nerve growth factor; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; Trk, tyrosine kinase receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; MTT, (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl)tetrazolium bromide; PI, propidium iodide; RT, reverse transcription; IP-10, interferon-inducible protein 10; 1-TAC, interferon-inducible T cell-α chemoattractant; GAP-43, growth-associated protein 43; GPCR, G protein-coupled receptor; Trk, tyrosine kinase receptors.

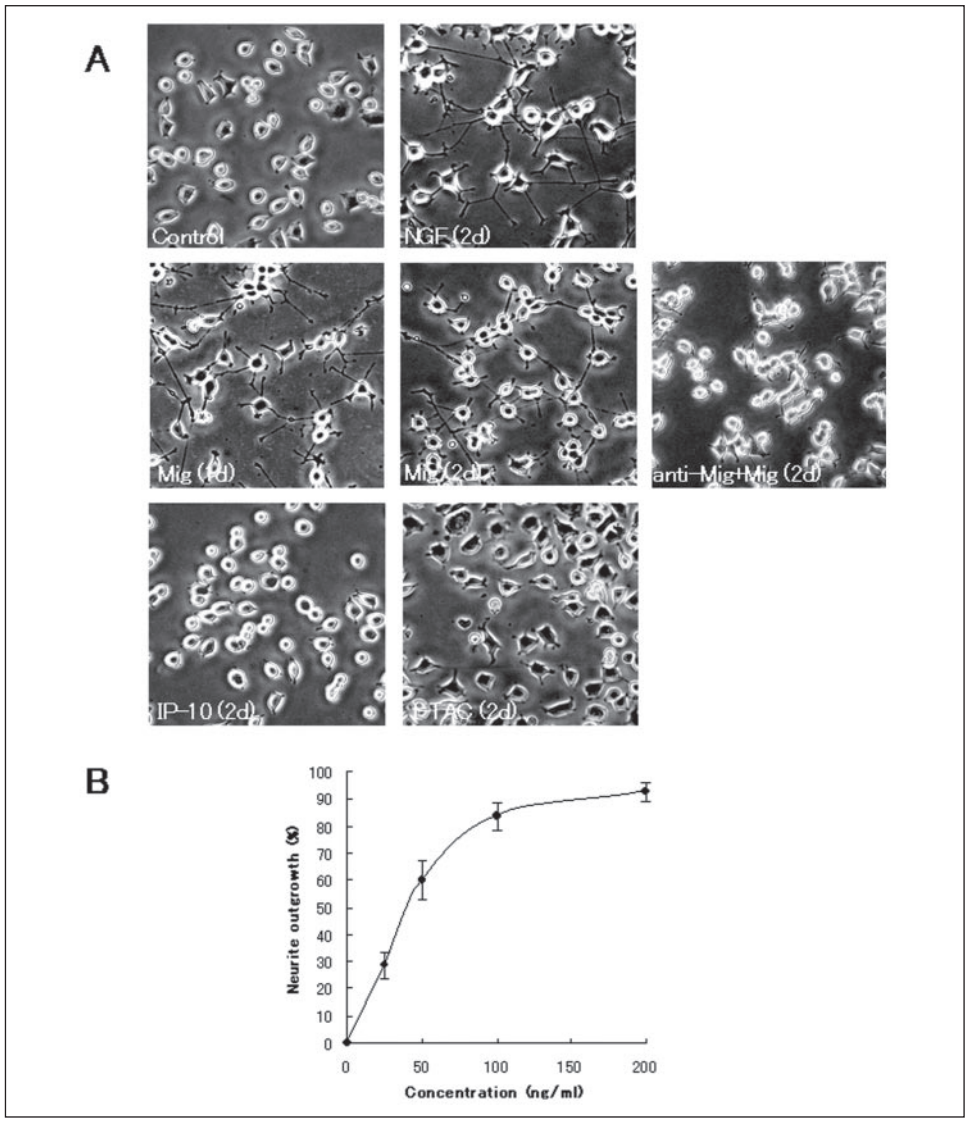

FIGURE 1. **Induction of neurite outgrowth in PC12 cells by Mig.** *A*, morphological examination of the effect of Mig on PC12 cells. PC12 cells were treated with 100 ng/ml Mig, 100 ng/ml Mig plus anti-Mig antibody, 100 ng/ml IP-10, 100 ng/ml I-TAC, or 100 ng/ml NGF for the indicated periods of time. The experiment shown was repeated five more times, with similar results. *B*, dose-dependent curve showing neurite outgrowth activity of Mig. PC12 cells were treated with varying concentrations of Mig for 24 h. The percentage of cells with neurites is plotted against the concentration of Mig. Each value is the mean ± S.E. for ~100 cells sampled from three independent experiments.

Determination of Neurite Outgrowth—PC12 cells were maintained at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 5% heat-inactivated horse serum (Invitrogen). To measure neurite outgrowth, 2×10^4 cells were seeded in each well of a 24-well plate that had been coated with mouse collagen type IV (BD Biosciences) in DMEM containing 1% heat-inactivated fetal bovine serum and 0.5% heat-inactivated horse serum with or without ligand (Mig, NGF) as indicated. After incubation for 24 h, for quantification of neurite outgrowth, random photographs were taken (three or four per well) and process-bearing neurites were counted, scoring as possible processes those of length greater than the cell's diameter. Three independent experiments were performed in duplicate for each data point presented. Neurites were identified and counted from ~100 cells per photograph.

To investigate the effect of a neutralizing Mig antibody on Mig-induced neurite outgrowth of PC12 cells, Mig-containing medium (100 ng/ml) was preincubated with the antibody (1 μg/ml) for 60 min at 4 °C, and the resulting medium was used for PC12 neurite extension assay as described above.

Confocal Microscopy—PC12 cells were seeded on poly-D-lysine/mouse laminin-coated culture slides and were incubated with or without Mig or NGF. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 100% methanol for 30 min, and blocked with 5% donkey serum/PBS for 30 min. After incubation with primary antibody (anti-GAP43 mAb, Sigma) for 1 h, the cells were rinsed with PBS and incubated with Alexa488-labeled secondary antibody (Molecular Probes) for 1 h. Propidium iodide (Molecular Probes) was used to visualize the nucleus. Fluorescent images were obtained by FV300 laser scanning confocal microscopy (Olympus).

Ras and Rap1 Activation Assay—Ras and Rap1 GTP loading were measured using Ras and Rap1 activation assay kits (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, after stimulation, cells were rinsed rapidly in ice-cold PBS and solubilized at 4 °C for 10 min in lysis buffer. Lysates were clarified by centrifugation, and supernatants were incubated with glutathione-Sepharose beads coupled to C-RafRBD/GST to isolate RasGTP or RalGDSRBD/GST to isolate Rap1GTP. Protein complexes were allowed to form for 1 h at 4 °C. Precipitates were washed three times with lysis buffer. Finally, precipitates were resuspended in Laemmli sample buffer, and denatured proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with an anti-Ras or anti-Rap1 antibody.

Cell Survival Assay—Cells were seeded on mouse collagen type IV coated 96-well plates at 5×10^3 cells/well. At 24 h after plating, the cells

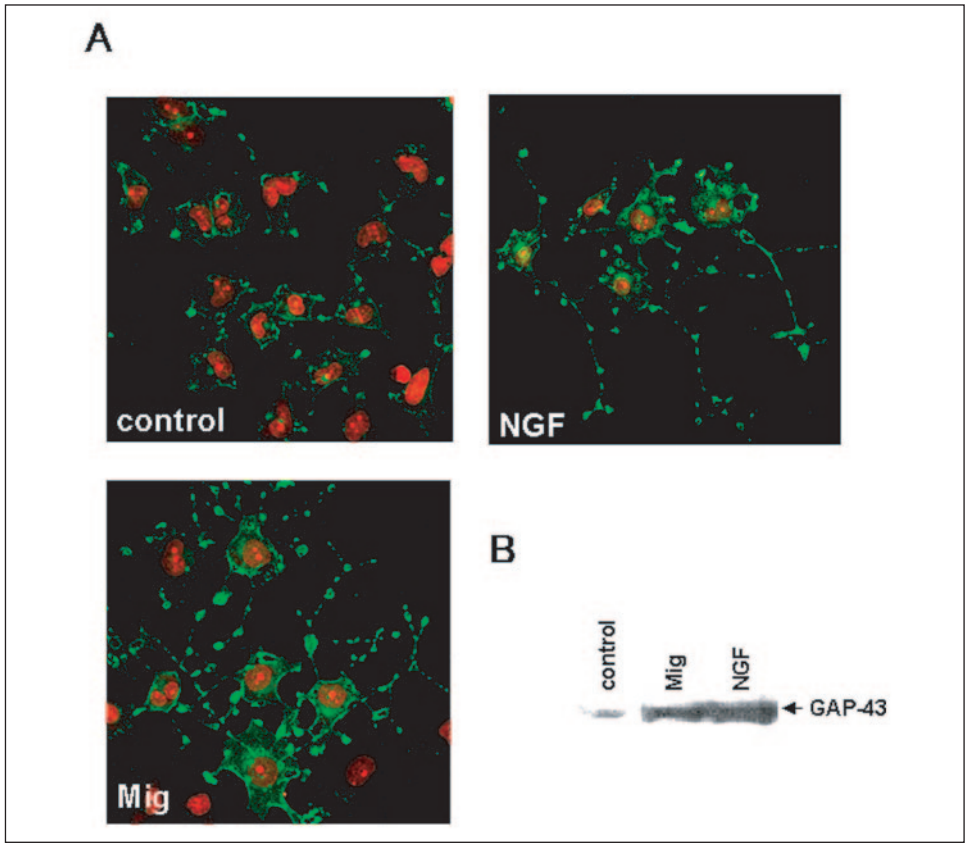

FIGURE 2. **Mig augments the amount of GAP-43 protein in PC12 cells.** *A*, distribution of GAP-43 in PC12 cells. PC12 cells were treated with 100 ng/ml Mig or 100 ng/ml NGF for 24 h. After 24 h, Mig, NGF-treated and untreated cells were fixed, and then immunostained with a monoclonal antibody for the GAP-43 (*green*). Nuclei were stained with propidium iodide (*red*). GAP-43 was detected by confocal microscopy. The experiment shown was repeated three more times, with similar results. *B*, immunoblot analysis of GAP-43. PC12 cells were plated in 24-well plates at a density of 2×10^4 cells/well in DMEM and treated with DMEM containing various ligand (100 ng/ml, respectively). After 24 h, lysates were prepared and subjected directly to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-GAP-43 antibody. The experiment shown was repeated three more times, with similar results.

were washed three times with PBS and grown in serum-free DMEM treated with or without NGF or Mig at the indicated concentration. In experiments including LY294002, the PI3K inhibitor was added at 20 μM to the serum-free media for 15 min, and 100 ng/ml NGF or 200 ng/ml Mig was added. Cell survival was determined using the (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl)tetrazolium bromide (MTT) assay-based Cell Counting Kit-8 (Dojindo), and relative cell numbers were determined in triplicate by estimating the value of day 0 as 100.

Detection of Apoptosis—Apoptosis was examined by flow cytometry analysis of the cell cycle after DNA staining with propidium iodide (PI). Cells were grown in 6-well plates that had been coated with mouse collagen type IV until they reached ~50% confluence. The medium was then replaced with complete or serum-free medium in the presence or absence of indicated ligand (Mig, NGF). After 48-h incubation, the cells attached to the plates and floating (detached) dead cells were harvested in PBS/EDTA (5 mM EDTA in PBS). The cells were then centrifuged, and the cell pellet was suspended in 0.5 ml of PBS. The cell suspension was transferred to 4.5 ml of 70% cold ethanol and kept at −20 °C for a minimum of 2 h. Ethanol-fixed cells were centrifuged and washed once with 10 ml of PBS. Finally, the cell pellet was suspended in 1 ml of staining solution (20 μg/ml PI, 0.2 mg/ml RNase in PBS) and incubated for 30 min at room temperature. The samples were analyzed by flow cytometry (Epics XL, Beckman Coulter), including a first selection (gate 1) in which events of the appropriate size (forward scatter) and complexity (side scatter) were selected. The selected events were then analyzed to discard doublets using a PI intensity-width *versus* PI intensity-area dot plot (gate 2). Finally, events (cells) present in gates 1 and 2 were plotted as a histogram representing the number of events (cells) containing a specific PI intensity-area (specific amount of DNA). Apoptosis was measured as the percentage of cells with a sub-G_0/G_1 DNA content in the PI intensity-area histogram plot.

Sympathetic Neuronal Cultures and Neuronal Death Survival Assay—Primary cultures of sympathetic neurons from superior cervical ganglion were prepared by dissecting tissue from embryonic day 21 or postnatal day 0 rats. The ganglia were placed in DMEM, digested with 1 mg/ml collagenase (Sigma) for 30 min at 37 °C, followed by digestion with trypsin (Invitrogen) for another 30 min. Ganglia were then dissociated into a suspension of individual cells. The cell suspension was plated on poly-D-lysine/mouse laminin-coated culture slides (BD Biosciences). Cultures were then incubated at 37 °C under 5% CO_2 atmosphere in medium composed of minimal essential medium (Invitrogen), containing 2 nM NGF, 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, and 10 μM aphidicolin. After 6 days, the neuronal cultures were deprived of NGF by switching to a medium lacking NGF and containing an anti-NGF antibody (Roche Applied Science). After 12 h, this medium was replaced with medium in the presence or absence of indicated ligand (Mig, NGF). Neurons were fixed with 4% paraformaldehyde/PBS overnight at 4 °C and rinsed in PBS and stained briefly with 0.1% crystal violet. The neurons were destained in H_2O, dehydrated in increasing ethanol concentrations, and then transferred to xylene. Neurons staining darker than debris with a clearly defined cellular outline and a well defined nucleus were scored as viable. Random photographs were taken (three or four per well), and the average number of viable cells per field was determined. Three independent experiments were performed in duplicate for each data point presented.

RNA Isolation and Northern Blot Analysis—Poly(A)$^+$ RNA was isolated from PC12 cells. Rat spleen, fetal brain (Sprague-Dawley, postnatal day 0), and adult brain (Sprague-Dawley, 8 weeks old) mRNAs were purchased from Clontech. Poly(A)$^+$ RNA, 5 μg, was fractionated on 1% agarose gel containing 2.2 M formaldehyde and then transferred to a Hybond N+ filter membrane (Amersham Biosciences). Hybridization was carried out at 65 °C in ExpressHyb solution (Clontech). The probe for rat CXCR3 was prepared by reverse transcription (RT)-PCR using

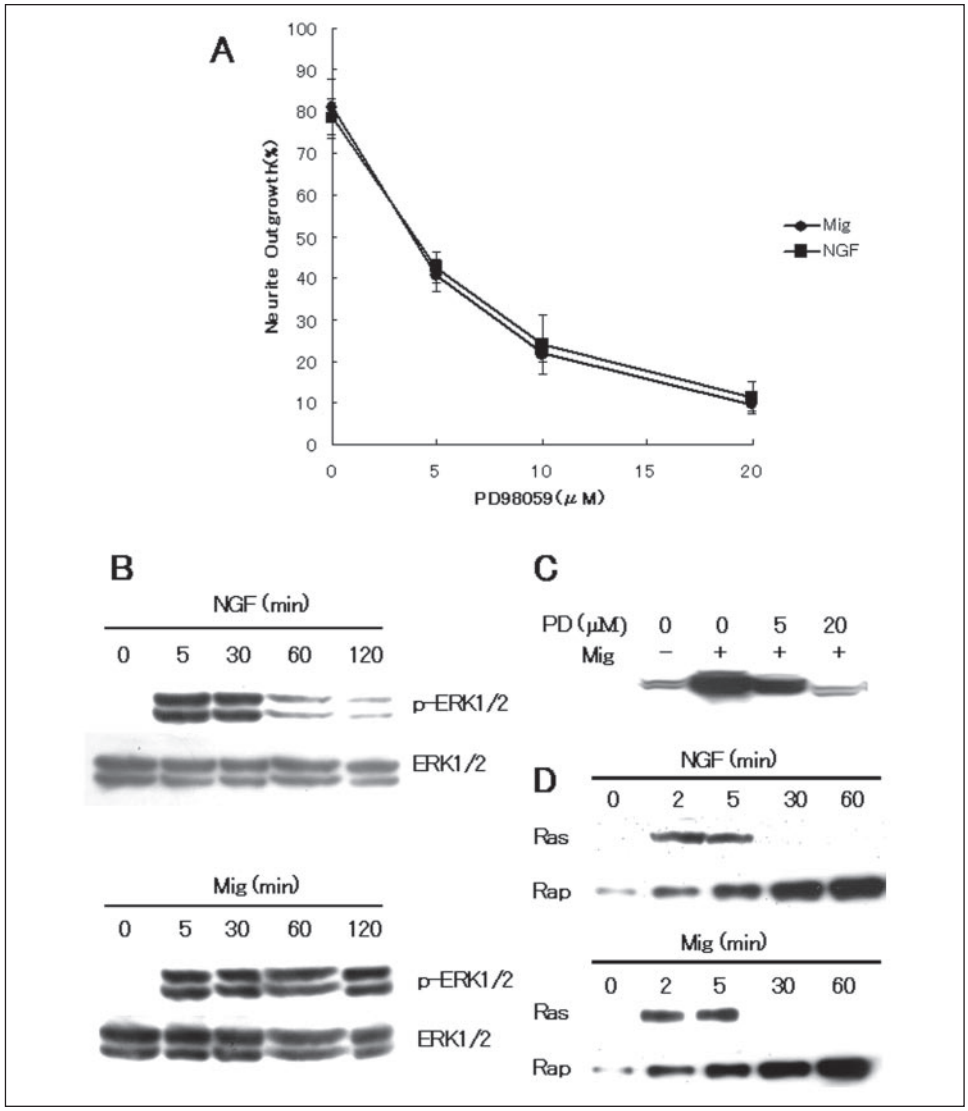

FIGURE 3. **Activation of Ras, Rap1, and ERK by Mig.** *A*, effect of PD98059 on neurite outgrowth induced by Mig. PC12 cells were plated and preincubated in DMEM containing the indicated concentration of PD98059 for 15 min, and then 100 ng/ml Mig or 100 ng/ml NGF was added. After further incubation for 24 h, the percentage of cells with neurites was determined. Each value is the mean ± S.E. for about 100 cells sampled from three independent experiments. *B*, time course of ERK phosphorylation in PC12 cells stimulated by Mig or NGF. PC12 cells (2×10^4 cells) were treated with DMEM containing 100 ng/ml Mig or 100 ng/ml NGF. At the indicated times, the cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with an anti-phospho-ERK antibody (*p-ERK*). The blots were stripped and reprobed with anti-ERK antibody (*ERK*) to verify that the protein levels were uniform. The experiments were repeated three times with similar results. *C*, effect of PD98059 on ERK phosphorylation in PC12 stimulated by Mig. PC12 cells were plated and preincubated in DMEM containing the indicated concentration of PD98059 for 15 min, and 100 ng/ml Mig was added, followed by further incubation for 5 min. ERK phosphorylation was analyzed by immunoblotting. Data shown are representative of three experiments. *D*, kinetic analysis of Ras and Rap1 activation. Equal numbers of serum-starved PC12 cells were treated with either Mig (100 ng/ml) or NGF (100 ng/ml). At different time points, cells were lysed and analyzed with *in vitro* Ras and Rap1 activation assays employing C-RafRBD/GST and RalGDSRBD/GST. Data shown are representative of three experiments.

rat spleen mRNA and a primer set of 5′-GAGCAGCACAGACACCTTCCTGCTCCACCT-3′ (sense) and 5′-GGATATGGGCATAGCAGTAGGCCATGACTA-3′ (antisense). The probe for human Mig was prepared by RT-PCR using human spleen mRNA and a primer set of 5′-CCACATCCCACTCACAACAG-3′ (sense) and 5′-GCTGAGCAAACATCCTGTC-3′ (antisense). The probe for rat Mig was prepared by RT-PCR using rat spleen mRNA and a primer set of 5′-TTTCCTAAATAAATATGACC-3′ (sense) and 5′-CGAGTTGTGTATTAAAGGGA-3′ (antisense). The β-actin probe was purchased from Clontech. The DNA fragments were labeled with ^{32}P using the Multiprime DNA labeling system (Amersham Biosciences). After washing at 60 °C with 0.2 × standard saline citrate (SSC) and 0.1% SDS, the filter was exposed to an x-ray film (Kodak) at −80 °C with an intensifying screen.

Binding Assay—The purified recombinant Mig was radiolabeled using ^{125}I-labeled Bolton-Hunter reagent (Amersham Pharmacia Biotech). For displacement experiments, cells were incubated for 1 h at room temperature with ^{125}I -labeled Mig in the presence of increasing concentrations of unlabeled chemokines in 200 μl of binding buffer (0.5 M NaCl, 50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% bovine serum albumin, pH 7.2). For saturation experiments, cells were incubated with increasing concentrations of radiolabeled Mig in the presence or absence of 1 μM unlabeled Mig. At the end of the incubation, the cells were washed five times with binding buffer and then lysed in 0.5 ml of 1 N NaOH. Radioactivity was determined using a gamma counter. Each data point was determined in duplicate, and binding data were analyzed according to the method of Scatchard.

RESULTS

Mig Increases Neurite Extension in PC12 Cells—We examined the effect of over 200 small secreted proteins such as cytokines, chemokines, and bioactive peptides on neurite outgrowth in PC12 cells. Our results indicated that one of the CXC chemokines, monokine induced by interferon-γ (Mig, CXCL9) stimulates neurite extension in PC12 cells. Mig treatment of PC12 cells induced their differentiation, as shown clearly by the appearance of neurite outgrowth within 24 h (Fig. 1A). In contrast, interferon-inducible protein 10 (IP-10), interferon-inducible T cell-α chemoattractant (I-TAC) that belongs to the CXC chemokine (non-ELR motif) subfamily did not stimulate neurite extension in PC12 cells (Fig. 1*A*). We next attempted to block Mig-induced neurite outgrowth of PC12 cells using a neutralizing Mig antibody. When the medium containing Mig was preincubated with a neutralizing Mig antibody, the neurite outgrowth activity of Mig was dramatically reduced

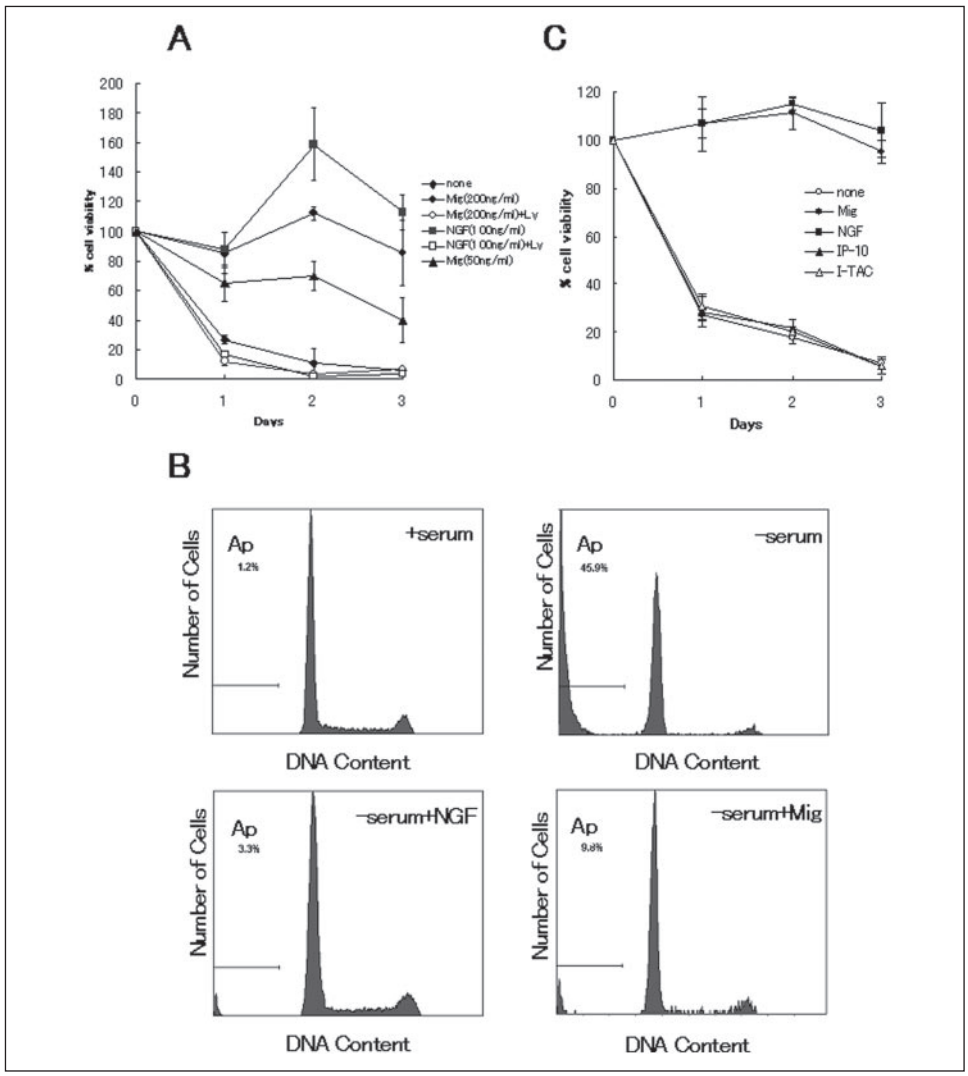

FIGURE 4. **Mig promotes PC12 cell survival.** *A*, PC12 cells were cultured in serum-free DMEM with or without either 200 ng/ml, 50 ng/ml Mig, or 100 ng/ml NGF in the absence or presence of 20 μM LY294002. Cell viability was measured at the indicated times by the MTT metabolism assay. Relative cell numbers were determined in triplicate by estimating the value of day 0 as 100. Results are the means ± S.E. of one representative experiment performed three times with similar results. *B*, PC12 cells were tested for cell survival by flow cytometry analysis. PC12 cells were treated as indicated and as described under "Experimental Procedures." Cells were harvested after 48 h and analyzed for DNA content by flow cytometry. The percentage of cells at the sub-G_1 phase of the cell cycle is indicated. This cell fraction represents cells undergoing apoptosis. The experiments were repeated three times with similar results. *C*, PC12 cells (5×10^3 cells/well) were induced to differentiate by 100 ng/ml NGF treatment for 7 days in DMEM containing 1% heat-inactivated fetal bovine serum and 0.5% heat-inactivated horse serum. The cells were then washed twice with PBS and cultured in serum-free DMEM with or without either 100 ng/ml Mig or 100 ng/ml NGF. The extent of cell viability was determined daily as described in *A* using the differentiated untreated cells at the start point as control $t = 0$. Results are the means ± S.E. of one representative experiment performed three times with similar results.

(Fig. 1*A*). In contrast, preincubation of NGF with this antibody had no effect on NGF-induced neurite outgrowth (data not shown).

Mig promoted the extension in PC12 cells in a dose-dependent manner according to quantitative analysis of neurite extension (Fig. 1*B*). Substantial neurite outgrowth was observed at 50 ng/ml Mig or higher concentrations. With 100 ng/ml Mig, neurite outgrowth was the maximum and about the same level as that by 100 ng/ml NGF. After 2 days of treatment, the cultures treated with Mig and NGF still appeared comparable. The rate of neurite extension was the same for NGF for the first 48 h but after that, neurite length in the presence of Mig remained relatively constant while that caused by NGF increased. The amino acid sequence of rat Mig shares 65% sequence identity (or 77% similarity) to its human ortholog. Like human Mig, rat Mig also strongly stimulated neurite outgrowth in PC12 cells in a dose-dependent manner (supplemental Fig. S1).

The growth-associated protein 43 (GAP-43) is frequently used as a marker for sprouting, because it is located in growth cones, maximally expressed during nervous system development, and re-induced in injured and regenerating neural tissues (23). Immunocytochemistry showed that GAP-43 immunoreactivity was much stronger in Mig-differentiated PC12 cells and NGF-differentiated PC12 cells than in untreated PC12 cells (Fig. 2*A*) in accordance with the result of Western blotting analysis of GAP-43 (Fig. 2*B*). GAP-43 protein in Mig-differentiated PC12 cells was intensely distributed in the neuritic processes as in NGF-differentiated PC12 cells (Fig. 2*A*).

Neurite Outgrowth Action of Mig Is Mediated by ERK Signal Transduction—NGF-induced sustained activation of ERK pathway has been suggested to be crucial to the neuronal differentiation of PC12 cells (24). This led us to investigate whether Mig induces neurite outgrowth via sustained activation of the ERK pathway. ERK kinase inhibitor PD98059 markedly inhibited neurite outgrowth activity of NGF in a dose-dependent manner, supporting previous findings (Fig. 3A). Mig-induced neurite outgrowth was also inhibited by PD98059 in a similar dose-dependent manner (Fig. 3*A*), suggesting that activation of the ERK pathway is critical to the neurite outgrowth activity of Mig. We then used immunoblot analysis with anti-phospho-ERK1/2 antibody to detect activated ERK in PC12 cells stimulated by NGF or Mig. NGF stimulated activation of ERK in PC12 cells, and Mig also stimulated it (Fig. 3*B*). This induction of ERK activation in PC12 cells appeared within 5 min by Mig as well as NGF. ERK activation by Mig, however, lasted for 2 h at least, which was much longer than that by NGF (Fig. 3*B*). These findings suggest that sustained activation of ERK is critical for Mig-induced neurite outgrowth of PC12 cells as well as NGF. IP-10 and I-TAC did not activate ERK1/2 (supplemental Fig. S2). We confirmed that PD98059 was able to inhibit ERK activation by Mig (Fig. 3*C*).

It has been suggested that activation of ERK by NGF involves two distinct pathways: the initial activation of ERK requires the small G protein Ras, but its activation is sustained by the small G protein Rap1 (24). Thus we investigated whether Mig activates Ras and Rap1 in PC12 cells. First, we examined Ras activation via an established method that takes advantage of specific binding of a C-RafRBD/GST fusion protein to the activated, GTP-bound, form of Ras. When serum-starved PC12 cells were treated with Mig, Ras activation was induced transiently in a pattern similar to that for NGF (Fig. 3*D*). Ras was activated maximally at 2–5 min and was down-regulated rapidly thereafter (Fig. 3*D*). We next investigated the effect of Mig on Rap1 activation. Similar to the assay used for activated Ras, a RalGDSRBD/GST fusion protein was used to analyze Rap1 activation. Like other small GTP-binding proteins, Rap1 is active in the GTP-bound state. Mig treatment elicited a marked increase in $Rap1^{GTP}$ (Fig. 3*D*). Like NGF, Mig-induced Rap1 activation was detected as early as 2 min and sustained for 60 min at least, an effect that matched the sustained activation of ERK pathway. These results suggest that Ras and Rap1 are involved in ERK activation by Mig.

Induction of Cell Survival by Mig through PI3K Pathway—PC12 cells are not viable in medium lacking serum. However, supplementation of serum-free medium with NGF promotes the survival of PC12 cells (25–27). To test the possibility of Mig also promoting the survival of PC12 cells, the cells were cultured in serum-free medium with or without Mig for up to 3 days. The relative number of surviving cells was determined at different time points after serum deprivation. In contrast to the exponential decrease in the number of PC12 cells in serum-free medium, the number of Mig-treated PC12 cells was maintained or even increased in serum-free medium for up to 3 days (Fig. 4A). The same result was obtained with the experiment using NGF. Mig displays dose-dependent survival activity on PC12 cells (Fig. 4*A*). IP-10 and I-TAC did not prevent cell death (data not shown).

To further analyze the effect of Mig on survival of PC12 cells, we determined the fraction of cells that underwent apoptosis in serum-starved condition by performing flow cytometry. After 48-h serum deprivation, apoptosis was measured as the percentage of cells with a content of DNA lower than G_0/G_1 by analysis of the cell cycle by flow cytometry. The fraction of sub-G_1 cells, which represents cells undergoing apoptosis, was increased by serum deprivation to 45.9%. Addition of NGF or Mig reduced the percentage of apoptotic cells to 3.3% and 9.8% in serum-deprived cells, respectively (Fig. 4*B*). These results indicate that Mig protects serum-starved PC12 cells from apoptosis.

We next examined whether Mig can also protect differentiated PC12 cells that exhibit a sympathetic neuronal cell phenotype from cell death induced by serum withdrawal. For this aim, we performed experiments using PC12 cells that were induced to differentiate by NGF treatment for 1 week. As shown in Fig. 4*C*, incubation of differentiated PC12 cells with Mig or NGF inhibited neuronal cell death induced by serum withdrawal. In contrast, IP-10 and I-TAC did not prevent neuronal cell death (Fig. 4*C*).

Activation of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of the downstream signaling effector Akt has previously been implicated in the survival signaling caused by NGF in serum-deprived PC12 cells (26, 27). For this reason, we analyzed the kinetics of Akt activation following NGF or Mig treatment of PC12 cells, and tested whether LY294002, a specific inhibitor of PI3K, blocks the activity of Akt. Activation of Akt can be easily detected by Western blotting with antibodies that specifically recognize Akt molecules phosphorylated at serine 473, because this phosphorylation is correlated with Akt activity (28). Western blotting analysis showed that NGF and Mig induced prolonged Akt activation, which lasted for 2 h in PC12 cells (Fig. 5*A*), and

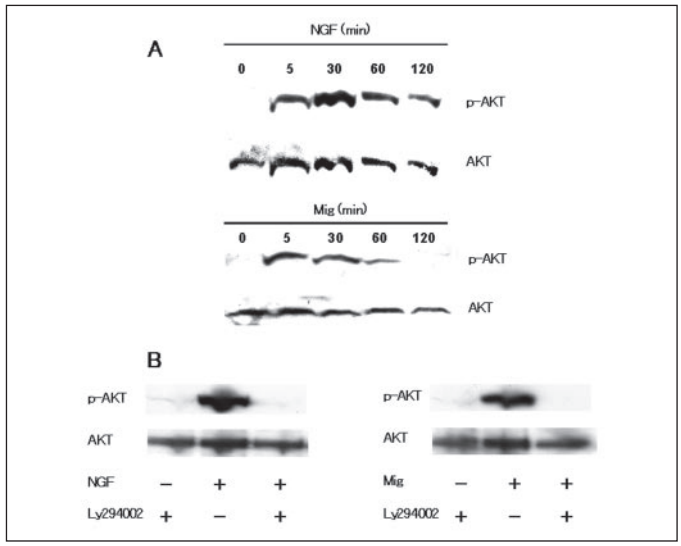

FIGURE 5. **Activation of PI3K/Akt pathway by Mig.** *A*, PC12 cells (2×10^4 cells) were treated with DMEM containing 100 ng/ml Mig or 100 ng/ml NGF for the indicated time points. Whole cell lysates were analyzed by immunoblot analysis using anti-phospho-Akt antibody (*top panel*), stripped, and reprobed with anti-Akt antibody (*bottom panel*). Representative data from three independent experiments are shown. *B*, PC12 cells were plated and preincubated in DMEM containing 20 μM LY294002 for 15 min, and 100 ng/ml Mig or 100 ng/ml NGF was added, followed by further incubation for 5 min. Akt phosphorylation was analyzed by immunoblotting. Representative data from three independent experiments are shown.

LY294002 was able to inhibit Akt activation by Mig or NGF (Fig. 5*B*). In contrast, IP-10 and I-TAC did not induce Akt activation (supplemental Fig. 3). LY294002 caused cell death of PC12 cells in serum-free medium in the presence of Mig or NGF (Fig. 4*A*). These findings indicate that activation of PI3K and subsequent activation of the downstream signaling effector Akt is necessary for Mig-and NGF-induced cell survival.

Mig Prevents NGF Deprivation-induced Apoptosis of Sympathetic Neurons—Sympathetic neurons from the rat superior cervical ganglion provide a well characterized model for studying NGF-mediated neuronal survival. If NGF is withdrawn from cultured sympathetic neurons, they undergo apoptosis (29). Therefore, we questioned whether Mig would rescue embryonic rat sympathetic neurons deprived of NGF. Primary neuronal cells cultured with NGF for 6 days were deprived of NGF for 12 h and then treated with NGF or Mig for 4 days. In control cultures the removal of NGF caused about 50% death by 24 h and about 75% death by 48 h. In contrast, only 5% of neurons treated with 200 ng/ml Mig died by 48 h, with 20% dying by 96 h (Fig. 6A). Neurons cultured with either NGF or Mig exhibited phase-bright cell bodies and extensive neuritic networks characteristic of viable neurons. In contrast, apoptotic neurons were found in the cultures incubated with no factors (Fig. 6*B*).

Mig Does Not Act on CXCR3, TrkA—Mig (CXCL9) along with IP-10 (CXCL10) and I-TAC (CXCL11) belong to the CXC chemokine (non-ELR motif) subfamily and share the same receptor CXCR3, which is preferentially expressed on Th1 lymphocytes in the immune system (30). Nevertheless, PC12 neurite extension assay revealed that only Mig activated ERK and stimulated neurite extension. To solve this puzzle, we investigated whether CXCR3 is expressed in PC12 cells. Northern blot analysis revealed that the rat CXCR3 transcript was not detected in PC12 cells (Fig. 7A). Because the probe used for Northern blot analysis covers the entire rat CXCR3 cDNA, it would detect any of the alternative spliced forms of CXCR3.

To characterize the Mig receptor on PC12 cells, we investigated the specific binding of Mig to PC12 cells. Radiolabeled Mig specifically

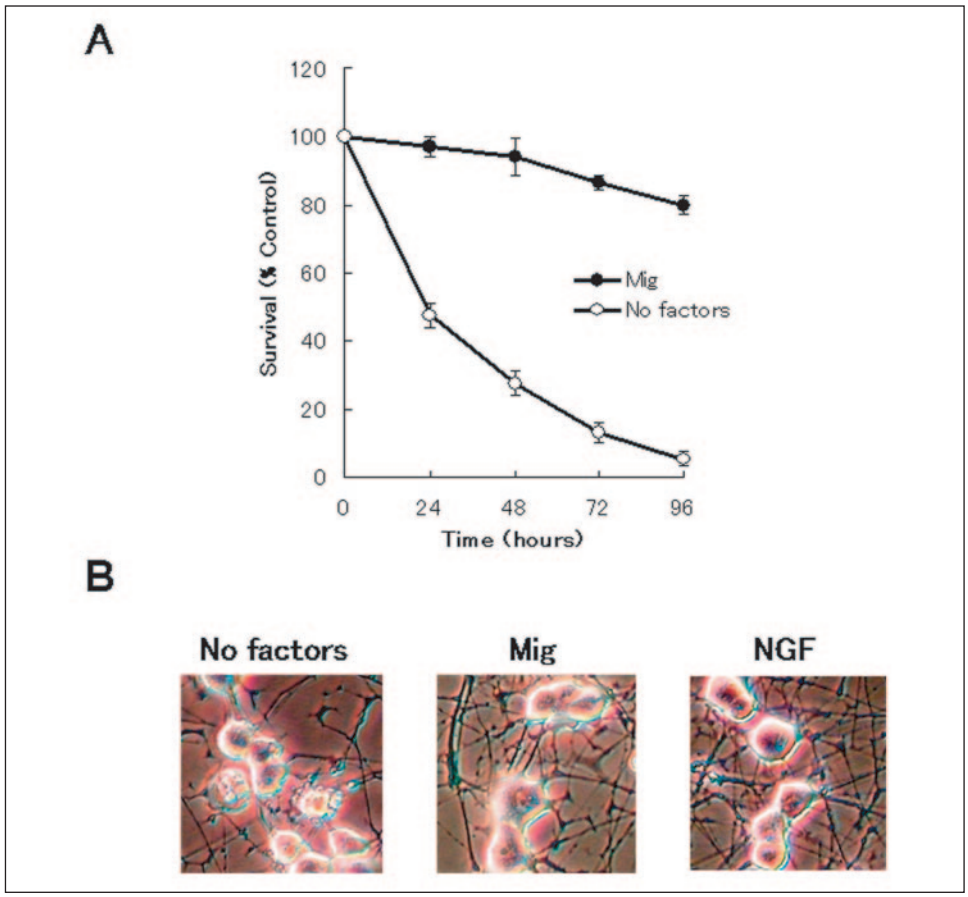

FIGURE 6. **Mig prevents the death of NGF-deprived sympathetic neurons.** *A*, 6-day neuronal cultures were deprived of NGF for 12 h, and then treated with no factors, 200 ng/ml Mig, or 100 ng/ml NGF. Survival was assayed at 24, 48, 72, and 96 h after treatment by counting Nissl-stained neurons, as described under "Experimental Procedures." Results are the means ± S.E. from three independent experiments and are presented as a percentage of the survival in control NGF-maintained cultures. *B*, 6-day neuronal cultures were deprived of NGF for 12 h, then maintained for an additional 24 h with no factors, 200 ng/ml Mig, or 100 ng/ml NGF. Healthy sympathetic neurons maintained in the presence of Mig or NGF showed phase-bright appearance in phase-contrast photographs.

bound to PC12 cells in a dose-dependent manner (Fig. 7*B*). A typical set of binding curve and corresponding Scatchard analyses are shown in Fig. 7 (*B* and *C*). These gave a linear plot characteristic of a single high affinity binding site with 670 receptors/cell and a dissociation constant (K_d) of 2.9 nM. The absence of detectable CXCR3 mRNA expression in PC12 cells suggests that the Mig receptor on PC12 cells is not CXCR3. We tested this idea by attempting to compete radiolabeled Mig binding with unlabeled IP-10, another non-ELR-containing CXC chemokine that binds to CXCR3 with the same affinity as Mig (30). Fig. 7*D* shows that IP-10 was not able to displace ^{125}I-Mig from PC12 cells even when its concentration exceeded that of Mig by 500-fold, whereas Mig efficiently displaced itself under the same conditions. These findings suggest that the receptor for Mig on PC12 cells is not CXCR3.

CXCR3 is a pertussis toxin-sensitive G protein-coupled receptor (GPCR), indicating that it is coupled to the G_i class of heterotrimeric G proteins (3). Mig-induced neurite outgrowth of PC12 cells was not blocked by pertussis toxin pretreatment, suggesting that this response does not involve CXCR3 (Fig. 7*E*).

Several studies have demonstrated that NGF mediates neuronal survival and differentiation processes in PC12 cells through its receptor, TrkA (26, 31, 32). This led us to investigate whether TrkA and its signal transduction are involved in Mig-induced neurite outgrowth. For this purpose, we used K252a to block tyrosine autophosphorylation of TrkA and its downstream signal transduction. PC12 cells were incubated with the indicated concentrations of K252a for 15 min prior to Mig or NGF addition. K252a at concentrations higher than 50 nM markedly inhibited NGF-induced neurite outgrowth but did not show any inhibition of Mig-induced neurite outgrowth (Fig. 7*F*). Furthermore, immunoblot analysis showed that Mig had no effect on TrkA autophosphorylation at Tyr-490 (data not shown). These results suggest that the neurite outgrowth action of Mig is independent of TrkA activation in PC12 cells. Thus, Mig and NGF actions are mediated by distinct upstream receptors sharing a common signaling pathway via ERK cascades. Mig probably acts on PC12 cells through an unknown receptor distinct from CXCR3 or TrkA.

Expression of mig mRNA in the CNS—There are few studies on the expression of *mig* under physiological condition in the CNS. For this reason we examined the expression of *mig* mRNA in various parts of human brain. Northern blot analysis showed an ~3-kb *mig* mRNA was found to be at high levels in spinal cord and, to a lesser extent, in medulla oblongata (Fig. 8*A*). In rat fetal brain, two transcripts, ~1.6 and 3 kb long, were observed, whereas in rat adult brain these two transcripts were detected at very low levels (Fig. 8*B*). RT-PCR analysis showed that rat *mig* mRNA is also expressed in spinal cord and medulla oblongata (data not shown).

DISCUSSION

Chemokines are small, soluble proteins that have been recognized to regulate leukocyte migration, adhesion, and proliferation in the immune system (1–3). Recent evidences suggest that chemokines and their receptors are expressed in the CNS and that their functions extend beyond their roles in inflammation. However, little is known about their physiological roles in the CNS.

In this report, we described one of these CXC chemokines, Mig (CXCL9), which acts as a neurotrophic factor on PC12 cells and rat primary sympathetic neurons. With regard to neurite extension activity, Mig strongly stimulated neurite outgrowth in PC12 cells in a dose-dependent manner. GAP-43, a marker for sprouting, was boosted by addi-

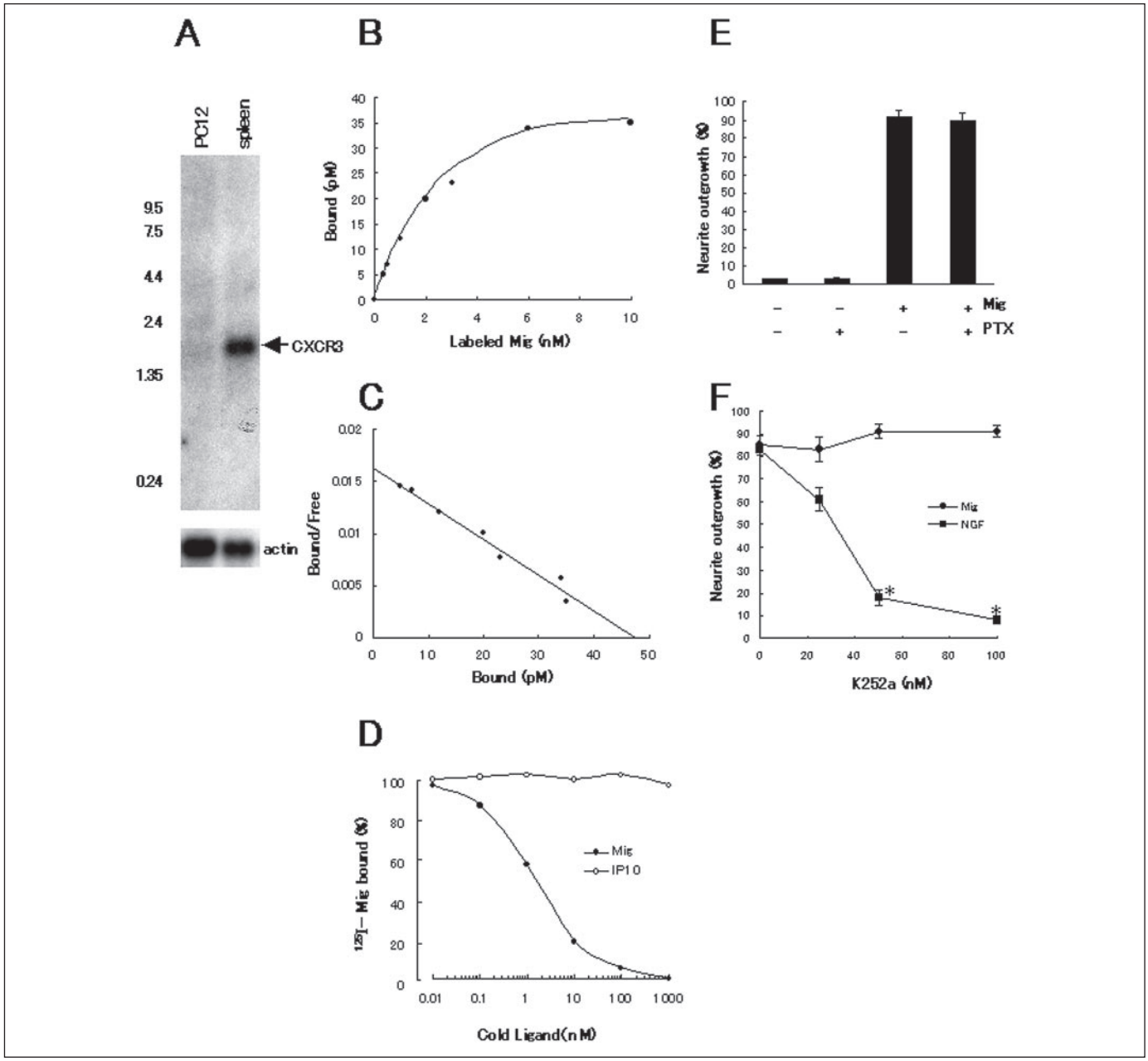

FIGURE 7. **Mig does not act on CXCR3, TrkA.** *A*, Northern blot analysis of rat CXCR3. Poly(A)$^+$ RNA samples derived from rat spleen and PC12 cells were subjected to Northern blot analysis using ^{32}P-labeled rat CXCR3 probes. After autoradiography, the filter was rehybridized with the β-actin cDNA probe. Positions of markers (kilobases) are shown on the *left*. Representative data from three independent experiments are shown. *B*, specific binding of ^{125}I-labeled Mig to PC12 cells (4×10^6 cells) with increasing concentrations of ^{125}I-Mig. Representative results from three separate experiments are shown. *C*, Scatchard analysis of the binding data in *B*. *D*, displacement of ^{125}I-Mig with unlabeled Mig or IP-10. PC12 cells (4×10^6 cells) were incubated with 2 nM ^{125}I-Mig in the presence of increasing concentrations of unlabeled Mig or IP-10. Representative results from three separate experiments are shown. *E*, effect of pertussis toxin (*PTX*) on Mig-induced neurite outgrowth of PC 12 cells. PC12 cells were preincubated for 60 min with 1 μg/ml PTX and 100 ng/ml Mig was added, followed by further incubation for 24 h. Each value is the mean $\pm$ S.E. for $\sim$100 cells sampled from three independent experiments. *F*, effect of K252a on PC12 cell neurite outgrowth induced by Mig. PC12 cells were preincubated in DMEM containing the indicated concentration of K252a for 15 min, and 100 ng/ml Mig or 100 ng/ml NGF was added, followed by further incubation for 24 h. The percentage of cells with neurites was determined. Each value is the mean $\pm$ S.E. for about 100 PC12 cells sampled from three independent experiments. *, $p < 0.001$, by Student's *t* test.

tion of Mig in PC12 cells. PC12 cells treated with Mig were differentiated into sympathetic-like neurons and induced prolonged activation of ERK. Because ERK kinase inhibitor PD98059 inhibited blocking of ERK activation and inhibited neurite outgrowth, sustained activation of ERK is considered to be crucial for Mig-induced neurite outgrowth of PC12 cells as well as NGF. It has been suggested that, in PC12 cells, NGF activates Ras and Rap1 to elicit the rapid and sustained activation of ERK, respectively (24). The sustained activation of ERK via Rap1 has been proposed to participate in NGF-dependent PC12 cells differentiation (24). Like NGF, Mig stimulated a transient activation of Ras but sustained Rap1 activation. These results suggest that Ras and Rap1 are involved in ERK activation and neuronal differentiation by Mig.

Several studies have demonstrated that NGF mediates neuronal survival and differentiation processes in PC12 cells through its tyrosine kinase receptor, TrkA (26, 31, 32). Therefore, we investigated whether TrkA and their downstream signal transduction were involved in Mig-induced neurite outgrowth. K252a, an inhibitor of tyrosine autophosphorylation of TrkA, inhibited neurite extension by NGF but did not

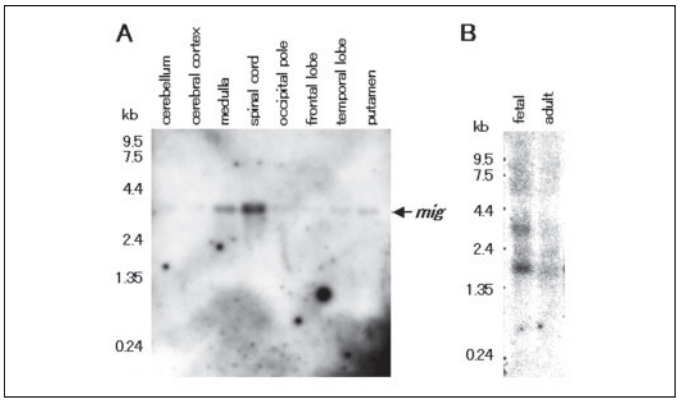

FIGURE 8. **Northern blot analysis of *mig* mRNA expression in the CNS.** *A*, Northern blot filter (Clontech) was hybridized with ^{32}P-labeled human *mig* cDNA. The experiment was repeated once using another batch of the commercial Northern filter, with similar results. *B*, Poly(A)$^+$ RNA samples derived from rat fetal and adult brain were subjected to Northern blot analysis using ^{32}P-labeled rat *mig* probes. Similar results were obtained in at least two more experiments.

inhibit that by Mig. This result suggests that neurite extension activity by Mig is not mediated by TrkA on PC12 cells. Mig and NGF actions are mediated by distinct upstream receptors sharing a common signaling pathway via ERK cascades.

In addition to neurite extension activity, Mig showed dose-dependent survival activity on PC12 cells in serum-free medium. It also promoted survival of rat primary sympathetic neurons that die when deprived of NGF. NGF and Mig induced prolonged Akt activation that is involved in cell survival. LY294002, a specific inhibitor of PI3K, inhibited Akt activation and caused apoptosis of PC12 cells. These results indicate that PI3K, as well as its downstream effector Akt, can be the signaling pathway responsible for Mig-induced cell survival.

Chemokines mediate their biological activities through G protein-coupled cell-surface receptors (1–3). Binding of chemokine to its receptor triggers the activation of several molecules and signaling pathways, including the activation of PI3K and ERK pathways (1–3). In this study, we have provided evidence that Mig stimulates activation of ERK and PI3K signal transduction pathway in PC12 cells. IP-10 (CXCL10) and I-TAC (CXCL11), along with Mig (CXCL9), belong to the CXC chemokine (non-ELR motif) subfamily and share the same receptor CXCR3, which is preferentially expressed on Th1 lymphocytes in the immune system (30). However, IP-10 and I-TAC could not cause neurite outgrowth or neuroprotective actions. We have found that PC12 cells have abundant levels of a receptor that binds Mig with a K_d of 2.9 nM, but the binding of ^{125}I-labeled Mig to PC12 cells was not competed by another high affinity ligand for CXCR3, IP-10. Furthermore, Northern blot and RT-PCR analyses revealed that CXCR3 mRNA expression did not occur in PC12 cells. In addition, although CXCR3 is a pertussis toxin-sensitive G protein-coupled receptor, Mig-induced neurite outgrowth was not inhibited by pertussis toxin pretreatment. A recent study indicates that a functional IP-10-specific receptor, which is not CXCR3 and does not bind Mig, is expressed by epithelial and endothelial cells (33). Therefore, a novel Mig-specific receptor, other than CXCR3, may exist on PC12 cells and contribute to Mig-induced signal transduction. In contrast to I-TAC and IP-10, Mig has an extended basic C-terminal region (34). The role of this extension is unknown, but it is speculated that this region interacts with cell surface glycosaminoglycans such as heparin sulfate that might be important in the binding of Mig to its receptor (35–37). The highly basic C-terminal region might be necessary to bind to the novel receptor in PC12 cells.

Chemokine receptors belong to the GPCR family and exhibit common structural features (1–3). RT-PCR using degenerate primers targeting the conserved DRY box and seventh transmembrane (TM7) consensus sequences showed that three GPCRs (NLR, GPRN1, and D6) are expressed in PC12 cells. NRL and GPRN1 are orphan GPCRs (38, 39). D6 is a chemokine binding molecule with homology to chemokine receptors but defective signaling function (40). These receptor cDNAs were transfected into HEK293EBNA cells, and transfectants were tested for binding with ^{125}I-labeled Mig. Radiolabeled Mig bound specifically to CXCR3 transfectants, while NLR, GPRN1, and D6 transfectants showed virtually no specific binding (supplemental Fig. S4). This suggests that the Mig receptor expressed in PC12 cells is unlikely to be encoded by these GPCR cDNAs. It is possible that the receptor for Mig on PC12 cells is not a GPCR.

In the CNS, Mig is secreted from macrophages and reactive astrocytes under pathological conditions such as multiple sclerosis and viral infection, and its expression is augmented by interferon-γ produced by activated T-cells (6). However, few studies have been done on the expression of Mig under physiological conditions in the CNS, except for a recent study showing that cultured human fetal and simian adult astrocytes express mRNA for Mig (41). Our Northern blot and RT-PCR analyses showed that Mig is expressed in human and rat adult spinal cord and medulla oblongata. We found the expression of Mig in rat fetal brain. Because the expression of Mig is much stronger than in adult brain, it is speculated that Mig plays an important role in developmental brain. We have to broadly examine the expression of Mig in both developing and adult brains to obtain clues on the function of Mig. Recently mice deficient in Mig were generated by targeted gene deletion mutagenesis (42). The *mig*−/− mice, born at the expected Mendelian ratios, showed no overt developmental abnormalities. Microscopic examination of hematoxylin and eosin-stained tissues, including among them lymph nodes, spleen, and Peyer's patches, showed no differences in histology between *mig*−/− and *mig*+/+ mice (42). Because these studies are focused on the immune system, it may be important to examine the CNS in Mig-deficient mice.

In conclusion, we have demonstrated in this report that Mig is able to induce the neuronal differentiation and prevent the neuronal cell death of PC12 cells through ERK and PI3K signaling pathways. It has been reported that NGF and a pituitary adenylate cyclase-activating polypeptide (PACAP) induce differentiation with sympathetic neuron-like characteristics and survival of PC12 cells (21, 22, 43–45). These molecules have a neurotrophic effect on the CNS. NGF plays a crucial role in promoting growth, differentiation, and function in sympathetic nerve cells (46). Pituitary adenylate cyclase-activating polypeptide has neurotrophic activity on the rat cerebellar cortex during development (47). In this report, we have reported that Mig promotes cell survival of rat primary sympathetic neurons. Our findings offer clues for elucidating the novel functions of chemokines in the CNS.

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