The RON receptor-type tyrosine kinase, a member of the hepatocyte growth factor receptor family, is a receptor for macrophage-stimulating protein (MSP). Recently, we observed that MSP induces morphological changes in interleukin (IL)-3-dependent Ba/F3 cells ectopically expressing RON. We show here that stimulation of those cells with either MSP or IL-3 increases tyrosine phosphorylation of proteins of 130, 110, 90, 62, and 58 kDa and induces similar morphological changes, accompanied by unique nuclear shape and redistribution of F-actin. A tyrosine kinase inhibitor, genistein, blocked both the increase in tyrosine phosphorylation and morphological changes. Upon stimulation with either MSP or IL-3, prominent tyrosine-phosphorylated pp90 was similarly co-immunoprecipitated with the common β chain of IL-3 receptor (βc). Unlike IL-3, stimulation with MSP increased tyrosine phosphorylation of βc without activation of JAK2, resulting in morphological changes with modest cell growth. Confocal immunofluorescence analyses showed colocalization of RON, βc, and tyrosine-phosphorylated proteins. In vitro kinase assays revealed that autophosphorylated RON phosphorylated βc. These results suggest that the signaling pathway for morphological changes through βc and its associated protein pp90 is distinct from the pathway for cell growth in the IL-3 signal transduction system.

Receptor-type tyrosine kinases (RTKs) play a critical role in pleiotropic cell functions such as proliferation and differentiation (1, 2). All RTKs are composed of three major domains, an extracellular ligand binding domain, a single membrane-spanning domain, and a cytoplasmic domain that contains a tyrosine kinase catalytic domain (3, 4). After ligand binding, RTKs undergo dimerization leading to activation of their intrinsic tyrosine kinase activity and autophosphorylation (5). The tyrosine-phosphorylated regions of the receptor function as high-affinity binding sites for proteins containing Src homology 2 and phosphotyrosine-binding domains, which transduce receptor function as high-tyrosine kinase activity and autophosphorylation (5). The tyrosine kinase catalytic domain (3, 4). After ligand binding, RTKs

The RON receptor-type tyrosine kinase is a member of a subfamily of RTK that includes the c-Met hepatocyte growth factor receptor and c-Sea (7). The human RON gene was cloned from human keratinocytes (8), and the mouse homolog, formerly known as STK (gtem cell-derived tyrosine kinase), was derived from mouse hematopoietic stem cells (9). RON, as well as the hepatocyte growth factor receptor, is synthesized as a single-chain precursor, then cleaved to a mature disulfide-linked heterodimer composed of an extracellular α chain and a transmembrane β chain with intrinsic tyrosine kinase activity (10, 11). RON has been identified as a receptor for macrophage-stimulating protein (MSP) and binding of MSP to RON stimulates autophosphorylation of RON to transmit signals intracellularly (10–13).

MSP is an 80-kDa serum protein that belongs to a family characterized by the presence of a highly conserved tripeptide disulfide loop structure (Kringel domain). The family includes prothrombin, plasminogen, urokinase, and hepatocyte growth factor (14–17). MSP was shown to induce murine resident peritoneal macrophages to become responsive to chemoattractant C5a (18). MSP has multiple biological effects. In murine resident peritoneal macrophages it induces cell spreading and migration (19, 20), direct chemotaxis (19), stimulates ingestion of complement-coated erythrocytes (15), and inhibits endotoxin- or cytokine-induced expression of inducible nitric oxide synthase mRNA (20). MSP also plays roles in chemotaxis of keratinocytes (21), bone resorption in osteoclasts (22), and in cilary motility in bronchial epithelial cells (23).

It has been established that the IL-3 receptor shares a common β(3) subunit with the IL-5 receptor and the granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor and that each receptor is composed of a unique α subunit and the βc subunit (24–26). Each α subunit specifically exhibits low-affinity binding to IL-3, IL-5, or GM-CSF, whereas βc subunit lacks direct ligand binding but confers high affinity binding to the α subunit. Binding of IL-3 to its receptor complex induces activation of the JAK2 tyrosine kinase. In turn, autophosphorylated JAK2 kinase activates βc to recruit various signaling molecules to the tyrosine-phosphorylated βc and triggers signal transduction events mediated by the cytoplasmic domain of βc (27–30).

Recently, we found that stimulation with MSP induces cell growth through phosphorylation of two C-terminal tyrosine residues in the multifunctional docking site of RON (31). Stimulation with MSP also induces cell shape changes (11). To gain further insight into signal transduction events required for morphological changes and cell growth, we have compared MSP- and IL-3-induced tyrosine phosphorylation in IL-3-dependent Ba/F3 cells that ectopically express RON on the cell surface. Binding of MSP to RON activates βc without activation of JAK2 to produce morphological changes. Our findings sug-
suggest that IL-3 stimulation activates two distinct signaling pathways, one involved in morphological changes and the other in cell growth.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Recombinant human MSP and recombinant mouse IL-3 were kindly provided by Drs. M. Hagiya and T. Takehara (Toyobo Co. Ltd, Shiga, Japan) and Dr. T. Sudo (Toray Industries, Inc., Kamakura, Japan), respectively. A rabbit polyclonal and a biotinylated rat monoclonal antibody against RON recognize the intracellular C-terminal region and the extracellular domain, respectively (11). Anti-phosphotyrosine (4G10 and FITC-conjugated 4G10) and anti-mouse JAK2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-mouse β chain common to IL-3, IL-5, and GM-CSF receptors (βc), and Texas red-conjugated streptavidin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and PharMingen (San Diego, CA), respectively. Horseradish peroxidase-conjugated F(ab')2 fragments of anti-mouse Ig and of anti-rabbit Ig (Amersham Pharmacia Biotech) and rhodamine-phalloidin (Molecular Probe, Eugene, OR) were also used.

**Plasmids and Cells**—cDNAs for murine full-length RON and RON-F1330/F1337, carrying combined mutations of tyrosine 1330 and 1337 to phenylalanine, were described previously (31). The murine IL-3-dependent pro-B cell line Ba/F3 stably expressed with either wild-type mouse JAK2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-mouse β chain common to IL-3, IL-5, and GM-CSF receptors (βc), and Texas red-conjugated streptavidin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and PharMingen (San Diego, CA), respectively. Horseradish peroxidase-conjugated F(ab')2 fragments of anti-mouse Ig and of anti-rabbit Ig (Amersham Pharmacia Biotech) and rhodamine-phalloidin (Molecular Probe, Eugene, OR) were also used.

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**FIG. 1.** Morphological changes accompanied by F-actin reorganization upon stimulation with MSP or IL-3. 

A, phase-contrast micrographs of IL-3-dependent long-term cultured bone marrow cells. Cells were cultured in the presence of 100 units/ml IL-3 at 37 °C (a). The cells became round after IL-3 deprivation for 7 h (b) and were restimulated with 100 units/ml IL-3 for 1 h (c). Arrowheads indicate cells morphologically changed. Bar, 20 μm. B, phase-contrast micrographs of either wild-type RON- or RON-F1330/F1337-expressing Ba/F3 cells. Cells deprived of IL-3 for 12 h were stimulated with 100 ng/ml MSP (b and c) or 100 units/ml IL-3 (d and f) in the presence of 1 mM Na3VO4 for 1 h at 30 °C after preincubation with 1 mM Na3VO4 for 5 min at 30 °C as described under “Experimental Procedures.” Bar, 20 μm. C, Nomarski differential-interference-contrast and confocal fluorescence microscopic analyses of either wild-type RON- or RON-F1330/F1337-expressing Ba/F3 cells. After stimulation with 100 ng/ml MSP, 100 units/ml IL-3, or medium alone as above, cells were stained with propidium iodide for nuclei and rhodamine-phalloidin for F-actin. Bar, 5 μm.
For detection of colocalization of tyrosine-phosphorylated proteins and with rhodamine-phalloidin for F-actin or propidium iodide for nuclei. BSA and 0.1% saponin for 20 min, as described (32), and then stained cells were permeabilized with phosphate-buffered saline containing 3% paraformaldehyde at room temperature for 20 min. After washing, the MSP or IL-3 as described above. The cells were fixed with 3.7% formaldehyde at 37 °C for 30 min and then stained for 30 min as above. The results represent the mean ± S.D. from three different experiments (%).

RON or RON-F1330/F1337 was cultured in serum-free medium containing 100 units/ml IL-3, as described (31).

**Morphological Changes**—Wild-type RON- or RON-F1330/F1337-expressing Ba/F3 cells were deprived of IL-3 for 12 h before stimulation. Cells were adjusted to 1.6 × 10^6 cells/ml in RPMI 1640 medium supplemented with 25 mM HEPES, pH 7.2, and 0.1% bovine serum albumin (BSA), preincubated in the presence of 1 mM Na_3VO_4 for 5 min, and then stimulated with 100 ng/ml MSP or 100 units/ml IL-3 in the presence of 1 mM Na_3VO_4 for the indicated periods at 30 °C. Treatment of cells with Na_3VO_4 specifically enhanced MSP- or IL-3-induced morphological changes. Morphological changes were quantitated by counting the number of cells characterized by distortion, bending, stretching, and branching of the cell body under a phase-contrast microscope in three randomly selected high-power fields (magnification, ×200). Cells having filopodia and lamellipodia around the cell periphery were not counted. For treatment with various inhibitors, cells (1.6 × 10^6 cells/ml) were pretreated with various concentrations of genistein or forskolin dissolved in 0.25% Me_2SO, 20 μg/ml actinomycin D, or 10 μg/ml cycloheximide in 0.5% ethanol at 37 °C for 30 min, and then stimulated with MSP or IL-3 as described above. Murine bone marrow cells were obtained from C57BL/6 mice and long-term cultured in α-minimum essential medium containing 10% fetal bovine serum in the presence of 100 units/ml IL-3 for 14 days. Cells were deprived of IL-3 for 7 h and then stimulated with 100 units/ml IL-3 for 1 h at 37 °C.

**Immunofluorescence**—Cells (1.6 × 10^6 cells/ml) were stimulated with MSP or IL-3 as described above. The cells were fixed with 3.7% paraformaldehyde at room temperature for 20 min. After washing, the cells were permeabilized with phosphate-buffered saline containing 3% BSA and 0.1% saponin for 20 min, as described (32), and then stained with rhodamine-phalloidin for F-actin or propidium iodide for nuclei. For detection of colocalization of tyrosine-phosphorylated proteins and RON-F1330/F1337-expressing cells (a) were pretreated with Me_2SO alone (upper panels) or 50 μg/ml genistein (lower panels) for 30 min and then stimulated with MSP or IL-3 in the presence of Me_2SO or genistein as described in Fig. 1. Bar, 20 μm. B, quantification of morphological changes. Wild-type RON-expressing cells (a) were stimulated with MSP (hatched bars), IL-3 (stippled bars), or medium alone (open bars) for 1 h as described in Fig. 1. The number of cells morphologically changed in the presence of the indicated concentrations of genistein (μg/ml), 20 μg/ml actinomycin D (ActD) and 10 μg/ml cycloheximide (CHX) were examined in three randomly selected high-power fields (magnification, ×200). The results represent the mean ± S.D. from three different experiments (%). RON-F1330/F1337-expressing cells (b) were pretreated with Me_2SO alone or 50 μg/ml genistein for 30 min and then stimulated for 30 min as above. The results represent the mean ± S.D. from three different experiments (%).

RON, cells were fixed as above, blocked with phosphate-buffered saline containing 3% BSA for 20 min, and then stained with biotinylated anti-RON for 1 h. After permeabilization with saponin, the cells were stained with FITC-conjugated anti-phosphotyrosine antibody for 1 h and Texas red-conjugated streptavidin for 30 min. To prevent dephosphorylation, 10 mM Na_3VO_4 was included throughout the staining procedure, as described (33). To detect colocalization of RON and β_c, cells were fixed as above, blocked with phosphate-buffered saline containing 3% BSA for 20 min, and then stained with biotinylated rat anti-RON for 1 h, followed by staining with Texas red-conjugated streptavidin for 30 min. After permeabilization with saponin, cells were stained with anti-β_c (upper panels) and reprobed with rabbit anti-RON (lower panels). The RON blot, the upper band represents the cytoplasmic single-chain precursor of RON. B, the upper panel shows pp90 (pp86, pp87, pp91, and pp94) upon stimulation with medium alone (lane 1), MSP (lane 2), and IL-3 (lane 3); the lower panel shows schematic locations of pp90 subtypes (pp86, pp87, pp91, and pp94).

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INCUBATION AT 37 °C FOR 3 H, CELLS MIGRATING THROUGH THE MEMBRANE WERE FIXED AND STAINED WITH MAY-GIEMSA. THE CELLS WERE COUNTED UNDER A MICROSCOPE IN FIVE RANDOMLY SELECTED HIGH-POWER FIELDS (MAGNIFICATION, ×400). RESULTS WERE EXPRESSED AS CELL NUMBER/S-HIGH-POWER FIELDS (5 HPF).

DETECTION OF TYROSINE PHOSPHORYLATION BY WESTERN BLOTTING AND IMMUNOPRECIPITATION—Cells (2 × 10⁵ cells/ml) were stimulated in the presence of 1 mM Na₃VO₄ at 30 °C as described above, unless stated. The cells were washed with HEPES-buffered saline (50 mM HEPES, pH 7.2, 150 mM NaCl and 10 mM Na₃VO₄) at 4 °C, and then lysed with Triton X-100 lysis buffer (50 mM HEPES, pH 7.4, 1% Triton-X-100, 4 mM EDTA, 100 mM NaF, and 10 mM Na₃VO₄) containing 50 μg/ml aprotinin, 200 μM leupeptin, 50 μM pepstatin A, and 2 μM phenylmethylsulfonyl fluoride at 4 °C. Lysates were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes. Immunodetection was performed by enhanced chemiluminescence, as described (31, 34, 35). Immunoprecipitations were performed with anti-RON, anti-βc, and anti-JAK2, and the immune complexes were washed with Triton X-100 lysis buffer at 4 °C and analyzed as described (31, 34, 35).

In Vitro Kinase Assay—Protein-G Sepharose beads (Pharmacia) were precoated with both anti-RON reactive to the epitope in the extracellular domain of RON and anti-βc antibodies. First, βc was immunoprecipitated from the Triton X-100 lysate of parental Ba/F3 cells on the precoated beads. After washing with Triton X-100 lysis buffer, RIPA buffer (50 mM HEPES, pH 7.4, 1% Triton-X-100, 4 mM EDTA, 1% deoxycholate, 0.1% SDS, 10 mM Na₃VO₄), and Triton X-100 lysis buffer containing 1 M NaCl, the beads were incubated with equal amounts of Triton X-100 lysates of either COS-7 cells or COS-7 cells transiently transfected with RON. RON kinase expressed in COS-7 cells was highly autophosphorylated without exposure to MSP, probably due to aggregation of abundantly expressed RON. After washing as above, equal amounts of each immunoprecipitate were subjected to in vitro kinase assays. The immunoprecipitates were suspended with 70 μl of kinase buffer (50 mM HEPES, pH 7.4, 0.1% Triton-X-100, 5 mM MgCl₂, and 5 mM MnCl₂) containing 0.2 mM Na₃VO₄ and 100 μM ATP. After incubation at 30 °C for the indicated periods, reactions were terminated by addition of 2× SDS sample buffer and boiling for 3 min. SDS-polyacrylamide gel electrophoresis and immunodetections were performed as described above. Phosphorylated bands detected with anti-pTyr (4G10) were quantified with a densitometer (ATTO, Tokyo).

RESULTS

Morphological Changes Induced by MSP or IL-3—It is often observed that exposure of bone marrow cells and IL-3-dependent cell lines to IL-3 leads to changes in cell morphology and cell growth. Fig. 1A shows an example of cell shape changes of IL-3-dependent long-term cultured bone marrow cells. Our previous finding (11) indicates that MSP stimulation induces morphological changes in the IL-3-dependent pro-B cell line Ba/F3 ectopically expressing wild-type RON. We therefore compared the effects of MSP and IL-3 on cell morphology in these cells. MSP and IL-3 were found to induce similar morphological changes. A decrease in incubation temperature from 37 to 30 °C and addition of Na₃VO₄, a tyrosine phosphatase inhibitor, increased the proportion of cells morphologically changed (Fig. 1B, upper panels). Morphological changes were detected at 15 min and peaked at ~60 min of stimulation (data not shown). Phosphorylation of two C-terminal tyrosine residues, Tyr-1330 and Tyr-1337, in the multifunctional docking site of RON plays a critical role in RON-mediated signal transduction (31). Surprisingly, cells expressing RON-F1330/F1337, which carried mutations of tyrosine 1330 and 1337 to phenylalanine, responded to MSP with striking changes of morphology (lower panels).

To characterize MSP- or IL-3-induced shape changes, cells were stained with propidium iodide for nuclei and rhodamine-phalloidin for F-actin, and analyzed by Nomarski differential-interference-contrast and confocal fluorescence microscopy. Fig. 1C shows that stimulation with MSP or IL-3 induced drastic morphological changes accompanied by unique nuclear shape changes and redistribution and an increase in F-actin. Most cells showed uropod-like structures. Note that lobulated nuclei were preferentially observed upon MSP stimulation, suggesting that the activity of MSP is stronger than that of IL-3. These results indicate that MSP and IL-3 induce similar shape changes, and that MSP-induced shape changes are not.
mediated through the multifunctional docking site of RON.

**Blockade of MSP- or IL-3-induced Shape Changes by Genistein**—Tyrosine phosphorylation is involved in MSP and IL-3 signaling. We examined whether genistein, a tyrosine kinase inhibitor, blocked morphological changes. Treatment of wild-type RON-expressing cells with genistein inhibited both MSP- and IL-3-induced morphological changes, whereas genistein alone had no effect on morphology and viability (Fig. 2, A and B). Another tyrosine kinase inhibitor, herbimycin A (20 µg/ml), also blocked morphological changes (data not shown). Morphological changes were not inhibited by actinomycin D (ActD), an RNA synthesis inhibitor, or cycloheximide (CHX), a protein synthesis inhibitor (Fig. 2B, a). MSP was more potent than IL-3 in both cell types (Fig. 2B, a and b), and the proportion of cells morphologically changed in RON-F1330/F1337-expressing cells was larger than that observed in wild-type RON-expressing cells (data not shown). In RON-F1330/F1337-expressing cells, morphological changes were also blocked by genistein (Fig. 2B, b). These results indicate that tyrosine phosphorylation is required for morphological changes.

**Tyrosine Phosphorylation of Cellular Proteins Induced by MSP or IL-3**—To determine whether MSP or IL-3 induced tyrosine phosphorylation of cellular proteins, Western blotting was performed using the anti-phosphotyrosine mAb 4G10 (Fig. 3). Stimulation of wild-type RON-expressing cells with MSP increased tyrosine phosphorylation of RON at 145 kDa and of proteins at 130, 110, 90, 62, and 58 kDa (Fig. 3A). A similar increase in tyrosine phosphorylation of proteins at 130, 110, 90, 62, and 58 kDa was observed upon stimulation with IL-3. Tyrosine phosphorylation of RON per se was not induced by stimulation with IL-3. Addition of Na3VO4 to cell suspensions throughout stimulation periods and the decrease in reaction temperature from 37 °C (Fig. 3A, lanes 4–6) to 30 °C (lanes 1–3) enhanced increases in the level of MSP- or IL-3-induced tyrosine phosphorylation probably due to inhibition of rapid dephosphorylation. These results are consistent with the morphological observations (Figs. 1 and 2) and suggest that RON-mediated signaling is closely related to the IL-3-induced signaling through tyrosine phosphorylation.

In addition, increased levels of tyrosine phosphorylation were apparent 5 min after stimulation, peaked at 15 min, and sustained for at least 60 min of stimulation (data not shown). These kinetics suggest that MSP- or IL-3-induced tyrosine phosphorylation precedes morphological changes. Furthermore, the levels of tyrosine phosphorylation induced by MSP were generally higher than those induced by IL-3, consistent with observations that MSP was more potent than IL-3 in promoting morphological changes (Fig. 2). pp90 was a prominent tyrosine-phosphorylated protein consisting of four subspecies (pp86, pp87, pp91, and pp94) (Fig. 3B, upper panel). Fig. 3B (lower panel) represents schematic locations of pp90 subspecies.

**Interaction of RON with the Common β Chain of IL-3 Receptor (βc)**—Because treatment with genistein inhibited MSP- or IL-3-induced morphological changes (Fig. 2), we examined whether genistein indeed inhibited MSP- or IL-3-induced tyrosine phosphorylation in both wild-type RON- (Fig. 4, A-C) and RON-F1330/F1337-expressing cells (E-G). Tyrosine phosphorylation was similarly induced in both cell types, and treatment with genistein inhibited increases in tyrosine phosphorylation of RON, pp130, pp110, pp90, pp62, and pp58 (Fig. 4, A and E). Inhibition of RON autophosphorylation (B and F) indicates that RON kinase activity is repressed by genistein.

The common β chain of IL-3 receptor (βc) is known to play a pivotal role in signal transduction mediated by the IL-3 receptor system (27). Because βc appeared to correspond to pp130 on anti-βc blots (data not shown; see Figs. 3A and 4, A and E), we therefore examined whether βc is involved in RON-mediated signaling. Fig. 4, C and G show by immunoprecipitation that MSP, like IL-3, induced an increase in tyrosine phosphorylation of the βc doublet. The levels of βc tyrosine phosphorylation induced by MSP were generally higher than those induced by IL-3. In addition, MSP- or IL-3-induced tyrosine phosphorylation of βc was inhibited by genistein (Fig. 4, C and G, lanes 4–6).

IL-3-induced receptor aggregation initiates autophosphorylation and activation of JAK2 tyrosine kinase, and activated JAK2 in turn phosphorylates βc to recruit various signaling molecules (36). We analyzed an increase in tyrosine phosphorylation of JAK2. Fig. 4, D and H show that MSP, unlike IL-3, was unable to increase tyrosine phosphorylation of JAK2, suggesting that activation of JAK2 is not required for RON-mediated signaling.

To examine whether RON kinase could directly tyrosine-phosphorylate βc, an in vitro kinase assay was performed with RON kinase immunoprecipitated from RON-transfected COS-7 cells. Fig. 5, A and B show that tyrosine phosphorylation of βc was clearly detected at 5 min (Fig. 5A, lane 5), and increased at 30 min (lane 6), whereas an increase in tyrosine phosphorylation of βc was not detected at 0 min (lane 4). βc was not tyrosine-phosphorylated without RON kinase (lanes 1–3). These results suggest that RON kinase could directly tyrosine-phosphorylate βc.

**Interaction among RON, βc, and pp90**—To examine whether βc could associate with tyrosine-phosphorylated proteins, co-immunoprecipitation analyses were performed with Triton X-100 lysates obtained from wild-type RON-expressing cells.
We found that \( \beta_c \) was specifically co-immunoprecipitated with pp90 but not with pp62 and pp58 (Fig. 6A; data not shown). Three pp90 subspecies, pp87, pp91, and pp94, were co-immunoprecipitated with MSP- or IL-3-activated \( \beta_c \), whereas pp86 was associated with control \( \beta_c \). The degree of association of pp87, pp91, and pp94 with \( \beta_c \) corresponded to that of tyrosine phosphorylation of \( \beta_c \) (see Fig. 6, shorter exposure). Neither pp86, pp87, pp91, nor pp94 was identified as STAT5, Vav, the p85 subunit of phosphatidylinositol 3-kinase, or ezrin (data not shown). These results suggest that tyrosine-phosphorylated \( \beta_c \) forms a novel complex with pp90.

RON-mediated tyrosine phosphorylation of \( \beta_c \) suggests that RON, \( \beta_c \), and tyrosine-phosphorylated proteins could be colocalized. To test this, confocal immunofluorescence microscopic analyses were performed. Fig. 6B (upper panels) shows a uniform distribution of tyrosine-phosphorylated proteins and RON in control cells. On the other hand, cells stimulated with MSP showed the redistribution and colocalization of RON and tyrosine-phosphorylated proteins as well as increases in tyrosine phosphorylation of cellular proteins. Colocalization of RON and tyrosine-phosphorylated proteins was prominently observed in regions containing many granules and uropod-like structures.
binding of IL-3 to its receptor induces activation of JAK2 and recruitment of signaling proteins, such as STAT5 and c-Myc. Moreover, stimulation with IL-3 leads to morphological changes through tyrosine phosphorylation of \( \beta_c \) and its associated protein pp90. RON kinase is activated through autophosphorylation. Subsequently, activated RON phosphorylates \( \beta_c \) and pp90 without activation of JAK2, leading to morphological changes. In contrast to IL-3, MSP stimulation does not activate JAK2 and induces modest cell growth probably due to tyrosine phosphorylation of two tyrosine residues in the multifunctional docking site of RON.

**Fig. 8.** A model for two distinct signaling pathways via \( \beta_c \), Panel A, ectopically expressed RON tyrosine kinase localizes with \( \beta_c \) in unstimulated cells. Panel B, when IL-3 binds to its receptor that consists of \( \alpha \) and \( \beta_c \) chains, JAK2 tyrosine kinase is activated to phosphorylate \( \beta_c \) and STAT5 triggering cell growth. In addition, stimulation with IL-3 leads to morphological changes through tyrosine phosphorylation of \( \beta_c \) and its associated protein pp90. Panel C, when MSP binds to its receptor RON, RON kinase is activated through autophosphorylation. Subsequently, activated RON phosphorylates \( \beta_c \) and pp90 without activation of JAK2, leading to morphological changes. In contrast to IL-3, MSP stimulation does not activate JAK2 and induces modest cell growth probably due to tyrosine phosphorylation of two tyrosine residues in the multifunctional docking site of RON.

**Fig. 7.** Cell growth. After IL-3 deprivation for 12 h, wild-type RON-expressing Ba/F3 cells were stimulated with MSP or IL-3 as described in Fig. 1. Viable cells were counted by trypan blue exclusion, and the data represent the mean ± S.D. from three different experiments.

- **Panel A**, ectopically expressed RON tyrosine kinase localizes with \( \beta_c \) in unstimulated cells. Panel B, when IL-3 binds to its receptor that consists of \( \alpha \) and \( \beta_c \) chains, JAK2 tyrosine kinase is activated to phosphorylate \( \beta_c \) and STAT5 triggering cell growth. In addition, stimulation with IL-3 leads to morphological changes through tyrosine phosphorylation of \( \beta_c \) and its associated protein pp90. Panel C, when MSP binds to its receptor RON, RON kinase is activated through autophosphorylation. Subsequently, activated RON phosphorylates \( \beta_c \) and pp90 without activation of JAK2, leading to morphological changes. In contrast to IL-3, MSP stimulation does not activate JAK2 and induces modest cell growth probably due to tyrosine phosphorylation of two tyrosine residues in the multifunctional docking site of RON.

**DISCUSSION**

In this study, we use IL-3-dependent cells ectopically expressing RON to characterize distinct pathways involved in cell growth and changes in morphology seen following IL-3 and MSP stimulation.

Our model for the mechanism of IL-3 receptor-mediated morphological changes is shown in Fig. 8. In unstimulated cells (Fig. 8A), ectopically expressed RON kinase localizes with the IL-3 receptor \( \beta_c \). Neither JAK2 kinase that constitutively associates with \( \beta_c \) nor RON kinase is activated in the absence of IL-3 or MSP. When cells are stimulated with IL-3 (Fig. 8B), JAK2 is activated to phosphorylate both \( \beta_c \) at multiple sites and recruited STAT5 to trigger cell growth. Moreover, activation of JAK2 increases tyrosine phosphorylation of several proteins including pp90, which associates with \( \beta_c \). When cells are stimulated with MSP (Fig. 8C), RON kinase activated by autophosphorylation triggers increased tyrosine phosphorylation of \( \beta_c \) and several proteins including pp90, resulting in morphological changes. In contrast to IL-3, MSP stimulation does not activate JAK2 or promote strong proliferative responses. Modest cell growth induced by MSP results from phosphorylation of two tyrosine residues in the multifunctional docking site of RON. pp90 is located downstream of activated JAK2 in the IL-3 signal transduction pathway. Activated RON kinase presumably phosphorylates p90 without activation of JAK2; this path leads to \( \beta_c \)-mediated morphological changes but not to proliferation.

It has been well established that tyrosine phosphorylation of \( \beta_c \) is crucial for IL-3 signal transduction (24–30, 36, 38, 39). Binding of IL-3 to its receptor induces activation of JAK2 tyrosine kinase and triggers tyrosine phosphorylation of the cytoplasmic domain of \( \beta_c \) phosphorylation sites, resulting in recruitment of signaling proteins, such as STAT5 and c-Myc.
Stimulation of the c-Kit receptor-type tyrosine kinase with the Kit ligand phosphorylates and activates the erythropoietin receptor, which associates with JAK2 (40). The activated β subunit of the IL-6 receptor and the ErbB2 receptor-type tyrosine kinase interact directly with each other (41). The results presented here provide another example of cross-talk between a receptor-type tyrosine kinase and a signal-transducing subunit/domain of the cytokine receptor family. Based on our observations of crosstalk between RON and βc in cell shape changes, we hypothesize that ectopically expressed RON preferentially phosphorylates some of tyrosine residues on βc that can be phosphorylated by activated JAK2 following stimulation with IL-3. Identification of the βc tyrosine residues responsible for morphological changes will help further our understanding of this signaling pathway.

We recently showed that two C-terminal tyrosine residues, Tyr-1330 and Tyr-1337, in the multifunctional docking site of RON that are conserved among the hepatocyte growth factor family receptors are principally responsible for the signal transduction involved in cell growth (31). After MSP binds to RON, signaling molecules such as Shc, phospholipase Cγ, phosphatidylinositol 3-kinase, Grb2, and others are recruited to the two C-terminal tyrosine phosphorylation sites. We therefore compared morphological changes and tyrosine phosphorylation in wild-type RON-expressing cells with those in RON-F1330/ F1337-expressing cells. Surprisingly, the F1330/1337 mutation enhanced the morphological responses, indicating that the multifunctional docking site is not required. However, the kinase activity of RON is required for morphological changes.

Intriguingly, treatment of cells with Na3VO4 at 30 °C during stimulation periods greatly augments not only the morphological responses but also the level of tyrosine phosphorylation of cellular proteins, because this treatment prevents tyrosine-phosphorylated proteins from rapid dephosphorylation by a yet unidentified protein-tyrosine phosphatase(s). We have evidence that activity of a protein-tyrosine phosphatase(s) is tightly associated with RON (data not shown), although the site of interaction of RON with the phosphatase(s) is not known. It is possible that the F1330/1337 mutation enhances activity of a protein-tyrosine phosphatase(s) by an unknown mechanism. Moreover, treatment with the protein-tyrosine kinase inhibitors, genistein and herbimycin A, completely blocks the morphological changes observed in both wild-type RON-expressing or RON-F1330/F1337-expressing cells. The delicate balance between tyrosine phosphorylation and dephosphorylation of specific proteins plays an important role in MSP- or IL-3-induced morphological changes.

pp90 consists of 4 subspecies, pp86, pp87, pp91, and pp94. pp86 is associated with βc in the unstimulated state. Upon stimulation with MSP or IL-3, pp87, pp91, and pp94 are tyrosine-phosphorylated and associated with βc, and the levels of tyrosine phosphorylation of these proteins correspond to those of tyrosine phosphorylation of βc. In addition, using sucrose density gradient fractionation analyses we found that pp90 was localized in the higher density lysate fractions at ~400 kDa (data not shown), consistent with the results that pp90 can form a protein complex with βc. Treatment of cells with MSP relocates RON, βc, and tyrosine-phosphorylated proteins to uropod-like structures where F-actin is redistributed and concentrated. These results suggest that phosphorylation of pp90 could control distribution and reorganization of actin filaments. Despite efforts to purify pp90, at present we are unable to immunoprecipitate pp90 with the anti-phosphotyrosine antibody 4G10, probably because phosphotyrosine residues are not exposed to the surface of the protein complex. Although we do not know that the migration of pp90 subspecies on SDS-polyacrylamide gels stems from one molecule having four different phosphorylation states or from four distinct molecules, identification of pp90 will help us more fully understand how activation of βc initiates morphological changes.

Previous data with LyD9 IL-3-dependent pro-B cells, which ectopically express the IL-2 receptor, and FDC-P1 IL-3-dependent hematopoietic precursor cells show that stimulation of the former with IL-3 or IL-2 and the latter with IL-3 induces tyrosine phosphorylation of an ~90-kDa protein accompanied by early cell shape changes and later cell growth (42). It is possible that the IL-2 receptor, which associates with JAK1 and JAK3, also interacts with βc. Given that morphological change-related pp90 is induced in IL-3-dependent Ba/F3 cells, we hypothesize that a tyrosine-phosphorylated protein at ~90 kDa seen in other IL-3-dependent LyD9 and FDC-P1 cells is identical to pp90 seen in Ba/F3 cells.

In conclusion, we have elucidated a novel IL-3 signaling pathway involving pp90, which associates with βc, and leading to morphological changes and subsequent cell migration. Our findings demonstrate that the signaling events of this pathway are distinct from those required for cell growth. Cell migration is accompanied by induction of morphological changes (43). To move through small openings in various tissues, cells must be able to change their shapes drastically. Because the receptors for IL-3, IL-5, and GM-CSF each consist of a ligand-binding α chain associated with a common signal transducer β chain (24–27), it is reasonable to predict that, in addition to IL-3 in immature hematopoietic cells, IL-5 and GM-CSF can induce pp90 required for morphological changes of eosinophils and neutrophils/macrophages, respectively.

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Cross-talk between RON and JAK2 on $\beta_c$

27. Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K. (1992) Annu. Rev. Immunol. 10, 295–332
28. Sakamaki, K., Miyajima, I., Kitamura, T., and Miyajima, A. (1992) EMBO J. 11, 3541–3549
29. Sato, N., Sakamaki, K., Terada, N., Arai, K., and Miyajima, A. (1993) EMBO J. 12, 4181–4189
30. Kinoshita, T., Yokota, T., Arai, K., and Miyajima, A. (1995) EMBO J. 14, 266–275
31. Iwama, A., Yamaguchi, N., and Suda, T. (1996) EMBO J. 15, 5866–5875
32. Yamaguchi, N., and Fukuda, M. N. (1995) J. Biol. Chem. 270, 12170–12176
33. Tada, J., Omine, M., Suda, T., and Yamaguchi, N. (1999) Blood, in press
34. Hirao, A., Hamaguchi, I., Suda, T., and Yamaguchi, N. (1997) EMBO J. 16, 2942–2951
35. Hirao, A., Huang, X. L., Suda, T., and Yamaguchi, N. (1998) J. Biol. Chem. 273, 10004–10010
36. Ihle, J. N. (1995) Nature 377, 591–594
37. Warringa, R. A. J., Koenderman, L., Kok, P. T. M., Kreukniet, J. K., and Bruijnzeel P. L. B. (1991) Blood 77, 2694–2700
38. Taniguchi, T. (1995) Science 268, 251–255
39. Ihle, J. N. (1996) Cell 84, 331–334
40. Wu, H., Klingmuller, U., Besmer, P., and Lodish, H. F. (1995) Nature 377, 242–246
41. Qiu, Y., Revi, L., and Kung, H.-J. (1998) Nature 393, 83–85
42. Sabe, H., Kuno, J., Kolomilas, A., Saito, Y., Kinashi, T., Ueda, M., Takamatsu, T., Hamaguchi, M., Kawakami, T., and Honjo, T. (1991) Int. Immunol. 3, 1137–1148
43. Bretscher, M. S. (1996) Cell 87, 601–606