Midkine Induces Tumor Cell Proliferation and Binds to a High Affinity Signaling Receptor Associated with JAK Tyrosine Kinases∗

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The G401 cell line derived from a rhabdoid tumor of the kidney secretes the heparin-binding growth factors midkine and pleiotrophin. Both proteins act as mitogens for diverse cells, but only midkine serves as an autocrine mitogen for G401 tumor cells. We show that midkine specifically binds a protein or complex of molecular mass greater than 200 kDa with high affinity (Kd = 0.07 ± 0.01 nm). Midkine, but not pleiotrophin, stimulates tyrosine phosphorylation of several cellular proteins with molecular mass of 100, 130, and 200+ kDa. Upon midkine binding, the midkine-receptor complex associates with the Janus tyrosine kinases, JAK1 and JAK2. MK stimulates tyrosine phosphorylation of JAK1, JAK2, and STAT1α. Our initial characterization of the midkine receptor suggests that midkine autocrine stimulation of tumor cell proliferation is mediated by a cell-surface receptor which in turn might activate the JAK/STAT pathway.

Mesenchymal-epithelial interactions during development involve reciprocal inductive stimuli that are critically important in the regulation of cellular proliferation, differentiation, and tissue morphogenesis. The elucidation of the molecular basis for these events is a major goal relevant to advancing our understanding of basic developmental processes. Kidney organogenesis depends upon a set of molecular signaling events that form the basis for the induction of nephron formation in the metanephric mesenchyme by the ureteric bud (1, 2). Midkine (MK),1 a recently identified growth factor, may play an important regulatory role at sites of mesenchymal-epithelial interaction during tooth development and during organogenesis of the kidney (3, 4). MK and pleiotrophin (PTN) are developmentally regulated heparin-binding proteins that regulate cell growth, survival, and differentiation (5–8). Both MK and PTN are products of retinoic acid-responsive genes (8). Expression of PTN is also regulated by platelet-derived growth factor (9). Mature MK and PTN are basic, cysteine-rich polypeptides of 123 and 136 amino acids, respectively, with approximately 50% homology to each other (5, 10, 11). MK and PTN are conserved between mammalian species, and both are distinct from other heparin binding growth factors such as basic and acidic fibroblast growth factors (6, 10, 12).

MK is mitogenic to a number of cell lines and induces neurite outgrowth of embryonic brain cells, PC 12 cells, and dorsal root ganglion cells (12–15). MK also promotes survival of retinal cells in vitro, astrocytes and mesencephalic neurons in culture (17–16). MK stimulates differentiation of P19 embryonic carcinoma cells into nerve cells, and this stimulation is inhibited by anti-MK antibodies (17). In addition, MK enhances plasminogen activator and plasmin activity in a dose- and time-dependent manner, implying a role for MK in tissue repair and angiogenesis (18–20).

MK is expressed in a characteristic pattern in the developing embryo and may play a role in neurogenesis, kidney organogenesis, and in mesodermal-epithelial interactions (3, 4, 19–22). MK is absent in the mouse embryo until day 5 of gestation. MK expression then increases until it is widely expressed at 7–9 days of gestation; its expression then decreases. In the mid-gestation period (days 11–13) MK is expressed in the brain and kidney and in epithelial cells of the small intestine, pancreas, lung, and stomach. At day 15 of gestation MK expression is limited to the kidney (22–26). The expression of MK decreases in later embryogenesis but then increases again postnatally in certain organs and tissues. In adult mice and humans, MK is expressed in the kidney, testis, stomach, and small intestine (24–28).

MK is expressed in various human cancers, including neuraloblastomas, hepatocellular carcinomas, gastric, colorectal, pancreatic, esophageal, lung, and breast carcinomas, and kidney cancers (27–31). MK is thought to be an autocrine tumor growth factor, since the G401 cell line derived from a rhabdoid kidney tumor expresses MK and since anti-MK antibodies partially inhibit the in vitro growth of these cells (27, 31). However, definitive evidence for a direct role of MK as an autocrine mitogenic factor in tumor cells has not yet been established.

Tyrosine phosphorylation of growth factor receptors upon ligand binding is an important signaling mechanism for cellular activation, proliferation, and differentiation (32). MK and PTN might exert their functions through interaction with specific cell-surface signaling receptors and induction of tyrosine phosphorylation of cellular proteins. Previous studies have...
shown that PTN induces tyrosine phosphorylation of cellular proteins (33). However, the cellular signaling receptors for MK and PTN have not yet been identified and characterized. Study of the molecular basis for signal transduction pathways of MK and PTN will further enhance the understanding of their roles in development and cancer. We report that MK, but not PTN, stimulates tumor (G401) cell proliferation in a dose-dependent and time-dependent manner. MK specifically binds to a high-affinity cell-surface receptor, which is tyrosine-phosphorylated after MK binding and stably associates with the Janus non-receptor tyrosine kinases JAK1 and JAK2. MK also stimulates tyrosine phosphorylation of JAK1, JAK2, and STAT1α. Our studies support the hypothesis that this newly identified MK receptor is the signal transduction receptor that mediates the MK-dependent autocrine stimulation of this tumor cell line in vitro.

EXPERIMENTAL PROCEDURES

Cultures, Media, and Antibodies—Human pediatric rhabdoid tumor kidney-derived G401 cell line (CRL 1441), normal rat kidney cells (CRL 6509), and NIH 3T3 mouse embryo fibroblasts (CRL 1658) were obtained from the American Type Culture Collection. G401 cells (passages 15–45) were cultured in McCoy’s 5A medium (Sigma) with 5% fetal calf serum. Human adult kidneys were obtained from the deceased patient (pH 7.4, for 60 min at 4 °C). A cell-free duplicate plate with identical protein fractions and media was assayed as a control. Both cell-free plates and cell containing plates were analyzed on plate reader Labsystems Multiscan MC (Labsystems, Cambridge, MA) within 24 h of harvest in Collins solution (without cross-reactivity) and monoclonal antibodies to STAT1 kinase, JAK1, JAK2, STAT2, gp130, and antibodies to MK and to PTN (without cross-reactivity) and monoclonal antibodies to STAT1α were purchased from Santa Cruz Biotechnology. Streptavidin-Sepharose was purchased from Pierce.

Expression of MK and PTN in COS1 Cells—Total cellular RNA was extracted from human adult kidney (34). cDNA was synthesized from total RNA (5–10 μg) in 50 μl of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, and 1 μg/ml nuclease-free bovine serum albumin with an oligo (dT)₁₅ primer in the presence of RNasin (1 unit/ml). First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Gibco BRL). cDNAs were cloned in the pCMV vector (Promega). The resulting constructs were subcloned into pCR 2.1 vector (InVitrogen). The expression vector pCMV-MCC/340 was a gift from Dr. Matthew Longo, University of California, San Francisco. The expression vector pCMV-MCC/340 was digested with XbaI and EcoRI and the resultant DNA fragment was subcloned into the XbaI and EcoRI sites of the pCMV vector. The resulting plasmid was sequenced and the expression cassette for MK was confirmed.

Labeling of ligands—Recombinant MK (0.5 μg) with Na₁²⁵I (1.3–1.5 μCi, 16.8 mCi/mg, Amersham Corp.) during 1 min at 4 °C in the presence of charcoal (1 mg/ml) and PBS, then stopping the reaction with sodium metabisulfite, followed by desalting chromatography on a PD-10 column (Pharmacia Biotech Inc.) in PBS (38). Specific activity of iodinated ligands was 1.1–1.3 × 10⁶ cpm/μg. ²⁵¹MK and ²²⁵PTN were stored at 4 °C and used within 2 weeks of preparation. For detection of cell-surface receptor, 0.5 nM ²⁵¹MK or 0.5 nM ²²⁵PTN was incubated with intact G401 cells (2–5 × 10⁵ cells) in 1 ml of Ca²⁺/Mg²⁺-free PBS, pH 7.0, and 150 mM NaCl to inactive DSS (33). Lipid-receptor binding experiments were done in the absence or presence of a 100-fold excess of unlabeled MK, PTN, or control protein fraction. Cell pellets were treated with liquid nitrogen and extracted with 1 ml/10⁶ cells of lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% of Nonidet P-40, 1 mM FMSF, 4 μg/ml aprotinin, 1 mM ρ-mercaptoethanol, and 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 4 μg/ml aprotinin, and 1 mM NaF). Post-nuclear supernatants were incubated with 1% Nonidet P-40 for the next 30 min and cleared by centrifugation for 15 min at 15,000 × g. Supernatants 40–60 μl were boiled with SDS and β-mercaptoethanol and resolved by denaturing 6% SDS-PAGE; dried gels were examined by autoradiography (38).

Saturation and Scatchard Analysis of the MK Cell-surface Receptor—Confluent intact G401 cells (3 × 10⁵) were incubated for 1 h at 4 °C with ²²⁵MK or ²⁵¹MK at 0.005–1.0 nM, cross-linked, and treated as above; the samples were resolved by denaturing 6% SDS-PAGE; dried gels were autoradiographed and sliced into 1-cm³ pieces in regions of radioactive bands and background. The slices were counted in a Beckman 5500 gamma counter for 1 min. Scatchard analysis of saturation isotherms was performed using a linear least-squares regression LIGAND program (37). Scatchard analysis of MK ligand-receptor binding was also performed on intact G401 cells in Eppendorf tubes (without subsequent SDS-PAGE and excision of the radioactive receptor band). G401 cells (5 × 10⁵) were incubated in 100 μl of PBS for 60 min at 4 °C with increasing amounts (0.01 to 1 nM) of ²²⁵MK-cross-linked for 20 min at 4 °C with PBS (as above), spun down, and washed 5 times with 1 ml of PBS, and the pellets were dried and counted in a gamma counter for 1 min. The binding experiments were repeated 4 times, normalized for cell number, and averaged.

Tyrosine Phosphorylation Analysis—Confluent G401 cells (2 × 10⁵) were incubated overnight in serum-free McCoy’s 5A medium, washed with the same media containing 1% Na₂VO₃ and 50 mM NaF, and treated with MK or PTN or control proteins at 37 °C. Stimulation was
stopped with ice-cold PBS containing 1 mM Na3VO4 and 50 mM NaF. Cells were scraped off the flasks with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1% Nonidet P-40 containing 1 mM Na3VO4, 50 mM NaF, and protease inhibitors mixture, as above). The postnuclear supernatants were treated with 1.0% Nonidet P-40 on ice for 30 min and spun at 15,000 × g for 15 min. Supernatants containing 20–100 μg per lane were boiled with 1% SDS and β-mercaptoethanol, resolved by SDS-PAGE, and transferred onto Immobilon P membrane sheets. Sheets were blocked with 10% non-fat milk, PBS, 0.05% Tween 20, probed with antibody to phosphotyrosine (PT-66) (1: 5,000), followed by washing in PBS, 0.05% Tween 20. Then sheets were incubated with goat anti-mouse antibody conjugated with horseradish peroxidase (1:10,000), washed with PBS, 0.05% Tween 20 and visualized with ECL (Amersham Corp.). Quantitative densitometry of phosphotyrosine signals was performed as follows; slides of films with immunoblot data were made, scanned, and plotted as diagrams using the Macros (NIH Image 1.58b 39f) computer program.

**Streptavidin Precipitation of Biotinylated Ligand-Receptor Complexes and Western Blot Analysis—PTN and MK were prepared from conditioned media of transfected COS1 cells as above. For biotinylation, 10 μg of MK or 10 μg of PTN or 10 μg of control proteins were incubated with 1 mg/ml sulfoconjugated biotin (AmershammbH) and 1 mg/ml streptavidin (Amersham Corp.) for 30 min at 4 °C. Then sheets were incubated with 50 mM HEPES buffer, pH 7.5, with gentle shaking (36). The reaction was stopped by 0.01 M glycine and dialyzed against 10 mM PBS through 3656 K membrane sheets. Sheets were blocked with 10% non-fat milk, PBS, 0.05% Tween 20, probed with antibody to phosphotyrosine (PT-66) (1: 5,000), followed by washing in PBS, 0.05% Tween 20 and visualized with ECL (Amersham Corp.). Quantitative densitometry of phosphotyrosine signals was performed as follows; slides of films with immunoblot data were made, scanned, and plotted as diagrams using the Macros (NIH Image 1.58b 39f) computer program.

Intact G401 cells (5–8 × 104 cells) were cross-linked with 1 nM biotinylated MK or 1 nM biotinylated PTN or biotinylated control proteins for 30 min at 4 °C in serum-free McCoy’s 5A media (1 ml/107 cells). Cells were centrifuged and resuspended in 1 ml/107 cells of ice-cold PBS with 0.5 mM DSS for 20 min at 4 °C, then washed with ice-cold PBS. The cells were solubilized (1 ml/107 cells) in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% Bej-50, 1 mM PMFSF, 4 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM Na3VO4, 50 mM NaF for 30 min on ice, and supernatants were recovered by centrifugation at 15,000 × g for 15 min. Then 500-μl supernatants were pre-cleared with 10 μl of normal rabbit serum for 30 min and incubated with 50 μl of protein A-Sepharose 4B (Pharmacia) for 30 min (36). Supernatants were recovered by centrifugation and then used for subsequent precipitation. 40 μl of a 50% suspension of streptavidin-Sepharose (Pierce) was added to 500 μl of supernatant and incubated for 4 h at 4 °C with rotation, washed three times with 1 ml of cold 20 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM Na3VO4, 50 mM NaF, 1 mM EDTA, 0.5% Nonidet P-40, 0.2 mM PMFSF (34). Samples were boiled with SDS and β-mercaptoethanol, resolved by denaturing 6% SDS-PAGE, and transferred onto Immobilon P sheets. Blocked sheets were incubated for 2 h at room temperature with antibodies directed against phosphotyrosine (PT-66) (1:5,000), MK, JAK1, JAK2, focal adhesion kinase, gp130 (each in dilution 1:1,000), washed, incubated for 1 h with goat anti-mouse antibodies (1:10,000) or protein A (1:10,000) coupled to horseradish peroxidase (Sigma), and visualized with ECL.

**Immunoprecipitation—** For immunoprecipitation experiments, G401 cells were starved for 24 h in serum-free media, incubated for 20 min with MK (0.5 nM), PTN (0.5 nM), or control (concentrated conditioned media from COS1 cells transfected with pCB6 vector alone). Cells extracts were prepared as above, including pre-clearing with protein A-agarose (Sigma) beads or goat anti-mouse immunoglobulin-bound agarose beads. Supernatants were precipitated with primary antibodies directed to JAK1, JAK2, STAT2, and STAT1 for 16–18 h at 4 °C and then the secondary antibodies (protein A or goat anti-mouse immunoglobulin) coupled to Sepharose/agarose beads. Antigen-antibody complexes were washed three times with 0.05% PBS, Tween 20, boiled in sample buffer containing SDS and β-mercaptoethanol, and separated by SDS-PAGE followed by transfer onto Immobilon P membrane, and probed with antibody to phosphorytosine (PT-66) and antibody to JAK1, JAK2, STAT2, or STAT1. Immunoreactive bands were visualized by ECL as above.

**RESULTS**

**MK, but Not PTN, Stimulates G401 Cell Proliferation—** To analyze the mitogenic effect of MK and PTN on G401 cells, we cloned human MK and PTN using reverse transcription-polymerase chain reactions with human kidney RNA as a template. The MK (522 base pairs) and PTN (542 base pairs) cDNA fragments were ligated into the pCB6 vector, and COS1 cells were transfected with the pCB6-MK or pCB6-PTN expression constructs. COS1 cells transfected with pCB6-MK secrete MK, and COS1 cells transfected with pCB6-PTN secrete PTN, in contrast to COS1 cells transfected with pCB6 vector alone (Fig. 1A). MK, PTN, and control proteins were prepared from concentrated conditioned media of COS1 cells transfected with pCB6-MK, pCB6-PTN, or pCB6 alone, respectively, and purified by heparin affinity column chromatography (Fig. 1B).

We then studied the mitogenic properties of MK and PTN in the pediatric tumor kidney-derived G401 cell line, using the non-radioactive colorimetric MTS/PSMS proliferation assay. Since NIH 3T3 fibroblasts proliferate in response to both MK and PTN, and normal rat kidney cells proliferate in response to PTN only (6, 13, 39), we used these cell lines as positive controls in our experiments (Fig. 2, A and B). MK stimulates G401 cell proliferation in a time-dependent and dose-dependent fashion (Fig. 3, A and B); PTN and control protein have no effect. These data (Fig. 1 and Fig. 3, A and B) demonstrate that MK might regulate G401 cell proliferation in an autocrine manner. Titration of MK from 0.05 to 5 nM indicates that half-maximal stimulation of G401 cell proliferation occurs at approximately 0.3–0.5 nM.

**MK, but Not PTN, Binds a G401 Cell-surface Receptor—** To test our hypothesis that MK mitogenic activity is mediated by its binding to a high affinity cell-surface receptor, we performed chemical cross-linking assays. Intact G401 cells were incubated with 125I-MK or 125I-PTN in the absence or presence of a 100-fold excess unlabeled MK or PTN, followed by cross-linking with DSS. Cell lysates were resolved by denaturing SDS-PAGE and examined by autoradiography. MK but not PTN is cross-linked to a protein (Fig. 4); the total mass of the MK-protein complex is between 200 and 250 kDa (200–250 kDa linked to a protein (Fig. 4); the total mass of the MK-protein complex is between 200 and 250 kDa (200–250 kDa linked to a protein). This mass is consistent with a cell-surface receptor for MK, but not for PTN.

To quantitate the binding of MK to its receptor, we cross-linked intact G401 cells with increasing amounts of 125I-MK and resolved ligand-receptor complexes by SDS-PAGE. Saturation and Scatchard analysis showed that G401 cells displayed receptor molecules for MK with high ligand binding affinity (200 ± 200 kDa; average data from four experiments; Kd = 0.072 nM, 7,700 receptors/cell) (Fig. 5, A and B). Similar data (average data from four experiments; Kd = 0.056 nM, 6,600 receptors/cell) were obtained for the G401 cell-surface MK receptor using a complementary approach of cross-linking of 125I-MK with intact G401 cells without subsequent lysis and

**FIG. 1.** Expression of human MK and PTN. Western blot assay of media from G401 cells (lanes 1 and 2) and from COS-1 cells transfected with pCB6-PTN (lane 3) or pCB6-MK (lane 4) or pCB6 vector alone (lane 5), using antibody to PTN (lanes 1, 3, and 5) or to MK (lanes 2, 4, and 5), B, electrophoresis of 125I-PTN (lane 1) and 125I-MK (lane 2) purified on heparin affinity chromatography column were separated by 15% SDS-PAGE.
MK Induces Tyrosine Phosphorylation of Its Cell-surface Receptor That Is Associated with JAK1 and JAK2 Tyrosine Kinases—We next analyzed the ligand-induced tyrosine phosphorylation pattern of G401 cell proteins using immunoblotting with anti-phosphotyrosine antibodies (see “Experimental Procedures”). Stimulation with MK, but not PTN or control proteins, resulted in tyrosine phosphorylation of several cellular proteins, including 100-, 130-, and 200-kDa proteins (Fig. 7A). Immunoblotting with antibody to phosphotyrosine shows that the MK-induced tyrosine phosphorylation was time-dependent (Fig. 7B). Immunoblotting and quantitative densitometry show that the MK-induced tyrosine phosphorylation is dose-dependent (Fig. 7C and Fig. 8).

To determine the identity of proteins binding MK and tyrosine phosphorylated upon MK treatment, we used streptavidin-Sepharose to precipitate complexes of biotinylated MK and MK-binding proteins. Intact G401 cells (1 ml/10⁷ cells) were incubated with 1.0 nM biotinylated-MK or 1.0 nM biotinylated-PTN or biotinylated-control proteins for 15 min at 4 °C, followed by DSS cross-linking. Cells were lysed as above, and cross-linked complexes were precipitated with streptavidin-Sepharose beads followed by blotting with antibodies to phosphotyrosine (lanes 1–3), MK (lanes 4–6), JAK1 (lanes 7–9), or JAK2 (lanes 10–12) (Fig. 9). The antibody to phosphotyrosine recognizes three streptavidin-precipitated, biotinylated MK-MK receptor complex components of 100, 130, and 200-kDa. These data also demonstrate that MK binds to the 200-kDa receptor that becomes tyrosine-phosphorylated (Fig. 9, lanes I and 4).

Moreover, antibodies to JAK1 and JAK2 detected JAK1 (126 kDa) and JAK2 (130 kDa) tyrosine kinases associated with the protein complex bound to biotinylated MK (but not to biotinylated PTN or biotinylated control proteins) (Fig. 9, lanes 7 and 10). Probing the same blots with antibody to JAK3 or anti-gp130 or focal adhesion kinase failed to detect any proteins...
associated with MK-MK receptor complex (data not shown).

By comparing data presented on lanes 1, 7, and 10 (Fig. 9), we show that the apparent molecular mass of JAK1 and JAK2 are identical to the 130-kDa phosphoproteins that co-precipitate with biotinylated MK-MK receptor complexes. Taken together, these findings suggest that MK but not PTN stimulates G401 cell proliferation by binding to a high affinity cell-surface receptor that undergoes tyrosine phosphorylation and that appears to activate and recruit the downstream signaling molecules JAK1 and JAK2.

**MK Activates the JAK/STAT Pathway**—We next analyzed the phosphorylation of various members of the JAK and the STAT family. G401 cells were stimulated with MK, PTN, and control as above for 20 min. Cell extracts were precipitated separately with antibodies against JAK1, JAK2, STAT1α, or STAT2. Precipitated complexes were electrophoresed, transferred to a membrane, and then hybridized with antibodies against JAK1, JAK2, STAT1α, or STAT2, as well as antibodies against phosphotyrosine as above. Stimulation of G401 cells with MK, but not PTN or control proteins, resulted in tyrosine phosphorylation of JAK1, JAK2, and STAT1α (Fig. 10) but not STAT2 (not shown).

**DISCUSSION**

The pediatric tumor kidney-derived G401 cell line was chosen to elucidate molecular mechanisms underlying MK signal transduction because these cells produce high amounts of both MK and these cells also proliferate in response to MK. Garvin et al. (40) suggest the G401 cell line is of rhabdoid tumor, rather than Wilms’ tumor origin. Regardless of its origin, the G401 cell line is a useful tool to study MK signal transduction.

Our results demonstrate that although G401 tumor cells secrete both MK and PTN, MK but not PTN stimulates G401 tumor cell proliferation in a time-dependent and dose-dependent fashion. These data also show that MK, but not PTN, interacts with a G401 cell-surface receptor and induces tyrosine phosphorylation of several cellular proteins. A quantitative analysis of MK-induced G401 cell proliferation (Fig. 3), MK/MK receptor binding affinity (Figs. 5 and 6), and MK-stimulated tyrosine phosphorylation of G401 cellular proteins (Fig. 8) strongly suggests that MK acts as an autocrine mitogen through its interaction with its cell-surface high affinity receptor identified in our studies.

Ligand-binding assay of MK cell-surface receptor was assessed by two independent techniques and demonstrates that this receptor binds MK with high affinity ($K_d = 0.056-0.072$).
nm). This $K_d$ value fits nicely within the range of values for other cytokine/growth factor receptors as follows: interleukin-3 and granulocyte-monocyte colony-stimulating factor (0.14 nM); interleukin-4 (0.16 nM); interleukin-5 (0.03 nM); interferon-α (0.02 nM); interferon-γ (0.1 nM); insulin-like growth factor (0.06 nM); and transforming growth factor-β (0.07 nM) (41–47). The precise identity of the 200+-kDa high affinity MK receptor is unknown. Our experiments demonstrate that a 200+-kDa protein binds MK and becomes tyrosine-phosphorylated upon MK binding; cross-linking of MK pulls down a 200+-kDa protein (Fig. 4), and anti-phosphotyrosine immunoblot of total proteins from MK-stimulated cells also identifies a 200+-kDa protein (Fig. 7). Although the species identified by cross-linking could represent multiple polypeptides cross-linked to MK, this anti-phosphotyrosine immunoblot demonstrates a single 200+-kDa polypeptide chain. However, we cannot exclude the possibility of a multi-subunit composition of the high affinity MK cell-surface receptor, as has been demonstrated for number of cytokine receptors.

To identify the proteins phosphorylated upon MK exposure, we precipitated biotinylated MK cross-linked to an MK receptor, followed by Western analysis with various antibodies. This set of experiments demonstrates that MK binds to a 200+-kDa G401 cell-surface receptor complex that is associated with the non-receptor tyrosine kinases JAK1 and JAK2 (Fig. 9). Furthermore, protein species of the same molecular mobility as JAK1 and JAK2 are tyrosine-phosphorylated upon MK stimulation. To prove that MK induces tyrosine phosphorylation of specific members of the JAK/STAT pathway, we performed further immunoprecipitation experiments, with antibodies against JAK/STAT family members, and probing these complexes with anti-phosphotyrosine antibodies (Fig. 10). These experiments show that MK stimulates phosphorylation of specific members of the JAK/STAT pathway, namely JAK1, JAK2, and STAT1α. These data suggest that the mitogenic effect of MK on G401 cells is mediated by binding MK to a G401 cell-surface receptor; the MK receptor then becomes tyrosine-phosphorylated upon ligand-receptor interaction.

Previous studies have not yet identified a high affinity signaling receptor for either MK or PTN. Cross-linking studies with PTN performed on NIH 3T3 cells detected 155- and 127-kDa PTN binding proteins ($K_d = 0.6$ nm, 5000 molecules/cell) that are not yet purified or completely characterized (48). It was reported that MK and PTN in vitro also bind to the proteoglycans N-syndecan (syndecan-3) and ryudocan (syndecan-4), respectively (49, 50). MK was also shown to bind nucleolin (100 kDa), a major nucleolar protein that acts as a shuttle between the nucleus and the cytoplasm (51, 52). Perhaps the MK-nucleolin complex can be translocated to the nucleus where it might function in MK signal transduction, as recently suggested for platelet-derived growth factor and fibroblast growth factor (52, 53). However, our results suggest that MK signal transduction at the cell surface appears to involve a cell-surface receptor distinct from nucleolin with activation of the JAK/STAT pathway.

Growth factors and cytokines bind to cell-surface receptors with different affinities. For example, fibroblast growth factor and transforming growth factor-β both bind to signaling receptor tyrosine kinases with high affinity (54). However, the same growth factors bind with low affinity to cell-surface proteoglycans. Growth factors and cytokines bind to cell-surface receptors with different affinities. For example, fibroblast growth factor and transforming growth factor-β both bind to signaling receptor tyrosine kinases with high affinity (54). However, the same growth factors bind with low affinity to cell-surface proteoglycans. Growth factors and cytokines bind to cell-surface receptors with different affinities. For example, fibroblast growth factor and transforming growth factor-β both bind to signaling receptor tyrosine kinases with high affinity (54). However, the same growth factors bind with low affinity to cell-surface proteoglycans.
cans that cannot transmit signals alone but modulate the ability of these growth factors to generate a biological response through other high affinity signaling receptors (57). Both ryu-
docan and N-syndecan, which interact with PTN and MK, are members of a type I integral membrane heparan sulfate proteoglycan family and were also reported to bind the basic fibro-
blast growth factor and tissue factor pathway inhibitor (49, 50, 55–58). Our studies have not yet identified a type I proteogly-
can as an MK receptor in G401 cells. However, given the previous work suggesting the potential importance of proteo-
glycans in signal transduction of heparin binding growth fac-
tors, we cannot exclude that the high affinity cell-surface MK receptor identified here may interact with as yet unidentified proteoglycans.

Autocrine control mechanisms that regulate cell proliferation have been found to be important in tumorigenesis in many model systems (59). Our studies have demonstrated that the G401 cell line both secretes MK and expresses a high affinity cell-surface receptor for MK that appears to be directly in-
volved in the regulation of cell proliferation in vitro. We have now demonstrated that the candidate MK signal transduction receptor is a 200-kDa plasma membrane protein that is ty-
rosine-phosphorylated promptly after MK binding. In addition, we have shown that MK stimulation results in association of JAK1 and JAK2 with the MK ligand-receptor complex in the plasma membrane, followed by tyrosine phosphorylation of JAK1 and JAK2 and STAT1a. However, the architecture of the MK receptor complex as well as the specific molecular mecha-
nisms underlying MK-induced signal transduction remain to be identified.

Janus non-receptor tyrosine kinases can be recruited by both growth factor receptors with intrinsic kinase activity and also by cytokine receptors that are associated with separate tyro-
sine kinase molecules only upon ligand binding (60, 61). Cross-
talk between signaling pathways of receptor tyrosine kinases and cytokine receptors that both involve JAK kinases might be an important molecular mechanism mediating regulation of cell proliferation, differentiation, and development (60, 61). We cannot yet ascertain whether the MK high affinity receptor belongs to the receptor tyrosine kinase superfamily or cytokine receptor superfamily, but our data support the possible involve-
ment of the JAK/STAT pathway in MK signal transduction. Additional studies, including the cloning of the 200-kDa MK receptor to assess its functional role in signal transduction, are needed to elucidate the molecular events associated with MK regulation of cell proliferation.

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