Monomeric Midkine Induces Tumor Cell Proliferation in the Absence of Cell-Surface Proteoglycan Binding†

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ABSTRACT: Midkine (MK), a retinoic acid-inducible heparin-binding protein, is a mitogen which initiates a cascade of intracellular protein tyrosine phosphorylation mediated by the JAK/STAT pathway after binding to its high affinity p200$^+$/MKR cell surface receptor in the G401 cell line [Ratovitski, E. A. (1998) *J. Biol. Chem. 273,* 3654−3660]. In this study, we determined the biophysical characteristics of purified recombinant murine MK and analyzed the requirements for ligand multimerization and cell surface proteoglycan binding for the G401 cell mitogenic activity of MK. Our studies indicate that the secreted form of MK (M = 13 kDa) exists in solution as an asymmetric monomer with a frictional coefficient of 1.48 and a Stokes radius of 23.7 Å. By constructing bead models of MK using the program AtoB and the program HYDRO to predict the hydrodynamic properties of each model, our data suggest that MK has a dumb-bell shape in solution composed of independent N- and C-terminal domains separated by an extended linker. This asymmetric MK monomer is a biologically active ligand with mitogenic activity on G401 cells in vitro. Neither heparin-induced formation of noncovalent MK multimers nor tissue transglutaminase II covalent multimerization of MK enhanced MK mitogenic activity in this system. Since neither heparin competition nor cell treatment with chondroitinase ABC or heparinase III abolished the mitogenic effects of MK on G401 cells, cell-surface proteoglycan binding by MK does not appear to be a requirement for its observed mitogenic effects. These results provide strong evidence that the MK-specific p200$^+$/MKR has distinctive biochemical properties which distinguish it from the receptor tyrosine phosphatase cell-surface proteoglycan PTPζ/RPTPβ and support the hypothesis that the diverse biological effects of MK are mediated by multiple cell-specific signal transduction receptors.

Heparin-binding[1] growth factor proteins, including the fibroblast growth factor (FGF) family, are involved in the regulation of cellular growth, differentiation, and development (*1*, *2*). Recently, a new family of heparin-binding growth factors comprised of Midkine (MK) and pleiotrophin (PTN) has been described (*3*, *4*), which may have important regulatory functions in development, angiogenesis, and cancer. MK, originally isolated as a product of a retinoic acid-responsive gene in an embryonal carcinoma cell differentiation system (*5*), shares 50% amino acid sequence identity with PTN (*6*, *7*) and both are basic and cysteine-rich cytokines (*8*) encoded by genes with similar genomic organization including open-reading frames encoded by four exons (*9*−*11*).

A major objective in understanding the molecular basis of MK and PTN action is the identification of the signal transduction receptors which mediate the diverse biological effects of these polypeptides. Recently, it has been demonstrated that PTN binding to N-syndecan (*12*) enhances tyrosine phosphorylation of both c-Src and cortactin, which bind to the cytoplasmic domain of this receptor (*13*, *14*). MK promotes neurite outgrowth and the survival of various embryonic neuron types (*6*, *15*, *16*), is mitogenic for NIH 3T3 and PC12 cells (*17*, *18*), results in transformation of NIH3T3 cells with tumor formation in nude mice (*19*, *20*), and has potent angiogenic effects (*21*) associated with stimulation of endothelial cell plasminogen activator release (*3*, *4*, *22*), which may reflect association with multiple cell surface signaling receptors. MK is strongly expressed in embryonic periods, especially in the mid-gestation stage (*7*, *11*, *23*) and may play critical roles in neurogenesis, kidney organogenesis and in the mesenchymal-epithelial interactions necessary for limb bud and tooth development.

Current evidence suggests that there are multiple cell surface MK receptors including the integral membrane heparan sulfate proteoglycan ryudocan (*24*), the chondroitin sulfate proteoglycan PTPζ/RPTPβ (a receptor protein tyrosine phosphatase) (*25*), and a 200$^+$ kDa MK receptor (p200$^+$/MKR), which demonstrates ligand-dependent phos-

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[1] Abbreviations: MK, Midkine; PTN, pleiotrophin; aFGF, fibroblast growth factor 1, FGF1; bFGF, fibroblast growth factor 2, FGF2; TG2, type 2 tissue transglutaminase; uPA, urokinase plasminogen activator; BAEC, bovine aortic endothelial cells; PA, plasminogen activator; p200$^+$/MKR, MK cell-surface 200−250 kDa receptor expressed in G401 cells; PTPζ/RPTPβ, protein tyrosine phosphatase ζ/receptor-like protein tyrosine phosphatase β; NGF, nerve growth factor; PDGF-BB b chain homodimer of platelet derived growth factor; TGFβ2, transforming growth factor β-2; JAK1 and JAK2, Janus kinase family members; STAT1α, signal transducer and transcriptional activator family member; DSS, disuccinimidyl suberate cross-linking reagent.

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phorylation and activation of JAK1, JAK2, and STAT1α (*26*) in a renal tumor cell line for which MK is a potent mitogen. An important distinction between the PTPζ/RPTPβ and the $p200^+$/MKR is that both MK and PTN bind to PTPζ/RPTPβ [MK $K_d = 3.0$ and 0.58 nM (low vs high affinity site)], while MK alone, and not PTN, binds the $p200^+$/MKR receptor ($K_d = 70$ pM) (*26*).

MK consists of two domains, the structures of which have been determined individually by NMR (*27*). The two domains are similar in structure and both consist of three antiparallel β strands forming a triple-stranded β sheet in the N- and C-terminal domains of the molecule each of which features intradomain disulfide bonds (three in the N-terminal domain, two in the C-terminal domain). However, the orientation of these two domains one to the other and the dimensions of the intact molecule composed of both the N- and C-terminal domains separated by the linker has not been determined, although NMR data suggest that interactions between the two domains are minimal. MK has two distinct charged clusters of amino acids in its C-terminal half (*27*), which are heparin-binding sites, and one of these may mediate MK binding to PTPζ/RPTPβ (*25*) and appears to provide crucial interactions for signaling of neuronal cell migration. Indeed, the C-terminal domain alone is sufficient for activation of fibrinolysis by endothelial cells and stimulation of neurite outgrowth while the N-terminal domain (which binds heparin weakly) lacks these activities (*25* and references therein). Heparin induces the formation of noncovalent MK dimers (*27*), which lends support to the hypothesis that MK dimerization after binding to cell-surface integral membrane proteoglycans may be required for the neurite-outgrowth promoting activity of MK (*28*). These results suggested that MK, like FGF (*29*), might require proteoglycan induced dimerization for biological activity (*28*).

A requirement for ligand dimerization or oligomerization for biological activity is common to many polypeptide cytokines and growth factors including the cystine-knot family members [disulfide linked dimers in the case of PDGF−BB (*30*), but noncovalent dimers in the case of NGF (*31*)], and the cytokine TNFβ where the formation of noncovalent trimers is required for productive interactions with its signal transduction receptor (*32*). Chemically synthesized human MK forms noncovalent dimers and higher order oligomers in the absence of heparin (*33*), which potentially have important biological effects. Recently, MK stimulation of urokinase plasminogen activator (uPA) secretion by cultured bovine aortic endothelial cells (BAEC) was found to require covalent dimerization of MK by type II tissue transglutaminase (TG2) (*33*) [which catalyzes formation of a new peptide bond between an ε-amino nitrogen of lysine of one cytokine to the γ-carboxyamide carbonyl group of glutamine of another (*34*, *35*)]. It therefore appears that MK, like many other cytokines and growth factors, requires oligomerization for at least some of its biological activities induced either by heparin (or cell surface proteoglycan) binding or by TG2 catalyzed covalent oligomerization.

In this study, we investigate whether covalent modification of MK by TG2, cell surface proteoglycan binding, or formation of MK dimers in solution is required for its function as a growth factor in tumor cell proliferation. Our results suggest that purified recombinant MK exists in solution as an asymmetric monomer with a high frictional coefficient and a larger than expected Stokes radius. Using coordinates from the NMR structures of the N- and C-terminal domains of MK (*27*) and the programs AtoB and HYDRO, we have devised and validated a model which predicts that these hydrodynamic properties derive from an extended dumb-bell shape of this macromolecule. Our studies also show that MK noncovalent dimerization induced by heparin or covalent oligomerization induced by TG2 is not required for the mitogenic activity of MK on the G401 cell line. This suggests that MK, a molecule with two structurally similar N- and C-terminal domains, may potentially have two receptor-binding sites [as is true for growth hormone (*32*)], on its surface sufficient to induce $p200^+$/MKR receptor activation. Our studies also emphasize the markedly different molecular determinants of MK interaction with the PTPζ/RPTPβ receptors and the signaling receptor(s) associated with endothelial cell responses versus the proteoglycan-independent interactions with the $p200^+$/MKR implicated in the mitogenic response of G401 cells to MK. These differences in MK signaling observed in different cell types predict that the diverse biological effects of MK are probably mediated by multiple cell-specific signal transduction receptors.

EXPERIMENTAL METHODS

Tissue Culture, Reagents, and Antibodies. The Wilms' tumor cell line, G401, was obtained from the American Tissue and Cell Culture Association. G401 cells were cultured in McCoy's 5A medium (Cellgro catalog no. 10-050-CV) containing heat-inactivated 1−10% fetal bovine serum, penicillin 100 units/mL, and streptomycin 100 units/mL (Gemini). An MK-producing CHO cell line (gift of Dr. J. Milbrandt, Washington University School of Medicine, St. Louis) was used to generate conditioned media as starting material for MK purification. A goat polyclonal antibody raised against the mouse MK C-terminal region (amino acid epitope corresponds to residues 101−118 of the 118 amino acid secreted MK polypeptide) was purchased from Santa Cruz Biotechnology (catalog no. sc1398). CellTiter 96 Aqueous MTS reagents were purchased from Promega (catalog no. G5421). Disuccinimidyl suberate (DSS) was purchased from Pierce (catalog no. 21555). Heparin (sodium salt isolated from porcine intestinal mucosa) (catalog no. H3149), type 2 tissue transglutaminase (catalog no. T5398), cystamine (catalog no. C8707) and BSA fraction V powder, endotoxin-free (catalog no. A2058), *N*-*N*′-dimethylcasein (catalog no. C9801), dithiothreitol (catalog no. D0632), and putrescine (catalog no. P7630) were all obtained from Sigma. [^{3}H]Putrescine (specific activity = 21 Curies/mmol) was obtained from Amersham (catalog no. TRK 414).

Purification of MK. The secreted, mature form of the MK polypeptide was purified from serum-free conditioned medium of an MK-expressing CHO cell line (MK-CHO) using heparin affinity chromatography. MK-CHO cells were cultured in DMEM (Cellgro catalog no. 15-013-CV) containing 10% FBS and 25 nM methotrexate for 48−72 h until reaching confluence. Confluent cells were washed twice with serum-free DMEM. The medium was then changed to 1:1 DMEM/F12 (Cellgro catalog no. 10−092-CV) containing 15 mM Hepes, 25 μg/mL heparin, 0.5 mg/mL bovine serum albumin, and 1× insulin-transferrin-selenite supplement

(Sigma), and the cells were cultured for an additional 48 h. Conditioned media were collected, centrifuged, and filtered using a 0.22 μM Millipore vacuum filtration system. All subsequent steps were carried out at 4 °C. The conditioned medium was loaded onto a 5 mL of HiTrap heparin-affinity column (Pharmacia catalog no. 17-0407-01) preequilibrated with 50 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl, using a peristaltic pump at a flow rate of 1 mL/min. The column was connected to a Waters 600 FPLC system and washed with the same buffer (wash volume approximately 20 mL, until the baseline was reestablished). After preclearing the column with 0.5 M NaCl in 50 mM sodium phosphate, pH 7.4 (volume approximately 15 mL until the baseline was reestablished), MK was eluted with 1 M NaCl in the same buffer. Fractions were collected in 1 mL aliquots and dialyzed [dialysate, 50 mM sodium phosphate (pH 7.4)] using a microdialysis system, pooled, and then repurified with a second round of heparin affinity chromatography as described above. Fractions containing full-length MK purified to homogeneity, as determined by Coomassie staining, silver staining, and Western blot following SDS−PAGE, were dialyzed against 50 mM sodium phosphate (pH 7.4) buffer, pooled, and concentrated using a Centricon 10 filtration device (Amicon, catalog no. 4206). Aliquots of purified MK were stored in 50 mM sodium phosphate, pH 7.4, at 4 or −80 °C. To confirm CHO cell secretion of the predicted mature 118 amino acid MK polypeptide, and a lack of proteolysis during purification, the N-terminal sequence of purified MK was determined by protein microsequencing and found to be: KKKEKVKKGS (as predicted by cleavage of the 22 amino acid signal sequences, Genbank accesssion M19662).

G401 Cell Proliferation Assays. G401 cells were prepared from confluent monolayers by brief dissociation with 0.25% trypsin-0.1% EDTA (Mediatech, catalog no. 25053CI) and were plated in triplicate at a concentration of 2×10^4/mL cells onto Corning 96-well plates (2000 cells/well) in McCoy's 5A medium containing 10 μg/mL transferrin, 10 mM Hepes, pH 7.4, and either 0.5% fetal calf serum (for experiments with MK) or serum-free (for experiments with bFGF). Cells were stimulated with 5−100 nM purified MK or 0.2−40 ng/mL of bFGF in the presence or absence of heparin (12.5 μg/mL), 0.5 units/mL of heparinase III (Sigma, catalog no. H8891), 0.5 units/mL of chondroitinase ABC (Sigma, catalog no. 3667), TG2 (1.6 μM), or cystamine (200 μM) at 37 °C, 5% CO_2 for 48 h. Alternately, cells were first cultured in McCoy's 5A media (1% FBS) supplemented with 20 mM of sodium chlorate at 37 °C and 5% CO_2 for 48 h prior to use in mitogenesis assays. Chlorate-treated or control cells were then plated in the 96-well plates as above and stimulated with MK (5−100 nM) or bFGF (0.2−40 ng/mL) in the presence or absence of chlorate (20 mM), respectively, for 48 h. Following cell culture, 20 μL of combined MTS/PMS (20:1) reagent (CellTiter 96 Aqueous, Promega) was added to each of the 96 wells, and the cells were further incubated for 4 h at 37 °C (5% CO_2). 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 read at absorbance of 490 nm (A490) on a plate reader; data shown represent the results obtained after subtracting the A490 readings of the cell-free plates from the cell-containing plates.

Gel Filtration Analysis of MK. Gel filtration was used to provide an estimate of the MK Stokes radius. Homogeneously pure MK purified by heparin affinity chromatography (10 μg) or gel filtration protein standards (Pharmacia, catalog no. 17-0442-01) were applied to a Protein-Pak 200SW, 8 mm × 300 mm column (Waters) preequilibrated with 50 mM sodium phosphate, pH 7.4, buffer containing either 0.15 or 1.0 M NaCl at a flow rate of 1 mL/min. Proteins were eluted with the same buffer, the elution profile was monitored with a 280 nM detector, and 1 mL fractions were collected. MK-containing fractions were determined by Western dot-blotting. K_{av} values were determined from elution positions of MK or protein standards using the following equation: $K_{av} = V_e - V_o/V_t - V_o$, where V_e = elution volume for each protein, V_o = column void volume, V_t = total bed volume. The Stokes radius of MK was estimated by interpolation from the calibration curve of protein standards with known Stokes radii.

MK Sedimentation Velocity Experiments and Utilization of AtoB and HYDRO Programs To Predict the Hydrodynamic Properties of MK. A total of 250 μg of mouse MK purified to homogeneity was used for sedimentation velocity experiments conducted with a Beckman XL-1 analytical ultracentrifuge (AT-60 rotor, 60 000 rpm, $T = 20$ °C, buffer, 50 mM sodium phosphate, pH 7.4) using 12 mm double sector aluminum centerpieces. Scans were taken every 2 min using the absorbance detection system at 280 nm. Data sets were interpreted using either the time derivative of the concentration profile g(s*) with the program dcdt (*36*) or by direct fitting of the concentration profiles using Svedberg (*37*), which is better for low molecular weight proteins. The hydrodynamic modeling of MK was done using the program HYDRO (*38*) using 5 Å bead models of MK created from the atomic coordinates by the program AtoB (*39*). The model consisted of the NMR structures of the two halves of Midkine (MK1−59) and MK62−104) (*27*) linked together with a dialanine insert created using the program "O" (*40*). The AtoB bead models shown in Figure 3 were drawn using the program MACBEADS by Daniel Thomas, NCMH, University of Leicester.

Cross-Linking of MK by DSS or Type 2 Tissue Transglutaminase. Cross-linking experiments were performed using purified recombinant MK. MK (1 μM) was incubated with the covalent cross-linking agent DSS at a concentration of 0.25 mM in the presence or absence of heparin (12.5 μg/mL) for 25 min at room temperature. The reaction was quenched by 50 mM Tris-HCl and 100 mM glycine for 10 min. Cross-linking of MK by TG2 was performed by incubating MK (1 μM) with 0−4 μM type 2 tissue transglutaminase (Sigma catalog no. T5398, 2.2 units/mg protein; 1 unit = 1 μmol hydroxamate formed/min from *N*-αCBZ-Gln-Gly and hydroxylamine) in the presence or absence of 100 μM cystamine. The TG2 cross-linking reaction was also performed in the presence or absence of 1 mM $CaCl_2$ and was incubated for 1 h at 37 °C. The reaction was terminated by the addition of 50 mM EDTA. TG2 cross-linked products were analyzed by 15% SDS−PAGE, followed by Western immunoblot using mouse C-terminal MK antibody (1 μg/mL, Santa Cruz) and ECL detection as described below.

Western Immunoblotting. Protein samples were boiled for 5 min in Laemmli sample buffer under denaturing and reducing conditions. Proteins were separated by 15% poly-

acrylamide gel electrophoresis as described by Laemmli et al. (*41*). The gel was stained with either Coomassie Brilliant Blue solution or the NOVEX Silver Xpress silver staining kit. In Western blot experiments, following electrophoresis, proteins were transferred to a PVDF filter, and the filter was blocked with 5% nonfat dry milk in 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, and 0.2% Tween 20 (PBS-T). The filter was then incubated with 1 μg/mL goat MK polyclonal antibody (Santa Cruz) in blocking buffer for 1 h, washed, then incubated with 1:10000 dilution of horseradish peroxidase conjugated rabbit anti-goat IgG (KPL, catalog no.141306) for 1 h, at room temperature with gentle shaking. After a thorough washing with PBS-T buffer, the filters were developed and immunoreactive bands were visualized using the ECL system (Amersham, catalog no.RPN 2106).

Type 2 Tissue Transglutaminase Assay. Transglutaminase activity was measured as the Ca^{2+}-dependent incorporation of [^{3}H]putrescine into *N*,*N*-dimethylcasein as described by Nara et al. (*42*) with the following modifications. G401 cell membrane extracts were prepared by harvesting cells by scraping from three 25 cm^2 flasks (80% confluent) three times in CMFH buffer and resuspending the cell pellet in 500 μL of CMFH buffer (Hepes 25 mM, pH 7.4, 129 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 1 mM $NaHCO_3$, 5 mM glucose) followed by ultrasonic cell disruption and 15 min 15000*g* centrifugation. The pellet was washed once with 500 μL of CMFH, resuspended and extracted in 500 μL of CMFH containing 10 mM Chaps, 40 mM leupeptin, and 2 mM PMSF; followed by centrifugation at 15000*g* for 15 min. The supernatant soluble membrane extract was assayed for protein concentration using the BCA assay (Pierce). For the TG2 assay, the reaction mixture consisted of 50 mM Hepes, pH 8.5, 20% glycerol, 1 mg/mL *N*,*N*′-dimethylcasein, 250 μM putrescine, 20 mM dithiothreitol, 1 mM $CaCl_2$, 1 μCi of [^{3}H]putrescine, and 17.5 μg of membrane protein extract, in a final volume of 100 μL. The reaction mixtures was incubated for 1 h at 37 °C, and reactions stopped by addition of 900 μL of 50% TCA. The precipitate was collected on Whatman GF/C glass filters using a Millipore filtration manifold. The filters were then washed three times with 3 mL of 50% TCA. Radioactivity was measured by liquid scintillation counting. Transglutaminase activity was expressed as nanomoles of [^{3}H]putrescine incorporated into *n*,*N*′-dimethylcasein in 1 h at 37 °C/mg of protein of sample. Using this assay, the specific activity of pure TG2 (Sigma, catalog no. T5389) was 2257 nmol of [^{3}H]putrescine incorporation/mg/h.

RESULTS

Purification and Biophysical Studies of Recombinant MK. We purified MK to homogeneity by two rounds of heparin-affinity chromatography from serum-free conditioned media collected from the MK-CHO cell line (see Experimental Methods). Figure 1 shows Coomassie blue staining (panel A, lane 2) and Western blotting (panel B, lane 4), respectively, of purified recombinant MK following reducing SDS−PAGE. The MK M_r was determined to be approximately 13 kDa, consistent with the predicted molecular mass of 13 066 Da for the secreted mature polypeptide of 118 amino acid residues (confirmed by N-terminal sequence analysis, see Methods). Purified MK was then applied to a 200SW 8 mm × 300 mm gel filtration column (Waters) to obtain an estimate of its Stokes radius (Figure 2). We found that MK eluted as a single peak corresponding to an unexpectedly large Stokes radius of 22.8 Å, which was significantly larger than that expected of a 13 kDa globular protein as shown by the measured Stokes radius for ribonuclease A of 16.4 Å (M = 13.7 kDa) and for chymotrypsinogen of 20.9 Å (M = 25 kDa) (see also ref *43*). We obtained the identical estimate of the MK Stokes radius using either high ionic strength (1.0 M NaCl) or low ionic strength (0.15 M NaCl) gel filtration buffer for these experiments, which suggests that this estimate of the Stokes radius was not influenced by nonspecific binding of MK to the matrix of the gel filtration column media. These results, combined with the estimate of the MK M_r of 13.0 kDa by SDS−PAGE after sample reduction with β-mercaptoethanol at 100 °C, suggested that MK was either a noncovalent dimer in solution or an asymmetric monomer.

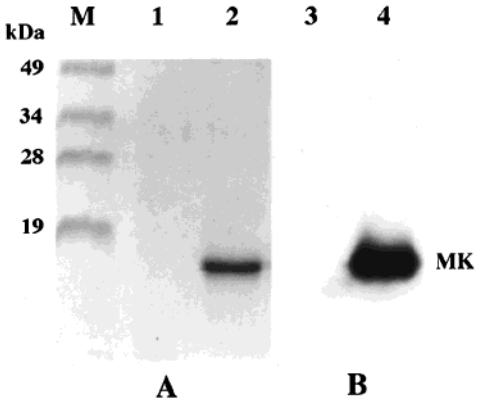
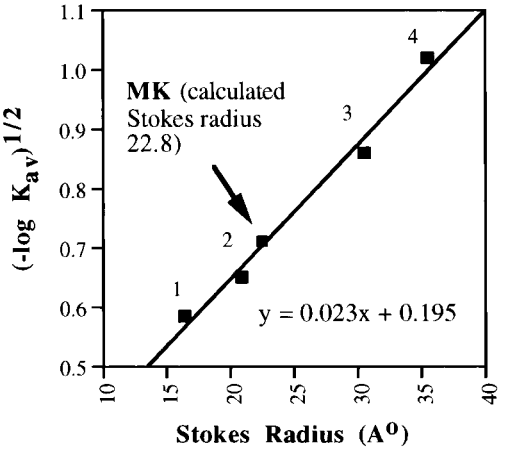


FIGURE 1: Purification of recombinant MK. MK was purified from CHO-MK cell line (gift of Milbrandt) conditioned media by two rounds of heparin affinity chromatography. The most highly purified pooled MK fractions were concentrated on a Centricon-10 (MWCO = 10 kDa) ultrafiltration device and analyzed by 15% SDS−PAGE with Coomassie blue staining (A) and Western blotting (B). Lanes 1 and 3: Ultrafiltrate from Centricon 10 concentration step; lane 2: 5 μg of MK after reduction with β-mercaptoethanol; lane 4: 0.5 μg of MK after reduction with β-mercaptoethanol.

Using analytical ultracentrifugation sedimentation velocity analysis, we further characterized the biophysical and hydrodynamic properties of MK. MK had an unexpectedly low sedimentation coefficient of 1.373×10^{-13} s balanced by a lower than expected diffusion coefficient of 9.33×10^{-7} cm^2/sec to yield a calculated molecular mass M of 13.48 kDa using the Svedberg equation [MK partial specific volume $\upsilon = 0.730$ mL/g and $M = sRT/D(1 - \upsilon\rho)$]. Using an assumption of a globular shape, the calculated MK Stokes radius by this method was 23.73 Å [from $R_S = kT/(6\pi\eta D)$], which was close to the 22.8 Å value obtained by gel filtration analysis. These results indicated that the MK molecule exists in solution as an asymmetric monomer with a frictional coefficient f/f_0 of 1.48 and resembled a prolate whose axial ratio is about 8:1. In addition, we did not observe any evidence of aggregation of MK into oligomers during this experiment.

To develop a model of the macromolecular solution conformation of purified MK, we used the atomic coordinates for the human N-terminal (residues 1−59) and C-terminal domains (residues 62−104) (*27*) determined by NMR (kindly



Name	MW (kDa)	Stokes radius (A^O)
1 ribonuclease A	13.7	16.4
2 chymotrypsinogen A	25	20.9
3 ovalbumin	43	30.5
4 albumin	67	35.5
MK	**13**	**22.8**

FIGURE 2: Calculation of the MK Stokes radius by gel filtration. MK or gel filtration protein standards (shown in table) were applied to a Protein-Pak 200 SW 8 mm × 300 mm column (Waters) at a flow rate of 1 mL/min. K_{av} values were calculated from the elution volumes (V_e) of MK and the protein standards of known Stokes radius. The Stokes radius of MK was calculated from the calibration curve. In comparison to the globular protein ribonuclease A (M 13.7 kDa) with a Stokes radius of 16.4 Å, MK (M 13.1 kDa) had an unexpectedly high Stokes radius (22.8 Å) even larger than the 20.9 Å Stokes radius of chymotrypsinogen (M 25 kDa).

provided by Dr. F. Inagaki, Tokyo) and the AtoB program (*39*) to construct a bead model at 5 Å resolution required for input into the program *HYDRO* (*38*). *HYDRO* generates a predicted sedimentation coefficient, a translational diffusion coefficient, and a radius of gyration of a solvated molecule which can be compared to that obtained from experimental observations which can then be used to validate and refine the model. On the basis of the prediction that the N- and C-terminal domains should exist independently in solution (*27*) with little interaction, we used the AtoB algorithm to construct a bead model (consisting of 86 beads with a radius of 3.36 Å) of an MK macromolecular conformation with an extended linker allowing the maximal separation between these two domains for analysis by *HYDRO* (Figure 3). The predicted sedimentation coefficient of 1.357×10^{-13} s and diffusion coefficient of 1.05×10^{-6} cm^2/s for this model using HYDRO analysis are very similar to the observed values of 1.373×10^{-13} s and 9.33×10^{-7} cm^2/s obtained by the sedimentation velocity method. An alternate MK AtoB model showing close packing of the N-terminal and C-terminal domains (Figure 3) resulted in a HYDRO-predicted sedimentation coefficient of 1.60×10^{-13} s and a diffusion coefficient of 1.47×10^{-6} cm^2/s which are signifcantly different from the observed hydrodynamic properties of the molecule in solution. The extended dumb-bell model for MK suggests that the linker between the N- and C-terminal domains is fully extended and predicts that the MK molecule has a molecular length of approximately 79 Å in solution and about 16 Å in the shortest dimension.

Dimerization of the MK Molecule Induced by Heparin Is Not Required for MK Mitogenic Activity on G401 Cells. Previous studies of FGF interactions with FGF cell surface receptor tyrosine kinases have demonstrated that heparin induces oligomerization of FGF molecules, which is required for FGF receptor dimerization, activation, and cell proliferation in BAF3 and mutant CHO-pgsD-677 cells (*29*, *44*, *45*), which are defective in heparan sulfate expression at the cell surface (*29*). In contrast, growth factor dimerization stabilized by covalent disulfide bonds is required for biological activity of the cystine knot family ligands PDGF-BB, and TGFβ2 (*30*) although another cystine-knot family member, NGF, is thought to exist in solution as a noncovalently associated

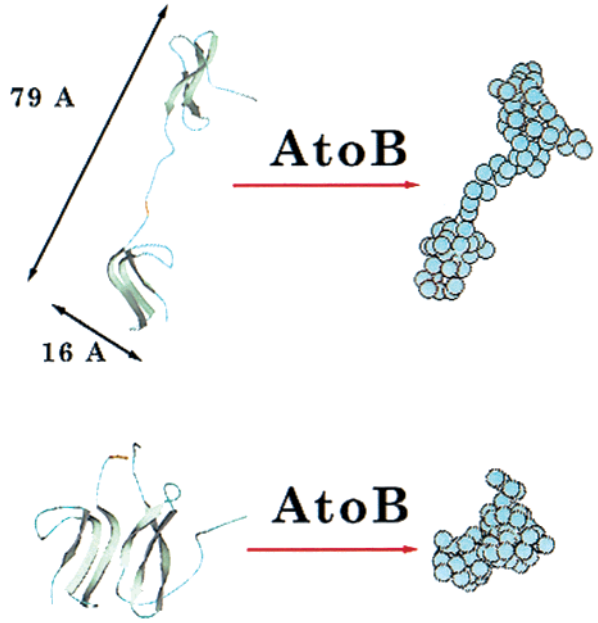


FIGURE 3: Molecular model for MK. Using the coordinates from the NMR structure of the N-terminal (residues 1−59) and C-terminal (residues 62−104) halves of MK (*27*), we constructed two bead models of the molecule with the program AtoB which predicted different hydrodynamic properties in solution (see Methods). The model showing the extended linker with dimensions of 79 Å × 16 Å most closely conforms to the observed hydrodynamic properties of Midkine (see text).

dimer (*31*). For MK, it appears that heparin-induced dimerization is required for the stimulation of neurite outgrowth (*27*) and that interactions with chondroitin sulfate residues associated with the PTPζ/RPTPβ receptor tyrosine phosphatase (*25*) may also be required for biological activity of neuronal cell migration and neurite process formation.

To analyze the effects of heparin on the mitogenic activity of MK, we first carried out chemical cross-linking experiments using disuccinimidyl suberate (DSS) to determine whether heparin binding resulted in the formation of MK dimers or higher-order oligomers. In Figure 4A, purified recombinant MK was incubated with heparin (12.5 μg/mL) in the presence or absence of DSS (0.25 mM), and the reaction mixture was analyzed by 15% SDS−PAGE. In the

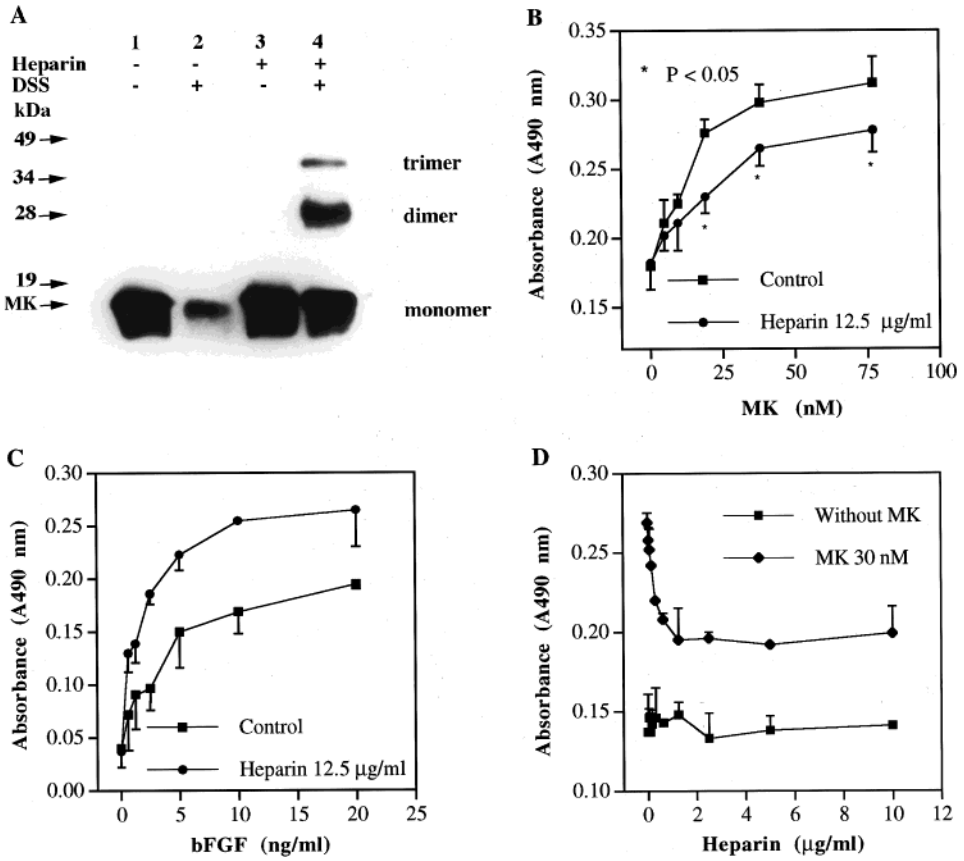


FIGURE 4: Heparin induces formation of MK oligomers and partially inhibits MK stimulation of G401 cell proliferation. (A) MK was incubated with or without heparin (12.5 μg/mL) in the presence or absence of the cross-linking reagent DSS (0.25 mM) for 25 min at room temperature. The reaction was quenched by 50 mM Tris-HCl and 100 mM glycine for 10 min. Cross-linked MK products were analyzed by 15% SDS−PAGE followed by Western blotting. Both dimers and trimers were detected after incubation with heparin and DSS as shown in lane 4, but were not found when DSS was omitted (lane 3), when both heparin and DSS were absent (lane 1) or when heparin was omitted (lane 2). (B and C) G401 cells were plated in 96-well plates (2000 cells/well) and stimulated by MK (B) or by bFGF (C) in the presence or absence of heparin (12.5 μg/mL) for 48 h. Heparin induced a moderate inhibition of the MK mitogenic effect which was statistically significant, but resulted in stimulation of the mitogenic effect of bFGF. (D) G 401 cells were plated in a 96-well plate (2000 cells/well) and coincubated with heparin over a concentration range of 0−10 μg/mL in the presence or absence of MK (30 nM) for 48 h; (heparin concentrations: 0, 39, 78, 156, 313, 625 ng/ml and 1.25, 2.5, 5, 10 μg/ml). Cell number after mitogenic stimulation was assayed using the MTS/PMS assay (A490 is corrected for A490 of control cell-free wells); data shown are mean ± SD (n = 3); if SD bars are absent, the SD is contained within the data symbol).

absence of heparin, MK migrated with an M_r of approximately 13 kDa, (lane 1). Addition of heparin alone did not result in formation of MK dimers in SDS−PAGE under reducing conditions (lane 3), but addition of both heparin and DSS resulted in MK dimerization and formation of lesser amounts of trimer and tetramer (lane 4). Treatment with DSS alone (lane 2) failed to demonstrate any dimers or trimers; although there was reduction in the amount of the monomer band detected by immunoblotting in this experiment. However, we failed to detect any higher order multimers with DSS treatment in the absence of heparin and believe that this reduction in Western blot sensitivity may reflect epitope masking by DSS preventing optimal binding of the MK antibody to the C-terminus of the mature protein (amino acid residues 101−118). This seems to be a reasonable interpretation given that sedimentation velocity analysis showed that recombinant murine MK exists in solution as a 13 kDa monomer (see above) and does not associate spontaneously into stable noncovalent multimers. These results showed that heparin stimulates the formation of MK noncovalent oligomers and extend previously published studies showing that heparin induced dimerization of chemically synthesized human MK by light-scattering photometry experiments (*27*).

Next, we analyzed whether heparin incubation with MK would modify its mitogenic effects on the G401 cell line that we used previously to characterize MK activation of cell proliferation and to identify the p200$^+$/MKR receptor (*46*). G401 cells were treated with MK in the presence or absence of heparin (12.5 μg/mL) and the mitogenic activity of MK was determined using the MTS/PMS cell proliferation assay. As shown in Figure 4B, heparin did not increase the response of G401 cell proliferation to MK stimulation, but instead modestly inhibited the mitogenic effect of MK on G401 cells. In contrast, heparin addition (12.5 μg/mL) stimulated the mitogenic effects of FGF-2 [FGF-2 was recently identified as another autocrine mediator of G401 cell proliferation (*47*)] over a range of concentrations of this growth factor (Figure 4C). Since previous studies have shown that much lower concentrations of heparin (in the range of 30−100 ng/mL) might be needed to detect a stimulatory effect of heparin on the mitogenic response to heparin-binding growth factors (*48*), we carried out a titration of heparin over a concentration range of 0−10 μg/mL to determine the effects on the mitogenic response to 30 nM Midkine. As shown in Figure 4D, we observed a progressive decrease in the inhibitory effect of heparin on MK-dependent

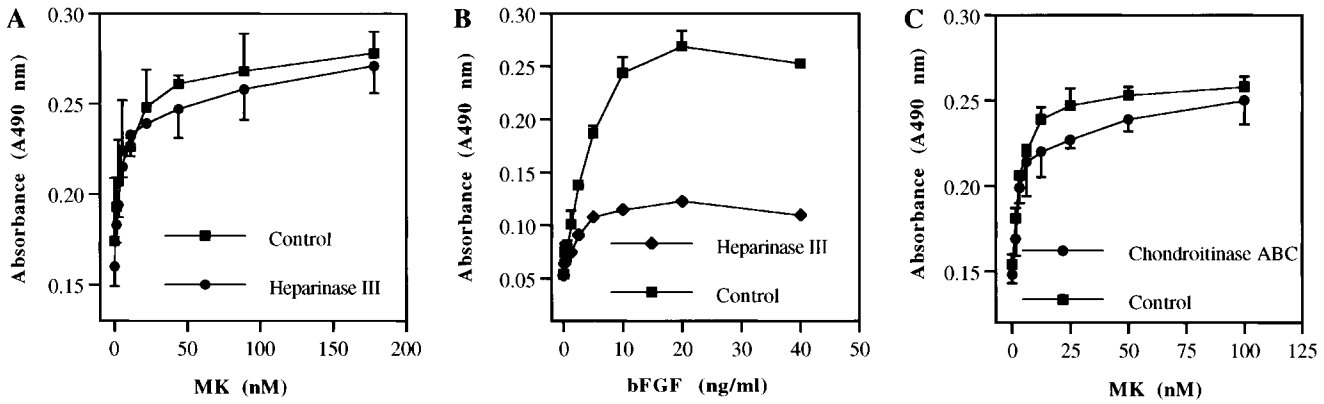


FIGURE 5: Cell surface proteoglycans are not required for MK stimulation of G401 cell proliferation. G401 cells were plated in 96-well plates at a concentration of 2000 cells/well and stimulated with purified MK in the presence or absence of 0.5 units/L of heparinase III (A) or 0.5 units/mL chondroitinase ABC (C), or stimulated with bFGF in the presence or absence of 0.5 units/mL of heparinase III (B) for 48 h. Cell number after mitogenic stimulation was assayed using the MTS/PMS assay (A490 is corrected for A490 of control cell-free wells). As shown, heparinase-III treatment inhibited the mitogenic effect of bFGF, but not of MK, on G401 cells. (Data shown are mean ± SD, $n = 3$; if SD bars are absent, the SD is contained within the data symbol).

cell proliferation as its concentration was decreased through this range. Since we were able to demonstrate a stimulatory effect for heparin addition to the FGF-2 mitogenic response of G401 cells in this system, but not for Midkine, these results suggested that the dimerization of MK molecules induced by heparin treatment in vitro is not required for MK receptor activation of cellular proliferation in G401 cells.

Binding of the MK Ligand to Cell Surface Proteoglycans Is Not Required for MK Mitogenic Activity on G401 Cells. It has been reported that cell surface heparan sulfate is necessary for FGF receptor (FGFr) signaling after FGF binding (*44*, *49*, *50*) and that this requirement can be overcome in cells which fail to express cell surface heparan sulfate by the addition of heparin (*48*). Although our results showing that heparin addition stimulated the mitogenic response to FGF-2 suggested that G401 cells might express a suboptimal amount of heparan sulfate at the cell surface for FGF-2 signaling, we could not exclude the possibility that heparan sulfate expression of G401 cells might still be sufficient to bind and activate MK by inducing its dimerization. Thus, our failure to observe any stimulatory effect with the addition of heparin to Midkine might be explained by adequate cell-surface heparan sulfate (or chondroitin sulfate) binding and dimerization of Midkine. To test this hypothesis, G401 cells were stimulated with MK in the presence or absence of heparinase III (see refs *49*, *51*, and *52*), which cleaves heparan sulfate in low sulfation domains (*14*). As shown in Figure 5A, cotreatment with Heparinase III (0.5 units/mL) (which is known to block FGF-2 signal transduction (*53*), had little effect on the observed G401 cell proliferation stimulated by MK. To demonstrate a biologically significant reduction in cell-surface heparan sulfate with heparinase III cotreatment, we analyzed the effects of this treatment on the mitogenic response to FGF-2 in this cell line. As shown in Figure 5B, using the same conditions as used for the Midkine experiment in Figure 5A, we observed a highly significant reduction in the mitogenic effects of FGF-2 with heparinase III cotreatment. Since MK and PTN bind chondroitin sulfate glycosaminoglycans, we determined whether cell treatment with chondroitinase ABC [which interferes with MK binding to PTPζ/RPTPβ (*25*)] would inhibit the mitogenic response of G401 cells to MK. These experiments showed that cotreatment with chondroitinase ABC did not have statistically significant effects on the mitogenic activity of MK (Figure 5.C).

To further confirm our hypothesis that Midkine binding to cell-surface cell surface glycosaminoglycans is not required for activation of the mitogenic response, we also cultured G401 cells in media supplemented with 20 mM sodium chlorate to diminish proteoglycan sulfation and thereby inhibit association with heparin-binding growth factors (including FGF-2, see ref *48*). As can be seen in Figure 6A, the mitogenic response of G401 cells to FGF-2 was diminished in cells cultured in sodium chlorate in comparison to untreated cells. However, as shown in Figure 6B, the mitogenic response to Midkine was identical in cells cultured in sodium chlorate supplemented media or in control media.

Taken together, these results suggest that cell-surface proteoglycan binding is not required for the mitogenic effects of MK in the G401 cell line although we cannot absolutely exclude that such interactions may provide a modest enhancement of MK signaling at low ligand concentrations. We therefore conclude that although heparin induced the formation of MK dimerization in vitro as previously reported, MK dimerization either by heparin or binding to cell-surface proteoglycans does not appear to be required for MK mitogenic activity in G401 cells.

Tissue Transglutaminase Induces the Formation of MK Multimers and Inhibits Mitogeneic Activity of MK on G401 Cells. It has been reported that MK is a substrate for type 2 tissue transglutaminase (TG2) and that TG2 enhances the stimulation of plasminogen activator release by MK treated bovine aortic endothelial cells (BAEC) by cross-linking MK into covalent multimers (*22*, *33*). To determine whether TG2 is important in mediating MK mitogenic effects, we performed the following experiments. First, purified recombinant MK was incubated with TG2 over a concentration range (0−4 μM) in the presence or absence of cystamine (200 μM), an inhibitor of TG2. The cross-linked products were then applied to 15% SDS−PAGE, and the reaction products analyzed by Western blotting. We found that TG2, in the presence of 1 mM $CaCl_2$, resulted in the formation of high molecular weight MK multimers in a dose-dependent manner (as previously reported in ref *22*) and that cystamine 100 μM completely abolished TG2 activity (Figure 7A). The

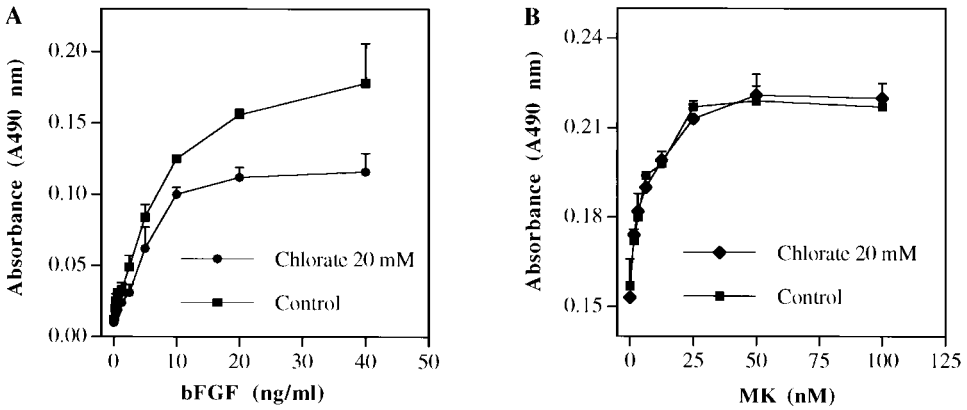


FIGURE 6: Sodium chlorate inhibited G401 cell proliferation stimulated by bFGF but not by MK. G401 cells were cultured with either 20 mM sodium chlorate supplemented McCoy's 5A media or control media for 48 h prior to use in mitogenic assays. G401 cells were then plated in 96-well plates (2000 cells/well) and stimulated with bFGF (A) or MK (B) in the presence or absence of 20 mM sodium chlorate for 48 h. Cell number after mitogenic stimulation was assayed using the MTS/PMS assay (A490 is corrected for A490 of control cell-free wells). As shown, chlorate inhibited the mitogenic effect of bFGF on G401 cells but had no effect on the mitogenic activity of MK. (Data shown are mean ± SD, $n = 3$; if SD bars are absent, the SD is contained within the data symbol).

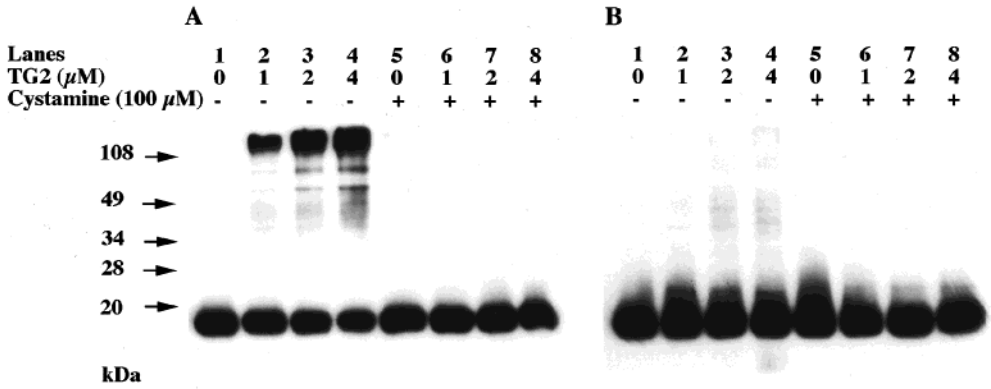


FIGURE 7: TG2 induces MK oligomerization in the presence of Ca^{2+} which can be blocked by the TG2 inhibitor cystamine. Purified MK was incubated with 0−4 μM TG2 in the presence or absence of 100 μM cystamine for 1 h at 37 °C. Reactions were terminated by the adding of 50 mM EDTA. (A) The reaction mixtures contained 10 mM $CaCl_2$. (B) The reactions were performed in the absence of Ca^{2+}. Cross-linked MK products were analyzed by 15% SDS−PAGE followed by Western blotting using MK antibody as described in the Experimental Methods.

effect of TG2 was also Ca^{2+} dependent since the reaction did not occur in the absence of Ca^{2+} (Figure 7B).

We then determined whether TG2 treatment affected the mitogenic activity of MK. G401 cells were stimulated with MK in the presence or absence of transglutaminase and the effects of this treatment are shown in Figure 8: TG2 at 1.6 μM significantly inhibited G401 cell proliferation stimulated by MK and this inhibitory effect was abolished by 200 μM of cystamine which alone had no effect on MK-stimulated G401 cell proliferation. Next, we measured TG2 activity in both membrane extracts of G401 cells using Ca^{2+}-dependent incorporation of [^{3}H]putrescine into *N*,*N*-dimethylcasein (*42*). We found that the level of TG2 activity in cultured G401 cells (3.8 nmol of [^{3}H]putrescine incorporation/h/mg membrane protein and 2.9 nmol of [^{3}H]putrescine incorporation/h/mg cytosolic protein) was more than 1 order of magnitude lower than the TG2 activity reported for many cell lines (*54*). Since it is possible that TG2 addition to cultured G401 cells might also covalently modify (and functionally inhibit) cell-surface signaling receptors including the p200/MKR receptor, we also analyzed whether the TG2 inhibitor cystamine might attenuate or abolish the mitogenic effects of MK. In these experiments, we found that cystamine at a concentration of 200 μM did not inhibit MK mitogenic action (Figure 8),

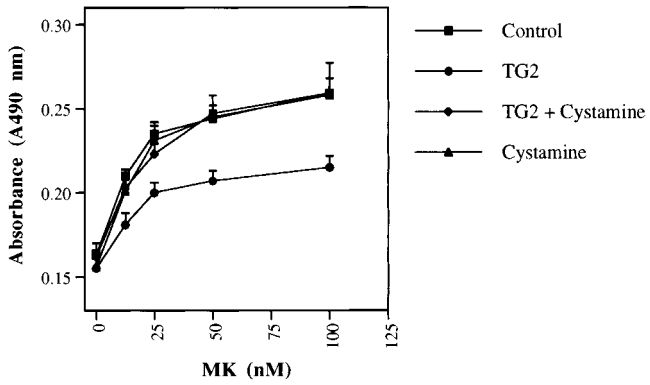


FIGURE 8: TG2 partially inhibits G401 cell proliferation stimulated by MK. G401 Cells (2000 cells/well) were stimulated with 5−100 nM purified MK in the presence or absence of TG2 (1.6 μM) or cystamine (100 μM) for 48 h at 37 °C, 5% CO_2 in a 96-well plate. Cystamine treatment alone had no effect but entirely blocked the inhibitory effect of TG2 which was present at all concentrations of MK [data shown are mean ± SD ($n = 3$)].

although this concentration completely abolished the formation of MK multimers by TG2 in vitro (Figure 7A) and interfered with TG2 activation of MK multimer formation in BAEC cells (*22*). These studies suggested that although TG2 covalent modification of MK results in oligomerization,

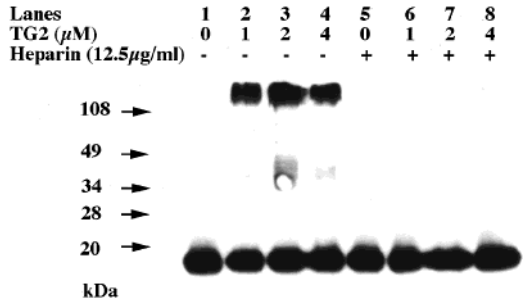


FIGURE 9: Heparin abolishes TG2 cross-linking and oligomerization of MK. MK was incubated with 0−4 μM TG2 in the presence or absence of heparin (12.5 μg/mL) for 1 h at 37 °C. Reaction mixtures contained 10 mM $CaCl_2$ and the reaction was terminated by addition of EDTA to a final concentration of 50 mM. Cross-linked MK products were analyzed by a 15% SDS−PAGE followed by Western blotting using MK antibody as described in the Experimental Methods.

TG2 multimerization of MK is not required for its mitogenic activity in vitro using this experimental system. Furthermore, we have determined that G401 cells have a low level of TG2 activity and have shown that the TG2 inhibitor cystamine does not inhibit the mitogenic effects of MK on G401 cells. Taken together these data provide strong evidence that neither heparin, nor TG2 oligomerization of MK is required for its mitogenic properties on cultured G401 cells.

Heparin Blocks TG2 Catalyzed MK Oligomerization and TG2 Oligomerization of MK Reduces Heparin-Binding Affinity of MK. Since heparin and TG2 each induced oligomerization of MK (noncovalent dimers and trimers with heparin addition, covalently cross-linked oligomers for TG2), we wondered whether these effects would be additive or mutually antagonistic. We therefore incubated MK with TG2 in the presence or absence of heparin followed by SDS−PAGE to determine the effects of these combined treatments. As shown in Figure 9, MK multimers formed by TG2 cross-linking were reduced to almost undetectable levels by inclusion of heparin (12.5 μg/mL) in the reaction mixture. This inhibition of TG2 cross-linking by heparin was specific, and contrasts to the stimulatory effects of heparin on DSS cross-linking shown in Figure 4A. Cross-linked MK oligomers produced by TG2 incubation were also analyzed by heparin affinity column chromatography using NaCl step gradient elution (50 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl loading buffer; step 1, 0.3 M NaCl; step 2, 0.5 M NaCl; step 3, 1.0 M NaCl). Although we found that the TG2 MK multimeric products bound quantitatively to a heparin affinity column at 0.15 M NaCl (50 mM sodium phosphate buffer, pH 7.4), these MK multimers were unexpectedly eluted efficiently by increasing the salt concentration to 0.3 M NaCl (data not shown). In contrast, monomeric MK was retained on the heparin affinity column under these conditions and was not eluted at salt concentrations lower than 0.8 M NaCl. These results were consistent with the interpretation that the heparin-binding charged amino acid clusters in the C-terminal half of the molecule (*25*, *27*) (putative cluster I, K^{76}, R^{78}, K^{99}; cluster II, K^{83}, K^{84}, R^{86} of the secreted mature mouse MK polypeptide) have reduced solvent accessibility for heparin binding after TG2 induced multimerization of MK. Since heparin incubation inhibited TG2 cross-linking of mouse MK, it is possible that either the potential carbonyl acceptor site Q^{92} (human Q^{95} proposed in ref *33*) or potential donor lysines in heparin-binding clusters I and II are rendered inaccessible for TG2 catalysis by the side-chain interactions of these residues with heparin which have been previously demonstrated by NMR (*27*).

DISCUSSION

An essential objective in elucidating the biological functions of growth factors is the molecular identification of cell-surface receptors, which mediate signal transduction. MK appears to have multiple cell surface receptors including the heparan sulfate proteoglycan ryudocan (*24*), the chondroitin sulfate proteoglycan receptor tyrosine phosphatase PTPζ/RPTPβ, and the signaling receptor p200$^+$/MKR identified in the G401 cell line (*26*). A major challenge at this point is identification of the essential ligand−receptor interactions which mediate specific biologic effects of MK in different cell types. It is not surprising that the paradigms established for ligand receptor interactions with other heparin-binding factors such as FGF (*29*) and VEGF (see review in ref *55*) establishing the importance of ligand binding both to polysaccharide sites of multiple cell surface proteoglycans as well as to the extracellular protein binding sites of multiple signal transduction receptors may be directly relevant to understanding the actions of MK and PTN on diverse cell types.

With regard to the stimulation of neuronal migration and neurite outgrowth by MK and PTN (*56*), it has now been established that interactions of *substrate-attached* MK or PTN by C-terminal domain interactions with chondroitin sulfate (and/or heparan sulfate) are essential for biological activity. Mutations in mouse MK (replacing Arg^{78}), which disrupt chondroitin sulfate binding, markedly impair neurite outgrowth, as does competition with multiple glycosaminoglycans including chondroitin sulfates B−E and heparin (*25*). In contrast, *soluble* MK stimulates plasminogen activator release from endothelial cells but these interactions depend on Lys^{83} and Lys^{84}, which are not essential for the effects on neurite outgrowth. These results are consistent with the existence of multiple and distinct independent cell-surface MK receptor-binding sites, one of which mediates the effects of MK on neurite outgrowth and neuronal migration [and is likely the glycosaminoglycan component of PTBζ/RPTPβ and/or N-syndecan (*13*)] and a second receptor-binding site present on BAEC cells which mediates activation of fibrinolysis by the stimulation of uPA release.

In our previous work using the G401 renal tumor cell line (*57*) for which *soluble* MK is an autocrine factor with mitogenic activity (*26*), we used cross-linking to identify the p200$^+$/MKR, which binds MK with very high affinity (K_D = 70 pM), is rapidly tyrosine phosphorylated within minutes of ligand binding and associates with phosphorylated JAK1 and JAK2 kinases. The specificity of the p200$^+$/MKR receptor for MK binding (PTN does not bind this receptor or stimulate its tyrosine phosphorylation in G401 cells) and its binding affinity (K_D of 70 pM), which is an order of magnitude higher than the binding affinity of MK to PTBζ/RPTPβ [K_D of 700 pM (*25*)] and 5-fold that of the binding affinity of MK to ryudocan [K_D = 300 pM (*24*)], clearly demonstrates that the interactions of MK with the p200$^+$/MKR are biochemically unique and quite different from the

interactions of this growth factor with either PTBζ/RPTPβ or ryudocan.

In the studies reported here, we have developed a model of the solution conformation of MK from the atomic coordinates for the N- and C-terminal domains using the AtoB algorithm (*39*) and the hydrodynamic modeling program *HYDRO* (*38*). We have tested and refined this model by experimental determination of the hydrodynamic properties of recombinant MK using gel filtration and sedimentation velocity analysis. The latter studies demonstrated that MK exists in solution as an asymmetric monomer with a Stokes radius of 22.8 Å (by gel filtration) and a sedimentation coefficient of 1.373×10^{-13} s. Our model predicts that MK exists in solution as an extended dumb-bell-shaped molecule with a surprisingly large maximal molecular diameter of 79 Å for a polypeptide with a molecular mass of 13 kDa. This unusual molecular shape predicted by our model results from an extended linker conformation, which keeps the N-terminal and C-terminal domains well separated. This might allow independent binding events between the N-terminal domain and C-terminal domains and two cell surface receptor macromolecules important for the biological function of MK in signal transduction.

In contrast to our results which demonstrate that recombinant mouse MK purified from a stably transfected CHO cell line exists in solution as an extended dumb-bell-shaped monomer, it has previously been reported that chemically synthesized human MK forms noncovalently associated dimers, tetramers, and higher order multimers (*33*) which can be detected by gel filtration. In our experiments with gel filtration, we detected only a single peak and our analytical ultracentrifugation analysis did not disclose evidence of a heterogeneous mixture of MK multimers. The absence of MK dimers and higher-order multimers in our purified MK fraction may result from amino acid differences between human and mouse MK (16/121 human residues of the secreted polypeptides are not shared by the 118 amino acid residue mouse sequence) or might reflect differences in the MK protein concentrations used for analysis. Alternatively, the chemical synthesis methodology (*58*) used for the preparation of MK in previous studies, or different storage conditions, might promote formation of MK multimers. Although we are unable to distinguish with certainty which of these hypotheses is correct, we believe that our data convincingly demonstrate that mouse MK can exist as a biologically active monomeric macromolecule in solution at physiological salt concentrations.

Since noncovalent dimerization by heparin in solution or by cell-surface heparan sulfate or chondroitin sulfate proteoglycans is required for the biological activities of many heparin-binding growth factors (*29*, *44*, *49*, *50*), and since MK is known to bind the cell surface proteoglycans ryudocan (*24*) and PTPζ/RPTPβ (*25*), we explored whether heparin might stimulate the mitogenic effects of MK on G401 cells. However, we found that although heparin did promote MK dimerization in cell-free conditions in vitro, the addition of heparin did not stimulate the mitogenic effects of MK on G401 cells but rather produced partial inhibition. The lack of robust competition by heparin of the mitogenic effects of MK on G401 cells suggested that cell surface proteoglycan binding of MK might not be required for its mitogenic effects. In contrast, the addition of 0.1−10 μg/mL heparin prevents PTPζ/RPTPβ binding and nearly abolishes MK induced neuronal migration (*25*). In subsequent experiments, we found that neither cell treatments with either heparinase III or chondroitinase ABC nor cell culture in sodium chlorate-containing culture media inhibited the mitogenic response to Midkine. In contrast, heparin addition stimulated the FGF-2 mitogenic effect, and heparinase III and sodium chlorate pretreatment of cells inhibited the FGF-2 stimulation of cell proliferation as predicted by many previous studies showing a requirement for noncovalent oligomerization of this mitogen following glycosaminoglycan binding (*29*). These results support the conclusion that MK binding to cell surface proteoglycans and noncovalent dimerization is not required for its mitogenic effects on G401 cells and strongly argue against a role for PTPζ/RPTPβ in this biological response.

It has previously been demonstrated that tissue transglutaminase II (TG2) covalent cross-linking of MK enhances its biological activity as an inducer of plasminogen activator release from endothelial cells (*22*, *33*). However, we found that neither TG2 addition to cultured G401 cells treated with MK nor cystamine inhibition of TG2 during MK stimulation had strong effects on its mitogenic activity. In contrast, it has been established that cystamine at 200 μM concentration blocks the multimerization and biological activity of MK induced by retinoic acid stimulation of endothelial cells in culture (*22*). We also found that G401 cells contain low levels of TG2 and secrete only monomeric, not multimeric MK (*26*). These results make it unlikely that the mitogenic effects of MK on G401 cells require TG2-dependent dimerization or multimerization and serve to emphasize the important differences between ligand receptor interactions required for stimulation of plasminogen activator release by endothelial cells (which require TG2 oligomerization of MK) on one hand and the mitogenic effects of MK on G401 cell proliferation on the other.

Our studies reported here support the notion that monomeric MK is the biologically active ligand which interacts with the $p200^{+}$/MKR signal transduction receptor associated with the G401 cell mitogenic response and argues against a requirement for binding to the plasma membrane proteoglycans ryudocan or to PTPζ/RPTPβ in this cell type. This implies that the mitogenic effects of MK in the G401 cell line are mediated by a different ligand−receptor interaction than those which mediate the respective effects of MK on neuronal and endothelial cells. In turn, this points to the existence of mutliple cell surface MK receptors as the underlying basis for the diversity of biological effects produced by this growth factor on different cell lineages. The proposed extended dumb-bell-shaped macromolecular conformation with independent N- and C-terminal domains for monomeric MK also raises the intriguing possibility that at least some MK-receptor interactions might depend on two receptor binding sites per ligand and not require ligand dimerization for receptor activation.

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