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Clusters of Basic Amino Acids in Midkine: Roles in Neurite-Promoting Activity and Plasminogen Activator-Enhancing Activity[1]

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The removal of N-terminally located clusters of basic amino acids (N-tail) or C-terminally located clusters of basic amino acids (C-tail) from the midkine (MK) molecule severely reduced its neurite-promoting activity. However, experiments involving chemically synthesized MK derivatives revealed that the roles of the N-tail and C-tail were mostly indirect ones, *i.e.* they probably maintain the steric arrangements of the N-terminal and C-terminal halves. In particular, the C-domain, which is the C-terminal half devoid of the C-tail, retained considerable neurite-promoting activity when it was uniformly coated on a dish. The removal of the N-tail or C-tail also reduced the enhancing activity of plasminogen activator (PA) in aortic endothelial cells, although the effect was lower. There are two heparin-binding sites in the C-domain, Clusters I and II. A mutation in Cluster I [$R^{78}\rightarrow Q$] affected the PA-enhancing activity only slightly, and a mutation in Cluster II [$K^{83}K^{84}\rightarrow QQ$] abolished the activity, while both mutations are known to reduce the neurite-promoting activity moderately. Therefore, the two heparin-binding sites in the C-domain play different roles in these two activities. Indeed, heparin exhibited different effects on these two activities. We also observed that intact MK was required for ordered neurite-promotion along the path of MK; one possible interpretation of this is that the N-terminal half is necessary for the stability of the molecule. Furthermore, K^{76} and K^{99} were found to be required for the secretion of MK; *i.e.* mutants in which one of these K residues was changed to Q were produced in the host cells, but not found in the medium.

Key words: growth factor, heparin, midkine, neurite outgrowth, plasminogen activator.

The heparin-like oligosaccharide structures located in cell-surface heparan sulfate proteoglycans play important roles in mediating growth factor signals to target cells (*1-3*).

Midkine (MK) (*4, 5*) and pleiotrophin (PTN) (*6-8*) form a distinct family of heparin-binding growth factors. They exhibit 45% sequence identity, and are unrelated to other heparin-binding growth factors such as fibroblast growth factors (*5, 7, 8*). MK and PTN have diverse activities; they promote the neurite outgrowth of embryonic neurons (*6, 9*), enhance plasminogen activator (PA) activity in aortic endothelial cells (*10, 11*), oncogenically transform NIH3T3 cells (*12, 13*), and induce postsynaptic specification of neuromuscular junctions (*14, 15*). In addition, MK enhances the survival of embryonic neurons (*16*), and neuronal differentiation (*17, 18*), and is chemotactic as to neutrophils (*19*). Accumulating evidence indicates their importance in tumor progression (*20, 21*), inflammation (*19*), and tissue repair (*22, 23*).

MK and PTN bind with high affinity to syndecans (*24-26*), a family of cell-surface heparan sulfate proteoglycans. Upon neurite outgrowth promotion of embryonic neurons, *N*-syndecan may well serve as a receptor for PTN (*24*). For high affinity binding, both MK and PTN require all of the 2-*O*, 6-*O* and 2-*N*-sulfates in the heparin-like structure (*27, 28*); they bind to the highly sulfated domain of cell-surface heparan sulfate proteoglycans.

MK and PTN are largely composed of two domains, each of which is held through 2 or 3 intradomain disulfide bridges (*29, 30*) (Fig. 1). Chemically synthesized half molecules of MK, which have only one domain, were previously used to analyze the structure and function relationship of MK (*31*). We found that the C-terminal half (C-half) of MK exhibits strong and conformation-dependent heparin-binding activity, neurite-promoting activity (*31*), and PA enhancing activity (*32*). On the other hand, the N-terminal half of MK (N-half) exhibits relatively weaker heparin binding activity, and is devoid of neurite-promoting activity and PA

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Abbreviations: MK, midkine; PA, plasminogen activator; PTN, pleiotrophin.

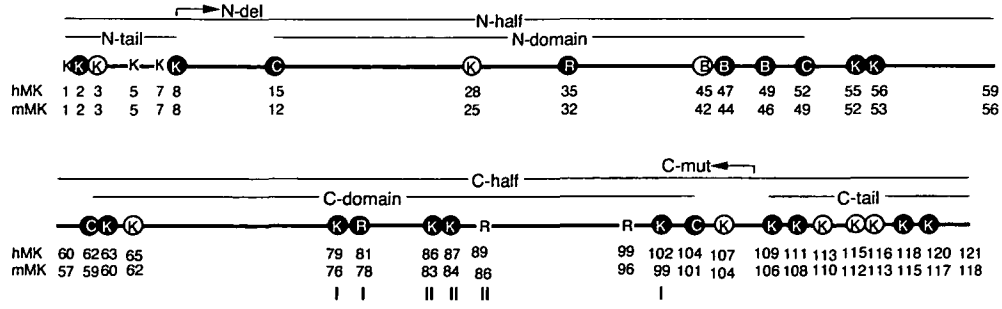

Fig. 1. **Location of conserved basic amino acids in segments of MK.** Numbers indicate the amino acid numbers in human MK (hMK) or mouse MK (mMK). ⊗ indicates basic amino acids conserved in MK of all species but not in PTN: ⊘ indicates those conserved in MK and PTN. X indicates those conserved in mouse and human MK and C indicates conserved cysteines at the boundaries of domains. I or II shows the location of basic amino acids in Cluster I or II (*33*). Arrows, N-del and C-mut, indicate the start of retained amino acid residues in MK mutants devoid of the N-tail and C-tail, respectively (*cf.* "MATERIALS AND METHODS"). B, basic amino acid (R, K, or H).

enhancing activity (*31, 32*).

More recently, the three-dimensional structure of each domain of MK was clarified by NMR spectroscopy (*33*). In the C-terminally located domain (C-domain), there are two clusters of basic amino acids, which can potentially serve as heparin-binding sites (*33*). NMR spectroscopy has revealed that these clustered amino acids indeed interact with a heparin oligosaccharide (*33*). *In vitro* mutagenesis of some of the basic amino acids in the clusters lowered the heparin-binding activity of the mutants, this being further evidence that these amino acids constitute heparin-binding sites (*34*). Furthermore, these mutants showed reduced neurite-promoting activity (*34*). In the experiments reported here, we systematically analyzed the roles of individual heparin binding sites in neurite promotion and in enhancement of PA activity.

MATERIALS AND METHODS

Construction of Transfer Vectors Carrying Various MK Mutants—Site-directed mutagenesis was performed by PCR with wild-type mouse MK cDNA as a template, following the method described by Ito *et al.* (*35*). Briefly, the first PCR was performed using two primer pairs, as follows: primer I (sense), containing a vector sequence in the 5′ region, and primer IV (antisense), containing a cDNA sequence including a site-specific mutation, and primer III (sense), which corresponds to the hinge region between the multiple cloning sites of the vector and the 5′ end of the cDNA, and contains a mutation in a cloning site (in the present case, *Bam*HI), and primer II (antisense) containing the 3′ sequence of a cDNA and an additional *Xba*I site. The PCR conditions were 94°C, 2 min; 2 cycles of 94°C, 1 min, 42°C, 2 min, and 72°C, 2 min; and 30 cycles of 94°C, 1 min, 60°C, 1.5 min, and 72°C, 2 min. The PCR products with both primer pairs were mixed to generate a heterodimer, which was further used as a template for the second PCR using primers I and II. The products were then cut with *Bam*HI/*Xba*I to obtain site-directed mutagenized cDNA fragment, followed by insertion into pBluescript KSII$^+$ (Stratagene, CA) cut with *Bam*HI/*Xba*I. The sequences were confirmed by the dideoxy termination method using an automated fluorescence DNA sequencer (model 373A, Applied Biosystems, CA). Each mutagenized MK cDNA was excised as a *Bam*HI-*Xba*I fragment, and ligated into the *Bam*HI/*Xba*I-cleaved transfer vector, pVL1393 (Pharmingen, CA).

Primer I (sense): 5′AGCTCGGAATTAACCCTCACTAAAG3′; Primer II (antisense): 5′GCTCTAGACCGTTCAGGCTCCAGGCGAGG3′; Primer III (sense): 5′GGCGATCCTATAAATATGCAGCACCGAGGCTTCT3′; Primer IV (antisense): for MK(K76R), 5′CCCTTGGCGGGCTCTGGTGCCAGT3′; for MK(K76Q), 5′CTTGGCGGGCCTGGGTGCCAGTGCTC3′; for MK(K99R), 5′GGAGGTGCAGGGCCTAGTCACGCG3′; for MK(K99Q), 5′AGGTGCAGGGCTGAGTCACGCGGATG3′; for MK(KK83,84QQ), 5′GTACCGCGCCTGCTGCAGGTCCCTTG3′; and for MK(R78KK83,84QQQ), the same primer as used for MK(KK83,84QQ), but the template was MK(R78Q), which was previously constructed (*34*).

DNA Transfection and Selection of Mutant Virus Clones—For the generation of a recombinant virus, *Spodoptera frugiperda* Sf-21 cells were cotransfected with 0.1 μg of a BaculoGold DNA (Pharmingen, CA) and 1 μg of transfer vector containing mutagenized MK cDNA by means of the lipofectin method (Life Technologies, MD). Conditioned medium containing recombinant baculovirus was harvested after 5-day incubation at 27°C. In order to isolate positive clones, Sf-21 cells seeded at 2×10^4/well in a 96-well plate were infected with the conditioned medium, which was serially diluted 10^2 to 10^5 times, and incubated for 5 days. Each lot of conditioned medium was collected and kept at 4°C until the next infection. Cells were lysed in 2 M NaOH to isolate viral DNA. The lysates were blotted onto a nitrocellulose membrane, and then hybridized with a ^{32}P-labeled mouse MK cDNA probe. Then, individual clones were selected and subjected to the next amplification.

Expression and Purification of Mutant MKs—For the production of mutant MK proteins, *Trichoplusia ni* High Five (Tn 5) cells (Invitrogen, CA) infected with a recombinant virus were incubated for 72 h at 27°C in Ex-cell 400 serum-free medium (JRH Bioscience, KS). The culture medium was collected and centrifuged at 7,000 rpm for 10 min, followed by ultracentrifugation at 35,000 rpm ($100,000\times g$) for 30 min to remove virus particles. The supernatant (500 ml) thus obtained was directly applied to a HiTrap heparin column (column size, 1 ml; Pharmacia Biotech, Uppsala), pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.8, containing 0.2 M NaCl. After washing the column with the same buffer, proteins were eluted with a linear NaCl gradient (0.2 to 2 M NaCl in 80 ml of 50 mM sodium phosphate buffer, pH 6.8). All column operations were performed using an FPLC system (Pharmacia Biotech, Uppsala) at the flow rate of 0.5 ml/min at 4°C. The protein eluted in the peak fractions was pooled,

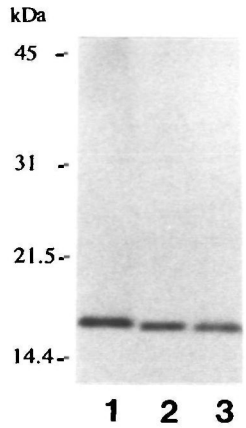

Fig. 2. **Purity of mutant MKs.** Mutant MKs (about 100 ng) purified by heparin-agarose affinity chromatography were analyzed on 15% running gels, followed by silver staining. Lane 1, wild-type MK; lane 2, MK(KK83,84QQ); lane 3, MK(R78KK83,84QQQ). The positions to which standard substances migrated are shown, with their weights in kDa: 45, ovalbumin; 31, carbonic anhydrase; 21.5, soybean trypsin inhibitor; and 14.4, lysozyme. MK is known to migrate slower than expected from its actual molecular weight, due to high its basic charge content.

concentrated with Centricon-3 (Amicon), and stored at −80°C. The yields of MK(KK83,84QQ) and MK(R78KK-83,84QQQ) were 1.4 and 1.6 mg/liter, respectively. They gave single bands upon SDS-PAGE (Fig. 2).

For simultaneous analysis of secreted MK and intracellular MK, infected Tn 5 cells (2.5×10^6) were lysed in 0.5 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 2 mM pepstatin A, 2 mM leupeptin, and 1% Triton X-100, and then centrifuged at $19,000\times g$ for 40 min. The medium after culturing of infected cells was centrifuged as mentioned above. To the supernatant heparin-Sepharose CL-6B (Pharmacia) was added to 10% (v/v), and then the mixture was rotated overnight at 4°C. After centrifugation, the resin was washed successively with 1.0 ml of 50 mM sodium phosphate buffer, pH 6.8, containing 0.2 M and 0.4 M NaCl. Then, strongly binding proteins were eluted with the buffer containing 1 M NaCl.

Other MK Mutants and Derivatives—Mouse MK devoid of the N-terminal cluster of basic amino acids (amino acid numbers 1-7) (N-del) and that devoid of the C-terminal cluster of basic amino acids (amino acid numbers 106-118) with the addition of SLID (C-mut) were produced in L cells and purified as described previously (*31*).

The human MK N-terminal half (N-half; amino acid numbers 1-59), C-terminal half (C-half; amino acid numbers 60-121), C-terminal tail (C-tail; amino acid numbers 109-121), and C-half without the C-tail (C-domain; amino acid numbers 62-104) were chemically synthesized (*36*), and used as in the previous studies (*31, 32*).

Limited proteolysis using α-chymotrypsin was also performed to generate the mouse MK C-half and N-half through cleavage at F^{55} (*37*). The digestion was performed as described previously (*37*) in a reaction mixture comprising 50 μg/ml of MK and 0.125 μg/ml of α-chymotrypsin at 37°C for 1 h.

Assaying of Biological Activities of MK—The assay for neurite outgrowth on MK tracks was performed as described previously (*38*). Briefly, various purified mutant MKs were coated on a 24-well culture plate (Falcon 3057; Becton Dickinson, NJ) at a concentration of 10 μg/ml. A grid pattern of MK on the plastic-culture plate was formed by irradiation with UV light at 315 nm for 30 min (*39*). Single cells were isolated from 17-day embryonic cerebral cortex from a SD rat as described previously (*38*). Cells were then seeded at a density of 1×10^6 cells per well, followed by culturing for 20 h at 37°C under an atmosphere of 5% CO_2 and 95% air. Neurite outgrowth on dishes uniformly coated with MK at 6 μg/ml was monitored as described previously (*40*). The number of neurite-extending cells was determined by taking 5 pictures of randomly selected fields (307×470 μm) and counting the single neurons with neurites. The average number of neurite-extending neurons is shown.

PA-enhancing activity was determined by measuring the enzymatic activity after culturing bovine aortic endothelial cells with MK derivatives for 16 h as described previously (*10*).

Analytical Procedures—SDS-polyacrylamide gel electrophoresis (13.5% gels) was carried out by the method of Laemmli (*41*). The gels were then stained with silver reagent, or subjected to Western blotting (*42*) using a specific anti-MK antiserum as described previously (*40*). The protein concentration was measured by means of the micro BCA assay (Pierce, IL) using bovine serum albumin (BSA) as a standard.

RESULTS

Some Conserved Basic Amino Acids Are Important in the Secretion of MK—There are 25 basic amino acids conserved in MK of four species (man, mouse, chicken, and *Xenopus*); among them 17 are also conserved in PTN (*cf.* Fig. 1). We have already prepared MK devoid of the N-terminal tail (N-del) or the C-terminal tail with some amino acids in the tail (C-mut), and a mutant in which R^{78} was changed to Q[MK(R78Q)] (*cf.* Fig. 1), and histidine-tagged MK in which K^{83} and K^{84} were changed to Q-[His-MK(KK83,84QQ)].

We next tried to prepare MK mutants in which K^{76} or K^{99} was changed to Q[MK(K76Q) or MK(K99Q)]. However, such mutants were not detected in the culture medium (Fig. 3, A and B, lanes 7 and 9). When the cells were dissolved in a detergent and then analyzed by Western blotting, we detected a doublet of 20.2 and 19 kDa bands reacting with the anti-MK antibody. Of these bands, the 20.2 kDa one appears to correspond to MK with a signal sequence (*5*). Thus, we conclude that K^{76} and K^{99} are required for the secretion of MK, mostly in the step of transport into the lumen of the endoplasmic reticulum. Previously, we mentioned that MK(K76Q) and MK(K99Q) were also not detected in the cells (*34*), but this was due to the small amounts of cells used for the analysis. We also produced a mutant in which K^{76} was converted to R^{76}. This mutated MK was secreted after 3 days (Fig. 3B, lane 5), but not after 2 days (Fig. 3A, lane 5) when normal MK was secreted by the insect cells. Thus, the basicity of K is important for secretion, but still K is better than R of this position.

Heparin-Binding Activity of an MK Mutant in Which K^{83}, K^{84}, and R^{78} Are Changed to Q—We previously prepared MK(R78Q) and His-MK(KK83,84QQ), and re-

ported that these mutants had reduced heparin-binding activity, as shown by lowered NaCl concentration required for elution from a heparin agarose column (*34*).

In the present study, we prepared a triple mutant [MK(R78KK83,84QQQ)] in which R^{78}, K^{83}, and K^{84} were all changed to Q. The mutant was eluted from the heparin column with 0.78 M NaCl (Fig. 4), this value being lower than those for MK(KK83,84QQ) (Fig. 4) and MK(R78Q). In the previous experiment, the value obtained for MK-(R78Q) was 0.80 M (*34*), but on use of the column directly after elution of the triple mutant, the value was 0.85 M. This difference is due to the use of different lots of heparin-agarose.

The fact that the triple mutant retained considerable heparin-binding activity can be interpreted in two different ways. Firstly, even after mutation of R^{78} in Cluster I, and K^{83} and K^{84} in Cluster II, other basic amino acids in Cluster I and II contributed to the residual heparin binding activity. Alternatively, our previous conclusion that the major heparin-binding sites are in the C-domain is not correct, and other major heparin-binding sites remains in the triple mutant. To examine the second possibility, we cleaved MK into the N- and C-halves by digestion with α-chymotrypsin (*37*).

The C-half derived from wild-type MK of mouse was eluted from the heparin-agarose column with 0.8 M NaCl, while the N-half was eluted with 0.42 M NaCl (Fig. 5A). This result is consistent with the data on human MK reported previously (*31*). The C-half derived from the triple mutant was eluted with 0.62 M NaCl (Fig. 5B).

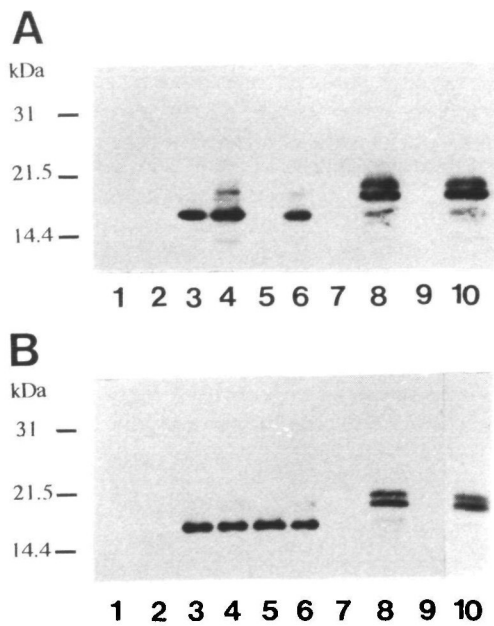

Fig. 3. **Analysis of secretion of MK and its mutants by Tn 5 cells.** Heparin-binding proteins in the culture medium (lanes 1, 3, 5, 7, and 9) or within the cells (lanes 2, 4, 6, 8, and 10) were separated by 15% SDS-PAGE and stained with an anti-MK antibody. A, 48 h culture after virus infection; B, 72 h culture after virus infection. Lanes 1 and 2, control (without virus); lanes 3 and 4, wild-type MK; lanes 5 and 6, MK(K76R); lanes 7 and 8, MK(K76Q); lanes 9 and 10, MK(K99Q). The positions of standard markers are shown with their molecular weights (kDa).

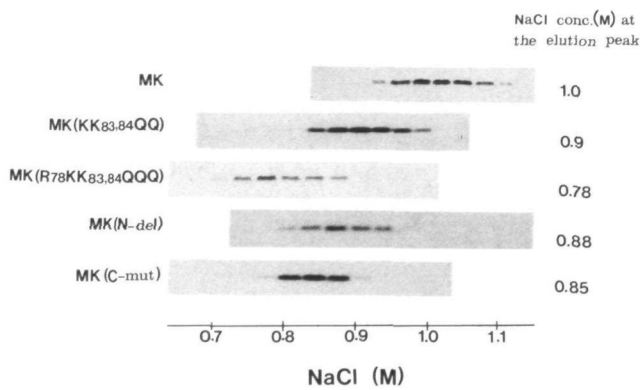

Fig. 4. **Altered heparin-binding activities of mutant MKs.** Heparin-agarose affinity chromatography was performed as described under "MATERIALS AND METHODS," and fractions, 1.5 ml each, were collected. The elution positions of wild-type and mutant MKs were determined by SDS-PAGE on 15% gels, followed by silver staining. The NaCl concentrations at which the peaks of MK and its mutants were eluted are indicated on the right of the figure.

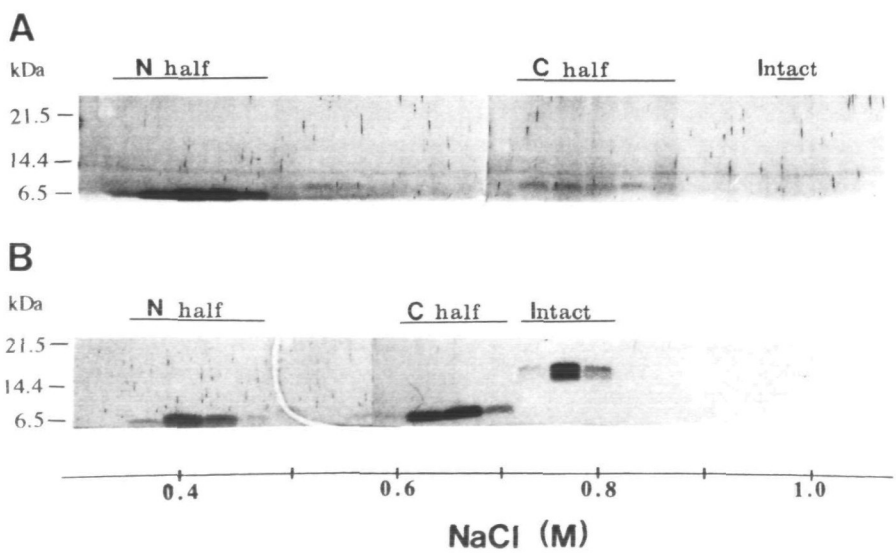

Fig. 5. **Heparin-binding activity of MK half molecules generated by α-chymotrypsin digestion.** Wild type MK and MK-(R78KK83,84QQQ) (50 μg) were digested with α-chymotrypsin as described under "MATERIALS AND METHODS," and then applied to a heparin-agarose column (1 ml, HiTrap™ Heparin), which was eluted with a linear NaCl gradient (0.2 M to 1.5 M NaCl in 45 ml of 50 mM sodium phosphate buffer, pH 6.8). Each fraction was subjected to 15% SDS-PAGE, followed by silver staining. A, wild-type MK. Since the C-half was easily degraded on proteolysis, the elution position of the C-half as well as that of the intact one were confirmed by chromatography of chemically synthesized human C-half and the undigested MK. B, MK(R78KK83,84QQQ).

Thus, the major heparin-binding activity was due to the C-half even in the mutant. Although there are clusters of basic amino acids in both the N-tail and C-tail, complete removal of a tail resulted in only a modest reduction in the heparin-binding activity (*31*). The previous experiment involved step-wise elution, and we confirmed the conclusion by gradient elution (Fig. 4). C-mut, which had lost the whole C-tail, was eluted from the column with a NaCl concentration of 0.15 M lower than for the intact MK. As mentioned above, the C-tail remaining in the triple mutant cannot be responsible entirely for the remaining heparin-binding activity of the C-half. Thus, we confirmed that the C-domain carries the major heparin-binding sites, and the residual activity due to the C-domain contributes to the heparin-binding activity of the triple mutant.

Roles of the N-Tail and C-Tail in Neurite Promotion— We previously showed that MK(R78Q) and His MK(KK83, 84QQ) exhibit reduced neurite-promoting activity (*34*). We confirmed that MK(KK83,84QQ) also exhibits neurite-promoting activity (data not shown). Then, we examined the triple mutant, MK(R78KK83,84QQQ). The triple mutant exhibited reduced neurite-promoting activity (Fig. 6D), but the level of the activity was similar to that in the single mutant MK(R78Q) (Fig. 6C) (*34*) or MK(KK83, 84QQ). Interestingly, C-mut had lost the ability to support neurite promotion almost completely (Fig. 6E), and N-del also exhibited greatly reduced activity (Fig. 6F).

The result for N-del apparently contradicted our previous result, since the C-half retained neurite-promoting activity (*31*). We noted a difference in the methods used to evaluate neurite-promoting activity; in the above experiments MK was coated in a grid-like pattern, while in the previous experiment (*31*) it was uniformly coated on the dish. Thus, we evaluated neurite promoting activity of the C- and N-halves by means of the present method, namely,

TABLE I. **Neurite-promoting activity of mutant MKs.**

MK mutant	Neurite-promoting activity (relative number of neurite-promoting cells[a])
Wild-type	100
C-half	76.6
N-half	24.0
C-domain	40.6
C-mut	12.4
N-del	11.2
R78Q	26.0
KK83,84QQ	42.0
R78KK83,84QQQ	32.0

[a]The numbers of neurite-promoting cells in an area of 307×470 μm on dishes coated with wild-type MK, 193, being taken as 100.

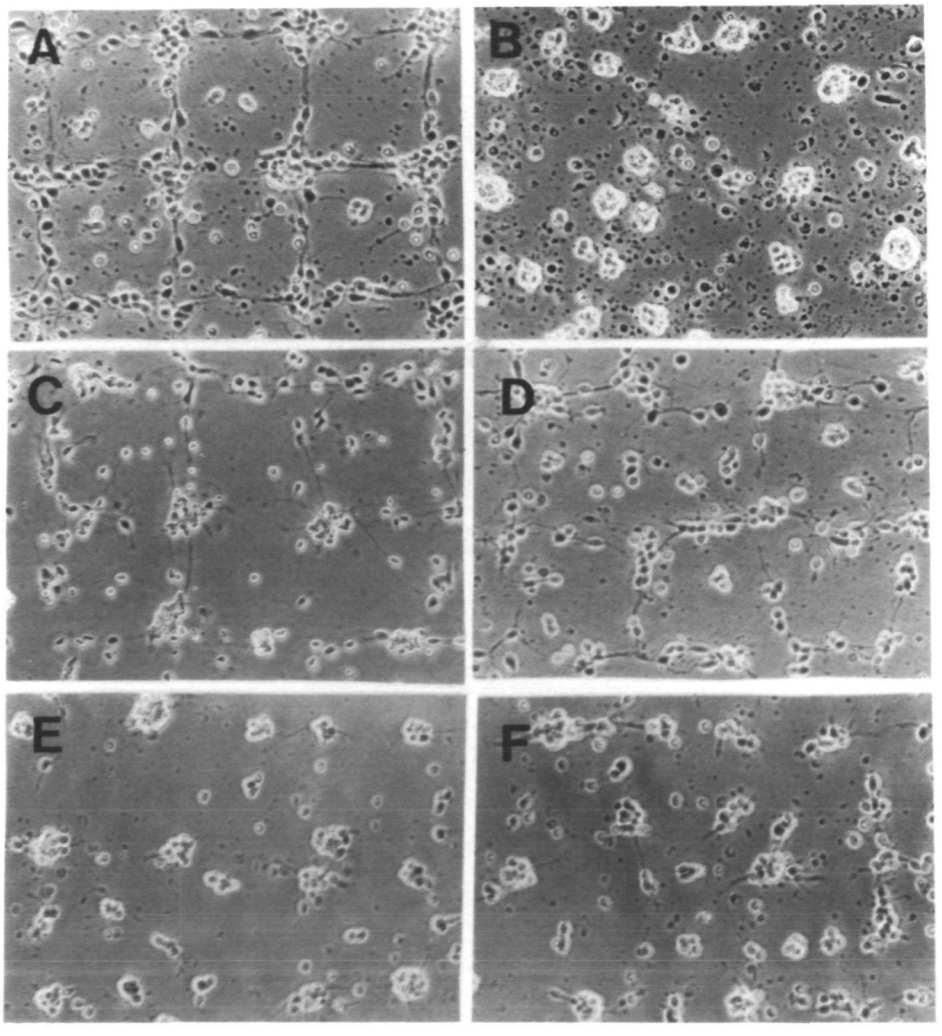

Fig. 6. **Neurite-promotion along the path of MK or one of its mutants.** Embryonic rat brain cells were cultured on a grid pattern of MK or a mutant for 20 h as described under "MATERIALS AND METHODS." The pitch of the grids was 125 μm. A, wild-type MK; B, no coating; C, MK-(R78Q); D, MK(R78KK83,84QQQ); E, C-mut; F, N-del.

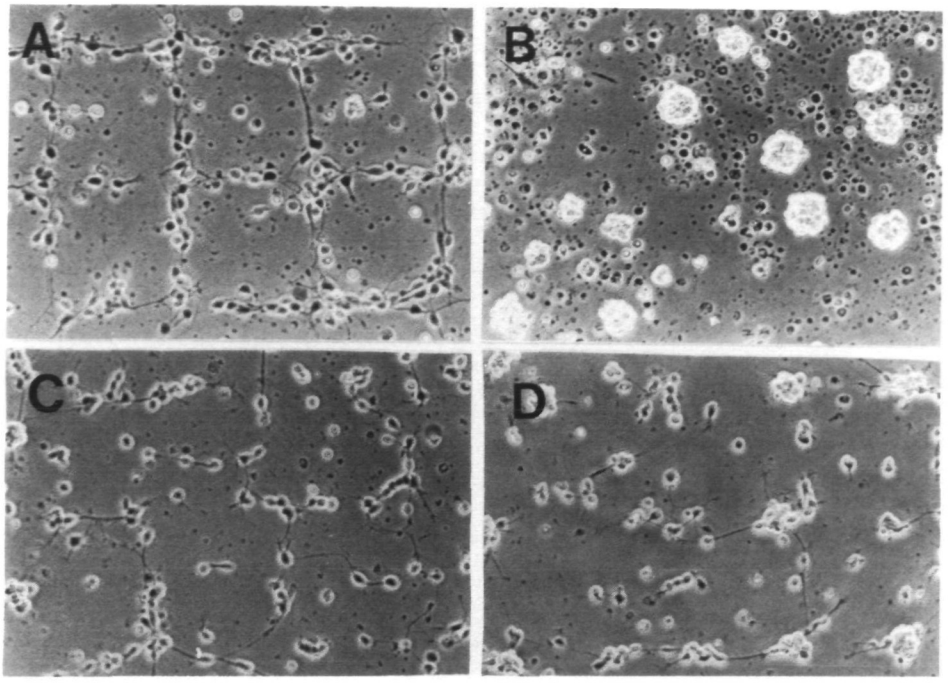

Fig. 7. **Neurite-promotion along the path of MK or its half molecule.** The assay was performed as described in Fig. 6. The pitch of the grids was 125 μm. A, intact MK; B, no coating; C, C-half; D, N-half.

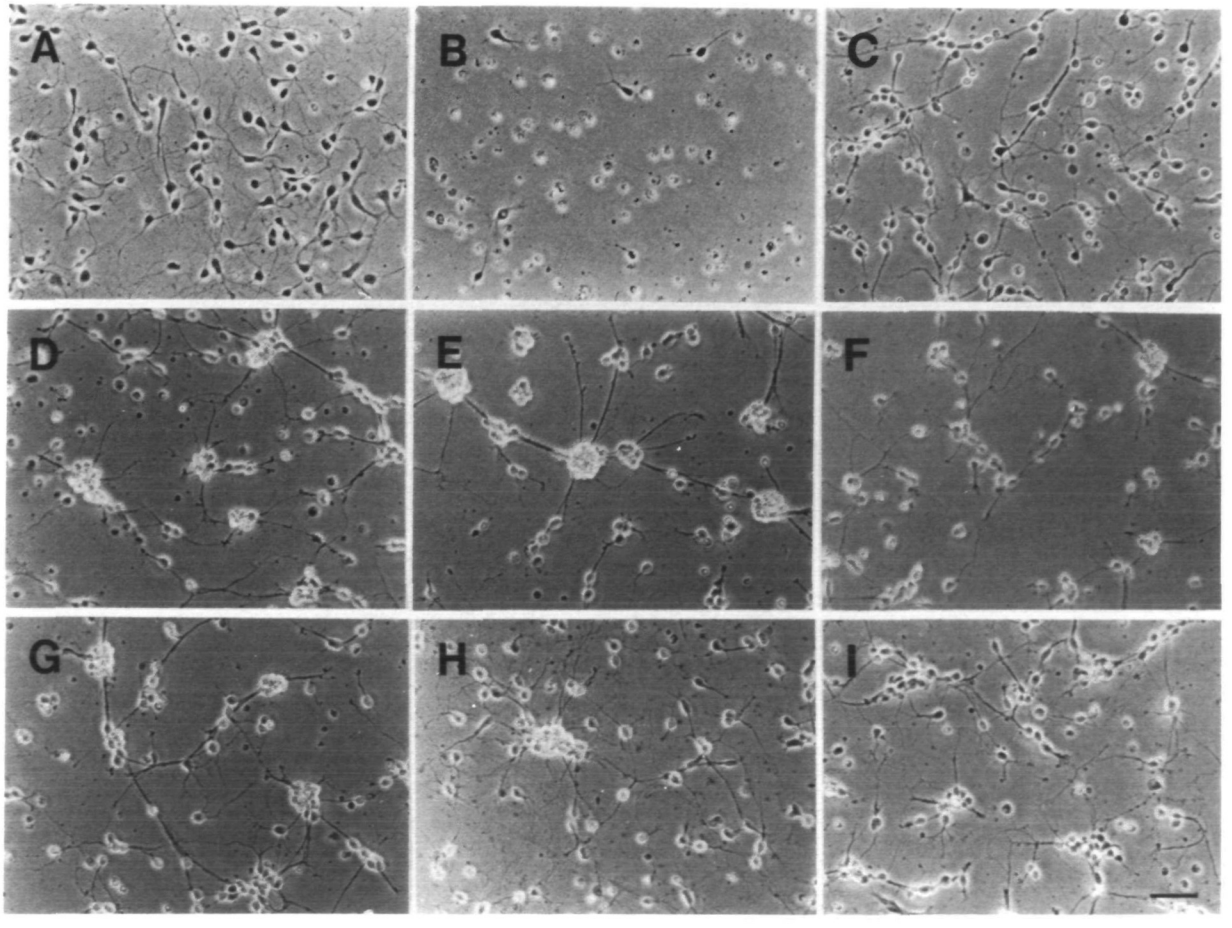

Fig. 8. **Neurite-promotion on dishes uniformly coated with MK and its derivatives.** Embryonic rat brain cells were cultured on dishes coated with MK or one of its derivatives for 48 h. A, MK; B, poly-D-lysine; C, C-half; D, N-half; E, C-mut; F, N-del; G, MK-(R78Q); H, MK(R78KK83,84QQQ); I, C-domain. The assay was performed in duplicate, and 5 different fields were scanned for each dish to confirm the reproducibility of the results. Bar, 50 μm.

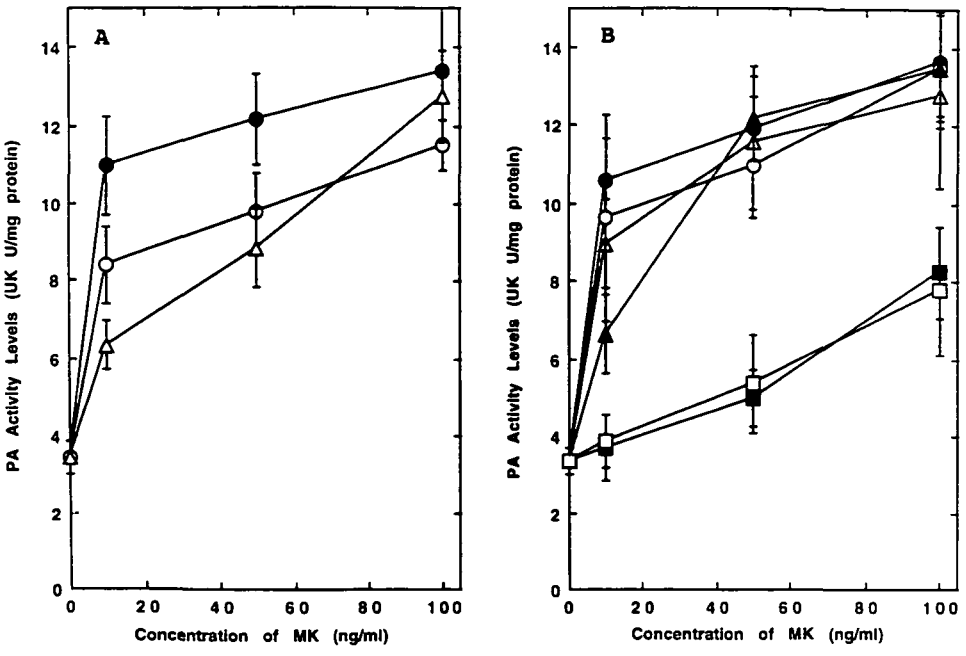

Fig. 9. **PA-enhancing activities of various mutant MKs.** Confluent cultures of bovine aortic endothelial cells were incubated for 16 h with increasing concentrations of MK or one of its mutants in serum-free α-minimal essential medium containing 0.1% BSA. Cell lysates were prepared, and the PA activity level of each cell lysate was determined using the chromogenic substrate, S-2403, and expressed as urokinase (UK) units/mg of protein in the sample. A, deletion mutants. ●, wild-type MK; ○, N-del; △, C-mut. B, point mutated mutants. ●, wild-type MK; △, MK(R78Q); ○, MK-(K83Q); ▲, MK(K84Q); ■, MK-(KK83,84QQ); □, MK(R78KK83, 84QQQ).

TABLE II. **Schematic comparison of neurite-promoting activity and PA-enhancing activity of MK mutants.**

Mutant	Neurite-promoting activity	PA-enhancing activity
C-half	↘	↓
N-half	↓↓	↓↓↓
C-domain	↓	↓↓
C-mut	↓↓↓	↓↓
N-del	↓↓↓	↘↘
R78Q	↓↓	↘
KK83, 84QQ	↓	↓↓↓

Neurite-promoting activity was taken from Table I, and PA-enhancing activity at 10 ng/ml or a corresponding molar concentration was taken from Fig. 9 or Ref. *32*. As compared to wild-type MK, ↘, more than 75%; ↘↘, 75-60%; ↓, 60-40%; ↓↓, 40-15%; ↓↓↓, less than 15%. ⌒ on the left shows the N-half; ⌒ on the right shows the C-half.

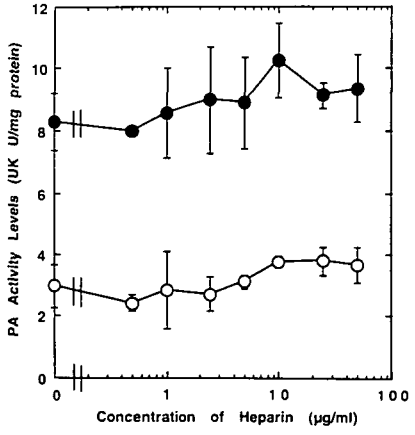

Fig. 10. **Effect of heparin on MK-induced enhancement of cellular PA activity.** Confluent cultures of bovine aortic endothelial cells were incubated for 16 h with the designated concentrations of heparin in the presence (●) and absence (○) of 10 ng/ml recombinant murine MK as before. Cell lysates were prepared and the PA activity level of each cell lysate was determined.

by forming a grid-like pattern of the test molecule. Interestingly, both the C-half (Fig. 7C) and the N-half (Fig. 7D) exhibited reduced the neurite-promoting activity.

We also performed the "uniformly coating assay" used previously. In this assay, we determined the number of neurons which extended neurites (Table I). The C-half of MK showed sufficient neurite-promoting activity (Fig. 8C), while the N-half showed much less activity (Fig. 8D), confirming the previous data (Table I). Mutant MKs, MK(R78Q) (Fig. 8G) and MK(R78KK83,84QQQ) (Fig. 8H), exhibited reduced neurite-promoting activity: a clump of neurons was observed in both cases (Table I). Surprisingly, not only C-mut (Fig. 8E) but also N-del (Fig. 8F) showed disturbed neurite-promoting activity (Table I). The N-terminal half should not be required for neurite-promoting activity in this assay. Probably, the N-domain of the N-del mutant might not be in the proper steric position relative to the C-half, resulting in interference with the action of the C-half as to neurite-promoting activity.

Similar inference is possible for the role of the C-tail. To examine this possibility, we examined whether or not the C-domain (human MK 62-104), which lacks the C-tail, exhibits neurite-promoting activity when it is coated uniformly on a dish. We found that it had considerable activity, although it was less active than the C-half (Fig. 8I and Table I). Furthermore, the C-tail, at the concentration of 20-250 μg/ml, did not inhibit neurite-promotion on a MK-coated dish or on a grid of MK (data not shown). Thus, the effect of C-tail deletion might be principally an indirect one.

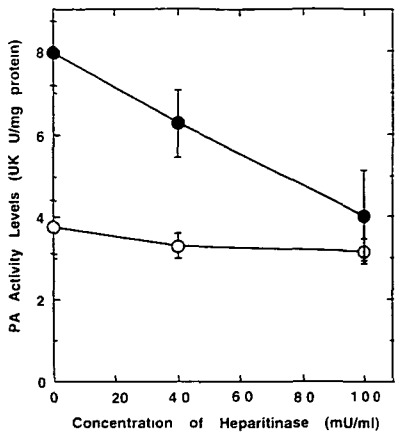

Fig. 11. **Attenuation of PA-enhancing activity of MK on digestion of endothelial cells with heparitinase.** Confluent cultures of bovine aortic endothelial cells were incubated for 3 h with the indicated concentrations of *Flavobasterium heparinum* heparitinase (Seikagaku Kogyo) in serum-free medium containing 0.1% bovine serum albumin. Thereafter, to half of the dishes recombinant murine MK (final, 10 ng/ml) was directly added, and then the cultures were further incubated for 16 h with (●) or without (○) MK. Cell lysates were prepared and the PA activity level of each cell lysate was determined.

Roles of Heparin-Binding Sites in PA-Enhancing Activity in Bovine Aortic Endothelial Cells—MK enhances the PA activity of aortic endothelial cells, leading to enhanced fibrinolytic activity of these cells (*10*). This effect is caused by transcriptional activation of the PA gene and suppression of transcription of the PA inhibitor gene (*10*). We have already reported that the C-half of MK, but not its N-half has PA-enhancing activity (*32*). C-mut, in which the C-tail has been removed, showed reduced activity [*32* and Fig. 9A (△)]. The C-domain showed activity similar to the C-half (*32*). In this study, we observed that N-del had relatively reduced activity [Fig. 9A (○)]. Thus, the removal of the tails reduced both the neurite-promoting activity on uniformly coated dishes and PA-enhancing activity, although the effect was stronger on the neurite-promoting activity (Table II).

We further examined MK mutants, and found that MK(R78Q) and MK(K83Q) showed almost unchanged activity, while MK(KK83,84QQ) and MK(R78KK83, 84QQQ) exhibited drastic decreases in the PA-enhancing activity (Fig. 9B). Thus, mutation of heparin-binding sites had different effects on neurite-promoting activity and PA-enhancing activity (Table II).

Consistent with the different effects of the mutation, heparin did not inhibit the PA-enhancing activity (Fig. 10), while the neurite-promoting activity was abolished by heparin (*38*). The PA-enhancing activity of MK still requires interaction with heparin-like saccharides on the cell surface, since heparitinase treatment of bovine aortic endothelial cells decreased the response to MK (Fig. 11).

DISCUSSION

MK can be regarded to be composed of 4 segments, *i.e.* N-tail, N-domain, C-domain, and C-tail (Fig. 1). Each of the two domains is largely composed of 3 β-sheets tightly held through 2 or 3 disulfide bridges, while both tails do not form stable steric structures (*33*). Of the 4 segments, the C-domain is the most interesting one from the functional viewpoint, since it has conformation-dependent heparin-binding sites (*31*), and retains PA-enhancing activity (*32*). The neurite-promoting activity, determined by coating a culture dish with MK derivatives, has been shown to be in the C-half (C-domain plus C-tail) (*31*), and the present results revealed the principal role of the C-domain also in this activity.

The role of the N-half (N-domain plus N-tail) remained rather unclear, although many amino acids in the N-half are conserved in MK of different species, and shared by MK and PTN. Chymotrypsin digestion indicated that the N-half assists the stability of the C-half (*37*). Although intact MK is required for the promotion of neuronal survival for a long period (*31*), this effect can be attributed to the increased stability of the active C-half in the presence of the N-half. In the present investigation, we observed that the presence of the N-half is required for the neurite-promoting activity along the path of MK, as determined with the "grid assay." One possibility is that the grid assay is more sensitive and clearly detects the decreased neurite-promoting activity of MK devoid of the N-half. The other and more likely possibility is that in the grid assay the local density of neuronal cells is high as compared to in the conventional coating assay, leading to unstability of the C-half through the attack by cellular proteases.

Mutational studies and NMR spectroscopy revealed four heparin-binding sites in the MK molecule. In the C-domain, there are two heparin-binding sites, Cluster I, which is composed of $K^{76}R^{78}$ and K^{99} (numbering of mouse MK), and Cluster II, which is composed of $K^{83}K^{84}R^{86}$ (numbering of mouse MK) (*33*) (Fig. 1). The other two heparin-binding sites are in the N-tail and C-tail respectively. As reported previously, the main heparin-binding site is in the C-domain (*31*). There are no apparent heparin binding sites in the N-domain (*33*).

The present and previous investigations revealed the roles of each heparin binding site in neurite outgrowth and enhancement of PA activity. Removal of the N-tail or C-tail severely reduced the neurite-promoting activity and moderately reduced the PA-enhancing activity (Table II). However, the fact that a chemically synthesized C-domain retained significant PA-enhancement activity (*32*) and neurite-promotion activity (this study) indicates the secondary roles of the tails; these tails appear to facilitate the steric arrangement of the N-domain and C-domain so that the function of the C-domain is not disturbed. Probably, the C-domain and N-domain interact with each other in the absence of the C-tail or N-tail. Generally speaking, the roles of individual segments of MK (N-tail, N-domain, C-domain, and C-tail) may be similar in neurite-promoting activity and PA-enhancing activity.

Mutations in heparin-binding sites in the C-domain had different effects on the two activities. R^{78} (in Cluster I) and $K^{83}K^{84}$ (in Cluster II) were both involved in neurite-promotion. However, R^{78} was not significantly involved in PA-enhancing activity, while K^{83} and K^{84} were essential for the activity (Table II). The different effects of heparin on neurite-promotion and enhancement of PA-enhancing activity suggest a difference in the involvement of heparin-like saccharides in the MK action. In the enhancement of PA activity, heparin-like oligosaccharides may play secon-

dary roles, such as the promotion of dimerization, as in the case of fibroblast growth factors (*3, 43*), and external heparin can substitute for the endogeneous heparan sulfate but does not inhibit it. In the case of neurite-promotion, the interaction between MK and heparin-like oligosaccharides may be of prime importance, and exogeneously added heparin inhibits the activity. In both cases, interaction with heparin-like carbohydrates is important for the MK action, as shown by the effect of heparitinase digestion of the target cells (Ref. *38* and this study). Probably, the difference in the role of the interaction with heparin-like carbohydrates is reflected in the different effects of mutations of heparin-binding sites.

Finally, few studies have been performed on the structure-functional relationship of PTN. However, because of the overall structural homology of MK and PTN, we believe that most of the conclusions drawn from our research are also applicable to PTN.

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