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Limited Proteolysis by Chymotrypsin of Midkine and Inhibition by Heparin Binding

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When digested with a low concentration of chymotrypsin, midkine (MK) underwent limited proteolysis and produced two fragments with Mr of 11,000 and 6,000 Da. The cleavage site was identified as on the carboxyl side of Phe55. This limited proteolysis was specifically inhibited by heparin, but not by other glycosaminoglycans. Using various heparin-derived oligosaccharides with different chain lengths or chemically desulfated heparin derivatives, it was shown that a minimum of 6 monosaccharide units was necessary for the inhibition, and that sulfonyl groups of the heparin disaccharide unit were required for inhibition. The present study showed that MK consists of two domains, N- and C-domains, and that Phe55 localized to the hinge region is exposed on the surface of the molecule. It was also suggested that the N-domain may function as a stabilizing domain against proteolytic degradation of the C-domain in the intact molecule.

Midkine (MK) is a heparin-binding growth/differentiation factor with Mr of 13,000 Da, which is rich in basic amino acids and Cys residues, and constitutes a new family of heparin-binding growth/differentiation factors named the MK family (1-4). MK has pleiotropic effects such as promotion of neurite outgrowth and survival of spinal cord, dorsal root ganglion and mesencephalic neurons (5-8). It also has nerve cell adhesion and guidance activity for neurite outgrowth (9,10), and activates plasminogen activator in bovine aortic endothelial cells (11). Amino acid sequence analysis and localization of disulfide linkage revealed that all ten Cys residues are cross-linked, and that three and two disulfide linkages are localized in the N- and C-terminal halves of the molecule, respectively (12). This finding suggested that MK consists of two domains of almost the same size. To confirm this possibility, we digested intact MK with various proteolytic enzymes. Of the enzymes examined, chymotrypsin was found to cleave the molecule approximately at the center of the whole sequence, leading to the generation of two fragments which was corresponding to N- and C-terminal halves of the molecule. Furthermore, we found that this limited proteolysis was specifically inhibited by binding with heparin.

MATERIALS AND METHODS

Materials. Recombinant mouse MK was produced in the baculovirus expression system, and was purified to homogeneity on a heparin column (Hi-Trap heparin, Pharmacia Biotech, Uppsala) as described previously (9, 10).

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Abbreviations used: MK, midkine; 2-O-DS-, 2-O-desulfated; 6-O-DS-, 6-O-desulfated; NDSNAc-, N-desulfated and N-acetylated; CDSNS-, completely desulfated and N-sulfated; CDSNAc-, completely desulfated and N-acetylated; PVDF, polyvinylidene difluoride.

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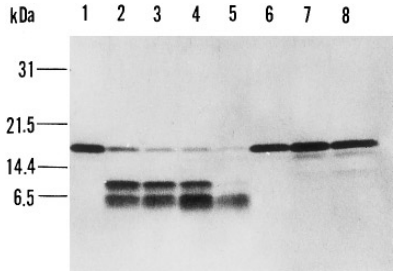

FIG. 1. Limited proteolysis by chymotrypsin, and the effects of heparin. MK (60 μg/ml) was digested with chymotrypsin for 1 h at 37°C in 50 mM Tris-HCl, pH 8.0. Digestion was carried out at different enzyme/substrate (w/w) ratios. Lane 1, intact MK; lane 2, 1/400; lanes 3 and 6, 1/150; lanes 4 and 7, 1/50; lanes 5 and 8, 1/20. In lanes 6-8, heparin (600 μg/ml) was added to the reaction mixture. The products were analyzed by SDS-PAGE (12.5% gel), and visualized by silver staining.

Chymotrypsin was obtained from P-L Biochemical Inc. (Milwaukee). Heparin purified from porcine intestine was obtained from Wako Chemicals (Tokyo). Various glycosaminoglycans including heparan sulfate from bovine kidney, chondroitin sulfate A from whale cartilage, chondroitin sulfate C from shark cartilage, dermatan sulfate from porcine skin, keratan polysulfate from shark cartilage and hyaluronic acid from porcine skin were purchased from Seikagaku Corporation (Tokyo). Chemically 2-O-desulfated (2-O-DS-) and 6-O-desulfated (6-O-DS-) heparin derivatives were prepared as described previously (10). The removal of sulfate groups from a specific site was confirmed by disaccharide composition analysis as described (13). The 2-O- and 6-O- desulfations were 76.7% and 88.9% effective, respectively, without any detectable depolymerization or other desulfations (data not shown). N-desulfated and N-acetylated (NDSNAc-), completely desulfated and N-sulfated (CDSNS-), and completely desulfated and N-acetylated (CDSNAc-) heparin derivatives were obtained from Seikagaku Corporation (Tokyo). Heparin-derived oligosaccharides with different chain lengths were prepared by digestion of heparin (LAOB, Brazil) with *Flavobacterium heparinum* heparinase (Seikagaku Corporation, Tokyo) as described previously (10). The molecular weight of size-fractionated heparin was estimated by gel-permeation HPLC connected to a combined column of TSK gel 4000, 3000 and 2500 PWXL (TOSO Co. Tokyo), using heparin standards as described previously (14, 15).

Limited proteolysis of MK by chymotrypsin. Chymotryptic digestion was carried out at 37°C for 1 h in a reaction mixture containing 60 μg/ml of MK, 0.15-3.0 μg/ml of chymotrypsin (enzyme/substrate ratio of 1/400 to 1/20, w/w), and 50 mM Tris-HCl, pH 8.0. Chymotryptic digestion was terminated by addition of an equal volume of sample buffer consisting of 0.25 M Tris-HCl, pH 6.8, 20% glycerol, 0.005% bromophenol blue, 4% SDS, and 10% 2-mercaptoethanol, and the product was analyzed by SDS-PAGE (16) followed by silver staining using a Sil-Best Stain kit (Nakalai Tesque, Kyoto). Chymotryptic digestion in the presence of heparin or heparin derivatives (60-600 μg/ml) was carried out under the same conditions as described above, in which 0.3 μg/ml of chymotrypsin was used. Chymotrypsin activity using *N*-benzoyl-*L*-tyrosine *p*-nitroanilide as a substrate was measured as described (17). Protein concentration was measured by means of the micro BCA assay (Pierce, IL) using BSA as a standard.

N-terminal amino acid sequence of chymotryptic peptides. Determination of N-terminal amino acid sequences of chymotryptic peptides was carried out according to the method of Ploug *et al.* (18). Briefly, after chymotryptic digestion, the reaction mixture was subjected to SDS-PAGE (12.5% gels) followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes. The membranes were stained with 0.1% coomassie brilliant blue R-250 in 50% methanol. After a brief wash with water, the membranes were destained with a solution of 40% methanol and 10% acetic acid. The regions of the membranes containing stained peptides were excised and directly applied to a gas-phase protein sequencer (Model 372A, Applied Biosystems, CA).

RESULTS AND DISCUSSION

Limited proteolysis by chymotrypsin. To investigate the possible domain structure of MK, purified MK was subjected to proteolytic digestion with various enzymes, and the products were analyzed by SDS-PAGE. As shown in Fig. 1 (lanes 2-4), when MK was treated with low concentrations of chymotrypsin, two bands with Mr of about 11,000 and 6,000 Da were detected. The sum of these values was in good agreement with the apparent Mr of the intact molecule on SDS-PAGE (17,000; lane 1) (9). These fragments were produced depending on

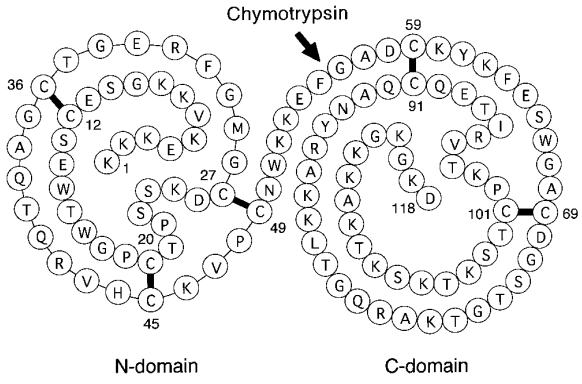

FIG. 2. Schematic representation of domain structure of MK molecule. MK is comprised of the N-domain (1-49), C-domain (59-118), and hinge region (50-58) connecting these two domains. Chymotrypsin cleaves specifically at Phe55 of the intact molecule.

the ratio of enzyme to MK. The two fragments were most effectively produced under the experimental conditions used at a chymotrypsin/MK ratio of 1/400 (w/w). However, when the concentration of the enzyme was increased, the upper band became relatively faint, and finally at a ratio of 1/20 (w/w), the upper band almost disappeared (lane 5). In contrast, the lower band remained undegraded even at relatively high enzyme concentrations, indicating that this fragment was resistant to chymotryptic digestion (lanes 2-5).

Identification of the cleavage site. To determine the chymotrypsin cleavage site, the chymotryptic peptides were separated by SDS-PAGE, electroblotted onto PVDF membranes, and then subjected to peptide sequencing after staining with coomassie brilliant blue R-250. The lower band with Mr of about 6,000 Da had the amino acid sequence NH_2-Lys-Lys-Lys-Glu-Lys-Val-Lys-, which was identical to the N-terminal sequence of intact molecule. On the other hand, the amino acid sequence of upper band was NH_2-Gly-Ala-Asp-X-Lys-Tyr-Lys-, where X denotes an unidentified residue. This sequence corresponded to the sequence from residue 56 to 62 of the original MK molecule. This indicates that chymotryptic cleavage occurred specifically at the carboxyl side of Phe55 (Fig. 2). This seems reasonable considering the substrate specificity of the enzyme; chymotrypsin primarily cleaves peptides at the carboxyl side of aromatic amino acid residues. From the amino acid sequence and the localization of disulfide linkages of MK, it was shown that three disulfide linkages are located from residue 1 to 49, and the remaining two from residue 59 to 118 (12). The clustering of these disulfide linkages suggested the presence of a domain structure in the MK molecule; N- and C-domains having almost the same size and connected by a hinge region from residue 50 to 58 (Fig. 2). This means that Phe55 is localized to the hinge region. The present results strongly suggest that MK consists of two domains, and these domains are proteolytically separated by chymotrypsin at the hinge region. The finding that Phe55 is susceptible to limited proteolysis shows that Phe55 is exposed to the outer surface, and is not folded on the inside of the molecule. As shown in Fig. 1, the upper band (C-domain) became highly unstable after cleavage at Phe 55 in the presence of high concentrations of chymotrypsin. This suggests that the N-domain functions as a stabilizing domain against proteolytic degradation of the C-domain in the intact molecule.

Effect of heparin on limited proteolysis. Since MK can bind strongly with heparin, we investigated the effect of heparin binding on limited proteolysis by chymotrypsin. As shown in Fig. 1 (lanes 6-8), the addition of heparin (60-600 μg/ml) completely inhibited proteolysis.

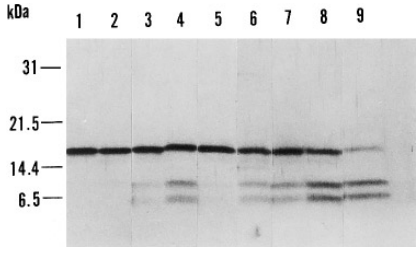

FIG. 3. Effects of heparin-derived oligosaccharides with different chain lengths on limited proteolysis by chymotrypsin. Limited proteolysis was carried out as described in FIG. 1 at the enzyme/substrate (w/w) ratio of 1/400 in the presence of oligosaccharides. Lanes 1 and 2, 12 monosaccharide units; lanes 3 and 4, 8 monosaccharide units; lanes 5 and 6, 6 monosaccharide units; lanes 7 and 8, 4 monosaccharide units. Lanes 1, 3, 5 and 7, oligosaccharide/MK (w/w) = 1; lanes 2, 4, 6 and 8, oligosaccharide/MK (w/w) = 1/10. Lane 9, no oligosaccharide.

The absence of inhibitory activity of heparin toward chymotrypsin was confirmed using *N*-benzoyl-*L*-tyrosine *p*-nitroanilide as a substrate (data not shown). Therefore, the observed inhibition by heparin was due to its binding to MK, and not due to interference with the enzyme activity. The effects of other glycosaminoglycans including heparan sulfate, chondroitin sulfate A and C, dermatan sulfate, keratan polysulfate and hyaluronic acid were also investigated. The limited proteolysis of MK was specifically inhibited by addition of heparin, but not by other glycosaminoglycans (data not shown), suggesting that the interaction is highly specific to heparin.

To investigate the minimum polysaccharide unit of heparin necessary for inhibition, the effects of heparin-derived oligosaccharides with different chain lengths were examined. As shown in Fig. 3, oligosaccharides longer than 12 monosaccharide units inhibited the limited proteolysis to almost the same extent as that observed with native heparin. Oligosaccharides with 8 or 6 monosaccharide units also inhibited proteolysis, but larger amounts of oligomers were required. On the other hand, oligosaccharides with 4 monosaccharide units had no effect on the proteolysis. These observations suggest that a minimum of 6 monosaccharide units is required for binding to MK, but the affinity is much less than that of 12 monosaccharide units. The inhibitory mechanism of heparin is not clear. However, it is likely that steric hindrance by heparin binding is predominant rather than a conformational change of MK, because no significant change was observed in intrinsic fluorescence intensity or fluorescence spectra of MK upon binding with heparin (data not shown).

The contribution of sulfate groups in the heparin molecule to the inhibitory effect was investigated using selectively desulfated heparins, namely 2-O-DS-, 6-O-DS- and NDSNAc-heparins. As shown in Table 1, each of these mono-desulfated derivatives exhibited inhibitory activity similar to that of native heparin. CDSNS-heparin showed markedly reduced activity, and complete desulfation and N-acetylation resulted in complete loss of the activity. These observations indicate that sulfate groups of the heparin disaccharide unit are significantly involved in the inhibitory activity. When we assayed interaction of heparin derivatives with MK by inhibition of MK-induced neurite outgrowth, the loss of any sulfate groups resulted in significant decrease of the activity (10). This difference suggests that in the latter case strong affinity to MK is required for the inhibition.

In previous studies, we demonstrated that a chemically synthesized C-terminal half polypeptide with reconstituted disulfide linkages exclusively exhibited heparin binding and neurite outgrowth activities (19), and activation of plasminogen activator (20), suggesting that the C-domain is a functionally important domain. However, the role of the N-domain has not yet

TABLE 1
Effects of Various Desulfated Heparins on Limited Proteolysis by Chymotrypsin

	Native or desulfated heparin/MK (w/w)	
Heparin derivatives	10	1
Native heparin	+	+
2-O-DS-heparin	+	+
6-O-DS-heparin	+	+
NDSNAc-heparin	+	+
CDSNS-heparin	+	−
CDSNAc-heparin	−	−

+, limited proteolysis was inhibited; −, limited proteolysis was not inhibited.

been defined. The present study suggested a possible role of the N-domain in protection of the molecule from proteolytic degradation. Since many proteases are present in the extracellular matrix, where MK is also secreted and may be stored, the stabilizing role of the N-domain will be important to keep the molecule intact. In accordance with this hypothesis, we previously observed in a baculovirus expression system that the C-domain was not successfully expressed in culture medium whereas the N-domain was expressed (unpublished data). This might be due to the extreme instability of the C-domain. The observation that heparin exhibited specific inhibitory activity against chymotryptic degradation is suggestive of the biological significance of heparin-like molecules on the cell surface or in the extracellular matrix for stabilization of the MK molecule. The limited proteolysis of purified MK by chymotrypsin may provide a useful alternative to prepare the N- and C-domains for further investigations.

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