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A functional role of the glycosylated N-terminal domain of chondromodulin-I

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Abstract Chondromodulin-I (ChM-I) is a 25-kDa glycoprotein that specifically localizes in the extracellular matrix of cartilage and negatively regulates angiogenesis. ChM-I comprises two domains: an N-terminal hydrophilic domain (Domain 1) containing an N-linked glycosylation site and a C-terminal hydrophobic domain (Domain 2) with all four disulfide bonds that are present in this protein. We generated a non-glycosylated recombinant human ChM-I (NG-hChM-I), and compared its bioactivity with that of the glycosylated form of human ChM-I (G-hChM-I) expressed in CHO cells *in vitro*. NG-hChM-I exhibited the growth factor/inhibitor activity in the cultures of chondrocytes and vascular endothelial cells, but required markedly higher doses. Although Domain 1 is predicted to be hydrophilic *per se* on the basis of its amino acid sequence, NG-hChM-I remains insoluble in aqueous solution as much as ΔN-hChM-I that lacks the N-terminal 37 amino acids containing an N-glycosylation site. CD (circular dichroism) measurements revealed that the content of α-helix was calculated to be 34% in G-hChM-I, whereas the content of the characteristic secondary structures in NG-hChM-I was distinctly lower than those in G-hChM-I. These results indicate that glycosylation in Domain 1 is critical for the structural integrity for biological functions of ChM-I *in vitro*.

Key words: chondromodulin-I; glycosylation; angiogenesis inhibitor; vascular endothelial cells; chondrocytes

Introduction

Chondromodulin-I (ChM-I) is a cartilage-specific 25-kDa glycoprotein that was originally purified as a growth-promoting component of fetal bovine cartilage extracts [1, 2], and found to stimulate both DNA synthesis and colony formation in cultured chondrocytes in the presence of fibroblast growth factor-2 (FGF2) [1, 3]. Thereafter, ChM-I was identified as a cartilage-derived inhibitor of angiogenesis as it inhibited DNA synthesis and tube morphogenesis in cultured endothelial cells [4, 5]. Gene targetting revealed that the loss of ChM-I gene (*Chm1*) leads to pathogenic angiogenesis and unusual calcification in the cardiac valves [6], which are normally maintained in an avascular state and are one of the major sites of *Chm1* expression. Moreover, the null mice exhibited a phenotype of imbalanced bone remodeling with reduced bone turnover [7].

The cloning of ChM-I cDNA suggested that the mature human protein (120 amino acid residues) was encoded as the C-terminal part of a larger type II transmembrane precursor (334 amino acid residues) [1]. The human ChM-I gene (*Chm1*) encoding ChM-I maps to 13q14-21, and consists of seven exons and covers a genomic region of approximately 40 kb with a TATA-less type promoter [2, 8]. The last two exons of this gene encode the mature ChM-I protein. As depicted schematically in Fig. 1, exon 6 encodes both the furin processing signal sequence and the N-terminal hydrophilic domain (Domain 1), which harbors the conserved N-glycosylation site. Moreover, N-terminal amino acid sequencing revealed that naturally occurring bovine ChM-I contained two O-oligosaccharide moieties at Thr9 and Thr22 [1], suggesting that Domain 1 is heavily glycosylated. However, the functional importance of Domain 1 has

been poorly understood. Exon 7 encodes the domain (Domain 2) containing all four disulfide bonds and the C-terminal hydrophobic sequence (Fig. 1). The amino acid sequences in Domain 2 are conserved among species [9].

The expression of human ChM-I precursor cDNA in Chinese hamster ovary (CHO) cells successfully resulted in the recovery of a mature glycosylated form of this protein (G-hChM-I) in the culture media [10]. N-terminal amino acid sequencing of G-hChM-I revealed that the expressed ChM-I precursor was properly cleaved at the predicted processing signal for the furin endoprotease. However, the produced G-hChM-I was recovered as an aggregated form with the apparent molecular size over 200 kDa due to improper intermolecular disulfide bonds. Therefore, the resultant G-hChM-I preparation was then reduced by β-mercaptoethanol or dithiothreitol in the presence of 6 M urea, diluted under reoxidation conditions, and purified to homogeneity as a monomer form with four intramolecular disulfide bridges [10]. Thus obtained recombinant G-hChM-I stimulated the matrix synthesis of cultured chondrocytes and inhibited tumor angiogenesis and growth in a xenograft model of human tumor cells *in vivo* [10, 11]. The reduced form of G-hChM-I failed to show any bioactivity on cultured chondrocytes and endothelial cells. Reduction of naturally occurring ChM-I loses its bioactivity as well [1, 4].

In our present study, we expressed the mature human ChM-I protein in *E. coli*, and purified the resulting recombinant product in its non-glycosylated form (NG-hChM-I) with correctly formed disulfide bridges. Using NG-hChM-I, we evaluated the functional significance of each domain within the mature ChM-I protein.

Materials and methods

Materials

Recombinant G-hChM-I was expressed in CHO cells and purified from the culture supernatants as described previously [10]. *Escherichia coli* BL21 (DE3) cells and the pET-11a vector were purchased from Novagen (Madison, WI). Lysozyme and TPCK treated trypsin were obtained from Sigma Chemical (St. Louis, MO). V8 protease was purchased from Pierce (Rockford, IL).

Bacterial production and purification of NG-hChM-I

The sequence encoding the mature form of hChM-I was amplified by PCR from pCRII-hChM-I [10] and cloned into *Nco*I and *Bam*HI sites of pET-11a to generate pET11ChM-I. *E. coli* BL21 (DE3) cells were then transformed with pET11ChM-I and NG-hChM-I expression was induced by the addition of 1 mM β-D-thiogalactopyranoside. After culturing for a total of 5 h, bacterial cells were harvested and the cell pellets (4.4 g in wet weight) were resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA, 0.2 mg/ml lysozyme). The insoluble crude debris was suspended in 200 ml of solubilization buffer (20 mM Tris-HCl, pH, 9.0, 6 M urea, 1 mM EDTA, and 50 mM DTT) and incubated at room temperature overnight with stirring. This solution was then centrifuged at 30,000 × g for 20 min at 4˚C, and the supernatant was loaded on Q Sepharose FF anion-exchange column (2.6 × 32 cm, Amersham Pharmacia Biotech, Buckinghamshire, UK) equilibrated with a starting buffer containing 20 mM Tris-HCl, pH 9.0, 6 M urea, 1 mM EDTA, and 1 mM DTT, and then an SP Sepharose FF cation-exchange column (1.6 × 13 cm, Amersham Pharmacia Biotech) equilibrated with a starting buffer

comprising 20 mM acetate-NaOH, pH, 5.0, 50 mM NaCl, 6 M urea, 1 mM EDTA, and 1 mM DTT. The reduced form of the NG-hChM-I protein was eluted with a linear gradient of 0-300 mM NaCl and 50-500 mM NaCl, respectively. The eluate was then dialyzed against 5% acetic acid and applied to a Butyl-Toyopearl 650 M (1.6 × 13 cm, Tosoh, Tokyo, Japan) equilibrated with 0.1% trifluoroacetic acid (TFA). The reduced NG-hChM-I was subsequently eluted with a linear gradient from 0 to 80% ethanol and the purified protein was worked up by evaporation. A final yield of 59.6 mg of NG-hChM-I in the reduced form was recovered from 1200 ml culture of the bacterial cells. Protein concentrations were determined by the method of Bradford (Bio-Rad Laboratories, Hercules, CA).

Reoxidation of reduced NG-hChM-I

An evaporated sample of purified NG-hChM-I in its reduced form was solubilized in 0.5 ml of buffer containing 10 mM acetate-NaOH, pH 4.7, 6 M urea, and 1 mM EDTA to yield a 10 mg/ml protein solution. This solution was diluted into 100 ml of reoxidation buffer (50 mM Tris-HCl, pH 9.1, 0.1 M NaCl, 1 mM EDTA, 2 mM oxidized glutathione, 0.2 mM reduced glutathione, and 2% CHAPS) to initiate disulfide reconstruction. After 4 h incubation at room temperature, acetic acid (5 ml) was added to the mixture to stop the reaction. The reoxidized NG-hChM-I was purified using a Butyl-Toyopearl 650M (1.6 × 12 cm) and analyzed by reversed phase HPLC as described previously [4, 5].

Trypsin digestion and peptide mapping of reoxidized NG-hChM-I

Fifty microliters of a 20 mg/ml preparation of reoxidized NG-hChM-I in 50% ethanol was diluted into 950 µl of digestion buffer (0.1 M HEPES-NaOH, pH 6.5 and 2 M urea),

and mixed with 36 µg of trypsin. This mixture was then incubated at 37˚C for 12 h with gentle mixing, followed by the addition of 50 µl of acetic acid to stop the digestion. The fragments were separated by reversed phase HPLC.

Limited proteolysis of NG-hChM-I with V8 protease

A 1 ml aliquot of a 10 mg/ml solution of NG-hChM-I in 50% ethanol was added to a 50 ml volume of reaction buffer (0.1 M ammonium acetate, pH 4.4, and 1 mM EDTA) containing 100 µg of V8 protease. After incubation at 37˚C for 30 min, this reaction was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. The digested products were then purified and separated by Butyl-Toyopearl 650M (1.6 × 13 cm) and reversed phase HPLC.

Cell culture

Mouse spleen derived vascular endothelial cell line (MSS31) were a generous gift from Dr. Y. Sato (Institute of Development, Aging, and Cancer, Tohoku University) and were cultured in alpha-Modified Eagle's Medium (αMEM) containing 10% fetal bovine serum (FBS) at 37˚C under 5% CO_2 in air [12]. Human umbilical vein endothelial cells (HUVEC) were obtained commercially (Lonza, Walkersville, MD) and cultured in endothelial cell growth medium (EGM, Lonza, Walkersville, MD) at 37˚C under 5% CO_2 in air. Chondrocytes were isolated from rib cartilage of rats by collagenase digestion as described previously [13, 14].

Bioassays

DNA synthesis assays were performed using a 5'-bromo-2'-deoxy-uridine (BrdU) Labelling and Detection Kit (Roche, Mannheim, Germany). The cells were starved in low serum medium and incubated with 2 ng/ml of human fibroblast growth factor-2 and

the indicated test molecules. Cells were cultured and labeled with BrdU (25 μl/well) for the periods indicated in the figure legends. BrdU incorporation was then assayed using an ELISA BrdU chemiluminescence kit according to the manufacturer's instructions. Each experiment was performed three times in quadruplicate. The colony formation assay was performed using rat growth plate chondrocytes as previously reported [3, 15]. Colony formation was examined on day 8 under a phase-contrast microscope. *In vitro* tube morphogenesis assays were performed in triplicate using Growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA) as previously described [16, 17], except in the case of HUVECs which were incubated with test samples for 20 min and allowed to form tube-like structures for 10 h.

CD (circular dichroism) measurement

Far-UV CD measurements were carried out at 10°C on a J-720WI spectrophotometer (JASCO, Tokyo) in the range of 190-270 nm, by using a cell with a light-path of 1 mm. G-hChM-I (1 mg/ml) was dissolved in PBS, whereas NG-hChM-I (0.8 mg/ml) was dissolved in two different solutions (in 0.1 M sodium acetate buffer, pH 3.8 with 1.4 mM L-arginine and in 50% ethanol) because of its low solubility in PBS. The solvent spectrum obtained under the same conditions was subtracted. CD spectra shown were average of five measurements, and the results were expressed as the mean residue ellipticity [θ] (deg cm^2 dmol^{-1}).

Results and discussion

Non-glycosylated human chondromodulin-I (NG-hChM-I) was expressed in *E. coli* BL21 cells and was produced as inclusion bodies in these bacteria. The expressed proteins were therefore extracted using a buffer containing 6 M urea. Without the use of a reductant, most of the NG-hChM-I molecules were recovered in a heavily polymerized form probably due to intermolecular disulfide bonds. To avoid this, 50 mM DTT was included in the extraction buffer. After extraction, NG-hChM-I was purified with Q Sepharose, SP Sepharose and Butyl-Toyopearl (Supplemental Fig. 1).

To convert the reduced NG-hChM-I into its disulfide-bridged form, a reoxidation reaction was carried out using oxidized glutathione as a catalyst. The reoxidation of NG-hChM-I was monitored using reverse phase HPLC. The reduced form of this protein is indicated by a sharp peak at a retention time of 22.9 min (Fig. 2A). When the proteins were analyzed by HPLC immediately after dilution into the reoxidation buffer, no discernible peaks could be observed (0 h in Fig. 2A). Over a short incubation time, however, a sharp peak at a retention time of 19.9 min was visible and reached a maximum area after 4 h of incubation. Chemical modification analysis further indicated that no detectable free sulfhydryl groups were present in this protein component (data not shown). The reoxidized product was recovered, and digested with trypsin to identify the disulfide bridges. These digested fragments were then separated, again using reverse phase HPLC (Fig. 2B). The peaks were subsequently collected and identified by N-terminal amino acid sequence analysis. These identified sequences are shown in Fig. 2C and no fragments containing either Cys79 or Cys103 were identified. Hence, as no free Cys residues were evident in the recovered NG-hChM-I preparation, this indicated that these two cysteines had formed a disulfide bond. These disulfide

pairings are consistent with those expected from previous structural analysis of naturally occurring bovine ChM-I [4, 18].

We have shown that naturally occurring ChM-I is a bifunctional protein that inhibits DNA synthesis in vascular endothelial cells, and that promotes DNA synthesis and colony formation of cultured chondrocytes synergistically in the presence of FGF2 [1, 3, 4]. Purified bovine ChM-I stimulates sulfated proteoglycan synthesis in a dose-dependent manner in cultured chondrocytes as well [3, 4]. In either type of cells, bioactivity of ChM-I could be discernible at a concentration of several tens ng/ml, became evident at 100-300 ng/ml *in vitro*. At a dose range of 0.5-1 μg/ml, ChM-I gives a maximal effect. Recombinant G-hChM-I derived from CHO cells, which was prepared by reduction and reoxidation after denaturation in 6 M urea, has shown a dose-dependency of bioactivity similar to that of naturally occurring bovine ChM-I [10]. In our present study, we examined the effects of NG-hChM-I on DNA synthesis in quiescent rat growth plate chondrocytes in culture. As shown in Fig. 3A, NG-hChM-I stimulates DNA synthesis in rat primary chondrocytes in the presence of recombinant human FGF2 (hFGF2) in a synergistic and dose-dependent manner. The incorporation of BrdU into the DNA was found to have increased 4-fold over the basal levels when the cells were treated with 30 μg/ml of NG-hChM-I and 2 ng/ml hFGF2. Moreover the same dose range of NG-ChM-I (3-10 μg/ml) was found to stimulate the colony formation of chondrocytes in agarose cultures in the presence of hFGF2 (1 ng/ml) (Figs. 3B-E) [3]. However, an approximately 10-fold higher dose of NG-ChM-I is required for stimulation of DNA synthesis compared with our previous calculations for naturally occurring bovine ChM-I or G-hChM-I [1, 4].

Human umbilical vein endothelial cells (HUVECs) spontaneously form

capillary-like structures on Matrigel and this reflects *in vitro* angiogenic processes in which cells migrate and join together (Fig. 4A). G-hChM-I derived from CHO cells was found to significantly impair the ability of these cells to form capillary-like structures at a concentration of 100 ng/ml (Fig. 4B) and 1 μg/ml (Fig. 4C). These data are comparable with those of our previous study that used cultured bovine carotid artery endothelial cells (Fig. 4G) [10]. In the present study, we further found that NG-hChM-I impairs the formation of capillary-like structures in a dose-dependent manner (Figs. 4D-G). A concentration of 10 μg/ml NG-hChM-I also resulted in a significant inhibition of *in vitro* tube morphogenesis (Figs. 4F and 4G). However, a concentration of NG-hChM-I greater than 10 μg/ml did not cause any further inhibition of this process (data not shown). These findings indicate that the maximal inhibitory effects of NG-hChM-I appear to require a 10-fold higher dose compared with G-hChM-I.

To study the structural requirements for the functional activity of ChM-I, we carried out a limited proteolysis of NG-hChM-I. Incubation of this protein with V8 protease resulted in its rapid digestion and the accumulation of comparatively larger fragments than those generated using other proteases such as trypsin or chymotrypsin (Fig. 5A). After about 30 min of digestion, almost all of the NG-hChM-I molecules had been fully cleaved and a fragment which migrated slightly faster than the 14-kDa marker had accumulated. N-terminal sequencing analysis of this fragment revealed an Asp-Ser-Gln-Ala-Phe- sequence, which indicates that the fragment starts from Asp38 in the N-terminus of the full length protein (Fig. 1). This digested product (ΔN-hChM-I) was then purified by HPLC. To explore the functional significance of the glycosylation of ChM-I in Domain 1, a 1 mg aliquot of G-hChM-I or purified NG-hChM-I was separately suspended in both PBS and 5% ethanol. After centrifugation, the protein

concentrations in the supernatants were measured (Fig. 5B). All three types of recombinant ChM-I could be maintained at a 1 mg/ml concentration in 5% ethanol. G-hChM-I was found to be soluble in PBS. However, NG-hChM-I was only marginally soluble in PBS. It gave only a solution at a concentration of less than 50 μg/ml in PBS. ΔN-hChM-I was even less soluble than NG-hChM-I in PBS. Hence, glycosylation in Domain 1 enhances the solubility of the ChM-I protein under physiological conditions.

To assess the bioactivity of ΔN-hChM-I, the mouse spleen-derived vascular endothelial cell line, MSS31, was cultured in αMEM containing 10% FBS [12]. These cultures were then made quiescent by incubation in low serum medium (αMEM containing 0.1% FBS) for 48 h after reaching confluence. As shown in Fig. 6, the stimulation of DNA synthesis by hFGF2 (2 ng/ml) was inhibited by G-hChM-I in a dose-dependent manner. The concentration of G-hChM-I required for 50% inhibition was found to be within the 1-5 μg/ml range. In contrast, the low solubility of NG-hChM-I and ΔN-hChM-I hampered the examination of the maximal inhibitory doses of these peptides. However, at the near maximal soluble concentration (50 μg/ml) of NG-hChM-I or ΔN-hChM-I, the BrdU uptake in MSS31 cells was suppressed by up to 75% in comparison with the control cells treated with hFGF2 (2 ng/ml) alone. These dose-dependency measurements suggested that an approximately 10-fold or higher dose of NG-hChM-I or ΔN-hChM-I than G-hChM-I was required for inhibition of the FGF2-induced DNA synthesis (Fig. 6).

In a similar manner to naturally occurring chondromodulin-I, NG-hChM-I was found to be able to exhibit biological activities in chondrocytes and vascular endothelial cells, even though this required higher doses when compared with glycosylated recombinant protein G-hChM-I (Figs. 3 and 4). This high

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dose-requirement for NG-hChM-I is likely related to the lower solubility of this molecule. Figure 7 compares the CD spectra of G-hChM-I in PBS and NG-hChM-I in 0.1 M sodium acetate buffer (pH 3.8) with 1.4 mM L-arginine, and in 50% ethanol. The negative bands at 208 nm and 222 nm indicate that G-hChM-I in PBS has an α-helical structure. The fraction of an α-helix of G-hChM-I is calculated to be 34 % according to the equation proposed by Chen and coworkers [19]. In contrast, the CD spectra of NG-hChM-I in both solutions do not show distinct characteristic bands for typical secondary structures, suggesting that hChM-I molecule does not have a stable three-dimensional structure without glycosylation. Even though ΔN-hChM-I that lacks most of hydrophilic Domain 1 had an even lower solubility in PBS than the parent NG-hChM-I molecule, its growth inhibitory action was indicative in the culture of MSS31 cells (Fig. 6). The hydrophobic Domain 2 that contains all four disulfide bonds of ChM-I thus appears to be responsible for the biological activities of this protein. This is compatible with the fact that reducing agents such as mercaptoethanol or dithiothreitol abolished biological activities of G-hChM-I as well as naturally occurring bovine ChM-I [4, 10]. We thus conclude that chondromodulin-I contains two structural domains, and that each plays a distinct role in transmitting the cellular functions of this protein *in vitro*.

Acknowledgments

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Figure Legends

FIG. 1. The structure of human Chondromodulin-I. Amino acid sequence of the human ChM-I protein and configuration of its disulfide bonds are shown. The disulfide bond arrangements are assumed to be identical with those of ChM-I purified from bovine fetal cartilage [1, 4, 14, 18]. The residues indicated in green circles indicate the amino acids that are not conserved in the mouse ChM-I protein [14].

FIG. 2. Reverse-phase chromatographic analysis of NG-hChM-I during reoxidation. (A) The catalyzed reoxidation of NG-hChM-I was carried out using glutathione as described in "Materials and methods." After this reaction was stopped by the addition of acetic acid, 10 μg of protein was injected into a YMC-Pack C4-AP HPLC column. Elution of the proteins (at a flow rate of 1 ml/min) was monitored by the measurement of absorbance at 210 nm. The retention times between 17 min and 25 min are shown in the figure. The reduced and oxidized forms of NG-hChM-I were eluted at 23 and 20 min, respectively. (B) A reverse phase chromatogram of tryptic fragments of NG-hChM-I is shown. The numbered peaks correspond to fragments containing disulfide bridges. The peaks labeled with an asterisk have the same N-terminal sequence, LNNETRPSVQED. (C) N-terminal sequencing of the numbered fragments shown in (B). Fragment #1 was found to contain the same N-terminal sequence as fragment #2 except for digestion at Tyr94-Asn95.

FIG. 3. Stimulation of DNA synthesis and colony formation in rat chondrocytes by NG-hChM-I in the presence of hFGF2. (A) Quiescent rat primary chondrocytes in 48-multiwell culture plates were stimulated by 2 ng/ml of hFGF2 with or without the

indicated concentration of recombinant NG-hChM-I for 22 h. Thereafter BrdU was added to the cultures followed by incubation for another 4 h. Incorporation of BrdU into the cells was determined using an ELISA BrdU chemiluminescence kit according to the manufacturer's instructions. This experiment was repeated three times with similar results. (B-E) Rat primary chondrocytes (5×10^3 cells/well) were suspended in 0.41% agarose in F-12 medium containing 5% FBS, 2×10^{-7} M hydrocortisone, and 60 µg/ml transferrin. The cell suspensions were then overlaid on a base agarose layer. After incubation overnight, the indicated factors were added to the cells. On day 8, colony formation was examined under a phase-contrast microscope. Cells were treated with 0.1% BSA alone (B), 1 ng/ml hFGF2 (C), 3 µg/ml NG-hChM-I in the presence of 1 ng/ml hFGF2 (D), and 10 µg/ml NG-hChM-I in the presence of 1 ng/ml hFGF2 (E). Bar, 100 µm.

FIG. 4. Inhibition of tube morphogenesis *in vitro* by recombinant human ChM-I. (A-F) Quiescent HUVECs were plated on growth factor-reduced Matrigel with αMEM containing 0.5% FBS with or without test samples. (A) HUVECs were plated on Matrigel without ChM-I. (B, C) Cells were cultured in the presence of G-hChM-I (panel B, 0.1 µg/ml; panel C, 1.0 µg/ml). (D-F) Cells were cultured in the presence of NG-ChM-I (panel D, 0.1 µg/ml; panel E, 1.0 µg/ml; panel F, 10 µg/ml). All photographs are representative of four standardized fields from three separate experiments. Bar, 200 µm. (G) Tube morphogenesis of HUVECs was quantitatively evaluated by measuring the total lengths of the tube-like structures in randomly selected fields with NIH image version 1.61. Four standardized-fields in each experiment were randomly chosen for measurement. Each value is the mean ± S.D. of data from three

separate experiments.

FIG. 5. Generation and solubility of the purified N-terminal truncated fragment of ChM-I (ΔN-hChM-I). (A) Limited proteolysis of NG-hChM-I by V8 protease. The reaction conditions are described in "Materials and methods." At a given time period, an aliquot of the reaction mixture was withdrawn and added to SDS-PAGE sample buffer to stop the reaction. Then samples were then resolved in a 15% polyacrylamide gel and the proteins were stained with Coomassie Brilliant Blue R250. Samples were taken at 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 15 min (lane 4), 20 min (lane 5), 25 min (lane 6), 30 min (lane 7), 40 min (lane 8), 50 min (lane 9), and 60 min (lane 10). In lane 11, the N-terminal truncated fragment that was purified by preparative HPLC was loaded. Molecular weight markers are shown in lane 12. (B) One milligram each of NG-hChM-I, ΔN-hChM-I, and G-hChM-I was dissolved in 1 ml of PBS (closed bars) or 5% ethanol (open bars), and after centrifugation the protein concentrations of the supernatant were measured using the Bradford assay. All of the derivatives were completely soluble in 5% ethanol. The solubilities of these products in PBS are shown relative to in those in 5% ethanol.

FIG. 6. Inhibition of DNA synthesis of MSS31 cells by recombinant human ChM-I and its derivatives. Quiescent MSS31 cells in 48-multiwell culture plates were stimulated with a 2 ng/ml dose of hFGF2 and then cultured with the indicated concentrations of recombinant human ChM-I for 24 h. Thereafter, BrdU was added to the cultures, which were incubated for a further 12 h. The incorporation of BrdU into the cells was subsequently determined using ELISA BrdU chemiluminescence. These

experiments were repeated three times and gave similar results.

FIG. 7. Far-UV CD spectra of G-hChM-I and NG-hChM-I. CD spectra of G-hChM-I in PBS (black solid line), and of NG-hChM-I in 0.1 M sodium acetate buffer pH 3.8 with 1.4 mM L-arginine (gray solid line) and in 50 % ethanol (black dotted line) are shown.

Legends for Supplemental Data

Supplemental Fig. 1. SDS-PAGE analysis (15%) of NG-hChM-I under reduced conditions during purification. Proteins were stained with Coomassie Brilliant Blue R250. Lane 1, total cells; lane 2, extracts with urea and DTT; lane 3, Q Sepharose eluate; lane 4, SP Sepharose eluate; lane 5, Butyl-Toyopearl eluate; lane 6, molecular weight markers.

Fig. 1.

Domain 1 (the glycosylation domain)

V8-digestion

N-Glycosylation site

HOOC-

Domain 2 (the cysteine-rich domain)

Fig. 2.

A

B
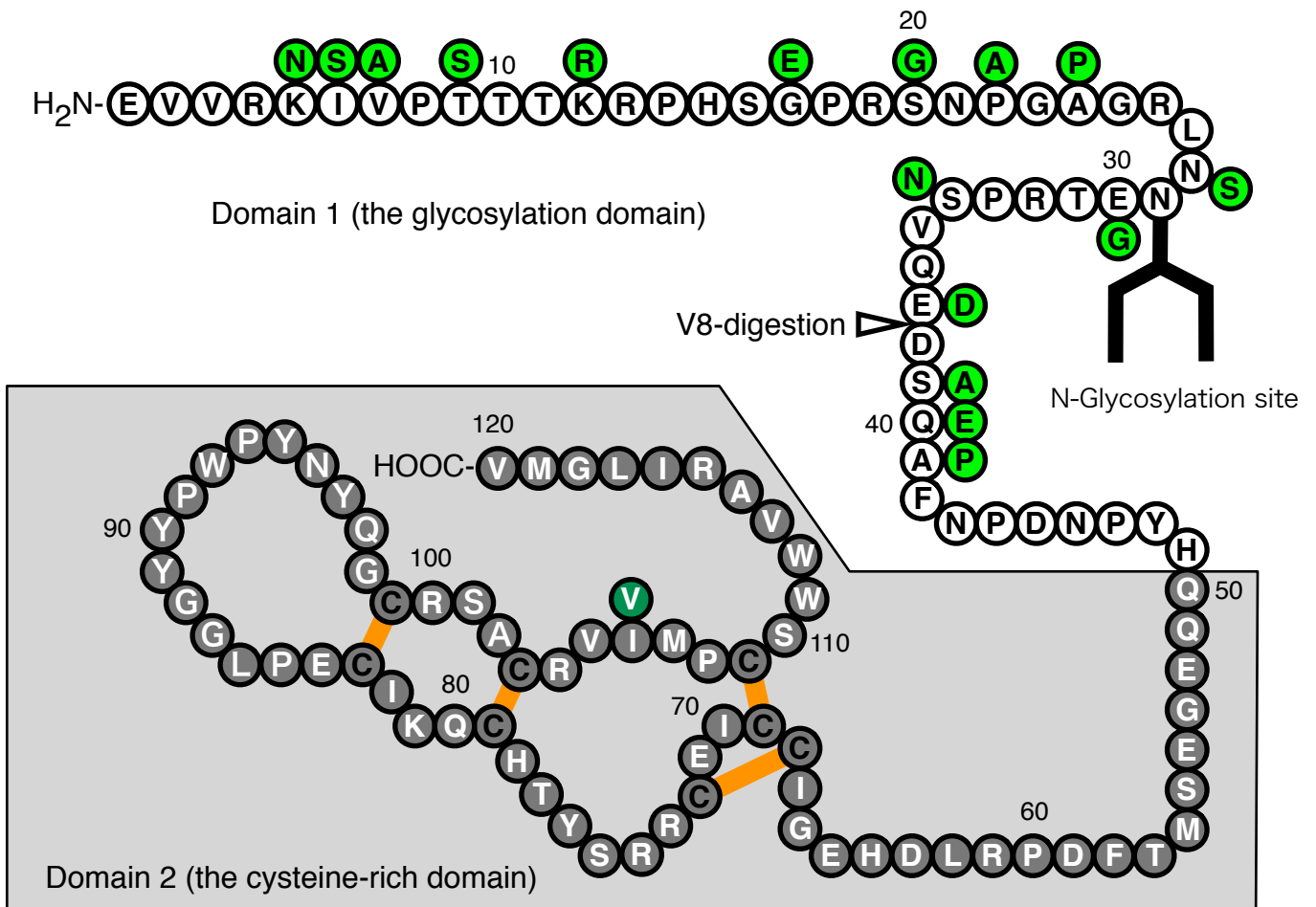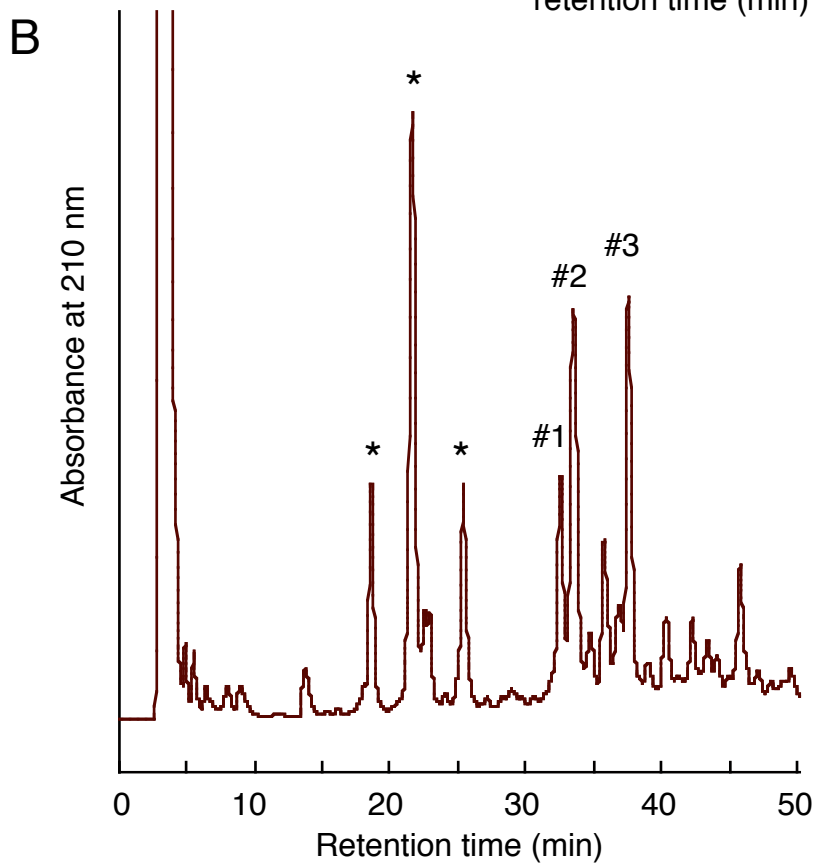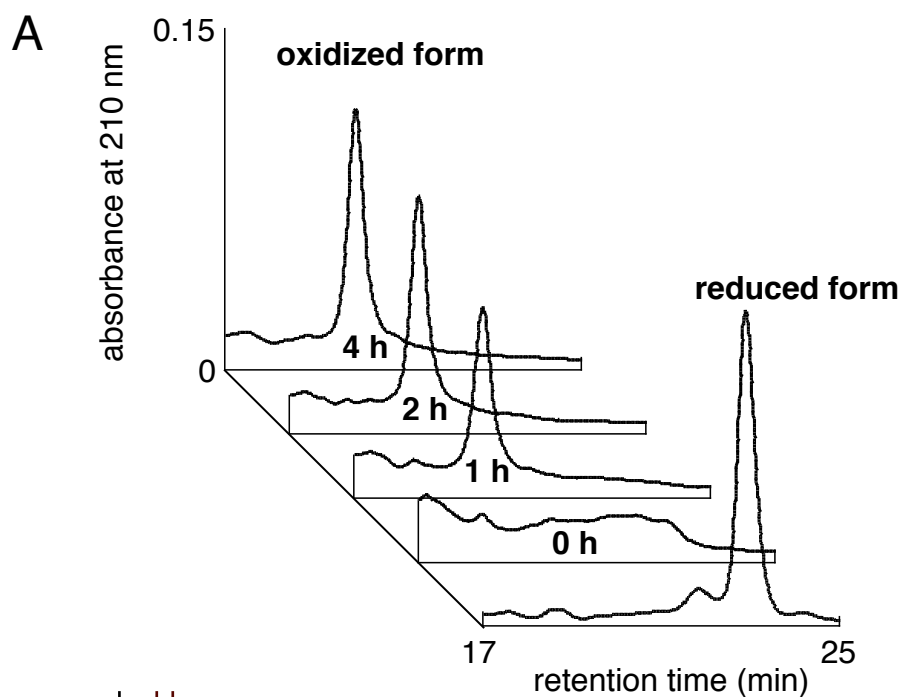
C

#1:   82   100
      ICEPLGGYYPWPYNYQGCR

#2:   82   94
      ICEPLGGYYPWPY

#3:   62   73
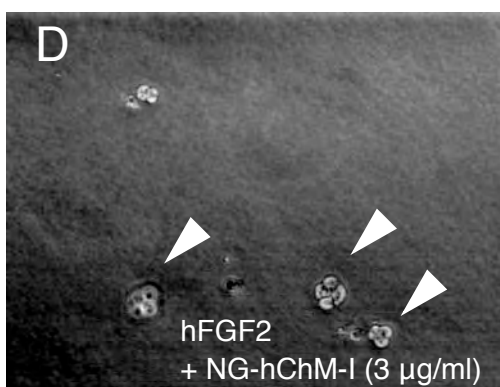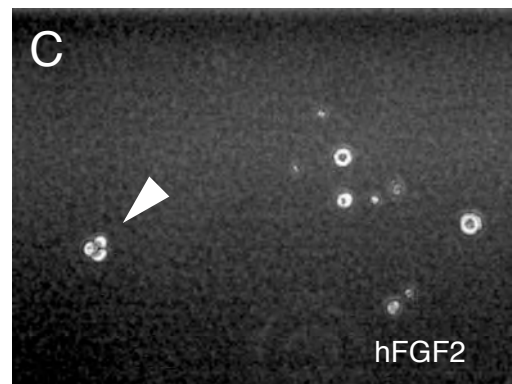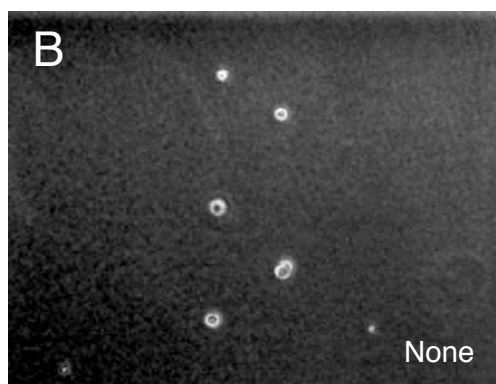   LDHEGICCIECR

      105   111
      VIMPCSW

Fig. 3.

A

BrdU incorporation (Absorbance ratio, A$_{405}$ nm/A$_{492}$ nm)

| | | | | | |
| 0.3% FBS | 0.1% BSA | 1.0 | 3.0 | 10 | 30 (µg/ml) |

NG-hChM-I

hFGF2 (2 ng/ml) + 0.3% FBS

B — None

C — hFGF2

D — hFGF2 + NG-hChM-I (3 µg/ml)

E — hFGF2 + NG-hChM-I (10 µg/ml)

Fig. 4.

G

(μm)

Length of capillary-like structures

120

80

40

0

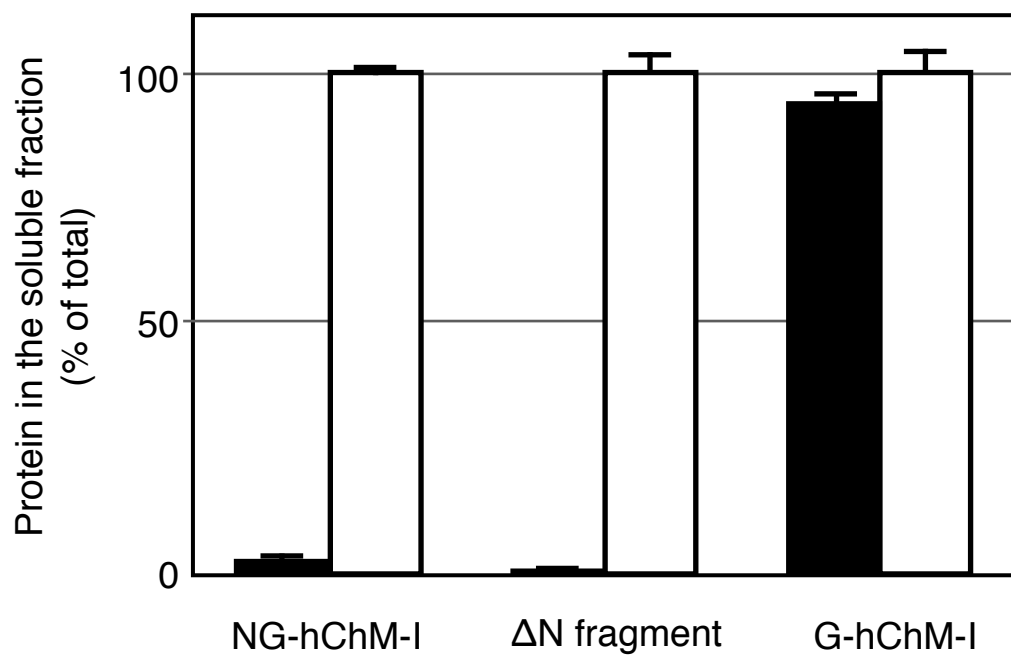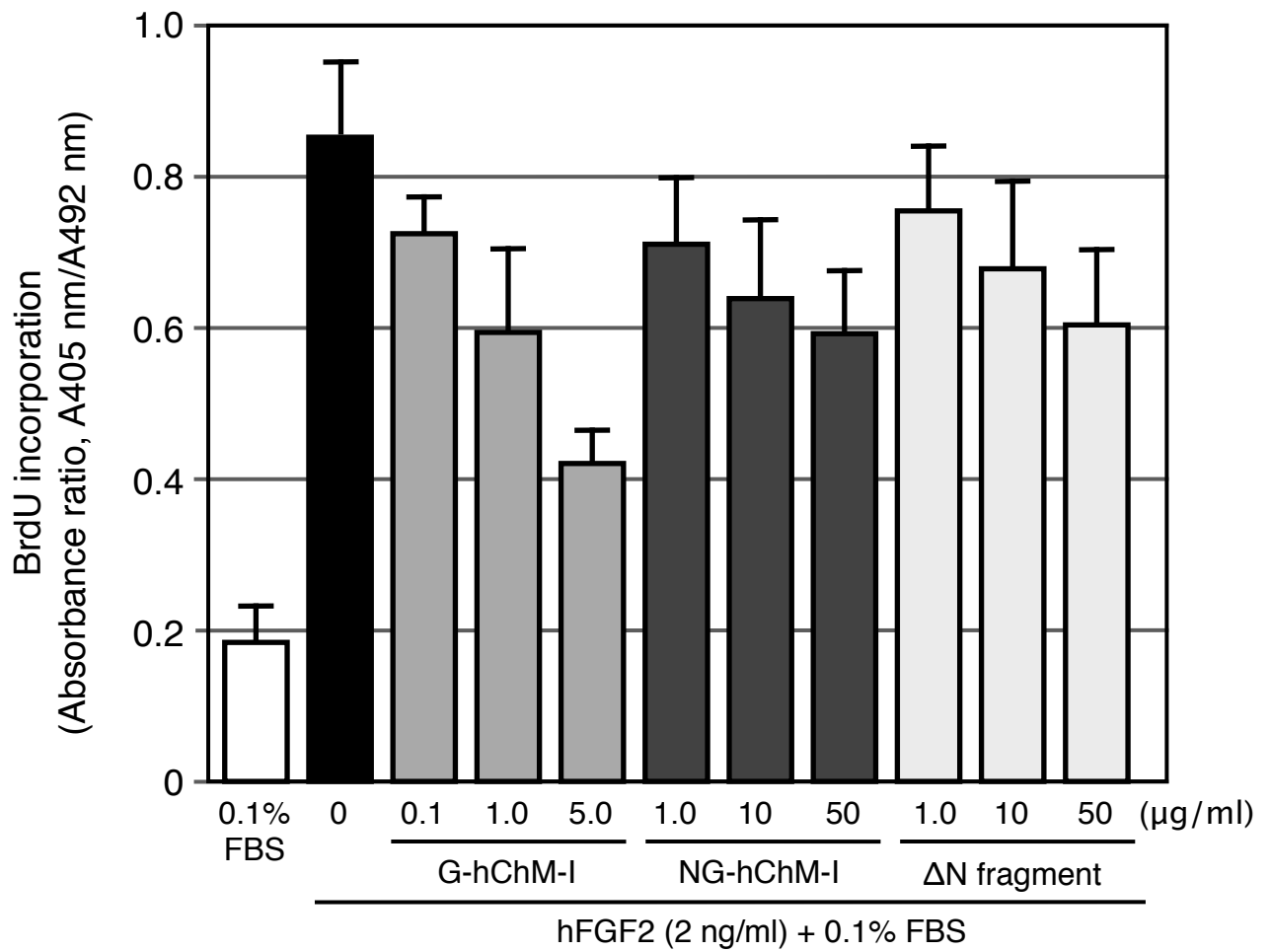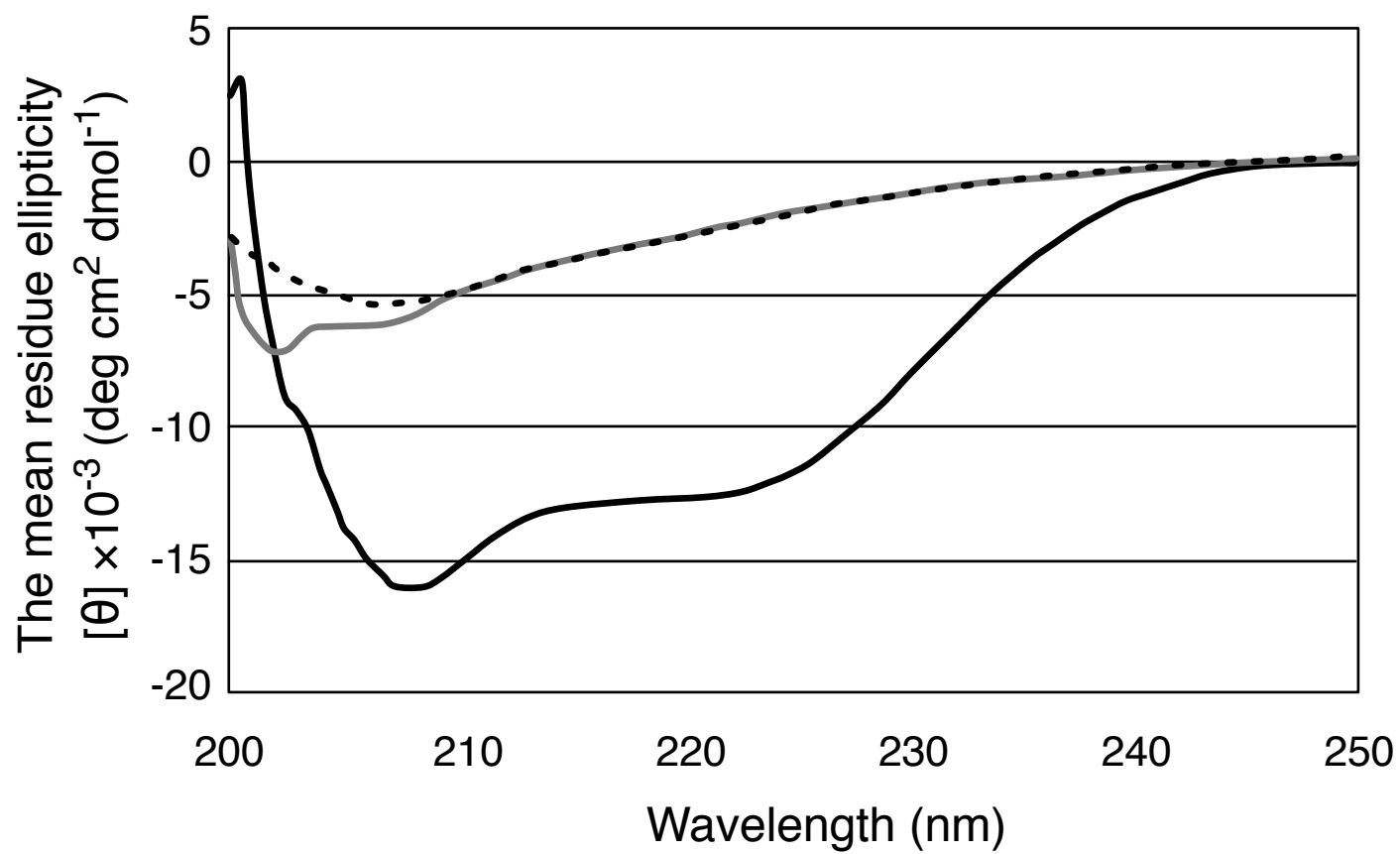Control | 0.1 | 1.0 | 0.1 | 1.0 | 10 | (μg/ml)

G-hChM-I   NG-hChM-I
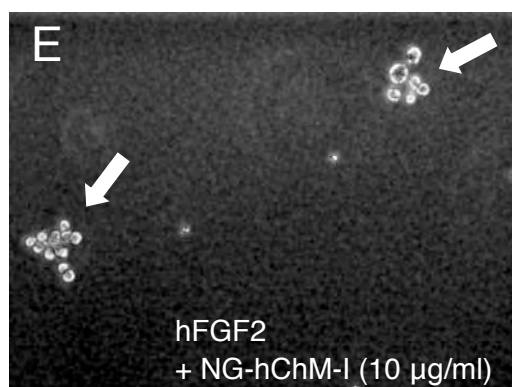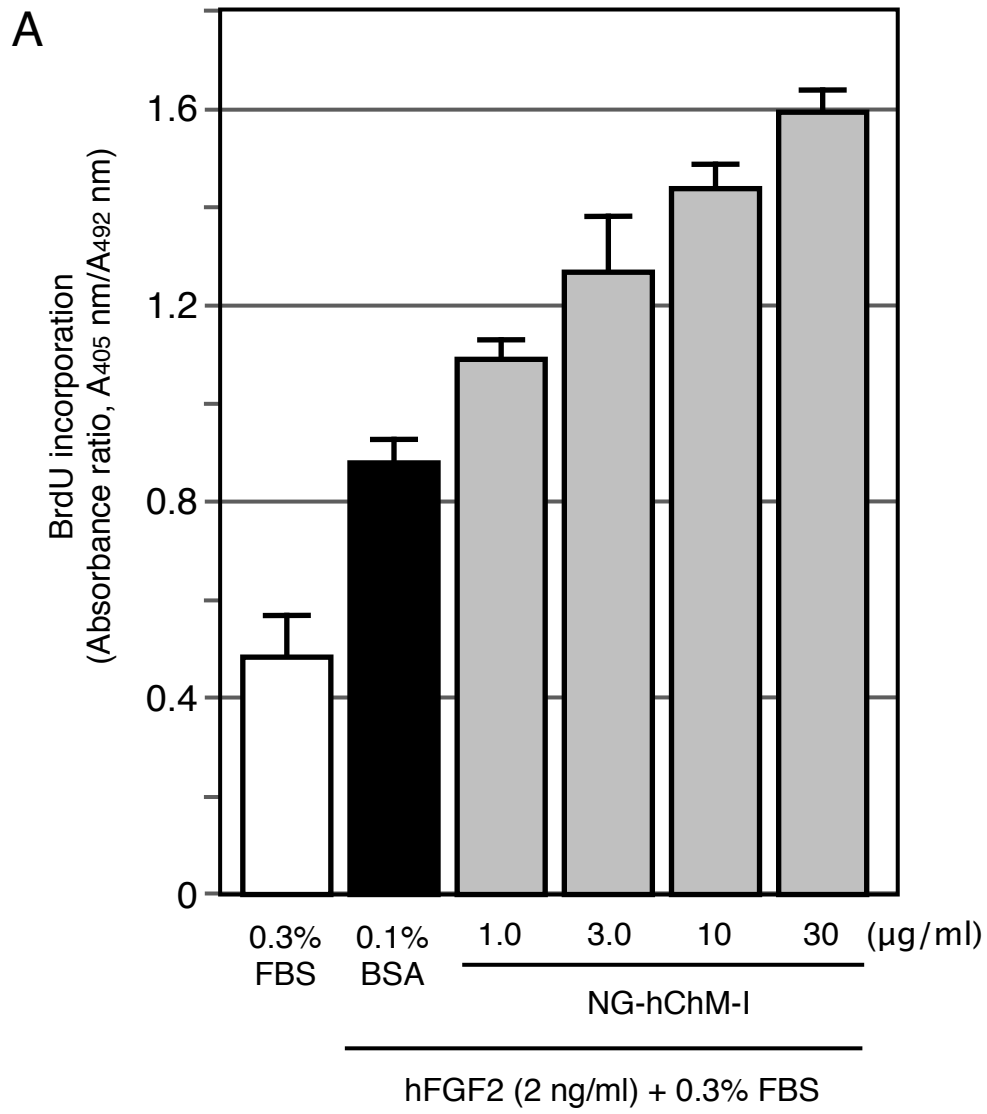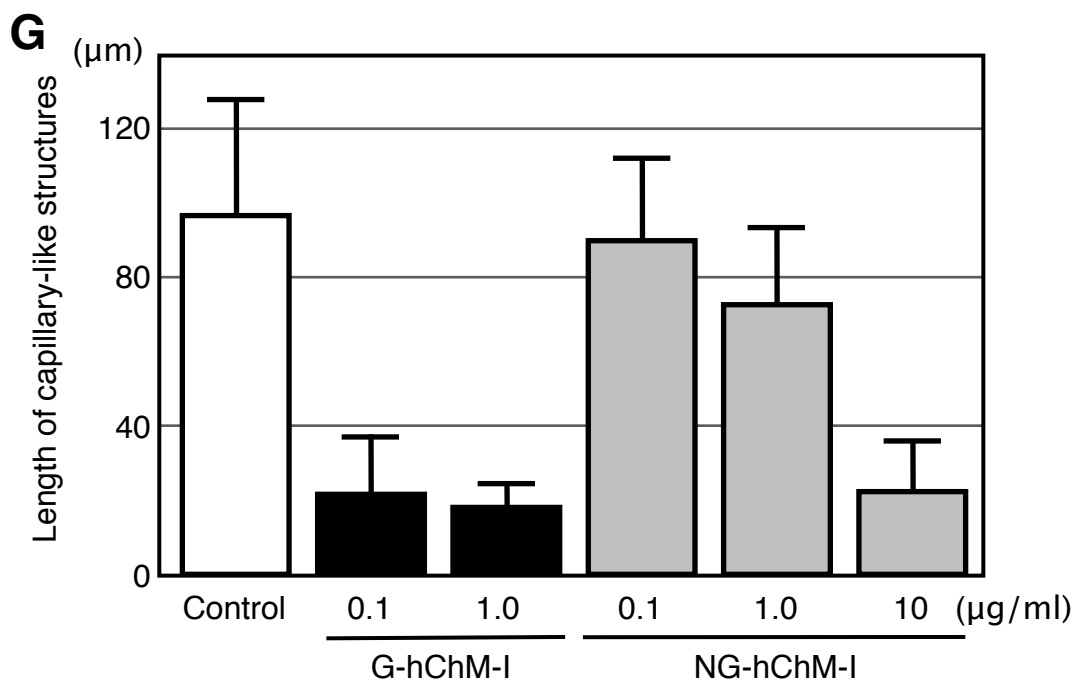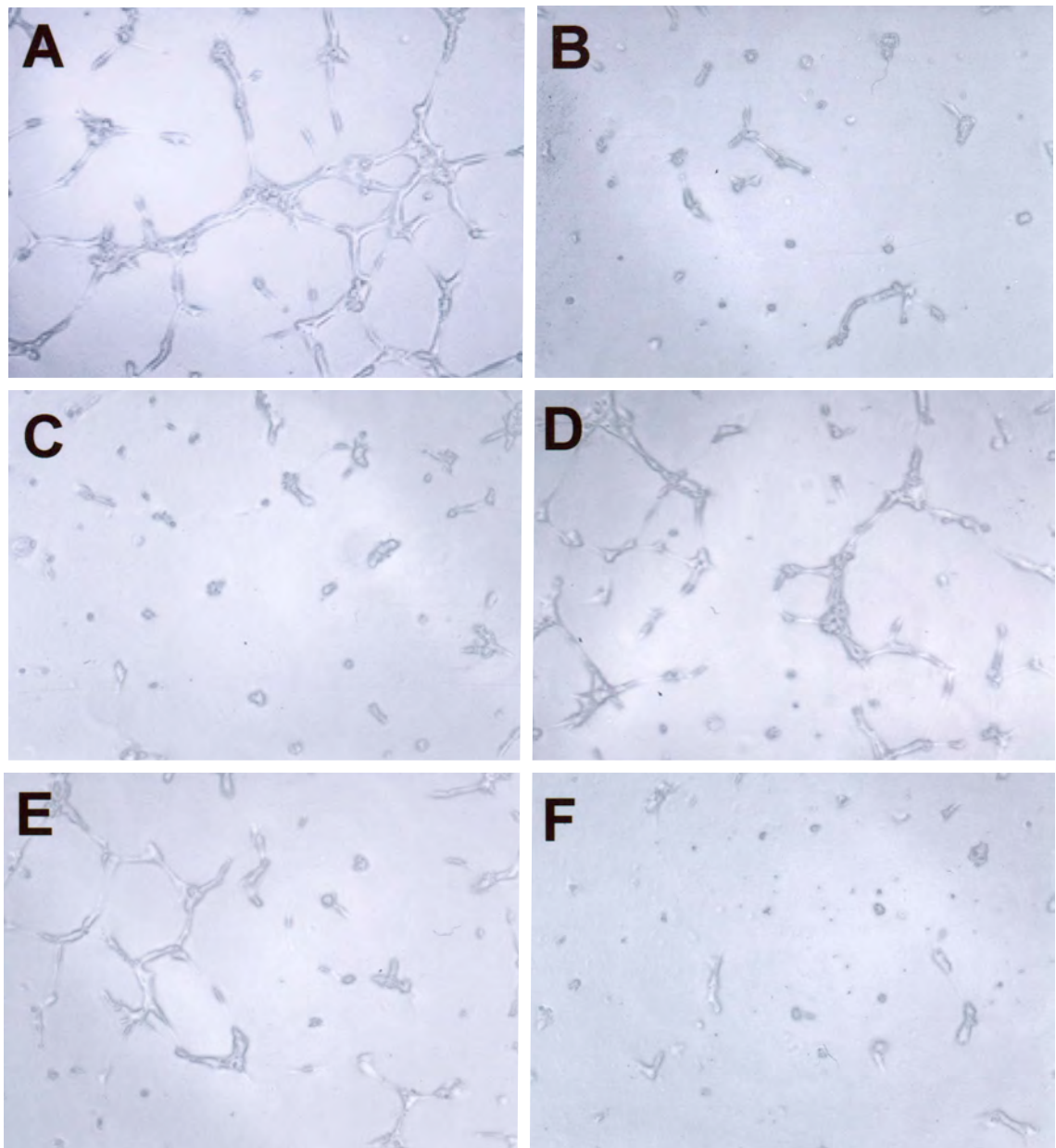
Fig. 5.

A
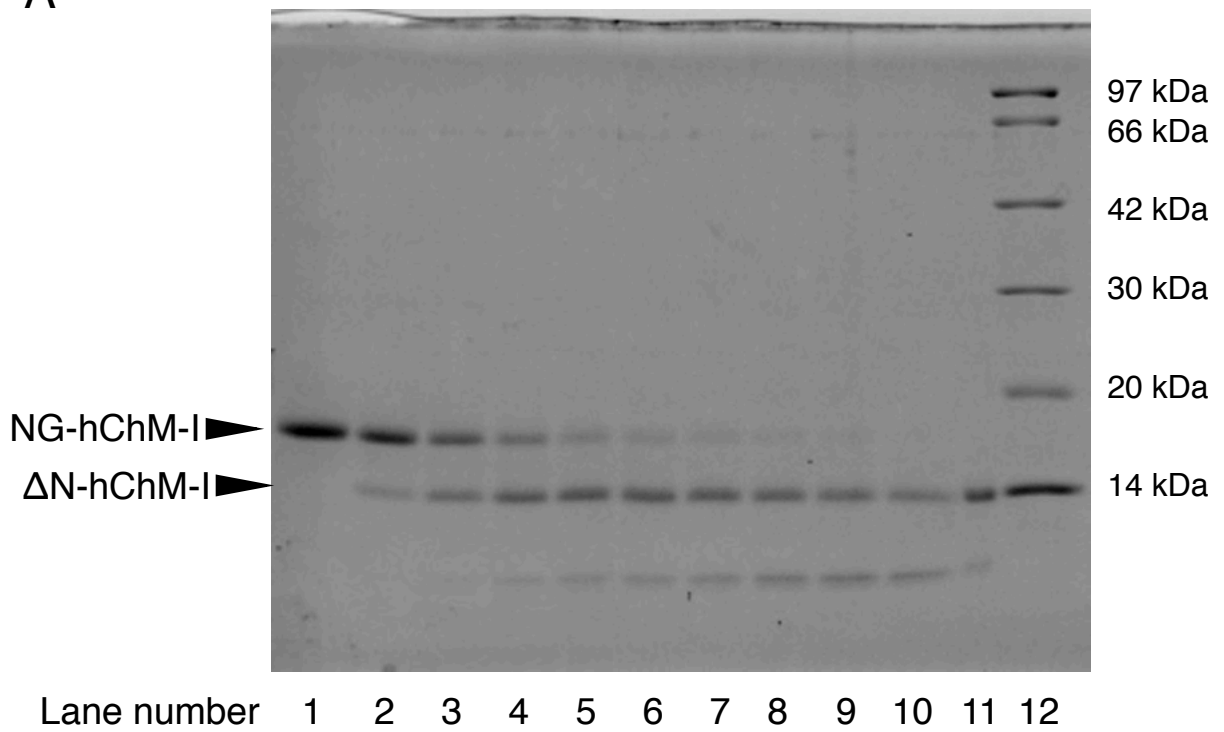
B

Fig. 6.

Fig. 7

Supplemental Fig. 1.

NG-hChM-I ►

					97 kDa
					66 kDa
					42 kDa
					30 kDa
					20 kDa
					14 kDa

Lane number   1   2   3   4   5   6