主　論　文

Abnormal occurrence of a large chondroitin sulfate proteoglycan, PG-M/versican in osteoarthritic cartilage

(大型のコンドロイチン硫酸プロテオグリカンであるPG-M/versicanの変形性関節症軟骨における異常発現)

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Running title: PG-M/versican in osteoarthritic cartilage

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Summary

We have investigated the expression of PG-M in osteoarthritic cartilage. Cartilage from 5 patients with osteoarthritis and normal cartilage from 5 patients with post-traumatic injury were obtained and analyzed with anti-PG-M antibodies. Normal cartilage showed no staining, but in osteoarthritic cartilage there was strong staining of both the cytoplasm of chondrocytes with abnormal morphology and that of inflammatory cells invading the osteoarthritic cartilage matrix. These findings prompted us to determine the sequence of PG-M core protein. The deduced amino acid sequence and homology analysis indicated that PG-M had a complement regulatory protein-like domain, a lectin-like domain, two EGF-like domains from the carboxyl terminal with an extremely high homology to the respective domains of versican, a large proteoglycan expressed by human fibroblasts. The anti-PG-M antibodies crossreacted with Ver-27b fusion protein which was expressed by a cDNA clone coding the N-terminal portion of versican core protein. Thus, the immunological and sequencing data suggest that PG-M is a molecule similar to or identical with human versican, and that the material in cartilage reactive to the anti-PG-M antibodies is versican. These findings suggest that PG-M/versican is expressed in osteoarthritic cartilage.

Index terms: osteoarthritic cartilage
PG-M, versican
large chondroitin sulfate proteoglycan

Introduction

PG-M is a large chondroitin sulfate proteoglycan that was first isolated from chick limb buds at stage 22-23 and characterized as one of the major extracellular matrix components in the limb buds (1). It has also been found to be produced by cultured chick embryonic fibroblasts (2). PG-M is very similar in molecular properties to aggrecan, another large chondroitin sulfate proteoglycan that is characteristically expressed in cartilage. However, PG-M is immunologically distinct from aggrecan and is expressed in a variety of differentiated mature tissues such as aorta (Yamagata M, Sinomura T, Kimata K. Unpublished observations) as well as being temporarily detected in various embryonic tissues such as cartilage primordia (3). For example, in the developing limb buds, when the mesenchymal condensation that is thought to be essential for the differentiation of limb cartilage occurs at stage 23, the expression of PG-M increases at the site of condensation (3). During the development of cartilage, however, PG-M disappears from the matrix in contrast to a dramatic increase of aggrecan. In normal human articular cartilage, aggrecan is a major proteoglycan. Osteoarthritic cartilage consists of a mixture of abnormal hyaline cartilage, fibrocartilage, and fibrous tissues (4, 5). Among many structural alterations that characterize osteoarthritis, the most prominent one is a decrease in the aggrecan content of the cartilage matrix. The reduced proteoglycan content of osteoarthritic cartilage compared with normal cartilage may result from an imbalance between proteoglycan synthesis and degradation. Increased proteolytic degradation of proteoglycans in osteoarthritic cartilage has been reported (6), but their increased synthesis has also been reported, possibly as a "repair mechanism" that attempts to overcome the loss of proteoglycans (7). However, some authors have found unchanged or even decreased proteoglycan synthesis in osteoarthritic cartilage (8, 9). Since PG-M is produced by mesenchymal cells and chondroblasts (2), this proteoglycan may be one of the molecules expressed during the repair of cartilage. In this study we have investigated PG-M expression in osteoarthritic cartilage using anti-PG-M

polyclonal antibodies. We have observed the abnormal presence of PG-M in osteoarthritic cartilage, and then have characterized this molecule by cDNA cloning. The results have suggested that PG-M may be a proteoglycan similar to or identical with versican, which is a chondroitin sulfate proteoglycan synthesized by cultured human fibroblasts and may be one of the molecules specifically expressed in osteoarthritic cartilage.

Materials and Methods

Cartilage

For the preparation of osteoarthritic cartilage, we used human femoral heads obtained at total hip replacement surgery. Five femoral heads were selected with at least a 2-cm^2 area of yellowish, soft, and surface-fibrillated articular cartilage on the proximal, anterior and lateral aspects. The average thickness of the cartilage samples was 2.5 mm. Areas of hard white cartilage with an intact surface and a similar appearance to normal cartilage were not included.

Control normal cartilage was obtained from the condylar resions of 5 knees at surgery after trauma. All control tissues were of a normal white-to-yellowish color, with an intact surface and a hard consistency.

Preparation of antibodies against PG-M core protein

The method used to raise the antibodies has been described previously (8). In brief, polyacrylamide gel slabs containing the 550-kDa protein-enriched core fraction of PG-M (1) were homogenized in phosphate-buffered saline (PBS; 145 mM NaCl, 2.8 mM NaH_2PO_4, 7.2 mM Na_2HPO_4, pH 7.2) and mixed with an equal volume of complete Freund's adjuvant. This mixture was injected subcutaneously into a rabbit and the antiserum obtained was subjected to affinity chromatography on a column of Sepharose 4B gel coupled with intact PG-M molecules. The bound antibodies were eluted with 3M KSCN in PBS, and then dialyzed against PBS.

Immunohistochemistry

Samples were fixed in 10% formalin, and then embedded in paraffin for sectioning. Deparaffined sections were treated with chondroitinase ABC (1 units/ml) in PBS. After washing with PBS, the sections were exposed to a 1 : 200 dilution of the normal goat serum for 30 min, and then the polyclonal rabbit anti-chick PG-M antibodies were applied to the sections overnight at a concentration of 1 : 800 at 4°C. The sections were then washed three times for 5 min with PBS, and

incubated for 30 min with a 1 : 500 dilution of a peroxidase-conjugated goat anti-rabbit IgG antibodies. After further washing with PBS, the sections were reacted with aminoethyl carbazol (AEC) using the AEC substrate Kit (Seikagaku Co.,Japan), rinsed in distilled water, and counterstained with hematoxylin.

Preparation and screening of a λgt11 cDNA library

Poly $(A)^+$ RNA was isolated from stage 22-23 chick limb buds by a guanidine isothiocyanate method (10) followed by oligo (dT)-cellulose affinity chromatography. An oligo (dT)-primed cDNA library was constructed in λgt11 using the cDNA Synthesis Kit (Pharmacia LKB Biotechnology Inc.) as described previously (11). The library comprised 4.8×10^7 independent recombinants when transfected to Escherichia coli Y1090. A total of 5×10^5 independent clones was initially screened with the antibody to PG-M according to the methods described by Young and Davis (12). Positive plaques were detected by visualizing the bound antibodies using peroxidase-conjugated protein A (E-Y Laboratories Inc, San Matero, CA). After the cDNA clones, λMa and λMb, were isolated by immunoscreening, subsequent clones were obtained by plaque hybridization using ^{32}P-labeled cDNA probes. Labeling of cDNA probes was done by the random priming method (13).

DNA sequencing and analysis

The inserts from λgt11 cDNA clones were purified and subcloned into the pGEM3Zf(-) plasmid vector (Promega Corporation, Madison, WI). The nucleotide sequences of the isolated cDNAs were determined by the dideoxy chain termination method (14) using oligonucleotide primers synthesized on the basis of the preceeding sequences. Then the cDNA sequences thus obtained were compiled and analyzed using a DNASIS computer program (Hitachi Software Engineering Co., Japan), and the deduced amino acid sequence was compared with other protein sequences in the data base compiled by the National Biomedical Research Foundation.

Immunological relationship between PG-M and versican

Truncated cDNA inserts from PG350 (TERIOS Pharmaceuticals Inc., San Diego, CA) and Ver-27b (a kind gift of Dr. E. Ruoslahti, La Jolla Cancer Res. Foundation), which are cDNA clones coding the C-terminal and N-terminal portions of versican core protein (residues 2214-2409 and 31-386), respectively, were inserted into a Glutathione-S-Transferase (GST) Gene Fusion vector, pGEX-3X (Pharmacia LKB Biotechnology, Uppsala, Sweden). The plasmids thus constructed were introduced into E. coli JM109 cells as recommended by the manufacturer. Transformation was confirmed by colony hybridization using the PG350 and Ver-27b cDNA fragments as probes. Fusion proteins were purified from the cell lysates by affinity chromatography on Glutathione-Sepharose 4B column, and the purified fusion proteins were subjected to SDS-polyacrylamide gel (9%) electrophoresis. Proteins in the gels were blotted onto nitrocellulose membranes and stained with the rabbit anti-PG-M antibodies. As a control, duplicate blots were stained with a mouse anti-GST monoclonal antibody (a kind gift from Dr. M. Yamagata in our laboratory).

Results

Immunohistochemistry

Sections of normal articular cartilage from adult post-traumatic knees showed no reaction with the anti-PG-M antibodies (Fig. 1a). In the 5 cartilage samples from the femoral heads of patients with osteoarthritis, chondrocytes were not arranged in a normal pattern typical of articular cartilage as described previously (15), and the specimens showed destructive changes of various grades (abnormal hyaline cartilage , fibrocartilage, and fibrous tissues). The osteoarthritic cartilage taken from the lateral posterior region of the femoral heads had a yellowish and slightly fibrillated surface. The cytoplasm of the chondrocytes in this cartilage was stained strongly (Fig. 1b), and pericellular deposition of the antigen suggested its active synthesis of PG-M by the chondrocytes (Fig. 1d). The same sections showed no reaction when treated with non-immunized rabbit anti-serum(Fig. 1c and 1e). In more severely osteoarthritic cartilage samples composed of fibrocartilage and fibrous tissues, hypertrophic cells and proliferating chondrocytes were strongly stained both intracellularly and pericellularly (Fig. 1f and 1g). Figures 1g and 1h show that the antigen was also prominent in the extracellular matrix of severely degraded osteoarthritic cartilage, fibrocartilage, and fibrous tissues, suggesting that fibroblasts in these tissues may synthesize PG-M. The same results were obtained with or without chondroitinase ABC digestion.

DNA sequencing and homology analysis of PG-M

Because the PG-M antigen was strongly expressed in osteoarthritic cartilage and thus might be related to pathogenesis of osteoarthritis, we attempted to further characterize this molecule by cDNA cloning and protein homology analysis. We isolated two cDNA clones (λMa and λMb) reactive to the anti-PG-M antibodies from 2.5 x 10^5 independent clones of a λgt 11 expression library derived from stage 22-23 chick limb buds. λMa was 3243 nucleotides long (Fig. 2), including 819 nucleotides of the 3'-noncoding sequence, while Mb had the identical 3'

sequence but was 47 nucleotides shorter than λMa at the 5' end. λMa encoded 808 amino acids from the carboxyl terminus of the polypeptide. Homology analysis of the sequences revealed a complement regulatory protein-like domain (residues 704-764) , a lectin-like domain (residues 575-703) , and two EGF-like domains (residues 498-536 and 537-574) from the carboxyl end. The same set of domain elements has been identified in the carboxyl terminal portion of versican, a large chondroitin sulfate proteoglycan expressed by human fibroblasts (16). The homologies of PG-M and versican were high, being 93% for the complement regulatory protein-like domain, 96% for the lectin-like domain, and 68 and 84% for the two EGF-like domains (Fig. 3). This high level of homology suggested that PG-M may be the molecular equivalent of versican. Taken together with the immunohistochemistry data, our findings indicate that PG-M/versican is abnormaly expressed in osteoarthritic tissues. In addition, the presence of domains with evolutionarily conserved primary sequences suggests that these domains may be important for the function of PG-M.

Immunological relationship between PG-M and versican

The PG350 and Ver-27b fusion proteins purified from E. coli transfected with fused cDNAs coded the carboxyl and amino terminal portions of versican core protein, respectively. They were blotted onto nitrocellulose membranes and stained with the anti-PG-M antibodies. As a control, duplicate blots were stained with a mouse anti-GST monoclonal antibody (Fig. 4). The anti-PG-M antibodies strongly stained the Ver-27b fusion protein, but did not stained the PG350 fusion protein. The molecular weights of the Ver-27b fusion protein, the PG350 fusion protein, and GST were expected to be about 67,000, 49,000, and 23,000, respectively. The monoclonal antibody against GST not only stained both proteins but also stained GST itself. The extremely high homology of the deduced amino acid sequences of the carboxyl terminal domains of PG-M and versican might have caused their loss of antigenicity, and could explain the lack of cross-reactivity between the PG350 fusion protein and the anti-PG-M antibodies.

Discussion

We have investigated the expression of PG-M in osteoarthritic cartilage by immunohistochemistry and found that osteoarthritic cartilage of various grades was all positive for this protein. The cytoplasm of the chondrocytes in hyaline cartilage with osteoarthritic changes was strongly stained. In the case of fibrocartilage and fibrous tisssues, not only the cytoplasm of the mesenchyme-like cells invading the cartilage but also the extracellular matrix were stained by the anti-PG-M antibodies. These results suggest that chondrocytes synthesize PG-M in osteoarthritic cartilage. Chondrocytes usually show increased proliferation in osteoarthritic cartilage (17, 18), and this may eventually result in their differentiation into hypertrophic cells (19) that are no longer able to synthesize functional cartilage matrix consisting of aggrecan and collagen (types II, VI, IX, and XI) (20). Changes of collagen synthesis in osteoarthritic cartilage have also been reported (20, 21). For example type X collagen appears to be localized around chondrocyte clusters in fibrillated osteoarthritic cartilage, but is not detected in the noncalcified part of normal articular cartilage (20). Other studies have shown that the proteoglycans appearing in osteophytic cartilage are similar to those found in fetal cartilage, and may be a marker of immature cartilage (22, 23, 24). This is consistent with the fact that PG-M was first isolated from chick limb buds, which contain immature cartilage. These reports also support our suggestion that PG-M/versican is synthesized by the chondrocytes in osteoarthritic cartilage.

Our study also showed that the deduced amino acid sequence of the PG-M core protein included two EGF-like sequences, a C-type lectin-like sequence, and a complement regulatory protein-like sequence at the carboxyl terminal. Computer-assisted homology analysis revealed that the carboxyl terminal portion of PG-M showed an extremely high homology to the corresponding portion of versican, an embryonic human fibroblast proteoglycan (16), and immunological analysis confirmed this finding. Although the function and distribution of versican in human articular cartilage is not well known, our studies indicate that PG-

M/versican is expressed in osteoarthritic cartilage and may perhaps be useful as a marker for evaluating osteoarthritis.

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Legends for Figures

Fig. 1.

Immunolocalization of PG-M in normal (a) and osteoarthritic (b-i) human cartilage. (a), Normal articular cartilage shows no staining by the anti-PG-M antibodies. (x 100.). (b), Macroscopically yellowish and slightly fibrillated cartilage. The cytoplasm of the chondrocytes is stained strongly. (x 400). (c), The same section as shown in (b) is not stained by non-immunized rabbit serum. (x 400). (d, f, g, h), More severely degraded osteoarthritic cartilage. The PG-M antigen is prominent in the cytoplasm and the extracellular matrix. (d x 400, f x 200, g x 200, h x 100). (e) and (i), The same sections as shown in (d) and (h), respectively. They are not stained by the non-immunized rabbit serum. (e x 400, i x 100).

Fig. 2.

Partial nucleotide sequence of the cDNA encoding PG-M and the predicted amino acid sequence. A complement regulatory protein-like domain (residues 704-764), a lectin-like domain (residues 575-703), and two EGF-like domains (residues 498-536 and 537-574) are present from the carboxyl terminal. These are indicated by the dotted line, the thick line, and the thin line, respectively. Arrows and triangles indicate potential sites for N-glycosylation and chondroitin sulfate attachment, respectively.

Fig. 3.

The deduced amino acid sequence based on analysis of the λMa cDNA clone was compared with the sequences of the corresponding parts of versican using the DNASIS program (Hitachi Co., Tokyo).

Fig. 4.

The reactivity of amino terminal and carboxyl terminal versican fusion proteins with the antibodies to PG-M. The fusion proteins expressed by plasmids constructed for the Ver-27b clone (lanes 1 and 3) and for the PG350 clone (lanes 2 and 4), and by pGEX-3X constructed as the control (lane 5), were stained with the antibodies to PG-M (lanes 1 and 2) and with a monoclonal antibody to GST (lanes 3, 4, and 5) after transfer onto a nitrocellulose membrane,

Fig. 1.

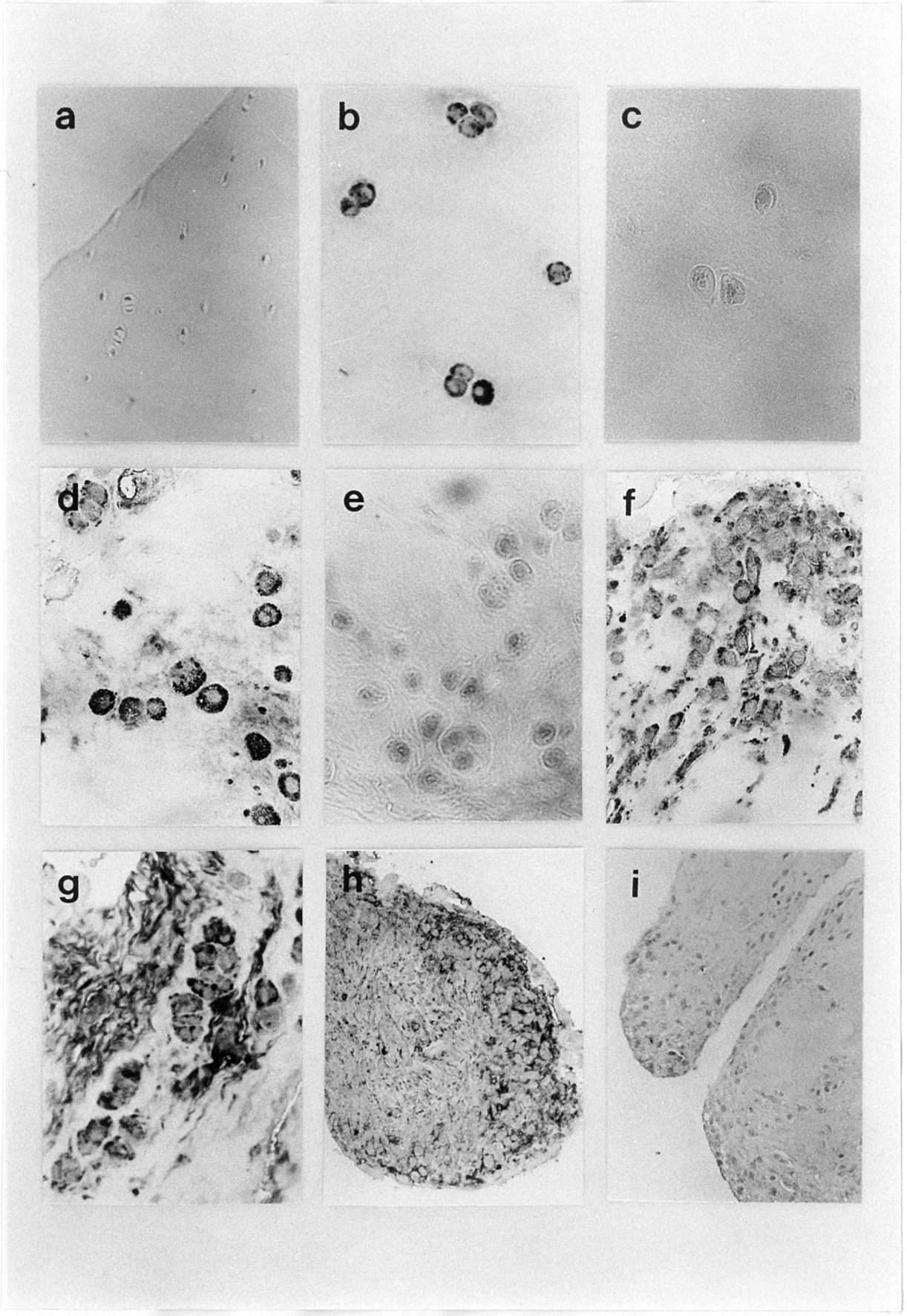

Fig. 2.

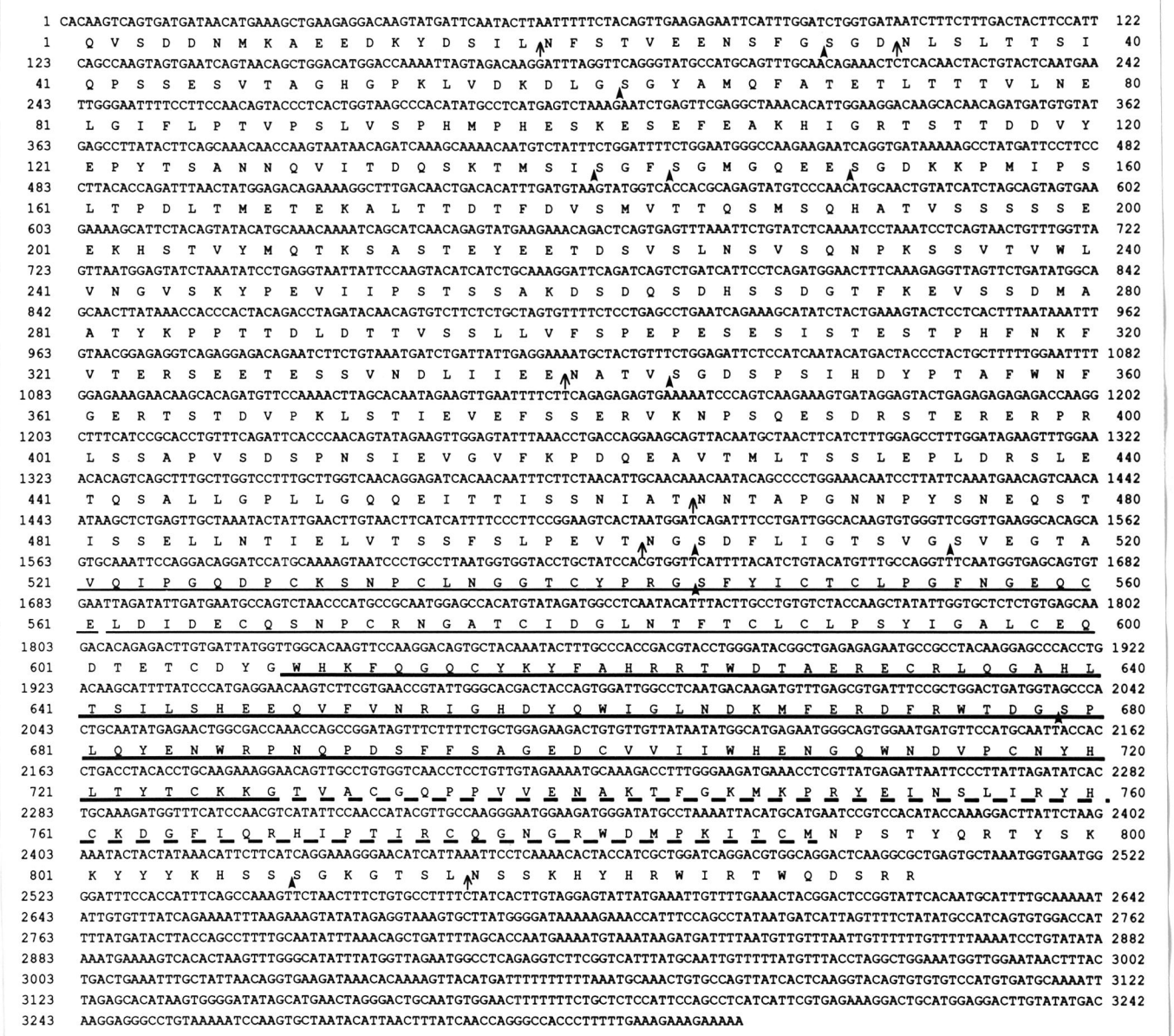

Fig. 3.

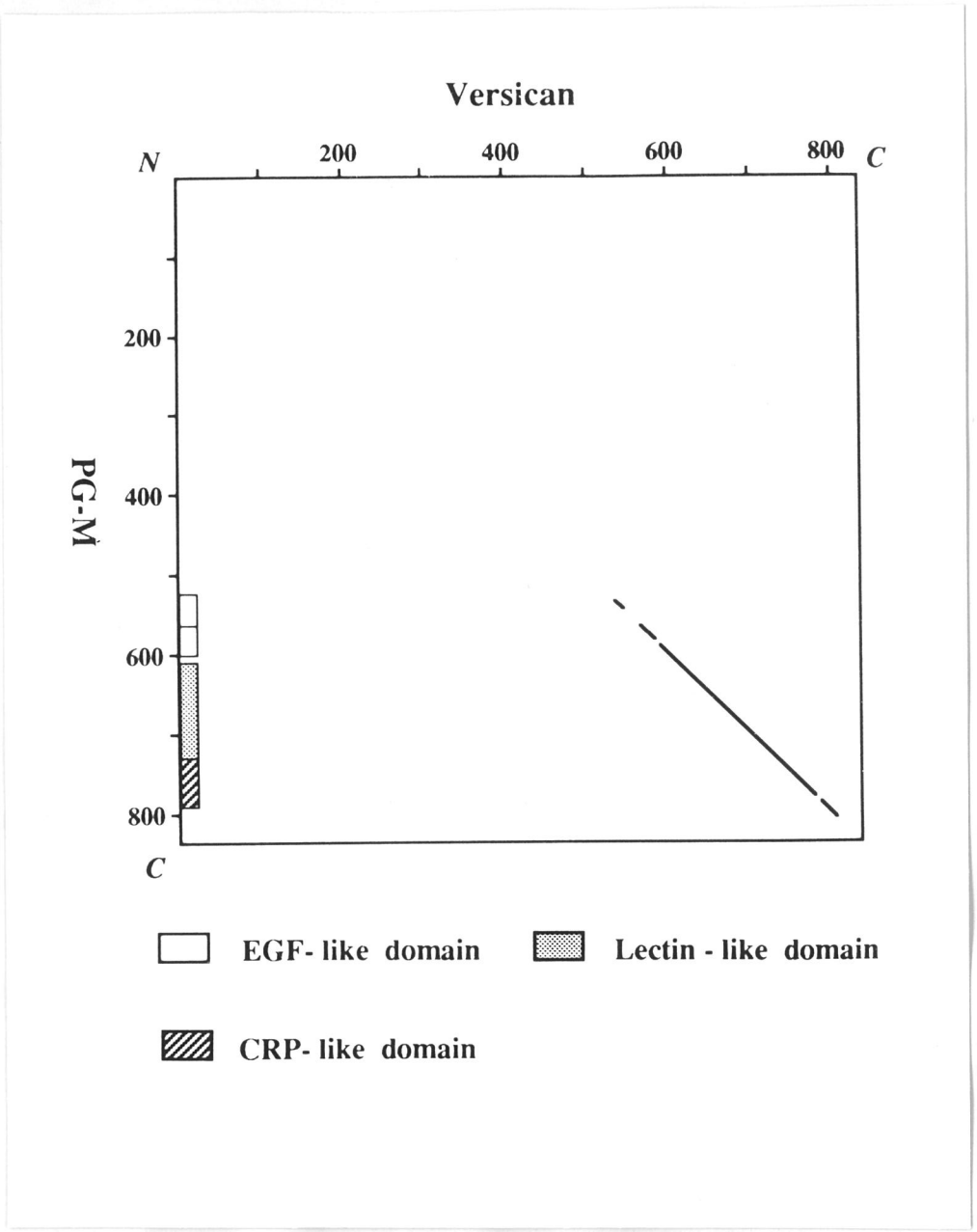
Versican
N
200
400
600
800
C
200
400
600
800
C
PG-M
EGF- like domain
Lectin - like domain
CRP- like domain

Fig. 4.