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Isolation and Characterization of GBP28, a Novel Gelatin-Binding Protein Purified from Human Plasma

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By use of its affinity to gelatin-Cellulofine, a novel protein, GBP28 (gelatin-binding protein of 28 kDa), was obtained from human plasma. GBP28 bound to gelatin-Cellulofine could be eluted with 1 M NaCl. By analysis of its amino-terminal amino acid sequences and the peptides obtained by protease digestion, GBP28 was identified as a novel protein. After repeated gel chromatography of the 1 M NaCl eluate from gelatin-Cellulofine, about 50 μg of GBP28 was purified from 500 ml of human plasma. On gel chromatography, the protein migrated as a molecule of about 420 kDa. On SDS-PAGE, its molecular mass was 28 kDa under reducing conditions and 68 kDa under nonreducing conditions. Recently, human mRNA specific to adipose tissue, cDNA clone apM1, has been registered [Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. (1996) *Biochem. Biophys. Res. Commun.* 221, 286-289]. The assumed amino acid sequence of cDNA clone apM1 contained all the sequences of GBP28 and its peptides. Therefore, it is evident that the cDNA clone apM1 encodes GBP28 and the protein is specific to adipose tissue. The clone encodes a polypeptide of 244 amino acids with a secretory signal sequence at the amino terminus, a small non-helical region, a stretch of 22 collagen repeats and a globular domain. Thus, GBP28 appears to belong to a family of proteins possessing a collagen-like domain through which they form homo-trimers, which further combine to make oligomeric complexes. Although its biological function is presently unclear, its adipocyte-specific expression suggests that GBP28 may function as an endogenous factor involved in lipid catabolism and storage or whole body metabolism.

Key words: adipocyte, collagen-like domain, extracellular matrix, GBP28, gelatin-binding.

The extracellular matrix (ECM) is composed largely of collagen fibers and glycosaminoglycans. Until recently, ECM was thought to serve only as a scaffold to stabilize the physical structure of tissues. Now it is becoming clear that ECM plays a more active and complex role in regulating the behavior of the cells that contact it. ECM secreted by fibroblasts and endothelial cells has been shown to function as a reservoir of growth factors, enzymes and inhibitors. Among glycosaminoglycans, heparan sulfate and heparin bind to many cytokines that affect cell growth and differentiation. Heparin affinity chromatography is hence commonly used for their purification (*1*). Gelatin affinity chromatography is commonly used for purification of matrix metalloproteinases, which are secreted by resident cells of tissues, like fibroblasts and epithelial cells, or by cancer cells (*2, 3*). These ECM-degrading metalloproteinases play key roles in the turnover of ECM components during tissue remodeling, wound healing and pathophysiological processes, such as tumor invasion and metastasis (*4-6*).

Recently, ECM-stabilizing activity of inter-α-trypsin inhibitor (ITI) family members has been reported (*7, 8*) and heavy chains of the ITI family have been shown to bind to hyaluronic acid, which is a common ECM component (*9*). The ITI family proteins are soluble in plasma and the above characteristics suggest that this family has a role in wound healing. Many proteins in plasma have also been reported to bind to heparin (*10*). In view of these interesting findings, we assumed that there might be plasma proteins that bind to collagen fibers, another major component of ECM, and searched for such novel plasma proteins using gelatin affinity chromatography.

EXPERIMENTAL PROCEDURES

Materials—TPCK-trypsin, KLH (keyhole limpet hemocyanin), Nitroblue tetrazolium, 5-bromo-4-chloro-3-indonyl phosphate, and PVP-40 (Polyvinylpyrrolidone-40) were purchased from Sigma Chemicals, St. Louis, MO.

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Abbreviations: GBP28, gelatin-binding protein of 28 kDa; ECM, extracellular matrix; OVA, ovalbumin; FN, fibronectin; Acrp30, adipocyte complement-related protein of 30 kDa; HP, hibernation-associated plasma protein; C1q-C, C subunit of complement factor C1q; SP-A, pulmonary surfactant-associated protein A; MBP-C, mannose-binding protein C.

Achromobacter lyticus protease I (lysylendoprotease) was from Wako Pure Chemicals, Osaka. A column for HPLC, SynChropak RP-P (250×2.1 mm ID), was from SynChrom, Lafayette, IN. Gelatin-Cellulofine and FMP (2-fluoro-1-methylpyridinium toluene-4-sulfonate)-activated Cellulofine were from Seikagaku, Tokyo. A gel chromatography column, HiPrep 16/60 Sephacryl S-300 HR (600× 16 mm ID), and activated thiol Sepharose 4B were obtained from Pharmacia LKB Biotech, Uppsala. Sulfo-MBS (sulfo-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) was purchased from Pierce, Rockford, IL. Renaissance™ Western blot chemiluminescence reagent was from DuPont NEN, Boston, MA. PVDF membrane, ProBlott™, was from Perkin Elmer Applied Biosystems, CA. Natural *N*-glycanase enzyme was obtained from Genzyme, Cambridge, MA.

Purification of GBP28—EDTA was added at a final concentration of 5 mM to human plasma (500 ml). The plasma was applied to a gelatin-Cellulofine column (25 ml) equilibrated with 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl (TBS). After thorough washes with TBS containing 0.1% NP40 and with TBS to remove NP40, proteins were eluted with 1 M NaCl in 10 mM Tris-HCl, pH 7.4, and with 6 M urea, 0.2 M NaCl in 10 mM Tris-HCl, pH 7.4. The fractions containing GBP28 were then applied to HiPrep 16/60 Sephacryl 300-HR column equilibrated with TBS containing 1 mM EDTA. By repeating the gel chromatography, GBP28 was further purified.

SDS-PAGE—SDS-PAGE was performed according to Laemmli's method using 10 or 11% polyacrylamide slab gel (*11*). After electrophoresis, proteins in the gels were stained with Coomassie Brilliant Blue using RAPID KANTO CBB (Kanto Chemical).

Preparation of Anti-GBP28 Rabbit Antisera—Two peptides corresponding to the amino-terminal 20 amino acids, ETTTQGPGVLLPLPKGACTG, and to the carboxyl-terminal 20 amino acids with one extra cysteine at its amino-terminal, CYADNDNDSTFTGFLLYHDTN, were synthesized with an Applied Biosystems 432A peptide synthesizer. According to manufacturer's instructions, each sulfhydryl-containing peptide was conjugated to the carrier protein, KLH, using Sulfo-MBS. Then rabbits were immunized with each peptide conjugated to KLH as an emulsion with Freund's complete adjuvant.

Western-Blot Analysis—After SDS-PAGE, proteins in the gel were transferred electrically onto a nitrocellulose membrane. The membrane was rinsed twice with 10 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS) and then blocked with 3% non-fat dry milk in PBS containing 0.05% tween 20 (TPBS) for 15 min. Then the membrane was incubated with rabbit antiserum solution (7,500-fold dilution in TPBS containing 3% non-fat dry milk) for 15 min at room temperature. After washes with TPBS for 5 min three times, the membrane was incubated with a peroxidase-conjugated anti-rabbit IgG or a horseradish peroxidase-conjugated anti-rabbit IgG solution (2,000-fold dilution in TPBS containing 1% non-fat dry milk) for 15 min at room temperature. The membrane was rinsed twice and washed three times with TPBS, then color was developed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indonylphosphate or chemiluminescence reagent.

Sequence Analysis—GBP28-containing fractions were electrophoresed and transferred to PVDF membrane (ProBlott™). Protein bands were detected with Coomassie Brilliant Blue and the membrane was rinsed thoroughly with water and dried. Then each protein band was excised and its amino-terminal amino acid sequence was analyzed with an Perkin Elmer Applied Biosystems 473A Protein Sequencer. Cys was determined after the derivatization to *S*-4-pyridylethyl-cysteine as described in Protein Sequencer User Bulletin (Perkin Elmer Applied Biosystems) except that the derivatization was done in the gas phase. The excised band containing GBP28 was used for preparation of peptide fragments by the method described by Shohet *et al.* (*12*). Briefly, after washes with water, the band was incubated with PVP-40 for 30 min at room temperature. The excess PVP-40 was removed by extensive washes with water and the membrane was treated with lysylendopeptidase or trypsin in 0.1 M Tris-HCl, pH 8.8, at 37°C overnight. Assuming that 5 μg of protein was contained in the preparative band, GBP28 in the band was treated with each enzyme at an enzyme to substrate ratio of 1 : 50 (w/w). After digestion, the supernatant was removed, and peptides were eluted from the band with 40% acetonitrile and then 40% acetonitrile with 0.1% trifluoroacetic acid (TFA). All the elution supernatants were combined with the original supernatant. Proteolytic fragments in the pooled supernatant were fractionated by HPLC on a SynChropak RP-P column equilibrated in 5% acetonitrile and 0.1% TFA using a linear gradient of acetonitrile (5-70%). The amino acid sequence of each peptide was analyzed as above.

Sugar Analysis—GBP28 was digested with *N*-glycanase overnight according to the manufacturer's instructions (Genzyme). Briefly, GBP28 (50 ng) in 5 μl of TBS containing 0.5% SDS, 50 mM 2-mercaptoethanol (2-ME), and 50 mM EDTA was boiled for 5 min. After the addition of 2.5 μl of 7.5% NP-40, 6.5 μl of water, and 1 μl (0.05 unit) of *N*-glycanase, the mixture was incubated at 37°C overnight. Two mixtures without *N*-glycanase were also prepared. One was incubated at 37°C overnight and the other was kept at −20°C. As a positive control, 5 μg of ovalbumin (OVA) in 5 μl of the same buffer was treated as above. The digested samples were analyzed by SDS-PAGE followed by Western-blot analysis with the anti-amino-terminal peptide of GBP28 rabbit antiserum and alkaline phosphatase-conjugated anti-rabbit IgG.

Affinity Precipitations—The synthetic peptide (1 mg) in 0.1 M Tris-HCl, pH 7.4, 0.5 M NaCl, and 1 mM EDTA was applied to activated thiol-Sepharose 4B (4 ml) in the same buffer and coupling was monitored by measuring the absorbance at 343 nm. Then the resin was washed thoroughly with TBS. From rabbit antisera, anti-amino-terminal peptide and carboxyl-terminal peptide of GBP28 antibodies (anti-N and anti-C antibodies) were affinity-purified using the respective peptide-coupled Sepharose columns.

Human fibronectin (FN) was purified from human plasma with a gelatin-Cellulofine column. After GBP28 had been eluted with 10 mM Tris-HCl, pH 7.4, containing 1 M NaCl, FN was eluted with 10 mM Tris-HCl, pH 7.4, containing 6 M urea and 0.2 M NaCl.

Each affinity-purified antibody and FN in 10 mM sodium carbonate, pH 8.5, was coupled to FMP-activated Cellulofine (2 mg protein/ ml gel) according to the manufacturer's instructions.

Human plasma, mouse plasma, or purified GBP28 was incubated with one of the above resins for 10 min at 4°C.

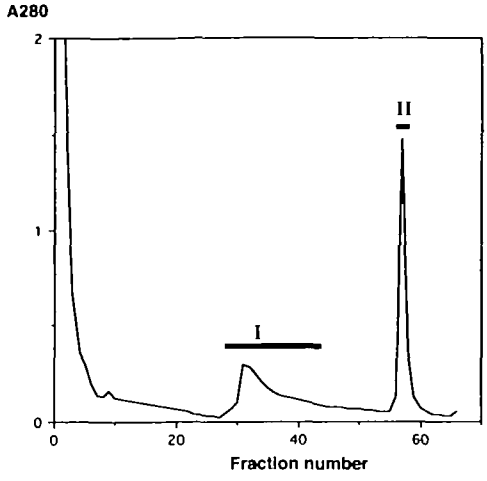

Fig. 1. **Affinity chromatography of human plasma proteins using gelatin-Cellulofine.** Pooled human plasma (500 ml) containing 5 mM EDTA was applied to a gelatin-Cellulofine column (25 ml) in TBS. Washes and elutions were performed as described in "EXPERIMENTAL PROCEDURES." Elution with 1 M NaCl started at fraction 29, and that with 6 M urea at fraction 56. Fractions of 10 ml per tube were collected.

The affinity resin was then washed thoroughly with TPBS and boiled in Laemmli's sample buffer containing 2-ME. The samples were analyzed by SDS-PAGE followed by Western-blot analysis with the anti-N or anti-C antibody.

RESULTS

Purification of GBP28 from Human Plasma—Human pooled plasma was applied to the column of gelatin-Cellulofine (Fig. 1). Fractions were analyzed by SDS-PAGE. The eluate with 1 M NaCl was pooled as pool I and the eluate with 6 M urea was pooled as pool II. Pools I and II were electrophoresed and transferred to ProBlot membrane. The protein bands stained with Coomassie Blue were excised and their amino-terminal amino acid sequences

TABLE I. **N-terminal amino acid sequence of GBP28 and its fragments produced with lysylendopeptidase or trypsin.** N-terminal amino acid sequences were determined with the 473A Protein Sequencer (Perkin Elmer Applied Biosystems). P represents hydroxyproline.

GBP28:	ETTTQGPGVLLPLPKGACTG
L10:	IFYNQQNHYDGSTGK
L11:	VSLFKK
L12:	VSLFK
T8 :	KGEPGEGAYVYR
T10:	GFPGIQGR

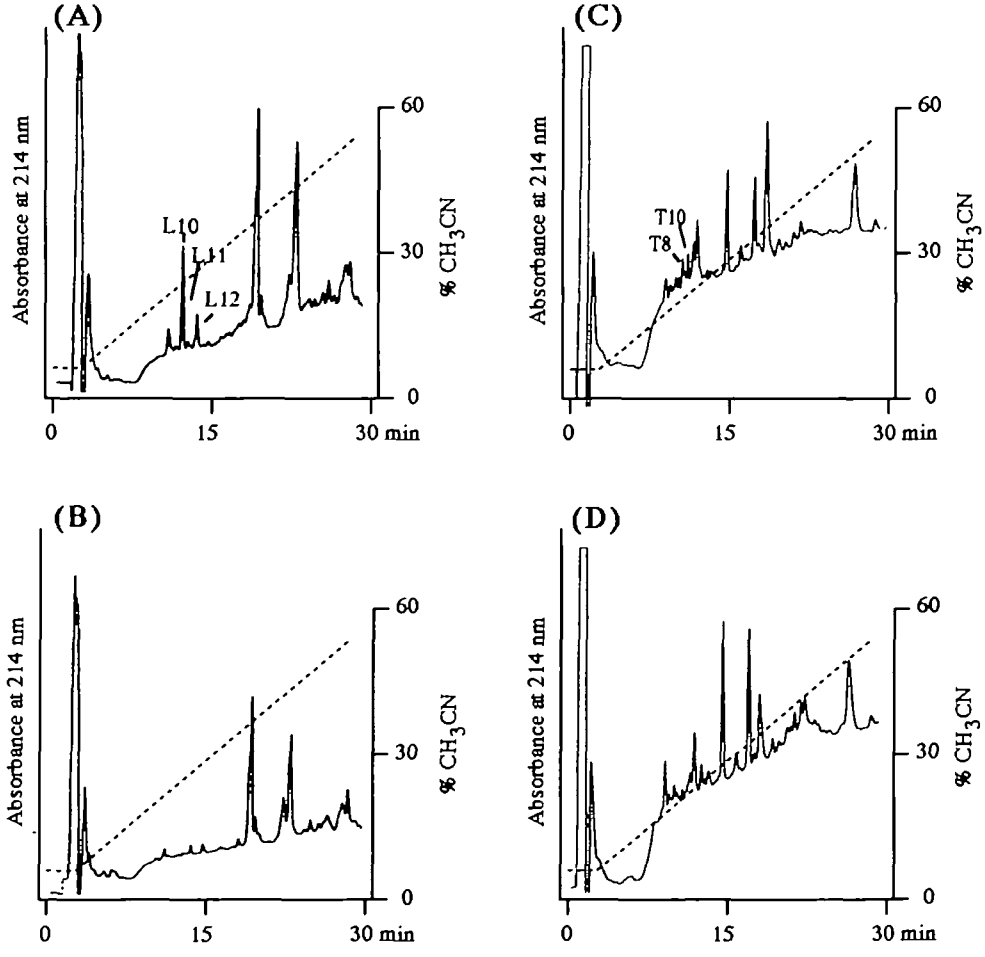

Fig. 2. **HPLC of the lysylendopeptidase digest and trypsin digest of GBP28.** Each enzyme digest of GBP28 was prepared and separated as described in "EXPERIMENTAL PROCEDURES." As a control, a slip of PVDF membrane was treated with the same reagents. (A), the lysylendopeptidase digest of GBP28; (B), the control slip treated with lysylendopeptidase; (C), the tryptic digest of GBP28; (D), the control slip treated with trypsin.

were analyzed with the protein sequencer. In pool I, we found one novel protein that had the amino-terminal sequence ETTTQGPGVLLPLPKGACTG (Table I) and the molecular mass of about 28 kDa on SDS-PAGE. This protein band was digested with lysylendopeptidase and the resulting peptides were fractionated by HPLC (Fig. 2A). Although several peaks resulted from reagents (Fig. 2B), three peaks, L10, L11, and L12 in Fig. 2A, were sequenced. The same membrane band was treated with PVP-40 again and further digested with TPCK-trypsin. Two peaks, T8 and T10, were selected and analyzed (Fig. 2C). Peaks resulting from reagents are shown in Fig. 2D. From the sequences of these peptides (Table I), this protein was confirmed to be novel. For further purification, gel chromatography using HiPrep Sephacryl 300-HR was performed (Fig. 3A). Fractions from 47 to 57 contained IgM as a predominant protein. Fractions from 72 to 87 contained IgG. Fractions from 62 to 72 contained fragments of FN. Fractions from 55 to 61 were pooled because of the presence of 28-kDa protein on SDS-PAGE (Fig. 3E), and by repetition of gel chromatography, the 28-kDa protein was purified (Fig. 3, B–D and F–H). When fractions from 55 to 61 in Fig. 3A were applied to the same column, fractions from 54 to 64 in Fig. 3B contained the 28-kDa protein (Fig. 3F). Fractions from 54 to 58 contained IgM as a major contaminant and fractions from 59 to 66 contained FN fragments as a major contaminant (Fig. 3F). Fractions from 52 to 58 and fractions from 59 to 66 were pooled and each pooled fraction was applied to the same column separately (Fig. 3, C and D). As shown in Fig. 3G, fractions from 53 to 61 in Fig. 3C contained IgM, which was hard to remove completely. As shown in Fig. 3H, fractions from 62 to 67 in Fig. 3D contained FN fragments. Avoiding fractions containing IgM (Fig. 3G) and FN fragments (Fig. 3H), only a portion of GBP28 could be isolated. Finally, from 500 ml of human plasma, about 50 μg protein was obtained. Because of its molecular mass and affinity to gelatin, this protein was tentatively named GBP28 (gelatin-binding protein of 28 kDa).

SDS-PAGE and Western-Blot Analyses of GBP28—When GBP28 was boiled in Laemmli's loading buffer for 3 min, it showed a single band of 28 kDa on SDS-PAGE under reducing conditions and a single band of 68 kDa under nonreducing conditions (Fig. 4A). Surprisingly, when

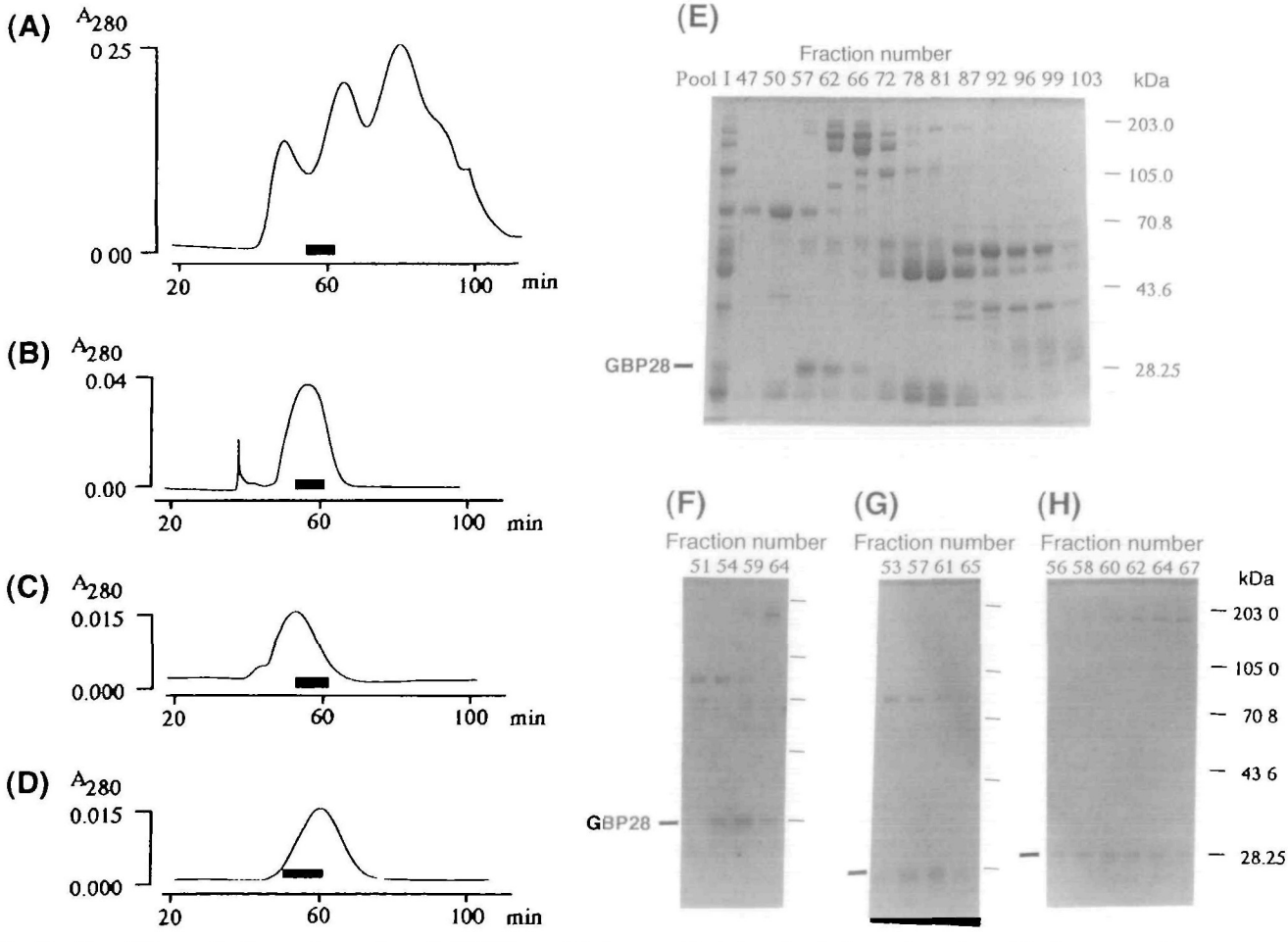

Fig. 3. **Repetitive gel chromatography of GBP28-containing pool obtained from affinity chromatography.** (A) Pool I from gelatin-Cellulofine was applied to a HiPrep 16/60 Sephacryl 300-HR column in TBS. Fractions of 0.7 ml per tube were collected. GBP28 was detected by SDS-PAGE, as shown in (E), and its position is indicated by a horizontal bar. (B) Fractions from 55 to 61 in (A) were collected and gel chromatography with the same column was performed. Its SDS-PAGE profile is shown in (F). (C) Fractions from 52 to 58 in (B) were applied to the same column and analyzed by SDS-PAGE (G). (D) Fractions from 59 to 66 in (B) were applied to the same column and analyzed by SDS-PAGE (H).

GBP28 in Laemmli's loading buffer was not boiled, the protein band shifted from 28 to 70 kDa under reducing conditions, and from 68 to 150 kDa and the bands near gel top under nonreducing conditions. When these samples in the loading buffer or in the loading buffer containing 6 M urea were incubated overnight at room temperature, the same patterns were obtained on SDS-PAGE. The 70-kDa band under reducing conditions was transferred to ProBlot membrane and the protein band was excised for sequence analysis. The amino-terminal amino acid sequence of the 70-kDa band was identical to that of GBP28 (data not shown).

As shown in Fig. 4B, when GBP28 was heat-denatured at 100°C with or without 2-ME, the bands could be detected by both anti-N and anti-C antibodies. However, when the proteins were not heat-denatured, the bands could not be detected by the antibodies. Because high-molecular-weight proteins are known to be inefficiently transferred to blot membranes, the low transfer might be the reason that bands of 150 kDa and near 440 kDa could not be detected by the antibodies. On the other hand, although the 70-kDa band could be transferred to a membrane, it could not be detected by either antibody. As shown in Fig. 7A, lane 1, when a membrane was dried by hot air after transfer of the protein, the 70-kDa band became detectable by the antibodies, and a weak band of 70 kDa appeared. These results indicate that neither amino-terminal nor carboxyl-terminal portions of GBP28 were on a surface of the intact molecule, or that they had secondary structures different from the peptide antigens.

Structure of GBP28—Using the sequences in Table I, we tried to generate a cDNA probe by the polymerase chain reaction using human liver cDNA (QUICK-Clone™ cDNA, Clontech, Palo Alto, CA) but could not obtain such a probe. Recently, human mRNA specific to adipose tissue, cDNA clone apM1, has been registered by Maeda *et al.* (*13*). We found that its assumed amino acid sequence contained all the sequences in Table I (Fig. 5). Therefore, it is evident that the mRNA encodes GBP28. Maeda *et al.* reported that the mRNA was detected only in subcutaneous fat and visceral fat. More recently, Scherer *et al.* have reported Acrp30 (adipocyte complement-related protein of 30 kDa) (*14*). They obtained the clones from a subtractive cDNA library enriched in mRNAs which were induced during adipocyte differentiation of mouse 3T3-L1 fibroblasts, in order to identify novel adipocyte-specific proteins. Because the amino acid sequence of Acrp30 showed 82.7% homology to that of GBP28 (Fig. 6), Acrp30 appears to be the mouse counterpart of GBP28. By comparison of our results of the amino-terminal sequence analysis of GBP28 with the assumed amino acid sequence of cDNA clone apM1, it is evident that the proGBP28 has a signal sequence of 18 amino acids, and that the mature GBP28 consists of an amino-terminal small non-helical region of 23 amino acids, a stretch of 22 collagen repeats with 8 "perfect" Gly-X-Pro repeats and 14 "imperfect" Gly-X-Y repeats, and the carboxyl-terminal 137 amino acids that probably form a globular domain. The mature GBP28 contains 226 amino

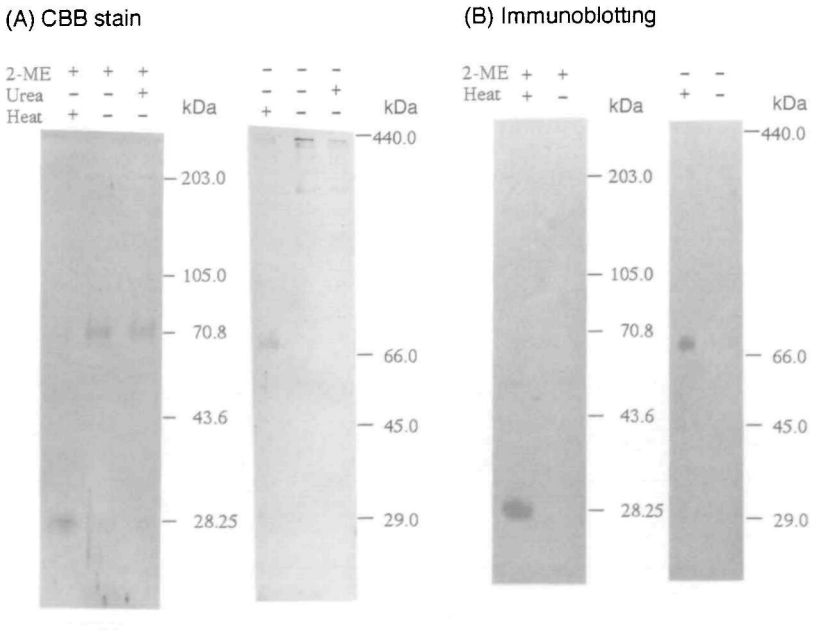

Fig. 4. **SDS-PAGE and Western-blot analyses of GBP28.** (A) Purified GBP28 (300 ng) was analyzed by SDS-PAGE (10% gel) with or without 6 M urea, heat-denaturing at 100°C and 2-ME as described in "EXPERIMENTAL PROCEDURES." (B) Purified GBP28 (50 ng) was electrophoresed with or without heat-denaturing and 2-ME, and after transfer into a nitrocellulose membrane, GBP28 was detected with anti-N antibodies and alkaline phosphatase-conjugated anti-rabbit IgG. The migration distances of molecular size markers under reducing and nonreducing conditions are indicated and the migration distance of FN under nonreducing conditions is marked as 440 kDa.

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1                   20                  40                  60
MLLLGAVLLLLALPGHDQETTTQGPGVLLPLPKGACTGWMAGIPGHPGHNGAPGRDGRDG

                    80                  100                 120
TPGEKGEKGDPGLIGPKGDIGETGVPGAEGPRGFPGIQGR KGEPGEGAYVYRSAFSVGLE
                                    T10         T8
                   140                 160                 180
TYVTIPNMPIRFTKIFYNQQNHYDGSTGKFHCNIPGLYYFAYHITVYMKDVKVSLFK KDK
                        L10                         L12,11
                   200                 220                 240
AMLFTYDQYQENNVDQASGSVLLHLEVGDQVWLQVYGEGERNGLYADNDNDSTFTGFLLYHDTN*
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Fig. 5. **Deduced amino acid sequence of GBP28.** The deduced amino acid sequence of cDNA clone apM1 is presented (*13*). The amino-terminal amino acid sequences of mature GBP28 and its enzymatic fragments in Table I are underlined. A putative *N*-glycosylation site is double-underlined, and **P** represents hydroxyproline.

acids with a predicted molecular mass of 24.5 kDa.

Several proteins are known to contain an amino-terminal collagen-like domain with a carboxyl-terminal globular domain (Fig. 10). Among them, complement factor C1q (*15*), type VIII and type X collagens (*16, 17*), and hibernation-associated plasma proteins (HP27, HP25, and HP20) (*18*), showed significant homology to GBP28. By computer analysis of the overall sequence identity using a program Genetyx (SDC, Tokyo), GBP28 has 83% amino acid identity with Acrp30, 39% with C subunit of complement factor C1q (C1q-C), 28% with HP27, and 41% and 36% with the 233 and 196 carboxyl-terminal amino acids of type X collagen and type VIII collagen, respectively (Fig. 6). Especially in the carboxyl-terminal globular domain, there are apparently conserved sequences among those proteins. Thus, GBP28 is suggested to be a member of a protein family that has a collagen-like domain through which they form oligomeric complexes of homo- or hetero-trimers. Beside the above proteins, precerebellin and multimerin, which have no collagen-like domain but a carboxyl-terminal globular domain similar to C1q, also showed significant homology to GBP28 (*19, 20*).

pH Effect on the Conformation of GBP28—GBP28 contains one Cys residue before and after the collagen-like domain as shown in Fig. 5. The disulfide-bonding pattern in one trimer remains to be elucidated. The 1 M NaCl eluate from gelatin-Cellulofine was prepared from fresh plasma containing 0.1 M ICH_2COOH and analyzed by SDS-PAGE. Surprisingly, when a final concentration of 0.1 M ICH_2COOH was added to the eluate, which was then electrophoresed under reducing conditions without heat-denaturing, the band of GBP28 was shifted from 70 to 28 kDa and detectable by both anti-N and anti-C antibodies (Fig. 7A, lane 4). This change was also observed with CH_3COOH treatment instead of ICH_2COOH treatment (Fig. 7A, lane 5). Under nonreducing conditions without heat-denaturing, regardless of whether treated with ICH_2COOH or CH_3COOH, the band of GBP28 shifted to 68 kDa and became detectable by the antibodies (Fig. 7A, lanes 8 and 9). When the ICH_2COOH was added after Na_2CO_3 addition, the shift was inhibited (Fig. 7A, lane 10). These results suggested that the pH of the solution affects the GBP28 oligomer. When the pH of the solution was adjusted between 6 and 2.5, the band of GBP28 in the solution below pH 6 was shifted to 68 kDa under nonreducing conditions without heat denaturing, although at pH 6, this effect was small (Fig. 7A, lanes 11 to 14). When the size of purified GBP28 treated with ICH_2COOH or CH_3COOH was also analyzed,

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GBP28     1:           MLLLGAVLL LLALPGH  DQETTTQG  PGVLLPLPKGACTGWMAGIPGHPGHNGA
Acrp30    1:           ----Q- --F--I--S-AE-DV---EELA-A -V-P---T-A--------------T
C1q-C     1: MDVGPSSLPHLG-KLLL--L----R-QAN   -GCY-I --MPG -- --P -KD -YD-LP-PK-E
X       484:                                                        -LN-PT-P
VIII    513:                             GIPGPKGEPGLPGPPGF-GIGKPGVAGLH-P--K--AL-P
HP27      1:                                      MYEAGKRASFM-GAGI-ILALSVLM -VVC

GBP28    53: PGRDGRDGTPGEKGEKGDPGLI    GPKGD IGETGVPGAEGPRGFPGIQGRKGEPGEGAYVYRSA
Acrp30   56: ------------------A--L    ----E T-DV-MT----------TP--------A--M----
C1q-C    60: --IPAIP-IR-P--Q--E---P    -HP-KN -PM-P--MP-VP-PM--P-EP--E-RYKQKFQ-V
X       492: --PP-PR-PS--P-LP-P--PP    --P-QAVMPE-FIK-GQRPSLS-TPLVSANQ-VTG MPV--
VIII    553: Q-QP-LP-P--PP-PP-P-AVMPPTPP-Q-E YLPDMGL-ID-VKP-HATGAK--KN-GP--E MP-
HP27     30: SETQ-NPESCNVP-PQ-P--MR    --P-TP -KP-P--WN-FP-L--PP-PP-MTVNCHSKGT--
conserved               G  G           G

GBP28   115: FSVGLETY VTIPNMPIRFTKIFYNQQNHYDGSTGKFHCNIPGLYYFAYHITVYMKDVKVSLFKKDK
Acrp30  118: -------R --V--V----------------------Y---------S-------------------
C1q-C   122: -T-TRQ-HQPPA--SL---NAVLT-P-GD--T-----T-KV------V--ASHTANLC -L-YRSGV
X       554: -T-I-SK AYPAIGT--P-D--L--R-Q---PR--I-T-Q---I---S--VH-KGTH-W-G-Y-NGT
VIII    618: -TAE- -APFPPVGG-VK-N-LL--GRQN-NPQ--I-T-EV--V------VHCKGGN-W-A---NNE
HP27     92: -A-KANEL PPA-SQ-VI-KEALHDA-G-F-LA--V-T-PV----Q-GF--EAVQRA-----MRNGT
conserved   F                 F              TG F C  PG Y F  H         V L

GBP28   181: AMLFTYDQYQE NNVDQASGSVLLHLEVGDQVWLQVYGEGERNGLYADNDNDSTFTGFLLYHDTN:244
Acrp30  184: -V----------K ------------------------D-DH-------V---------------:247
C1q-C   188: KVV -FCGHTSKT-Q-N --G---R-Q--EE---A-   NDYYDMVGIQGS--V-S----FP-  :245
X       620: PVMY---E-TKGYL ------AIID-TEN------L PNA-S----SSEYVH-S-S---VAPM  :680
VIII    684: PVMY---E-KKGFL ------AV-L-RP--R-F--MPS- QAA----GQYVH-S-S-Y---PM  :744
HP27    158: QVM EREAEAQDGY EHI--TAI-Q-GME-R---E  NKLSQTD- ERGTVQAV-S---IHEN  :215
conserved                    SG     L     V L                    F G L
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Fig. 6. **Comparison of the amino acid sequences of GBP28 and proteins that show significant homology to GBP28.** Standard single-letter symbols are used for amino acid residues. "-" indicates identical amino acid residues with GBP28. Conserved indicates the residues identical in all six sequences. X, type X collagen; VIII, type VIII collagen $\alpha 1$ chain.

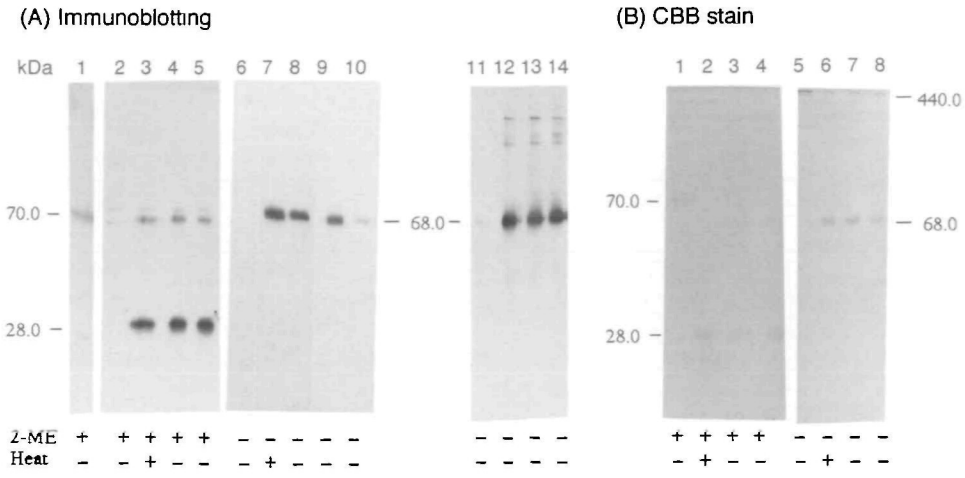

Fig. 7. **pH effect on GBP28.** (A) Fresh human plasma containing 0.1 M ICH_2COOH was applied to gelatin-Cellulofine and GBP28 was eluted with 1 M NaCl. Portions of 9 μl of the eluate corresponding to 200 μl of plasma were treated as below and electrophoresed with or without heat-denaturing and 2-ME. After transfer to a nitrocellulose membrane, a piece of membrane corresponding to lane 1 was dried with hot air, and GBP28 was detected with anti-C antibodies and alkaline phosphatase-conjugated anti-rabbit IgG. Lanes 1, 2, 3, 6, and 7, the sample without any treatment; lanes 4 and 8, 1 μl of 1 M ICH_2COOH was added to the sample, then the loading buffer and 1 μl of 2 M Na_2CO_3 were added; lanes 5 and 9, 1 μl of 1 M CH_3COOH was added to the sample, which was then treated as lanes 4 and 8; lane 10, 1 μl of 2 M Na_2CO_3 and the loading buffer were added to the sample, then 1 μl of 1 M ICH_2COOH was added. For lanes 11 to 14, the pH of each sample was changed, then the loading buffer was added; lane 11, 2.5 μl of 0.2 M sodium phosphate, pH 6.0, was added to the sample; lane 12, 1 μl of 1 M sodium acetate, pH 4.6, was added to the sample; lane 13, 1 μl of 1 M sodium acetate, pH 3.8, was added to the sample; lane 14, 2.5 μl of 0.2 M Gly-HCl, pH 2.5, was added to the sample. (B) Purified GBP28 (300 ng) was treated as above, and after electrophoresis, the protein bands were stained with Coomassie Brilliant Blue. Lanes 1, 2, 5, and 6, GBP28; lanes 3 and 7, 1 μl of 1 M ICH_2COOH was added to GBP28, then the loading buffer and 1 μl of 2 M Na_2CO_3 were added; lanes 4 and 8, 1 μl of 1 M CH_3COOH was added to GBP28, which was then treated as lanes 3 and 7.

without heat-denaturing, the band of 70 kDa shifted to 28 kDa under reducing conditions, while the bands of 150 kDa and near 440 kDa shifted to 68 kDa under nonreducing conditions, as shown in Fig. 7B. Therefore, at acidic pH, some interaction between GBP28 monomers and trimers could be destroyed.

Size Analysis of GBP28 in Plasma—The purified GBP28 migrated as a molecule of about 420 kDa by gel chromatography. To analyze the size of GBP28 in plasma, 1 ml of human pooled plasma was fractionated by the HiPrep 16/60 Sephacryl 300-HR (Fig. 8A). The molecule that bound to gelatin-Cellulofine migrated with an apparent molecular mass of 420 kDa (Fig. 8B). However, another molecule that could not bind to gelatin-Cellulofine was also detected by anti-N and anti-C antibodies; it was found in fractions 66 and 73 and had an apparent molecular mass of 300 kDa (Fig. 8C). It migrated as a 68-kDa molecule on SDS-PAGE under nonreducing conditions. From the results of Fig. 8, GBP28 was suggested to exist in two molecular forms: a minor one of 420 kDa and a major one of 300 kDa.

Although the anti-N antibody-conjugated Cellulofine and anti-C antibody-conjugated Cellulofine were prepared to purify both forms of GBP28, immunoprecipitation of GBP28 using the conjugated Cellulofine was unsuccessful. As shown in Fig. 4B, the molecules without heat-denaturing could not be detected with anti-N and anti-C antibodies, suggesting that these antibodies did not react with the native molecules. In contrast to antibody-conjugated Cellulofine, with gelatin-Cellulofine, about 7 ng of GBP28 was precipitated from 20 μl of plasma (data not shown). This yield indicated that 1 ml of human plasma contained about 0.35 μg of the form having the gelatin-binding activity. From the bands in Fig. 8C, another form of GBP28 was expected to exist at a concentration of a few micrograms per ml of plasma.

Affinity Precipitations of GBP28—For further characterization, the purified GBP28 was affinity-precipitated (Fig. 9A). GBP28 could be reprecipitated with gelatin-Cellulofine, and the supernatant contained no detectable amount of GBP28. GBP28 was also precipitated with FN-Cellulofine.

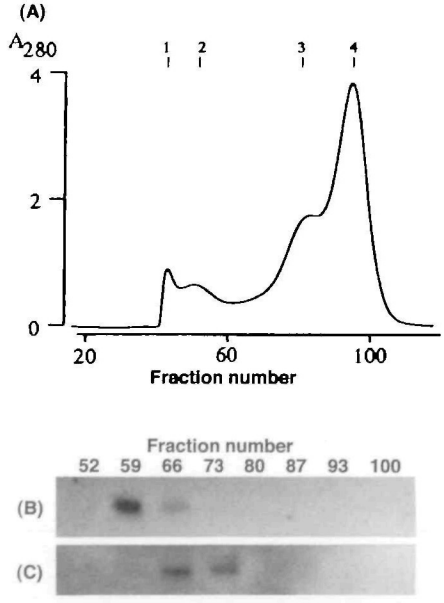

Fig. 8. **Analysis of GBP28 in plasma.** (A) Human pooled plasma (1 ml) was fractionated by HiPrep Sephacryl 300-HR and fractions of 0.7 ml per tube were collected. Elution positions of molecular-mass markers are indicated by arrows: 1, IgM (971 kDa); 2, FN (440 kDa); 3, IgG (160 kDa); 4, Alb (66 kDa). (B) An aliquot (100 μl) of each fraction was incubated with gelatin-Cellulofine (5 μl) for 10 min at 4°C. After washing with TPBS, the resulting resin was analyzed by SDS-PAGE under reducing conditions and Western blotting using anti-N antibodies and chemiluminescence system. (C) An aliquot (10 μl) of each fraction was electrophoresed under reducing conditions and analyzed by Western blotting using anti-N antibodies and chemiluminescence system.

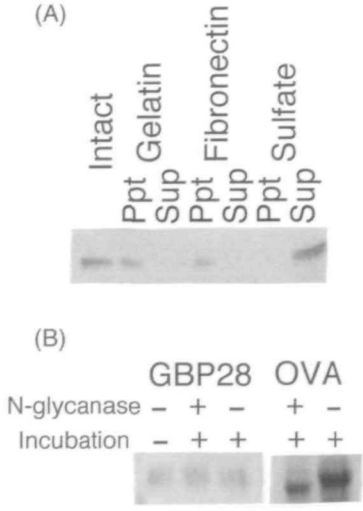

Fig. 9. **Affinity precipitation and sugar analysis of GBP28.** (A) Purified GBP28 (50 ng) was incubated with 5 μl each of gelatin-Cellulofine, FN-Cellulofine, and sulfate-Cellulofine for 10 min at 4°C. After washing with TPBS, each resin (Ppt) and wash solution (Sup) was electrophoresed under reducing conditions and proteins were detected with anti-N antibodies and alkaline phosphate conjugated anti-rabbit IgG. (B) GBP28 (50 ng) and OVA (5 μg) were digested with *N*-glycanase as described in "EXPERIMENTAL PROCEDURES." The digested samples of GBP28 were analyzed by SDS-PAGE under reducing conditions and Western blotting using anti-N antibodies and alkaline phosphate conjugated anti-rabbit IgG. The digested sample of OVA was electrophoresed under reducing conditions and stained with RAPID KANTO CBB.

Since FN contains a collagen-binding domain, GBP28 might be caught by this domain. When plasma was fractionated by gel chromatography, FN migrated with a molecular mass of 440 kDa in fractions from 50 to 56, while GBP28 migrated in fractions from 55 to 75 (Fig. 8). Hence GBP28 does not bind to FN in plasma. In ECM, FN exists in an insoluble multimeric form and GBP28 might interact with this species of FN. When sulfate-Cellulofine was tested, GBP28 did not bind and was recovered in the supernatant. GBP28 passed through the hyaluronan-Sepharose column (data not shown). These results suggest that GBP28 did not interact with glycosaminoglycans such as hyaluronan, heparin, and heparan sulfate.

Sugar Analysis of GBP28—As shown in Fig. 5, GBP28 contains one potential *N*-glycosylation site in the carboxyl-terminal globular domain. To determine whether GBP28 is glycosylated or not, GBP28 was treated with *N*-glycanase (Fig. 9B). *N*-Glycanase treatment of OVA that contains one *N*-glycoside caused a shift in molecular mass, but the molecular mass of GBP28 did not change by this treatment, indicating that GBP28 is not glycosylated. The possibility remains that *N*-glycanase could not approach due to steric hindrance by the polypeptide moiety of GBP28. Acrp30 also contains one potential *N*-glycosylation site and because Endo H treatment did not cause a shift in molecular mass at any time during a metabolic pulse-chase experiment, Acrp30 was also concluded not to be glycosylated (*14*).

DISCUSSION

We isolated and characterized a human plasma protein tentatively named GBP28. GBP28 is a large molecule of 420 kDa that is composed of identical chains. Since GBP28 has a collagen-like domain, three identical chains are suggested to oligomerize into one homo-trimer. Thus, the 70-kDa band under reducing conditions without heat-denaturing in Fig. 4A should represent a homo-oligomer composed of three identical chains rather than two chains. Without heat-denaturing, the trimer structure could not be dissociated into monomers. The stability of collagen trimers under reducing conditions without heat denaturing was also observed by Chan *et al.* with the type X collagen trimers formed during cell-free translation, and their stability was reduced by increasing the temperature of sample denaturation prior to electrophoresis (*21*). The 68-kDa band under nonreducing conditions in Fig. 4A could be a dimer or a trimer. Since the 70-kDa band under reducing conditions was assumed to be a trimer, the 68-kDa band is likely to be a trimer. The bands of 150 kDa and near 440 kDa under nonreducing conditions without heat-denaturing in Fig. 4A might represent complexes of two and six sets of homo-trimers. The effect of increasing temperature was mimicked by acidic pH. The 70-kDa band and the bands of 150 kDa and near 440 kDa in Fig. 4A, which appeared when samples were not heat-denatured, were shifted to 28 and 68 kDa, respectively, by acidic pH treatment as shown in Fig. 7. This result indicates that there is no disulfide bond between trimers, and the interactions between chains in one trimer and between trimers could be disrupted by heat and acidic pH. Taken together, these results suggest that the 420-kDa protein is a hexamer of the homo-trimer.

GBP28 has two Cys residues in each chain. When three chains construct one homo-trimer, the amino-terminal non-helical region and the carboxyl-terminal globular domain of the trimer each contain three Cys residues. The disulfide-bonding pattern in one trimer remains to be elucidated, but GBP28 migrates as one trimer under nonreducing conditions with heat-denaturing. The macrophage scavenger receptor, which is a trimeric protein with a collagen-like domain, comprises one noncovalently associated monomer and one disulfide-linked dimer, and only a receptor of trimeric, not dimeric or monomeric, form could bind ligands (*22*). This protein has only one Cys residue per chain, which forms a disulfide bond between chains and on SDS-PAGE, it migrates as monomers and dimers under nonreducing conditions with heat-denaturing. In GBP28, three chains contain at least two disulfide bonds per trimer, and the resulting trimer forms no disulfide bonds between trimers. The collagenous domain of macrophage scavenger receptor was supposed to be a ligand-binding site (*23*). Hence, GBP28 might also interact with this collagenous domain and influence its ligand uptake.

Many proteins contain a collagen-like domain. The proteins that show significant homology to GBP28, contain a collagen-like domain and have a similar size to GBP28 are schematically presented in Fig. 10. All proteins in Fig. 10 form homo- or hetero-trimers and many of them consist of four or six sets of trimers (*24-32*). Acrp30 in mouse serum migrated as a trimer and the larger nonamer or dodecamer by velocity gradient centrifugation (*14*). At least a part of Acrp30 forms larger oligomers than the trimer. When mouse serum was incubated with gelatin-Cellulofine, about one-tenth of the protein, which could be detected by anti-C

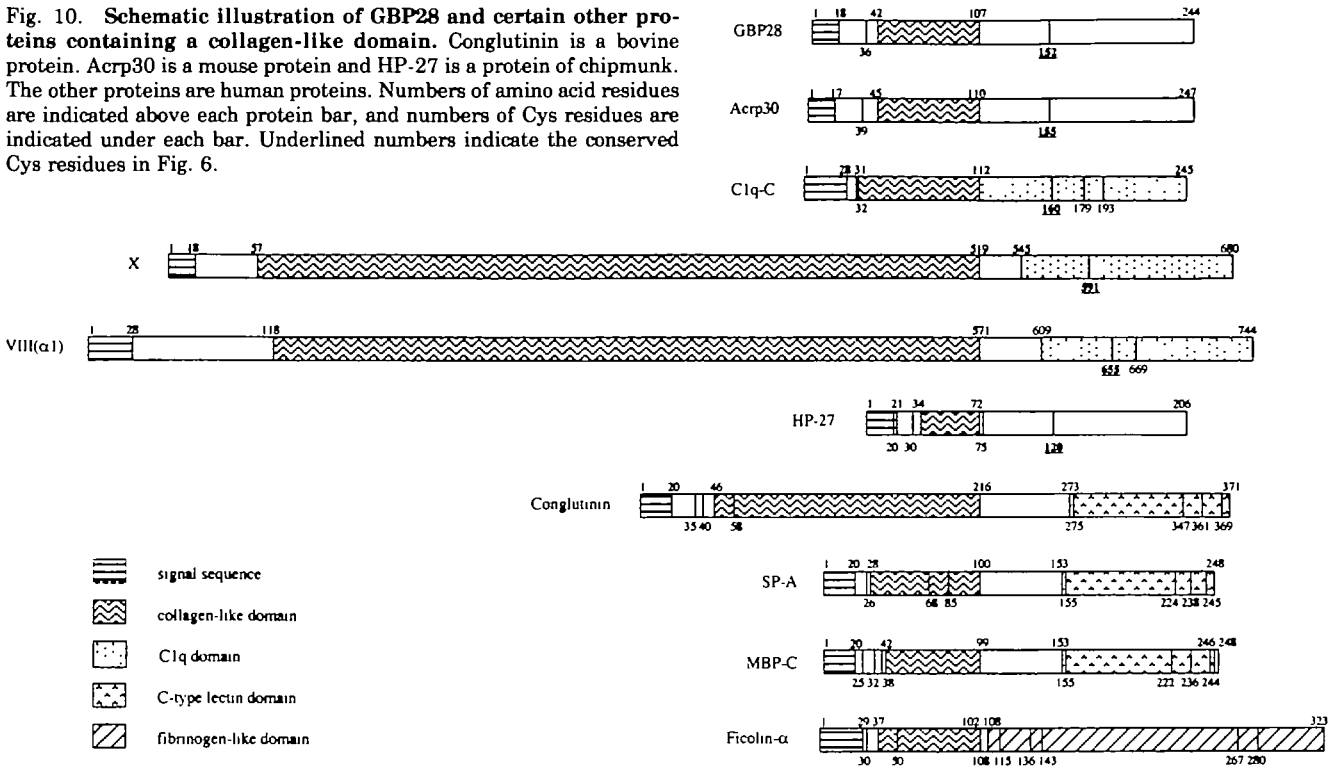

Fig. 10. Schematic illustration of GBP28 and certain other proteins containing a collagen-like domain. Conglutinin is a bovine protein. Acrp30 is a mouse protein and HP-27 is a protein of chipmunk. The other proteins are human proteins. Numbers of amino acid residues are indicated above each protein bar, and numbers of Cys residues are indicated under each bar. Underlined numbers indicate the conserved Cys residues in Fig. 6.

antibodies, bound to it (data not shown). The mouse protein detected by anti-C antibodies had a similar molecular weight to GBP28 and existed at about 10-fold concentration of that of GBP28 in human plasma. A small portion of Acrp30 might bind to gelatin.

Although the function of human GBP28 is yet unknown, two possible functions can be considered for GBP28. First, it may function as a special component of fat tissue ECM that binds to collagen fibrils like type X collagen in cartilage (*31, 32*). Type X collagen has been reported to be important for cellular proliferation, angiogenesis, and matrix mineralization, besides providing structural support. Among the proteins in Fig. 10, C1q, conglutinin, MBP-C, and SP-A were bound by C1q-receptor (*33*). Within the collagen-like region, these proteins show a characteristic bend halfway along their collagen-like triple helical regions due to a disruption of Gly-X-Y repeating pattern, and near the "bend" region, a cluster of similar charged residues is observed. This region has been suggested to be associated with receptor binding, and using a truncated form of conglutinin, this region is supposed to be a binding site (*34*). An assumed binding site of C1q-C is the residues from 46 to 60 in Fig. 6. Although GBP28 lacks a bend region, it does contain a cluster of similar charged residues like those in the above proteins (residues from 54 to 67 with a carboxyl-terminal Lys instead of Pro in C1q-C in Fig. 6). This cluster in GBP28 might function as a binding site to C1q-receptor on macrophages, and in this way, GBP28 might mediate the removal of collagen fibrils in fat-tissue ECM. Second, GBP28 may be an endogenous factor that binds to a collagen-like domain of a special receptor or a collagen-binding protein and influence lipid catabolism or storage or whole body metabolism. To understand the functions of GBP28, its localization and amount in plasma of many individuals in normal and pathological states should be elucidated.

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