

Processing and Transport of Matrix γ -Carboxyglutamic Acid Protein and Bone Morphogenetic Protein-2 in Cultured Human Vascular Smooth Muscle Cells

EVIDENCE FOR AN UPTAKE MECHANISM FOR SERUM FETUIN*

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Nadeem Wajih[‡], Terete Borrás[§], Wei Xue[§], Susan M. Hutson[¶], and Reidar Wallin^{‡||}

From the Departments of [‡]Internal Medicine and [¶]Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27517 and the [§]Department of Ophthalmology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Matrix γ -carboxyglutamic acid protein (MGP) is a member of the vitamin K-dependent protein family with unique structural and physical properties. MGP has been shown to be an inhibitor of arterial wall and cartilage calcification. One inhibitory mechanism is thought to be binding of bone morphogenetic protein-2. Binding has been shown to be dependent upon the vitamin K-dependent γ -carboxylation modification of MGP. Since MGP is an insoluble matrix protein, this work has focused on intracellular processing and transport of MGP to become an extracellular binding protein for bone morphogenetic protein-2. Human vascular smooth muscle cells (VSMCs) were infected with an adenovirus carrying the MGP construct, which produced non- γ -carboxylated MGP and fully γ -carboxylated MGP. Both forms of MGP were found in the cytosolic and microsomal fractions obtained from the cells by differential centrifugation. The crude microsomal fraction was shown to contain an additional, more acidic Ser-phosphorylated form of MGP believed to be the product of Golgi casein kinase. The data suggest that phosphorylation of MGP dictates different transport routes for MGP in VSMCs. A proteomic approach failed to identify a larger soluble precursor of MGP or an intracellular carrier protein for MGP. Evidence is presented for a receptor-mediated uptake mechanism for fetuin by cultured human VSMCs. Fetuin, shown by mass spectrometry not to contain MGP, was found to be recognized by anti-MGP antibodies. Fetuin uptake and secretion by proliferating and differentiating cells at sites of calcification in the arterial wall may represent an additional protective mechanism against arterial calcification.

(Gla)¹ protein (MGP), have provided new insight into the protective mechanism (2). Mice lacking the MGP gene develop severe calcification of arteries and cartilage, suggesting that MGP is an important inhibitor of ectopic calcification. This function of MGP has been shown to be dependent upon the vitamin K modification of the protein, a modification that converts Glu residues in the protein to Gla calcium-binding residues (3).

Several hypotheses have been launched about mechanisms by which MGP may work as a calcification inhibitor (1). We demonstrated by ligand blotting that a binding interaction exists between MGP and bone morphogenetic protein-2 (BMP-2) (4), a potent growth factor (5) that transforms undifferentiated cells and subpopulations of vascular smooth muscle cells (VSMCs) into bone-forming cells (6–8). In support of this finding are 1) the demonstration by Zebboudj *et al.* (9) of a complex between MGP and BMP-2 in cell culture medium from MGP- and BMP-2-producing cells and 2) observations that the activities of growth factors are often regulated by specific binding proteins through formation of protein complexes (10). Complexes between BMP-2 and its soluble binding protein noggin are known to be formed (11). On the other hand, MGP is extremely insoluble in physiological solutions (12), which poses the question of whether soluble complexes between MGP and BMP-2 are being formed.

All precursors of vitamin K-dependent proteins are equipped with a propeptide that is the site recognized by the vitamin K-dependent γ -carboxylase for post-translational γ -carboxylation of the precursors (13). The N-terminal propeptide is normally released in the *trans*-Golgi apparatus before the precursors become mature proteins (14). MGP is unique among the members of the vitamin K-dependent protein family by having the propeptide as part of the mature protein sequence (15). It is possible that this unusual design of the protein gives MGP properties that are not shared by the other members of the vitamin K-dependent protein family, which include poor solubility of MGP. The unique properties of MGP have hindered an understanding of the intracellular processing and intracellular transport of MGP to become an extracellular binding protein

Formation of bone outside the skeleton (ectopic calcification) has been recognized for >100 years, but we still have no clear picture of the events that lead to this pathological condition and the mechanism(s) that opposes onset of the pathology (1). However, findings in mice deleted of the gene for a 14-kDa vitamin K-dependent protein, matrix γ -carboxyglutamic acid

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|| To whom correspondence should be addressed: Dept. of Internal Medicine, Section on Rheumatology, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157. Tel.: 336-716-6166; Fax: 336-716-9821; E-mail: rwallin@wfubmc.edu.

¹ The abbreviations used are: Gla, γ -carboxyglutamic acid; MGP, matrix Gla protein; BMP-2, bone morphogenetic protein-2; VSMCs, vascular smooth muscle cells; Gla-MGP, fully γ -carboxylated MGP; Glu-MGP, non- γ -carboxylated or incompletely γ -carboxylated MGP that cannot attain the secondary structure of fully γ -carboxylated MGP in the presence of Ca²⁺; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; MS/MS, tandem mass spectrometry; TRITC, tetramethylrhodamine isothiocyanate.

for BMP-2. A main goal of this work has been to understand the intracellular processing and intracellular transport of MGP.

In contrast to MGP, the intracellular processing and intracellular transport of BMP-2 have been studied extensively. BMP-2 is synthesized as a 60-kDa precursor that is processed in the secretory pathway to a small 18-kDa monomer. Two monomers associate to form the active homodimer of the growth factor (16). In contrast to processing of BMP-2, processing of MGP includes its post-translational vitamin K-dependent modification step in the endoplasmic reticulum. If the vitamin K-dependent γ -carboxylation system is inhibited by coumarin anticoagulant drugs (17) or the cell is deficient in vitamin K (17), MGP may escape this modification step, and a non- γ -carboxylated form of MGP is produced (17).

The physical properties of fully γ -carboxylated MGP (Gla-MGP) are quite different from those of non- γ -carboxylated MGP (Glu-MGP) in that Gla-MGP undergoes a conformational change upon Ca^{2+} binding (18). We have shown that this conformational change is necessary for formation of the MGP-BMP-2 complex (4, 19), and Price *et al.* (20) have shown that the fully γ -carboxylated protein, but not the non- γ -carboxylated protein, is carried in plasma by fetuin, suggesting that the vitamin K modification of MGP is also essential for fetuin binding. Furthermore, the majority of MGP proteins produced in calcified lesions in the arterial wall appear as non- γ -carboxylated proteins that are inactive as binding proteins for BMP-2 (4), a finding that may reflect the inability of MGP to neutralize the growth factor activity of BMP-2 at the site of pathology. The processing and transport of the non- γ -carboxylated form of MGP are also poorly understood. Therefore, to understand the MGP/BMP-2 system as a regulatory system of ectopic calcification, it is imperative that we understand the cellular processing and transport of both forms of MGP.

VSMCs are believed to be the source of the MGP/BMP-2 system in the arterial wall (1, 9). In support of this hypothesis is the recent demonstration in transgenic mice that Gla-MGP produced in the vessel wall, and not systemic MGP, inhibits arterial calcification (21). To study the cellular processing and transport of Gla-MGP and Glu-MGP, we have used in this work a primary culture of human VSMCs infected with an adenovirus carrying the MGP construct. Overproduction of MGP by the infected cells exceeded the capacity of the γ -carboxylation system, and the cells produced a mixture of Gla-MGP and Glu-MGP, allowing us to follow both forms of MGP in the cell. Both forms of MGP, but not BMP-2, were found in the cytosolic fraction obtained by subcellular fractionation of the cell homogenate. However, an additional, more acidic form of MGP was found in the microsomal fraction. This additional form of MGP was identified as the phosphoserine-modified form of MGP (22). Fetuin was identified in both the cytosolic and microsomal fractions. An endocytotic mechanism for fetuin uptake by the cultured VSMCs is demonstrated. Furthermore, fetuin is shown to harbor one or more epitopes that are recognized by anti-MGP antibodies. This finding raises concerns about antibody-based assays used to quantify MGP in serum and tissue extracts.

EXPERIMENTAL PROCEDURES

Preparation of an Adenovirus-MGP Construct—Adenovirus carrying the coding cDNA of human MGP (adenovirus-MGP) was constructed by site-specific bacterial transposition using the Transpose-Ad adenoviral system (QBIgene, Montreal, Canada). The coding region of the human MGP cDNA (nucleotides 13–349, GenBank™/EBI accession number M58549) was obtained by PCR amplification of plasmid HTMI-296 from a human trabecular meshwork library (23) using high proof *Taq* polymerase and primers 5'-TGCAGGACGAAACCATGAAG-3' (forward) and 5'-TTCCCTCAGTCTCATTGGC-3' (reverse). These primers were designed to contain KpnI and XbaI sites at their 5'-ends,

respectively. The amplified insert was digested with KpnI/XbaI, purified, and cloned into a KpnI/XbaI-predigested pCR259 transfer vector (pWX1, QBIgene) under the transcriptional control of the cytomegalovirus promoter-enhancer. Upon sequencing confirmation, pWX1 was transfected into Transpose-Ad294-competent *Escherichia coli* cells and then transposed into the Ad294 plasmid, disrupting the *lacZ* gene.

High titer viral stocks were obtained by propagation in 293 cells and purification by CsCl density centrifugation as described (24, 25). Purified viruses were titered by spectrophotometric reading at A_{260} and stored in aliquots at -80°C . The absence of contaminant wild-type viruses was tested in each stock by PCR amplification with E1A-specific primers 5'-TCGAAGAGGTACTGGCTGAT-3' and 5'-TGACAAGACCTGCAACCGTG-3'.

Infection of VSMCs with the Adenovirus-MGP Construct—Human VSMCs (cc2571, Cambrex, Boston, MA) were cultured in SmGM-2 (Cambrex). At 70% confluency, cells were infected with the adenovirus-MGP construct (multiplicity of infection of 20) and harvested 48 h after infection. All cultures contained $10\ \mu\text{g}$ of vitamin K_1 (Sigma)/ml of medium. For collection of proteins secreted from the cells, cells were washed with phosphate-buffered saline (PBS) and cultured for an additional 16 h in serum-free SmBM (basal medium; catalog no. CC-3177; Cambrex). Proteins in the serum-free medium were concentrated in a Centricon concentrator with a 3.5-kDa cutoff.

Biotin Labeling of Fetuin—Ten mg of fetuin (molecular biology grade) was dissolved in 1 ml of PBS. A 20-fold molar excess of EZ-Link sulfosuccinimidobiotin was added to the fetuin solution, and the mixture was reacted for 30 min at room temperature in the dark on a rotating shaker. The reaction mixture was gel-filtrated on Sephadex G-25 in PBS, and the void volume fraction containing biotin-conjugated fetuin was collected. The biotin-conjugated fetuin was dialyzed against PBS overnight at 4°C and sterile-filtrated before added to the cell culture medium.

Immunohistochemistry—VSMCs cultured in SmGM-2 were depleted of endogenous fetuin by incubating the cells for 15 min at 37°C in serum-free SmBM as described by Lorenzo *et al.* (26). Fetuin binding to the VSMC membrane was investigated by incubating depleted cells with biotin-labeled fetuin ($50\ \mu\text{g}/\text{ml}$) in serum-free medium for 30 min at 4°C (26). Cells were washed with cold PBS and fixed for 10 min in 4% paraformaldehyde. For competition studies, depleted cells were incubated for 30 min at 4°C in serum-free medium containing a 100-fold excess of unlabeled fetuin over biotin-labeled fetuin. For uptake studies, cells incubated for 30 min at 4°C in the presence of $5\ \mu\text{g}/\text{ml}$ biotin-labeled fetuin were exposed to serum-free medium at 37°C for an additional 30 min before being fixed in paraformaldehyde (26). For fluorescent staining, fixed cells were incubated at room temperature for 30 min with biotin-specific monoclonal antibodies (1:100 dilution), followed by incubation with rhodamine-labeled donkey anti-mouse IgG (1:200 dilution) for 30 min at room temperature. Rhodamine epifluorescence images were obtained using a Zeiss Axioskop equipped with a digital camera and Axovision imaging software. Final images were processed using Adobe Photoshop Version 7.0.1. Images of rhodamine epifluorescence were also obtained by confocal microscopy. A Zeiss Model 510 laser scanning confocal microscope equipped with a $\times 40$ water immersion objective was used. The optical slice thickness was $2.1\ \mu\text{m}$ for each image.

Cell Disruption and Subcellular Fractionation—Cells were washed with cold PBS, and the cell lysate was prepared using modified radioimmune precipitation assay (RIPA) buffer (50 mM Tris, 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) containing the protease inhibitors aprotinin, leupeptin, and pepstatin ($1\ \mu\text{g}/\text{ml}$ each) as recommended by Upstate Biotechnology, Inc. (Lake Placid, NY). Subcellular fractionation was carried out as described previously (27) with some modifications. Cells were washed with cold PBS and disrupted by sonication in isotonic buffer containing 25 mM imidazole, 250 mM sucrose, 10 $\mu\text{g}/\text{ml}$ Sigma protease inhibitor mixture for use with mammalian cells and tissues, 5 $\mu\text{g}/\text{ml}$ orthovanadate, and 10 $\mu\text{g}/\text{ml}$ NaF, pH 7.5. Cell disruption was carried out on ice in a Fisher Scientific Sonic Dismembrator (Model 100) at setting 4. Twenty pulses of 2-s duration were used. The protein concentration was 8 mg/ml. The sonicate was centrifuged at $10,000 \times g$ for 10 min to remove cell debris. A microsomal pellet was obtained by subjecting the supernatant to centrifugation at $229,000 \times g$ for 60 min in a Ti-70.1 rotor (Beckman Instruments). The microsomal pellet and the supernatant (cytosolic fraction) were stored frozen at -85°C until used in the experiments.

Electrophoresis and Western Blotting—Prior to SDS-PAGE, proteins in cell microsomes were dissolved in 250 mM phosphate, 0.5 M KCl, 20%

glycerol, and 0.75% CHAPS, pH 7.85, containing 10 μ l/ml Sigma protease inhibitor mixture and subsequently precipitated with cold acetone (-20°C) by mixing 1 part protein solution with 5 parts acetone. Cytosolic proteins and cell proteins extracted with RIPA buffer were also precipitated with acetone as described above. The mixtures were left overnight at -20°C , and precipitated proteins were harvested by centrifugation. The precipitated proteins were washed consecutively with 5 ml of cold 10% trichloroacetic acid (4°C) and 5 ml (v/v) of ice-cold ether/ethanol (-20°C). The protein pellets from the ether/ethanol washes were dried by N_2 aspiration. For two-dimensional SDS-PAGE, the dried proteins were dissolved in 8 M urea, 2% CHAPS, 50 mM dithiothreitol, and 2% pH 3–10 ampholyte and absorbed onto Bio-Rad pH 3–10 gradient strips overnight according to the instruction manual for the Protean isoelectric focusing cell (Bio-Rad). SDS-PAGE in the second dimension was carried out on Criterion 8–16% gradient gels (Bio-Rad) also according to the Bio-Rad instruction manual. One-dimensional SDS-PAGE was carried out on Criterion 8–16% gradient gels by dissolving the dried cell proteins in SDS-PAGE running buffer containing 5% mercaptoethanol. Gels were stained with Coomassie Brilliant Blue. Western blotting was carried out as described (28) after transfer of proteins to polyvinylidene difluoride membranes.

Immunoprecipitation—Affinity-purified polyclonal or monoclonal antibodies were added to cell extracts in RIPA buffer and left overnight at 4°C on a rotating shaker. Immune complexes were captured with protein A-Dynabeads (Dynal Biotech) and washed on a magnetic rack as described in the instruction manual for Dynabeads. Proteins were released from the beads with 8 M urea, 2% CHAPS, 50 mM dithiothreitol, and 2% pH 3–10 ampholyte and absorbed onto Bio-Rad pH 3–10 isoelectric focusing gradient strips overnight. For one-dimensional SDS-PAGE, proteins were released from the beads by boiling the beads in SDS-PAGE running buffer containing 5% mercaptoethanol.

Assays— γ -Carboxylase activity was assayed as described for VSMCs (27) as ^{14}C incorporation into the synthetic peptide FLEEL. The reaction was triggered by adding chemically reduced vitamin K_1H_2 (100 $\mu\text{g}/\text{ml}$) to the assay mixture.

Mass Spectrometry—In-gel digestion of Coomassie Blue-stained proteins on SDS-polyacrylamide gels was carried out with trypsin, and sequence analysis was performed at the Harvard Microchemistry Facility (Cambridge, MA) by microcapillary reverse-phase HPLC/nanospray tandem mass spectrometry (MS/MS) on a Finnigan LCQ DECA XP quadrupole ion trap spectrometer.

Materials—The anti-MGP N-terminal peptide antibodies were raised in rabbits against a synthetic peptide covering residues 6–37 of the human MGP sequence (19). These antibodies recognize MGP independently of the γ -carboxylation status of the MGP protein (19). The anti-Glu-MGP and anti-Gla-MGP antibodies were raised in rabbits against synthetic peptides covering the Gla region of MGP. Synthesis of these peptides, antibody production, and purification of conformation-specific antibodies against the Gla region of MGP have been described (4). The anti-Glu-MGP and anti-Gla-MGP antibodies recognize the non- γ -carboxylated and fully γ -carboxylated forms of MGP, respectively (19). Goat anti-human BMP-2 polyclonal antibodies were prepared as described (4). All of the anti-MGP peptide antibodies prepared by our laboratory and also the anti-BMP-2 antibodies (4) were affinity-purified by our laboratory and stored frozen at -85°C in 50% glycerol/water as described (4, 19). A monoclonal antibody made against human MGP epitope 3–15 (N-terminal residues 3–15 of the human MGP sequence) was purchased from VitaK Inc. (University of Maastricht, Maastricht, The Netherlands). Affinity-purified rabbit antiphosphoserine polyclonal antibodies (Poly-Z-PS1) were from Zymed Laboratories Inc.. Mouse anti-biotin, horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L), horseradish peroxidase-conjugated goat anti-mouse IgG (H + L), and TRITC-conjugated donkey anti-mouse IgG (H + L) antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse anti-human Golgi apparatus monoclonal antibodies (clone AE-6) were from Calbiochem. Anti-bovine fetuin polyclonal antibodies (IgG fraction) were from Acris (Hiddenhausen, Germany). These antibodies were affinity-purified on a bovine fetuin-Sepharose affinity resin prepared by our laboratory and stored frozen at -85°C in 50% glycerol/water. Bovine fetuin (molecular biology grade) was from USBiological (Swampscott, MA). A_2 -hs-glycoprotein from human plasma and fetuin from fetal calf serum were from Sigma. EZ-Link sulfo succinimidyl 6-(biotinamido)hexanoate was from Pierce. Centriprep 3000 NMWL centrifugal filter units were from Millipore Corp. All other chemicals were chemical grade or better.

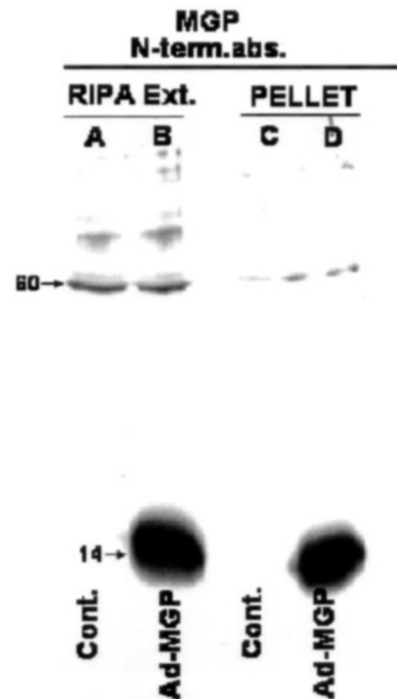


FIG. 1. Human VSMCs infected with the adenovirus-MGP construct. Uninfected (control (*Cont.*)) and infected (*Ad-MGP*) VSMCs were harvested, and cell extracts were made with RIPA buffer (*RIPA Ext.*) as described under "Experimental Procedures." Cell debris not soluble in RIPA buffer (*PELLET*) was collected by centrifugation. Proteins from control and infected cells were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane for Western blotting with the affinity-purified anti-MGP N-terminal peptide antibodies (*MGP N-term.abs.*) prepared by our laboratory (see "Experimental Procedures").

RESULTS

Adenovirus-MGP Infection of VSMCs—VSMCs infected with the adenovirus-MGP construct resulted in overexpression of MGP. Fig. 1 shows Western blots with the anti-MGP N-terminal peptide antibodies of proteins present in a RIPA buffer extract (*RIPA Ext.*) of control cells (*Cont.*) and infected cells (*Ad-MGP*) and in the pellets of cell debris left over after RIPA buffer extraction (*PELLET*) of control and infected cells. A 14-kDa protein recognized by the antibodies appeared both in the RIPA buffer extract of infected cells (*lane B*) and in the proteins in the infected cells that were not soluble in RIPA buffer (*lane D*). An immune reaction with a 60-kDa protein was also seen. VSMCs are known to synthesize MGP (29). We could detect MGP in the control cells when a longer exposure time was used for imaging Western blots for chemiluminescence (data not shown).

Searching for an Intracellular MGP Carrier Protein(s) or Precursor—The unique properties of MGP and the poor solubility of the protein prompted a search for the cellular mechanism(s) by which MGP is transported intracellularly and released from the cell. Initially, we considered two possible mechanisms. 1) Although the mRNA for MGP does predict translation of a protein <23 kDa (20), we did not exclude the possibility that a larger soluble precursor of MGP could be made and processed to a smaller protein before secretion from the cell; and 2) MGP could be carried through the cell attached to a carrier protein to assure solubility of MGP.

VSMCs infected with the adenovirus-MGP construct were extracted with RIPA buffer, and the extracted proteins were separated by two-dimensional SDS-PAGE (Fig. 2A). Proteins on identical two-dimensional gels were transferred to polyvinylidene difluoride membranes for Western blotting with the

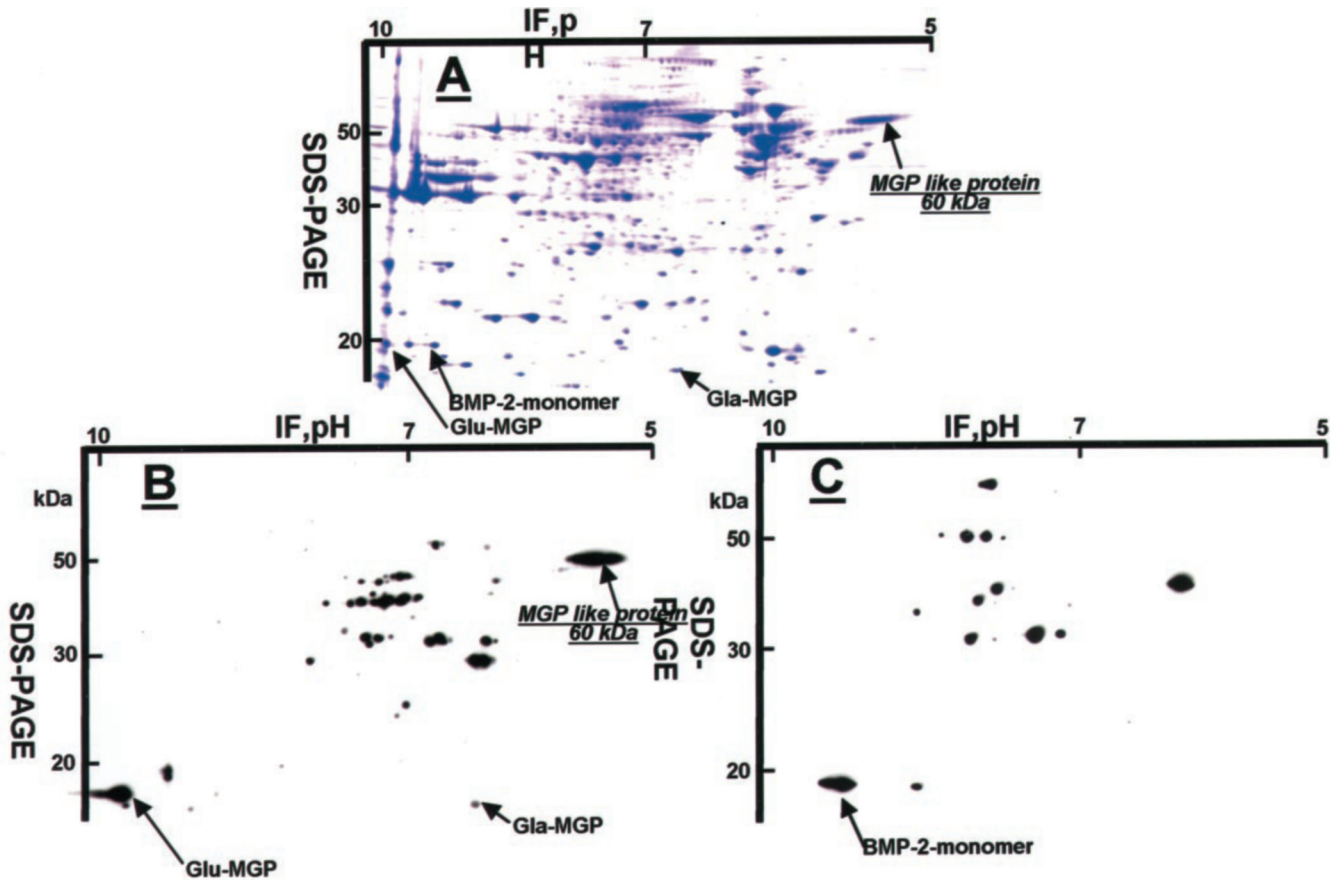


FIG. 2. Two-dimensional SDS-PAGE and Western blotting of proteins in RIPA buffer extracts of VSMCs infected with the adenovirus-MGP construct. Human VSMCs were cultured, infected with the virus, and harvested as described under "Experimental Procedures." *A*, Coomassie Blue-stained two-dimensional SDS-polyacrylamide gel of proteins present in a RIPA buffer extract of infected cells; *B* and *C*, Western blots of the same proteins with affinity-purified anti-MGP N-terminal peptide and anti-BMP-2 antibodies, respectively. The positions of Glu-MGP and Gla-MGP on the gel (*A*) and on the membrane (*B*) were determined with the anti-Glu-MGP and anti-Gla-MGP antibodies as described under "Experimental Procedures" (not shown). *IF*, isoelectric focusing.

anti-MGP N-terminal peptide antibodies (Fig. 2*B*) and the anti-BMP-2 antibodies (Fig. 2*C*). As shown in Fig. 2*B*, the anti-MGP N-terminal peptide antibodies recognized a 60-kDa protein spot (*MGP like protein, 60 kDa*), several proteins of intermediate size, the basic 14-kDa non- γ -carboxylated form of MGP (Glu-MGP), and the more acidic fully γ -carboxylated form (Gla-MGP). The positions of Glu-MGP and Gla-MGP on the two-dimensional gel were determined by Western blotting with the anti-Glu-MGP and anti-Gla-MGP peptide antibodies, respectively (data not shown). Fig. 2*C* shows the Western blot obtained with the anti-BMP-2 antibodies. Consistent with the known processing events of BMP-2 (16), several immunoreactive proteins were identified in the RIPA buffer extract along with the basic 18-kDa monomer of the growth factor. These data show that MGP and BMP-2 were produced by the cultured human VSMCs.

Identification of the 60-kDa MGP-like Protein—The Coomassie Blue-stained protein spot labeled *MGP like protein, 60 kDa* in Fig. 2*A* was excised from the two-dimensional gel and subjected to in-gel digestion with trypsin, and peptides were analyzed by MS/MS for protein identification. The identities of five proteins present in the excised spot are shown in Table I. No tryptic peptides from the MGP sequence could be identified. This negative finding strongly suggested that none of the proteins identified in the Coomassie Blue-stained *MGP like protein, 60 kDa* spot could be a MGP precursor or a protein carrying the MGP protein. The most abundant protein present in the spot was bovine fetuin. The characteristic shape of the spot is also typical of the appearance of bovine fetuin on two-dimen-

TABLE I
Tryptic in-gel digestion of proteins and their identification by microcapillary reverse-phase HPLC/nanospray MS/MS
The Coomassie Blue-stained spot labeled *MGP like protein, 60 kDa* in Fig. 2 was excised from the gel and subjected to analysis. Proteins identified in the gel piece are shown.

Protein name	Accession no.	Total no. of MS/MS spectra
Bovine fetuin	NP_776409	28
Type II transmembrane protein p63	NP_006816	15
Myristoylated alanine-rich C kinase substrate	AAA_59555	9
Albumin	P_02769	9
Microtubule-associated protein-1B isoform 2	NP_114399	7

sional SDS-polyacrylamide gels (Die Fetuin Homepage, 132.130.13.185/Fetuin-HP/ebene31.html).

Cellular Uptake of Fetuin—The conclusive identification of bovine fetuin in the RIPA buffer extract of cultured human VSMCs showed that the fetuin protein came from fetal bovine serum in the cell culture medium. Active uptake mechanisms for fetuin have been documented in embryonic, undifferentiated, and tumor cells (31, 33), but have never been shown to exist in primary cultures of VSMCs. Therefore, we set out to determine whether our primary culture of human VSMCs could internalize fetuin by endocytosis.

To investigate the mechanism, VSMCs were incubated first with biotin-labeled bovine fetuin at 4 °C, which maintains re-

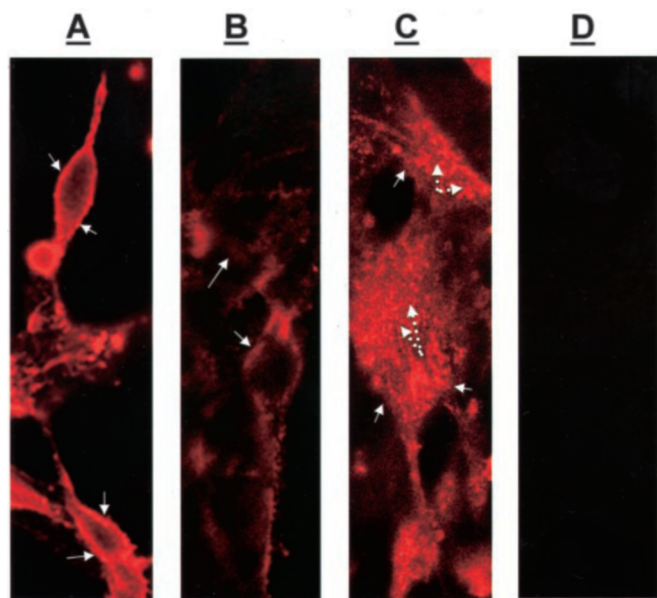


FIG. 3. Biotin-labeled fetuin binding and uptake by cultured human VSMCs. VSMCs were depleted of endogenous fetuin and incubated at 4 or 37 °C in serum-free medium containing biotin-labeled fetuin or biotin-labeled fetuin plus fetuin. Incubations, paraformaldehyde fixation, and rhodamine fluorescent staining are described under "Experimental Procedures." *A*, cells incubated with biotin-labeled fetuin for 30 min at 4 °C; *B*, cells incubated for 30 min at 4 °C in the presence of a 100-fold excess of unlabeled fetuin over biotin-labeled fetuin; *C*, cells incubated with biotin-labeled fetuin for 30 min at 4 °C, followed by incubation at 37 °C for an additional 30 min; *D*, same experiment as in *C*, except that biotin-labeled fetuin was absent. Rhodamine epifluorescence images were obtained using a Zeiss Axioskop equipped with a digital camera and Axovision imaging software. Final images were processed using Adobe Photoshop Version 7.0.1. *Scale bar* = 25 μ m.

ceptor binding but prevents endocytosis (34). Next, the cells were incubated at 37 °C, which triggers endocytosis of receptor-bound fetuin (35). As shown in Fig. 3*A*, cells incubated with biotin-labeled fetuin at 4 °C showed intense rhodamine fluorescent staining of biotin-labeled fetuin attached to the cell membrane, but only faint staining of the interior of the paraformaldehyde-fixed cells could be seen. In a parallel experiment, the VSMCs were incubated at 4 °C with biotin-labeled fetuin in the presence of a 100-fold excess of unlabeled bovine fetuin. As shown in Fig. 3*B*, excess unlabeled bovine fetuin significantly prevented rhodamine fluorescent staining of the cell membrane, suggesting competition between unlabeled and biotin-labeled fetuin for binding sites on the VSMC membrane. Fig. 3*C* shows rhodamine staining when the cells were first incubated for 30 min at 4 °C with biotin-labeled fetuin, followed by a 30-min incubation at 37 °C. Clearly, raising the temperature allowed biotin-labeled fetuin to be internalized, and the protein then appeared in intracellular organelles with little staining of the cell membrane. The control experiment shown in Fig. 3*D* was identical to the experiment shown in Fig. 3*C* except that the experiment was carried out in the absence of biotin-labeled fetuin. No noticeable background staining was seen. Omitting the anti-biotin antibody from the experiments resulted in no fluorescent staining (data not shown). We also imaged this experiment using confocal microscopy. The confocal image of biotin-labeled fetuin attached to the cell membrane at 4 °C is shown in Fig. 4*A*. Incubation with biotin-labeled fetuin at 4 °C followed by incubation at 37 °C resulted in the image shown in Fig. 4*B*. Again, biotin-labeled fetuin appeared in intracellular organelles, consistent with endocytosis of the protein. The confocal image provided conclusive evidence for an intracellular localization of the rhodamine-stained organelles.

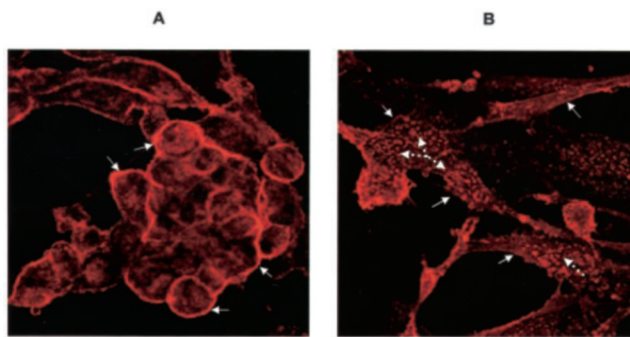


FIG. 4. Confocal microscopy of biotin-labeled fetuin binding and uptake by cultured human VSMCs. The experiments were identical to those described in the legend to Fig. 3. *A*, cells incubated with biotin-labeled fetuin for 30 min at 4 °C; *B*, cells incubated with biotin-labeled fetuin for 30 min at 4 °C, followed by incubation at 37 °C for an additional 30 min. Images of rhodamine epifluorescence were obtained using a Zeiss Model 510 laser scanning confocal microscope equipped with and $\times 40$ water immersion objective. The optical slice thickness was 2.1 μ m for each image. *Scale bar* = 25 μ m.

Recognition of Fetuin by Anti-MGP Peptide Antibodies—The finding that our anti-MGP N-terminal peptide antibodies recognized the *MGP like protein*, 60 kDa spot shown on the two-dimensional SDS-polyacrylamide gel in Fig. 2*A* suggested that the anti-MGP N-terminal peptide antibodies recognized bovine fetuin. This was indeed confirmed by Western blotting of commercially available purified fetuin. As shown in Fig. 5*A*, our affinity-purified anti-bovine fetuin antibodies recognized bovine and human fetuin, reflecting the high degree of sequence homology (70%) between the two proteins. Fig. 5*B* shows that our affinity-purified anti-MGP N-terminal peptide antibodies recognized human and bovine fetuin purchased from two different suppliers (*Bovine* (Sigma) and *Bovine** (USBiological)). We also purchased an anti-MGP monoclonal antibody (VitaK Inc.) made against an N-terminal peptide containing residues 3–15 of the MGP sequence. As shown in Fig. 5*C*, the VitaK monoclonal peptide antibodies also recognized fetuin. The horseradish peroxidase-conjugated secondary antibodies used for immunodetection did not recognize fetuin. Since Price *et al.* (20) have shown that serum fetuin carries MGP noncovalently, we overloaded a two-dimensional SDS-polyacrylamide gel with bovine fetuin purchased from Sigma (see legend to Fig. 5) to search for the 14-kDa mature form of MGP. The stained image of the two-dimensional gel is shown in Fig. 6. A variety of different proteins were shown to be present in the commercial fetuin preparation. However, no stained protein spot with a molecular mass of 14 kDa was seen. The *arrow* in Fig. 6 indicates where purified mature MGP would have appeared on the two-dimensional SDS-polyacrylamide gel (4). The absence of MGP on the gel supports the MS/MS data and the conclusion that the anti-MGP N-terminal peptide antibodies recognize one or more epitopes present on the fetuin protein.

Fetuin Secreted from VSMCs Is Recognized by Anti-MGP N-terminal Antibodies—VSMCs cultured in SmGM-2 were rinsed with PBS, and culture was continued for 16 h in serum-free SmBM. Proteins in the serum-free media from uninfected cells (Fig. 7, *Control*) and infected cells (Fig. 7, *Infected*) were subjected to SDS-PAGE, followed by Western blotting with the anti-MGP N-terminal peptide antibodies. As shown in Fig. 7, the antibodies recognized a 60-kDa protein in the media from control and infected cells. The protein was identified as fetuin. No protein with a molecular mass corresponding to MGP (14 kDa) was seen. The result is consistent with secretion of internalized fetuin by VSMCs (26) and adds further support to the finding that the anti-MGP N-terminal peptide antibodies recognize fetuin.

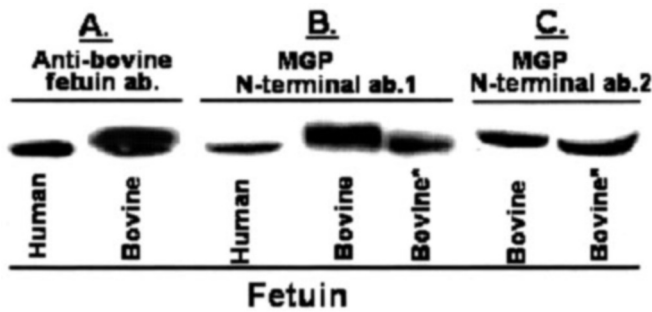


FIG. 5. Anti-MGP N-terminal peptide antibodies recognize fetuin. Commercially available purified fetuin from human and bovine sera was subjected to SDS-PAGE and Western blotting with affinity-purified anti-bovine fetuin antibodies (*ab*; A), affinity-purified anti-MGP N-terminal peptide antibodies (B), and monoclonal peptide antibodies prepared against the N-terminal part of human MGP (C) (for details, see "Experimental Procedures"). *Bovine* and *Bovine** represent serum fetuin from two different suppliers (see "Experimental Procedures").

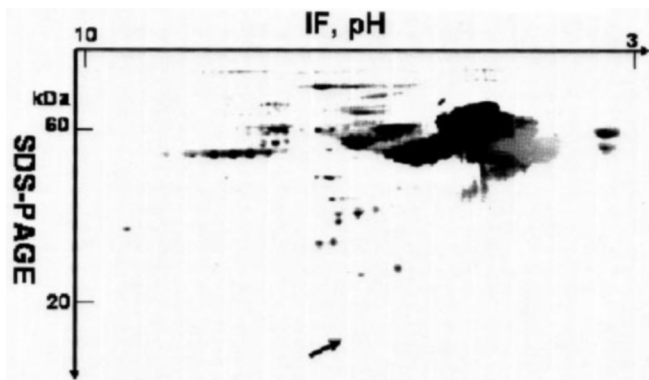


FIG. 6. Two-dimensional SDS-PAGE of purified serum fetuin. Purified serum fetuin from Sigma (catalog no. F 3004) was subjected to two-dimensional SDS-PAGE as described under "Experimental Procedures." Shown is a Coomassie Blue-stained Criterion 8–16% gradient gel used in the second dimension. The gel was overloaded with protein. *IF*, isoelectric focusing.

Fetuin Internalized by VSMCs Is Not a Carrier of Intracellular MGP but Is Recognized by MGP Conformation-specific Antibodies—Since serum fetuin has been shown to carry mature Gla-MGP noncovalently (20), we investigated whether fetuin endocytosed by the cultured VSMCs could carry MGP intracellularly in a similar way. A RIPA buffer extract of infected cells was incubated with affinity-purified anti-fetuin antibodies, and immune complexes were isolated with protein A-Dynabeads as described under "Experimental Procedures." Fig. 8 shows a Western blot with the anti-MGP-Gla peptide antibodies of captured immune complexes subjected to two-dimensional SDS-PAGE. To our surprise, a strong immune reaction of fetuin was seen with affinity-purified conformation-specific anti-Gla-MGP antibodies (19), but no 14-kDa immunoreactive band was visible on the blot. This finding eliminated the possibility of co-immunoprecipitation of a noncovalently linked complex between mature MGP and fetuin. On the other hand, the anti-Glu-MGP antibodies did not recognize fetuin on the two-dimensional SDS-PAGE blot (data not shown). We could conclude from these results that fetuin internalized by the VSMCs is not a cellular carrier of MGP but share epitopes recognized by antibodies raised against MGP synthetic peptides.

MGP in Subcellular Fractions of VSMCs—Since a RIPA buffer extract of cells will contain proteins from the cytosol as well as from cellular compartments and organelles, we carried out subcellular fractionation of cell homogenates from virus-infected cells in isotonic buffer to more specifically localize

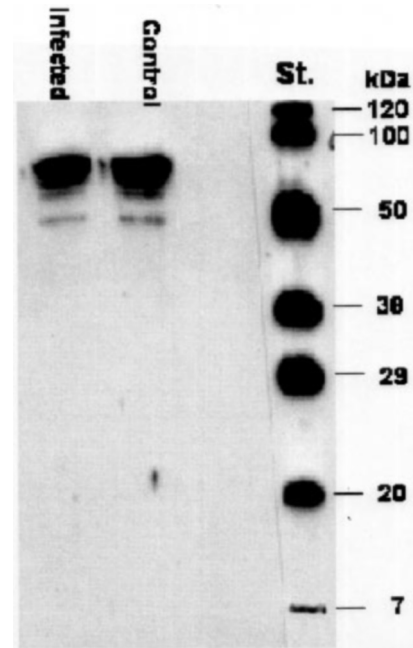


FIG. 7. Cultured VSMCs secrete fetuin, but not detectable amounts of MGP, into the cell medium. Cultured human VSMCs were exposed to serum-free medium for 16 h for collection of secreted proteins. Proteins were concentrated on a 3.5-kDa cutoff filter and subjected to SDS-PAGE and Western blotting with affinity-purified anti-MGP N-terminal peptide antibodies. *Infected* and *Control* lanes contain proteins from adenovirus-MGP-infected and uninfected VSMCs, respectively. The protein recognized by the antibodies was identified as fetuin. *St.*, standards.

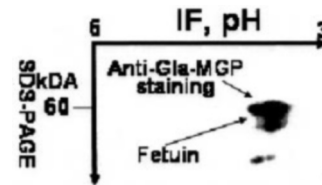


FIG. 8. Intracellular fetuin is not a carrier of intracellular MGP but is recognized by MGP conformation-specific antibodies. VSMCs infected with the adenovirus-MGP construct were extracted with RIPA buffer, and the extract was used for immunoprecipitation of endocytosed fetuin and coprecipitating proteins. Immune complexes were captured on protein A-Dynabeads and subjected to two-dimensional SDS-PAGE and Western blotting as described under "Experimental Procedures." The two-dimensional blot was developed with the anti-Gla-MGP antibodies, which recognized fetuin (see *arrows*), but the 14-kDa MGP protein was not detected on the blot. *IF*, isoelectric focusing.

MGP in VSMCs. The microsomal fraction was shown to contain endoplasmic reticulum vesicles, as γ -carboxylase activity could be measured. However, the fraction also contained vesicles derived from the Golgi apparatus, as anti-Golgi marker antibodies (see "Experimental Procedures") recognized a 50-kDa Golgi resident protein (data not shown). Fig. 9 (A and C) shows Coomassie Blue-stained polyvinylidene difluoride membranes containing cytosolic proteins and proteins from the microsomal fraction, respectively, after their transfer to the membranes for immunoblotting. Fig. 9 (B and D) shows Western blots of the proteins in Fig. 9A (cytosolic) and Fig. 9D (microsomal) with anti-fetuin antibodies. Thus, fetuin could be identified both in the cytosolic and microsomal fractions. Fig. 10 (A and B) shows Western blots of the 14-kDa forms of MGP appearing in the cytosolic and microsomal fractions, respectively. Glu-MGP and Gla-MGP were identified in both fractions, but the microsomal fraction (Fig. 10B) contained an additional, more acidic form of Gla-MGP. As shown in Fig. 10C, anti-phosphor-Ser antibodies

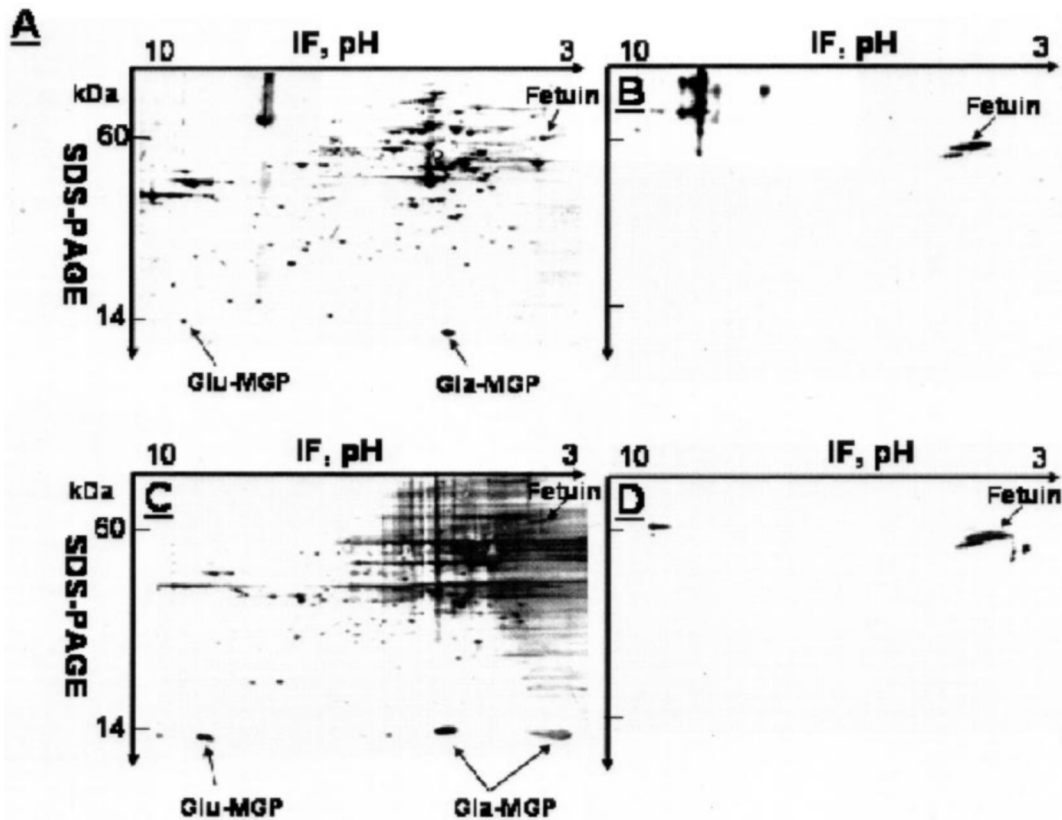


FIG. 9. MGP and fetuin in subcellular fractions from adenovirus-MGP-infected VSMCs. Infected cells were disrupted in isotonic buffer and subjected to subcellular fractionation by differential centrifugation as described under "Experimental Procedures." A and C, Coomassie Blue-stained proteins in the cytosolic and microsomal fractions, respectively; B and D, Western blots with anti-fetuin antibodies of the proteins shown in A and C, respectively. The positions on the membranes of proteins that reacted with the anti-Glu-MGP and anti-Gla-MGP antibodies are indicated. IF, isoelectric focusing.

identified the more acidic form of MGP as the phospho-Ser-modified form of MGP. No phosphorylated forms of MGP were found in the cytosolic fraction. BMP-2 could also not be identified in the cytosolic fraction.

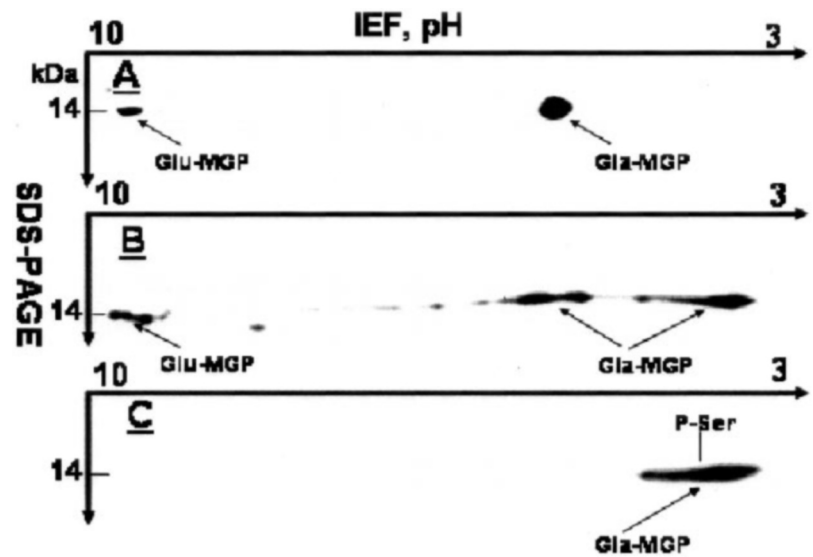
DISCUSSION

This work is the first attempt to understand the intracellular processing and intracellular transport of the vitamin K-dependent protein MGP to become an extracellular matrix protein capable of forming a complex with the potent bone-promoting growth factor BMP-2. The cellular processing of the other members of the vitamin K-dependent protein family, except for two newly discovered transmembrane proteins (37, 38), has been studied extensively. These water-soluble proteins are known to follow the secretory pathway, which includes the endoplasmic reticulum and the Golgi compartments, for their exit from the cell. MGP, as it is purified from bone or cartilage, is a highly insoluble protein. Poor solubility has also been shown to be a property of MGP made by chemical synthesis (39). If MGP should leave the ribosome as a larger soluble precursor, this means that elongation of the polypeptide must have extended through the UGA stop codon in the mRNA sequence, which has been shown to occur with selenocysteine-containing proteins (40). Although the mRNA for MGP (30) does not predict synthesis of a larger precursor, we still decided to use a proteomic approach for identification of all MGP-like proteins that possibly could be a MGP precursor or a protein carrying the smaller MGP protein. We searched for such proteins in RIPA buffer extracts of adenovirus-MGP-infected human VSMCs. Our anti-MGP N-terminal peptide antibodies recognized a 60-kDa protein and several intermediate sized proteins in addition to the 14-kDa non- γ -carboxylated (Glu-

MGP) and fully γ -carboxylated (Gla-MGP) forms of MGP. Surprisingly, mass spectrometry conclusively showed that the 60-kDa protein does not contain any sequences that could be attributed to MGP. This conclusion was based on two independent analyses of the MS/MS data. The dominant protein present in the 60-kDa immunoreactive spot was identified as bovine fetuin. The intermediate sized proteins were difficult to isolate from the gel by excision (see Fig. 1), and these spots were not analyzed by MS/MS. We have shown that fetuin could be internalized by the cultured human VSMCs and secreted from these cells when surrounded by a medium deficient in fetuin. Our data are consistent with the presence of a receptor-mediated uptake mechanism, a mechanism shown to exist in embryonic and proliferating cells, but not in resting differentiated cells (32). Serum fetuin carries calcium phosphate (41) and has been shown to play a major role in preventing calcium phosphate precipitation from serum and deposition of the salt in extrahepatic tissues (42). Indeed, arterial calcification has been shown to be inversely correlated with the serum concentration of fetuin (43). Our demonstration of an uptake mechanism for serum fetuin by proliferating human VSMCs and its secretion by the cells could reflect a protective mechanism against calcification at pathological sites in the vessel wall. At such sites in atherosclerotic disease and Monckeberg's disease, VSMCs are known to proliferate and differentiate (29). Fetuin uptake and secretion by cells in calcifying lesions may contribute to protection against pathological calcification. The extent of this protective mechanism by fetuin appears to be less than the protective mechanism exerted by MGP (2), as fetuin-deficient mice develop only minor calcified lesions (41).

Fetuin is synthesized by the liver (44) but is found as a major

FIG. 10. Identification of Ser-phosphorylated MGP. Proteins in the cytosolic (A) and microsomal (B) fractions from adenovirus-MGP-infected VSMCs were subjected to two-dimensional SDS-PAGE for identification of the different 14-kDa forms of MGP present in the fractions. A and B, Western blots developed with affinity-purified anti-MGP N-terminal peptide antibodies. The positions of Glu-MGP and Gla-MGP on the blots are indicated. C, proteins in the microsomal fraction as visualized with anti-phosphoserine antibodies (P-Ser). The anti-phosphoserine antibodies recognized the most acidic form of MGP present in the microsomal fraction. Phosphorylated MGP was not found among the cytosolic proteins (A). IEF, isoelectric focusing.



protein in bone matrix (45). Price *et al.* (20) have identified a calcium phosphate-fetuin complex in serum that carries Gla-MGP. The fetuin complex is believed to be formed in bone and has been demonstrated to release MGP when subjected to SDS-PAGE. Since free MGP has never been identified as a protein in serum (4), the fetuin complex could have been the source of MGP when measured in plasma and serum by antibody-based detection methods (46). We purchased serum fetuin from independent suppliers and were unable to find the 14-kDa MGP protein associated with either product. The reasons for the discrepancies between the data presented by Price *et al.* (20) and the results shown in this work are unclear. Furthermore, we have shown that anti-peptide antibodies raised against the N terminus and the Gla region of MGP recognized the 60-kDa fetuin protein. A homology search failed to reveal any sequence homology between the two proteins. Since MS/MS analyses eliminated the presence of MGP in the fetuin protein that was recognized by these antibodies, it appears that fetuin harbors false epitopes that mimic epitopes on MGP. Although antibodies are generally accurate and specific in their antigen detection, they can find epitopes similar to the desired epitope on other structures. Indeed, it is not an uncommon finding that antibodies cross-react with antigens other than the ones that originally elicited them. Our finding raises questions about the specificity of immunological assays that have been used to quantify MGP in serum and tissue extracts.

Immunoprecipitation of fetuin from a RIPA buffer extract of infected VSMCs showed that internalized fetuin is not an intracellular carrier of MGP. However, Glu-MGP and Gla-MGP were detected in the cytosolic fraction, which also harbored fetuin. BMP-2 was not found in the cytosolic fraction, in agreement with its known confinement to organelles of the secretory pathway (16). Earlier electron microscopy studies carried out by our laboratory on cartilage chondrocytes also showed a cytosolic location of MGP (47). Cytosolic MGP appears to be associated with vesicular or membrane-bound structures (47). Since cytosolic MGP must exit from the secretory pathway intracellularly or is taken up by endocytosis by an unknown mechanism, our data suggest that the highly insoluble MGP protein is carried through the cytosol packed in vesicles. These vesicles may be precursors of matrix vesicles known to initiate bone formation in cartilage (48).

The microsomal fraction, which was also shown to contain vesicles derived from the Golgi apparatus, contained a third, more acidic form of MGP. This acidic form could not be detected in the cytosolic fraction. The acidic form was identified as the

Ser-phosphorylated form of MGP. Price *et al.* (22) have shown that the motif in MGP recognized for Ser phosphorylation is Ser-X-Glu. Procino *et al.* (49) have shown that Ser phosphorylation of aquaporin-2 at its Ser-X-Glu motif is carried out by Golgi casein kinase, a membrane kinase located in the Golgi apparatus (50). Phosphorylation of aquaporin-2 is essential for its insertion into the plasma membrane. Non-phosphorylated aquaporin-2 exits the Golgi apparatus in vesicles that appear in the cytosol. The analogy to intracellular sorting of MGP, as our work indicates, is striking. We propose that MGP, phosphorylated by Golgi casein kinase in the Golgi apparatus, exits VSMCs via the secretory pathway. Dephosphorylated MGP or MGP that escapes phosphorylation is released, as is aquaporin-2, from the Golgi apparatus in vesicles that appear in the cytosol. Several studies have shown that protein kinases and phosphatases control sorting of proteins within the Golgi apparatus/endosomal system (18, 36). To understand Ser phosphorylation as a sorting signal for MGP and the relevance of the signal for MGP function, we are currently conducting a detailed study of this system in our laboratory.

In none of the experiments reported in this work were we able to demonstrate a form of MGP that was soluble in the cell medium or physiological buffers. The insoluble properties of MGP strengthen our view of MGP as an insoluble matrix protein. Since one function of MGP as a matrix protein is to bind BMP-2 via its vitamin K-dependent Gla region, we predict that the N- or C-terminal part of the protein is attached to the matrix, leaving the Gla region free for BMP-2 binding.

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