Periostin, a Member of a Novel Family of Vitamin K-dependent Proteins, Is Expressed by Mesenchymal Stromal Cells*

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The modification of glutamic acid residues to γ-carboxyglutamic acid (Gla) is a post-translational modification catalyzed by the vitamin K-dependent enzyme γ-glutamylcarboxylase. Despite ubiquitous expression of the γ-carboxylation machinery in mammalian tissues, only 12 Gla-containing proteins have so far been identified in humans. Because bone tissue is the second most abundant source of Gla-containing proteins after the liver, we sought to identify Gla proteins secreted by bone marrow-derived mesenchymal stromal cells (MSCs). We used a proteomics approach to screen the secretome of MSCs with a combination of two-dimensional gel electrophoresis and tandem mass spectrometry. The most abundant Gla-containing protein secreted by MSCs was identified as periostin, a previously unrecognized γ-carboxylated protein. In silico amino acid sequence analysis of periostin demonstrated the presence of four consensus γ-carboxylase recognition sites embedded within fasciclin-like protein domains. The carboxylation of periostin was confirmed by immunoprecipitation and purification of the recombinant protein. Carboxylation of periostin could be inhibited by warfarin in MSCs, demonstrating its dependence on the presence of vitamin K. We were able to demonstrate localization of carboxylated periostin to bone nodules formed by MSCs in vitro, suggesting a role in extracellular matrix mineralization. Our data also show that another fasciclin I-like protein, βGlh-3, contains Gla. In conclusion, periostin is a member of a novel vitamin K-dependent γ-carboxylated protein family characterized by the presence of fasciclin domains. Furthermore, carboxylated periostin is produced by bone-derived cells of mesenchymal lineage and is abundantly found in mineralized bone nodules in vitro.

Bone marrow stroma contains a population of adherent cells referred to as mesenchymal stromal cells (MSCs), which have the capacity to differentiate into many mesenchymal cell lineages, including osteoblasts, chondrocytes, and adipocytes (1–3). To date, no single marker that is able to identify and localize MSCs in vivo has been described. MSCs are therefore identified in vitro based on their abilities to differentiate into mesenchymal lineage cells and express specific membrane-bound surface antigens that distinguish them from hematopoietic, endothelial, and fibroblastic cells (4, 5). It is widely accepted that one of the main biological functions of marrow MSCs is to provide the microenvironment necessary for support of hematopoiesis, but their role in development, maintenance, and repair of bone is also well established (6–8). It is also known that a subset of proteins post-translationally modified in a vitamin K-dependent manner with γ-carboxylated glutamic acid (Gla), such as osteocalcin (OC) and matrix Gla protein (MGP), play a pivotal role in normal bone development and repair (9). OC is the most abundant noncollagenous protein in bone, suggesting an important role in bone homeostasis. The physiologic role of the Gla-containing proteins in mineralized tissues is still a matter of debate. MGP is recognized as a potent inhibitor of the calcification of soft tissues, whereas OC is involved in the homeostatic regulation of bone mass (10, 11). There is, however, little doubt that γ-carboxylation of these proteins and possibly others is essential for normal bone development, as is exemplified by the well described congenital bone defects observed in warfarin embryopathy (warfarin being a vitamin K antagonist blocking γ-carboxylation of proteins) (12). Since MSCs give rise to all nonhematopoietic cell lineages in the bone (stromal fibroblasts, osteocytes, chondrocytes, and adipocytes) and are also known to support hematopoiesis, we sought to identify which Gla-containing proteins are produced by these cells. To identify the Gla-containing proteins secreted by MSCs, we used a proteomic approach, which permitted an unbiased screening of the entire secretome of MSCs. Two-dimensional gel electrophoresis and tandem mass spectrometry (MS/MS) analysis unambiguously identified periostin (PN) as

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the most abundant Gla-containing protein secreted by MSCs. This was a surprising and unexpected finding, since periostin is not known as a Gla-containing protein. We further show that β1g-h3, another fasciclin 1-like protein highly homologous to periostin, is also carboxylated. Thus, periostin is a member of a new family of Gla-containing proteins characterized by tandem repeat fasciclin-like domains with canonical carboxylase recognition sites (CRS) embedded in them.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and Cell Lines—Antibodies used were as follows: mouse anti-Gla (American Diagnostica Inc.), goat anti-murine periostin (R&D Systems) (this antibody also reacts with human periostin), anti-His (clone H3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rabbit anti-mouse heterogeneous nuclear ribonucleoprotein A1 (generously provided by William Rigby (Dartmouth Medical School, Hanover, NH). The following were from Invitrogen (Molecular Probes): donkey anti-rabbit Alexa Fluor 633 and donkey anti-goat Alexa Fluor 594. All reagents used were from Sigma unless otherwise specified. 293 cells and A549 cells were obtained from the ATCC.

Murine MSC Isolation and Characterization—MSCs from 4-week-old C57Bl/6 mice (Harlan) were isolated as previously described (13). Adherent cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Wisent) plus antibiotics and allowed to expand for at least five passages to obtain a homogenous population before being immunophenotyped by fluorescence-activated cell sorting using the following antibodies: anti-mouse CD31, CD44, CD45, CD73, and CD90. Multipotentiality was determined as described (13). Vitamin K1 (7 μg/ml) was added to the media when stimulation of γ-carboxylation was needed. To block carboxylation, the cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal bovine serum, antibiotics, and sodium warfarin (50 μM).

Human MSC Culture and Differentiation—Passage two human MSCs from two different donors were obtained from the Tulane Center for Gene Therapy, where they were immunophenotyped and assessed for multipotentiality. The cells were cultured and differentiated according to the Center’s instructions.

Secretome Analysis—Murine MSCs or A549 cells were grown on a T75 tissue culture flask (Flacon) and allowed to expand to 80% confluence in the presence of vitamin K. The cells were then washed extensively with phosphate-buffered saline, and serum-free Dulbecco’s modified Eagle’s medium was added. Conditioned medium (CM) was harvested after 48 h. After being 50-fold concentrated on an Amicon ultrafiltration unit (molecular weight cut-off 10,000 kDa; Millipore), the samples were precipitated with 80% cold acetone overnight, washed once with 100% cold acetone, air-dried, and redissolved in DeStreak rehydration solution (Amersham Biosciences). Protein concentration was determined using the two-dimensional Quant kit (Amersham Biosciences).

Two-dimensional SDS-PAGE and Isoelectric Focusing—50 μg of total protein samples in 155 μl of Destreak rehydration buffer supplemented with 1% IPG buffer (3–10NL; Amersham Biosciences) were placed in ZOOM IPGRunner cassette chambers (Invitrogen). ZOOM dry strips (7 cm, pH range 3–10NL; Invitrogen) were then placed in the chambers, sealed, and left for rehydration for 16 h. The cassette was inserted in ZOOM IPGRunner (Invitrogen), and a voltage gradient (200–2000 V) was applied, as recommended by the manufacturer. A total of 2000 V-h was applied. After isoelectric focusing, strips were equilibrated with SDS using NuPAGE LDS sample buffer (1×; Invitrogen), reduced with 2% dithiothreitol (Amersham Biosciences), and alkylated with iodacetamide (Sigma). The procedure was done at room temperature for 15 min with each component (dithiothreitol and iodacetamide). Reduced and alkylated strips were placed on 4–12% gradient BisTris precast minigels (Invitrogen) and immobilized with 1% agarose. Second dimension separation was performed using the XCell SureLock (Invitrogen) module. After completion of the run, one of the gels was fixed overnight in 50% methanol, 10% acetic acid fixation solution and stained with silver nitrate by modified Shevchenko’s protocol. Gel image was acquired on ImageScanner (Amersham Biosciences).

Sample Preparation for MS Analysis, Chromatography, and Mass Spectrometry—Images of silver-stained gel and anti-Gla immunoblot were superimposed, and the spots giving positive signals were selected. The spots were cut manually from the gel and subjected to trypsin digestion on robotic MassPREP Station (PerkinElmer Life Sciences). Sample injection and HPLC separation was done using an Agilent 1100 series system. 20 μl of digest solution was loaded onto a Zorbax 300SB-C18 5 μm 3.0-mm trapping column and washed for 5 min at 15 μl/min with 3% acetonitrile, 0.1% formic acid. Nano-HPLC peptide separation was done using a Biobasic C18 10×0.075-mm pico-frit analytical column (New Objective). The gradient was 10% acetonitrile, 0.1% formic acid to 95% acetonitrile, 0.1% formic acid in 15 min at 200 nl/min. The instrument used for mass spectrometry was a QTRAP 4000 (Sciex-Applied Biosystems). Information-dependent MS/MS analysis was done on the three most intense ions selected from each full scan MS with dynamic exclusion for 90 s. The survey scan was used for an enhanced MS scan from 3500 to 1600 m/z at 4000 atomic mass units/s using Dynamic Fill time. MS/MS data were acquired for three scans from 70 to 1700 m/z, using a fixed 25 ms trap fill time and with Q0 trapping activated.

MS Data Base Search and Protein Sequence Alignments—Peaklists were generated with mascot distiller 1.1 (Matrixscience). Searches of sequences from the NCBI nr database using a rodent taxonomy filter (157,986 sequences) were performed with Mascot 1.9, using trypsin as digestion enzyme, carboxyamidomethylation of cysteines as fixed modification, methionine oxidation as variable modification, and 1.5-Da precursor and 0.8 Da fragment search tolerances. Protein sequences were obtained from NCBI, and alignments were performed using the CLC Free Workbench version 3.1.2 (available on the World Wide Web).

Immunoprecipitation—30 μl of protein G-Sepharose Fast Flow (Sigma) was coupled with anti-Gla antibody or an isotypic control for 2 h. Concentrated CM was incubated with unconjugated protein G-Sepharose beads for 1 h. The precleared samples were then diluted in radioimmune precipitation buffer...
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(Right) and boiled for 3 min to denature the proteins and dissociate protein complexes. After cooling on ice, samples were added to conjugated beads and incubated for 1–2 h. Beads were then washed extensively with radioimmune precipitation buffer and then phosphate-buffered saline. Antigen was dissociated by boiling the beads in sample buffer, and the immunoprecipitate was analyzed by SDS-PAGE followed by Western blotting using the anti-periostin antibody.

Generation of His-PN-overexpressing 293 Cells—The human periostin cDNA fused to a hexahistidine tag cloned in the pcdNA3.1 plasmid was a generous gift from Dr. Xiao-Fan Wang (Duke University) (14). 293 cells were transfected with the plasmid using Polyfect reagent (Qiagen), and stable transfectants (293PN) were selected for using G418 sulfate (Wisetn) at 650 μg/ml.

Recombinant Periostin Purification—293PN cells were grown in the presence of vitamin K, and conditioned medium was collected and concentrated as above. Purification of recombinant His-tagged periostin under native or denaturing conditions was done on an Ni²⁺-nitrilotriacetic acid column (Novagen), as specified by the manufacturer.

Barium Sulfate Precipitation of Periostin—40 mg/ml BaSO₄ was added to concentrated CM from 293PN cells and incubated for 60 min at room temperature. The BaSO₄ crystals were centrifuged briefly, and the pellet was washed three times with large volumes of cold 0.5 M sodium acetate. Carboxylated periostin was eluted using 0.17 M sodium citrate.

Analysis of Periostin Produced by Osteoblasts—MSCs were differentiated into osteoblasts as above. When mineralized nodules covered 50% of the tissue culture vessel, serum-free CM was prepared as above. After collecting the CM, the clarified ECM was dissolved using 0.5 M EDTA and incubated for 30 min at room temperature with gentle shaking. The soluble fraction was centrifuged to remove cells, debris, and insoluble material for Western blotting.

Immunostaining and Laser-scanning Confocal Imaging Analysis—MSCs were grown and differentiated into osteoblasts on coverslips. The immunofluorescence staining has been described elsewhere (15). Briefly, cells were fixed using 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% dry milk in phosphate-buffered saline before being incubated with the primary antibodies and appropriate secondary fluorophore-conjugated antibodies. Images were acquired using a Zeiss LSM 5 PASCAL laser-scanning confocal microscope. Alexa Fluor 633 and 594 antibodies were scanned using excitation wavelengths of 633 and 543 nm, and emission spectra were filtered with 650-nm long pass and 560 – 615-nm band pass filters, respectively and sequentially.

Three-dimensional Structural Modeling of Human Periostin—The crystal structure of fasciclin I (Protein Data Bank entry 1o70), which contains two domains, was used as the template for FAS1 and FAS2 as well as for FAS3 and FAS4. Based on sequence alignment, the structural fragment from Ala⁴⁵⁹ to Ala⁴⁷⁴ in the fasciclin I crystal structure (1o70) was used as a template for the region connecting domains FAS2 and FAS3. Next, 100 structural models of periostin were generated using the MODELLER version 8 software (University of California at San Francisco) (16). The structural model with the lowest objective function was selected for further analysis. Both the stereochemical quality and packing quality of the human periostin model were evaluated as excellent using software WHAT IF version 4.99 (Radboud University Nijmegen, Netherlands) (17).

RESULTS

Identification of Gla-containing Proteins Secreted by MSCs—To identify the γ-carboxylated proteins secreted by murine MSCs, we used a two-dimensional gel electrophoresis approach, which allowed screening the entire secretome. We expanded murine MSCs in the presence of vitamin K to 80% confluence, and serum-free CM was collected. Two parallel gels were made using the CM (Fig. 1A); one of them was transferred to a membrane and probed by Western blotting using an anti-Gla antibody (left), whereas the other was silver-stained (right). The images from the Western blot film and the silver-stained gel were overlaid, and positive spots on the silver-stained gel were cut out and analyzed by MS/MS. The major carboxylated protein (Fig. 1A, spot 2) seen by Western blot as well as a minor species (Fig. 1A, spot 1) migrated at ~90 kDa. MS/MS analysis and Mascot search of these two spots yielded periostin as a high confidence hit with probability-based Mowse scores above 40. As shown in Fig. 1B, total ion score for spot 1 was 592, and eight of the 11 ions identified had scores above 40 (not shown). Importantly, the peptides identified covered most of the protein’s length, with 17% total coverage. This increased our confidence in the positive identification of periostin. Periostin was also found in spot 2 (Fig. 1C), with identified ions covering 6% of the protein’s total length and a total ion score of 192. Because of the strong signal in the anti-Gla immunoblot, spot 2 was difficult to excise precisely, and contaminants were simultaneously excised. This accounts for the low score and poor coverage obtained. The presence of periostin at two different isoelectric points (pI) probably reflects heterogeneity in the extent of γ-carboxylation. Taken together, these results identified periostin, a previously unrecognized vitamin K-dependent protein, as the major γ-carboxylated protein secreted by murine MSCs.

In Silico Primary Sequence Analysis of Periostin—According to Furie et al. (18), the CRS is both necessary and sufficient to drive γ-carboxylation of adjacent glutamic acid residues in a protein. When the CRS of known Gla-containing proteins are aligned, four amino acid residues are highly conserved, and the second of the four (a phenylalanine and an alanine) are most highly conserved. Therefore, we analyzed the published primary sequence of periostin (accession number NM_015784) and found a sequence that contained, properly organized, all of the important amino acids found in the CRS of the other known Gla-containing proteins (Fig. 2A). This sequence was positioned at the N terminus of the first fasciclin-like domain and was repeated (with some variability) in all four fasciclin domains (Fig. 2B). The CRS from the third fasciclin-like domain is the least well conserved. The observed molecular weight of periostin matches its predicted molecular weight, suggesting that periostin does not contain a CRS propeptide that is cleaved during protein maturation. Furthermore, this suggests that all four fasciclin-like domains in periostin may be carboxylated independently through their own CRS.
To further confirm the presence of Gla on periostin, we immunoprecipitated proteins secreted by MSCs with the anti-Gla antibody or an isotypic control antibody. To ensure that we were not precipitating a Gla-containing protein to which periostin binds, we boiled the samples in the presence of detergents (radioimmune precipitation buffer) in order to denature them and break down protein complexes in conditions still amenable to antibody binding. In this case, the SDS present in the buffer prevents the boiled proteins from refolding, but the low concentration of detergents does not impede binding to antibodies. The immunoprecipitates were analyzed by Western blotting using the anti-periostin antibody. As shown in Fig. 3A, periostin could be immunoprecipitated with the anti-Gla antibody but not with the isotypic control antibody.

We next examined whether we could purify recombinant carboxylated periostin. To do this, we obtained the human periostin cDNA fused to a hexahistidine tag, and we stably transfected 293 cells with this construct. The secretion of γ-carboxylated, His-tagged periostin from these 293PN cells was analyzed by Western blotting (Fig. 3B). The results demonstrate that only transfected cells produced detectable levels of His-tagged periostin and that this protein was most likely carboxylated. To confirm the latter, we purified recombinant PN using immobilized metal affinity chromatography. We ran the CM from the 293PN cells on a nickel-nitritoltriacetic acid His-bind column under both native and denaturing conditions. Surprisingly, only under denaturing conditions could the carboxylated variant of periostin be purified (Fig. 3C). This result indicates that carboxylation of periostin most likely induces conformational changes in the protein, making the His tag inaccessible for binding to nickel.

One of the main characteristics of Gla residues is their ability to bind divalent metal cations, such as calcium, magnesium, and barium. This feature has been historically used for the purification of plasma-derived Gla-containing proteins, such as coagulation factor IX. In this case, barium salt crystals are used to precipitate Gla-containing proteins as a first step in protein purification. We thus examined whether barium sulfate could be used to precipitate periostin. Fig. 3D shows that carboxylated periostin does bind BaSO₄ crystals with sufficient affinity to allow for its precipitation and thus possesses an affinity for divalent metal ions similar to that of other Gla-containing proteins.

**γ-Carboxylation of Periostin by MSCs**—To further confirm the presence of Gla on periostin, we immunoprecipitated proteins secreted by MSCs with the anti-Gla antibody or an isotypic control antibody. To ensure that we were not precipitating a Gla-containing protein to which periostin binds, we boiled the samples in the presence of detergents (radioimmune precipitation buffer) in order to denature them and break down protein complexes in conditions still amenable to antibody binding. In this case, the SDS present in the buffer prevents the boiled proteins from refolding, but the low concentration of detergents does not impede binding to antibodies. The immunoprecipitates were analyzed by Western blotting using the anti-periostin antibody. As shown in Fig. 3A, periostin could be immunoprecipitated with the anti-Gla antibody but not with the isotypic control antibody.

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Finally, to demonstrate that the carboxylation of periostin was dependent on the presence of vitamin K, we expanded murine MSCs in medium supplemented with charcoal-stripped fetal bovine serum, which removes most of the vitamin K present in fetal bovine serum, and in the presence of the vitamin K antagonist sodium warfarin. The MSCs grown under these conditions produced similar levels of periostin, but the protein was only poorly carboxylated (Fig. 3E), demonstrating that the post-translational modification is vitamin K-dependent.

Periostin Expression and Carboxylation by Human MSCs—We next determined whether the carboxylation of periostin was also operative in human MSCs and their differentiated progenies of mesenchymal lineages in vitro. Human MSCs were obtained from two different donors and grown to confluence in 6-well plates. The cells were then differentiated to either osteocytic, adipocytic, or chondrocytic lineages by using standard differentiation media supplemented with vitamin K. Histological assessment of differentiation was performed, and CM was sampled. Representative results from one of the donors are presented in Fig. 4. We observed that cultures of undifferentiated human MSC do secrete periostin as did cultures induced to differentiate into adipocytes and osteoblasts (left). However, in cultures where chondrogenic differentiation was induced, no periostin expression could be detected at the protein level. Interestingly, secreted periostin appeared carboxylated in all instances except when produced by osteoblasts (right), although the latter secreted similar amounts of the protein.

It is known that carboxylated OC tightly binds the calcium-rich extracellular matrix in bones (19, 20); therefore, we...
hypothesized that carboxylated periostin produced by osteoblasts would be sequestered within the calcified matrix and would not be detectable in the CM of those cells. To test this hypothesis, we collected CM from MSC-derived osteoblasts and also decalcified the mineralized matrix using the calcium chelator EDTA. The proteins released from the ECM by this treatment were analyzed by Western blotting (Fig. 4F). This finding shows that osteoblasts are able to carboxylate periostin but that the protein is sequestered within the ECM in a calcium-dependent manner.

To confirm the presence of periostin in the mineralized matrix produced by osteoblasts, we performed confocal microscopy analysis of bone nodule formation by MSCs. Although we could detect intracellular periostin in the endoplasmic reticulum of cells both outside and inside the nodules (not shown), our data demonstrate extensive deposition of periostin only in the mineralized matrix of bone nodules in vitro (Fig. 4G). The adjacent extracellular matrix outside the nodules did not stain for periostin (Fig. 4H). It can also be noted that osteocytic cells throughout the nodule are embedded in a compact extracellular matrix, which stains densely for periostin (Fig. 4, I at 11.7 &mu;m within the nodule) and J). The donut shape of the staining observed for periostin on the bone nodule is probably due to the incapacity of the antibody to penetrate the highly mineralized nodule, since nuclei within this nodule also failed to stain with the heterogeneous nuclear ribonucleoprotein A1 antibody. Therefore, whether periostin locates only near the surface of the nodule or also within it remains to be determined.

Three-dimensional Structural Modelling of Human Periostin—In order to obtain structural information about periostin, we built a tridimensional model of human periostin based on its homology with the insect fasciclin I protein, for which a crystal structure is available. The best fit model is presented in Fig. 5, where...
the four fasciclin domains are shown in different colors. There is, however, one major limitation to the model. Although the stereochemical quality and packing quality were evaluated as excellent, we cannot exclude the possibility that calcium binding to the Gla residues on periostin induces profound conformational changes in the tertiary structure of the protein. The model is nevertheless still useful to analyze the spatial organization of the protein before its modification by the carboxylase enzyme. The predicted model shows that all four CRS present in human periostin have a similar secondary structure consisting of a helix-turn-helix motif (shown as red ribbons in Fig. 5). This helical secondary structure is consistent with the secondary structure of the prothrombin CRS as assessed by NMR (21). Moreover, the model predicts that all four CRS are solvent-exposed and can thus interact with the \( \gamma \)-glutamyl carboxylase. This is particularly important, since \( \gamma \)-carboxylation is known to be a post-translational modification as opposed to a co-translational one.

In Gla-containing proteins, the CRS is believed to be the primary docking site of the carboxylase enzyme, and this binding is thought to bring the Gla domain in proximity to the catalytic pocket. The \( \gamma \)-carboxylated residues in all known Gla-containing proteins are found within 40 amino acids of the CRS. Matrix Gla protein was previously the only other Gla protein in which the propeptide-like sequence is not removed during maturation, and this made possible the observation that residues N-terminal of the CRS can also be carboxylated (22). There is also experimental evidence that glutamic acid residues located within the propeptide sequence can be carboxylated, such as that of coagulation factor VII, which contains a diglutamate pair (17). Therefore, we identified all glutamic acid residues in the human periostin primary sequence that were located within 40 amino acids of both the N and C terminus of the four CRS, since these represent candidate targets for carboxylation. The 28 identified residues are listed in Table 1 with their location relative to the nearby CRS. The same residues are shown as white spheres in the three-dimensional model (Fig. 5). There is a striking heterogeneity in the number and spatial distribution of these candidate glutamic acid residues in the different fasciclin domains. Furthermore, no consensus could be found in the primary sequence alignments (not shown) or spatial organization of those candidate residues. The number of potential carboxylation sites ranges from 4 to 10 in fasciclin domains 3 and 2, respectively. Also, some candidate Glu residues are solvent-exposed, whereas others appear sequestered within the protein core. Clearly, the heterogeneity observed in the distribution of

![FIGURE 5. Fasciclin I-like proteins; a novel family of vitamin K-dependent proteins. A, three-dimensional modeling of the four fasciclin domains of periostin. The predicted tridimensional structural model of human periostin is colored according to domains FAS1, FAS2, FAS3, and FAS4. The CRS motifs are shown in red ribbons. The glutamic acids within 40 amino acids of the CRS motifs are shown in white spheres. B, sequence alignment of some CRS sequences found in the Fas domains of all known fasciclin-like proteins in humans. Sequence analysis demonstrated the presence of a CRS-like sequence in most fasciclin domains analyzed for all of the proteins. Representative examples are shown. The fasciclin domain from which the CRS was derived is given for each protein. ST1, stabilin-1; ST2, stabilin-2. C, identification of Gla on \( \gamma \)-h3. A method similar to that used in Fig. 1 was used to analyze the seceretome of A549 cells secreting \( \gamma \)-h3. The spots on the silver-stained two-dimensional gel that showed immunoreactivity for the Gla antibody were analyzed by MS/MS, and all four spots were shown to contain \( \gamma \)-h3. D, Mascot search of the ions obtained from MS/MS analysis of spot 4 identified six peptides (in boldface type) covering 15% of the \( \gamma \)-h3 sequence.]
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TABLE 1

Positions of the candidate glutamic acid residues that could be targeted for carboxylation in human periostin

| Position of candidate Glu residues | Position relative to CRS |
|-----------------------------------|--------------------------|
| Fas domain 1                      |                          |
| 124                               | N-terminal               |
| 125                               | N-terminal               |
| 127                               | N-terminal               |
| 140                               | Within                   |
| 154                               | C-terminal               |
| 160                               | C-terminal               |
| Fas domain 2                      |                          |
| 242                               | N-terminal               |
| 244                               | N-terminal               |
| 261                               | N-terminal               |
| 277                               | Within                   |
| 280                               | Within                   |
| 288                               | C-terminal               |
| 298                               | C-terminal               |
| 313                               | C-terminal               |
| 322                               | C-terminal               |
| 325                               | C-terminal               |
| Fas domain 3                      |                          |
| 375                               | N-terminal               |
| 402                               | N-terminal               |
| 442                               | C-terminal               |
| 450                               | C-terminal               |
| Fas domain 4                      |                          |
| 496                               | N-terminal               |
| 501                               | N-terminal               |
| 517                               | N-terminal               |
| 523                               | N-terminal               |
| 547                               | Within                   |
| 548                               | Within                   |
| 550                               | C-terminal               |
| 578                               | C-terminal               |

DISCUSSION

We have demonstrated that marrow-derived MSCs secrete periostin and that a subset of this secreted protein is γ-carboxylated in a vitamin K-dependent manner. This unique biochemical feature was not predicted by what is known to date of periostin. There is a considerable body of work that has defined the role of periostin in health and disease. Periostin is an ECM-associated protein whose expression is developmentally regulated and is associated with bone, heart, and dental ligament ontogeny (23). It has been shown to directly bind some integrins and has been found to promote cell migration and angiogenesis (24, 25). It is also highly expressed in remodeling tissues both under physiological and pathological conditions, and its expression by tumor cells is associated with a poor prognosis (26). The periostin knock-out mouse demonstrated a role for periostin in the postnatal maintenance of periodontal ligaments, trabecular bones, and heart valves (23). Moreover, it has been shown in these mice that periostin plays an important role in the physiological response to vascular injury (27), and these findings defined its role as a key player in the pathological remodeling of the myocardium following ischemic injury. Although the precise function of periostin in the infarcted heart is still unclear, it definitely has an important role in remodeling processes like fibrosis and cardiomyocyte hypertrophy (28, 29). The function of periostin in cancer development is mostly attributed to its role in cell migration and its proangiogenic properties. Although periostin was first described as OSF-2 (osteoblast-specific factor 2) in 1993 (30), ours is the first description of this protein being amenable to γ-carboxylation. At present, virtually all published in vitro studies conducted to unveil the functions of periostin have used recombinant periostin devoid of γ-carboxylated Glu residues due to its being produced in insect cells and/or without vitamin K. Hence, the significance of periostin γ-carboxylation remains unexplored in vitro as well as in vivo.

Since we only observed the full-length 90-kDa periostin by Western blotting, we can only speculate at this time whether other isoforms of periostin (such as the splice variant periostin-like factor) (31) are also carboxylated. However, because these isoforms are produced by alternative splicing at the C terminus, they must also contain the CRS and thus should also be carboxylated.

Based on strong experimental evidence, Furie et al. (18) defined the minimal criteria for a protein to undergo carboxylation as follows: 1) the protein possesses a CRS allowing binding to the carboxylase; 2) the protein translocates to the endoplasmic reticulum during its maturation; 3) the cells express the γ-carboxylase enzyme; 4) glutamic acid residues are present in the 40- amino acid region adjacent to the CRS; and 5) vitamin K is present in the system. When all of these requirements are met, the presence of the CRS is necessary and sufficient to drive carboxylation of the protein. Accordingly, we analyzed the primary amino acid sequence of periostin for the presence of the CRS. We found a sequence within the first fasciclin domain of periostin that matched the consensus sequence of other known GRS. This sequence was repeated in all four fasciclin domains found within periostin. To our knowledge, this is the first Gla-
containing protein identified that contains more than one CRS. Whether all four CRS are functional in periostin remains to be determined. However, we can speculate that the four fasciclin domains are carboxylated independently. Interestingly, the CRS found in periostin fasciclin domains partially overlap the H1 region, which is one of the two conserved regions defining a fasciclin domain. This led us to search for the CRS in the other mammalian fasciclin-like proteins, namely fasciclin domain. This led us to search for the CRS in the other mammalian fasciclin-like proteins, namely βlg-H3, stabilin-1, and stabilin-2. Sequences similar to the periostin CRS were also found in all of these proteins (Fig. 5B), and we confirmed that at least one of them was also carboxylated. This observation strongly suggests that all mammalian proteins bearing fasciclin-like domains can be γ-carboxylated, defining a new family of γ-carboxylated proteins. It is also worth noting that mutations in the CRS-like region of βlg-H3 are known to cause corneal dystrophies in affected individuals (32).

The electrophoretic migration pattern of periostin, according to its pl, indicates that it is produced by MSCs in two major forms, one that is highly carboxylated and another that is poorly carboxylated. The carboxylation of a glutamic acid residue introduces an acidic carboxyl group, which adds only 40 Da to the total molecular mass of the protein but has a substantial impact on its isoelectric point (33). It is, however, difficult to estimate the number of Gla residues on a protein based on a variation of its charge, since other posttranslational modifications may also influence its pl. The dichotomic pattern of migration observed in periostin, however, from a very basic pl to a very acidic pl, seems to indicate that periostin is decorated with many Gla residues in a nonrandom manner, which could suggest distinct biological roles for highly and poorly carboxylated variants of periostin. This could otherwise represent experimental limitations in vitro. The latter would reflect the situation seen with OC and MGP in vivo, of which a significant fraction of the total antigen circulates as an incompletely carboxylated form in healthy individuals because of subclinical vitamin K deficiency (9).

Only 12 vitamin K-dependent Gla-containing proteins have so far been unambiguously identified in humans. Periostin and βlg-H3 would be the 13th and 14th, respectively. According to the organization of the Gla domain and subcellular localization, these proteins can be subdivided into four classes (9, 18, 35): 1) typical Gla proteins, which are mainly involved in blood coagulation (coagulation factors II, VII, IX, and X, protein C, and protein S); 2) endothelial homeostasis (Gas6); 3) Gla-containing proteins found in mineralized tissues, such as bone and teeth (OC and MGP); and 4) transmembrane Gla-containing proteins (proline-rich Gla proteins 1 and 2 and transmembrane Gla proteins 3 and 4). Periostin would fall in the bone-associated Gla-containing protein group due to its known association with skeletal development and secretion into the extracellular space akin to that seen with OC and MGP. This is supported by our observation that carboxylated periostin associates with the calcified matrix produced by osteoblasts and is deposited in high abundance on bone nodules forming in vitro.

Bone-associated Gla proteins, OC and MGP, differ from the typical Gla-containing proteins by the fact that they do not possess the prototypical N-terminal Gla domain initially defined in vitamin K-dependent blood clotting proteins. OC and MGP each contain 3 and 5 Gla residues, respectively. However, MGP differs significantly from OC and other known Gla-containing proteins in that its propeptide-like sequence containing the CRS is present in the mature secreted protein, and Gla residues are located in both the N terminus and C terminus of its CRS. Accordingly, Price et al. (36) suggested that the CRS could be located anywhere in a Gla-containing protein to direct carboxylation to both upstream and downstream Glu residues, a feature possibly shared by MGP and periostin. Periostin and MGP are also unique among Gla-containing protein in that they do not contain a propeptide sequence. Although their function is not completely understood, knock-out experiments with OC and MGP demonstrated their role in regulating the normal and pathological mineralization of tissues (19, 20). MGP is known as one of the most potent inhibitors of soft tissue mineralization (9). However, its mechanisms of action are still being debated. The fact that it is abundantly found in nonmineralized cartilage suggests that it does not require calcium for binding to the ECM. However, calcium binding to the Gla residues in MGP triggers conformational changes in the tertiary structure of the protein, allowing protein-protein interactions with BMP-2 and modulating its activity (37). Osteocalcin, on the other hand, needs calcium to associate with the ECM. Binding of calcium to OC also triggers conformational changes in the protein. The known functions of OC include inhibition of hydroxyapatite nucleation and inhibition of the brushite to hydroxyapatite metamorphosis. It is also a potent chemoattractant to osteoclast precursors and can activate mature osteoclasts (38).

It is interesting to note the similarities shared by periostin and these other bone-derived Gla proteins. Our data indicate, for instance, that periostin had an affinity for the mineralized matrix of osteoblasts. Also, our attempts at purifying the recombinant carboxylated form of periostin suggest that the carboxylated form does not bind the nickel column, as opposed to the noncarboxylated variant. This could be attributed to a difference in the conformation of the protein, although a change in the surface charges of the protein due to the carboxylation could also explain this low affinity to the nickel column. Finally periostin, like osteocalcin and MGP (20, 39), is also known as an ECM-associated protein with chemoattractant and cell adhesion properties for many cell types.

The predicted three-dimensional model of human periostin provided important structural information about the protein. First, it showed that the secondary structures of all four CRS were similar and that they are all exposed to solvent. This indicates that periostin may be the first Gla-containing protein characterized to date with more than one functional CRS. Second, the model predicts that the spatial distribution of glutamic acid residues surrounding the CRS is not the same in all four fasciclin domains. We can only speculate on the relevance of this last observation. Clearly, more biochemical data are needed to define the function of γ-carboxylation in periostin. However, the elucidation of the role of each fasciclin domain and the identification of the specific residues that are carboxylated, in conjunction with the three-dimensional model of the protein, will help to rationalize the role of Gla in periostin. For instance, in typical Gla-containing proteins, the Gla residues are seques-
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terred within the protein core. Calcium binding by these Gla residues forms a linear calcium-carboxylate network, which triggers conformational changes in the proteins and allows binding to phospholipid membranes. On the other hand, in OC, the Gla residues are exposed to solvent and are part of a helical structure thought to be involved in the interaction with the calcium-phosphate lattice of hydroxyapatite (38).

In order to study whether the carboxylation status of periostin by murine MSCs was also observed in humans, we investigated its expression by human MSCs. We also examined whether carboxylated periostin was produced by the adipogenic, osteogenic, and chondrogenic differentiatied progeny of human MSCs. Although differentiation of MSCs in vitro never yields 100% of differentiated cells and thus results in a non-homogenous cell population, we were able to derive interesting observations from this assay. The undifferentiated human MSCs as well as all of their progeny except chondrocytes did secrete similar levels of periostin. This latter observation could be accounted for by the sensitivity of our assay, since others have found chondrocytes to express periostin in vivo (40). Because chondrocytic differentiation of MSCs is done in micromass cultures with high density but low total cell numbers, the detection method used may have missed low levels of the protein. Surprisingly, we were not able to detect the carboxylated form of periostin in the conditioned medium of human MSC-derived osteoblasts, whereas the periostin secreted by normal derived osteoblasts, whereas the periostin secreted by normal MSCs and MSC-derived adipocytes was carboxylated. We thus examined whether carboxylated periostin could be found associated with the calcified matrix of osteoblasts and found this to be the case. By confocal microscopy, periostin was found to be abundantly deposited in bone nodules, in areas were osteoblastic cells are tightly embedded in mineralized extracellular matrix.

The data presented here demonstrate that bone marrow-derived MSCs are a major source of periostin. Although the exact function of periostin in bones remains elusive, its continuous expression from development to postnatal life as well as up-regulation during fracture healing underlines its importance in bone maintenance and regeneration (41). Because MSCs represent a specific bone stem cell pool participating in bone development and repair (42, 43), we propose that MSC-derived periostin is a key player in these processes. MSCs are also known to be able to migrate and home to distant sites of tissue injury (24). Periostin is a key player in these processes. MSCs are also known to present a specific bone stem cell pool participating in bone development from development to postnatal life as well as up-regulation by mesenchymal fibroblast-like cells in the heart after myocardial infarction. Thus, it is conceivable that some of these periostin-expressing cells are MSCs that have homed to the infarcted heart and are participating in tissue remodeling. Again, the specific role of periostin in the infarcted heart is still a matter of debate. Whereas some have shown that periostin knock-out mice show less fibrosis and cardiomyocyte hypertrophy after myocardial infarction (28), others have shown that exogenous supplementation of periostin after the infarct decreases fibrosis and hypertrophy (29). In light of the present data, it will be important to assess whether the carboxylation status of periostin affects these results. In all of these cases, however, it would theoretically be possible to modulate periostin function by using clinically available vitamin K antagonists.

In summary, our results demonstrate that periostin is a novel γ-carboxylated protein that is highly expressed by MSCs. Periostin is known to be an extracellular matrix-associated protein and is also known to interact with integrins on the cell surface by its fasciclin domains (24, 26). We hypothesize that some of those fasciclin domains are the sites of γ-carboxylation and that this modification modulates cell-ECM interactions. Since periostin knock-out mice show skeletal and dental phenotypes (23), Gla residues on periostin may also provide hydroxyapatite binding properties that may have an important structural role. Alternatively, γ-carboxylation of periostin could modulate binding to integrins. Periostin is believed to possess important proangiogenic activity and is also expressed in nonmineralized tissues and in tumor cells. At the cardiovascular level, it is also implicated in pathological conditions, such as restenosis after balloon injury and myocardial remodeling after infarction. It will be important to address the role of carboxylation of periostin on angiogenesis, tumor metastasis, and cardiovascular pathologies, especially since its carboxylation can be readily modulated by vitamin K antagonists, such as warfarin.

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