A Novel Interaction between Perlecan Protein Core and Progranulin

POTENTIAL EFFECTS ON TUMOR GROWTH*

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In an in vivo search of novel partners for perlecan, a major heparan sulfate proteoglycan of basement membranes and cell surfaces, we identified progranulin, a secreted growth factor, as a strong interacting protein. Unambiguous interaction, first observed with the yeast two-hybrid system, was corroborated by co-immunoprecipitation studies using cell-free transcription/translation and transient cell transfection assays. The interaction of progranulin with perlecan domain V involved the first two laminin- and epidermal growth factor-like repeats. Within progranulin, the subdomains interacting most with perlecan harbored granulins F and B. Kinetics analysis of the interaction using surface plasmon resonance showed a saturable binding of relative low affinity (Kd ~ 1 μM). These results were supported by significant expression overlap of these two proteins in a series of ovarian tumor tissue microarrays. Progranulin was present within proliferating blood vessels of ovarian carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan. Notably, both progranulin and domain V stimulated the growth of adrenal carcinomas and perivascular matrices, with a distribution similar to perlecan.

Perlecan, a heparan sulfate proteoglycan found in nearly all basement membranes and cell surfaces, has a complex structure based on seven protein modules arranged in five distinct domains (1). The individual domains of perlecan interact with a variety of macromolecules. For example, the C-terminal domain V, which harbors three laminin G (LG) modules separated by four epidermal growth factor (EGF) repeats, interacts with laminin and collagen Type IV and serves as an attachment substrate for various cells (2). Domain V interacts with fibulin-2, β1-integrin, heparin (3), collagen Type XVIII (4), and extracellular matrix protein-1 (5). Perlecan is deposited in all vascularized tissues where it maintains structural integrity through its interaction with other basement membrane components (6, 7). Genetic evidence for the role perlecan of in vasculogenesis derives from the complex phenotype of perlecan null mice. The majority of these mutant mice perish at day 10.5 of embryogenesis because of increased blood vessel pressure (8). Surviving mice die shortly after birth and exhibit extreme vascular and cephalic abnormalities (8, 9), as well as a high incidence of cardiac outflow malformations (10).

In addition to these structural functions, perlecan affects cell proliferation, tumor invasion, and angiogenesis primarily by modulating the activity of growth and angiogenic factors (11). For instance, perlecan protects bioactive molecules from proteolytic degradation and misfolding, and the subsequent regulated cleavage by proteases provides a mechanism by which vital growth factors are released to induce multiple signaling cascades (12). Perlecan is highly enriched in various tumorogenic cell lines and tumors (13, 14), and blocking its endogenous production suppresses the autocrine and paracrine functions of FGF2 and impairs tumor growth and invasion (15–17). These multiple roles of perlecan correlate with its widespread tissue distribution (7). Notably, a similar distribution (18) and some overlapping functions are shared by progranulin (19), also known as epithelin precursor (20), PC cell-derived growth factor (21, 22), or acrogranin (23). Progranulin is a large, secreted precursor of 593 amino acids, which contains seven and one-half protein repeats of ~6 kDa called granulins (24). The granulins are characterized by a highly conserved motif of 12 cysteine residues consisting of four cysteine pairs flanked by two single cysteine residues near their respective N and C termini. Individually, several granulins are biologically active (24). However, recent studies have shown that the entire progranulin is functional (25, 26). Progranulin is a mitogen for various epithelial and mesenchymal cells (21, 25–28) and promotes in vivo tumor growth (25), wound healing, and angiogenesis (29). In the present study we discovered a novel interaction between the perlecan domain V and progranulin, an interaction that could modulate tumor growth.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Library Screening—The screening was done with the Matchmaker GAL4 system (Clontech, Palo Alto, CA). Assays for α- and β-galactosidase, growth in quadruple minus (Trp-, Leu-, His-, Ade-) media, and liquid assay quantification of β-galactosidase activity were performed as described previously (5, 30).

In vitro Transcription/Translation, Co-immunoprecipitation, and Immunoblotting—In vitro transcription/translation was performed with the Tnt® reticulocyte lysate system (Promega) using the pGBK7-T domain V and pGADT7-progranulin constructs and [35S]methionine as the labeled precursor. Co-immunoprecipitation with anti-Myc or anti-hemagglutinin (HA) antibodies (Clontech) was as described previously (5). For co-precipitation in mammalian cells, we generated domain V-expressing 293-EBNA cells by culturing in puromycin (1 μg ml−1) for at least 4 weeks. Following verification of domain V expression by

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The abbreviations used are: LG, laminin G-like module; EGF, epidermal growth factor; HA, hemagglutinin epitope.
enzyme-linked immunosorbent assay and microsequencing (4), three domain V-expressing clones were pooled and transiently transfected with a full-length progranulin cDNA cloned into pcDNA3.1 using LipofectAMINE 2000 (Invitrogen). As a further control, we transfected wild type 293-EBNA cells with the progranulin construct. After 48 h, the conditioned media were collected and filtered, and a mixture of protease inhibitors (CompleteTM, Roche Applied Science) was added. Rabbit polyclonal anti-domain V antiserum was added (1:200 dilution), and the proteins were immunoprecipitated at 4°C for 16 h. Sepharose-G beads (Amersham Biosciences) were added and incubated for 4 h, washed four times, boiled, and analyzed by SDS-PAGE and immunoblotting (4).

### Purification of Reconstituted Domain V and Progranulin

**Immunohistochemistry**—Recombinant full-length progranulin and domain V were purified from the secretion of 293-EBNA cells (4). Purity was verified by SDS-PAGE and immunoblotting with specific antibodies. Binding of domain V to progranulin was determined with a BicoreTM 2000 (Biacore) using previously described parameters (31). Initially, the progranulin was captured on individual channels of the surface of a CM5 sensor chip by amine coupling. Solutions containing domain V in elution buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) at the required concentration were then injected over each surface, and the response was measured as a function of time. After subtraction of the contribution of bulk refractive index changes and nonspecific interactions with the CM5 chip surface (typically <1%), the individual association (k_a) and dissociation (k_d) rate constants were obtained by global fitting of data to a 1:1 Langmuir binding model, using BIAevaluationTM. These values were then used to calculate the dissociation constant (K_D). Growth was assayed with CellTiter 968 proliferation assay (Promega).

**Immunohistochemistry** of ovarian cancer tissue (Fig. 1C) says, which utilize a sensitive chemiluminescence detection system, which utilizes the substrate Galacto-StarTM (Clontech). The values represent the mean ± S.D. (n = 5) and are expressed as relative light units normalized on cell content (OD600). Experiments were repeated three times.

### RESULTS AND DISCUSSION

To identify novel interacting partners for the perlecan protein core, we employed perlecan domain V as bait (Fig. 1A) and screened a human keratinocyte cDNA library using the yeast two-hybrid system (32). Domain V was initially cloned into the pGBK-T7 vector and was tested by itself to ensure that it did not activate the reporter genes HIS3 and ADE. As a control for positive interactions, all constructs were also tested as either bait or prey and for growth in double minus (Trp–/Leu–) media. One of the fastest growing clones (clone A12) contained a ~0.9-kb cDNA insert (Fig. 1B) which encoded the 17-amino acid signal peptide and the first 254 residues (Thr^1–Leu^254) of the mature progranulin (19), including the partial granulin p, and granulins G, F, and B (Fig. 1C). The cDNA was in frame with the activating domain of the plasmid, further supporting the concept of a real protein-protein interaction. We then subcloned clone A12 into the pGBK-T7 vector, and the interaction with domain V was tested with the yeast two-hybrid system on a one-to-one basis. The growth of the cells in quadruple minus media was comparable with the positive control and also with the activity of α-galactosidase (Fig. 1D).

Next, we analyzed the precise site of progranulin interaction within perlecan domain V. To this end, we generated seven deletions of domain V in the pGBK-T7 (bait) vector (Fig. 2A). The plasmids were co-transfected with the pGADT7-progranulin (prey) vector into AH109 yeast cells, and the cells were plated on selective media. Robust growth was observed in cells co-transfected with progranulin and either full-length domain V, Δ1, or Δ2. Moderate growth was observed only with Δ5, but minimal or no growth was observed with the other deletion mutants (Fig. 2A). These results were strengthened by qualitative (not shown) and quantitative liquid β-galactosidase assays, which utilize a sensitive chemiluminescence detection system (Fig. 2B). The results indicate that LG1 and EGF1–2 together are the strongest binding sites for progranulin, based on the robust reaction of Δ2. However, individually, they do not interact significantly with progranulin because Δ3 and Δ4 showed minimal growth (Fig. 2A) and β-galactosidase activity (Fig. 2B). However, Δ5 was consistently reactive and showed lower, but still significant levels of β-galactosidase activity. Thus, we conclude that the major site of progranulin interaction resides within LG1 and EGF1–2, but LG2/EGF3–4 can substitute reasonably effectively.
Progranulin Interacts with Perlecan

Next, we determined the precise site of perlecan domain V interaction within progranulin. First, we generated a full-length progranulin by ligating into clone A12 two expressed sequence tags that encode granulins A, C, and D (NCBI 2171326) and E (NCBI 6007270), respectively. The sequence matched the 593 amino acid residues of progranulin (19, 20).

We then subcloned progranulin into the pGADT7 vector (bait) and generated three additional C-terminal deletions (Fig. 3A). The plasmids were co-transfected with the pGADT7-domain V (prey) vector into AH109 cells, and the cells were plated on selective media. Strong growth was observed in cells co-transfected with domain V and either full-length progranulin or Δ1 (Fig. 3A). Interestingly, Δ2 gave reduced growth and Δ3 minimal growth. These results, corroborated by quantitative β-galactosidase assays (Fig. 3B), clearly show that the main binding site resides within granulins B and F.

To strengthen the interaction obtained with the yeast two-hybrid system, the two proteins were in vitro transcribed and translated and subjected to co-immunoprecipitation. The bait construct conferred a Myc epitope to perlecan domain V, whereas the prey construct conferred an HA epitope to progranulin. The translated proteins were first analyzed on SDS-PAGE, and bands corresponding to the predicted sizes of the molecules, i.e., ~81 kDa for domain V and ~34 kDa for progranulin (clone A12), were obtained (Fig. 3C, left panel). Notably, anti-Myc and anti-HA monoclonal antibodies co-precipitated both proteins (Fig. 3C, right panel). In contrast, no protein was precipitated when empty plasmids were used.

The yeast two-hybrid system is an established method for investigating protein-protein interactions involved in intracellular events. However, only recently has the method been used to study interactions between secreted proteins (4, 5, 26), in part because glycosylation and disulfide bond formation might not be correctly reproduced in the yeast cytosol. This is particularly critical for the proteins in this study, insofar as progranulin and domain V contain 88 and 30 cysteine residues, respectively. Thus, we confirmed the specificity of the interaction using transient transfection in human 293-EBNA cells, harboring the Epstein-Barr virus nuclear antigen, that previ-
Ovarian cancer (Fig. 4, co-localized within the stroma and the perivascular spaces of constant concentration (100 nM) of domain V. There was neither an additive nor a synergistic effect; on the contrary, interaction between progranulin and domain V led to a marked suppression of their respective mitogenic activities (Fig. 4C).

Because perlecans and progranulin are strongly expressed in the ovary and in ovarian cancer (2, 27), we investigated the expression of these two proteins in a series (n = 50) of tissue microarrays derived from various types of ovarian tumors. Progranulin was present within proliferating blood vessels of ovarian carcinomas (Fig. 4, D and F), with a distribution similar to perlecans (Fig. 4, G and J). Progranulin and perlecans also co-localized within the stroma and the perivascular spaces of ovarian cancer (Fig. 4, E and H, *asterisk*). In addition, progranulin was localized within scattered ovarian cancer cells (Fig. 4F). Collectively, these studies substantiate the protein-protein interaction reported above and further indicate that the perlecans and progranulin core could interact in vivo with progranulin within the newly formed tumor blood vessels.

In this study, we provide several independent lines of evidence for a physiological interaction between the C terminus of perlecans protein core and progranulin. First, when screening a human keratinocyte library using perlecans domain V as bait, we identified a novel molecule interacting with the perlecans protein core. Once we had corroborated the interaction using the yeast two-hybrid system and one-to-one transfections, we then identified the interactive regions within the respective molecules. Using a battery of deletion mutants, we demonstrated that the major site of interaction within perlecans was complex and included the first two LG and EGF modules. Within progranulin, the subdomains that interact most with perlecans harbored granulins F and B. Second, the specificity of the interaction was substantiated by co-immunoprecipitation experiments using cell-free transcription/translation systems as well as transient cell transfection. A nonspecific electrostatic interaction between progranulin and perlecans domain V is less likely insofar as they have a very similar net negative charge of −17.53 and −17.05, respectively, at pH 7.5. Third, the potential physiologic relevance of complex formation between progranulin and perlecans was corroborated by an interaction using a biosensor chip and by their overlapping tissue distribution. Fourth, we discovered that although both progranulin and domain V caused enhanced growth of SW-13 cells, when supplemented together, their mitogenic activity was neutralized.

Notably, progranulin was associated with blood vessels similarly to perlecans, and it has recently been shown that recombinant progranulin not only stimulates the growth of various tumor cells but also the proliferation and migration of endothelial cells (29). We recently discovered that perlecans domain V is a potent anti-angiogenic factor and have named it endorepellin, to signify its anti-endothelial function (4). Because proteolytic processes of several basement membrane proteins can directly affect blood vessel formation and tumor growth, a biological consequence of progranulin/perlecans interaction is that they might tune each other's biological activity, which could contribute to a fine regulation of tumour angiogenesis and ultimately affect cancer growth and metastasis.

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