Progranulin Gene Expression Regulates Epithelial Cell Growth and Promotes Tumor Growth in Vivo

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ABSTRACT

Progranulin is a 593-amino acid glycoprotein, the mRNA of which is expressed by many epithelial cells both in vitro and in vivo, but the biological significance of this expression is unclear. In this study, we demonstrate that overexpression of the progranulin gene in SW-13 adrenal carcinoma cells and MDCK nontransformed renal epithelia results in the transfection-specific secretion of progranulin, acquired clonogenicity in semisolid agar, and increased mitosis in monolayer culture, whereas diminution of progranulin gene expression impairs growth of these cells. Purified recombinant progranulin reproduces the effects of forced progranulin expression, being clonogenic in soft agar and mitogenic in monolayer culture to SW-13 and MDCK cells and other epithelia of various origins such as GPC16 colonic epithelium and A549 lung carcinoma cells. Progranulin overproduction in SW-13 cells markedly increases its tumorigenicity in nude mice, demonstrating that it can regulate epithelial proliferation in vivo. We propose that the rate of growth for some epithelia, such as SW-13 and MDCK, is proportional to the level of intrinsic progranulin gene expression, and that elevated progranulin gene expression confers a transformed phenotype on epithelial cells including anchorage independence in vitro and growth as tumors in nude mice.

INTRODUCTION

Carcinomas are the commonest of all malignant tumors (1) and are, by definition, epithelial in origin. Healthy epithelial tissues typically undergo highly ordered programs of sequential mitosis and differentiation, which become deregulated in carcinomas. These processes are triggered by growth factors (2) and cell-cell (3) and cell-matrix (4) interactions, all of which are important in the transition from normal to aberrant growth in tumors. Granulins have been shown to modulate cell growth in vitro, and the progranulin gene is expressed in a number of epithelial cell lines, but it is not known whether the level of granulin gene expression can favor a transformed phenotype in epithelial cells, and if so, whether it can support tumor growth in vivo. Granulins, which are also called epithelins, were first identified as peptides of \( M_f \sim 6000 \) that were isolated from human neutrophils and rat bone marrow (5) and in rat kidney extracts (6). Granulin/epithelins have no sequence homology with any other protein family and are structurally characterized by a unique motif of 12 cysteinyl residues (5, 6). Several granulins have been isolated, all of which are encoded within a common glycoprotein precursor, which consists of a secretory signal peptide and seven and a half repeats of the granulin motif, separated by short intervening spacer sequences (7–10). Two granulins, granulinA/epithelin1 and granulinB/epithelin2, are biologically active and have multiple, and some times opposing, effects on the growth of cells. GranulinA/epithelin1 promotes the growth of keratinocytes (6), and in conjunction with TGF-\(\beta\), supports anchorage-independent growth of normal rat kidney fibroblasts. GranulinB/epithelin2 inhibits keratinocyte growth and antagonizes the proliferative actions of granulinA/epithelin1. Both peptides inhibit the growth of other epithelial cells including A431 (6). Intact progranulin has been identified as a potential autocrine growth-promoting factor for the PC cell line, a highly tumorigenic murine teratoma (11), and for R- cells, a murine embryonic fibroblast cell line with deleted IGF-1 receptor gene (12). The intact precursor is also found in the acrosome of guinea pig sperm (9), where it is referred to as acrogain, although its activity in this context is unknown. Most epithelial cell lines that have been tested express high levels of progranulin mRNA in vitro, and in situ hybridization studies show that progranulin mRNA is predominantly expressed in epithelial and hemic cells in healthy adult rat tissue (10). Putative high-affinity receptors for the \( M_f \) 6000 peptides have been identified on the MDA-MB-486 breast tumor cell lines (13) and for the precursor on CCL64 mink lung epithelial cells, murine PC cells, and mouse embryo fibroblast 3T3 cells (14); however, the nature of these receptors and whether they can transduce mitogenic or antimitogenic signals in epithelial cells remains unknown.

Cumulatively, the evidence favors a role for progranulin gene products in epithelial homeostasis (15); however, the functional consequences of intrinsic progranulin gene expression by epithelial cells is not known. In particular, given the antagonistic actions of individual granulin moieties (6, 8) encoded within the precursor, it is unclear whether expression of progranulin would favor or attenuate cell division, or if different domains cosynthesized within the precursor would be mutually antagonistic, resulting in no proliferative outcome. A previous report indicated that the precursor is inactive as a rodent keratinocyte mitogen, as a growth inhibitor of A431 epidermoid carcinoma cells, and as a costimulant with TGF-\(\beta\) on rat kidney fibroblasts (8), all assays in which \( M_f \) 6000 granulinA/epithelin1 is active (6). To investigate the role of progranulin expression in epithelial cell proliferation and transformation, we have tested the effects of manipulating progranulin mRNA levels on the epithelial proliferative phenotype both in cell culture and when grown as tumors in athymic nude mice. Two principal indicator cell lines were chosen, SW-13 cells, which are derived from a human adrenal small cell carcinoma (16), and MDCK cells, which are untransformed epithelial cells from the kidney (17). SW-13 cells respond to very few known growth factors with the exception of members of the fibroblast growth factor family (18), pleiotrophin (19), and a partially characterized protein called TGFe (18, 20), which has some similarity with granulin. A previous report indicated that the precursor is inactive as a rodent keratinocyte mitogen, as a growth inhibitor of A431 epidermoid carcinoma cells, and as a costimulant with TGF-\(\beta\) on rat kidney fibroblasts (8), all assays in which \( M_f \) 6000 granulinA/epithelin1 is active (6). To investigate the role of progranulin expression in epithelial cell proliferation and transformation, we have tested the effects of manipulating progranulin mRNA levels on the epithelial proliferative phenotype both in cell culture and when grown as tumors in athymic nude mice. Two principal indicator cell lines were chosen, SW-13 cells, which are derived from a human adrenal small cell carcinoma (16), and MDCK cells, which are untransformed epithelial cells from the kidney (17). SW-13 cells respond to very few known growth factors with the exception of members of the fibroblast growth factor family (18), pleiotrophin (19), and a partially characterized protein called TGFe (18, 20), which has some similarity with granulin (21). Although derived from a tumor, SW-13 cells grow poorly in athymic mice. They therefore provide a good model to study the ability of progranulin gene expression to favor epithelial tumor growth. MDCK cells are immortalized, but untransformed epithelial cells retain many differentiated features, including tight junctions and cellular polarity. They are anchorage dependent but will grow as colonies in semisolid medium in response to appropriate growth.
factors, such as hepatocyte growth factor and epidermal growth factor (22). The results reported here indicate that the level of progranulin mRNA expressed by epithelial cells such as SW-13 and MDCK is an important determinant of their rate of proliferation in vitro, their clonogenicity in soft agar, and the ability of SW-13 cells to form tumors in vivo.

MATERIALS AND METHODS

Construction of Plasmids. cDNA encoding the full-length granulin precursor was cloned into the polylinker sites of the mammalian expression vectors pRc/RSV (Invitrogen, San Diego, CA) and pcDNA3-myc (pcDNA3 with a 14-amino acid c-myc epitope downstream of an EcoRI restriction site, which was a generous gift from Dr. J. Henderson, Department of Medicine, McGill University, respectively). The vectors consist of replication origin, RSV (pRc/RSV) and CMV (pcDNA3) promoters, and they also contain the neomycin-resistant gene, which is suitable for selection. The orientation of the granulin precursor inserts were confirmed by nucleotide sequencing using a T7-Sequencing kit (Amersham Pharmacia Biotech, Baie d’Urfé, Québec, Canada), according to the manufacturer’s instructions. For the sense or antisense Progranulin/pcDNA3 construct, the myc epitope is always downstream of the insert.

Cell Culture and the Establishment of Stable Transfectants. SW-13, COS7, MDCK, GPC16, A431 A549, and NIH3T3 cells were purchased from American Type Cell Collection (Manassas, VA). They were maintained in DMEM (Life Technologies Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 20 μg/ml Gentamicin. The cells were grown in a 37°C incubator supplied with 5% CO2. Progranulin/ pRc/RSV (sense orientation), pRc/RSV, Progranulin/pcDNA3 (sense and antisense orientation), and pcDNA3 were used to establish stable transfectants of SW-13 and MDCK cells. Ten μg of plasmid DNA and 100 μl of Lipofectamine (Life Technologies Inc. Rockville, MD) were mixed and added to the cells according to the protocol suggested by the manufacturer. After 48 h, the cells were subjected to selection for stable integrants by exposure to 800 μg/ml G418 (Life Technologies, Inc.) in medium with 10% fetal bovine serum. Selection was continued until monolayer colonies formed. The transfectants were then maintained in medium supplemented with 10% fetal bovine serum and 400 μg/ml G418.

Isolation of RNA and Northern Blot Analysis. Around 2 x 10⁶ cells were harvested and washed with ice-cold PBS, pH 7.4. Total cellular RNA was isolated by the guanidinium thiocyanate method (23). Fifteen μg of total RNA were denatured with glacial acetic acid (1% acetic acid in 10 mM NaHPO4, pH 7.0), transferred to nylon membrane, and hybridized with a 32P-labeled granulin cDNA probe as described (7). The transcripts of 28S and 18S rRNA were used as the loading control.

Western Analysis of Recombinant Progranulin. Concentrated CM from the transfectants was electrophoresed in 10% SDS-PAGE gel under reducing conditions and transferred to Nitrocellulose membrane (Boehringer-Mannheim). Western hybridization was performed using a specific monoclonal antibody 9E10 against the c-myc tag (Research Diagnostic, Inc., Flanders, NJ) following instructions from ECL Western Blotting Detection Kit (Amersham Life Science, Buckinghamshire, United Kingdom).

Soft Agar Assay. McCoy medium (2%; Life Technologies, Inc.) supplemented with 10% fetal bovine serum was mixed with an equal volume of Sea Plaque agar. One ml of this mixture was solidified per 35-mm Petri dish. Cells (7500) were mixed well with 0.7 ml of 1× McCoy with 5% serum, 0.35 ml of 2× McCoy with 10% serum, 0.35 ml agar, and overlayed on the bottom gel; this was allowed to set at room temperature and then grown in a 37°C incubator. Colony formation was monitored under the microscope, and 10 cells or more in a cluster were defined as a colony.

Recovery of the Transfectants from the Colonies in Soft Agar. Three colonies visible to the naked eye were randomly chosen from which to isolate cells. These colonies were picked with 200-μl Pipette tips under a microscope; they were then incubated with 200 μl of 2% Trypsin-EDTA (Life Technologies, Inc.) at 37°C for 5 min and spun at 1000 rpm (DuPont, Wilmington, DE). The cell pellets were then resuspended in 10% DMEM medium and cultured in a 37°C incubator supplemented with 5% CO2. These cells were maintained in growth medium with 400 μl/ml G418.

Cell Growth Assay. To compare the growth capacity of different transfected, 1.5 x 10⁴ cells were seeded in 12-well plates (Corning Costar, Cambridge, MA) and then maintained in DMEM in the presence or absence of 10% fetal bovine serum at 37°C, in a 5% CO2 humidified incubator for 7 days, with the medium replaced every 3 days. After 7 days, the cells were trypsinized and counted in a hemocytometer. To evaluate the growth-promoting activity of recombinant progranulin on cells grown in monolayer culture, 1.5 x 10⁶ cells were incubated with increasing concentration of purified recombinant progranulin in 24-well plates (Corning Costar) in serum-free DMEM for 3 days, the cells were then trypsinized and counted in a hemocytometer.

Tumor Formation in Nude Mice. SW-13 cells (2 x 10⁶) transfected with either Progranulin/pcDNA3-myc and overexpressing progranulin or empty pcDNA3-myc were s.c. inoculated bilaterally into the abdomen of 10 nude mice (Charles River Breeding Laboratories, St. Constant, Quebec, Canada). The mice were maintained in a sterile animal facility and monitored for tumor growth. Tumor volume was calculated as (short axis x long axis)/2. All experimental procedures were approved by the University Animal Care Committee and performed in accordance with the guideline of the Canadian Council on Animal Care.

Recovery of SW-13 Transfectants from Mice Tumor. Tumors were excised and chopped to fine pieces (0.5 mm³) with a surgical scalpel; these pieces were then incubated with stirring in 26 ml of digestion buffer containing 50 mg of Collagenase A (Boehringer-Mannheim), 50 mg of hyaluronidase (Boehringer-Mannheim), and 5 mg of Dnase I (Boehringer-Mannheim) at 37°C. Fifteen min later, the suspensions were spun at 200 rpm for 10 min; cell and tissue pellets were resuspended in 10% DMEM medium and spun at the same speed; then the supernatant was removed. This process was repeated three times. The cells were then grown in 10% DMEM containing 800 μg/ml G418 for selection of progranulin/pcDNA3-myc or mock transfectants, which contained the neomycin-resistant gene.

Purification of the Recombinant Granulin Precursor. Progranulin/pcDNA3, or empty vector as a control, was transiently introduced into COS7 cells via Lipofectamine-mediated transfection following the manufacturer’s instructions (Life Technologies, Inc.). Forty-eight h after transfection, the cells were incubated with 0.05 mlCi of 35S-labeled cysteine in serum-free medium for 24 h. The CM was collected and concentrated using a Centricron Plus-20 ultrafiltration unit (cutoff factor, M1, 30,000; Millipore, Bedford, MA) following the manufacturer’s instructions. The acidified concentrated media were further fractionated by Vydac C4 reversed-phase HPLC using an isocratic phase of 0.1% trifluoroacetic acid for 10 min, followed by a linear gradient from 0% acetonitrile to 80% acetonitrile in 0.1% trifluoroacetic acid throughout over 60 min at 1.5 ml/min, collecting 1-mn fractions. The column eluate was monitored for UV absorbance at 215 nm. Two % aliquots of all of the fractions were counted for radioactivity by liquid scintillation. Two % aliquots were assayed in duplicate for their colony-promoting activity on SW-13 cells in soft agar.

SDS-PAGE Gel Electrophoresis. Thirty μl of the protein sample (2% of total activity) were boiled and dissolved in 20 μl of reducing loading buffer; the mixture was boiled for 5 min and then electrophoresed in 10% SDS-PAGE gel and exposed to Fuji film for autoradiography at −70°C for 24 h.

Fluorescence-activated Cell Sorting Analysis. Cells (2 x 10⁶) were washed twice with ice-cold PBS (pH 7.4) and fixed in ice-cold 70% ethanol for 30 min. They were then washed three times in Dulbecco’s PBS (Life Technologies, Inc.) and blocked in Dulbecco’s PBS supplemented with 2.5% FCS for 15 min. The cells were washed three times in Dulbecco’s PBS and incubated with 0.03 mg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and 50 mg/ml RNase A (Amersham Pharmacia Biotech, Baie d’Urfé, Quebec, Canada) for 5 min at 37°C. Cell cycle status was identified on a FACScan (Becton Dickinson Canada, Inc.) performed by K. McDonald (Department of Microbiology & Immunology, McGill University).

Statistical Analysis. The differences in the mean value of different groups were measured by ANOVA followed by alternative t test; significance is reported as two-sided P.

RESULTS

Effects of Constitutive Overexpression of Progranulin mRNA on Cell Growth in Culture. SW-13 (human adrenal carcinoma) and MDCK (canine renal epithelium) cells were transfected with cDNA
encoding the full-length human progranulin and selected in G418 for stable integration of the construct into the genome. To minimize possible nonspecific effects, the experiments were duplicated using two independent neomycin-selectable vector systems under the control of the RSV- and CMV-promoter, respectively. Northern blot analysis confirmed the overexpression of the progranulin gene in progranulin transfectants (Fig. 1A). To demonstrate that elevated progranulin mRNA directed production of progranulin protein products and to find out whether they were secreted, we used the pcDNA3 vector to incorporate a myc-epitope tag to the COOH-terminus of the progranulin sequence. When CM from SW-13 cells was analyzed for myc-tagged proteins by Western blotting, a single myc-tagged protein of Mr 80,000, corresponding to the expected mass of glycosylated human progranulin (11), was detected in the medium of progranulin-transfected, but not mock-transfected, cells (Fig. 1B). The medium was collected over a 24-h period, and no myc-tagged degradation products were observed.

Both SW-13 and MDCK progranulin transfectants showed enhanced clonogenicity in semisolid agar compared either with mock-transfected/G418-selected cells or untransfected control cells, regardless of the expression vector used (Fig. 2). The rate of colony formation for SW-13 progranulin transfectants was approximately twice that for MDCK cells. To confirm that the cells within the colonies continue to overexpress progranulin mRNA, individual colonies of SW-13 cells were recovered from the agar using a sterile pipette tip and then propagated in monolayer culture. The recovered cells showed markedly elevated progranulin mRNA expression compared with the vector transfectants and untreated control cells (Fig. 2C).

Overproduction of progranulin increases the proliferation of SW-13 and MDCK cells in monolayer culture. After propagation for 7 days with or without serum, the number of progranulin/pcDNA3-transfected SW-13 cells was 2.5- and 5-fold greater than control and mock transfectant cells with or without serum, respectively (Fig. 3A). Unlike mock-transfected and control SW-13 cells, which respond well to serum (Fig. 3A), progranulin-transfected SW-13 cells have lost their sensitivity to serum, with cell proliferation being identical in serum-supplemented or serum-free media (Fig. 3A). MDCK-progranulin transfectants showed a 2.5-fold increase in cell number over control cells or mock transfectants in medium both with or without serum (Fig. 3B). The proportion of cells in S-phase was quantitated using fluorescence-activated cell sorting of cells labeled with propidium iodide. Mock-transfected and untransfected control cells grown in medium with serum had equivalent proportions of cells in S-phase, 10.1 and 9.5% for SW-13, and 2.9 and 2.9% for MDCK. Both cell lines responded to elevated progranulin expression by an increase in the percentage of cells in S-phase to 18.7% for SW-13 and 10.2% for MDCK (n = 3; for SW-13 transfectants, P < 0.05; for MDCK cells, P < 0.001). SW-13 cells grown as subconfluent monolayers showed a marked tendency to form growth foci (data not shown), which were absent from the mock transfectant and control SW-13 cultures and MDCK cell monolayers.

Tumorigenicity of Progranulin Overexpression in Nude Mice. To determine whether overproduction of progranulin confers accelerated growth in vivo, 2 × 10⁶ SW-13 progranulin transfectants (in pcDNA3) or vector-transfected controls were injected s.c. into nude mice at two sites/mouse on opposite sides of the abdomen, with five mice/treatment group. Progranulin expression was strongly mitogenic in vivo. Growths formed bilaterally in all 10 mice at the sites of injection, but tumor onset was earlier (first palpable growths by day 7), and the volume was markedly greater (7-fold by day 60) in mice injected with progranulin transfectants compared with mice carrying...
cells transfected with the empty vector (Fig. 4A). Upon autopsy, there was no gross evidence of metastasis. To confirm that the SW-13 progranulin transfectants continue to express elevated levels of progranulin mRNA after growth in vivo, cells were recovered from the tumors by digestion with collagenase and hyaluronidase and grown in monolayer culture. Contaminating murine cells, which do not carry the neomycin-resistance gene, were eliminated by treating the cultures with G418. The tumor-derived SW-13 progranulin transfectants continue to show elevated progranulin mRNA expression compared with empty-vector transfectants (Fig. 4B). The recovered cells are highly clonogenic in soft-agar (Fig. 4C) and exhibit enhanced proliferation in monolayer culture (Fig. 4D).

Purification and Mitogenicity of Recombinant M<sub>r</sub> 80,000 Progranulin. If the altered growth profiles observed for the progranulin transfectants are due directly to the secreted M<sub>r</sub> 80,000 progranulin product, the effects of overexpressing progranulin mRNA should be reproduced by exogenous addition of progranulin to the culture medium. Recombinant M<sub>r</sub> 80,000 progranulin was generated in COS-7 cells. Progranulin production was followed by metabolically labeling proteins with 35S-labeling cysteine. A protein of M<sub>r</sub> 80,000, corresponding to the intact granulin precursor, was present in CM from progranulin transfectants but not in CM from mock-transfected cells (Fig. 5A). Serum-free CM from both progranulin and mock transfectants were assayed for colony formation by SW-13 cells in semisolid agar. Both CM stimulated anchorage-independent SW-13 cell growth in a dose-dependent manner, with the medium from progranulin transfectants being considerably more active than that from mock-
transfected cells (Fig. 5B). To correlate this activity with a specific protein, progranulin- and mock-transfectant CM were concentrated and subjected to C₄-reverse phase HPLC fractionation. A bioassay across the UV absorbing region of the chromatogram showed maximum colony stimulation of SW-13 (approximately eight times greater than unstimulated control cells) in fraction 37 from the progranulin transfectant CM (data not shown). This corresponds to a peak of progranulin-CM-specific 35S-labeled cysteine incorporation (Fig. 6A), which was identified as the M₆ 80,000 protein by autoradiography (Fig. 6A, insert). To unambiguously establish the identity of this

Fig. 5. Transiently transfecting COS7 cells with progranulin/pCDNA3 results in the secretion of M₆ 80,000 progranulin and CM containing progranulin stimulate colony formation of SW-13 cells in a dose-dependent manner. A, SDS-PAGE electrophoresis and autoradiography of 35S-cysteine-labeled proteins from medium conditioned by COS7 transfectants. B, colony-promoting activity on SW-13 cells of CM from progranulin transfectants and mock transfectants (n = 4 independent experiments each performed in duplicate; data are means; bars, SE).
Mitogenicity of Purified Progranulin in Monolayer Cultures. Purified recombinant progranulin was further tested for mitogenicity on monolayers of SW-13, MDCK, A549 (human lung adenocarcinoma), GPC16 (guinea pig colonic carcinoma), A431 (human epidermoid carcinoma), and NIH 3T3 (mouse embryonic fibroblast) cells. All cells except A431 and NIH3T3 responded to recombinant progranulin in serum-free medium with a concentration-dependent increase in cell number measured after 72 h (Fig. 6B). SW-13 and GPC16 cells showed the strongest response with a maximal increase of ~6-fold in cell number. Thus, $M_0$ 80,000 progranulin is mitogenic for several epithelial cell lines of different origins, even in the absence of serum. Because SW-13 cells do not respond to most growth factors (18, 19, 20) but do respond to recombinant progranulin, and because 3T3 cells respond to most classic growth factors but not to recombinant progranulin, it is highly unlikely that the proliferative activity observed for progranulin is due to contamination with other growth-promoting proteins.

Effects on Cell Proliferation of Progranulin Antisense Targeting. Progranulin mRNA was expressed in the antisense orientation under the control of the CMV-promoter (pCDNA3) in SW-13 and MDCK cells. This treatment markedly lowered the level of progranulin mRNA in both cell lines (Fig. 1A). Cells were then grown as monolayers for 7 days with or without serum. The number of SW-13 antisense transfectants was reduced by 47% in medium supplemented with 10% serum compared with the mean value for untransfected control and mock-transfected cells, and by 80% without serum (Fig. 7A). When cells expressing the antisense vector were challenged with purified recombinant progranulin, they showed an accelerated rate of proliferation (Fig. 7C), which is consistent with a reduction of secreted progranulin being the cause of their lower rate of growth. Proliferation of SW-13 cells is therefore strongly dependent on the intrinsic expression of the progranulin gene. Serum only partially compensates for progranulin mRNA depletion, with a greater recovery of proliferation achieved using recombinant progranulin (Fig. 7C). MDCK cells expressing antisense progranulin transcripts showed a statistically significant decrease in cell number of ~30% compared with controls with or without serum (Fig. 7B).

DISCUSSION

Peptide products of the progranulin gene modulate epithelial cell growth, and epithelial cells express the gene both in tissue culture and in vivo. Although the available evidence favors a role for various progranulin gene products in epithelial homeostasis, these effects are complex and in some cases mutually antagonistic. The functional consequence of progranulin gene expression by epithelial cells, and in particular its ability to favor transformation and tumor growth, remains unknown. In this report, we demonstrated that: (a) forced overexpression of the progranulin gene in SW-13 and MDCK cells resulted in the secretion of an $M_0$ 80,000 intact progranulin, enhanced the growth capacity of these cells, and promoted their anchorage-independent growth in vitro; (b) purified recombinant progranulin is a mitogen for certain epithelial cells in vitro; (c) the SW-13 progranulin transfectants became highly tumorigenic in nude mice compared with those transfected with empty vector; (d) SW-13 and MDCK cells with reduced progranulin gene expression have retarded growth phenotype.

SW-13 cells do not proliferate in response to the majority of growth factors, such as epidermal growth factor or TGF-α (18, 20), insulin-like growth factor (24), or TGF-β (18, 20) but as shown here will proliferate in response to progranulin. The parental SW-13 cells are serum sensitive, whereas transfectants that overexpress progranulin have a similar growth capacity regardless of the presence or absence of 10% FCS, indicating that progranulin expression can bypass the serum requirement of SW-13...
cells, also fail to respond to a number of growth factors but proliferate in response to progranulin (11). The retention of sensitivity to progranulin in diverse cells such as SW-13, R^+, and PC, which are to varying degrees refractory to most other growth factors, implies a fundamental role for progranulin in cell growth regulation and suggests that progranulin can circumvent aspects of the usual growth factor-signaling pathways. The mitogenicity of progranulin was independently confirmed using highly purified recombinant progranulin, the identity of which was verified by NH2-terminal, gas-phase microsequence analysis. Exogenous progranulin duplicates the effects of vector-driven overexpression of the progranulin gene and is mitogenic to several epithelial cells of diverse origin. Interestingly, the epidermoid carcinoma A431 and the embryonic fibroblast NIH-3T3 are unresponsive to progranulin under the conditions tested, implying cell specificity in the mitogenic response to progranulin.

Because both SW-13 and MDCK express the progranulin mRNA endogenously, we investigated whether the expression of the progranulin gene contributes to their basal proliferative rates. Expression of the antisense progranulin vector lowered progranulin mRNA levels (Fig. 1A) and attenuated proliferation in both cell lines (Fig. 7, A and B). The effect was most prominent in SW-13 cells, the proliferation of which in serum-free medium fell to one-fifth of the controls. Serum partially restored cell growth but was unable to completely compensate for the down-regulation of progranulin mRNA levels and was less effective than recombinant progranulin (Fig. 7C), implying a strong dependence on autocrine progranulin in these cells. Thus, given that elevated progranulin transcription accelerated cell division and attenuated progranulin mRNA levels reduced growth, we conclude that for epithelial cells such as SW-13 and MDCK, the rate of proliferation is proportional to the level of expression of the progranulin gene.

To test for transformation, we grew the cells suspended in soft agar because efficient anchorage-independent growth is commonly considered to indicate a highly transformed phenotype. Parental SW-13 and MDCK cells are only weakly clonogenic at low cell density when cultured in semisolid soft agar (Fig. 2, A and B). In contrast, SW-13 and MDCK progranulin transfectants showed enhanced clonogenicity in soft agar (Fig. 2, A and B). The ability of progranulin to stimulate mitosis and clonogenicity in SW-13 cells is particularly striking because, as discussed above, they are refractory to most classic growth factors. Anchorage independence can be promoted in MDCK cells by hepatocyte growth factor and epidermal growth factor (22) in addition to progranulin as shown here. Experiments using mutant tyrosine kinase receptors have shown that the signaling pathways that stimulate growth in monolayer culture and confer anchorage independence are not identical (27); thus, it is significant that progranulin overexpression augments both growth patterns.

SW-13 cells, which show the strongest responses to progranulin, were chosen to test whether the enhanced proliferation of progranulin transfectants in vitro correlates with accelerated tumor growth in nude mice. Although derived from a carcinoma, SW-13 cells are poorly tumorigenic in nude mice, unless genetically manipulated to express pleiotrophin (19) or secreteable fibroblast growth factors such as fibroblast growth factor 4 (28), neither of which, unlike progranulin, are normally expressed by SW-13 cells. All mice developed palpable growths at the site of injection; however, the tumors grew much more rapidly in mice carrying SW-13 progranulin transfectants than the control group, demonstrating that progranulin expression is highly mitogenic in vivo (Fig. 4A) and is capable of sustaining tumor development. SW-13 progranulin transfectants that were isolated from the resected tumors retained elevated progranulin expression and accelerated growth in monolayer and soft agar. The progranulin gene, therefore, fulfills several of the traditional criteria relating a gene product to tumorigenesis, being highly expressed in many transformed epithelial cell lines (7, 8), mitogenic in vitro, supporting...
colony formation in soft agar, and as shown here, promoting tumor growth in vivo.

Zhang and Serrero (29) have demonstrated recently that PC cells in which intrinsic progranulin expression was blocked using an antisense progranulin vector exhibit greatly diminished tumorigenicity. Thus, PC cells clearly require high levels of progranulin gene expression to maintain malignancy. Here we show that as well as requiring the gene product, elevating progranulin gene expression in SW-13 cells is by itself directly capable of promoting malignancy. Epithelial tumors are thought to originate as hyperproliferative epithelium that progresses through adenomatous intermediates to highly tumorigenic carcinomas (30). To some extent, the SW-13 progranulin constructs recapitulates this series, with the antisense cells being poorly proliferative, the parental cells being more active mitotically but not anchorage independent or able to form tumors, and the progranulin-overexpressing cells being highly proliferative, clonogenic, and tumor forming in vivo. It will be important to determine whether the levels of progranulin gene expression vary in a corresponding fashion during the pathological evolution of tumors in vivo.

Progranulin acts as a transforming factor in SW-13 and MDCK cells. Early studies on transforming growth factors identified a biological activity called TGFα, unrelated to TGF-α, TGF-β, or any other classic growth factor based on bioassay, chromatographic data, and ligand displacement studies (18, 20). TGFα was proposed to be an autocrine factor for SW-13 cells (18, 20, 24). Little attention has been given to TGFα. TGFα-like bioactivities of various molecular weights have been reported from CM of SW-13 cells and milk (31) and kidney extracts (32, 33). The first 13 residues of the NH2 terminus and an internal peptide of TGFα have been reported (21). Eleven of the NH2 terminal residues are identical to those of human granulinA, but the internal peptide shows no sequence identity. As isolated from bovine kidneys, TGFα has a molecular weight of $M_t \sim 25,000$ and is therefore distinct from the $M_t \sim 6,000$ granulin peptide or the intact progranulin molecule, but given the overlapping biological activities of progranulin and TGFα and their apparent structural similarities, it is likely that the progranulin protein products and TGFα are related entities.

In conclusion, our data strongly suggest that the level of progranulin mRNA expression is a determinant of the rate at which certain epithelial cells proliferate because it is expressed by epithelial cells; it is secreted intact; overproduction of progranulin promotes epithelial progression through the cell cycle via an autocrine action, confers anchorage independence in vitro, and accelerates tumor growth in mice; purified progranulin protein is mitogenic for several epithelial cell lines and duplicates the effects of elevated progranulin gene expression; and targeting the progranulin mRNA results in decreased progranulin transcripts and impairs epithelial proliferation. Progranulin behaves as a novel transforming growth factor in these systems and may be related to the elusive TGFα. The identification of progranulin levels as a novel rate-determining parameter in epithelial proliferation that favors tumor growth in some cell lines establishes a critical role for progranulin in epithelial proliferative homeostasis and may have important consequences in the development and growth of carcinomas.

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