Advances in Brief

Inhibition of Furin-mediated Processing Results in Suppression of Astrocytoma Cell Growth and Invasiveness[1]

Javier Mercapide, Ricardo Lopez De Cicco, Daniel E. Bassi, Javier S. Castresana, Gary Thomas, and Andres J. P. Klein-Szanto[2]

Department of Pathology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [J. M., R. L. D. C., D. E. B., A. J. P. K-S.]; Departamento de Genética, Universidad de Navarra, Pamplona, Spain 31080 [J. S. C.]; and The Vollum Institute, Oregon Health Sciences Center, Portland, Oregon 97201 [G. T.]

Abstract

Purpose: **Astrocytoma arises in the central nervous system as a tumor of great lethality, in part because of the invasive potential of the neoplastic cells that are able to release extracellular matrix-degrading enzymes. Furin convertase activates several precursor matrix metalloproteases involved in the breakdown of the extracellular matrix. In the present study inhibition of furin was achieved by gene transfer of α_1-antitrypsin Portland (PDX) cDNA.**

Experimental Design: **This furin inhibitor was transfected into two tumorigenic astrocytoma cell lines. The inhibitory effect was evaluated using *in vivo* tumorigenicity, invasion, and proliferation assays, as well as by investigating impairment of furin substrate processing.**

Results: **Expression of PDX prevented the s.c. growth of the transfected cells. Invasion assays demonstrated that PDX-transfected cells exhibited a reduced invasive ability *in vitro* and *in vivo*. Furthermore, s.c. growth of PDX transfectant xenotransplants showed a significant reduction in size that coincided with a significant decrease of the *in vitro* doubling time and of the *in vivo* cell proliferation ability. Additional studies showed that the furin substrates insulin-like growth factor IR, transforming growth factor β and membrane type 1-matrix metalloprotease were not activated in PDX-expressing astrocytoma cells.**

Conclusions: **PDX expression in astrocytoma cells demonstrated a direct mechanistic link between furin inhibition, and decreased astrocytoma proliferation and invasive ability. Because furin inhibition inhibits both invasiveness and cell growth in astrocytoma, furin should be considered a promising target for glioblastoma therapy.**

Introduction

Limited proteolysis aimed at removing the initial propeptide fragment in the NH_2-terminal end of proproteins is a molecular event required by many latent protein precursors to acquire biological activity. A family of Ca^{2+}-dependent serine endoproteases named PCs,[3] which participate in processing latent substrates to fully active enzymes, has been identified during the last decade. The PCs share a common phylogenetic origin with bacterial subtilisin and the yeast protease kexin, and their catalytic domains exhibit a high proportion (50–75%) of conserved residues (1, 2). To date, several members have been characterized in mammals, mostly in nervous and endocrine tissues. Among these, furin, PACE4, PC6B, and PC7 are ubiquitous enzymes expressed in many different tissues. Proteolytic cleavage by PCs occurs behind motifs of basic pairs KR $\downarrow$ or RR $\downarrow$, although basic residues at 4th and 6th upstream positions also contribute to substrate recognition (2).

The up-regulated expression of PACE4 and furin in some types of cancer supports a possible functional role in tumorigenesis (reviewed in Ref. 3). Enhanced expression of the PC PACE4 was found in invasive mouse skin squamous cell carcinomas, whereas levels remained low in the less aggressive tumors (4). Among the most attractive substrates of PCs that might link proprotein processing with progression of cancer disease are MMPs, synthesized as inactive precursors, because of their role in the proteolytic digestion of the ECM that anticipate tumor invasion. Stromysin-3 is processed into its active form by furin (5, 6), and the group of MT-MMPs also contains insertions between the propeptide and the catalytic domains that include the paired basic amino acid recognition site for furin processing (7). Recently, a variant α 1-antitrypsin was constructed, which contains in its reactive site Arg-X-X-Arg-, the minimal sequence required for efficient processing by furin (8). This furin-specific inhibitor called PDX, unlike other related serine protease inhibitors, does not inhibit either elastase or thrombin. PDX is a potent competitive inhibitor of furin (IC_{50} = 0.6 nM), and when expressed in cells (either by stable or transient transfection), blocks the processing of HIV-1 gp160 and measles virus-Fo inhibiting virus spread. PDX is also 10-fold more effective than currently used antiherpetic agents in cell-culture models. The requirement of furin for the processing of envelope glycoproteins from many pathogenic viruses and for the activation of several bacterial toxins suggests that selective inhibitors of furin have potential as broad-based antipathogens (8). Furthermore, furin substrate processing was inhibited by PDX in four (furin-overexpressing) epithelial-derived

malignant cell lines resulting in reduced tumorigenicity and invasiveness (9, 10).

Although to our knowledge, furin expression levels have not been investigated in brain tumors, low levels of expression have been detected in neurons and glial cells of murine and human normal brain by *in situ* hybridization (11–13).

High grade astrocytomas are characterized by enhanced proliferation and invasiveness, and their lethality is because of local brain tissue destruction rather than distant metastases. Invasion of glioma cells in the brain is preceded by a process of proteolysis and solubilization of the ECM that enables tumor cells to spread (14). Because local invasiveness is the major cause of glioma mortality and because furin is expressed in the nervous tissue together with several MMPs that contain sequences for cleavage and activation by furin, astrocytoma cells are excellent candidates to investigate the effects of furin inhibition as a possible new approach to astrocytoma therapy.

Herein we demonstrate a very significant reduction in cell proliferation, tumorigenicity, and invasiveness after introduction of a cDNA plasmid for expression of the furin inhibitor PDX into furin-expressing anaplastic astrocytoma cell lines U87MG and U118MG.

Materials and Methods

Expression Plasmids. The *EcoR*I/*Sal*I fragment encompassing the full length cDNA of PDX was directionally cloned into the pCI.neo vector (Promega) to yield pCIN.PDX.

Cell Culture and Transfection. Parental U87MG and U118MG astrocytoma cells (American Type Culture Collection, Manassas, VA) were maintained in MEM supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and DMEM, respectively, with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Both lines were stably transfected by lipofection (LipofectAMINE PLUS, Life Technologies, Inc., Gaithersburg, MD) with either pCIN.PDX or pCI.neo vector alone (pCIN). G418 (1 mg/ml)-resistant colonies were picked, cloned, and screened for high α_1-PDX expression by Western analysis. Transfectants were propagated in medium supplemented with 400 μg/ml G418. Subconfluent cells were serum-starved 1 day before harvesting. Primary cultures of NHAs from Clonetics (San Diego, CA) were used as control cells.

Protein Sample Preparation and Western Blot Analysis. For cell lysates, cell monolayers were harvested, washed, and disrupted with radioimmunoprecipitation assay (PBS containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1% NP40, plus freshly added protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml aprotinin, and 1 mM Na$_3$VO$_4$) for 30 min at 4°C. The soluble part was used as protein extract. Furin was first immunoprecipitated from lysates with MON-148 (Alexis Biochemicals, San Diego, CA), a monoclonal antibody raised against an epitope in the catalytic domain. Total protein (100 μg) was cleared with normal mouse IgG/protein A/G PLUS agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and the cleared rest was then incubated with MON-148 and protein A/G PLUS agarose.

Extracellular proteins were assessed on 24 h-conditioned medium concentrated 50-fold with Millipore centrifugal filter devices.

For expression analysis, proteins were subjected to SDS-PAGE using Tris-glycine gels, electrotransferred overnight onto nitrocellulose membranes, and probed with antibody against either furin (MON-152; Alexis Biochemicals), PDX (α1-antitrypsin antibody; Sigma, St. Louis, MO), TGF-β affinity-purified goat antihuman latent-associated peptide IgG (R&D, Minneapolis, MN), MT1-MMP (AB815; Chemicon International, Inc., Temecula CA), and IGF-IR (sc-711; Santa Cruz Biotechnology) were used as primary antibodies. Immunocomplexes were revealed with enhanced chemiluminescence based on the use of peroxidase-labeled IgG (enhanced chemiluminescence; Amersham Pharmacia Biotech).

Immunohistochemistry. Furin immunohistochemistry was performed using paraffin-embedded material. All of the paraffin sections were subjected to antigen retrieval for 10 min in distilled water. MON 152 (Alexis) was used as primary antibody at 1/100 dilutions. An avidin-biotin-peroxidase kit (Vectastain Elite, Vector, Burlingame, CA) was then used followed by the chromagen 3'3'-diaminobenzidine to develop the immunostain. Negative controls, not incubated with furin antibodies, were incubated with normal mouse IgG. All of the sections were counterstained with hematoxylin and mounted. Normal brain tissue (frontal cortex) from three individuals, eight high-grade astrocytomas, and tracheal xenotransplants of U87MGpCIN cells (see below) were used.

Doubling Time. Cells were grown in 12-well plates and counted every other day. Log of cell numbers *versus* days was plotted to obtain the doubling time for each transfected cell line.

Zymography. Cells (1×10^6) were grown overnight in serum-free S-MEM medium containing 2 mM L-glutamine and Pen-Strep. The conditioned media were concentrated down to 200 μl using Amicon centripreps (Fisher, Springfield, NJ), and 20 μl of each sample was loaded on a 10% Novex precast zymogram (gelatin) gel. The gel was run, renatured, and developed according to the manufacturer's instruction. Gelatinase Zymography Standards were purchased from Chemicon International, Inc.

***In Vitro* Invasion Assay.** The ability of cells to degrade ECM *in vitro* was assessed using Biocoat Matrigel invasion chambers (Becton Dickinson, Bedford, MA) according to the manufacturer's instructions (15).

***In Vivo* Invasion Assay.** Tracheal transplants were prepared as described previously (10, 15). Cells (5×10^5) from each transfected cell line were inoculated into de-epithelialized rat trachea (Zivic-Miller, Pittsburgh, PA). Six to ten tracheas were used for each cell line. After cell inoculation, the tracheas were sealed and transplanted into the dorsal s.c. tissues of Scid mice. Tracheal transplants were removed surgically at 8 weeks, sectioned into 3-mm thick rings, and fixed in 10% formalin. After H&E staining, the degree of invasion of the tracheal wall was determined by measuring the length of maximum penetration of the tumor cells into the tracheal wall (10, 15). All of the microscopic images of cross-sections of tracheal transplants were digitized at a magnification of $\times$40. Using an adequate reference micrometric scale, the lengths were determined by measuring the distance between the luminal center and the most distant point of tumor invasion. If the lumen was obliterated, the

distance measured was between the geometric center of the tumor mass inside the tracheal lumen and the most distant point of tumor invasion either in or outside the tracheal wall. Each tracheal transplant was represented by two to six measurements corresponding to the number of available cross-sections containing tumor cells. A mean was calculated for each tracheal transplant and for each group of transfected cells. The results were expressed in μm of penetration depth. Paraffin sections of tracheal transplants containing transfected tumor cell lines were used for the immunohistochemical detection of Ki-67 (Mib-1). A mouse monoclonal Mib-1 (Immunotech, Westbrook, ME) and an avidin-biotin-peroxidase kit (Vectastain Elite; Vector) were used. Negative controls were incubated with normal mouse IgG. Labeling indices (percentage of Mib-1-positive cells) were calculated by counting stained and unstained cells in tracheal transplants containing either vector alone- or α_1-PDX transfected cells (at least 500 cells/tracheal transplant were counted).

***In Vivo* Tumorigenicity.** Either PDX-transfected or vector alone-transfected cells (5×10^6) were injected into the s.c. tissues of Scid mice. Tumors were measured twice a week after the appearance of the first tumor for each pair of PDX- and vector alone-transfected cells using a Vernier caliper. Volumes (V) of the tumors were obtained using the following equation: $V = [(L_1 + L_2)/2] \times L_1 \times L_2 \times 0.526$, where L_1 and L_2 are the length and width of the s.c. tumor.

Results

Furin and PDX Expression. Two astrocytoma cell lines, U87MG and U118MG, were selected for these studies because of their known tumorigenicity in immunocompromised mice (16). Furin expression was higher in the two parental astrocytoma cell lines than in normal astrocytes (Fig. 1*A*). The expression of PDX was confirmed by Western analysis of astrocytoma cell lysates (Fig. 1*B*). PDX did not affect cell survival and was secreted into the culture medium (data not shown) as a product of $M_r \sim 60,000$. The transfection of PDX into the astrocytoma cell lines did not change their respective furin expression levels (Fig. 1*C*).

Immunohistochemical demonstration of furin showed that normal brain cells, including glial cells, showed very little or no furin expression (Fig. 2*A*). Conversely, six of eight high-grade astrocytomas examined showed moderate to intense immunostain (Fig. 2*B*). Control-transfected U87MG cells grown in tracheal xenotransplants showed intense furin immunostain (Fig. 2*C*). PDX transfection did not change this staining pattern (data not shown).

Expression of IGF-IR and TGF-β. Western analysis of IGF-IR showed that vector-alone transfected cells expressed only one low molecular weight band ($M_r \sim 105,000$) corresponding to the processed form of the receptor. Conversely, the PDX-transfected astrocytoma cells showed the predominance of a higher molecular weight band ($M_r \sim 200,000$) corresponding to the proform and low expression of the processed form (Fig. 3*A*). Similarly, Western analysis of TGF-β showed that the processed form of this growth factor ($M_r \sim 40,000$) was present in the vector alone-transfected astrocytoma cells, whereas the PDX transfectants did not express this lower molecular weight
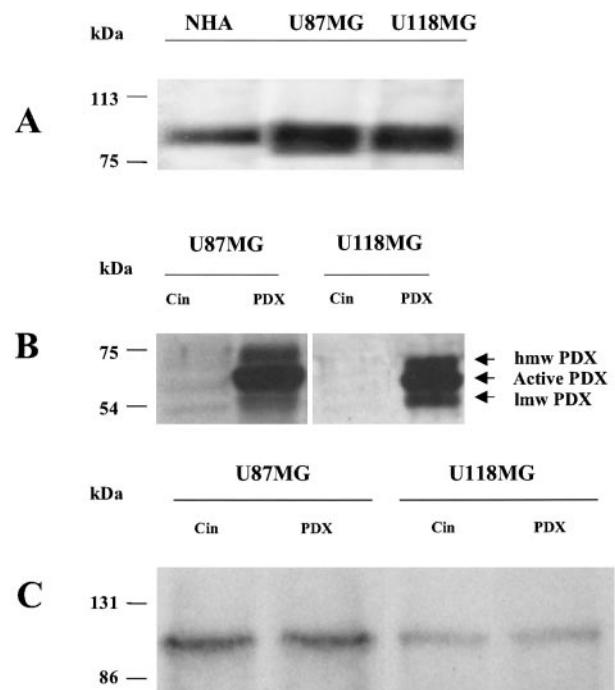
Fig. 1 Detection of furin and PDX in cell extracts of astrocytoma cells. *A,* furin was immunoprecipitated from cell lysates using the antifurin monoclonal antibody MON-148 and analyzed by Western blot. Expression of endogenous furin in the parental astrocytoma cells (U87MG and U118MG) as compared with NHAs is shown. The M_r 85,000 active form of furin was immunodetected with MON-152, which recognizes the Cys-rich region of the enzyme. *B,* Western blots for detection of PDX in stably transfected astrocytoma clones. Both U87MG.pCIN.PDX and U118MG.pCIN.PDX (*right lanes*) show presence of PDX. The vector-alone transfected clones (pCIN) are negative. In addition to the M_r 60,000 active PDX product, two additional forms were immunodetected. Abbreviations: hmw and lmw PDX, high (M_r 65,000) and low molecular weight (M_r 56,000) PDX, respectively. *C,* Western blot analysis of furin expression in vector alone (*Cin*) and PDX-transfected U87MG and U118MG cell lines. Similar levels of furin expression were noted before and after PDX transfection.

form and expressed the higher molecular weight band corresponding to the unprocessed form ($M_r \sim 50,000$; Fig. 3*B*).

Expression of MT1-MMP and MMP-2. Western analysis indicated that PDX gene transfer resulted in the impairment in MT1-MMP activation (Fig. 3*C*). PDX-transfected astrocytoma cells retained the latent MT1-MMP proform and failed to exhibit the mature form of $M_r \sim 63,000$. Conversely the vector alone-transfected cells showed both the proform and the mature form of MT1-MMP (Fig. 3*C*). Because MT1-MMP intervenes in the activation of MMP2, the possible effects of furin inhibition on the extracellular gelatinase activity of astrocytoma transfectants were evaluated by zymography (Fig. 3). Processing of M_r 72,000 gelatinase-A (MMP-2) into the mature active form was inhibited by PDX transfection, as evidenced by the low expression levels of the processed forms of MMP2.

PDX Effects on Proliferative, Tumorigenic, and Invasive Potential of Astrocytoma Cells. PDX-transfected cells exhibited a marked increase in doubling time in regard to their vector alone-transfected counterparts. This effect of the furin
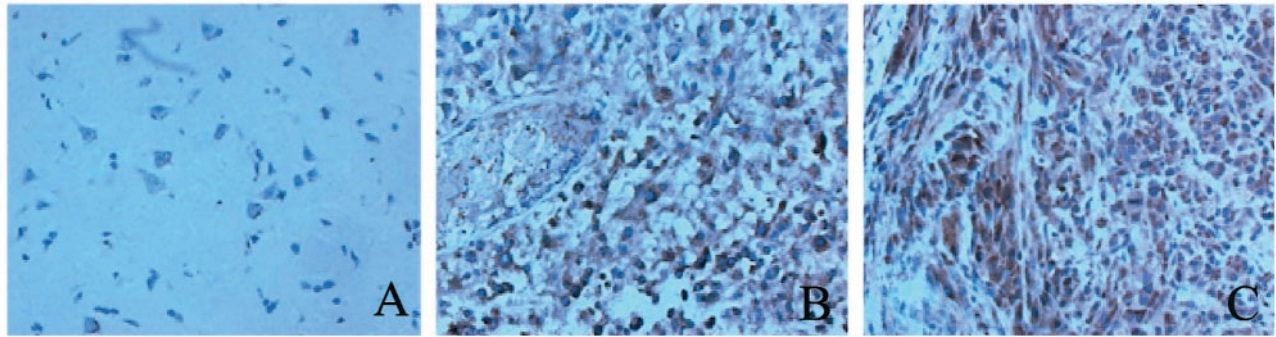
Fig. 2 Furin immunohistochemistry. *A,* very little or no furin expression could be detected in normal neurons and glial cells from human cerebral cortex. *B,* moderate furin immunostain is seen in most malignant cells of this astrocytoma grade III. *C,* tracheal xenotransplant containing U87MGpCIN cells. Note moderate to intense immunostain in the neoplastic cells. Furin immunohistochemistry and hematoxylin counterstain. ×90.
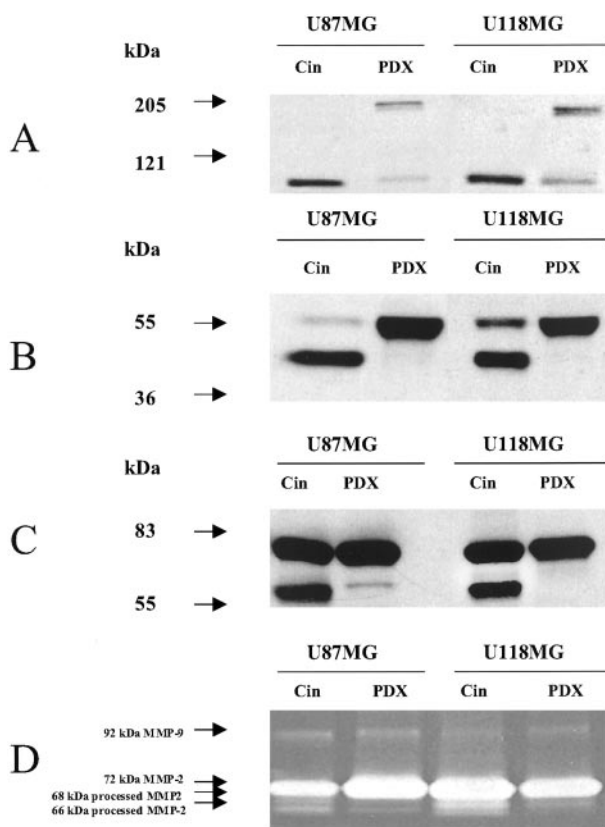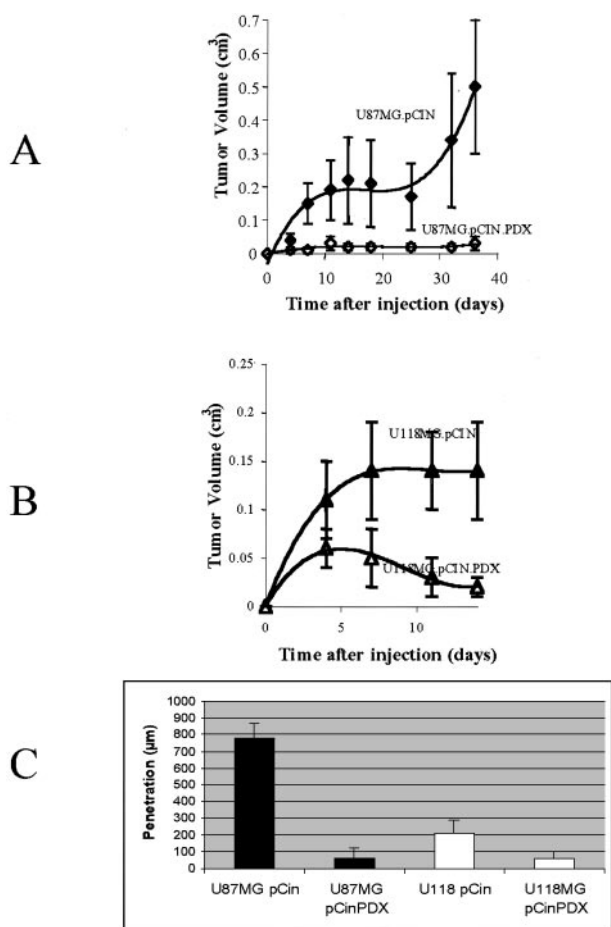

Fig. 3 Inhibition of furin substrate processing in U87MG and U118MG astrocytoma cells. *A,* Western blot showing practically no processing of IGF-IR in PDX-transfected cells. Conversely, the vector alone-transfected clones (*Cin*) show both the proform (*faint band*) and the processed form (*stronger lower band*). *B,* Western analysis of both PDX transfectants show the proform and the processed form of TGF-β, whereas the vector alone-transfected astrocytoma cells (*Cin*) only express the processed form. *C,* Western blots showing presence of processed MT1-MMP in vector alone-transfected cells (*Cin*), whereas the PDX transfectants conserve the inactive proform in addition to a *faint band* corresponding to the processed form. Marker sizes are seen on the *left* side. *D,* gelatinase zymogram depicting less MMP-2 activity and absent or less efficient processing in PDX-transfected astrocytoma cells (*PDX*) than in the respective vector alone-transfected cells (*Cin*). *Arrows* indicate from *top* to *bottom*: M_r 92,000 MMP-9, M_r 72,000 MMP-2, and processed MMP-2 forms of M_r 68,000 and 66,000, respectively.

inhibitor on cell division was more obvious in transfectants of U87MG, which exhibited an increase of ~50% (from 27 h to 42 h doubling time), whereas an increase of 30% was noted in U118MG cells (from 55 h to 72 h doubling time). The tumorigenic ability of the transfectants was evaluated in s.c. xenografts using Scid mice (Fig. 4). Growth of s.c. transfected U87MG cells was almost completely prevented by PDX expression. The growth of the vector alone-transfected cells was nearly exponential during the first week after inoculation. In the remaining 4 weeks the tumors increased very rapidly in size, forcing the termination of the *in vivo* experiment at 5 weeks after cell inoculation. Conversely, the cells transfected with furin inhibitor grew very little and remained practically unchanged during the course of the experiment (Fig. 4*A*). This inhibitory effect of PDX on s.c. tumor growth was also observed in tumors derived from U118MG cells (Fig. 4*B*). However, tumor masses produced after inoculation of U118MGpCIN.PDX cells shrunk and subsequently disappeared 15 days after cell inoculation. The vector-alone-transfected U118MG.pCIN cells tumors reached volumes of 0.3–0.6 cm^3 4 months after inoculation, whereas no tumor was noticed in animals injected with U118MG.pCIN.PDX cells even after 6 months of observation.

To assess PDX effects on invasiveness, both *in vitro* and *in vivo* assays were performed. The *in vitro* assay showed a 2–5-fold loss in the invasive ability of PDX transfectants when compared with their vector alone-transfected counterparts (Table 1). An *in vivo* invasion assay was performed to assess the invasive ability of PDX-transfected astrocytoma cell lines grown as xenotransplants in Scid mice. As shown in Fig. 4*C*, the PDX transfectants of each astrocytoma cell line exhibited a markedly decreased invasive ability when compared with their respective vector alone-transfected counterparts. U87MG cells had remarkably dissimilar invasion patterns depending on whether or not PDX was expressed. Control vector alone-transfected cells penetrated deeply into the tracheal wall and reached the outer part of the trachea (adventitia; Fig. 5*A*), whereas the PDX-transfected cells lacked an invasive front, and the cells remained in the tracheal luminal area (Fig. 5*B*). Vector alone-transfected U118MG cells grew with difficulty in the tracheal transplants and remained either in the luminal area or in a few cases invaded the *pars membranacea* (Fig. 5*C*). PDX-trans-

Table 1 *In vitro* invasion assay and *in vivo* proliferation rate of PDX-transfected cells

	In vitro invasion assay[a]		*In vivo* Mib-1 (Ki67) LI[b]	
	U87MG	U118MG	U87MG	U118MG
pCin transfected	833 ± 112	221 ± 19	43 ± 2	12.5 ± 2.5
pCin.PDX transfected	443 ± 32	40 ± 8	18 ± 3.6	0.5 ± 4

[a] Mean number of cells per filter ± SD. Invasion chambers were used to assess the ability of the cells to degrade ECM. A total of 12 filters (3/clone) were counted (approximately 1000–1600 cells/transfectant clone). One-sided *t* test indicated significant differences ($P < 0.05$).

[b] Labeling indices (percentage of Mib-1 (positive cells) in xenotransplanted cells growing in tracheal transplants (at least 500 cells/tracheal transplant were counted). Figures show mean ± SD. One-sided *t* test indicated significant differences ($P < 0.05$).

Fig. 4 PDX-induced inhibition of s.c., and intratracheal growth. Time course of sc. tumor development expressed in cm³ of tumor volume in Scid mice after injection of pCIN-(*solid symbols*) or pCIN.PDX-transfectant cells (*open symbols*). U87MG cells (*A*) and U118MG cells (*B*). *Plotted values* depict mean from 10 tumors. *In vivo* invasiveness was evaluated in tracheal xenographs (*C*); *bars,* ±SD. Invasiveness is depicted in μm of cell penetration from the tracheal middle point to the outermost point of astrocytoma cell invasion in the tracheal wall or beyond. One-sided *t* test showed that in both pair of cell lines PDX expression reduced significantly the invasive ability; $P < 0.05$.

fected U118 MG cells grew very little or not at all in tracheal transplants (Fig. 5D). Evaluation of Mib-1 LIs in the astrocytoma cells growing in tracheal transplants showed that U87 MG cells expressing PDX exhibited a reduction of >50% in their proliferative ability when compared with U87MG transfected with vector alone (Fig. 5, *E* and *F*). U118 MGpCIN cells had a lower LI than the other astrocytoma cells. Nevertheless the decrease in LI of PDX-transfected cells was also remarkable (Table 1).

Discussion

Expression and activation of MMPs are essential steps in the degradation and remodeling of the ECM. Furin is recognized as a fundamental component in the activation of several MMPs. MT-MMPs are proteolytic enzymes containing sites for furin recognition (6). Several growth factors and their receptors, such

as TGF-β and IGF-IR, involved in cell proliferation, are also well-recognized furin substrates (9, 17). Furin-mediated cleavage is essential in the processing of these proproteins into their active forms. Impairment of the processing of latent metalloproteases and growth factors/receptors by furin inhibition provides a rationale for antitumor strategies.

Furin is detected at very low levels in most normal tissues, suggesting that the potential deleterious effects of furin on the maintenance of cellular homeostasis under physiological conditions are avoided by very low cellular levels of expression. In certain types of cancer, expression of furin above that level is associated with the acquisition of a more aggressive phenotype (18, 19).

In this study we detected furin expression in primary glial cell cultures and even higher expression in two tumorigenic astrocytoma cell lines.

The two tumorigenic astrocytoma cell lines with demonstrable furin overexpression were transfected with the potent furin inhibitor PDX resulting in a decrease in cell growth, and an inhibition of tumorigenicity and invasive ability. These effects were associated with a decreased activation of MMPs combined with lower proliferative rates of the PDX-transfected cells.

MT1-MMP participates in the activation of the M_r 72,000 type IV collagenase, MMP-2 (20), a major ECM-degrading enzyme that is of paramount importance in human gliomas (21, 22). Although furin-dependent activation of MT1-MMP seems to vary among different cell types (23), astrocytoma cells effectively express the activated form (24). Our results demonstrate conclusively that PDX-transfected astrocytoma cells are unable to activate MT1-MMP. Consequently, activation of MMP-2 proenzyme into active ECM-degrading forms was also inhibited by PDX transfection. These effects of PDX strongly suggest that the impairment of furin-mediated metalloprotease processing results in the decreased invasive ability observed in PDX transfectants.

TGF-β promotes the proliferation of highly malignant, aneuploid gliomas and inhibits the growth of near diploid gliomas, normal astrocytes, and microglia (25). In addition, TGF-β1 and 2 are expressed by glioma cell lines, and PDX inhibited the *in vitro* TGF-β processing and release of two other glioma cell lines (LN-18 and T98G; Ref. 26). The IGF-IR has three major

Fig. 5 Tracheal cross-section showing the growth patterns of transfected astrocytoma cells. *A,* U87MGpCIN cells grow inside the tracheal graft and invade the *pars membranacea* (*arrow*) penetrating deep into the tracheal wall and extending into the extratracheal tissues (∗). *B,* U87MGpCIN.PDX cells remain inside the trachea (∗) and do not invade the tracheal wall. *C,* U118MG.pCIN cells grow inside the tracheal luminal area and occasionally invade the *pars membranacea* (*arrow*). *D,* U118MG.pCIN.PDX cells exhibited minimal or no growth. The ∗ marks the luminal area of the tracheal transplant. *E,* Mib-1 labeling of a tracheal xenograft containing U87MG pCIN cells. *F,* Mib-1 labeling of a tracheal xenograft containing U87MG.pCIN.PDX. Note the decrease in the number of positively stained cells. Also, cells seem to be larger with more prominent cytoplasm. *A–D,* H&E; *E* and *F,* Hematoxylin and Mib-1 immunostain. *A, B,* and *D,* ×14; *E* and *F,* ×120.
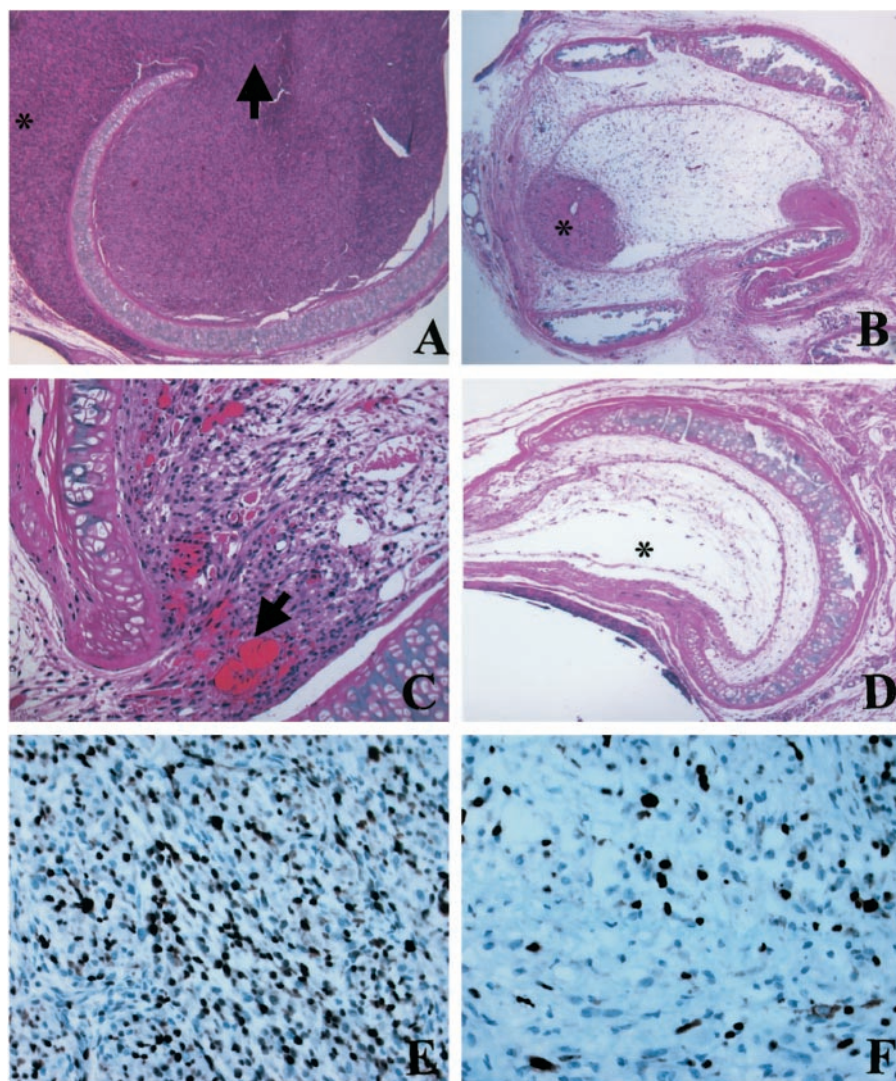
properties in terms of growth: it is mitogenic, it is required for the establishment and maintenance of the transformed phenotype in many cell types, and it protects cells from apoptosis (27).

Activation of TGF-β and IGF-IR are know to up-regulate cell proliferation in astrocytoma cells and other cell types. Inhibition of their furin-mediated processing could have a negative effect on mitogenesis. This was seen in a colon carcinoma cell line transfected with PDX (9) but could not be demonstrated in three transfectant head and neck cell lines (10). In the two PDX-transfected astrocytoma cell lines described herein, inhibition of cell proliferation plays a significant role as seen by the impressive reduction of the proliferation rate of PDX-transfected cells (50% reduction *in vitro* and 2–10 fold decrease *in vivo*). Furthermore, *in vivo* invasiveness was reduced 4-fold. Hence, it would be logical to conclude that substrates involved in loss of ECM integrity (MT1-MMP) together with cell cycle regulators, such as growth factors and receptors, play a central role in PDX-induced reduction of tumorigenicity and invasiveness of astrocytoma cells.

Astrocytoma is a type of brain tumor characterized by the strong tendency of neoplastic cells to emerge from the tumor margins and invade the normal brain (14). Because these tumors almost never metastasize, local growth and invasiveness should be the targets of innovative therapies. The present study shows that inhibition of a member of the family of PCs results in decreased tumor growth and invasiveness of astrocytoma cells through reduction of furin substrate (MMPs and growth factors) activation. Compared with carcinoma cells (10), astrocytomas showed a marked inhibition of cell proliferation after PDX transfection (little change in carcinoma cell lines *versus* a several-fold decrease in astrocytoma cells). The effect on *in vivo* invasiveness was similar in both cell systems. The difference in proliferation inhibition is probably because astrocytoma cells may express putative furin-substrates, such as nerve growth factor or glial cell line-derived neurotrophic factor (28, 29), which regulate cell proliferation and are not expressed at all in carcinoma cells.

In summary, furin inhibition results in a marked reduction

of cell proliferation and invasiveness in xenotransplanted astrocytoma cells. Because astrocytoma cell lines were very sensitive to the inhibitory effects of PDX, these findings provide a rationale for a new therapeutic strategy to limit astrocytoma cell growth based on the development of a small molecule targeting furin. In this cell system, in contrast to squamous cell carcinoma cells, both invasiveness and proliferation were affected by furin inhibition. Hence, this approach could have a remarkable impact on the survival of patients suffering from the usually chemoresistant glioblastomas.

References

1. Seidah, N. G., Chretien, M., and Day, R. The family of subtilisin/kexin-like proprotein and prohormone convertases: divergent or shared functions. Biochimie (Paris), *76:* 197–209, 1994.

2. Steiner, D. F., Smeekens, S. P., Ohagi, S., and Chan, S. J. The new enzymology of precursor processing endoproteases. J. Biol. Chem., *267:* 23435–23438, 1992.

3. Bassi, D. E., Mahloogi, H., and Klein-Szanto, A. J. P. The proprotein convertases furin and PACE-4 play a significant role in tumor progression. Mol. Carcinog., *28:* 63–69, 2000.

4. Hubbard, F. C., Goodrow, T. L., Liu, S. C., Brilliant, M. H., Basset, P., Mains, R. E., and Klein-Szanto, A. J. P. Expression of PACE4 in chemically induced carcinomas is associated with spindle cell tumor conversion and increased invasive ability. Cancer Res., *57:* 5226–5231, 1997.

5. Santavicca, M., Noel, A., Angliker, H., Stoll, I., Segain, J. P., Anglard, P., Chretien, M., Seidah, N., and Basset, P. Characterization of structural determinants and molecular mechanisms involved in prostromelysin-3 activation by 4-aminophenylmercuric acetate and furin-type convertases. Biochem. J., *315:* 953–958, 1996.

6. Pei, D., and Weiss, S. J. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature (Lond.), *375:* 244–247, 1995.

7. Nagase, H., and Woessner, J. F., Jr. Matrix metalloproteinases. J. Biol. Chem., *274:* 21491–21494, 1999.

8. Jean, F., Thomas, L., Molloy, S. S., Liu, G., Jarvis, M. A., Nelson, J. A., and Thomas, G. A protein-based therapeutic for human cytomegalovirus infection. Proc. Natl. Acad. Sci. USA, *97:* 2864–2869, 2000.

9. Khatib, A. M., Siegfried, G., Prat, A., Luis, J., Chretien, M., Metrakos, P., and Seidah, N. G. Inhibition of proprotein convertases is associated with loss of growth and tumorigenicity of HT-29 human colon carcinoma cells. Importance of insulin-like growth factor-1 (IGF-1) receptor processing in IGF-1-mediated functions. J. Biol. Chem., *276:* 30686–30693, 2001.

10. Bassi D. E., Lope Zde Cicco, R., Mahloogi, H., Zucker, S., Thomas, G., and Klein-Szanto, A. J. P. Furin inhibition results in absent or decreased invasiveness and tumorigenicity of human cancer cells. Proc. Natl. Acad. Sci. USA, *98:* 10326–10331, 2001.

11. Cullinan, W. E., Day, N. C., Schafer, M. K., Day, R., Seidah, N. G., Chretien, M., Akil, H., and Watson, S. J. Neuroanatomical and functional studies of peptide precursor-processing enzymes. Enzyme (Basel), *45:* 285–300, 1991.

12. Day, R., Schafer, M. K., Cullinan, W. E., Watson, S. J., Chretien, M., and Seidah, N. G. Region specific expression of furin mRNA in the rat brain. Neurosci. Lett., *149:* 27–30, 1993.

13. Schafer, M. K., Day, R., Cullinan, W. E., Chretien, M., Seidah, N. G., and Watson, S. J. Gene expression of prohormone and proprotein convertases in the rat CNS: a comparative *in situ* hybridization analysis. J. Neurosci., *13:* 1258–1279, 1993.

14. Chintala, S. K., Tonn, J. C., and Rao, J. S. Matrix metalloproteinases and their biological function in human gliomas. Int. J. Dev. Neurosci., *17:* 495–502, 1999.

15. Momiki, S., Baba, M., Caamano, J., Iizasa, T., Nakajima, M., Yamaguchi, Y., and Klein-Szanto, A. *In vivo* and *in vitro* invasiveness of human lung carcinoma cell lines. Invasion Metastasis, *11:* 66–75, 1991.

16. Fogh, J., Fogh, J. M., and Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst., *59:* 221–226, 1977.

17. Dubois, C. M., Blanchette, F., Laprise, M. H., Leduc, R., Grondin, F., and Seidah, N. Evidence that furin is an authentic transforming growth factor-β1-converting enzyme. Am. J. Pathol., *158:* 305–316, 2001.

18. Mbikay, M., Sirois, F., Yao, J., Seidah, N. G., and Chretien, M. Comparative analysis of expression of the proprotein convertases furin. PACE4, PC1 and PC2 in human lung tumours. Br. J. Cancer, *75:* 1509–1514, 1997.

19. Cheng, M., Watson, P. H., Paterson, J. A., Seidah, N., Chretien, M., and Shiu, R. P. Pro-protein convertase gene expression in human breast cancer. Int. J. Cancer, *71:* 966–971, 1997.

20. Yana, I., and Weiss, S. J. Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. Mol. Biol. Cell, *11:* 2387–401, 2000.

21. Abe, T., Mori, T., Kohno, K., Seiki, M., Hayakawa, T., Welgus, H. G., Hori, S., and Kuwano, M. Expression of 72 kDa type IV collagenase and invasion activity of human glioma cells. Clin. Exp. Metastasis, *12:* 296–304, 1994.

22. Sawaya, R. E., Yamamoto, M., Gokaslan, Z. L., Wang, S. W., Mohanam, S., Fuller, G. N., McCutcheon, I. E., Stetler-Stevenson, W. G., Nicolson, G. L., and Rao, J. S. Expression and localization of 72 kDa type IV collagenase (MMP-2) in human malignant gliomas *in vivo*. Clin. Exp. Metastasis, *14:* 35–42, 1996.

23. Sato, T., Kondo, T., Seiki, M., and Ito, A. Cell type-specific involvement of furin in membrane type 1 matrix metalloproteinase-mediated progelatinase A activation. Ann. N. Y. Acad. Sci., *878:* 713–715, 1999.

24. Yamamoto, M., Mohanam, S., Sawaya, R., Fuller, G. N., Seiki, M., Sato, H., Gokaslan, Z. L., Liotta, L. A., Nicolson, G. L., and Rao, J. S. Differential expression of membrane-type matrix metalloproteinase and its correlation with gelatinase A activation in human malignant brain tumors *in vivo* and *in vitro*. Cancer Res., *56:* 384–392, 1996.

25. Kjellman, C., Olofsson, S. P., Hansson, O., Von Schantz, T., Lindvall, M., Nilsson, I., Salford, L. G., Sjogren, H. O., and Widegren, B. Expression of TGF-β isoforms, and SMAD molecules at different stages of human glioma. Int. J. Cancer, *89:* 251–258, 2000.

26. Leitlein, J., Aulwurm, S., Waltereit, R., Naumann, U., Wagenknecht, B., Garten, W., Weller, M., and Platten, M. Processing of immunosuppressive pro-tgf-β1, 2 by human glioblastoma cells involves cytoplasmic and secreted furin-like proteases. J. Immunol., *166:* 7238–7243, 2001.

27. Adams, T. E., Epa, V. C., Garrett, T. P., and Ward, C. W. Structure and function of the type 1 insulin-like growth factor receptor. Cell. Mol. Life Sci., *57:* 1050–1093, 2000.

28. Singer, H. S., Hansen, B., Martinie, D., and Karp, C. L. Mitogenesis in glioblastoma multiforme cell lines: a role for NGF and its TrkA receptors. J. Neuro-Oncol., *45:* 1–8, 1999.

29. Wiesenhofer, B., Stockhammer, G., Kostron, H., Maier, H., Hinterhuber, H., and Humpel, C. Glial cell line-derived neurotrophic factor (GDNF) and its receptor (GFR-α 1) are strongly expressed in human gliomas. Acta Neuropathol., *99:* 131–137, 2000.

Inhibition of Furin-mediated Processing Results in Suppression of Astrocytoma Cell Growth and Invasiveness

Javier Mercapide, Ricardo Lopez De Cicco, Daniel E. Bassi, et al.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/8/6/1740

Cited articles	This article cites 27 articles, 10 of which you can access for free at: http://clincancerres.aacrjournals.org/content/8/6/1740.full#ref-list-1
Citing articles	This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/8/6/1740.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/8/6/1740. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.