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*

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Proprotein convertases (PCs) of the subtilisin/kexin family are responsible for the activation of prohormones, proteolytic factors, and their receptors. We sought to determine whether loss of PC-mediated activities might affect the malignant phenotypes of cancer cells. Stable transfectants of α1-antitrypsin Portland (α1-PDX) cDNA, coding for a potent PC inhibitor, were analyzed in model HT-29 cells (HT-29/PDX) and in other cell lines. Expression of α1-PDX resulted in a proinsulin-like growth factor-1 receptor (pro-IGF-1R) processing blockade, hence inhibiting the ability of exogenous IGF-1 to induce tyrosine phosphorylation of its β-subunit and insulin-related substrate-1. Coexpression of IGF-1R with four different PCs or the novel convertase SKI-1 in the furin-defective LoVo-C5 cells demonstrated that pro-IGF-1R (~200 kDa) cleavage into IGF-1R (β-subunit, ~105 kDa) can be achieved by furin and PC5A, but not by PACE4, PC7, or SKI-1. Expression of α1-PDX resulted in reduction of DNA synthesis and in anchorage-independent growth. Following serum deprivation, the α1-PDX transfectants exhibited an enhanced apoptotic phenotype and were insensitive to IGF-1-mediated [3H]thymidine incorporation and protection against apoptosis. These cells showed reduced invasiveness that paralleled decreased mRNA levels of urokinase-type plasminogen activator and its receptor, tissue-type plasminogen activator, and plasminogen activator inhibitor-1. Comparative subcutaneous inoculation of cells in nude mice revealed that animals injected with HT-29/PDX cells exhibited delayed and lower incidence of tumor development as well as reduced tumor size. Immunohistochemical analysis of CD31 antigen expression, a marker of endothelial cells, revealed reduced HT-29/PDX tumor vascularization. These findings indicate that PCs actively contribute to the growth and malignant phenotypes of HT-29 tumors, suggesting that PC inhibition strategies may be a useful adduct to the arsenal of colorectal anticancer gene therapies.

Proteins are the fundamental units from which bioactive proteins and peptides are derived by limited proteolysis. Pre-cursors are usually cleaved at the general motif (K/R)nXn(K/R)n, where n = 0, 2, 4, or 6 and X is usually not a Cys. Seven dibasic specific mammalian proprotein convertases (PCs) have been identified: furin, PC1/PC3, PC2, PC4, PACE4, PC5/PC6, and PC7/LPC/PC8. Each of these enzymes, either alone or in combination with others, is responsible for the tissue-specific processing of multiple polypeptide precursors. This combinatorial mechanism generates a large diversity of bioactive molecules in an exquisitely regulated manner (1–4). Some of these precursors include adhesion molecules, e.g. integrin α-subunits (5); matrix metalloproteinases (MMPs) such as stromelysin-3 and membrane-type MMPs (MT-MMPs) (6, 7); several growth factor precursors, including transforming growth factor-β (8, 9), insulin-like growth factor-1 (IGF-1), and IGF-2 (10–12); and some growth factor proreceptors such as the insulin receptor (13) and phosphotyrosine phosphatase μ (14).

Recently, the potential clinical and pharmacological role of the convertases fostered the development of both peptide- and protein-based PC inhibitors (for reviews, see Refs. 1–4). The most promising protein-based specific inhibitors of PCs are an α1-antitrypsin variant known as α1-antitrypsin Portland (α1-PDX) (15–17) and the individual PC prosegment-based inhibitors (18). Recent studies showed that inhibition of PCs by α1-PDX reduces the production level of the amyloid precursor protein, α-secretase product β-amyloid precursor protein-α (19) and blocks the activation of the pore-forming toxin proaerolysin (20), the cleavage of Notch (21), the proteolytic activation of bone morphogenic factor-4 (22), and the maturation of the surface glycoproteins of infectious viruses (15, 17, 23).

Multiple approaches, e.g. suppression of gene expression or

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§ The abbreviations used are: PCs, proprotein convertases; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; α1-PDX, α1-antitrypsin Portland; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; IRS-1, insulin-related substrate-1; FCS, fetal calf serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; bp, base pair; SKI-1, subtilisin kexin isozyme-1.

30686 This paper is available on line at http://www.jbc.org
enzyme inhibition, support the hypothesis that PCs play a role in the genesis and progression of different proliferative disorders, including cancer (27, 28–30). The critical nature of the furin processing of various precursors may explain the antiproliferative effect of furin blockade on H-500 rat Leydig tumor cells (27), GD6 mouse gastric mucus cells (31), and the pancreatic β-cell line MIN6 (32). Kayo et al. (34) showed that conditioned medium derived from furin-overexpressing MIN6 cells stimulated the growth of their parental control cells, whereas the medium from cells expressing α1-PI resulted in a lower growth rate. These results suggest that high furin expression stimulates growth through an autocrine/paracrine mechanism.

In recent years, IGF-1 and its receptor have emerged as key regulators of mitogenesis and tumorigenicity (35). It is well established that a functional IGF-1R is required for cell growth and plays a crucial role in survival of various transformed cells in vitro and in vivo (35, 36). In tumor cells, including colorectal cancer cells, IGF-1 alone or in combination with IGF-2 acts as an autocrine/paracrine growth factor (37) as well as an inhibitor of apoptosis (35, 38). Defects in IGF-1R expression and/or activation inhibit tumorigenicity, reverse the transformed phenotype, and cause massive apoptosis in vitro and in vivo (35–40). This anti-oncogenic effect of IGF-1R blockade likely involves the modulation of the levels of various effectors that play important roles in tumor growth and metastasis, e.g. urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and plasminogen activator inhibitor-1 (PAI-1) (39, 41). The mature IGF-1R is a transmembrane glycoprotein consisting of two pairs of intramolecularly disulfide-bonded α- and β-chains (αβ2). The latter are generated by intracellular cleavage of IGF-1R at the tetrabasic RKRR sequence (see Fig. 1A) (42) by one or more undefined processing enzymes, possibly PC-like in nature (43). Activation of IGF-1R induces Tyr phosphorylation of its β-subunit and of its insulin receptor substrate-1 (IRS-1), thereby triggering an intracellular signaling cascade (44).

In this report, α1-PI-producing colorectal tumor cells and other cells allowed us to define the processing enzymes of IGF-1R and to examine the consequences of their inhibition on the physiological functions of IGF-1R. The observed effect of α1-PI on the reduction of cellular proliferation, invasion, and tumorigenicity in nude mice implicated the PCs in these processes and suggested that their inhibition could form a basis for adjunct gene therapy in cancer.

MATERIALS AND METHODS

Cell Transfection and Culture—The control (pcDNA3 vector) and stably α1-PI-transfected Jurkat T human leukemia and AtT20 mouse tumor pituitary cell lines were previously described (16, 19, 21). The control (pBK-CMV vector, HT-29/CTL) and stably α1-PI (HT-29/ PDX)-transfected HT-29-D4 human colon adenocarcinoma cell lines were derived from a pool of Pseudomonas Exotoxin A-resistant clones as previously described (45). LoVo/C5 human colon adenocarcinoma cells were transiently transfected with the empty piRES2-EGFP vector (CLONTECH, Palo Alto, CA) or with one that expresses full-length human furin, PAC4E, SKI-1, mouse PC5A, or rat PC7 (4) using Effectene transfection reagent (QIAGEN Inc., Mississauga, Ontario, Canada) as recommended by the manufacturer. Jurkat cells were grown in RPMI 1640 medium, and HT-29, LoVo/C5, and AtT20 cells were grown in Dulbecco’s modified Eagle’s medium, both supplemented with 10% fetal calf serum (FCS). In both culture media, 100 units/ml penicillin, 100 mg/ml streptomycin (Life Technologies, Inc., Burlington, Ontario), and, for stable transfectants, 200 μg/ml G418 were added.

Western Blotting—Cells were lysed in phosphate-buffered saline (PBS) containing 2% Nonidet P-40; lysates were subjected to SDS-polyacrylamide gel electrophoresis on 8% gels; and proteins were blotted onto nitrocellulose membranes. The primary antibodies used were rabbit anti-human IGF-1R polyclonal antibody recognizing carboxy-terminal amino acids 1447-1366 (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-phosphotyrosine (2 μg/ml) and anti-actin (1:1000 dilution) antibodies (Sigma, Oakville, Ontario). Primary antibodies were revealed by horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution; Amersham Pharmacia Biotech, Baie-d’Urfe, Quebec, Canada) and enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Band intensities of the autoradiographs were quantitated by densitometry.

IGF-1R and IRS-1 Tyrosine Phosphorylation—Confuent HT-29/CTL and stabilized HT-29/PDX cells grown in 75-cm² flasks were maintained in serum-free Dulbecco’s modified Eagle’s medium for 24 h and incubated with or without 50 ng/ml IGF-1 for 2 min at 37 °C. Cells were washed twice in ice-cold PBS and lysed with 500 μl of lysis buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100, 2 mM vanadate, 100 mM NaF, and 0.40 mM phenylmethylsulfonyl fluoride). Equal amounts of proteins (1 mg) were immunoprecipitated overnight with anti-IGF-1R or IRS-1 polyclonal antibody (10 μg/ml; Santa Cruz Biotechnology, Inc.), and the whole pellets were analyzed by Western blotting.

Cell Growth Assay—[3H]Thymidine incorporation in 2 × 10⁴ cells/well in 96-well culture plates was assayed as follows. 24 h after plating, cells were incubated in a growth arrest medium (0.5% FCS) for the next 24 h and then incubated for an additional 24 h in fresh medium containing various concentrations of serum or IGF-1. At the end of incubation, 0.5 μCi/well [3H]methylthymidine (Amersham Pharmacia Biotech) was added. Cells were then harvested onto glass-fiber filters using a cell harvester (Amersham Pharmacia Biotech, Wallac Oy, Turku, Finland) and counted using a Betaplate liquid scintillation counter (Amersham Pharmacia Biotech). Results are expressed as percentages of the values obtained for vector-transfected cells in the absence of serum. Doubling times were assessed by cell counting on a hemocytometer following culture of 10,000 cells/well in 12-well plates for 3 days.

Soft Agar Assay—To assay anchorage-independent colony formation, 4 × 10³ cells were suspended in complete medium containing 0.3% agar and seeded in triplicate in six-well plates onto a base layer of complete medium containing 0.5% agar. Complete medium was added every 3 days. After 14 days, colonies >100 μm in diameter were counted by inverted microscopy.

Apoptosis Assays—To induce apoptosis, cells grown on 100-mm dishes to 80% confluency were placed in serum-free medium with or without 100 ng/ml IGF-1 for the indicated time periods. Apoptotic cells were analyzed by cell death and DNA ladder assays and propidium iodide staining. For the cell death assay, floating and attached cells were separately resuspended in 0.4% trypan blue (Life Technologies, Inc.), and cells that had taken up this dye were considered nonviable. The percentage of dead cells was calculated as a ratio of floating dead cells to the total number of cells/culture dish. For the DNA ladder assay, genomic DNA was isolated from attached cells using the apoptotic DNA ladder detection kit (Chemicon International, Inc., Temecula, CA) according to the manufacturer’s instructions and analyzed on 2% agarose gels. For propidium iodide cell staining, cells were incubated in propidium iodide for 5 min, and the percentage of stained cells was determined by fluorescence-activated cell sorter analysis (Becton Dickinson Inc., San Jose, CA). At least 10,000 cells were examined for each sample.

Matrigel Invasion Assay—HT-29/CTL and HT-29/PDX cell invasiveness was assessed in vitro using the reconstituted basement membrane (Matrigel) assay. Matrigel (0.25 mg/ml; Collaborative Research, Bedford, MA) in PBS was used to coat the filter (8-μm pore size), and human fibronectin (5 μg/ml; Sigma) was used as a chemottractant in the lower chamber (24-well Transwell plate, Corning Inc., Corning, NY). Cells (5 × 10⁶/6.5-mm filter) were incubated for 48 h, and cells that migrated to the underside of the filters were stained and counted using an inverted microscope.

Reverse Transcription-Polymerase Chain Reaction (PCR)—Using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions, total RNA was extracted, and predefined amounts (0.2–4 μg; see below) were reverse-transcribed in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 30 mM KCl, 6 mM MgCl₂, 1 mM DTT, 100 mM NaCl, and 0.2 units of avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech). In each case, the 3′-antisense oligonucleotide (2 μM) was used to initiate reverse transcription. The mixture was sequentially incubated for 10 min at 25 °C, for 60 min at 37 °C, and for 5 min at 95 °C. cDNAs were amplified by PCR using the following oligonucleotides: uPAR, CCTGGAGCTTGAAAATGC; and GGTGATGGTGGACTGAG (352 bp product), uPA, TGTGCTG.
GTGTGTATTTTGGC and CTCCCACTATTGCTAAGCTC (402-bp product); tPA, GAGTGCCTCCCTTTTGCTC and GAAAAGGGGAGGAGACTTG (504-bp product); PAI-1, TGGACTACGGCGGCTTACAG and AAGTGCGTGACCTTGAGA (554-bp product); and glyceraldehyde 3-phosphate dehydrogenase, TGGAATCCATCAACCATCCT and GTCTTGGTGAGGTCGTGAT (520-bp product). The PCRs (50 μL) included 10 μL of the cDNA sample, 5 μL each primer, 200 μM dNTPs, and 0.2 units of Taq polymerase (Amersham Pharmacia Biotech) in the buffer supplied by the manufacturer. PCR conditions were as follows: 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C for 25 cycles using a PerkinElmer Life Sciences thermocycler. The quantity of total RNA introduced in the reverse transcription reaction was set independently for each gene product to be below the saturation point of the reaction. Amplified PCR products were analyzed on a 1.5% agarose gel.

In Vivo Tumorigenicity Assay—Cells (1 × 10^6 HT-29/CTL or HT-29/PDX) were injected subcutaneously into 4-week-old male athymic mice. The animals were monitored for tumor formation every 3 days and killed 31 days after injection. Tumor volume was calculated using the formula described by Kyriazis et al. (46). Tumor volume = (width^3 × length^2) / 3.0, and stored at −80 °C.

Immunohistochemical Analyses—17 days following the subcutaneous injection of HT-29/CTL and HT-29/PDX cells, the developed tumors were frozen, cryosectioned to 10–15 μm at −20 °C, fixed in 4% PBS/paraformaldehyde, and incubated with 0.3% H_2O_2 in methanol for 20 min at room temperature to block endogenous peroxidase. Slides were rinsed with blocking solution (PBS supplemented with 5% skim milk and 0.1% Tween 20) and then incubated overnight at 4 °C with antimouse CD31 monoclonal antibody (Pharmingen, San Diego, CA) at 1:50 dilution. Following washes in PBS, sections were incubated with peroxidase-conjugated goat anti-rat IgG (Chemicon International, Inc.) at 1:100 dilution for 2 h, washed, and incubated with diaminobenzidine substrate. Staining was monitored under a microscope, and the reaction was stopped by washing with PBS. The corresponding frozen tumor sections were also stained with hematoxylin and eosin (Sigma).

RESULTS
Proteolytic Cleavage of Pro-IGF-1R—IGF-1R is processed at the RKRR^{αβ-} site into the α- and membrane-bound β-subunits (Fig. 1A) (42, 43). To affirm the involvement of PCs in IGF-1R maturation and to investigate which of them are likely to be the best convertases for this precursor, endogenous IGF-1R processing was examined in furin activity-deficient LoVo-C5 cells (47). In these cells, the majority of IGF-1R was expressed as an immature ~200-kDa form, and only a small amount of the receptor was cleaved to generate its β-subunit (apparent molecular mass of ~105 kDa), suggesting that PCs other than furin may contribute to this processing. LoVo-C5 cells were thus transfected with furin, PC5A, PC7, PACE4, or SKI-1 cDNA-containing vectors (Fig. 1B). IGF-1R/total signal intensity ratios, calculated as an indication of cleavage, were found to be 63 and 53% for furin and PC5A, respectively, whereas PC7, PACE4, and SKI-1 did not enhance the cleavage of IGF-1R. The processing of IGF-1R was then examined in control (CTL) and α,-PDX (PDX)-expressing HT-29 pool and Jurkat cells (Fig. 1C). In empty vector-transfected cells (CTL), both unprocessed and mature forms of IGF-1R were detected. However, in α,-PDX-transfected cells, the mature β-subunit was barely detectable. In these cells, pro-IGF-1R was also present as a slower migrating species believed to be a trans-Golgi network-associated form of pro-IGF-1R, as reported for transforming growth factor-β (9). Furthermore, only the lower band was observed in the presence of the fungal metalloproteinase brefeldin A (data not shown), suggesting that it is an endoplasmic reticulum-associated form. This drug results in the fusion of the Golgi apparatus (except for the trans-Golgi network) with the endoplasmic reticulum (48).

Tyrosine Phosphorylation of IGF-1R and IRS-1—To examine whether inhibition of IGF-1R processing in HT-29/PDX cells affects the IGF-1R signaling pathway, the tyrosine phosphorylation of IGF-1R and other endogenous proteins was examined following a 2-min incubation of HT-29/CTL and HT-29/PDX cells with or without IGF-1. As shown in Fig. 2A, addition of IGF-1 enhanced the tyrosine phosphorylation of several proteins. The species migrating with apparent molecular masses of ~180 and ~105 kDa are among the major Tyr phosphoproteins. On the basis of their molecular masses and characteristic response to IGF-1-induced tyrosine phosphorylation, these proteins are likely to be IRS-1 and the IGF-1R β-subunit, respectively. In HT-29/PDX cells, we observed a marked reduction of the tyrosine phosphorylation of several proteins (including IRS-1 and IGF-1R), and the Tyr phosphorylation of the latter was insensitive to IGF-1. Immunoprecipitation with anti-IGF-1R (Fig. 2B) or anti-IRS-1 (Fig. 2C) antibody followed by immunoblotting with anti-phosphotyrosine antibody confirmed the identities of these proteins (Fig. 2, B and C). HT-29/CTL cells treated with IGF-1 showed a marked increase in the tyrosine phosphorylation level of a protein identified as the IGF-1R β-subunit on the basis of its apparent molecular mass (~105 kDa). As expected, IGF-1 weakly induced the tyrosine phosphorylation of IGF-1R in HT-29/PDX cells. Similarly, HT-29/CTL cells treated with IGF-1 showed a marked increase in the tyrosine phosphorylation levels of IRS-1 (165–185 kDa) and of another protein with an apparent molecular mass of ~105 kDa. The latter is likely to be the IGF-1R β-subunit since, when phosphorylated, it associates with IRS-1 (44), hence rationalizing the observed co-immunoprecipitation of these phosphoproteins. Similar to IGF-1R, expression of α,-PDX in HT-29...
transfected cells, those expressing /H9251 10% FCS; and accordingly, cells displayededly reduced their ability to proliferate even in the presence of morigency (49). Consequently, we tested the effect of ony formation is the parameter that correlates best with tu-

Fig. 2. Tyrosine phosphorylation of IGF-1R and IRS-1. Confluent HT-29/CTL and HT-29/PDX cells were starved for 24 h and then treated with (+) or without (−) 50 ng/ml IGF-1 for 2 min at 37 °C. Equal amounts of cell lysates were subjected to Western blotting with an anti-phosphotyrosine (P-Tyr) antibody (A). Equal amounts of cell lysates were subjected to immunoprecipitation (IP) with either an anti-IGF-1R β-specific antibody (B) or an anti-IRS-1 antibody (C). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis on an 8% gel and Western blotting. The migration positions of the molecular mass markers are shown on the right. On the left, arrows indicate the positions of IRS-1 (~165–185 kDa), pro-IGF-1R (~200 kDa), and the IGF-1R subunit (~105 kDa).

cells prevented IGF-1-induced IRS-1 Tyr phosphorylation.

Growth Properties of α1-PDX-transfected Cells—Since IGF-1-induced IGF-1R and IRS-1 phosphorylation regulates cell growth and survival (35–40), we assessed the ability of HT-29/PDX and Jurkat/PDX cells to incorporate [3H]thymidine as –growth and survival (35 1-induced IGF-1R and IRS-1 phosphorylation regulates cell

Fig. 3. [3H]thymidine incorporation assay. Starved control (CTL) or α1-PDX (PDX)-expressing Jurkat and HT-29 cells were incubated for 24 h in medium containing different concentrations of serum (0–10% FCS), A or IGF-1 (0–100 ng/ml). [3H]Thymidine was added for the final 6 h of incubation. [3H]Thymidine incorporation was measured as described under “Materials and Methods.” Each value represents the mean ± S.E. of triplicates. The doubling times of tumor cells (seeded at 1 × 10⁵ cells/well) were measured as described under “Materials and Methods” (B). Results are shown as means ± S.E. of three experiments performed in quadruplicate.

Fig. 4. Soft agar assay. Control (CTL) and HT-29/PDX or Jurkat/ PDX cells (PDX; 4 × 10⁴ cells/well) were seeded in triplicate in six-well plates in soft agar as described under “Materials and Methods.” After 14 days, colonies > 100 μm in diameter were counted. Results are shown as means ± S.E. of three experiments performed in triplicate.

reduction in their anchorage-independent growth (Fig. 4). This observation suggests that inhibition of colony formation in soft agar by α1-PDX seems to a general phenomenon.

Serum Deprivation-induced Apoptosis—Given that previous studies implicated IGF-1 as a modulator of apoptosis (34, 35), we examined the effects of α1-PDX on serum deprivation-induced apoptosis in the presence or absence of IGF-1. Incubation in serum-free medium for 24, 48, and 72 h caused a gradual increase in the number of floating dead cells, an effect exacerbated in HT-29/PDX cells at all time points (Fig. 5A). Although addition of IGF-1 (100 ng/ml) to the medium resulted in an ~2-fold reduction of the number of dead control cells, it failed to rescue HT-29/PDX cells at the three times tested. Similar results were obtained in the presence of 1% FCS (data not shown). To confirm that adherent cells had undergone apopto-
sis, their DNA profile was analyzed. In the absence of IGF-1, a clear DNA degradation pattern characteristic of apoptosis was detected in both HT-29/CTL and HT-29/PDX cells, especially after 48 and 72 h of serum deprivation (Fig. 5B). We noted that the extent of apoptosis was significantly higher in HT-29/PDX cells at all time points. In addition, although treatment with IGF-1 largely prevented DNA degradation in HT-29/CTL cells, it had no effect on HT-29/PDX cells (Fig. 5B). The extent of apoptosis can conveniently be quantitated by fluorescence-activated cell sorter analysis of adherent cells stained with propidium iodide. At the three time points examined, a 2-fold increase in propidium iodide staining was observed for HT-29/PDX cells compared with control cells (Fig. 5C). Here also IGF-1 appeared to significantly protect only HT-29/CTL cells from apoptosis. Analyses of apoptosis using propidium iodide staining and DNA fragmentation assays following serum deprivation in Jurkat/PDX cells showed similar results (data not shown).

Cell Invasiveness—Cell invasiveness estimated by a Matrigel assay revealed that HT-29/PDX cells were ~50% less invasive compared with control cells (Fig. 6A). Among the trypsin-like serine proteinases described to be involved in invasion processes are the plasminogen activators uPA and tPA the receptor UPAR, and the uPA inhibitor PAI-1 (50, 51). The effect of α1-PDX on the mRNA level of these four molecules was thus analyzed by semiquantitative reverse transcription-PCR. In both Jurkat and HT-29 cells, expression of α1-PDX caused a reduction in the mRNA levels of all four gene products (Fig. 6B).

Tumorigenicity of HT-29/PDX Tumor Cells—To assess the effect of α1-PDX expression on the tumorigenicity of HT-29 cells, two groups of six male nude mice were subcutaneously singly inoculated with 1 × 10⁶ HT-29/CTL or HT-29/PDX cells. The size of the tumors that developed and their frequency were measured at 3-day intervals, and the data are summarized in Fig. 7. Animals injected with HT-29/PDX cells exhibited an 8-day delay in tumor appearance (day 12 as compared with day 4 in controls) (Fig. 7, A and B). Overall, the incidence of tumor development was markedly reduced in the presence of α1-PDX-containing cells. For example, at 31 days post-inoculation, maximally two out of six HT-29/PDX cell-inoculated mice developed tumors compared with five out of six control mice. On day 31, the total tumor volume that developed from the HT-29/PDX cells was ~3-fold smaller than that calculated for the tumors...
originating from HT-29/CTL cells (Fig. 7, B and C).

**Immunohistochemical Analysis of Tumor Vasculature**—The vessels within the HT-29 and HT-29/PDX 17-day post-injection developed tumors were stained with an antibody against CD31, a mouse endothelial cell marker that is involved in endothelial cell-cell adhesion and leukocyte transmigration. Indeed, HT-29 tumor sections showed extensive CD31 staining of vessels (Fig. 8, upper panels). Comparative sections from HT-29/PDX developed tumors showed a marked decrease in the CD31 staining pattern. Staining of HT-29 tumor sections with hematoxylin/eosin revealed a much more organized cellular pattern compared with that observed with HT-29/PDX tumor cells. The latter appeared quite disorganized, likely due to increased apoptosis and/or necrosis.

**DISCUSSION**

This report describes a functional link between inhibition of precursor processing achieved by PCs and the extent of colorectal tumor cell growth, invasion, and tumorigenicity. Thus, ex vivo studies of proliferation and cell transformation showed that \( \alpha_2 \)-PDX significantly altered the ability of tumor cells to incorporate \(^{3}H\)thyminidine and to form colonies in soft agar. In vivo, when nude mice were inoculated with a HT-29 human colon carcinoma cell line expressing \( \alpha_2 \)-PDX versus control cells, they showed delayed tumor development and a significantly decreased size, vascularity, and incidence of tumors.

The observed effect of \( \alpha_2 \)-PDX on the HT-29 tumor cell phenotype is probably due to the inhibition of the processing of various proteins, including growth factors and/or their corresponding receptors (8–14). The availability of growth factors is critical for malignant transformation and metastasis. These molecules mediate cell entry and progression through the cell cycle. They are divided into two major groups: factors such as basic fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor that enable cells to enter the G1 phase and factors such as IGF-1 that are required for progression from G1 into S phase, ultimately leading to cell division (52, 53). Many growth factors mediate their effects through receptor tyrosine kinases that transmit information to the nucleus through an intricate network of adapter and signaling molecules (54). Overexpression or mutations of growth factor receptors that result in high levels of active kinases are involved in tumor growth and progression (54, 55). The latter is often accompanied by constitutive high expression of the respective ligands, providing an autocrine mechanism for growth autonomy (56). Of these proteins, IGF-1 and its receptor (IGF-1R) emerged as key regulators of mitogenesis and tumorigenicity (35–41). A functional IGF-1R is known to be required for cell growth of various transformed cells (35–41). Overexpression and/or constitutive activation of IGF-1R in a variety of cell types leads to ligand-dependent growth in serum-free medium and to the establishment of a transformed phenotype such as the ability to form colonies in soft agar and tumors in mice (35–41). The anti-apoptotic function of IGF-1R is also substantial. Thus, an overexpressed IGF-1R allows the protection of
cells from apoptosis under a wide variety of conditions. These include growth factor withdrawal (57), serum deprivation (58), incubation with tumor necrosis factor (59), activation of interleukin-1β converting enzyme (ICE) protease (60), and irradiation with UV-B (61). Conversely, when the function of IGF-1R is blocked or otherwise impaired by antisense strategies, dominant-negative mutants, or triple-helix formation, tumor cells undergo apoptosis, leading to a dramatic inhibition of tumorigenesis and metastasis (35–40). The protective effect of an activated IGF-1R on apoptosis is apparently dependent on its ability to inhibit ICE-like caspases and to increase the activity and expression of the negative death regulator Bcl-xL (60, 62).

Taking advantage of the fact that pro-IGF-1R is poorly cleaved in furin-deficient LoVo-C5 cells (49), we first demonstrated that furin and PC5A are the major processing enzymes of this precursor in this cell line (Fig 1B). This conclusion was further confirmed in HK293 cells, a human kidney epithelial cell line, whereby overexpression of the inhibitory PC prosegment (18) of either furin or PC5 gave similar results, whereas pPC7 and pSKI-1 did not affect pro-IGF-1R processing (data not shown). The similar processing properties of furin and PC5A have been often observed with a number of precursors such as β-secretase β site APP cleaving enzyme (BACE) (63), α-integrins (5), bone morphogenic factors (22), and phosphotyrosine phosphatase μ (14).

Activated IGF-1R phosphorylates IRS-1, which subsequently binds to the cytosolic tail of IGF-1R (64) and recruits SH2 domain-containing proteins, thereby regulating a cascade of signaling pathways. These include phosphatidylinositol 3-kinase, Grb2, Nck, Crk, Fyn, SHP-2, Syp, and pp125FAK (65–67). Expression of the general serpin PC inhibitor α1-PDX (15, 16) blocked pro-IGF-1R processing in both Jurkat and HT-29 cells (Fig. 1C) and abrogated IGF-1-stimulated tyrosine phosphorylation of both the IGF-1R β-chain and IRS-1, its major substrate (Fig. 2). In agreement with the absence of IRS-1 Tyr phosphorylation in HT-29/PDX cells, loss of IGF-1R activation was evident by the inability of IGF-1 to stimulate [3H]thymidine incorporation in these cells (Fig. 3) and to protect them from apoptosis induced by serum deprivation (Fig. 5). IGF-1R is not necessary for the growth of cells in culture, whereas in its absence, the cell cycle division phases are all elongated, even at higher concentrations of serum (68). Our results describe similar observations in tumor cells expressing α1-PDX, suggesting that PCs are involved in the regulation of the cell cycle probably by processing IGF-1R and other cell cycle regulator proteins. The observed serum deprivation-enhanced apoptosis in HT-29/PDX cells may be explained by the α1-PDX-mediated blockade of an autocrine/paracrine mechanism involved in cell survival. A likely scenario may involve one or more secreted ligands and/or their receptors, all which require processing by PC-like enzymes. Examples include IGF-1 and IGF-2 that are synthesized and secreted by HT-29 cells (37, 69) and processed by furin (10, 11). Since overexpression of α1-PDX in HT-29 cells inhibited the processing of IGF-1R (Fig. 1C) and its furin-processed ligands, IGF-1 and IGF-2, it is liable to abrogate their autocrine/paracrine protective effects.

In addition to its proliferative and anti-apoptotic functions, IGF-1R activation was recently reported to mediate vascular endothelial growth factor production and to participate in angiogenesis of various tumors, including colon cancer (70). Immunohistochemical analysis of CD31 antigen expression revealed a reduced vascularization of HT-29/PDX tumors (Fig. 8), suggesting the importance of the PCs in tumor vessel formation through activation of IGF-1R and probably other proteins. Interestingly, hypoxia-inducible factor-1, an inducer of various genes involved in tumor progression, including vascular endothelial growth factor, was reported to be reciprocally and positively regulated by IGF systems (71). This suggests that inactivation of IGF-1R by α1-PDX may also inhibit tumor growth and vascularization via this autocrine loop.

Extracellular proteolytic enzymes such as serine proteinases and MMPs have been implicated in cancer metastasis by promoting cancer cell invasion into the surrounding normal tissue via the degradation of the extracellular matrix. To further investigate the influence of the endogenous inhibition of PCs on cell invasiveness, chemoinvasion assays with Matrigel-coated filters were performed. Our results demonstrated that the ability of these tumor cells to degrade and subsequently invade Matrigel was reduced by ~2-fold in HT-29/PDX cells (Fig. 6A). Consistent with previously reported studies (35, 41) showing that interfering with IGF-1R functions and/or expression reduces the level of tPA and uPAR mRNAs, we demonstrated that inhibition of IGF-1R cleavage by α1-PDX significantly decreased the levels of uPAR, uPA, tPA, and PAI-1 mRNAs (Fig. 6B). The matrix metalloproteinase MT1-MMP is known to activate pro-MMP-2, and both enzymes have been implicated in tumor invasion and metastasis (7). Although mature MT1-MMP could be detected in HT-29/CTL cells, expression of α1-PDX dramatically reduced its processing (data not shown). However and in agreement with others (72), we could not detect MMP-2 in HT-29 cells by gel zymography (data not shown), suggesting that this enzyme may have other functions
in these cells. Indeed, MT1-MMP was reported to be involved in extracellular matrix remodeling either directly or through activation of procollagenase (73, 74). Therefore, the observed reduction in expression of urokinases and inhibition of pro-MT1-MMP processing in HT-29/PDX cells suggest that inhibiting the PCs could alter the invasive and metastatic phenotypes of colorectal cancer cells.

In conclusion, our results strongly support the hypothesis that the growth and malignant phenotypes of tumor cells are regulated by PCs. Aside from the present data on IGF-1R, it is thought that the PCs could alter the invasive and metastatic phenotypes of colorectal cancer cells.

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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

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