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The Proteolytic Processing of Pro-Platelet-derived Growth Factor-A at RRKR[86] by Members of the Proprotein Convertase Family Is Functionally Correlated to Platelet-derived Growth Factor-A-induced Functions and Tumorigenicity[1]

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ABSTRACT

Although altered expression of platelet-derived growth factor (PDGF)-A is a hallmark of many cancers, the importance of pro-PDGF-A conversion to PDGF-A in tumorigenesis and the cognate protease(s) is unknown. Pro-PDGF-A processing occurs at pairs of basic residues, likely involving the proprotein convertases (PCs). In the colon carcinoma cell line LoVo, we found that Furin is the most potent PDGF-A convertase. Mutation of the PC-site *RRKR*[86] to *ARKA*[86] inhibited pro-PDGF-A processing, its receptor tyrosine phosphorylation, and cell proliferation. This processing is also blocked by the PC preprosegments (pps) ppFurin, ppPC5, and ppPACE4, and by the Furin-variants of α2-macroglobulin and α1-antitrypsin. Chinese hamster ovary cells overexpressing pro-PDGF-A (ARKA[86]) failed to induce tumors in nude mice. Thus, PC-directed inhibitors might represent new agents for therapy in neoplasia induced by PDGF-A.

INTRODUCTION

Many reports have cited the coexpression of PDGF[3] and its receptors in various tumor cells, suggesting both autocrine and paracrine mechanisms for PDGF-mediated tumor growth and invasion (1–3). The expression of PDGF and its receptors is up-regulated in diverse human cancers (1–3), and has been associated directly with metastases (4) and angiogenesis (5). PDGF is a disulfide-linked dimer composed of two polypeptide chains, denoted A and B, and represented *in vivo* by three PDGFs, PDGF-AA, PDGF-AB, and PDGF-BB (6–9). These isoforms bind to and activate two tyrosine kinase receptors, PDGF receptor-α and PDGF receptor-β (6–9). The α-receptor binds both the A and B chains of PDGF, but the β-receptor binds only the B chain. Two new members of the PDGF family, PDGF-C and PDGF-D were reported recently, exhibiting similar binding properties to PDGF-AB (10–14). Alternative splicing of the PDGF-A mRNA results in a long and short form. The longer variant (211 aa) is less common and differs from the shorter one (196 aa) by a COOH-terminal extension of 15 aa (8, 4, 15). After dimerization of PDGF-A monomers in the ER into a M_r ~50,000 form, this complex transits through the Golgi apparatus toward the *trans* Golgi Network where it is proteolytically cleaved at the sequence RRKR[86]$\downarrow$ and secreted as a M_r ~30,000 dimeric product (15). However, nothing is known about the enzymes involved in this processing event, likely leading to the activation of PDGF-A. The RRKR[86]$\downarrow$ cleavage site suggested that the dibasic-specific PCs could be implicated in this process (16). The mammalian subtilisin-like PCs constitute a family of seven known dibasic-specific proteinases, namely, Furin, PC1, PC2, PC4, PACE4, PC5, and PC7, as well as the two nonbasic specific convertase SKI-1 (16, 17) and NARC-1 (18). The PCs are implicated in the processing of multiple protein precursors, including proteases, growth factors, and receptors at multibasic recognition sites exhibiting the general motif (K/R)-(X)$_n$-(K/R)$\downarrow$ (n = 0, 2, 4, or 6; Refs. 16, 17). The enzyme SKI-1 recognizes the motif (R/K)-X-(L,V)-Z$\downarrow$, where Z is any aa except Pro, Cys, Glu, and Val (17) and NARC-1 prefers the motif Y-X-(V/I)-X-(L/M)$\downarrow$ (18). The purpose of this study was to identify the protease(s) involved in the processing of PDGF-A, and to evaluate the importance of this cleavage in PDGF-A-mediated *ex vivo* functions and *in vivo* tumor growth.

MATERIALS AND METHODS

Pro-PDGF-A Constructs. The human pro-PDGF-A cDNA was kindly provided by Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Pro-PDGF-A-V5 (with a COOH-terminal V5-tag) was cloned into *Xho*I/*Bam*HI-digested pIRES2-EGFP vector (Clontech, Palo Alto, CA) to generate pIRES2-EGFP-PDGF-A-V5. Mutagenesis was done by PCR using the primers: 5′CCCATTGCGAGGAAGGCAAGCATC3′ and 5′ GATGCTTGCCTTCCTCGCAATGGG3′ for the mutant RRKR[86] into ARKA[86], 5′CCCATTCGGAGGCTGAGAAGCATC3′ and 5′GATGCTTCTCAGCCTCCGAATGGG for the mutant RRKR[86] into RRKL[86], and 5′CCCATTCGGAGGCTGCTAAGCATC3′ and 5′GATGCTTAGCAGCCTCCGAATGGG3′ for the mutant RRKR[86] into RRLL[86]. The PDGF-A cDNA mutants were transferred into the pIRES2-EGFP-V5 vector and their integrity confirmed by DNA sequencing.

Transfections and Cell Culture. The Furin-deficient LoVo-C5 human colon adenocarcinoma cells were transiently cotransfected with the empty vector pIRES2-EGFP-V5, pIRES2-EGFP-PDGF-A-V5 construct or with the pIRES2-EGFP-PDGF-A-V5 and pIRES2-EGFP vector that expresses either full-length Furin, PACE4, PC5A, PC5B, PC7, or SKI-1 cDNAs (19). The human embryonic kidney (HK293) cells were transiently cotransfected with the pIRES2-EGFP-V5 empty vector, pIRES2-EGFP-PDGF-A-V5 construct, or with the pIRES2-EGFP-PDGF-A-V5 and pIRES2-EGFP that express PCs inhibitors including ppFurin, ppPACE4, ppPC5, ppPC7, ppSKI-1, (wild-type) or mutated α2-MG-F, and α1-PDX (19). In several experiments HK293 cells or CHO cells lacking SKI-1 (20) or the same cells stably expressing SKI-1 [SKI-1 (+) cells] were transiently transfected with pIRES2-EGFP-V5 vector expressing either wild-type or mutated PDGF-A cDNAs. Wild-type CHO-K1 cells were stably transfected with pIRES2-EGFP-V5 empty vectors, or pIRES2-EGFP-V5 vector containing wild PDGF-A or mutated PDGF-A (ARKA[86]) cDNAs. Pools of stably transfected cells were selected using G418 resistance, and controlled by Western blotting for wild-type and mutant PDGF-A expression. All of the transfections were carried out using the Effectene transfection reagent (Qiagen Inc., Mississauga, Ontario, Canada) as recommended by the manufacturer. Cells were grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Burlington, Ontario, Canada). For the stably transfected CHO cells 400 μg/ml G418 were added.

Biosynthetic Labeling and Immunoprecipitation. Two days after transfection, the cells were washed and then pulse-labeled for 2–3 h with 200 μCi/ml [^{35}S]Cys. After the pulse period, cells were lysed in buffer containing

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[3] The abbreviations used are: PDGF, platelet-derived growth factor; CHO, Chinese hamster ovary; aa, amino acid; α2-MG-F, α2-macroglobulin; α1-PDX, α1-antitrypsin; pp, preprosegment; PC, proprotein convertase; ER, endoplasmic reticulum; EGFP, enhanced green fluorescent protein.

150 mM NaCl, 50 mM Tris-HCl (pH 6.8), 0.5% NP40, and 0.5% sodium deoxycholate (Roche Molecular Biochemicals), and prepared for immunoprecipitations as described previously (19). Anti-V5 (1:1000 dilution; Invitrogen) was used as the primary antibody.

Western Blotting. Twenty-four h after transfection, the cells were lysed in PBS containing 2% NP40, Lysates were subjected to SDS-PAGE on 8% gels, and proteins were blotted onto nitrocellulose membranes. The primary antibodies used were: monoclonal antibodies directed against either the V5 epitope (1:1000 dilution; Invitrogen) or antiphosphotyrosine (2 μg/ml; Sigma-Aldrich Ltd., Oakville, Ontario, Canada).

Tyrosine Phosphorylation Assay. Confluent fibroblast NIH BALB/c-3T3 cells grown in 75-cm^2 flask dishes were maintained in serum-free DMEM for 24 h and incubated with or without medium derived from the indicated cells. Cells were washed twice in ice-cold PBS and lysed with lysis buffer [50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100, 2 mM vanadate, 100 mM NaF, and 0.40 mg/ml phenylmethylsulfonyl fluoride], and proteins were analyzed by Western blotting.

Cell Growth Assay. This assay was monitored as described previously elsewhere (21). Briefly, serum-starved BALB/c-3T3 cells were incubated for 24 h in medium derived from transfected HK293 cells. For the last 6 h of incubation, 0.5 μCi/well of [^{3}H]methyl-thymidine (Amersham) was added, and cells were harvested onto glass-fiber filters using a cell harvester (Pharmacia, Wallac Oy, Turku, Finland), and radioactivity was counted. Results were expressed as percentages of the values obtained for cells incubated with medium derived from HK293 cells transfected with empty vectors and medium derived from HK293 cells transfected with wild or mutated PDGF-A cDNAs.

***In Vivo* Tumorigenecity Assays.** For tumor growth measurement, pools of control CHO-K1 cells or CHO-K1 cells expressing wild-type or mutant PDGF-A cDNA (RRKR^{86} to ARKA^{86}) were assessed for their ability to proliferate as indicated above and injected s.c. into 4–6-week-old male athymic mice. Animals were monitored for tumor formation every 7 days as described previously (21), and tumors were cryosectioned and stained with H&E.

RESULTS

Processing of Pro-PDGF-A by the PCs. To determine which PC cleaves pro-PDGF-A, we transiently coexpressed in LoVo cells, a Furin-deficient cell line, both pro-PDGF-A C-terminally tagged with a V5-epitope (Fig. 1*A*) and each of the PCs. After transfection the cells were pulse-labeled for 2 h with [^{35}S]Cys, and the medium was immunoprecipitated with anti-V5 mAb (Fig. 1*B*). In parallel, supernatants collected 24 h after transfection were also analyzed for pro-PDGF-A processing by immunoblotting (Fig. 1*C*). As illustrated in Fig. 1*B*, LoVo cells cotransfected with pro-PDGF-A recombinant and the empty vector (Control) exhibited only one band with an apparent

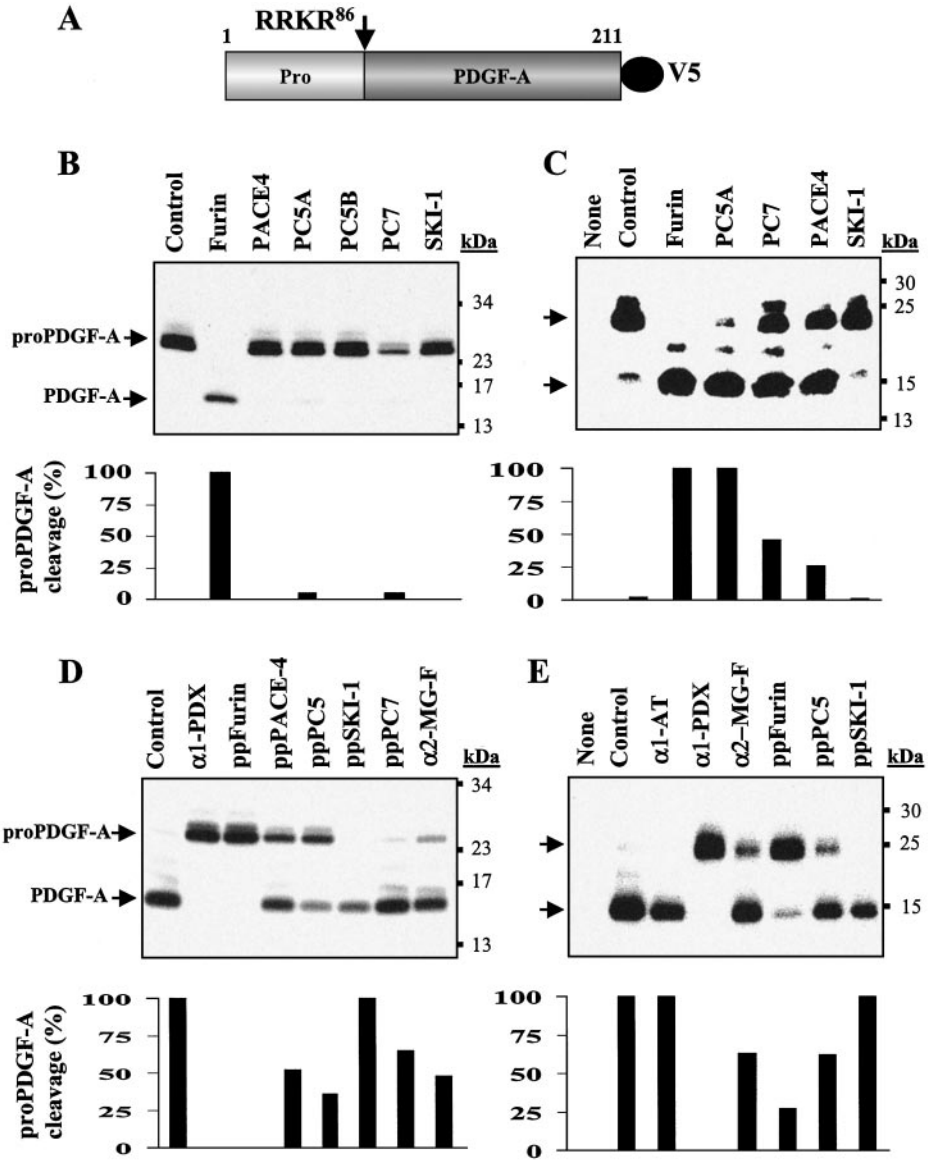

Fig. 1. Processing of pro-PDGF-A by the PCs family members. *A*, schematic representation of the primary structure of the 211-aa of human pro-PDGF-A, PC-processing site (RRKR^{86}), and the V5-tag attached to the COOH terminus. Pro-PDGF-A processing was analyzed by biosynthesis (*B*) and (*C*) Western blotting of LoVo-C5 conditioned medium obtained from transiently transfected cells with vector containing pro-PDGF-A cDNA (*Control*), or with vector containing pro-PDGF-A and vector that expresses full-length human Furin, PACE4, SKI-1, mouse PC5A, PC5B, or rat PC7. Inhibition of pro-PDGF-A processing by PC inhibitors was assessed by biosynthesis (*D*) and Western blotting (*E*) transiently transfected HK 293 cells with vector containing pro-PDGF-A cDNA alone (*Control*) or with vector that expresses ppFurin, ppPACE4, ppPC5, and ppPC7, wild-type or α2-MG-F, and α1-PDX. Band intensities of the autoluminographs were quantitated by densitometry, and the corresponding percentages of band intensities are deduced from the ratio of those of PDGF-A:(pro-PDGF-A+ PDGF-A). Shown are the results of a representative example of four independent experiments.

molecular weight of M_r ~25,000 corresponding to the intact monomeric PDGF-A precursor. Cotransfection of pro-PDGF-A with vectors encoding each of the PCs revealed that Furin, and to a much lesser extent PC5A and PC7, could process the M_r ~25,000 protein into a M_r ~15,000 product, corresponding to the mature form of monomeric PDGF-A. Western blot analysis of conditioned medium derived from LoVo cells cotransfected with pro-PDGF-A, and each of the PCs revealed that aside from Furin, PC5A > PACE4 and PC7 but not SKI-1 can process pro-PDGF-A under steady-state conditions (Fig. 1*C*).

Blockade of PDGF-A Processing by PC Inhibitors. To assess the possibility that PDGF-A is proteolytically activated by endogenous PC-like endoproteases, we cotransfected HK293 cells with vectors encoding pro-PDGF-A and each of the PC-inhibitors including the prosegments of PCs, namely, ppFurin, ppPACE4, ppPC5, ppPC7, and ppSKI-1, the Furin-motif variants of α2-MG-F, and of the serpin α1-PDX (19). Both biosynthesis (Fig. 1*D*) and Western blot (Fig. 1*F*) analyses concur that endogenous convertase(s) of HK293 cells are capable of complete processing of pro-PDGF-A into PDGF-A (Control). Cotransfection of cells with PC inhibitors revealed that processing of pro-PDGF-A is blocked by ppFurin (100%), ppPC5 (~60%), and ppPACE4 (~50%), as well as by α2-MG-F (~40%) and α1−PDX (100%). In contrast, ppSKI-1 and ppPC7 or wild-type α1-AT failed to inhibit processing. Because close to complete inhibition of pro-PDGF-A cleavage occurred only with ppFurin and α1-PDX, theses results suggest that most of the PDGF-A-converting activity found in HK293 cells is related to Furin, and that ppPC5 and ppPACE4 can partially inhibit Furin (19, 22, 23).

Cleavage Site Specificity of PDGF-A Processing in HK293 Cells. To assess the cleavage site specificity of PDGF-A processing we analyzed whether Arg for Ala or Lys substitutions at the P1 and/or the P4 positions at the PC-motif RRKR^{86} will affect processing of pro-PDGF-A by endogenous or exogenous Furin-like activity. The results of expression of these constructs in HK293 cells and their processing by endogenous enzymes or after overexpression of Furin are shown in Fig. 2. As described previously in Fig. 1, when wild-type pro-PDGF-A (RRKR^{86}) and EGFP vector are cotransfected in HK293 cells, one major band corresponding to mature PDGF-A is detected in the conditioned medium (Fig. 2*A*). When the PDGF-A mutant ARKA^{86} is transfected in these cells, the processing of pro-PDGF-A is completely blocked. These results highlight the importance of Arg at positions P1 and P4 for the processing of pro-PDGF-A. Transfection of HK293 cells with the pro-PDGF-A mutants RRKL^{86} or RRLL^{86} revealed that the processing of these mutants is not completely blocked (Fig. 2*A*). Overexpression of Furin in the presence of these pro-PDGF-A constructs revealed that only the processing of the mutant RRKL^{86} was increased from 25% to 100%. This suggested that Furin could process this mutant, possibly at the alternative dibasic RR^{84}↓KL site, containing a favorable Leu at P2′ (17). However, because Furin cannot process precursors with a P1′ Leu (17), it is not surprising that the RRLL^{86} mutant is not processed by Furin. However, expression of these PDGF-A cDNA constructs in SKI-1(+) cells mainly increased the processing of the pro-PDGF-A mutant RRLL^{86} and much less so of the RRKL^{86} one, without affecting that of the wild-type^{86} or ARKA^{86} mutant (data not shown).

Tyrosine Phosphorylation of the PDGF-A Receptor. To examine whether pro-PDGF-A processing by PCs is required for the mediation of its functions, conditioned media from transfected HK293 cells were tested for tyrosine phosphorylation of the PDGF-A receptor (PDGF-AR) and mitogenic activity. Fig. 3*A* shows that media derived from HK293 cells transfected with wild-type or mutants PDGF-A RRLL^{86} and RRKL^{86} enhanced the tyrosine phosphorylation of PDGF receptors and [^{3}H]methyl-thymidine incorporation (Fig. 3*B*) in NIH/BALB-c 3T3 cells. In contrast, media derived from cells transfected with the ARKA^{86} mutant were not effective.

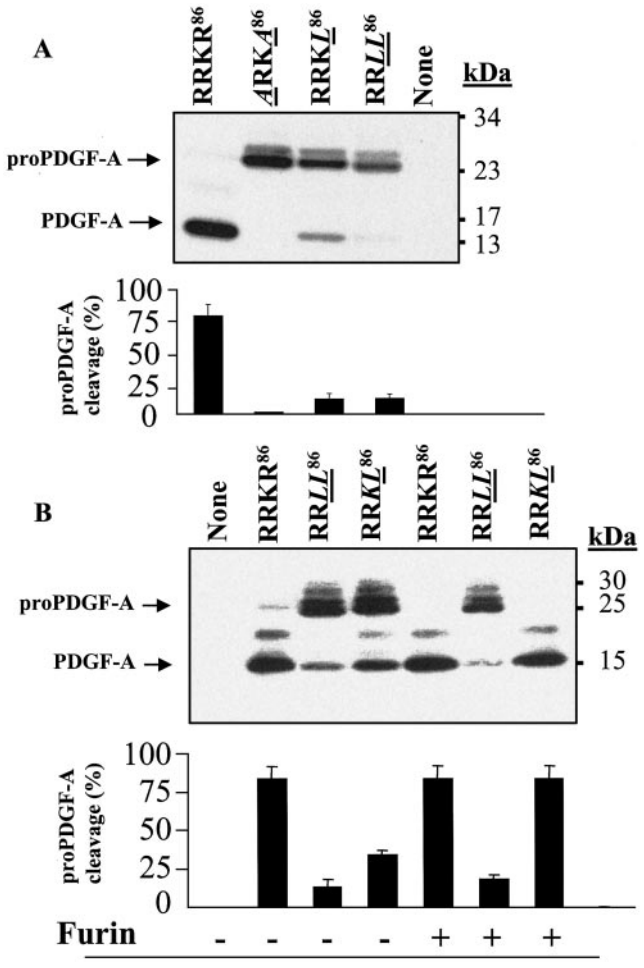

Fig. 2. Cleavage site specificity of pro-PDGF-A processing in HK293 cells. Conditioned medium obtained from transiently transfected HK293 cells with wild-type pro-PDGF-A (RRKR^{86}) or mutants pro-PDGF-A ARKA^{86}, RRKL^{86}, or RRLL^{86} and analyzed by biosynthesis (*B*) and Western blotting (*C*). In several experiments, HK293 cells were cotransfected with Furin and the pro-PDGF cDNA mutant constructs. Band intensities of the autoluminographs were quantitated by densitometry, and the corresponding percentages of band intensities are deduced from the ratio of those of PDGF-A:(pro-PDGF-A+PDGF-A). Shown are the results of one representative experiment of four performed; as means ± standard deviation (SD).

PDGF-A Processing and Tumorigenesis. Before any analysis, pools of CHO-K1 tumor cells stably expressing wild-type and mutant (ARKA^{86}) pro-PDGF-A were selected, and shown to efficiently produce and secrete the expected proteins, as verified by Western blotting of the media. Thus, whereas the mature PDGF-A is secreted from wild-type PDGF-A cell-pools, mostly unprocessed pro-PDGF-A is secreted from the ARKA^{86}-expressing cell pool (Fig. 3*C*). To assess the importance of pro-PDGF-A processing on the tumorigenicity of CHO cells, three groups of 6 male nude mice were s.c. inoculated with 4–5 × 10^6 cell pools of control CHO-K1 (empty vector), CHO-K1 cells expressing pro-PDGF-A, or the pro-PDGF-A mutant ARKA^{86}. As illustrated in Fig. 3*D*, expression of pro-PDGF-A in these cells increased tumor growth. Whereas tumor cells expressing mutant pro-PDGF-A developed tumors with reduced size as compared with CHO-K1 controls, analysis of the cell morphology using H&E staining revealed that tumors derived from either control or CHO-K1-PDGF-A (*ARKA*^{86}) cells showed increased apoptosis, and the tumor tissue exhibited necrotic areas (Fig. 3*E*).

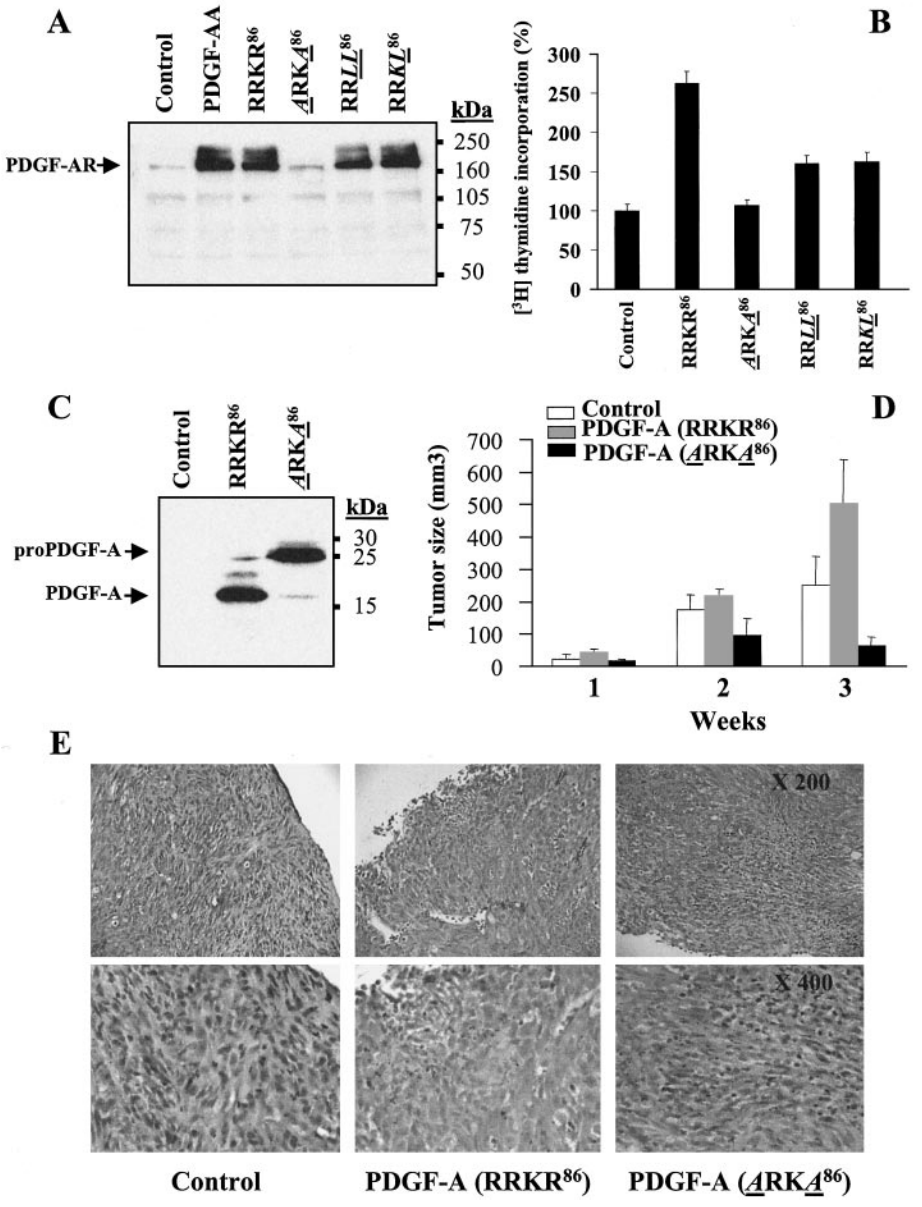

Fig. 3. Pro-PDGF-A processing blockade inhibit *in vitro* and *in vivo* cell growth. *A,* starved NIH 3T3/BALB-c cells were incubated for 10 min either with the PDGF-AA ligand (10 nM) used as a positive control for PDGF-A receptor (*PDGF-AR*) tyrosine phosphorylation, or with medium derived from HK293 cells transfected with empty vector, vector containing wild-type pro-PDGF-A (*RRKR*^{86}), or mutated pro-PDGF-A (*A*RK*A*^{86}; RR*LL*^{86}; and RRK*L*^{86}) cDNAs and cell lysates were analyzed by Western blotting for tyrosine phosphorylation. *B,* starved NIH 3T3/BALB-c cells were incubated for 24 h in medium derived from HK293 cells transfected with empty vector, or a cDNA vector containing wild-type or mutants of pro-PDGF-A in the presence of 2% FCS. [^{3}H]Thymidine was added for the final 6 h of incubation, and radioactivity was measured as described previously (21). *C,* CHO tumor cells were stably transfected with empty vector, or vectors containing either wild-type or mutant (*A*RK*A*^{86}) PDGF-A cDNA constructs. Pools of stably transfected cells were selected using G418 resistance and analyzed by Western blot using a V5 mAb. *D,* control CHO cells, expressing wild-type or mutant (*A*RK*A*^{86}) PDGF-A were injected s.c. into 4–6-week-old male nude mice (n = 6). The animals were monitored for tumor formation every 3 days as described previously (21). Results shown are representative of four experiments expressed as mean; *bars,*± SD. *E,* tumors developed after 17 days post-s.c. injection were cryosectioned and stained with H&E, and observed under ×200 and ×400 magnifications.

DISCUSSION

Many normal and tumor cells express PDGF-A that stimulates their own growth in an autocrine and/or paracrine manner (1–6). The importance of these PDGF-A functions is reinforced by the selective expression of PDGF-A and its α-receptor but not PDGF-B during the early stage of development (24, 25). Defects in PDGF-A interaction with its receptors during development results in anatomical defects leading to lethality (25). In addition, overexpression of PDGF-A mRNA in many cancers including brain and gastric carcinoma, and the lethal phenotype associated with cell hyperplasia in transgenic mice overexpressing PDGF-A (26) make this growth factor particularly interesting for proliferative disorder investigations. Upon synthesis of pre-pro-PDGF-A, the signal peptide is rapidly removed and pro-PDGF-A is then translocated to the Golgi network where other post-translational modifications occur. The newly synthesized PDGF-A chains are dimerized in the ER and thereafter transferred to the Golgi complex for proteolytic processing to produce a M_r~30,000 dimeric molecule that is carried in vesicles to the cell surface for release extracellularly by exocytosis (15). The presence of an optimum Furin consensus cleavage motif (RRKR ↓ SI) in PDGF-A (16, 17) and the ubiquitous expression of Furin suggested that this convertase is a good candidate for PDGF-A processing and activation. In pulse-chase experiments, using the Furin-deficient human colon carcinoma cell line LoVo (18), we found that Furin is the major candidate pro-PDGF-A convertase. However, Western blotting experiments revealed that under steady-state conditions other PCs such as PC5, PACE4, and PC7, were also able to processes PDGF-A. A similar conclusion was also reached with the transforming growth factor β (27). The endogenous processing of PDGF-A by PCs is confirmed by the inhibition of pro-PDGF-A processing in HK293 cells by the PC prosegments of Furin, PC5, PACE4, the Furin-motif variants of α2-MG-F, and serpin α1 pdx. However, the inhibitory prosegments of PC7, SKI-1, or wild-type α2-MG- and α1-antitrypsin did not significantly affect this cleavage. Expression experiments with pro-PDGF-A mutants containing substitutions at the potential cleavage site *RRKR ↓ SL* revealed that pro-PDGF-A is processed at Arg^{86} in HK293 cells. Experiments with additional mutants revealed that Arg^{83}, Lys^{85}, and Arg^{86} were also required for optimal endoproteoly-

sis, and underline the requirement for basic residues in the P1, P2, and P4 positions, which are characteristic of the substrate specificity of some members of the PC family including Furin (16, 17). The efficient secretion of the unprocessed pro-PDGF-A mutants indicates that the intracellular proteolytic cleavage is not a prerequisite for PDGF-A secretion. This was additionally confirmed by the accumulation of pro-PDGF-A in media derived from PDGF-A-transfected LoVo cells and HK293 cells transfected with PC inhibitors. The importance of the $RRKR^{86}$ sequence in pro-PDGF-A processing was reported previously by Mercola *et al.* (24). They demonstrated that alteration of the pro-PDGF-A cleavage site $RRKR^{86}$ to $RSNG^{86}$ resulted in the formation of a stable PDGF-A precursor. In agreement, our results demonstrated that the processing of pro-PDGF-A mutants $RRKR^{86}$ into $ARKA^{86}$ is completely blocked. However, transfections with the mutants $RRKR^{86}$ into $RRLL^{86}$ and $RRKR^{86}$ into $RRKL^{86}$ still produce the mature form of PDGF-A. The processing of the $RRLL^{86}$ mutant is not mediated by a PC-like activity, because overexpression of Furin did not significantly affect its processing (Fig. 2*B*), suggesting the involvement of another protease(s) in this process. Indeed, on cotransfection of these mutants with the novel convertase SKI-1/S1P in CHO cells deficient of this enzyme (20), only the mutants $RRLL^{86}$ and $RRKL^{86}$ were processed by SKI-1, suggesting that it is also the enzyme involved in the processing of the PDGF-A mutants $RRLL^{86}$ and to some extent $RRKL^{86}$ in HK293 cells. The convertase SKI-1 is a type I membrane-bound subtilisin-pyrolysin-like enzyme, identified to exhibit a specificity for precursor cleavage at the motif (R/K)-X-(hydrophobic)-(L,K,F,T) $\downarrow$, as deduced from its ability to process brain-derived neurotrophic factor, sterol regulatory element binding proteins, and recently the ER stress-induced transcription factor ATF6 (reviewed in Ref. 17). This conclusion cautions the indiscriminate mutation of processing sites in precursors, as this may result in a switching of the type of convertase involved that normally does not cleave at this site.

Like the other PDGFs, PDGF-A elicits its biological activity through interactions with transmembrane high-affinity receptors. Binding of PDGF-AA ligand to its receptor results in the autophosphorylation of the latter (9). In turn, the PDGF receptor activates an enzyme cascade that includes various phosphorylating enzymes, *e.g.*, protein kinase C, Ras, Raf, and mitogen-activated protein kinase, and ultimately triggers cell division (6–9). Similar to vascular endothelial growth factor and basic fibroblast growth factor, PDGF acts as a "competence" factor enabling cells to enter the G_1 phase, and participates with "progression" factors such as insulin-like growth factor I to move cells from the G_1 into S phase, ultimately resulting in cell division. Our studies demonstrate that complete inhibition of PDGF-A processing by mutagenesis (mutant $ARKA^{86}$) blocked the ability of PDGF-A to mediate PDGF-A receptor tyrosine phosphorylation and [^{3}H]thymidine incorporation in 3T3 cells. In contrast, although the processing the PDGF-A mutants $RRLL^{86}$ and $RRKL^{86}$ is dramatically reduced, the low level of the produced mature PDGF-A was enough to mediate PDGF-A receptor tyrosine phosphorylation and to stimulate [^{3}H]thymidine incorporation. To investigate the biological role of PDGF-A processing *in vivo* in tumor growth, we used CHO-K1 cells (that do not produce endogenous PDGF-A) to study the effects of wild and mutant PDGF-A ($ARKA^{86}$) on tumor growth in nude mice. Our results demonstrated that expression of PDGF-A in these cells increased the incidence and growth rate of the developed tumors. These results are in agreement with a previous report showing that PDGF-A overexpression in various tumor cells including mesothelioma increased tumor formation (28). In contrast, s.c. inoculation of tumor cells expressing the PDGF-A mutant $ARKA^{86}$ cDNA induced tumors with reduced size as compared with tumors obtained from control or wild-type PDGF-A transfected cells. This *in vivo* tumor growth inhibition by the PDGF-A mutant expressed in CHO-K1 cells could be explained by the action of pro-PDGF-A as a dominant negative (29). Like the other PDGF ligands, interaction of PDGF-A with their receptors induces the dimerization of the subunits and induces the formation of PDGFαr-PDGFαr homodimers. The absence of fully processed PDGF-A may affect the dimerization of the corresponding receptors leading to a loss of biological activity. In addition, the possible antagonist role of the PDGF-A mutant that may compete with the active PDGF-A for the PDGF receptors is not ruled out; however, additional studies are required to fully verify these hypotheses.

In conclusion, we have demonstrated that Furin, and to a lower extent PC5, PACE4, and PC7 are the cognate members of the PC family involved in the processing of pro-PDGF-A, and demonstrated that the biological functions of PDGF-A *ex vivo* and *in vivo* in tumors are critically dependent on the processing of pro-PDGF-A by the PCs. Our findings support the notion that targeting PDGF-A cleavage may provide a pharmacological complement that could be used for treatment of malignancies induced by this growth factor.

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