**Structural Requirements for Activation of Latent Platelet-derived Growth Factor CC by Tissue Plasminogen Activator**

Linda Fredriksson, Monika Ehnman, Christina Fieber, and Ulf Eriksson

From the Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S-171 77 Stockholm, Sweden

Platelet-derived growth factor C (PDGF-C) is one of four members in the PDGF family of growth factors, which are known mitogens and survival factors for cells of mesenchymal origin. PDGF-C has a unique two-domain structure consisting of an N-terminal CUB and a conserved C-terminal growth factor domain that are separated by a hinge region. PDGF-C is secreted as a latent dimeric factor (PDGF-CC), which undergoes extracellular removal of the CUB domains to become a PDGF receptor α agonist. Recently, the multidomain serine protease tissue plasminogen activator (tPA), a thrombolytic agent used for treatment of acute ischemic stroke, was shown to cleave and activate PDGF-CC. In this study we determine the molecular mechanism of tPA-mediated activation of PDGF-CC. Using various PDGF-CC and tPA mutants, we were able to demonstrate that both the CUB and the growth factor domains of PDGF-C, as well as the kringle-2 domain of tPA, are required for the interaction and cleavage to occur. We also show that Arg231 in PDGF-C is essential for tPA-mediated proteolysis and that the released “free” CUB domain of PDGF-C can act as a competitive inhibitor of the cleavage reaction. Furthermore, we studied how the PDGF-C/tPA axis is regulated in primary fibroblasts and found that PDGF-C expression is down-regulated by hypoxia but induced by transforming growth factor (TGF)-β treatment. Elucidating the regulation and the mechanism of tPA-mediated activation of PDGF-CC will advance our knowledge of the physiological function of PDGF-CC and tPA and may provide new therapeutic opportunities for thrombolytic and cardiovascular therapies.

Platelet-derived growth factor C (PDGF-C) was discovered a few years ago as the third member of the well characterized PDGF family of growth factors (1). The classical members of this family, PDGF-A and PDGF-B, have been intensively studied and are known to be important for connective tissue growth and maintenance, and overexpression has been observed in several pathological conditions, including malignancies and atherosclerosis (2). Since its discovery, PDGF-C has been shown to play a role in palate formation (3), fibrotic disease development (4, 5), and angiogenesis (6, 7). Recently a fourth member, PDGF-D, has been added to this family of growth factors (8, 9). The four PDGF chains assemble into five dimeric isofoms, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD, that exert their effects on cells through differential signaling via two known tyrosine kinase receptors, platelet-derived growth factor receptor (PDGFR)-α and PDGFR-β (10).

Unlike the classical members, PDGF-C and PDGF-D have a unique two-domain structure, with a so-called CUB domain N-terminal of the conserved growth factor domain (1, 8, 9). In order for the novel PDGFs to bind and activate the PDGFRs, the N-terminal CUB domains have to be removed through limited proteolysis by extracellular proteases. The origin of the protease involved in the activation of PDGF-DD still remains elusive, whereas the extracellular fibrinolytic protease tissue plasminogen activator (tPA) has been shown to be a potent activator of PDGF-CC (11).

tPA is a highly specific serine protease that consists of five structural domains, a finger domain, an epidermal growth factor-like domain, two kringle domains, and a trypsin-like protease domain (12). It is best known for its role in vascular fibrinolysis where it converts the zymogen plasminogen into plasmin, which in turn degrades the fibrin network in blood clots. The observation that tPA binds to fibrin via its finger and kringle-2 domains (13, 14), thus facilitating a localized generation of plasmin, has focused much attention on the use of tPA as a thrombolytic agent. In fact, tPA is currently used to treat acute myocardial infarction and is also approved for treatment of acute ischemic stroke (15). However, emerging evidence points at non-fibrinolytic functions of tPA, at least within the central nervous system, promoting events associated with synaptic plasticity and regulation of neurovascular permeability (16–18). Some of these studies claim the effect to be mediated by plasmin, whereas others show the effect to be independent of plasminogen activation (reviewed in Ref. 19). At present there are only two non-plasminogen substrates reported for tPA, namely PDGF-CC and the NR1 subunit of the N-methyl-D-aspartate receptor (11, 20).

A PDGF-CC/tPA stimulatory loop has recently been described to influence the growth of primary fibroblasts, which might have implications in the recruitment and growth of stromal fibroblasts into tumors and in wound-healing processes (11). It has also been shown that PDGF-CC can enhance delayed wound healing in diabetic mice (21) and revascularization of ischemic tissues (6), further emphasizing the therapeutic potentials of PDGF-CC. Clearly, it is of importance to determine the structural and regulatory requirements of PDGF-CC activation. Here, we describe the molecular mechanism of tPA-mediated cleavage of PDGF-CC. We demonstrate that both the CUB and the growth factor domains of PDGF-C...
and the kringle-2 domain of tPA are necessary for interaction of the two proteins and that Arg231 in the hinge region of PDGF-CC is needed for cleavage by tPA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—COS-1 cells and primary fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and porcine aortic endothelial (PAE) cells were kept in supplemented F12 medium. The cells were cultured at 37 °C in a humidified 5% CO2 atmosphere. Kidney primary fibroblast cultures were prepared as described previously, and experiments were performed on cells at passages 4–8 (11).

**Plasmid Construction**—The nucleotide sequences encoding the various PDGF-C and tPA truncation mutants, the CUB chimeric constructs (PDCUBPC and PCCUBPD), the CUB domain of PDGF-C (PCCUB), and the cleavage site mutants were amplified by PCR using gene-specific primers (see Table I) and Taq DNA polymerase (Invitrogen) if not stated otherwise. The PCR fragments of the PDGF-C and tPA truncation mutants, as well as the full-length tPA (tPAfl) lacking the signal sequence (used as control), were cloned in-frame with the signal sequence of the eukaryotic expression vector pSeqTag2B (Invitrogen). The tPA truncation mutants and tPAfl were then subcloned into the expression vector pcDNA3.1/Zeo(Invitrogen) accompanied by the Igγ/κ-chain leader sequence and c-myc epitope, but excluding the His6 tag, from pSeqTag2B. The amplified PDCUBPC and PCCUBPD fragments of the CUB regions (residues 1–172 of PDGF-D and 1–238 of PDGF-C, respectively) and the growth factor regions (residues 166–345 of PDGF-C and 261–370 of PDGF-D, respectively) were ligated and cloned into the eukaryotic expression vectors pSG5 (PDCUBPC) (22) or a modified pSeqTag2A (PCCUBPD; part of the multiple cloning site between SfiI and KpnI was removed by restriction; Invitrogen). The PCCUB PCR product (residues 1–165 of PDGF-C) was directionally cloned into pSG5.

To generate the PDGF-C cleavage site mutants, primers were designed to enable PCR amplification of the entire vector template, human PDGF-C in pSG5 (1), using Phusion DNA polymerase (Finnzymes). Point mutations and a HpaI site for in-frame cloning were included in the primer sequences. The 7.1-kb PCR products were cleaved and ligated. All primers used were purchased from Invitrogen, and all of the constructs were verified by nucleotide sequencing.

**Transfection, Immunoblotting, and Receptor Activation**—Subconfluent COS-1 cells were transfected with the various expression constructs using Lipofectamine Plus reagent in serum-free DMEM (Invitrogen). Transfection with empty vectors served as negative control (mock). After 4 h the transfection medium was replaced by supplemented DMEM overnight and thereafter by DMEM only. The conditioned serum-free medium was collected 48 h after transfection and used in receptor stimulation studies. Alternatively the proteins were precipitated using trichloroacetic acid as described previously (1). All precipitates were subjected to SDS-PAGE under reducing conditions, immunoblotted, and visualized by enhanced chemiluminescence plus reagent (ECL+, Amersham Biosciences). PDGF-C species and PCCUBPD were detected by immunoblotting using affinity-purified polyclonal rabbit antibodies against PDGF-C (1) and PDGF-D (8), respectively. tPA was detected using sheep polyclonal antibodies against human tPA (ab9030, Abcam) and tPA truncation mutants using rabbit polyclonal antibodies against human c-myc (sc-789, Santa Cruz Biotechnology).

To monitor growth factor-induced tyrosine phosphorylation of PDGFR-α and PDGFR-β, serum-starved PAE cells stably expressing the respective human PDGFRs, were incubated for 90 min on ice with conditioned medium from transfected COS-1 cells. PAE cells treated with either conditioned medium from COS-1 cells transfected with empty vector (mock) or with recombinant PDGF-BB (100 ng/ml), or alternatively the recombinant growth factor domain of PDGF-CC (100

### Table I

| Construct | Description | Oligonucleotides |
|-----------|-------------|------------------|
| PCCUB     | PDGF-C truncation mutants | Sense: 5'-CCCAAGGTGGTCTCTGCTGAACGGG-3' |
| PCCUBPD   | CUB region of PDGF-DD    | Sense: 5'-GGCGATTCGTTCTCTCCACAGGCTTC3' |
| PCUB      | CUB region of PDGF-DD    | Antisense: 5'-GGAATTCCTTCTGCTCCCTCTG-3' |

**REFERENCES**

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ng/ml) were used as controls. Following treatment the PAE cells were lysed in 20 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl, 5 mM EDTA, 200 μM orthovanadate, and complete protease and phosphatase inhibitor mixtures, and the PDGFRs were immunoprecipitated using specific antisera (23). Precipitated receptors were separated by SDS-PAGE under reducing conditions. Tyrosine-phosphorylated receptors were detected by immunoblotting using an anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology). Bound antibodies were visualized as described above.

**Protein-Protein Interaction Studies**—To determine which domain(s) of tPA and PDGF-CC are involved in the protein-protein interaction between the two proteins, His₆-tagged recombinant PDGF-CC protein species, expressed using the baculovirus expression system as described previously (1), were bound to nickel-nitrotriacetic acid (Ni-NTA)-agarose (Qiagen) and then incubated for 90 min at room temperature with conditioned serum-free media from COS-1 cells transfected with the tPA truncation mutants. Uncoated Ni-NTA beads were used as negative control. The beads were thoroughly washed, and the His₆-tagged PDGF-CC species were specifically eluted with 400 mM imidazole (Sigma). Eluted proteins were analyzed by SDS-PAGE under reducing conditions and immunoblotted with rabbit anti-PAI antibody (sc-8979, Santa Cruz Biotechnology). Using specific antibodies (see above), and PAI-1 was detected using a chromogenic assay. PDGF-C and tPA were detected as described above.

**Chromogenic Assay**—To confirm functional protease activity among the tPA truncation mutants, a chromogenic assay was developed. Conditioned serum-free media from COS-1 cells transfected with the tPA truncation constructs were subjected to size-exclusion chromatography using G-200 columns (Amersham Biosciences) to enable high-molecular-weight exchange to Tris-buffered saline. The protease activity analysis was performed in flat-bottomed microplates with 0.2 mM Spectrozyme tPA (American Diagnostica) as a chromogenic substrate for tPA. The formation of paranitroanilide (pNA), i.e. the amount of cleaved substrate, was measured photometrically at 405 nm. Comparable product amounts suggest functional protease activity. Buffer-exchanged conditioned media from mock-transfected cells were used as negative control. Purified human tPA was used to define maximal activity (T7776, Sigma).

**Regulation of PDGF-C, tPA, and Plasminogen Activator Inhibitor (PAI)-1 Expression**—To determine how PDGF-C, tPA, and its inhibitor PAI-1 are regulated by various growth factors and metabolic conditions, primary kidney fibroblasts were plated at subconfluence in 6-well plates. Following attachment, the medium was exchanged for serum-free DMEM in the absence or presence of tPA (5 ng/ml), fibroblast growth factor (FGF)-2 (5 ng/ml, R&D Systems), or high glucose (30 mM); alternatively the cells were placed in hypoxic conditions (1% oxygen). After 24 h the conditioned serum-free media were collected, and the proteins were trichloroacetic acid-precipitated and subjected to SDS-PAGE followed by immunoblotting. PDGF-C and tPA were detected using specific antibodies (see above), and PAI-1 was detected using a rabbit anti-PAI antibody (sc-8979, Santa Cruz Biotechnology).

**RESULTS**

**tPA-mediated Proteolysis Depends on Both Structural Domains of PDGF-CC**—We mapped the structural requirements for recognition of latent PDGF-CC as a substrate for tPA using mutated forms of PDGF-CC in a co-transfection assay. The mutants of PDGF-CC included chimeric forms of PDGF-C, one carrying the CUB domain of PDGF-D and the hinge region and growth factor domain of PDGF-C (PC_CUBPC) and the other one carrying the CUB domain and the hinge region of PDGF-C and the growth factor domain of PDGF-D (PC_CUBPD) (schematically illustrated in Fig. 1A). In addition, a truncation mutant lacking the CUB domain of PDGF-C was also employed (PC_D150). All mutants were properly expressed in transfected COS-1 cells, formed disulfide-linked dimers (data not shown), and were efficiently secreted in the conditioned medium (Fig. 1B). When co-transfected with tPA, the generation of a 22-kDa protected fragment from PC_CUBPC was significantly reduced as compared with wild-type PDGF-CC (PC_WT), whereas no cleavage product was detected in co-transfections with PC_CUBPD or with PC_D150 (Fig. 1B).

These results were verified in receptor stimulation experiments where conditioned media from transfected COS-1 cells were applied onto PAE cells with stable expression of PDGFR-α (upper panel) or PDGFR-β (lower panel), respectively (Fig. 1C). Following immunoprecipitation of the respective receptors, stimulation was measured as induction of receptor tyrosine phosphorylation. As shown previously, media from COS-1 cells co-expressing tPA and wild-type PDGF-CC induced strong PDGFR-α activation comparable with PDGF-BB-stimulated controls (11), whereas media from COS-1 cells co-expressing tPA with PD_CUBPC induced weaker PDGFR-α activation. Cells stimulated with conditioned media expressing the PDGF-CC mutants alone or co-expressing tPA with PC_CUBPD or the truncation mutant PC_D150, respectively, showed only background levels of PDGFR-α activation. As cleavage of PC_CUBPD would release the growth factor domain of PDGF-DD, a PDGFR-β agonist, the conditioned media were also applied to PDGFR-β expressing PAE cells, but none induced PDGFR-β stimulation. These findings indicate that both the CUB and the growth factor domains are necessary for efficient proteolytic cleavage of latent PDGF-CC by tPA.

**The Majority of the Hinge Region Is Removed in Active PDGF-CC**—The finding that the truncation mutant PC_D150...
fig. 2. The majority of the hinge region has to be removed for PDGF-CC to be a PDGFR-α agonist. A, illustration of the N-terminally truncated variants of PDGF-CC used to determine the structural requirements for PDGFR-α stimulation. B, immunoblot analysis of the truncation mutants in conditioned serum-free medium collected from transfected COS-1 cells. Equivalent expression of the PDGF-C species was detected using specific polyclonal antibodies. C, induction of tyrosine phosphorylation of PDGFR-α expressed in PAE cells. Conditioned serum-free media from transfected COS-1 cells were applied to the PAE cells, and the PDGFR-α (R-α) was immunoprecipitated (IP). Only the two shortest mutants induced efficient phosphorylation comparable with the positive control recombinant PDGF-CC (100 ng/ml, growth factor domain of PDGF-CC). failed to induce PDGFR-α activation indicates that not only the CUB domain but also parts of the hinge region have to be removed for receptor activation. To understand the structural requirements of PDGF-CC for receptor binding and activation, a series of truncated mutants of PDGF-C, lacking the CUB domain and increasing portions of the hinge region, was developed (schematically illustrated in Fig. 2A). All mutants formed disulfide-linked dimers (data not shown) and were efficiently secreted in the conditioned medium by transfected COS-1 cells (Fig. 2B). The multiple species of the truncated mutants seen in the immunoblots are possibly due to exposure and subsequent glycosylation of the putative N-glycosylation site present in the growth factor domain of PDGF-C (1). However, it cannot be ruled out that removal of the CUB domain leaves the otherwise protected hinge region vulnerable for degradation.

The truncation mutants were analyzed for their ability to activate PDGFR-α in PAE cells. Conditioned media containing equal amounts of the truncated mutant proteins of PDGF-CC (determined by enzyme-linked immunosorbent assay) were applied onto PAE cells, and the activation of PDGFR-α was monitored by induction of receptor tyrosine phosphorylation (Fig. 2C). The results showed that the two shortest mutants, PC320 and PC330, efficiently activated PDGFR-α, whereas mutants with additional parts of the hinge region, separating the CUB and the growth factor domains in PDGF-C, failed to do so. Thus, in order for PDGF-CC to be a receptor agonist, both the CUB domain and the majority of the hinge region have to be removed from the growth factor, allowing at most the last 40 amino acids of the hinge region to remain, indicating that the cleavage site resides in this region.

Arg231 in the Hinge Region of PDGF-CC Is Essential for tPA-mediated Cleavage—Within this stretch of amino acids we have previously identified a putative trypsin processing site (amino acid residues -Arg231-Lys232-Ser233-Arg234- in human PDGF-C) based on comparison with the well known processing sites in PDGF-A and PDGF-B (1). Recently we reported tPA to cleave mouse PDGF-CC in, or at least around, this conserved site (11). To better characterize which of the three basic amino acids, Arg231, Lys232, and Arg234, is important for cleavage to occur, the amino acids were individually mutated to alanine residues. The expression constructs encoding these PDGF-C mutants were separately co-transfected with tPA. The extent of PDGF-CC cleavage was monitored by immunoblotting as the presence of the 22-kDa band (Fig. 3A) and induction of PDGFR-α phosphorylation (Fig. 3B). Wild-type PDGF-C and a mutant resistant to tPA-mediated cleavage with the entire region Arg231–Arg234 replaced by alanine residues were used as controls. These experiments demonstrate that the cleavage site for tPA is confined to the Arg231–Arg234 segment in human PDGF-CC and that Arg231 is essential for cleavage to occur, whereas the other basic amino acid residues in the site are less important.

The CUB Domain of PDGF-C Acts as a Specific Inhibitor of tPA-mediated Cleavage—Based on our previous findings that the CUB domain of PDGF-C, but not that of PDGF-D, specifically interacts with tPA (11) and the above results showing that the CUB domain is necessary for specific cleavage, we hypothesized that the released free CUB domain of PDGF-C might act as a competitive inhibitor of tPA-mediated activation of PDGF-CC. To test this hypothesis we co-transfected COS-1 cells with wild-type PDGF-CC and tPA in the absence or presence of an expression construct expressing the free CUB domain of PDGF-C (PC320). We were able to show that the CUB domain of PDGF-C efficiently competed for the processing of latent PDGF-CC by tPA, as determined by immunoblot experiments (Fig. 4A), and activation, as determined by induction of PDGFR-α phosphorylation (Fig. 4B), thus suggesting that the CUB domain may indeed act as a competitive inhibitor of tPA-mediated proteolysis.

Krinlge-2 of tPA Is Necessary for Cleavage of PDGF-CC—To determine which of the structural domains of tPA is necessary for efficient cleavage of latent PDGF-CC we created truncated forms of tPA (schematically illustrated in Fig. 5A) and expressed them in transfected COS-1 cells (Fig. 5B). To ensure that the mutated tPA proteins were functionally active, a tPA Spectrozyme substrate was added to buffer-exchanged conditioned serum-free media from transfected COS-1 cells, and after 3 h the formation of pNA, indicative of the amount of cleaved tPA Spectrozyme substrate, was measured photometrically (Fig. 5C). All tPA mutants induced pNA formation in a similar fashion suggesting correct protein folding and intact protease activity. Purified tPA was used to define maximal structural requirements for PDGF-CC activation, 26859

Fig. 3. tPA-mediated cleavage of PDGF-CC is dependent on Arg231. A, immunoblot analysis of tPA-mediated proteolysis of the PDGF-CC cleavage site mutants. Mutant PDGF-C species, with any of three basic amino acid residues (Arg231, Lys232, and Arg234) in a conserved trypsin site, were co-expressed in COS-1 cells with tPA. The extent of PDGF-C cleavage was monitored by the presence of the 22-kDa band in immunoblots using PDGF-C-specific antibodies (arrow, upper panel). Cleavage was completely abolished in the PC320 mutant suggesting that Arg231 is essential for cleavage to occur. A mutant with the entire region Arg231–Arg234 replaced by alanine residues, PC333, was used as a cleavage-resistant control, whereas wild-type PDGF-CC (PCw) was used as a positive control. tPA expression was monitored using specific polyclonal antibodies (lower panel). B, receptor stimulation studies, measured as induction of tyrosine phosphorylation of PDGFR-α. Following stimulation, PDGFR-α (R-α) was immunoprecipitated (IP) using specific receptor antibodies, and phosphorylated receptors were detected by immunoblot analysis using an anti-phosphotyrosine antibody. Conditioned serum-free media from COS-1 cells co-transfected with tPA and either PC320 or PC333 failed to induce efficient phosphorylation of the receptor, thus confirming the results seen in A.
activity, and within 24 h all mutants had induced maximal formation of pRNA. Buffer-exchanged conditioned medium from cells transfected with empty vector (mock) was used as negative control.

To assess the structural requirements of tPA for cleavage of PDGF-CC, the tPA truncation mutants were co-expressed in COS-1 cells together with wild-type PDGF-CC, and cleavage was determined by the formation of the 22-kDa protected fragment of PDGF-C. Our results show that co-transfection of the shortest tPA mutant, tPA<sub>3300</sub>, containing only the trypsin-like protease domain, with PDGF-CC significantly reduced the generation of the 22-kDa band as compared with any of the other tPA mutants (Fig. 5D). These data suggest that, although tPA<sub>3300</sub> is expressed and functional, the kringle-2 domain is required for tPA to efficiently cleave PDGF-CC.

Interaction between tPA and PDGF-CC Is Mediated by kringle-2 in iPA—We explored the possibility that the kringle-2 domain of tPA mediates the reported protein-protein interaction between tPA and PDGF-CC (11). Ni-NTA beads were therefore coated with recombinant His<sub>6</sub>-tagged full-length PDGF-CC, and serum-free conditioned medium from COS-1 cells transfected with the different tPA truncation mutants was added. Following extensive washing, bound His<sub>6</sub>-tagged PDGF-CC protein was specifically eluted with an imidazole-containing buffer, and the eluates were analyzed by immunoblotting using specific antibodies. The results showed that full-length PDGF-CC-coated beads specifically bound all tPA mutants except the shortest tPA<sub>3300</sub> mutant lacking the kringle-2 domain (Fig. 6, two upper panels, co-eluted tPA above and eluted full-length PDGF-C below). Similarly, experiments using Ni-NTA beads separately coated with the recombinant His<sub>6</sub>-tagged CUB domain (Fig. 6, two middle panels, co-eluted tPA above and CUB below) or the recombinant growth factor domain of PDGF-C (Fig. 6, two lower panels, co-eluted tPA above and core PDGF-C below), showed that both domains failed to interact with tPA when kringle-2 had been removed. Uncoated beads were used to ensure specific interaction of the tPA mutants with PDGF-CC, illustrated here by incubation of uncoated Ni-NTA beads with tPA<sub>60</sub>. These data imply that the kringle-2 domain of tPA interacts with both the CUB and the growth factor domains of PDGF-C, thus properly positioning the trishastic -KRKR- cleavage site in the hinge region of PDGF-C and the protease domain of tPA closely together.

Regulation of PDGF-C, tPA, and PAI-1 Expression in Mouse Primary Fibroblasts—Our previous findings that PDGF-CC and tPA create a growth-stimulatory loop important for the establishment of primary fibroblast cultures might have implications in wound-healing processes, especially in the healing of chronic diabetic wounds known to have impaired granulation tissue formation probably because of reduced fibroblast activity (24). Interestingly, the expression of tPA and its inhibitor PAI-1 has been found to be altered in diabetic patients (25), but thus far, there are no reports on altered expression and activation of PDGF-CC in diabetes. To determine whether the expression of PDGF-C, and also tPA and PAI-1, is regulated in normal primary fibroblasts by metabolic conditions involved in the pathogenesis of diabetes, such as high glucose and hypoxia, we isolated primary murine fibroblasts, plated them at subconfluence, and treated them for 24 h in serum-free media. As fibroblast function is controlled by the intricate interaction of a number of growth factors, we also investigated whether the expression of PDGF-C, tPA, and PAI-1 in primary fibroblasts were regulated by such growth factors, namely TGF-β<sub>1</sub> and FGF-2. Following treatment the conditioned serum-free media were collected, proteins were precipitated, and the expression levels were analyzed by immunoblotting using specific antibodies and compared with a non-treated control (Fig. 7, representative blot of 5–7 individual experiments). The results showed that glucose did not affect the expression levels of PDGF-C, tPA, and PAI-1, whereas hypoxia decreased the expression of PDGF-C and tPA. On the other hand, stimulation of the primary fibroblasts with TGF-β<sub>1</sub> drastically enhanced the secretion of PDGF-C as well as PAI-1, whereas the expression of tPA was only modestly increased. In support of our findings it has previously been shown that PAI-1 transcripts are often up-regulated in hypoxic conditions and by TGF-β<sub>1</sub> treatment, whereas tPA transcripts are down-regulated by hypoxia and differentially regulated by TGF-β<sub>1</sub> in a cell-specific context (26–29). Treatment of the primary fibroblasts with FGF-2, which is known to induce expression of PDGF-C transcripts from vascular smooth muscle cells (30), did not alter the expression of PDGF-C but rather altered the expression of tPA. Taken together these results indicate that the fibroblastic PDGF-CC/tPA growth-stimulatory loop can be regulated by metabolic conditions and other growth factors, which are of importance in the pathophysiology of diabetes.

DISCUSSION

The discovery of the novel PDGFR-α ligand PDGF-CC (1) was not completely unexpected, as gene deletion studies of the classical PDGFs and the PDGFR-α had raised the possibility of an undiscovered ligand (reviewed in Ref. 31). However, the finding that PDGF-C had a unique two-domain structure and that the activity was regulated by extracellular cleavage was unpredicted (1). Until recently little was known about the protease responsible for the activation of PDGF-CC, but we have shown that the fibrinolytic serine protease tPA specifically cleaves and activates PDGF-CC (11). In this study we further investigated the molecular mechanism behind the tPA-mediated activation of PDGF-CC.

Gene deletion studies of PDGF-C have shown that PDGF-CC plays a specific role in PDGFR-α signaling and that PDGF-CC and PDGF-AA are the major PDGFR-α ligands in vivo (3). In our attempt to determine what is required for PDGF-CC to be a PDGFR-α agonist, we found that PDGF-CC truncation mutants with N-terminal extensions exceeding 40 amino acid residues from the growth factor domain could not activate PDGFR-α. This is consistent with the observation that mutant PDGF-AA, in which the propeptide could not be removed because of a mutation in the processing site, does not bind or activate the PDGFR-α receptor (32). Within the last 40 amino acids of the hinge region in PDGF-C we have previously shown
Fig. 5. The kringle-2 domain of tPA is necessary for the cleavage of PDGF-CC. A, illustration of the tPA truncation mutants used to determine the structural requirements of tPA for proteolysis of PDGF-CC. B, all mutants were efficiently expressed in the conditioned serum-free medium from transfected COS-1 cells as assessed by immunoblotting analysis using polyclonal anti-c-myc antibodies. C, functional analysis of the tPA truncation mutants. A tPA Spectrozyme substrate was added to buffer-exchanged conditioned media from COS-1 cells transfected with the tPA mutants, and after 3 h the formation of pNA was measured photometrically at 405 nm. All mutants induced the formation of pNA confirming preserved enzymatic activity of the truncated proteases. Purified tPA was used to define maximal pNA formation. D, COS-1 cells were co-transfected with the corresponding tPA truncation mutant in the presence of PCWT. Serum-free conditioned media were collected 48 h after transfection, proteins were trichloroacetic acid-precipitated, and PDGF-C was detected by immunoblotting using specific polyclonal antibodies. Co-expression of PCWT with all tPA truncation mutants, except the shortest, generated a 22-kDa protected PDGF-C fragment.

Fig. 6. Direct interaction of PDGF-CC with tPA is dependent on the kringle-2 domain of tPA. Ni-NTA beads were coated with recombinant His6-tagged full-length PDGF-CC (PCWT), CUB domain (CUBant/ant), and growth factor domain (Coreant/ant) of PDGF-C expressed using the baculovirus expression system. The coated beads were incubated with conditioned serum-free medium from COS-1 cells transfected with the tPA truncation mutants illustrated in Fig. 5A. Part of the transfected media was analyzed by immunoblotting before its addition to the coated beads to ensure that equal relative amounts of the tPA mutant proteins were being added (10% tPA input). Following incubation with tPA the beads were thoroughly washed and the His6-tagged proteins specifically eluted from the beads using a buffer containing 400 mM imidazole. Co-elution of interacting tPA species was analyzed by immunoblotting using polyclonal anti-c-myc antibodies. To detect elution of the PDGF-C species the blots were subsequently stripped and reblotted with PDGF-C-specific antibodies (for detection of PCant/ant and Coreant/ant) or alternatively anti-His antibodies (detection of CUBant/ant).

Fig. 7. The expression of PDGF-C in primary fibroblasts is modulated by hypoxia and TGF-β1. Primary murine fibroblasts were seeded at subconfluence, and after attachment the medium was changed to serum-free medium in the absence (Control) or presence of various growth factors/metabolic stimuli for 24 h. The serum-free medium was then collected, and the proteins were trichloroacetic acid-precipitated and subjected to SDS-PAGE under reducing conditions. Immunoblot analysis using specific antibodies against PDGF-C, tPA, or PAI-1 revealed that hypoxia and TGF-β1 modulate the PDGF-C/tPA axis.

That tPA-mediated cleavage of murine PDGF-CC occurs at, or at least around, a conserved trispecific region (amino acid residues Arg231-Lys232-Ser233-Lys234) (11). Here we confirm that this conserved trispecific region is also the site of cleavage in human PDGF-CC (amino acid residues Arg231-Lys232-Ser233-Lys234) and more specifically that the cleavage depends on Arg231. It is well known that tPA cleaves plasminogen at the Arg260-Val562 bond to produce plasmin (33), but previous findings have reported a lack of absolute specificity of tPA for an Arg-Val bond (34), and also the other non-plasminogen substrate for tPA, the NR1 subunit of the N-methyl-D-aspartate receptor, was recently shown to be cleaved at the Arg2560-Tyr2561 bond (35). Whether tPA specifically cleaves human PDGF-CC at the Arg231-Lys232 bond remains to be established.

A CUB domain is a common structural module found in many different kinds of proteins and is believed to participate in protein-protein or protein-carbohydrate interactions (36). The ability of the CUB domain of PDGF-C to interact with tPA and act as a competitive inhibitor of tPA-mediated proteolysis may explain the relatively low efficiency of the activation by tPA in the co-transfection assays. The stoichiometry of the activation reaction is such that generation of each molecule of receptor-active PDGF-C dimer will generate two molecules of the inhibitory free CUB domain. Whether this autoregulatory mechanism is used in vitro is unknown at present, but it may provide a potent regulatory mechanism controlling the activation of PDGF-CC. Apart from limited proteolysis, alternative splicing of the gene encoding PDGF-C can potentially also generate the free CUB domain of PDGF-C. However, bioinformatic efforts using the expressed sequence tag data base at NCBI have so far failed to provide any evidence of alternatively spliced PDGF-C transcripts encoding free CUB domains only. The inhibitory effect of CUB on tPA activity may have clinical implications, e.g. in management of the bleeding side-effects often seen when using tPA in thrombolytic treatment.

The different domains of tPA have been reported to mediate interaction between tPA and various proteins, e.g. the finger domain binds fibrin (14) and annexin II (37, 38), and the kringle domains, in particular the second kringle domain, also bind fibrin (13, 14). Our results show that the interaction of tPA with PDGF-CC is mediated through specific interaction of the kringle-2 domain. The kringle-2 interacts with both the CUB and the growth factor domains of PDGF-CC, possibly allowing the hinge region to loop out and thus positioning the cleavage site such that the protease domain in tPA can cleave. The kringle-2 domain has been demonstrated to inhibit FGF-2-induced endothelial cell proliferation and migration (39, 40), and recently PDGF-CC has been shown to have a direct stimulatory effect on endothelial cell migration (6). As FGF-2 up-

2 U. Erikkson, unpublished observation.
regulates PDGF-C transcription in vascular smooth muscle cells (30) it is possible that part of the inhibitory effect of the kringle-2 domain is through binding and subsequent blockade of PDGF-C activation. Furthermore, we could speculate that upon interaction of PDGF-C with kringle-2, the other domains of tPA could interact with molecules such as the low density lipoprotein receptor-related protein, known to both interact with tPA (41) and control PDGF signaling (42) and thereby facilitating a localized generation of active PDGF-C. Interestingly, tPA induces blood-brain barrier opening via interaction with the low density lipoprotein receptor-related protein and proteolysis of an as yet unidentified substrate (43).

Despite the similarities between PDGF-C and the novel PDGFR-β ligand, PDGF-DD, tPA fails to interact and induce cleavage of this latter factor (11). Thus far, less is known about the activation of PDGF-DD. We have suggested previously that the genes for the classical and novel PDGFs separated early during evolution and that the novel PDGFs then arose from a common ancestor (44). It is therefore not unlikely that the protease involved in PDGF-DD activation has a similar structural organization as tPA. Using a computer-based strategy we could identify several serine proteases with similar domain organization as tPA, including the other plasminogen activator kringle-2 domain is through binding and subsequent blockage of an as yet unidentified substrate (43).

Non-healing foot ulcers in diabetic patients are a common and expensive complication partially caused by reduced fibroblast activity (24). To develop rational therapeutic strategies it has become a major priority to characterize the pathophysiology of the delayed wound healing and the impaired fibroblast activity in diabetic patients. Considering that hyperglycemia regulates PDGF-C transcription in vascular smooth muscle cells (30) it is possible that part of the inhibitory effect of the kringle-2 domain is through binding and subsequent blockade of PDGF-C activation. Furthermore, we could speculate that upon interaction of PDGF-C with kringle-2, the other domains of tPA could interact with molecules such as the low density lipoprotein receptor-related protein, known to both interact with tPA (41) and control PDGF signaling (42) and thereby facilitating a localized generation of active PDGF-C. Interestingly, tPA induces blood-brain barrier opening via interaction with the low density lipoprotein receptor-related protein and proteolysis of an as yet unidentified substrate (43).

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