STRUCTURAL REQUIREMENTS FOR ACTIVATION OF LATENT PDGF-CC BY TISSUE PLASMINOGEN ACTIVATOR

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Running title: Structural requirements for PDGF-CC activation

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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 \( \alpha \) agonist. Recently, the multi-domain 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 domain 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 arginine 231 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. Further, 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 TGF-\( \beta \) 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 forth member, PDGF-D, has been added to this family of growth factors (8,9). The four PDGF chains assemble into five dimeric isoforms; 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, PDGFR-\( \alpha \) and PDGFR-\( \beta \) (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 domain (13,14), thus facilitating a localized generation of plasmin, has focused much attention into 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 CNS, 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 while others show the effect to be independent of plasminogen activation (reviewed in ref. 19). Although, at present there are only two non-plasminogen substrates reported for tPA, namely PDGF-CC and the NR1 subunit of the NMDA receptor (11,20).

A PDGF-CC/tPA stimulatory loop has recently been described to influence the growth of primary fibroblasts, which might have implication in 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 domain of PDGF-C, and the kringle-2 domain of tPA are necessary for interaction of the two proteins, and that arginine 231 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 Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and porcine aortic endothelial (PAE) cells were kept in supplemented F12 medium. Kidney primary fibroblast cultures were prepared as described previously and experiments were performed on cells at passages 4 to 8 (11).

Plasmid construction—The nucleotide sequences encoding the various PDGF-C and tPA truncation mutants, the CUB chimeric constructs (PDCUBPC and PC CUBPD), the CUB domain of PDGF-C (PC CUB) and the cleavage site mutants were amplified by PCR using gene specific primers (see Table 1) 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 pSecTag2B (Invitrogen). The tPA truncation mutants and tPAfl were then cloned into the expression vector pcDNA3.1/Zeo(+) (Invitrogen) accompanied by the Igk-chain leader sequence and c-myc epitope, but excluding the His6-tag, from pSecTag2B. The amplified PD_CUBPC and PC CUBPD fragments of the CUB regions (residues 1 to 172 of PDGF-D and 1 to 238 of PDGF-C, respectively) and the growth factor regions (residues 166 to 345 of PDGF-C and 265 to 370 of PDGF-D, respectively) were ligated and cloned into the eukaryotic expression vectors pSG5 (PD CUBPC) (22) or a modified pSeqTag2A (PC CUBPD; part of the multiple cloning site between SfiI and KpnI was removed by restriction; Invitrogen). The PC CUB PCR product (residues 1 to 165 of PDGF-C) was directionally cloned into pSG5. To generate the PDGF-C cleavage site mutants, primers were designed in order to enable PCR amplification of the entire vector template, human PDGF-C in pSG5 (1), using Phusion DNA Polymerase (Finnzymes). Point mutations and a Hpal 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 the constructs were verified by nucleotide sequencing.

Transfection, immunoblotting and receptor activation—Sub-confluent COS-1 cells were transfected with the various expression constructs using Lipofectamine plus reagent in serum-free DMEM (LifeTechnology). 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 (TCA) as previously described (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 PCUBPD 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 porcine aortic endothelial (PAE) cells stably expressing the human PDGFRs respectively were incubated 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) alternatively recombinant growth factor domain of PDGF-CC (100 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% desoxycholic acid, 150 mM NaCl, 5 mM EDTA, 200 μM orthovanadate and complete protease inhibitor cocktail 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). Bound antibodies were visualized as above.

Protein-protein interaction studies—To determine what domain(s) of tPA and PDGF-CC that 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 previously described (1), were bound to Ni-NTA-agarose (Qiagen) and then incubated 90 min, RT 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 polyclonal antibodies against human c-myc (see above) in order to detect co-eluted tPA truncation species. The membranes were subsequently stripped and reprobed with PDGF-C specific antibodies to detect input of full-length and core PDGF-C species, or an anti-His monoclonal antibody (C-terminal, Invitrogen) to detect input of CUB protein. Bound antibodies were detected as 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 NAP-10 columns (Amersham Biosciences) to enable buffer exchange to TBS. 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 paranitroaniline (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 was used as negative control. Purified human tPA was used to define maximal activity (T7776, Sigma).

Regulation of PDGF-C, tPA and the 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 sub-confluency in 6-well plates. Following attachment, the medium was exchanged for serum-free DMEM in the absence or presence of TGF-β1 (5 ng/ml), FGF-2 (5 ng/ml, R&D) or high glucose (30 mM), alternatively the cells were placed in hypoxic conditions (1% oxygen). After 24 h the conditioned serum-free media was collected, the proteins TCA-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 (PD\textsubscript{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\textsubscript{CUBPD}) (schematically illustrated in Fig. 1\textit{A}). In addition, a truncation mutant lacking the CUB domain of PDGF-C was also employed (PC\textsubscript{\Delta 150}). All mutants were properly expressed in transfected COS-1 cells, formed disulphide-linked dimers (data not shown), and were efficiently secreted in the conditioned medium (Fig. 1\textit{B}). When co-transfected with tPA, the generation of a 22-kDa protected fragment from PD\textsubscript{CUBPC} was significantly reduced as compared to wild-type PDGF-CC (PC\textsubscript{WT}), whereas no cleavage product was detected in co-transfections with PC\textsubscript{CUBPD}, nor with PC\textsubscript{\Delta 150} (Fig. 1\textit{B}).

These results were verified in receptor stimulation experiments where conditioned media from transfected COS-1 cells were applied onto porcine aortic endothelial (PAE) cells with stable expression of PDGFR-\textalpha (upper panel) or PDGFR-\textbeta (lower panel), respectively (Fig. 1\textit{C}). Following immunoprecipitation of the respective receptors, stimulation was measured as induction of receptor tyrosine phosphorylation. As previously shown, media from COS-1 cells co-expressing tPA and wild-type PDGF-CC induced strong PDGFR-\textalpha activation comparable to PDGF-BB-stimulated controls (11), whereas media from COS-1 cells co-expressing tPA with PD\textsubscript{CUBPC} induced weaker PDGFR-\textalpha activation. Cells stimulated with conditioned media expressing the PDGF-CC mutants alone, or co-expressing tPA with PC\textsubscript{CUBPD}, or the truncation mutant PC\textsubscript{\Delta 150}, respectively, showed only background levels of PDGFR-\textalpha activation. As cleavage of PC\textsubscript{CUBPD} would release the growth factor domain of PDGF-DD, a PDGFR-\textalpha agonist, the conditioned media were also applied to PDGFR-\textbeta expressing PAE cells, but none induced PDGFR-\textbeta stimulation. These findings indicate that both the CUB as well as the growth factor domain 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\textsubscript{\Delta 150} failed to induce PDGFR-\textalpha activation indicates that not only the CUB domain but also parts of the hinge region has to be removed for receptor activation. In order 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. 2\textit{A}). All mutants formed disulphide-linked dimers (data not shown) and were efficiently secreted in the conditioned medium by transfected COS-1 cells (Fig. 2\textit{B}). The multiple species of the truncated mutants seen in the immunoblotts 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-\textalpha 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 the PDGFR-\textalpha was monitored by induction of receptor tyrosine phosphorylation (Fig. 2\textit{C}). The results showed that the two shortest mutants, PC\textsubscript{\Delta 210} and PC\textsubscript{\Delta 230}, efficiently activated PDGFR-\textalpha, while mutants with additional parts of the hinge region, separating the CUB and the growth factor domain 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 has 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.

R231 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 tribasic processing site (amino acid residues R²³¹K²³²R²³⁴, 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, R²³¹, K²³² and R²³⁴, that are 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-[-phosphorylation (Fig. 3B). Wild-type PDGF-C and a mutant resistant to tPA-mediated cleavage, with the entire region R²³¹-R²³⁴ replaced by alanine residues, were used as controls. These experiments demonstrate that the cleavage site for tPA is confined to the R²³¹-R²³⁴ segment in human PDGF-CC and that R²³¹ is essential for cleavage to occur, while 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, and 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 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 “free” CUB domain of PDGF-C (PCU⁵). 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-[-phosphorylation (Fig. 4B), thus suggesting that the CUB domain may indeed act as a competitive inhibitor of tPA-mediated proteolysis.

Kringle-2 of tPA is necessary for cleavage of PDGF-CC—In order to determine which of the structural domains of tPA that 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 paranitroaniline (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 activity and within 24 h all mutants had induced maximal formation of pNA. Buffer-exchanged conditioned media 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, tPAΔ₃₀⁶, 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 tPAΔ₃₀⁶ is expressed and functional, the kringle-2 domain is required in order for tPA to efficiently cleave PDGF-CC.

Interaction between tPA and PDGF-CC is mediated by kringle-2 in tPA—We explored the possibility that the kringle-2 domain of tPA is mediating the reported protein-protein interaction between tPA and PDGF-CC (11). Ni-NTA beads were therefore coated with recombinant 6xHis-tagged full-length PDGF-CC, and serum-free conditioned medium from COS-1 cells transfected with the different tPA truncation mutants were added. Following extensive washing, bound His₆-tagged PDGF-CC protein was specifically eluted with an imidazole-containing buffer, and the eluates were analysed by immunoblotting using specific antibodies. The results showed that full-length PDGF-CC-coated beads specifically bound all tPA mutants except the shortest tPAΔ₃₀⁶ mutant lacking the kringle-2 domain (Fig. 6, upper two panels, co-eluted tPA above and eluted full-length PDGF-C below). Similarly, experiments using Ni-
NTA beads separately coated with recombinant “free” 6xHis-tagged CUB domain (Fig. 6, middle two panels, co-eluted tPA above and CUB below), or recombinant growth factor domain of PDGF-C (Fig. 6, lower two 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, here illustrated by incubation of uncoated Ni-NTA beads with tPAfl. These data imply that the kringle-2 domain of tPA interacts with both the CUB and the growth factor domain of PDGF-C, thus properly positioning the tribasic RKSR-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 creates a growth stimulatory loop important for the establishment of primary fibroblast cultures might have implication in wound healing processes, especially in healing of chronic diabetic wounds known to have impaired granulation tissue formation probably due to a reduced fibroblast activity (24). Interestingly, the expression of tPA and its inhibitor PAI-1 have 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. In order to determine whether the expression of PDGF-C, and also tPA and PAI-1, are 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 sub-confluency 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, and also tPA and PAI-1, are regulated by metabolic conditions and other growth factors, which are of importance in the pathophysiology of diabetes.

DISCUSSION

The discovery of the novel PDGFR-a ligand PDGF-CC (1) was not completely unexpected as gene deletion studies of the classical PDGFs and the PDGFR-a 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 cleaved and activated PDGF-CC (11). In this study we further investigate 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-a signaling, and that PDGF-CC together with PDGF-AA are the major PDGFR-a ligands in vivo (3). In our attempt to determine what is required for PDGF-CC to be a PDGFR-a 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-a. This is in consistence with the observation that mutant PDGF-AA, in which the pro-peptide could not be removed due to a mutation in the processing site, did not bind nor activate the PDGF-a-receptor (32). Within the 40
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last amino acids of the hinge region in PDGF-C we have previously shown that tPA-mediated cleavage of murine PDGF-CC occur at, or at least around, a conserved tribasic region (amino acid residues K^{231}-K^{232}-S^{233}-K^{234}) (11). Here we confirm that this conserved tribasic region is also the site of cleavage in human PDGF-CC (amino acid residues R^{231}-K^{232}-S^{233}-R^{234}) and more specifically that the cleavage depends on R231. It is well known that tPA cleaves plasminogen at the R^{561}-V^{562} bond to produce plasmin (33) but, previous findings have reported a lack of absolute specificity of tPA for a R-V bond (34), and also, the other non-plasminogen substrate for tPA, the NR1 subunit of the NMDA receptor, was recently shown to be cleaved at the R^{260}-Y^{261} bond (35). Whether tPA specifically cleaves human PDGF-CC at the R^{231}-K^{232} 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 vivo 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 EST database at NCBI have so far failed to provide any evidence of alternatively spliced PDGF-C transcripts encoding “free” CUB domains only (U. Eriksson, unpublished observation). 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 domain 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 have been shown to have a direct stimulatory effect on endothelial cell migration (6). As FGF-2 upregulates 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 blockage of PDGF-CC activation. Further, we could speculate that upon the interaction of PDGF-CC with kringle-2, the other domains of tPA could interact with molecules such as the LDL receptor-related protein (LRP), known to both interact with tPA (41) and to control PDGFR signaling (42), and thereby facilitating a localized generation of active PDGF-CC. Interestingly, tPA induces blood-brain barrier opening via interaction with LRP and proteolysis of an as-yet-unidentified substrate (43).

Despite the similarities between PDGF-CC 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 previously suggested 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 have 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, uPA. Whether any of these homologous proteases can cleave and activate PDGF-DD remains to be established.

Non-healing foot ulcers are a common and expensive complication in diabetic patients partially caused by reduced fibroblast activity (24). In order to develop rational therapeutic strategies it has become a major priority to characterize the pathophysiological mechanism of
the delayed wound healing and the impaired fibroblast activity in diabetic patients. Considering that the growth of primary fibroblasts in culture partially depends on a PDGF-CC/tPA stimulatory loop, we hypothesized that dysregulation of PDGF-CC signaling might be involved in the impaired function of fibroblasts seen in diabetic wounds. Our experiments demonstrate that hyperglycemia per se does not alter expression of the PDGF-C/tPA axis in normal primary fibroblasts, but that hypoxia, known to play an important role in all diabetes complications (45), decreases the expression of PDGF-C and thus a mitogenic signal for fibroblasts. It is worth noting that hyperbaric oxygen therapy is used to accelerate the rate of healing of diabetic foot ulcers (46). Further, we show that treatment with TGF-β greatly induced the expression of PDGF-C in the primary fibroblasts which is interesting considering that transgenic overexpression of either PDGF-C (4) or TGF-β, (47) in mouse heart results in fibrosis and cardiac hypertrophy. Although it is known that ectopically applied PDGF-CC can enhance delayed wound healing in diabetic mice (21) it still remains to be established whether PDGF-C expression is impaired in diabetic conditions.

In summary, the described molecular mechanism by which PDGF-CC becomes a PDGFR-a agonist through tPA-mediated proteolysis will assist the understanding of PDGF signaling in normal and pathological conditions.

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REFERENCES

FIGURE LEGENDS

FIG. 1. Cleavage of PDGF-CC by tPA is dependent on both the CUB and the growth factor domain. A, schematic illustration of the mutant proteins used to determine the structural requirements of PDGF-CC for proteolysis by tPA. B, COS-1 cells were transfected with the corresponding expression constructs in the absence or presence of tPA. 48 h after transfection serum-free conditioned media were collected and proteins TCA-precipitated. The precipitates were subjected to SDS-PAGE under reducing conditions, and the PDGF species were detected by immunoblotting using specific polyclonal antibodies. Co-expression of PC WT and, to a lesser extent, PD CUBPC with tPA generated a 22 kDa fragment (arrow). tPA expression was monitored using a polyclonal antibody against tPA (lower panel). C, similar results were obtained when conditioned serum-free media from transfected COS-1 cells were used to study induction of tyrosine phosphorylation of either PDGFR-α (upper panel) or PDGFR-β (lower panel) expressed in PAE cells. PAE cells were stimulated with the conditioned media on ice, lysed and then the PDGFRs were immunoprecipitated (IP) using specific antibodies. Phosphorylated receptors were detected by immunoblot analysis using an anti-phosphotyrosine antibody. Recombinant PDGF-BB (100 ng/ml) was used as positive control.

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 were 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 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).

FIG. 3. tPA-mediated cleavage of PDGF-CC is dependent on R231. A, immunoblot analysis of tPA-mediated proteolysis of the PDGF-CC cleavage-site mutants. Mutant PDGF-C species, with either of three basic amino acid residues (R231, K232, and R234) in a conserved tribasic site mutated to alanine, were co-expressed in COS-1 cells together 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 PC R231A mutant suggesting that R231 is essential for cleavage to occur. A mutant with the entire region R231-R234 replaced by alanine residues, PC AAAA, was used as a cleavage-resistant control whereas wild-type PDGF-CC (PC WT) 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 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 PC R231A, or PC AAAA, failed to induce efficient phosphorylation of the receptor, thus confirming the results seen in A.
FIG. 4. The CUB domain of PDGF-C can inhibit tPA-mediated cleavage of PDGF-CC. A, COS-1 cells were co-transfected with expression constructs of wild-type PDGF-C (PC<sub>WT</sub>) and tPA in the absence or presence of an expression construct expressing c-myc tagged “free” CUB domain of PDGF-C (PC<sub>CUB</sub>). TCA-precipitated proteins from conditioned serum-free media were immunoblotted using polyclonal antibodies against PDGF-C, tPA, and the human c-myc epitope (to detect the CUB domain), respectively. Co-expression of “free” CUB domain of PDGF-C with PC<sub>WT</sub> and tPA in COS-1 cells markedly reduced the tPA-mediated cleavage of PDGF-CC monitored as the presence of the 22 kDa band. B, 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 immunoprecipitated (IP). The presence of “free” CUB reduced phosphorylation of PDGFR-α as compared to when CUB was not co-expressed with PDGF-CC and tPA. Recombinant PDGF-CC (100 ng/ml, growth factor domain of PDGF-CC) and PC<sub>WT</sub> alone were used as controls.

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 paranitroaniline (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 PC<sub>WT</sub>. Serum-free conditioned media were collected 48 h after transfection, proteins TCA-precipitated and PDGF-C detected by immunoblotting using specific polyclonal antibodies. Co-expression of PC<sub>WT</sub> 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 His<sub>6</sub>-tagged full-length PDGF-CC (PC<sub>6xHis</sub>), CUB domain (CUB<sub>6xHis</sub>) and growth factor domain (Core<sub>6xHis</sub>) 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 were analysed by immunoblotting before addition to the coated beads in order to ensure that equal relative amount of the tPA mutant proteins were being added (10% tPA-input). Following incubation with tPA the beads were thoroughly washed and the His<sub>6</sub>-tagged proteins specifically eluted from the beads using a buffer containing 400 mM imidazole. Co-elution of interacting tPA species were analysed by immunoblotting using polyclonal anti-c-myc antibodies. To detect elution of the PDGF-C species the blots were subsequently stripped and rebotted with PDGF-C specific antibodies (for detection of PC<sub>6xHis</sub> and Core<sub>6xHis</sub>) alternatively anti-his antibodies (detection of CUB<sub>6xHis</sub>).

FIG. 7. The expression of PDGF-C in primary fibroblasts is modulated by hypoxia and TGF-β. Primary murine fibroblasts were seeded at sub-confluency and after attachment the medium was changed to serum-free medium in the absence (control) or presence of various growth factor/ metabolic stimuli for 24 h. The serum-free medium was then collected, the proteins TCA-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-β modulates the PDGF-C/tPA axis.
Table I  
Construct nomenclature and description of primers used.

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<th>Construct</th>
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Figure 1, Fredriksson et al.

A

B

C
Figure 2, Fredriksson et al.
Figure 3, Fredriksson et al.
Figure 5, Fredriksson et al.
Figure 6, Fredriksson et al.
Figure 7, Fredriksson et al.
Structural requirements for activation of latent PDGF-CC by tissue plasminogen activator
Linda Fredriksson, Monika Ehnman, Christina Fieber and Ulf Eriksson
J. Biol. Chem. published online May 23, 2005

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