PDGF-D is a specific, protease-activated ligand for the PDGF β-receptor

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The term 'platelet-derived growth factor' (PDGF) refers to a family of disulphide-bonded dimeric isoforms that are important for growth, survival and function in several types of connective tissue cell. So far, three different PDGF chains have been identified — the classical PDGF-A and PDGF-B[1,2] and the recently identified PDGF-C[3]. PDGF isoforms (PDGF-AA, AB, BB and CC) exert their cellular effects by differential binding to two receptor tyrosine kinases. The PDGF α-receptor (PDGFR-α) binds to all three PDGF chains, whereas the β-receptor (PDGFR-β) binds only to PDGF-B[1]. Gene-targeting studies using mice have shown that the genes for PDGF-A and PDGF-B, as well as the two PDGFR genes, are essential for normal development[4]. Furthermore, overexpression of PDGFs is linked to different pathological conditions, including malignancies, atherosclerosis and fibroproliferative diseases[1]. Here we have identify and characterize a fourth member of the PDGF family, PDGF-D. PDGF-D has a two-domain structure similar to PDGF-C[3] and is secreted as a disulphide-linked homodimer, PDGF-DD. Upon limited proteolysis, PDGF-DD is activated and becomes a specific agonistic ligand for PDGFR-β . PDGF-DD is the first known PDGFR-β-specific ligand, and its unique receptor specificity indicates that it may be important for development and pathophysiology in several organs.

We identified PDGF-D as a human expressed sequence tag (EST) in a BLAST search of the National Center for Biotechnology Information EST databases (accession no. AI488780). This EST encodes a polypeptide with ~50% identity to the carboxy-terminal portion of PDGF-C. Using a DNA probe derived from the EST sequence, we isolated several partial complementary DNA clones, and generated the extreme 5′ portion of the cDNA by rapid amplification of cDNA ends (RACE). The full-length cDNA for human PDGF-D encoded a polypeptide of 370 amino acids, which like PDGF-C has a two-domain structure with an amino-terminal CUB (InterPro IPR000859) domain (residues 56–167; reviewed in ref. 5) and a C-terminal PDGF/vascular endothelial growth factor (VEGF)-homology domain (residues 272–362, also known as the core domain). The identity shared by the full-length amino-acid sequences of PDGF-C and PDGF-D is ~43% (Fig. 1a). Similarity is highest in the distinct protein domains, whereas the N-terminal region, including the hydrophobic signal sequence, and the hinge region between the two domains have less identity. We identified a putative site for signal peptidase cleavage between residues 22 and 23; cleavage at this site would result in a secreted protein of 348 residues with a calculated relative molecular mass (M_r) of 40,270. We also identified a single putative site for *N*-linked glycosylation in the core domain of PDGF-D (residues 276–278).

The PDGF/VEGF domain of PDGF-D is ~50% identical to the corresponding domain in PDGF-C, and 20–23% identical to the core domains of the classical PDGFs and VEGFs (Fig. 1b). Only seven out of the eight invariant cysteine residues found in other PDGF/VEGF domains are present in PDGF-D (the fifth conserved cysteine residue is replaced by glycine). Similar to the unique insertion of three residues in PDGF-C, PDGF-D has an insertion of three amino acids (sequence NCG) between conserved cysteine residues three and four. In total, there are ten cysteine residues in the core domain, including the extreme C-terminal region, indicating a unique arrangement of the cysteines in the disulphide-bonded PDGF-D dimer. Phylogenetic analysis of the core domains of PDGFs and the VEGFs showed that the PDGF-D core domain forms a subgroup of the PDGFs together with PDGF-C (Fig. 1c).

We expressed the full-length form of PDGF-D, containing a C-terminal six-histidine (His_6) tag, in baculovirus-infected Sf9 insect cells. We purified the expressed protein on Ni–NTA–agarose columns and subjected it to SDS–polyacrylamide-gel electrophoresis (SDS–PAGE) under both reducing and non-reducing conditions (Fig. 2a). Non-reduced PDGF-D migrated with an M_r of 90,000 (90K), whereas the reduced protein migrated as a species of M_r 55K. We also expressed and purified a His-tagged version of full-length PDGF-D containing specific cleavage sites for the factor Xa protease in the hinge region that precedes the core domain (hereafter called fXPDGF-D, cleveage site IEGR × 2, replacing amino acids 251–258). The fXPDGF-D mutant showed similar electrophoretic properties to wild-type PDGF-D under reducing and non-reducing conditions (data not shown). Thus PDGF-D, like PDGF-A, B and C, forms disulphide-linked homodimers (PDGF-DD), and the introduced factor Xa cleavage sites did not affect the ability of fXPDGF-D chains to form such dimers.

It is known that full-length PDGF-CC, which has a domain structure similar to that of PDGF-DD, requires limited proteolysis to release the core domains of the protein, which then interact with its receptor[3]. Attempts to express independently the core domain of PDGF-D in baculovirus-infected insect cells were unsuccessful, as the truncated protein was retained intracellularly and was not secreted (E.B. and U.E., unpublished observations). We generated protease-treated preparations of wild-type PDGF-DD and fXPDGF-DD, analysed them by SDS–PAGE under reducing conditions, and immunoblotted them using an antipeptide antiserum raised against a sequence of PDGF-D just N-terminal to the first cysteine residue in the core domain. Plasmin digestion of wild-type PDGF-DD (data not shown) and factor Xa-digestion of fXPDGF-DD (Fig. 2b) generated distinct species with M_r values of 15K and 21K, respectively.

To investigate whether full-length PDGF-D is proteolytically processed *in vivo*, we overexpressed PDGF-D tagged with a Myc epitope in the hearts of transgenic mice using the α-myosin heavy chain (αMHC) promoter[6]. We analysed heart tissue extracts from

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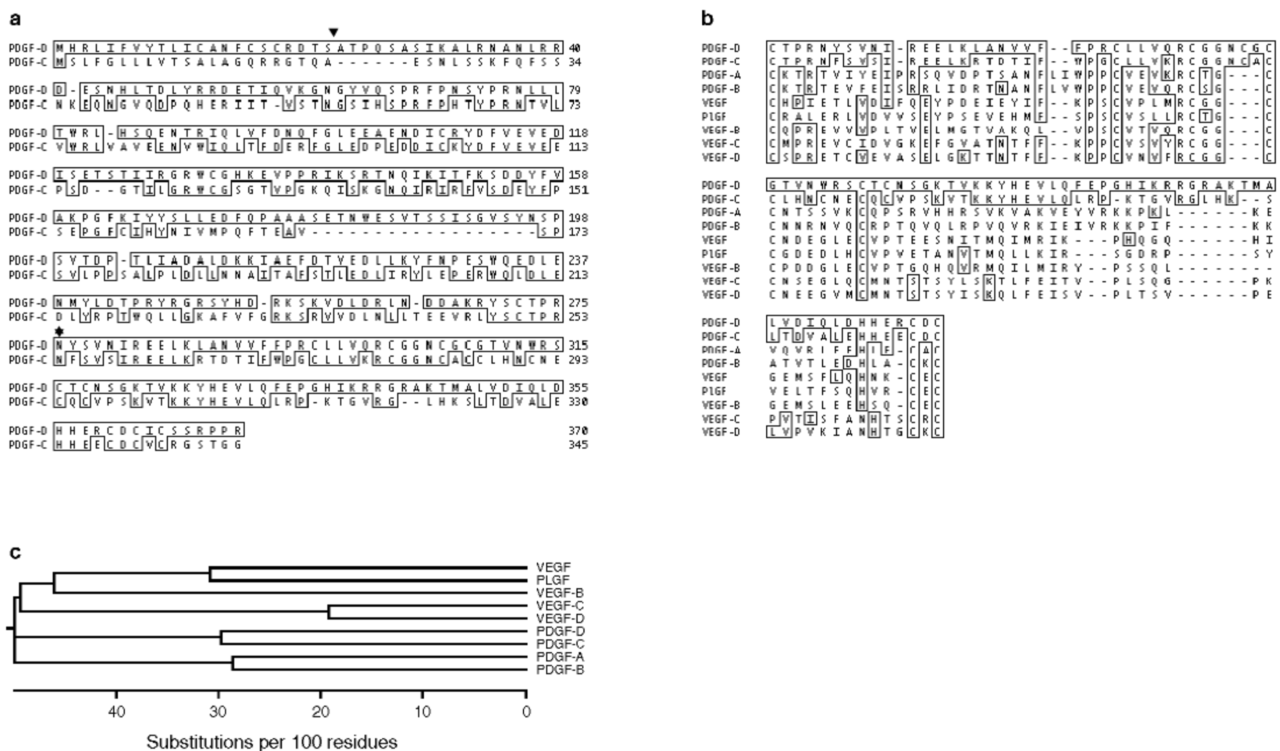

Figure 1 **Amino–acid sequence of human PDGF-D. a**, Alignment of the amino-acid sequences of human PDGFD and PDGF-C. Identical residues are boxed. The putative signal peptidase cleavage site between residues 22 and 23 (triangle) and the putative *N*-linked glycosylation site at residue 276 are marked (star). **b**, Alignment of the amino-acid sequences of members of the PDGF/VEGF family. Only regions that encompass the conserved cysteine-rich domain, which is involved in inter- and intra-disulphide bonds, are shown. Identical residues to those in PDGF-D are boxed, including the invariant cysteine residues found in all members of the PDGF/VEGF family. A unique feature of PDGF-D is that the fifth invariant cysteine residue is replaced by glycine. Similar to PDGF-C, PDGF-D has an insertion of three residues between cysteines three and four of the conserved cysteine motif. **c**, Phylogenetic analysis of growth-factor-homology domains in the PDGF/VEGF family. PDGF-D and PDGF-C form a subgroup of the PDGFs with an overall amino-acid sequence identity of ~43%.

transgenic and normal animals by immunoblotting using a monoclonal anti-Myc antibody to detect transgenic PDGF-D. Myc-tagged full-length PDGF-D (M_r 62K) was abundantly expressed in the transgenic hearts, as were several processed species, including two prominent fragments of M_r 36K and 30K (Fig. 2c). These results indicate that the myocardium of the transgenic mice may express enzymes that are capable of processing latent full-length PDGF-DD *in vivo*. A detailed phenotypic description of the transgenic animals will be published elsewhere.

We investigated the receptor specificity of full-length and plasmin-digested wild-type PDGF-DD and of full-length and factor Xa-cleaved fXPDGF-DD as competitors in PDGF receptor-binding assays. Using increasing concentrations of the two PDGF-DD preparations, we observed that full-length wild-type PDGF-DD and fXPDGF-DD failed to compete for binding of ^{125}I-labelled PDGF-BB to PDGFR-α and to PDGFR-β. In contrast, plasmin-digested PDGF-DD and factor Xa-digested fXPDGF-DD specifically competed for binding of ^{125}I-labelled PDGF-BB to PDGFR-β, but failed to compete for binding to PDGFR-α (Fig.2 d, e). Relative to PDGF-BB, protease-activated PDGF-DD was 10–12-fold less efficient as a competitor. As the core domain of PDGF-DD accounts for less than one-third of the full-length protein, these results indicate that activated PDGF-DD may be roughly threefold less efficient as a competitor in the ligand-binding assay, compared with PDGF-BB. Control experiments showed that plasmin and factor Xa present in the protease-digested PDGF-DD fractions did not affect the binding of ^{125}I-labelled PDGF-BB to PDGFR-β-expressing cells (data not shown). We also analysed the ability of the core domain of PDGF-DD to bind to VEGF receptors. No significant interaction was observed between the PDGF-DD core domain with VEGF receptors 1, 2 or 3 (M.U. and K. A., unpublished observations).

We next investigated the ability of full-length and protease-digested PDGF-DD to induce tyrosine phosphorylation of PDGFR-β. After stimulation of porcine aortic endothelial (PAE) cells expressing PDGFR-β with different preparations of PDGF-DD, we immunoprecipitated PDGFR-β from cell lysates, and subjected the samples to SDS–PAGE and immunoblotting with monoclonal antibodies against phosphotyrosine. Both plasmin-digested PDGF-DD and factor Xa-digested fXPDGF-DD stimulated tyrosine phosphorylation of receptors in a dose-dependent manner, as did PDGF-BB (Fig. 2f), whereas the full-length proteins failed to do so (Fig. 2f and data not shown). A similar analysis of receptor activation in human foreskin fibroblasts revealed strong tyrosine phosphorylation of the PDGFR-β, whereas PDGFR-α was only marginally phosphorylated (Fig. 2g). As a control, we stimulated heterodimeric receptor complexes with PDGF-AB heterodimers; both PDGF receptors were activated under these conditions. We conclude that PDGF-DD is a PDGFR-β-specific agonist and that proteolytic processing, releasing the core domains of PDGF-DD from the N-terminal CUB domains, is necessary for unmasking the receptor-binding epitopes of the core domain, as is the case for PDGF-CC[3].

We used a ^{32}P-labelled DNA probe to study the expression of PDGF-D transcripts in several human tissues by northern blotting (Fig. 3a). Highest expression of a principal 4.0-kilobase (kb) transcript occurred in heart, pancreas and ovary, whereas lower

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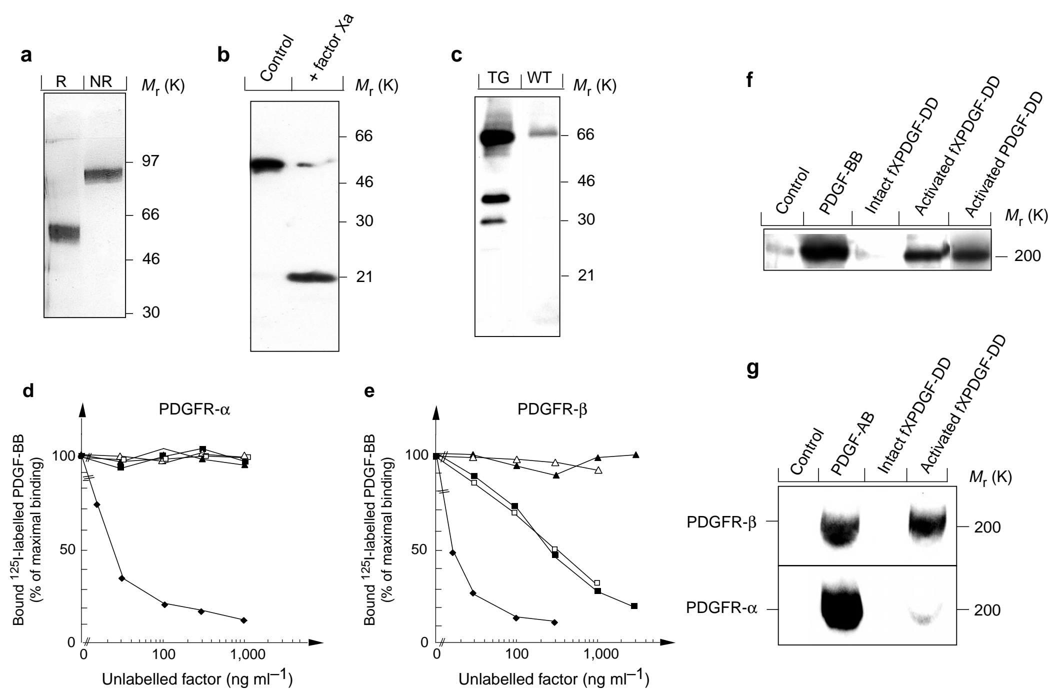

Figure 2 **Recombinant expression, *in vitro* and *in vivo* processing, and receptor analysis of PDGF-DD. a**, SDS–PAGE analysis of human recombinant PDGF-DD under reducing (R) and non-reducing (NR) conditions. PDGF-D was visualized by staining with Comassie brilliant blue. **b**, SDS–PAGE under reducing conditions and immunoblotting of human recombinant PDGF-DD containing introduced sites for factor Xa cleavage. Cleavage with factor Xa generates a species of M_r 21K, whereas the full-length protein has an M_r of 55K. The recombinant protein was detected using an antipeptide antiserum raised against a sequence N-terminal to the first cysteine residue in the core domain of PDGF-D. **c**, Mouse-heart tissue extracts were analysed by SDS–PAGE under reducing conditions and then immunoblotted. Transgenic PDGF-D (TG) was tagged at its C terminus with a Myc epitope and was detected using a specific anti-Myc antibody. A faint background band (M_r 66K) was observed in tissue extracts from wild-type heart (WT). The processed forms of PDGF-D have M_r values of 30K and 36K species. **d**, **e**, Competitive inhibition of binding of ^{125}I-labelled PDGF-BB to PAE cells expressing PDGFR-α (**d**) or PDGFR-β (**e**) by increasing concentrations of PDGF-BB (diamonds), full-length PDGF-DD (filled triangles), plasmin-treated PDGF-DD (filled squares), full-length fXPDGF-DD (open triangles) or factor Xa-treated fXPDGF-DD (open squares). **f**, Induction of tyrosine phosphorylation of PDGFR-β in PAE cells by full-length fXPDGF-DD, factor Xa-activated fXPDGF-DD or plasmin-activated PDGF-DD (300 ng ml^{-1}). Unstimulated PAE cells (control) and cells stimulated with 10 ng ml^{-1} PDGF-BB were used as controls. **g**, Induction of tyrosine phosphorylation of PDGF receptors in AG1519 human-foreskin fibroblasts stimulated with full-length fXPDGF-DD (300 ng ml^{-1}) or factor Xa-activated fXPDGF-DD (300 ng ml^{-1}). Unstimulated cells (control) or cells stimulated with PDGF-AB heterodimers (100 ng ml^{-1}) were used as controls. In **f** and **g**, PDGF receptors were immunoprecipitated from detergent-lysed cells, subjected to SDS–PAGE, and immunoblotted with monoclonal PY99 antibodies against phosphotyrosine.

expression levels were observed in several other tissues, including placenta, liver, kidney, prostate, testis, small intestine, spleen and colon. No expression was detected in brain, lung or skeletal muscle. In comparison, the 3.5-kb PDGF-B transcript was abundantly expressed in heart and placenta, whereas lower levels were observed in all other tissues. PDGF-D and PDGF-B were prominently co-expressed in heart, pancreas and ovary.

We analysed the tissue expression of PDGF-D in mouse embryos during mid-gestation (embryonic day 14.5) using immunohistochemistry with affinity-purified rabbit antibodies against full-length human PDGF-DD. These antibodies did not recognize PDGF-C in immunoblot analyses (Fig. 3b). Intense staining for PDGF-D was observed in several tissues, including the developing heart, lung, kidney and some muscle derivatives. Here we focus on the developing kidney; a broader expression analysis will be presented elsewhere. We observed intense staining of the highly vascularized fibrous capsule that surrounds the embryonic kidney, of the adjacent adrenal gland, and of the most peripheral aspect of the metanephric mesenchyme of the cortex (Fig. 3c, d). Staining was also present in cells located in the basal aspect of the branching ureter (Fig. 3e), whereas the developing nephron, including the ureter buds, glomeruli and Henle's loops, were devoid of staining. Previous analyses have shown that PDGFR-β is expressed by the metanephric mesenchyme and the developing vascular smooth-muscle cells and mesangial cells of the developing renal cortex[7,8]. In contrast, renal expression of PDGF-B is restricted to endothelial cells[7]. The non-overlapping patterns of expression of the two PDGFR-β ligands indicates that PGDF-B and PGDF-D may provide distinct signals to PDGFR-β-expressing perivascular cells. On the basis of their differential localization, it is appealing to speculate that PDGF-D may have a paracrine function in proliferation and/or commitment of PDGFR-β-expressing perivascular progenitor cells of the undifferentiated metanephric mesenchyme. As indicated by the phenotype of PDGF-B-deficient mice, PDGF-B may then provide proliferative signals and spatial cues for the branching vascular tree of the kidney, thus allowing proliferation and co-recruitment of PDGFR-β-expressing perivascular cells to form the mesangium of the glomeruli, and the smooth-muscle cells of the efferent and afferent arterioles[7,9].

The expression of PDGF-D partially overlaps with that of PDGF-C in the cortical area of the developing kidney[3]. The different receptor specificities of PDGF-C and PDGF-D, and their apparent inability to form heterodimers (X. L. and U. E., unpublished observations) indicate that these two recently identified PDGFs may provide distinct signals for migration and proliferation for at least two different cell populations in the undifferentiated metanephric mesenchyme — interstitial cell progenitors expressing PDGFR-α, and perivascular progenitor cells expressing PDGFR-β.

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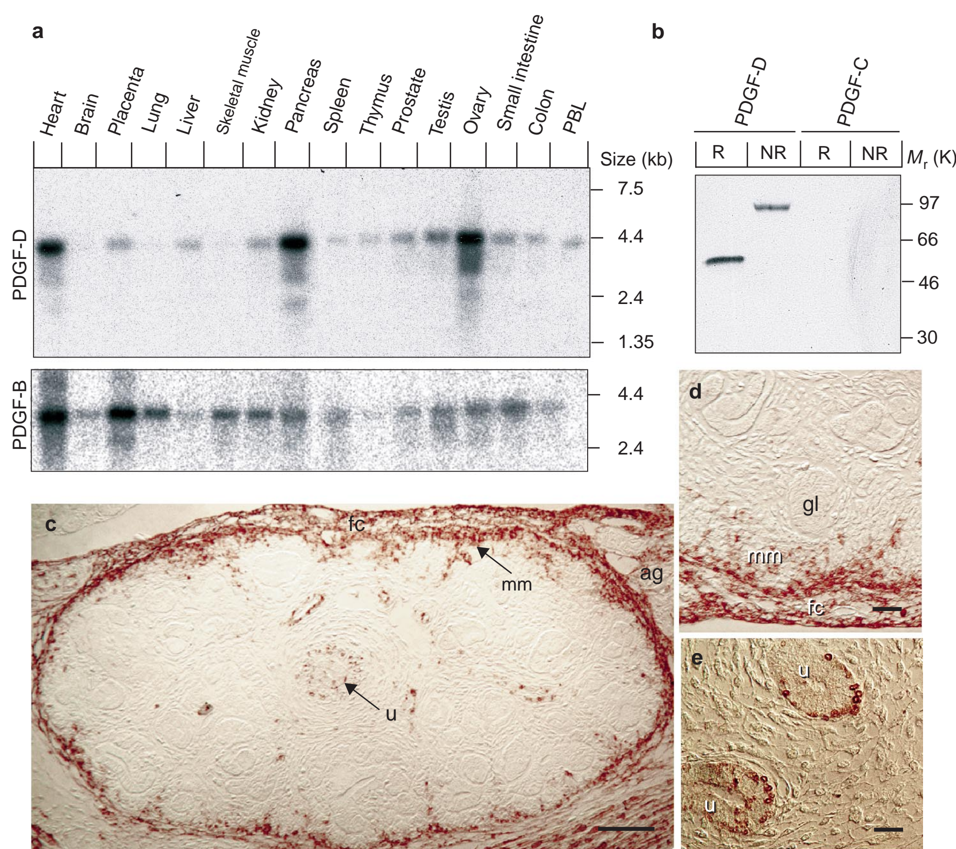

Figure 3 **Expression of PDGF-D in adult and embryonic tissues. a**, Northern-blot analysis of PDGF-D and PDGF-B transcripts in several adult human tissues. The blot was sequentially hybridized with ^{32}P-labelled probes for PDGF-D (upper panel) and PDGF-B (lower panel). PDGF-D transcripts are most highly expressed in heart, pancreas and ovary, whereas PDGF-B transcripts are most abundant in heart, placenta and lung. Co-expression of PDGF-D and PDGF-B was observed in several tissues, most notably heart, pancreas and ovary. PBL, peripheral blood leukocytes. **b**, Immunoblot analysis of purified PDGF-DD and PDGF-CC proteins using affinity-purified rabbit antibodies against PDGF-D. The analysis was carried out using reduced (R) and non-reduced (NR) proteins. Anti-PDGF-D antibodies do not cross-react with PDGF-C. **c–e**, Immunohistochemical localization of PDGF-D in the developing mouse kidney at embryonic day 14.5. PDGF-D (red-brown) is most abundantly found in the fibrous capsule (fc) surrounding the developing kidney and the adjacent adrenal gland (ag). Staining is also present in the metanephric mesenchyme (mm) in the cortical region of the kidney and in the branching ureter (u) in the medullar region. Notably, no PDGF-D staining was observed in the developing nephron, including the ureter buds, glomeruli (gl) and Henle's loops. Scale bars represent 100 μm (**c**) and 50 μm (**d**, **e**).

The phenotypic differences between the kidneys of mice lacking PDGFR-α and those lacking PDGF-A support the idea that another PDGFR-α ligand, such as PDGF-C, has a unique function in the formation of the renal mesenchyme[3]. Comparison of mice lacking PDGFR-β and those lacking PDGF-B has not revealed a similar phenotypic discrepancy, indicating that PDGF-D and PDGF-B may have at least partially redundant functions during the early stages of kidney development[9,10]. Targeted deletion of PDGF-D will be needed to test this hypothesis.

Our discovery of PDGF-D as a new PDGFR-β agonist provides further, and rather unexpected, insight into the complexity of the PDGF/PDGFR system during organogenesis, for instance of the kidney. PDGFR-α and PDGFR-β have different signalling capacities, for example, regarding chemotaxis of fibroblasts and smooth-muscle cells, which is stimulated by PDGFR-β and inhibited by PDGFR-α[1]. It is therefore possible that PDGF-D, which activates PDGFR-β but unlike other PDGF isoforms does not efficiently activate PDGFR-α, has important functions, for example in blood-vessel development and pathophysiology.

Note added in proof: The identification and partial characterization of PDGF-D are also described on page 517 of this issue by LaRochelle et al.[11]. □

Methods

Cloning of human PDGF-D cDNA.

Two primers, 5′-GTCGTGGAACTGCAACTGG (forward) and 5′-CTCAGCAACCACTTGTGTTC (reverse), derived from the identified human EST sequence (accession no. AI488780), were used in polymerase chain reactions (PCR) to amplify a 327-base pair (bp) fragment using DNA from a human fetal lung 5′-STRETCH PLUS λgt10 cDNA library (Clontech) as a template. The fragment was cloned into the pCR 2.1 vector (TA Cloning Kit, Invitrogen). The PCR fragment was labelled to high specific activity by random priming (Amersham) and the same cDNA library was screened by plaque hybridization to isolate several partial cDNA clones. The longest subcloned insert was 1,934 bp in length and encoded the C-terminal 322 amino acids of PDGF-D, whereas the 5′ part was missing. The 5′ part of the cDNA was then amplified by RACE using human-heart cDNA as a template (Marathon-Ready cDNA, Clontech) with adaptor primer 5′-CCATCCTAATACGACTCACTATAGGGC (forward) and a second primer derived from the partial cDNA clones, 5′-AGTGGGATCCGTTACTGATGGA-GAGTTAT (reverse). Amplification reactions were carried out using Advantage-GC cDNA PCR kit, (Clontech) and a 790-bp amplified fragment was cloned into the TOPO TA vector (TOPO TA Cloning Kit, Invitrogen). A full-length cDNA for PDGF-D was constructed by fusing the two partial cDNA clones at a common restriction site. Nucleotide sequences were determined using internal and vector-specific primers.

Expression of human PDGF-D in baculovirus-infected insect cells.

The part of the cDNA encoding amino acids 24–370 was amplified by PCR using *Taq* DNA polymerase (Amersham Pharmacia Biotech). The primers used were as follows: 5′-GATATCTAGAAG-CAACCCCGCAGAGC (forward, including an *Xba*I site for in-frame cloning, underlined), and 5′-GCTCGAATTCTAAATGGTGATGGTGATGATGTCGAGGTGGTCTTGA (reverse, including an *Eco*RI site, underlined, and sequences encoding a C-terminal His_6 tag). The amplified products were cloned in the pCR 2.1 vector (Invitrogen) and the *Xba*I–*Not*I-digested fragment was cloned into the baculovirus

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expression vector pAcGP67A (Pharmigen). For generation of mutant PDGF-D containing sites for factor Xa cleavage, the pCR 2.1 vector with the PDGF-D insert was modified at the position corresponding to amino acids 251–258 (sequence YHDRKSKV) into two tandem factor Xa cleavage sites (sequence IEGR × 2) by single-strand mutagenesis[12,13] using the primer 5′-CCTATCCAGGTCACGTCCTTCGATCCCGCCTTCGATTGACCTGCCTCG. After restriction digestion, the modified insert was subcloned into the baculovirus expression vector. Procedures used for generation of recombinant baculovirus expressing PDGF-D and for production, purification and analysis of recombinant PDGF-DD and PDGF-CC were as described[3].

Rabbit antisera against full-length PDGF-DD and against a synthetic peptide derived from the PDGF-D sequence (amino acids 254–272, sequence CRKSKVDLDRLNDDAKRYSC) were generated as described[3]. Antibodies against full-length PDGF-DD were affinity-purified on a column of purified full-length PDGF-DD coupled to CNBr-activated sepharose 4B (Pharmacia).

Receptor binding and receptor activation of PDGF-DD.

Ligand-binding competition experiments were carried out essentially as described[3,14], using PAE cells expressing human PDGFR-α or PDGFR-β. Aliquots of full-length or plasmin-digested PDGF-DD or of full-length or factor Xa-digested fXPDGF-DD (see below) were diluted in binding buffer and assayed for inhibition of binding of ^{125}I-labelled PDGF-BB to cells expressing PDGFR-α or PDGFR-β.

Growth-factor-induced tyrosine phosphorylation of PDGFR-β was analysed in PAE cells, essentially as described for PDGF-CC-induced stimulation of PDGFR-α[3]. Activation of PDGF receptors in fibroblasts was analysed using AG1519 human-foreskin fibroblasts (Coriell Cell Repository, Camden, New Jersey). Cells were stimulated with PDGF-AB heterodimers (100 ng ml^{-1}), full-length fXPDGF-DD (300 ng ml^{-1}) or factor Xa-activated fXPDGF-DD (300 ng ml^{-1}). Induction of PDGFR-α and PDGFR-β tyrosine phosphorylation was subsequently analysed as described above using specific antisera against the different PDGF receptors[15].

Full-length PDGF-DD was digested with plasmin as described[3]. The mutated version fXPDGF-DD, containing sites for factor Xa cleavage, was digested with factor Xa according to the manufacturer's instructions (Roche). The progression of the digestions was analysed by SDS–PAGE under reducing conditions, followed by immunoblotting using the antipeptide antiserum (see above). Receptor-binding and receptor-activation experiments were repeated at least three times with similar results.

Transgenic expression of full-length PDGF-D in mouse heart.

The sequence encoding the human Myc epitope was introduced at the 3′ end of the coding region of human PDGF-D cDNA by PCR mutagenesis using the following primers: 5′-GATAGTCGACTCCCAAATGCACCGG (forward) and 5′-AGTTCTGGTGGAGCTCTTGTTTTCGAATAAAGACTTCTTCTGAACATTCAGCTGATGC (reverse). Procedures used to generate the transgenic consruct driven by the α-MHC promoter[6] and to generate and analyse transgenic mice were essentially as described[3].

Northern blotting and immunohistochemistry.

A human multiple-tissue northern blot (MTN, Clontech) was sequentially hybridized, using the ^{32}P-labeled 327-bp PCR fragment of PDGF-D cDNA and full-length PDGF-B cDNA as probes at high stringency and ExpressHyb hybridization solution, according to the manufacturer's instructions (Clontech).

For immunohistochemistry, affinity-purified rabbit antibodies against human PDGF-DD (3–9 μg ml^{-1}), prepared as described above, were applied to tissue sections prepared from paraformaldehyde-fixed and paraffin-embedded mouse embryos. Staining was carried out as described[16]. In control experiments antibodies were pre-incubated with a 30-fold molar excess of full-length PDGF-DD. This blocked the staining, whereas similar pre-incubation with full-length PDGF-CC did not. Photomicrographs were obtained using a Zeiss microscope equipped with differential interference contrast optics.

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Correspondence and requests for materials should be addressed to U.E. The nucleotide and amino-acid sequences of human PDGF-D have been deposited at GenBank under accession no AF336376.

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