Identification of a cell retention signal in the B-chain of platelet-derived growth factor and in the long splice version of the A-chain

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The B-chain homodimer of platelet-derived growth factor (PDGF) is only very inefficiently secreted and remains largely associated with the producer cell; in contrast, the dimer of the short, and most common, splice variant of the A-chain is secreted. To identify the structural background to the differences in the secretory pattern between the different isoforms of PDGF, a set of chimeric PDGF A/B cDNAs was generated and expressed in COS cells. Analyses of the biosynthesis and processing of the corresponding products led to the identification of a determinant for cell association in the carboxy-terminal third of the PDGF B-chain precursor. Introduction of stop codons at various positions in the carboxy-terminal prosequence of the PDGF B-chain localized this determinant to an 11-amino-acid-long region (amino acids 219–229). This region contains an 8-amino-acid-long basic sequence that is homologous to a sequence present in an alternatively spliced longer version of the PDGF A-chain. In contrast to the short splice variant, the long splice A-chain version, like the B-chain, was found to remain predominantly cell associated. Thus, we have identified a conserved sequence that inhibits the secretion of some of the PDGF isoforms. Our data also suggest that switching of splicing patterns can be a mechanism to regulate the formation of secreted or cell-associated forms of PDGF-AA and possibly other growth factors.

Introduction

Platelet-derived growth factor (PDGF)¹ is a major mitogen in serum for connective tissue cells (Heldin and Westermark, 1990; Raines *et al.*, 1990). Structurally, PDGF is a disulfide-linked dimer composed of two homologous polypeptide chains, denoted A and B. All three dimeric isoforms of PDGF (-AA, -AB, and -BB) have been purified from natural sources (Heldin *et al.*, 1986; Hammacher *et al.*, 1988; Hart *et al.*, 1990).

Two types of PDGF receptors that differ in their ligand-binding specificities have been described. The PDGF α -receptor binds all isoforms of PDGF, whereas the PDGF β -receptor binds only PDGF-BB with high affinity (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1988, 1989; Hart *et al.*, 1988; Heldin *et al.*, 1988; Matsui *et al.*, 1989). Both PDGF α - and β -receptors have five immunoglobulin-like extracellular domains and intrinsic split tyrosine kinase domains; they both belong to the same subfamily of tyrosine kinase receptors as the CSF-1 receptor (Coussens *et al.*, 1986) and the *c-kit* protein (Yarden *et al.*, 1987).

Both the PDGF A- and B-chains are synthesized with amino-terminal hydrophobic leader sequences, and they show 60% amino acid identity in their mature parts (Josephs *et al.*, 1984; Betsholtz *et al.*, 1986b). The PDGF A-chain appears as two differently spliced versions having primary translations products of 196 and 211 amino acids (Bonthron *et al.*, 1988; Rorsman *et al.*, 1988). The longer version of the PDGF A-chain contains a carboxy-terminal sequence of basic amino acids derived from an alternatively spliced exon. A homologous basic sequence has also been identified in the carboxy-terminal prosequence of the PDGF B-chain and in one of two identified forms of vascular

¹ Abbreviations used: CHO, Chinese hamster ovary; CSF-1, colony stimulating factor-10; DTT, dithiothreitol; FCS, fetal calf serum; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

A **B**

Figure 1. Construction and design of expression vectors for six chimeric PDGF A/B-chains. (A) The expression vectors for the six chimeras shown at the lower part of the panel were derived from the top four plasmids. These contain cDNAs for the wild-type form of the short splice variant of the PDGF A-chain (pSV-PDGF-A102); the wild-type PDGF B-chain (pSV-PDGF-B1); the short splice variant of the PDGF A-chain, into which a *Bgl* II site was introduced at the indicated position (pSV-PDGF-A102Bg); and the PDGF B-chain into which a *Bgl* I site, as indicated, had been introduced (pSV-PDGF-B1Ba). From these four vectors the 12 restriction endonuclease fragments in the middle were prepared and ligated pairwise to give the six

endothelial growth factor/vascular permeability factor (VEGF/VPF) (Keck *et al.*, 1989; Leung *et al.*, 1989; Betsholtz *et al.*, 1990).

Studies of the biosynthesis of the different forms of PDGF have revealed that they differ in their processing pattern. In Chinese hamster ovary (CHO) cells transfected with either the short splice variant of the A-chain, the B-chain, or both chains, PDGF-AA and -AB were found to be processed to secreted dimeric forms of 30 kDa, whereas PDGF-BB formed predominantly a cell-associated form of 24 kDa; only small amounts of a structurally distinct secreted 30 kDa form were found (Östman *et al.*, 1988; Thyberg *et al.*, 1990). Likewise, studies on simian sarcoma virus-transformed cells showed that the fully processed product of *v-sis* (a transduced PDGF B-chain gene) is a cell-associated 24-kDa form that is both amino- and carboxy-terminally processed (Robbins *et al.*, 1983, 1985; Lokeshwar *et al.*, 1990).

To elucidate the structural background to the differences in processing between the short splice variant of the PDGF A-chain and the PDGF B-chain, we have analyzed chimeric PDGF A/B-chains. We present evidence that the region containing the basic sequence in the C-terminal part of the PDGF B-chain precursor is the major determinant for the formation of cell-retained forms. Analogously, the homologous sequence present exclusively in the long splice variant of the PDGF A-chain also confers retention of the product to the producer cell.

Results

To localize the region responsible for the differences in the processing pattern between the short splice variant of the PDGF A-chain and the PDGF B-chain, we generated a set of cDNAs for chimeric PDGF A/B-chains. The procedure for generating the chimeras and their structure and nomenclature are schematically shown in Figure 1, A and B. The chimeric cDNAs were cloned into the expression vector pSV7d (Truett *et al.*, 1985) and transfected into COS cells, and

media conditioned by transfected cells were assayed for PDGF receptor-competing activity. These analyses showed that the three chimeras that contained the A-chain C-terminal (upper 3 chimeras in Figure 1B) secreted high amounts of PDGF receptor-competing activity; in contrast, the three chimeras with the PDGF B-chain C-terminal secreted low amounts of PDGF receptor-competing activity (data not shown).

The chimeras PDGF(AAB) and PDGF(BBA) (Figure 1B) were further analyzed and compared with wild-type PDGF A- and B-chains. Cell lysates and conditioned media of transfected COS cells that had been metabolically labeled with ³⁵S-cysteine for 24 h were subjected to immunoprecipitations using a rabbit antiserum raised against human platelet PDGF, and immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis in the absence or presence of reducing agents (Figure 2). The main product detected in cells transfected with PDGF(AAB) and wild-type PDGF B-chain was cell-associated forms of 35–40 kDa (Figure 2, large arrows). In contrast, the main product of PDGF(BBA) was a secreted form of 30 kDa that was processed and secreted like the wild-type PDGF A-chain (Figure 2). After reduction, the major products of PDGF(AAB) and the wild-type B-chain were components of ~22 kDa, whereas PDGF(BBA) and the wild-type A-chain were processed to components of 16–17 kDa (Figure 2), confirming that the forms seen under nonreducing conditions represent disulfide-linked dimeric forms. None of these components were observed in untransfected cells, further indicating that they represent specific immunoprecipitations (Figure 2).

Thus, substitution of the A-chain carboxy-terminal with the B-chain carboxy-terminal in PDGF(AAB) conferred a processing pattern similar to wild-type PDGF B-chain, and conversely, substitution of the B-chain carboxy-terminal with that of the A-chain in PDGF(BBA) generated a chimera that was processed like the wild-type PDGF A-chain. We therefore concluded that the determinant in the PDGF B-

expression vectors schematically illustrated at the lower part. Numbers in front of the fragments refer to the plasmids from which they were derived. Designations at the end of the fragments refer to the restriction endonuclease sites used to isolate them. ■, PDGF A-chain cDNA sequences; □, PDGF B-chain cDNA sequences; and —, pSV7d vector sequences. ATG and TGA/TAG indicate positions of start and stop codons, respectively. The abbreviations used are E, *EcoRI*; Ba, *Bal* I; Bg, *Bgl* II; Sm, *Sma* I; Ss, *Ssp* I. (B) The structure of the short splice variant of the PDGF A-chain (Wt A_s), the B-chain (Wt B), and the six chimeric PDGF A/B-chains are depicted. Arrows delineate processing sites in the mature secreted 30-kDa forms. The positions of the conserved cysteine residues are indicated by C in the A-chain sequence. The positions of the *Bal* I site of the A-chain and the *Bgl* II site of the B-chain are indicated by arrowheads. ■, A-chain sequences; □, B-chain sequences.

Figure 2. Immunoprecipitations of transiently expressed PDGF chimeras. Wild-type short splice variant of PDGF A-chain (Wt A_s), wild-type B-chain (Wt B), and chimeras PDGF(AAB) and PDGF(BBA) were transiently expressed in COS cells. Two days after transfections, untransfected (control) and transfected cells were metabolically labeled with ³⁵S-cysteine for 24 h. Cell lysates and the corresponding media were immunoprecipitated and analyzed by SDS-gel electrophoresis, in the absence or presence of dithiothreitol (DTT), followed by fluorography.

chain responsible for the generation of predominantly cell-associated forms is localized within the last third of the PDGF B-chain precursor.

The carboxy-terminal third of the PDGF B-chain precursor contains sequences of the mature B-chain as well as carboxy-terminal prosequences (Figure 1B). To determine in which of the two parts the determinant for cell retention resides, we generated PDGF(Bstop191), a carboxy-terminally truncated form of the PDGF B-chain that lacks all of the carboxy-terminal prosequences (Figure 3A). PDGF(Bstop191) was expressed in COS cells and, after metabolic labeling, was analyzed by immunoprecipitations with an antiserum raised against recombinant PDGF-BB followed by SDS-gel electrophoresis. As shown in Figure 4, cells transfected with PDGF(Bstop191) expressed PDGF forms of 30, 35, and 40 kDa, most likely representing fully processed forms (30 kDa) and forms with one or two amino-terminal propeptides remaining (35 and 40 kDa, respectively). In contrast to wild-type PDGF-BB, these products were detected in the conditioned media. Thus, the major determinant for the formation of cell-associated

PDGF-BB resides in the carboxy-terminal prosequence of the PDGF B-chain.

Two additional carboxy-terminal mutants, PDGF(Bstop219) and PDGF(Bstop230), were made to localize the retention sequence in more detail (Figure 3A). Immunoprecipitations of cell lysates and conditioned media of metabolically labeled COS cells transfected with PDGF(Bstop219) and PDGF(Bstop230) revealed dimeric products with the predicted differences in size (Figure 4). These products differed strikingly in processing pattern; whereas PDGF(Bstop219) was secreted to a significant degree, PDGF(Bstop230), like the wild-type PDGF B-chain, remained cell associated (Figure 4). Thus, the major determinant for formation of cell-associated forms resides between amino acids 219 and 229.

The sequence between amino acids 219 and 229 of the PDGF B-chain is homologous to a part of the alternatively spliced exon 6 of the PDGF A-chain gene and also to a part of the longer version of VEGF/VPF (Figure 3, A–C). Therefore, the processing of the two variants of the PDGF A-chain was compared. Analyses of cell lysates and conditioned media by im-

Figure 3. Structure of the carboxy-terminally truncated mutants of the PDGF B-chain and the two alternatively spliced forms of the PDGF A-chains. (A) Schematic illustration of the structure of the wild-type PDGF B-chain and the three carboxy-terminally truncated forms PDGF(Bstop191), PDGF(Bstop219), and PDGF(Bstop230). Arrows indicate known processing sites of wild-type forms. The 11-amino-acid-long sequence that distinguishes PDGF(Bstop230) from PDGF(Bstop219) is indicated with the one-letter amino acid code. (B) Schematic illustration of the differences in the carboxy-terminal of the two alternatively spliced forms of the PDGF A-chain. The unique carboxy-terminal sequences of the two variants are shown with the one-letter amino acid code. (C) Alignment of the homologous sequences of the carboxy-terminals of the PDGF B-chain, the alternatively spliced sixth exon of the PDGF A-chain, and the insert of the longer variant of VEGF/VPF.

munoprecipitations with an antiserum raised against recombinant PDGF-AA of metabolically labeled COS cells transfected with the short or long splice variant of the PDGF A-chain confirmed that the main product of the short splice variant is a secreted dimeric form of 30 kDa. In contrast, the main product of the long splice variant is a cell-associated dimeric 35-kDa form (Figure 5, large arrows). Thus, the sequences of the alternatively spliced exon 6 of the PDGF A-chain gene are not only structurally but also functionally similar to the retention signal of the PDGF B-chain.

Discussion

Early studies on *sis*-transformed cells have shown that the *v-sis* product remains associated

with the producer cell (Robbins *et al.*, 1983, 1985), but the mechanism behind this retention has not been understood. We identify in this study a determinant in the PDGF B-chain between amino acids 219 and 229, which confers cell retention to the product. Furthermore, we show that the homologous region, found in the long but not the short splice version of the PDGF A-chain, also acts as a retention signal.

The mapping of the retention sequence of the PDGF B-chain to the C-terminal prosequence is somewhat unexpected because in most described cell systems the major products of the PDGF B-chain *v-sis* precursors are cell-associated 24-kDa dimers that lack the C-terminal prosequence (Robbins *et al.*, 1983, 1985; Beckmann *et al.*, 1988; Bywater *et al.*, 1988; Östman *et al.*, 1988). We therefore suggest that the con-

Figure 4. Immunoprecipitations of transiently expressed carboxy-terminally truncated forms of the PDGF B-chain. PDGF(Bstop191), PDGF(Bstop219), and PDGF(Bstop230) and wild-type PDGF B-chain were transiently expressed in COS cells. Two days after transfection, untransfected (control) and transfected cells were labeled for 3 h with ^{35}S -cysteine. Medium was then changed, and cells were chased for 3 h in the presence of an excess of unlabeled cysteine. Cell lysates and the chase media were then subjected to immunoprecipitation and analysis by SDS-gel electrophoresis, in the absence or presence of DTT, followed by fluorography.

Figure 5. Immunoprecipitations of the two alternatively spliced forms of the PDGF A-chain expressed transiently in COS cells. The short (Wt A_s) and long (Wt A_l) splice variant of the PDGF A-chain were transiently expressed in COS cells. Two days after transfection, untransfected (control) and transfected cells were metabolically labeled with ^{35}S -cysteine for 24 h; the cell lysates and the corresponding conditioned media were immunoprecipitated and analyzed by SDS-gel electrophoresis, in the absence or presence of DTT, followed by fluorography.

version to the 24-kDa form of PDGF-BB involves two steps. The first step would be a retention of the 40 to 55-kDa precursor form, which did or did not contain the amino-terminal prosequence. This retention would be mediated by the sequence identified in this study. The second step would be a proteolytic conversion to the 24-kDa form, which thereby, through an unknown mechanism, becomes excluded from the normal secretory pathway. As indicated by small arrows in Figure 2, PDGF-BB is also in COS cells, however incompletely, converted to smaller forms. The incomplete conversion to the 24-kDa form may be due to limiting amounts in these cells of the component(s) involved in that process. As shown in Figure 5 and indicated by small arrows, a minor part of the long splice variant of the PDGF A-chain also occurred as a smaller 28-kDa form (14 kDa after reduction). Because a corresponding form was not observed for the short splice version of the A-chain, processing to the smaller forms appears to be dependent on retention.

The fact that the two differently spliced forms of the PDGF A-chain are conserved in other species, including *Xenopus laevis*, indicates an important difference in the function of the two types (Mercola *et al.*, 1988; Matoskova *et al.*, 1989). Studies on the expression of mRNAs for the two forms of the A-chain in different cell lines have revealed that the short splice variant is the most abundant (Matoskova *et al.*, 1989; Mercola *et al.*, 1990). In further support of a functional significance of the presence of two splice variants of the PDGF A-chain is the fact that the recently discovered endothelial cell mitogen VEGF/VPF also occurs as two variants that differ by a sequence that is homologous to the retention sequences of PDGF (Figure 3C) (Keck *et al.*, 1989; Leung *et al.*, 1989).

The structural similarity between the carboxy-terminal sequence of the PDGF B-chain and of the longer splice variant of the PDGF A-chain has been noticed previously; this structural motif was found to be responsible for the nuclear accumulation of the long version of the PDGF A-chain (Maher *et al.*, 1989) and the B-chain (Lee *et al.*, 1987), which occurred when the signal sequences of the two chains were deleted or inactivated. In a recent study on the difference in processing between the PDGF isoforms, LaRochelle *et al.*, (1990) also found that substitution of the PDGF A-chain sequence carboxy-terminal of amino acid 177 with the corresponding B-chain sequence dramatically reduced the amounts of secreted PDGF. A

functional similarity between the basic carboxy-terminal sequence of the PDGF B-chain and the long splice version of the A-chain is also suggested by the findings that peptides containing this motif from the long A-chain, as well as from the B-chain, induced the release of PDGF from endothelial cells and smooth muscle cells (Raines and Ross, personal communication).

Previous characterization of the processing of the PDGF isoforms produced by a CHO cell line stably expressing high amounts of the PDGF B-chain and the short splice variant of the PDGF A-chain has shown that PDGF-AB, in contrast to PDGF-BB, is mainly secreted (Östman *et al.*, 1988). This suggests that the retention signal of the PDGF B-chain is only functional when present on both subunits of the dimeric PDGF molecules.

The functional consequence of the retention of certain isoforms of PDGF and, possibly, VEGF/VPF remains to be elucidated. Immunoelectron microscopical studies of a transfected PDGF-BB-producing CHO cell line showed that the cell-associated forms of PDGF-BB were localized mainly in the endoplasmic reticulum, the Golgi complex, and lysosomes (Thyberg *et al.*, 1990). This suggests that the retention sequence in PDGF-BB redirects the product from the secretory pathway to a degradatory compartment in those cells. It is also possible that the role of the retention signal in PDGF-BB is to facilitate the interaction of the growth factor with the cognate receptor in the producer cell by retarding the growth factor in a cellular compartment together with the receptor. This would provide a basis for an autocrine signal at the expense of a paracrine effect on neighboring cells. Indeed, there is unequivocal evidence for intracellular activation of the receptor tyrosine kinase in *v-sis*-transformed cells (Keating and Williams, 1988). There is, however, experimental evidence that the autocrine mitogenic signal in *v-sis*-transformed cells arises at the cell surface (Johnsson *et al.*, 1985; Betsholtz *et al.*, 1986a; Fleming *et al.*, 1989); a proportion of the ligand-receptor complexes may thus translocate to the cell surface and initiate the signal transduction pathway (reviewed in Heldin and Westermark, 1990). Because an autocrine-transforming activity of PDGF has been demonstrated only in cells in which the expression of PDGF is under control of viral or other strong promoters, the possibility remains that the autocrine response is elicited as a consequence of a leakage of the retention system. The small amounts of secreted 30-kDa PDGF-BB detected in the media

of transfected cells (Bywater *et al.*, 1988; Östman *et al.*, 1988) may thus also be an effect of an overloaded system.

The finding that the two splice variants of the PDGF A-chain differ in their compartmentalization is the first demonstration of a biological difference between the two forms and also an example of differential splicing as a mechanism to regulate the subcellular localization of a growth factor. Future studies will be aimed at defining in detail the structural motif responsible for cell retention and characterizing its interaction with cellular structures. Furthermore, it will be important to investigate whether alterations of the splicing pattern occur, e.g., during different stages of the development, and to identify the molecular mechanism that determines the splice pattern.

Materials and methods

Construction of expression vectors

The vectors for expression of the PDGF B-chain and the short splice version of the PDGF A-chain, pSV-PDGF-B1 and pSV-PDGF-A102, respectively, have been described previously (Östman *et al.*, 1988). For expression of the long version of the A-chain, a 1.3-kb long cDNA (Betsholtz *et al.*, 1986) was cloned into the vector pSV7d (Truett *et al.*, 1985).

To generate the vectors encoding the chimeric PDGF A/B chains, we cloned a 2.7-kb PDGF B-chain cDNA (Östman *et al.*, 1988) and a 1.3-kb PDGF A-chain cDNA (Betsholtz *et al.*, 1986), containing all the coding sequences of the PDGF B-chain and the short splice variant of the PDGF A-chain, respectively, as well as 5'- and 3'-untranslated sequences, into the *EcoRI* sites of M13mp19 to give M13mp19/PDGF-B and M13mp19/PDGF-A. Through oligonucleotide site-directed mutagenesis, a *Bgl* II site was introduced in the PDGF A-chain at the homologous position to the *Bgl* II site of the PDGF B-chain. In an analogous way, a *Bal* I site was introduced into the PDGF B-chain at the position corresponding to the *Bal* I site of the PDGF A-chain. Mutagenesis was performed using the Amersham mutagenesis kit (Amersham, Buckinghamshire, UK). Mutations were verified by sequencing across the mutated regions using internal sequencing primers. The mutated cDNAs were then cloned into the expression vector pSV7d (Truett *et al.*, 1985) and the resulting plasmids were designated pSV-PDGF-A102Bg and pSV-PDGF-B1Ba. From these two plasmids and the plasmids pSV-PDGF-B1 and -A102, 12 restriction endonuclease fragments were obtained and ligated together to give the six plasmids as illustrated in Figure 1A. The resulting vectors were characterized by restriction endonuclease digestions and, in addition, the DNA sequences encoding the chimeras PDGF(AAB) and PDGF(BBA) were analyzed by DNA sequencing.

The Amersham mutagenesis kit was also used to convert the codon encoding Arg-191 of the PDGF B-chain to a stop codon. Mutations converting Pro-219 and His-230 to stop codons were introduced by the method of Kunkel *et al.*, (1987). The expression vector pSV-PDGF(Bstop191) was generated by ligating a *Pvu* II/*Ssp* I fragment from the replicative form of the mutated M13mp19/PDGF-B into pSV-PDGF-B1 from which the corresponding sequences had been

excised. pSV-PDGF(Bstop219) and -PDGF(Bstop230) were constructed by ligating mutated *Bgl* II/*EcoRI* fragments into pSV-PDGF-B1 from which the corresponding wild-type sequences had been excised. The final vectors pSV-PDGF(Bstop191), -PDGF(Bstop219), and -PDGF(Bstop230) were analyzed and characterized by digestions with restriction endonucleases.

Transfection of COS cells

COS-1 cells (ATCC CRL 1650; American Type Culture Collection, Rockville MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Four hours before transfection, 0.5×10^6 cells were seeded in 60-mm culture dishes. A DNA/calcium phosphate precipitate was formed by first incubating 10 µg of plasmid DNA in 240 µl of 0.25 M CaCl_2 at room temperature for 10 min, and then adding dropwise while mixing 240 µl of (in mM) 276 NaCl, 10 KCl, 1.5 Na_2HPO_4 , 5.5 glucose, and 20 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid pH 6.85 and incubating for 15 min at room temperature. Cells were incubated with DNA precipitates overnight and then washed three times with 1 ml of (in mM) 25 tris-(hydroxymethyl)-aminomethane (pH 7.4), 138 NaCl, 5 KCl, 0.7 CaCl_2 , 0.5 MgCl_2 , and 0.6 Na_2HPO_4 .

Immunoprecipitations of metabolically labeled COS cells

Metabolic labeling of COS cells with ^{35}S -cysteine was started 2 d after transfection and was performed by growing the cells in cysteine-free medium supplemented with 0.1 mCi/ml of ^{35}S -cysteine, 10% dialyzed FCS, and antibiotics. The antisera used for immunoprecipitations have been described before and include a serum raised against PDGF purified from human platelets (Heldin *et al.*, 1981) and sera raised against recombinant PDGF-AA and -BB (Thyberg *et al.*, 1990). Immunoprecipitations, SDS-gel electrophoresis, and fluorography were performed as described (Östman *et al.*, 1988).

Acknowledgments

We thank Christer Wernstedt for synthesis of oligonucleotides and Ingegärd Schiller for excellent secretarial assistance.

Received: March 20, 1991.

Revised and accepted: April 29, 1991.

References

- Beckmann, M.P., Betsholtz, C., Heldin, C.-H., Westermark, B., Di Marco, E., Di Fiore, P.P., Robbins, K.C., and Aaronson, S.A. (1988). Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. *Science* 241, 1346–1349.
- Betsholtz, C., Johnsson, A., Heldin, C.-H., and Westermark, B. (1986a). Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. *Proc. Natl. Acad. Sci. USA* 83, 6440–6444.
- Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J., and Scott, J. (1986b). cDNA sequence and chromosomal localization of human platelet-

- derived growth factor A-chain and its expression in tumour cell lines. *Nature* 320, 695–699.
- Betsholtz, C., Rorsman, F., Westermark, B., Östman, A., and Heldin, C.-H. (1990). Analogous alternative splicing. *Nature* 344, 299.
- Bonthron, D.T., Morton, C.C., Orkin, S.H., and Collins, T. (1988). Platelet-derived growth factor A chain: gene structure, chromosomal location, and basis for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA* 85, 1492–1496.
- Bywater, M., Rorsman, F., Bongcam-Rudloff, E., Mark, G., Hammacher, A., Heldin, C.-H., Westermark, B., and Betsholtz, C. (1988). Expression of recombinant platelet-derived growth factor A- and B-chain homodimers in Rat-1 cells and human fibroblasts reveals differences in protein processing and autocrine effects. *Mol. Cell. Biol.* 8, 2753–2762.
- Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C., and Heldin, C.-H. (1988). cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. *Mol. Cell. Biol.* 8, 3476–3486.
- Claesson-Welsh, L., Eriksson, A., Westermark, B., and Heldin, C.-H. (1989). cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B type PDGF receptor. *Proc. Natl. Acad. Sci. USA* 86, 4917–4921.
- Coussens, L., Van Beveren, C., Smith, D., Chen, E., Mitchell, R.L., Isacke, C., Verma, I.A., and Ullrich, A. (1986). Structural alteration of viral homologue of receptor protooncogene fms at carboxyterminus. *Nature* 320, 277–280.
- Fleming, T.P., Matsui, T., Molloy, C.J., Robbins, K.C., and Aaronson, S.A. (1989). Autocrine mechanism for v-sis transformation requires cell surface localization of internally activated growth factor receptors. *Proc. Natl. Acad. Sci. USA* 86, 8063–8067.
- Hammacher, A., Hellman, U., Johnsson, A., Östman, A., Gunnarson, K., Westermark, B., Wasteson, Å., and Heldin, C.-H. (1988). A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. *J. Biol. Chem.* 263, 16493–16498.
- Hart, C.E., Bailey, M., Curtis, D.A., Osborn, S., Raines, E., Ross, R., and Forstrom, J.W. (1990). Purification of PDGF-AB and PDGF-BB from human platelet extracts and identification of all three PDGF dimers in human platelets. *Biochemistry* 29, 166–172.
- Hart, C.E., Forstrom, J.W., Kelly, J.D., Seifert, R.A., Smith, R.A., Ross, R., Murray, M.J., and Bowen-Pope, D.F. (1988). Two classes of PDGF receptors recognize different isoforms of PDGF. *Science* 240, 1529–1531.
- Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M., and Westermark, B. (1988). Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* 7, 1387–1394.
- Heldin, C.-H., Johnsson, A., Wennergren, S., Wernstedt, C., Betsholtz, C., and Westermark, B. (1986). A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A chains. *Nature* 319, 511–514.
- Heldin, C.-H., and Westermark, B. (1990). Platelet-derived growth factor: mechanism of action and possible in vivo function. *Cell Regul.* 1, 555–566.
- Heldin, C.-H., Westermark, B., and Wasteson, Å. (1981). Demonstration of an antibody against platelet-derived growth factor. *Exp. Cell Res.* 136, 255–261.
- Johnsson, A., Betsholtz, C., Heldin, C.-H., and Westermark, B. (1985). Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus. *Nature* 317, 438–440.
- Josephs, S.F., Guo, C., Ratner, L., and Wong-Staal, F. (1984). Human proto-oncogene nucleotide sequences corresponding to the transforming region of simian sarcoma virus. *Science* 223, 487–491.
- Keating, M.T., and Williams, L.T. (1988). Autocrine stimulation of intracellular PDGF receptors in v-sis transformed cells. *Science* 239, 914–916.
- Keck, P.J., Hauser, S.D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D.T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 246, 1309–1312.
- Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154, 367–382.
- La Rochelle, W. J., Giese, N., May-Siroff, M., Robbins, K. C., and Aaronson, S. A. (1990). Molecular localization of the transforming and secretory properties of PDGF and PDGF B. *Science* 248, 1541–1544.
- Lee, B.A., Maher, D.W., Hannink, M., and Donoghue, D.J. (1987). Identification of a signal for nuclear targeting in platelet-derived-growth-factor-related molecules. *Mol. Cell. Biol.* 7, 3527–3537.
- Leung, D.W., Cachianes, G., Kuang, W.-J., Goeddel, D.V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306–1309.
- Lokeshwar, V.B., Huang, S.S., and Huang, J.S. (1990). Intracellular turnover, novel secretion, and mitogenically active intracellular forms of v-sis gene product in simian sarcoma virus-transformed cells. *J. Biol. Chem.* 265, 1665–1675.
- Maher, D.W., Lee, B.A., and Donoghue, D.J. (1989). The alternatively spliced exon of the platelet-derived growth factor A chain encodes a nuclear targeting signal. *Mol. Cell. Biol.* 9, 2251–2253.
- Matoskova, B., Rorsman, F., Svensson, V., and Betsholtz, C. (1989). Alternative splicing of the platelet-derived growth factor A-chain transcript occurs in normal as well as tumor cells and is conserved among mammalian species. *Mol. Cell. Biol.* 9, 3148–3150.
- Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J., and Aaronson, S.A. (1989). Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* 243, 800–804.
- Mercola, M., Melton, D.A., and Stiles, C.D. (1988). Platelet-derived growth factor A chain is maternally encoded in *Xenopus* embryos. *Science* 241, 1223–1225.
- Mercola, M., Wang, C., Kelly, J., Brownlee, C., Jackson-Grusby, L., Stiles, C., and Bowen-Pope, D. (1990). Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Dev. Biol.* 138, 114–122.
- Östman, A., Rall, L., Hammacher, A., Wormstead, M.A., Coit, D., Valenzuela, P., Betsholtz, C., Westermark, B., and Heldin, C.-H. (1988). Synthesis and assembly of a functionally active recombinant platelet-derived growth-factor AB heterodimer. *J. Biol. Chem.* 263, 16202–16208.

- Raines, E.W., Bowen-Pope, D.F., and Ross, R. (1990). Platelet-derived growth factor. In: *Handbook of Experimental Pharmacology. Peptide Growth Factors and Their Receptors*, 95, Part I, ed. M.B. Sporn and A.B. Roberts, Heidelberg: Springer-Verlag, 173–262.
- Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W., and Aaronson, S.A. (1983). Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet derived growth factor. *Nature* 305, 605–609.
- Robbins, K.C., Leal, F., Pierce, J.H., and Aaronson, S.A. (1985). The *v-sis*/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. *EMBO J.* 4, 1783–1792.
- Rorsman, F., Bywater, M., Knott, T.J., Scott, J., and Betsholtz, C. (1988). Structural characterization of the human platelet-derived growth factor A-chain cDNA and gene: alternative exon usage predicts two different precursor proteins. *Mol. Cell. Biol.* 8, 571–577.
- Thyberg, J., Östman, A., Bäckström, G., Westermark, B., and Heldin, C.-H. (1990). Localization of platelet-derived growth factor (PDGF) in CHO cells transfected with PDGF A- or B-chain cDNA: retention of PDGF-BB in the endoplasmic reticulum and golgi complex. *J. Cell Science* 97, 219–229.
- Truett, M.A., Blacher, R., Burke, R.L., Caput, D., Chu, C., Dina, D., Hartog, K., Kuo, C.H., Masiarz, F.R., Merryweather, J.P., Najarian, R., Pachi, C., Potter, S.J., Puma, J., Quiroga, M., Rall, L.B., Randolph, A., Urdea, M.S., Valenzuela, P., Dahl, H.H., Favalaro, J., Hansen, J., Nordfang, O., and Ezban, M. (1985). Characterization of the polypeptide composition of human factor VIII:C and the nucleotide sequence and expression of the human kidney cDNA. *DNA* 4, 333–349.
- Yarden, Y., Escobedo, J.A., Kuang, W.-J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N., Francke, U., Friend, V.A., Ullrich, A., and Williams, L.T. (1986). Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* 323, 226–232.
- Yarden, Y., Kuang, W.-J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987). Human protooncogene *c-kit*: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J.* 6, 3341–3351.