The EMBO Journal vol.10 no.13 pp.4113-4120, 1991

Two PDGF-B chain residues, arginine 27 and isoleucine 30, mediate receptor binding and activation

J.M.Clements, L.J.Bawden, R.E.Bloxidge, G.Catlin, A.L.Cook, S.Craig, A.H.Drummond, R.M.Edwards, A.Fallon, D.R.Green, P.G.Hellewell, P.M.Kirwin, P.D.Nayee, S.J.Richardson, D.Brown[1], S.B.Chahwala[1], M.Snarey[1] and D.Winslow[1]

British Bio-technology Ltd, Watlington Road, Cowley, Oxford OX4 5LY and [1]Pfizer Central Research, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK

Communicated by T.L.Blundell

PDGF may be involved in the pathogenesis of a variety of disorders including atherosclerosis and certain types of cancer. There is currently little understanding of the molecular structure of PDGF and of the critical amino acid residues involved in receptor binding and cell activation. Two such PDGF-B chain residues, arginine 27 and isoleucine 30, have been identified by a site-directed mutagenesis programme. Substitutions in these positions can lead to PDGF mutants defective in both receptor affinity and cell activation as judged by displacement of [^{125}I]PDGF-BB, mitogenic assay and inositol lipid turnover. Circular dichroism and fluorescence spectroscopy show that such mutations do not disrupt the structure of PDGF.

Key words: mitogenesis/mutants/PDGF/receptor binding/structure

Introduction

Platelet-derived growth factor (PDGF) is the major serum mitogen for smooth muscle cells, fibroblasts and other cells of mesenchymal origin. As the name suggests, PDGF is released during activation of blood platelets, but in addition, is now recognized to be produced by a wide variety of other cell types, including endothelial cells and smooth muscle cells. Furthermore, PDGF stimulates a number of other biological responses such as chemotaxis and extracellular matrix production (Deuel *et al.*, 1982; Seppa *et al.*, 1982).

PDGF is a cationic disulphide-linked dimer (Raines and Ross, 1982) consisting of two polypeptides, designated the A-chain and the B-chain, which share ~60% homology (Betzholtz *et al.*, 1986). The three possible dimeric forms, AA and BB homodimers or AB heterodimers, have been described (Stroobant and Waterfield, 1984; Nister *et al.*, 1988; Sjolund *et al.*, 1988) and exhibit different functional effects, which appear to be due to the existence of two distinct cell surface receptors, each containing a tyrosine kinase in its intracellular domain. The α receptor binds PDGF AA, AB and BB with high affinity, whereas the β receptor binds only BB with high affinity (Heldin *et al.*, 1988; Seifert *et al.*, 1989). Binding of PDGF elicits dimerization of the receptor which activates a receptor intracellular tyrosine kinase and initiates the mitogenic response (Hammacher *et al.*, 1989; Heldin and Westermark, 1989).

Studies designed to investigate the active regions of PDGF-BB initially implicated the 89 residues of v-*sis* that are identical to the PDGF-B chain for transforming ability (King *et al.*, 1985). Recently, using chimeras of PDGF-A and -B chains, it has been shown that residues 24–63 contain the amino acids responsible for this activity (LaRochelle *et al.*, 1990). The present study was undertaken to identify the key receptor binding residues of PDGF-BB. A series of PDGF mutants with single amino acid substitutions was made by site-directed mutagenesis followed by expression in yeast. The biological effects of the mutants were assessed by ligand binding, inositol lipid turnover and mitogenesis studies. Since, in principle, single amino acid substitutions could significantly alter the conformation of PDGF and complicate data interpretation, the structural integrity of key PDGF mutants displaying altered function was confirmed by circular dichroism and fluorescence spectroscopy. Using these techniques, we have identified two residues, arginine 27 and isoleucine 30, that are critical for PDGF action.

Results

Mutagenesis of PDGF-B

As the basis of our mutation studies, we utilized a synthetic gene encoding a truncated, but mature form of PDGF-B (1–109) (Figure 1; Johnsson *et al.*, 1984). The mutants were produced using a yeast expression system which has been shown to produce authentic PDGF-BB homodimers having the same biological profile as PDGF-BB derived from natural sources (Cook *et al.*, 1991). In the course of our studies, we noted significant proteolysis of PDGF-BB following the consecutive arginines at positions 27 and 28. Substitution of Arg28 for the equivalent PDGF-A residue (Ser) increased expression levels 5- to 10-fold, and minimized proteolysis of PDGF-BB. PDGF-B chain molecules with this mutation have been shown to have similar biological activity (Cook *et al.*, 1991) and secondary structure (Craig *et al.*, 1991) to wild type (WT) PDGF-BB (see below).

A surface prediction algorithm (PREDICT) (Parker *et al.*, 1986), was used to select exposed residues and, in the first instance, mutations were focused on those surface residues which were shown to lie within turn or random coil regions. Ionizable amino acid residues were individually replaced by a residue of neutral or opposite charge, and hydrophobic residues were replaced by small polar groups. Figure 1A summarizes the position of all the substitutions made in PDGF-B. A number of the PDGF mutants were made in the protease-resistant Arg28 → Ser background molecule (Table I). Following this initial broad scan of the PDGF sequence, the individual residues implicated in the biological activity of PDGF were subjected to directed random

mutagenesis with redundant oligonucleotide primers to obtain a range of amino acid substitutions.

Biological properties of PDGF-B mutants

In the initial phase of the investigation, yeast supernatants containing variable amounts of the expressed PDGF mutants were screened in a number of immunological and biological assays. Two ELISAs were used to quantify mutant protein. Samples of yeast supernatants were routinely monitored using a sandwich ELISA but, given the possibility that mutants might be produced which are not recognized by one of the antibodies, a competition ELISA was used as back-up, particularly where expression levels of a mutant appeared very low. One mutant (Q64 → R) was not detected in the sandwich ELISA and the data shown are based on the competition ELISA. Expression levels varied considerably between mutants and a qualitative analysis is provided in Table I. The mutants were tested in three biological assays using Swiss 3T3 cells: receptor binding, as a measure of receptor affinity; mitogenesis, as a convenient functional assay; and inositol lipid (PI) turnover. The PI turnover assay, which has an incubation time of only 30 min, is complementary to the mitogenesis assay, which requires that the PDGF mutant is stable for 24 h in the presence of cells. Moreover, because PI turnover is closely linked to receptor occupancy, it represents a sensitive assay for the detection of PDGF partial agonists.

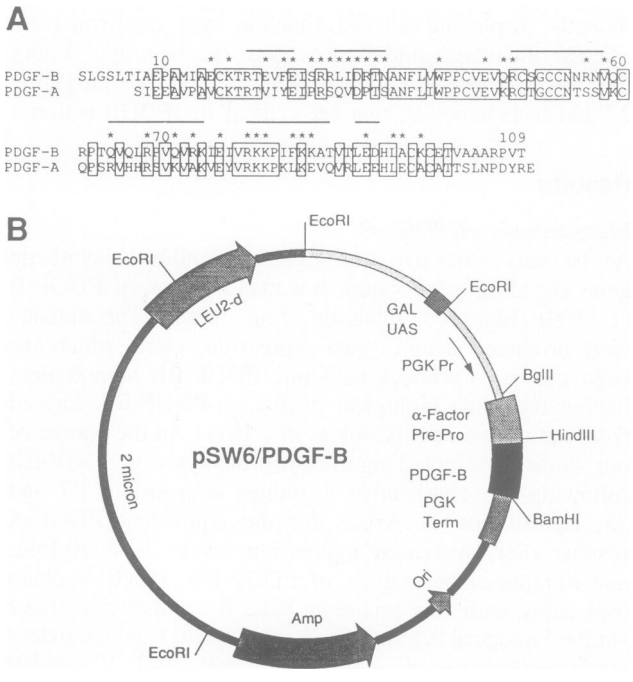

Fig. 1. PDGF-B sequence and yeast expression vector. (**A**) The sequence of PDGF-B is shown aligned with PDGF-A (Betzholtz *et al.*, 1986) and positions of identity are boxed. The position of amino acid substitutions made in PDGF-B are shown as an asterisk above the sequence. Predicted surface regions are indicated as a line above the sequence. (**B**) The expression vector pSW6 (Cook *et al.*, 1991) used to express PDGF-B and associated mutants. The position of the expression cassette and genes involved in the replication and selection of the plasmid in yeast and *E.coli* are indicated. Key restriction sites are shown on the outside of the plasmid. Transcription of PDGF-B is controlled by a hybrid yeast *PGK* and *GAL1-10* promoter. The PDGF-B coding sequence is fused in-frame with the pre-pro sequence of the yeast alpha factor gene (Kurjan and Herskowitz, 1982) at the *Hin*dIII restriction site and facilitates the secretion of PDGF-B from yeast.

Table I summarizes the data obtained from over 35 mutants having point mutations at individual amino acid residues. Although low expression levels precluded accurate quantification of mutant potency in a few cases, it is evident that the vast majority of PDGF mutants have activities that are comparable to the wild type molecule. The only exceptions are R27 → E, which shows low potency in the receptor binding and PI assays but appears wild type in the mitogenesis assay, and I30 → S, which exhibits low activity in all three assays. These latter PDGF mutants behave as full agonists when present in high concentrations (data not shown). Mutants constructed in a protease-resistant

Table I. Expression and biological activity of PDGF-B mutants

Mutation	Expression	ED_{50} (ng/ml)		
		RB	MITO	PI
E15V	+/−	>0.8	0.7	13
K17E	+	3.9	2.0	24
E21V	+	>3.4	0.8	11
F23W*	++	5.7	2.2	9.6
E24K	+++	6.2	1.7	6.3
S26P*	+++	1.6	0.9	7.6
S26Y*	+++	3.5	0.9	3.0
R27E*	++	>47	4.8	>500
L29A	++	6.8	2.1	5.0
I30S	++	>13	16	107
D31K	+/−	>1.0	>0.9	>11
R32P	+++	5.7	2.7	>5.2
T33E	++	24	3.1	13
N34E*	+++	50	1.4	7.3
W40H*	++	14	2.9	11
E45K	+/−	>5.0	ND	ND
Q47E	+	>9.0	3.2	>18
R48E	+/−	>5.2	ND	ND
R56Q	+	>2.4	0.8	6.1
R56S	++	13	3.2	11
V58S	+	4.5	1.7	12
Q64R	++	5.5	2.5	6.5
R68Q	+/−	>2.9	ND	ND
Q71E	++	0.9	0.9	4.0
R73A	++	6.8	2.1	8.7
E76Q*	+++	1.7	0.6	1.8
R79Q	+	2.1	0.8	4.3
K80E	+	2.1	1.7	>8.4
K81E	+/−	1.4	1.0	>4.2
F84S	+	4.5	2.0	26
K85E	+/−	0.5	0.5	>2
K86S	+	1.4	0.6	3.6
E92K	+	4.0	1.9	9.0
L95D*	+/−	>0.8	1.3	>5.4
A96E	+	2.5	2.4	12
K98S*	+++	1.7	8.2	13
WT	+/++	5.0 ± 0.7 (n = 11)	2.6 ± 0.2 (n = 24)	14 ± 1.2 (n = 10)
R28S	+++	1.9 ± 0.6 (n = 3)	2.0 ± 0.1 (n = 6)	3.6 ± 0.2 (n = 3)

Mutants were expressed in either wild type (WT) or protease-resistant (R28 → S; indicated by *) background. Expression levels are indicated as follows: +/−, <50 ng/ml; + 51−199 ng/ml; ++ 200−749 ng/ml; +++ >750 ng/ml. Data are ED_{50} values (ng/ml) determined in receptor binding, mitogenesis and PI turnover assays carried out on Swiss 3T3 cells. Values shown are one of duplicate expressions for each mutant (which was assayed two or three times) except for WT and R28 → S where means ± SEM are given.

background molecule (R28 → S), benefit from higher expression levels (Cook *et al.*, 1991); these mutants are indicated with an asterisk in Table I. Biophysical and biological analyses suggest that neither structure nor activity is significantly altered in R28 → S PDGF (Craig *et al.*, 1991; see also Figure 3). In addition, the data in Figure 2A indicate that PDGF mutants R27 → E and I30 → S produced in either the wild type or protease-resistant PDGF background molecule (R28 → S) are similarly defective in their biological activity.

Mutations at isoleucine 30

In the next phase of the study, a random mutagenesis strategy was adopted to produce further mutants with different substitutions at isoleucine 30 and arginine 27. The data obtained for I30 mutants, some of which are in the protease-resistant background, are shown in Table II. In general, three classes of mutants can be identified: the first, representing those with conservative substitutions at I30 (leucine, valine, alanine), have biological activity resembling, but slightly weaker than the wild type molecule. A second group, with more radical replacements for isoleucine (tyrosine, proline, serine) still retain moderate activity in the mitogenesis assay (2- to 5-fold less active than PDGF itself) but are much weaker in the PI and receptor binding assays. Lastly, replacement of isoleucine with a charged or polar amino acid (glutamic acid, lysine or asparagine) results in a PDGF molecule which is at least 20 times weaker than wild type in all assays. Thus the more radical the substitution replacing isoleucine at position 30, the less active the PDGF molecule.

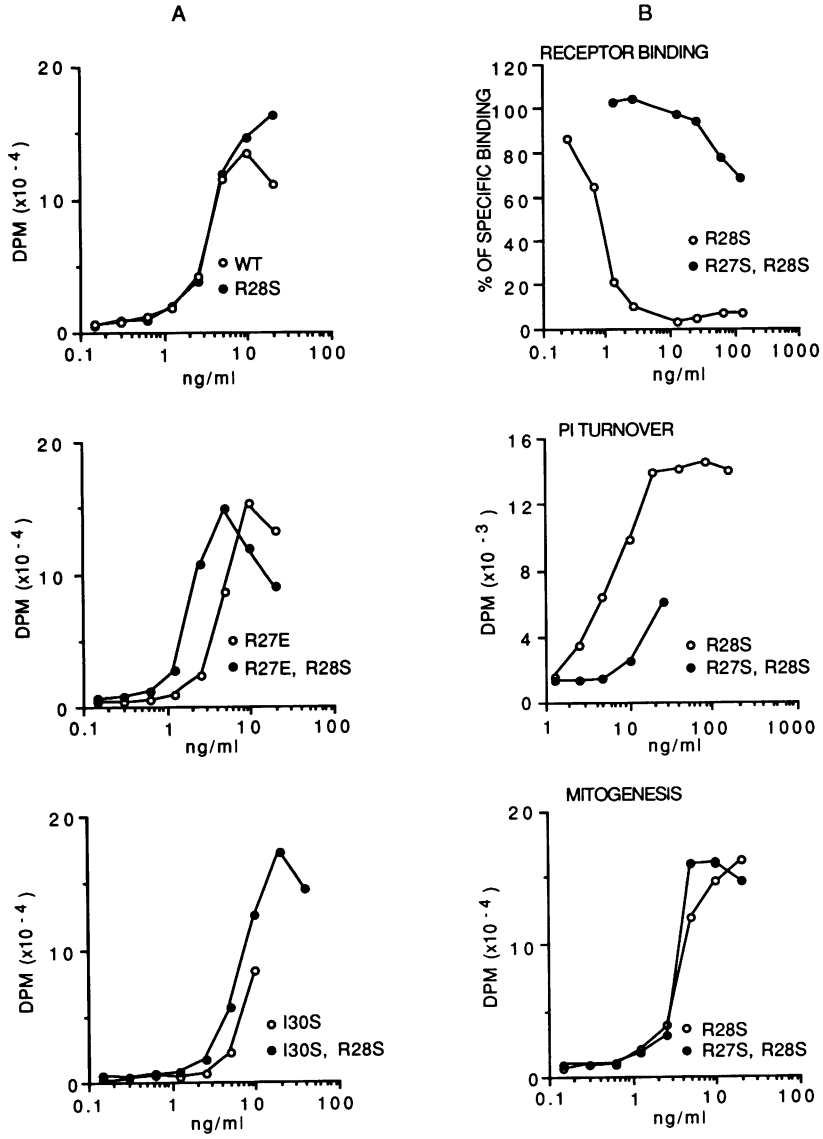

Fig. 2. (**A**) Mitogenic activity of PDGF-BB and two mutants R27 → E and I30 → S on 3T3 cells is comparable when expressed in the WT (○) or R28 → S (●) background molecule. Dose–response curves for PDGF and the mutant proteins were constructed based on their protein concentration as determined by ELISA. Mitogenesis of 3T3 cells was carried out as described in Materials and methods. Comparison of WT and R28 → S (top) and R27 → E mutant in WT and R28 → S background (middle), and I30 → S mutant in WT and R28 → S background (bottom). Values are the means of duplicate assays. (**B**) PDGF-B mutant R27 → S shows decreased biological activity in receptor binding (top) and PI turnover assays (middle), but no change in the mitogenesis assay (bottom). Receptor binding, mitogenesis and PI turnover assays were performed as described in Materials and methods. Receptor binding values are expressed as a percentage of maximal specific binding of [^{125}I]PDGF-BB. All values are the means of duplicate assays. Note that the presence of contaminating factors in yeast supernatants precluded the use of higher concentrations of R27 → S in the receptor binding and PI assays that are shown. Following purification and concentration, however, R27 → S behaves as a full agonist in the two functional assays (data not shown).

Table II. Substitution at I30 and R27, but not L29, results in altered biological activity of PDGF-B

	Mutation	ED_{50} (ng/ml)		
		RB	MITO	PI
Isoleucine 30	I30A	31	2.2	17
	I30V	5.0	4.7	13
	I30L	13	8.4	25
	I30Y	>25	13	>51
	I30S*	>100	5.0	>200
	I30P	>77	9.0	>183
	I30N	>88	53	>208
	I30K	>74	>76	>153
	I30E*	>140	>81	>132
Arginine 27	R27E	>7.8	2.6	144
	R27L*	>10	2.5	>95
	R27S*	>117	2.9	62
	R27P*	>198	1.9	23
Leucine 29	L29A*	1.5	2.1	3.2
	L29D*	4.2	2.7	10
	L29R*	1.5	1.9	14
	L29Y*	1.2	3.0	20
	L29P*	>113	3.0	32
	WT	5.2 ± 0.7 (n = 6)	3.1 ± 0.7 (n = 6)	14 ± 1.9 (n = 6)
	R28S	2.4 (n = 2)	1.9 (n = 2)	3.6 (n = 2)

Mutants were expressed in either the WT or R28 → S (indicated by *) background molecule. Data are ED_{50} values obtained in biological assays carried out as described in Table I. Values shown are means of 2–3 determinations except for WT and R28 → S where means ± SEM are given.

Mutations at arginine 27

A similar strategy was adopted to produce point mutations at residue 27. Like R27 → E (Table I), all other mutants showed decreased activity in the receptor binding and PI assays with no significant change in mitogenic potency (Table II). These are exemplified in Figure 2B by a more detailed comparison of the activity of PDGF mutants R27 → S, R28 → S and the protease-resistant background molecule R28 → S—the selective retention of mitogenic activity in this R27 mutant is evident. This biological profile is similar to some of the mutants with isoleucine 30 replacements, e.g. I30 → P, I30 → S, and indicates that mutations in this region of the PDGF-B chain can produce a molecule that is mitogenic at much lower levels of receptor occupancy than PDGF-BB itself. Swiss 3T3 cells express equal numbers of PDGF α and β receptors (Hosang *et al.*, 1989). Receptor binding assays on purified PDGF mutants R27 → E and I30 → S, unlike PDGF-AA, gave no indication of partial ligand displacement (Figure 3). This suggests that R27 and I30 mutants have reduced affinity on both α and β receptors. It seemed possible that the high mitogenic activity of R27 mutants in relation to their activity in other assays might have been due to the long incubation times necessary with the former assay. This possibility was tested by examining the activity of mutant R27 → E in a [^{3}H]uridine uptake assay. Uridine uptake is widely recognized as being a necessary early prelude to DNA synthesis (Jimenez de Asua *et al.*, 1974) which can be assayed within 30 min of PDGF addition

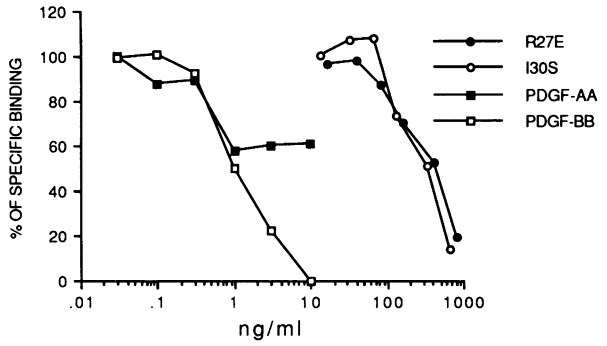

Fig. 3. Effect of PDGF-BB, PDGF-AA and R27 → E and I30 → S mutants on binding of [^{125}I]PDGF-BB in Swiss 3T3 cells. Receptor binding assays were carried out as described in Materials and methods. Values are expressed as a percentage of maximal specific binding of [^{125}I]PDGF-BB and are the means of duplicate observations. Mutants R27 → E and I30 → S were used as purified material.

Table III. Secondary structure content of PDGF measured by far UV-CD spectra

	Helix (%)	Sheet (%)	Remainder (%)
PDGF-BB	4	51	45
PDGF-BB R28 → S	4	48	47
PDGF-BB R27 → S, R28 → S	4	53	43
PDGF-BB I30 → K	3	47	50
PDGF-BB I30 → A	7	48	46
PDGF-BB L29 → Y	2	51	47

to 3T3 cells. The ED_{50} values in mitogenesis and uridine uptake assays were very similar, 2.6 ng/ml and 3.9 ng/ml respectively. Thus, as with its mitogenic activity, mutant R27 → E was more active in the uridine uptake assay than its receptor affinity would predict. The explanation for the discordant activity in the different assays remains unclear; obvious possibilities such as stimulation of receptors other than those for PDGF appear unlikely because of the high potency of the PDGF mutants and the fact that a variety of substitutions at R27 yield mutants with similar biological profiles. There is a precedent for separation of PDGF-induced mitogenesis and PI turnover: PDGF-B receptor mutants lacking 82 amino acids of the kinase insert sequence transduce early signals such as PI turnover, but are unable to mount a mitogenic response to PDGF (Escobedo and Williams, 1988). It is unclear whether this reflects a change in the catalytic efficiency of the tyrosine kinase or in substrate specificity (Severinsson *et al.*, 1990). By analogy, the R27 PDGF mutants described here must either have receptor efficacy in excess of the natural molecule, or be able to direct tyrosine kinase substrate specificity following binding to the external domain of the receptor in a manner different from PDGF-BB itself. Further work is necessary to clarify their mechanism of action, but the mutants undoubtedly represent interesting research reagents. Furthermore, the decreased activity of many of these mutants clearly implicate residue 27 in receptor binding and activation.

Mutants at leucine 29

It is evident from the data presented in Table I that non-conservative changes to amino acid residues neighbouring arginine 27 and isoleucine 30 (S26, R28, D31, R32, T33)

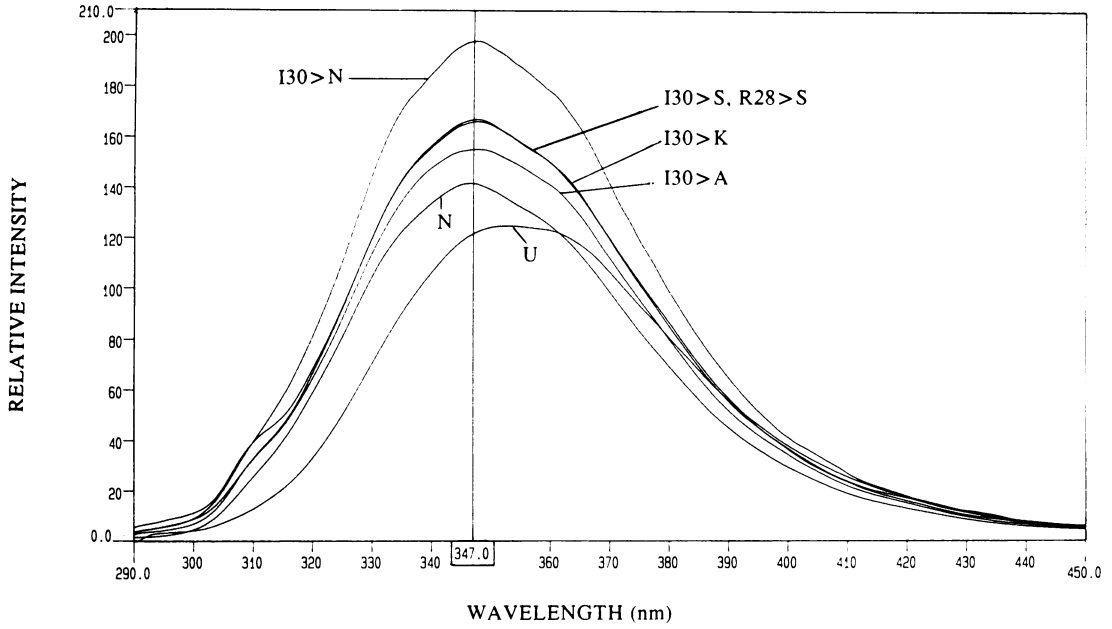

Fig. 4. Steady state fluorescence emission spectra native (N), unfolded/reduced (U) PDGF-BB and residue 30 mutants of PDGF-BB. Spectra are displayed with baselines subtracted; values were calculated as relative fluorescence. Protein concentration was 0.054–0.081 mg/ml in 10 mM acetic acid, pH 3.2, for native PDGF-BB and the mutants and 0.081 mg/ml in 7.8 M Gdn–HCl, 10 mM DTT, pH 9.2, for the unfolded/reduced PDGF-BB. Fluorescence emission was measured using a Perkin-Elmer LS-50 fluorimeter with excitation at 280 nm with a 2.5 nm bandwidth and 1 cm pathlength. The intensity of emission was measured at 2.5 nm bandwidth.

have no significant effect on biological activity. The change at L29 in the initial mutant screen (to alanine) is relatively conservative and, to examine more closely the importance of this residue in PDGF receptor binding and activation, a number of further mutants with more radical alterations at residue 29 were produced. The data obtained with these mutants are presented in Table II. In general, even radical substitutions for L29 lead to PDGF mutants that are similar to the wild type molecule. A modest decrease in potency in the PI assay is, however, evident for all but the original L29 → A mutant (Table II). An exception to this rule is L29 → P which like the R27 mutants, has impaired receptor affinity and decreased potency in the PI turnover assay though it retains wild type mitogenic activity. It seems likely that the proline substitution may cause a local structural perturbation that affects the orientation of neighbouring amino acid side chains essential for efficient receptor recognition and activation.

Structure of PDGF mutants

The analysis of biological data obtained with mutant proteins is complicated by the fact that observed changes in receptor binding or receptor-mediated events might be due to a general structural change in the molecule rather than to a local change in a critical region of the protein. Thus, residues distant from the real receptor binding region could be mistakenly implicated. Ideally, a study of the type described here should contain detailed structural information for both wild type and biologically altered PDGF molecules. Since neither NMR nor X-ray structures are available for PDGF, the mutants were examined by other physicochemical techniques which can provide useful data on the secondary and tertiary structure.

The secondary structure content of PDGF-BB has been determined previously using far ultra-violet circular dichroism (UV-CD) and Raman spectroscopy (Craig *et al.*, 1991). Given the high correlation in regular structure content calculated by these two independent methods, a single technique was deemed suitable for routine structural analysis of mutants. Far UV-CD was therefore used to analyse the folding of the polypeptide backbone of variant PDGF-BB molecules. Mutations at R27 → E, L29 → Y, I30 → S, K, A, N and Y all demonstrated far UV-CD spectra which superimposed with the wild type or protease-resistant R28 → S PDGF molecules. This superimposition is evidence for the fidelity of the backbone conformation in these variants. CONTIN analysis (Provencher and Gloeckner, 1981; Provencher, 1982) of the CD spectra of a range of key active or inactive I30 mutants indicated little variation in secondary structure content (Table III). Changes in activity arising from amino acid substitution at positions 27, 29 and 30 are not, therefore, caused by gross perturbation of the secondary structure.

Loss of enzyme activity in single site mutants of β-lactamase has been demonstrated to occur due to incorrect association of folded secondary structure elements (Craig *et al.*, 1985). The resulting non-native tertiary structure leads

Table IV. Steady state fluorescence anisotropy data for PDGF

Sample	Anisotropy
N-Acetyl tryptophanamide	0.005 ± 2e-3
PDGF-BB	0.084 ± 1e-3
PDGF-BB unfolded/reduced (U)	0.029 ± 7e-3
PDGF-BB R27 → E	0.082 ± 1e-3
PDGF-BB R27 → S, R28 → S	0.079 ± 1e-3
PDGF-BB R28 → S and I30 → S	0.080 ± 1e-3
PDGF-BB I30 → K	0.081 ± 8e-4
PDGF-BB I30 → A	0.081 ± 1e-3
PDGF-BB I30 → N	0.085 ± 2e-3

to disruption of the active site. In the same manner, incorrect folding of tertiary structure in mutant proteins may disrupt receptor binding regions. As previously described (Craig *et al.*, 1991), the only currently available probe of PDGF-BB tertiary structure is the specific environment of the single tryptophan residue at position 40. This residue displays no near UV-CD signal; however, both steady state and time-resolved fluorescence and anisotropy have been characterized. Tryptophan 40 is surface-exposed, yet exhibits complex emission characteristics which are very sensitive to local environment (Craig *et al.*, 1991). This fluorescence probe has been used to assess whether the functionally defective PDGF mutants exhibit altered tertiary structure.

The fluorescence emission spectra of native PDGF-BB, compared with the unfolded molecule and various mutants with substitutions at residue 30 are shown in Figure 4. The fluorescence emission spectrum of native PDGF (N) has a wavelength of maximum emission (λ_{max}) of 347 nm. On unfolding and reduction of the molecule (U), the intensity decreases (presumably due to quenching by vicinal residues) and λ_{max} red shifts to a higher wavelength (356 nm) consistent with tryptophan 40 being more exposed. All substitutions at position 30 (irrespective of the effect on biological activity) show dequenching of tryptophan emission with no change in the λ_{max}. These data indicate that no major change in tertiary structure has occurred with any of the I30 mutants but that minor side chain rearrangements which influence quenching of tryptophan 40 fluorescence have been caused by all substitutions.

Fluorescence anisotropy measurements yield information on the mobility (rotation) of the bulky tryptophan side chain relative to the overall movement (tumbling) of the whole protein molecule in solution. Information on the dynamic state of the side chain at position 40 in PDGF-BB can therefore be obtained. Despite being exposed on the surface of PDGF-BB, steady state and time-resolved fluorescence anisotropy measurements show the side chain of tryptophan 40 to be rigidly held (Craig *et al.*, 1991). In order to investigate the change in environment of tryptophan 40 further, the mutants containing substitutions at position 30 have been investigated using fluorescence anisotropy.

In the native state, the rate of rotation of the side chain appears to be the same as the rate of tumbling of the whole molecule. On reduction of the disulphide bonds and unfolding in denaturant the rate of rotation increases dramatically (Table IV) though collisions in the unfolded state presumably prevent the free rotation exhibited by *N*-acetyl tryptophanamide (NATA). The steady state anisotropy data for mutants containing substitutions R27 → E and S, I30 → K, N, A and S is shown in Table IV with values for freely rotating NATA standard and native and unfolded PDGF-BB for comparison. All of the substitutions demonstrate the same rotational restriction as wild type. The changes in the environment of tryptophan 40 which result in dequenching of fluorescence emission intensity do not therefore relax the conformational restriction to rotation of the aromatic ring.

The above results demonstrate that substitutions at position 30 which affect the biological activity of PDGF-BB do not disrupt structure formation and, moreover, have not radically altered the tertiary structure of the molecule in so far as it can be determined by the tryptophan 40 probe. The observed changes in biological activity in PDGF-BB mutants do not, therefore, arise from gross structural changes in the protein but, more likely, the nature of the side chain in these molecules is responsible for the altered activity.

Discussion

Key amino acid residues involved in binding and receptor activation have been identified for only a very small number of growth factors and cytokines, such as EGF (Engler *et al.*, 1990), IL-1 (Gehrke *et al.*, 1990) and IL-2 (Zurawski *et al.*, 1990). These studies indicate that multiple contact sites exist between the soluble protein and its receptor, often at some distance from each other on the molecule's surface. The key finding of the present study is two amino acids, arginine 27 and isoleucine 30, that are close neighbours on the PDGF-B chain are involved in receptor recognition. Immediately adjacent amino acids can, however, be altered without substantially modifying the receptor binding properties, suggesting that the polypeptide chain in this region of PDGF exhibits secondary structure appropriate for surface exposure of only arginine 27 and isoleucine 30. The nature of this structure, either helix, or more likely β-turn in view of the low helical content of PDGF (Craig *et al.*, 1991), awaits full solution structure determination. These data are in accord with, and extend substantially the results of, a study published while this report was in preparation which indicates that the region 25−37 contains residues important for the transforming ability of PDGF-BB (Giese *et al.*, 1990).

It is unlikely that this region contains the only receptor binding residues on the PDGF molecule. Loss of binding affinity for both β and α PDGF receptors must have occurred with all of the deficient mutants since binding to only one of the two receptors present on 3T3 cells would be sufficient to initiate a full biological response: the effect of PDGF-AA, which acts only at α receptors, to stimulate both mitogenesis and PI turnover in 3T3 cells, is identical to that of PDGF-BB (Hosang *et al.*, 1989; and unpublished). Moreover, the receptor binding data on the isoleucine 30 and arginine 27 mutants, unlike PDGF-AA, give no indication of partial ligand displacement that might signify receptor selectivity (Figure 3). Preliminary studies on cell lines expressing only the PDGF α receptor (A204 rhabdomyosarcoma) or PDGF β receptor (MBA fibrosarcoma) show isoleucine 30 and arginine 27 mutants produce similar mitogenic responses in each cell line. The most likely conclusion is that R27-I30 of PDGF-B (or R21-V24 on PDGF-A) represents a binding region common to the interaction of the growth factor with both its α and β receptors. It follows, therefore, that one or more other sites defining the receptor selectivities of PDGF-AA and -BB must exist elsewhere on the molecule.

It is noteworthy that no receptor antagonists or even partial agonists were produced during this study. All mutants with altered biological function exhibited reduced receptor affinity but could induce the full biological response found with the wild type molecule. A similar situation is, however, found for other receptors that exhibit tyrosine kinase activity, e.g. EGF, type 1 IGF and insulin where, despite considerable analysis, all mutants so far described are full agonists. Similar studies on cytokines such as IL-1 and IL-2 have, in contrast, readily revealed partial agonists and full antagonists (Gehrke *et al.*, 1990; Zurawski *et al.*, 1990). The reasons for these different general findings remain to

be elucidated. In conclusion, we have identified arginine 27 and isoleucine 30 as important residues involved in receptor recognition of PDGF-BB.

Materials and methods

Mutagenesis and expression of PDGF-BB

Mutations in PDGF were created by oligonucleotide site-directed mutagenesis of a M13mp19 clone containing PDGF-B on a *Hin*dIII–*Bam*HI restriction fragment using the method of Kunkel *et al.* (1987). Following mutagenesis, the PDGF-B mutants were cloned into the expression vector and transformed into *Saccharomyces cerevisiae* strain BJ2168 (*a prc1*-407 *prb1*-1122 *pep4*-3 *leu2 trp1 ura3*-52) by the method of Beggs (1978). All yeast media were as described by Sherman *et al.* (1986). Two independent transformants were picked and used for expression studies. Transformants were grown in synthetic complete medium (SC) minus leucine 1% glucose for 16 h at 30°C. The cells were harvested by centrifugation and inoculated into twice the original culture volume of SC minus leucine 1% galactose 0.2% glucose and grown at 30°C for 6 days. Cells were removed by centrifugation and the culture medium assayed for PDGF activity. As a quality control step, plasmid DNA was rescued from the yeast transformants into *Escherichia coli*. The DNA sequence of the PDGF-B mutant was then determined to ensure the sequence was as originally determined.

ELISA

Yeast supernatants were assayed both in a sandwich and in a competition ELISA. The sandwich assay was developed using two antibodies to PDGF: goat anti-human PDGF-AB (Collaborative Research) and rabbit anti-human PDGF-BB (Genzyme). A 96-well immunoplate (Nunc-Maxisorp) was coated overnight at 4°C with goat anti-PDGF antibody at 5 μg/ml in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6. Remaining protein adsorption sites were blocked by incubation with 0.1% (w/v) casein/PBS for 30 min at 20°C. The plate was then washed three times with 0.05% Tween 20 in PBS. Serial dilutions of the test samples and PDGF standard (c-*sis*, Amersham) were made by titration across the coated plate in PBS/Tween. The plate was incubated for 1 h at 20°C. After washing three times, 50 μl rabbit anti-PDGF antibody (5 μg/ml) was added to each well and incubated for 1 h at 20°C. The plate was washed a further three times followed by addition of HRP-conjugated goat anti-rabbit IgG (Tago), at a dilution of 1/1000, and incubation for 1 h at 20°C. After three washes, the chromogenic peroxidase substrate tetramethylbenzidine (TMB) was added at 100 μg/ml in 0.1 M sodium acetate buffered to pH 6.0 with 0.1 M citric acid with 0.04% (v/v) hydrogen peroxide. When sufficient colour had developed (typically 5–15 min), the reaction was stopped by addition of 2.5 M H_2SO_4 and the absorbance was read at 450 nm (Dynatech MR650 plate reader). The competition ELISA was developed using rabbit anti-human PDGF (Genzyme anti-PDGF-B or R and D Systems anti-PDGF-AB). All washes and dilutions were made using 0.05% (v/v) Tween 20 in PBS. A 96-well immunoplate (Nunc-Maxisorp) was coated with 100 μl of PDGF at 20 ng/ml in PBS, and incubated overnight at 4°C. Remaining protein adsorption sites were blocked by incubation with 0.1% (w/v) casein/PBS for 1 h at 20°C and the plate washed three times. In a separate v-well Titertek plate PDGF standard and samples were diluted in 2-fold serial dilutions at a final volume of 60 μl in PBS/Tween. Rabbit anti-human PDGF antibody, 60 μl of 10 μg/ml, was added to this plate and incubated for 1 h at 20°C. 100 μl was then transferred to the PDGF-coated plate and incubated for 1 h. The plate was washed a further three times followed by addition of HRP-conjugated goat anti-rabbit IgG as described above. After five washes, TMB was added, developed and absorbance quantified as described above.

Receptor binding assay

Receptor binding was carried out on Swiss 3T3 cells in accordance with the method of Williams *et al.* (1986). Amersham c-*sis* [^{125}I]PDGF-BB (50 000–60 000 c.p.m.) was incubated with cells (5×10^4 cells/0.5 ml) in a binding buffer (20 mM HEPES buffered PBS, pH 7.3, with 0.5% BSA) for 90 min at room temperature. Incubations were terminated by rapid filtration onto protamine sulphate coated Durapore HVLP filters, followed by three rapid washes (3 ml each) of the filters with binding buffer at room temperature. Non-specific binding to cells, estimated by the amount of binding in the presence of 200-fold excess unlabelled PDGF-BB (Amersham, c-*sis*), was usually $<10\%$ of the total binding. Unlabelled recombinant human PDGF-AA was obtained from Collaborative Research.

Mitogenic assay

The uptake of [^{3}H]thymidine by 3T3 cells was used as a measure of DNA synthesis. Cells were seeded onto microtitre plates (Falcon) at a density of 2×10^4 per well with DMEM containing 10% FCS. After incubation at 37°C for 6–8 days, when the cells were confluent and quiescent, diluted test samples (yeast supernatant or purified material) were added directly to the wells. After 16 h incubation, the cultures were pulsed with [^{3}H]thymidine (1 μCi/ml) for 6 h. Incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material was determined as described by Raines and Ross (1985). PDGF activity of the test samples was quantified by interpolation from the dose–response curve for a PDGF-BB standard, c-*sis* (Amersham).

Inositol lipid (PI) turnover

Swiss 3T3 cells were seeded into 24-well tissue culture plates (Falcon) at 1×10^5 per well. After 3 days, the cells were pre-labelled for a period of 72 h by adding [^{3}H]myoinositol at 3 μCi/ml directly to the growth medium. The monolayers were rinsed and subsequently incubated for 20 min at 37°C with 0.5 ml per well of Hanks' balanced salt solution (HBSS) containing the 10 mM LiCl to inactivate inositol-1-phosphatase (Berridge *et al.*, 1982). Dilutions of the samples and PDGF standard (c-*sis*, Amersham made up at 25 times the desired concentration) were added to the wells including the appropriate controls (e.g. yeast supernatant without PDGF). After 20 min stimulation, the reaction was stopped by aspiration of the medium and addition of 1 ml ice-cold 5% (v/v) perchloric acid per well. The plates were kept on ice for 30 min to extract the cellular inositol phosphates. Precipitated cellular protein was removed by centrifugation and perchloric acid quantitatively removed from the supernatant solution by the method of Khym (1975). Samples of the neutralized cell extracts (0.7 ml) were diluted to 5 ml with 5 mM potassium tetraborate/0.5 mM EDTA and the accumulated labelled inositol monophosphates were separated by the method of Bone *et al.* (1984) using AG 1-X8 anion exchange resin (Bio-Rad). Column fractions were counted for radioactivity (Beckman LS 5000CE) after mixing with Optiphase scintillant (LKB).

Purification of PDGF-BB

The purification scheme is a modification of the method of Raines and Ross (1982), which describes purification of PDGF from human platelets. Yeast supernatant (5–6 l) was clarified by centrifugation at 8600 *g* for 20 min at 4°C. The pH was adjusted to 6.0 with 20 mM Tris prior to 5- to 10-fold concentration by tangential flow ultrafiltration (Millipore Minitan, PTGC 10 000 membranes). The molarity of the concentrated supernatant was adjusted to 0.09 M with NaCl before chromatography on a 200 ml CM-Sepharose column (Pharmacia, 5 × 10 cm). The column was washed with 2–3 vol of 0.19 M NaCl, 20 mM Tris–HCl, pH 6.0, and fractions containing PDGF-BB were eluted with 1 M NaCl, 20 mM Tris–HCl, pH 7.4. The eluate was applied directly to a 20 ml phenyl-Sepharose column (Pharmacia, 1.6 × 10 cm) equilibrated in the same buffer, followed by washing with 60 ml of 1 M NaCl, 20 mM Tris–HCl, pH 7.4. PDGF-BB was eluted with 40–60 ml of 50% (v/v) ethylene glycol, 0.15 M NaCl, 20 mM Tris–HCl, pH 7.4. All chromatography steps were performed at 4°C. Fractions containing PDGF, assessed by ELISA or mitogenic assay, were pooled and stored at −20°C for up to 3 months.

For structural analysis, PDGF was transferred from phenyl-Sepharose elution buffer into 10 mM acetic acid using a two-step size exclusion chromatography process. The first step, using a 250 ml Sephadex G-25M column (Pharmacia, 5 × 12.5 cm), transfers PDGF into 0.5 M urea, 10 mM glycine–HCl, pH 3.0. Protein fractions from this column were pooled and applied to a further 250 ml Sephadex G-25M column and eluted with 10 mM acetic acid. Fractions containing PDGF-BB were pooled, concentrated by lyophilization and stored at −20°C.

The protein concentration was determined by absorbance at 280 nm. The specific absorption coefficient for PDGF-BB was calculated as 0.46 l/g/cm.

Circular dichroism spectra

Spectra in the 190–250 nm wavelength range were measured using a 0.1 mm pathlength and 2 nm bandwidth at 20°C. Protein samples were dissolved at a concentration of 0.357–0.867 mg/ml in 10 mM acetic acid. Spectra were analysed using the CONTIN program (Provencher and Gloeckner, 1981; Provencher, 1982); the standard error for the data is 1–3% for all of the samples.

Fluorescence anisotropy

Steady state fluorescence anisotropy was measured using a Perkin-Elmer LS-50 with polarizer attachment. Anisotropy at 347 nm (5 nm slit) was measured with sample excitation at 280 nm (5 nm slit) using a 1 cm pathlength at 20°C. Samples were dissolved at ~0.05 mg/ml in 10 mM acetic acid unless otherwise stated.

Acknowledgements

We would like to thank Dr Ken Brown for the frequent supply of Swiss 3T3 cells, Dr Mick Hunter for help in oligonucleotide design and Lindsay Stunt for assistance in preparing the manuscript.

References

Beggs,J.D. (1978) *Nature*, **275**, 104–109.
Berridge,M.J., Downes,C.P. and Hanley,M.R. (1982) *Biochem. J.*, **206**, 587–595.
Betzholtz,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. (1986) *Nature*, **320**, 695–699.
Bone,E.A., Fretten,P., Palmer,S., Kirk,C.J. and Michell,R.H. (1984) *Biochem. J.*, **221**, 803–811.
Cook,A.L., Kirwin,P.M., Craig,S., Bawden,L.J., Green,D.R., Price,M.J., Richardson,S.J., Fallon,A., Drummond,A.H., Edwards,R.M. and Clements,J.M. (1991) *Biochem. J.*, **280**, in press.
Craig,S., Hollecker,M., Creighton,T.E. and Pain,R.H. (1985) *J. Mol. Biol.*, **185**, 681–687.
Craig,S., Clements,J.M., Cook,A., Dryden,D.T., Green,D.R., Heremans,K., Kirwin,P., Price,M.J. and Fallon,A. (1991) *Biochem. J.*, **280**, in press.
Deuel,T.F., Senior,R.M., Huang,J.S. and Griffin,G.L. (1982) *J. Clin. Invest.*, **69**, 1046–1049.
Engler,D.A., Montelione,G.T. and Niyogi,S.K. (1990) *FEBS Lett.*, **271**, 47–50.
Escobedo,J.A. and Williams,L.T. (1988) *Nature*, **335**, 85–87.
Gehrke,L., Jobling,S.A., Paik,L.S.K., McDonald,B., Rosenwasser,L.J. and Auron,P.E. (1990) *J. Biol. Chem.*, **265**, 5922–5925.
Giese,N., LaRochelle,W.J., May-Siroff,M., Robbins,K.C. and Aaronson,A. (1990) *Mol. Cell. Biol.*, **10**, 5496–5501.
Hammacher,A., Mellstrom,K., Heldin,C.-H. and Westermark,B. (1989) *EMBO J.*, **3**, 2489–2495.
Heldin,C.-H. and Westermark,B. (1989) *Br. Med. Bull.*, **45**, 453–464.
Heldin,C.-H., Backstrom,G., Ostman,A., Hammacher,A., Ronnstrand,L., Rubin,K., Nister,M. and Westermark,B. (1988) *EMBO J.*, **7**, 1387–1393.
Hosang,M., Rouge,M., Wipf,B., Eggimann,B., Kaufmann,F. and Hunziker,W. (1989) *J. Cell. Physiol.*, **140**, 558–564.
Jimenez de Asua,L., Rozengurt,E. and Dulbecco,R. (1974) *Proc. Natl. Acad. Sci. USA.*, **71**, 96–98.
Johnsson,A., Heldin,C.-H., Wasteson,A., Westermark,B., Deuel,T.F., Huang,J.S., Westenburg,P., Gray,A., Ullrich,A., Scrace,G., Stroobant,P. and Waterfield,D.M. (1984) *EMBO J.*, **3**, 921–928.
Khym,J.X. (1975) *Clin. Chem.*, **21**, 1245–1252.
King,C.R., Giese,N.A., Robbins,K.C. and Aaronson,S.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5295–5299.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Kurjan,J. and Herskowitz,I. (1982) *Cell*, **30**, 933–943.
LaRochelle,W.J., Giese,N., May-Siroff,M., Robbins,K.C. and Aaronson, S.A. (1990) *Science*, **248**, 1541–1544.
Nister,M., Hammacher,A., Mellstrom,K., Siegbahn,A., Ronnstrand,L., Westermark,B. and Heldin,C.-H. (1988) *Cell*, **52**, 791–799.
Parker,J.M.R., Cro,D. and Hodges,R.S. (1986) *Biochemistry*, **25**, 5425–5432.
Provencher,S.W. (1982) *Comput. Phys. Commun.*, **27**, 229–242.
Provencher,S.W. and Gloeckner,J. (1981) *Biochemistry*,**20**, 33–37.
Raines,E.W. and Ross,R. (1982) *J. Biol. Chem.*, **257**, 5154–5160.
Raines,E.W. and Ross,R. (1985) *Methods Enzymol.*, **109**, 749–773.
Seifert,R.A., Hart,C.E., Phillips,P.E., Forstrom,J.W., Ross,R., Murray,M.J. and Bowen-Pope,D.F. (1989) *J. Biol. Chem.*, **264**, 8771–8778.
Seppa,H., Grotendorst,G., Seppa,S., Shiffman,E. and Martin,G.R. (1982) *J. Cell Biol.*, **92**, 584–588.
Severinsson,L., Ek,B., Mellstrom,K., Claesson-Welsh,L. and Heldin,C.-H. (1990) *Mol. Cell. Biol.*, **10**, 801–809.
Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sjolund,M., Hedin,U., Sejersen,T., Heldin,C.-H. and Thyberg,J. (1988) *J. Cell Biol.*, **106**, 403–413.
Stroobant,P. and Waterfield,M.D. (1984) *EMBO J.*, **3**, 2963–2967.
Williams,R.W. (1986) *Methods Enzymol.*, **130K**, 311–331.
Zurawski,S.M., Imler,J.-L. and Zurawski,G. (1990) *EMBO J.*, **9**, 3899–3905.

Received on July 8, 1991; revised on September 12, 1991