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Production of platelet-derived growth factor receptor (PDGFR-β) in *E. coli*

Mapping ligand binding domain

B.C. Rooney*, M. Hosang, W. Hunziker

Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4002 Basel, Switzerland

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Abstract

Portions of the extracellular domain of the platelet-derived growth factor receptor β (PDGFR-β) were expressed as fusion proteins with a hexa His tag in *E. coli*. Following purification by Ni chelate chromatography, the recombinant receptors were tested in cross-competition studies with ^{125}I-labelled PDGF-AA and -BB. Although of lower affinity than the native receptor (IC_{50} values of 10^{-8} M) the recombinant molecules retained ligand binding specificity and neutralised the mitogenic effect of PDGF-BB. These data indicate that the ligand binding region lies within the first four immunoglobulin-like domains on PDGFR-β. This *E. coli* expression system could be further used as a rapid and economical means to produce mutated receptors and map the ligand binding domain.

Key words: Platelet-derived growth factor receptor β; *E. coli* expression: Immunoglobulin-like domain

1. Introduction

Platelet derived growth factor (PDGF) is an important chemoattractant and mitogen for cells of mesenchymal origin and is thought to play an important role in embryogenesis and wound repair. PDGF has also been implicated in a number of pathological processes such as arteriosclerosis, tumourogenesis, glomerulonephritis, and rheumatoid arthritis (reviewed in [1]). PDGF is a disulphide-linked dimer of two related polypeptide chains, designated A and B, which are assembled as heterodimers (PDGF-AB) or homodimers (PDGF-AA or PDGF-BB) [2]. All three possible isoforms have been isolated and shown to bind with different affinities to two different but related receptor molecules, designated PDGFR-α and PDGFR-β [3,4]. The PDGFR-α binds all three isoforms of PDGF, while PDGFR-β exhibits high-affinity binding only for PDGF-BB [5]. Ligand-induced dimerisation of the PDGFRs has been shown to activate the receptor's intrinsic intracellular tyrosine kinase domains [6] which subsequently leads to phosphorylation of target intracellular signalling molecules and mediation of a cellular response [1].

Major advances have been made in our understanding of the structure of the PDGF ligand by solving the crystal structure [7], and the sites of interaction with the receptor by site-directed mutagenesis [8–10], but it is still unclear how the receptor interacts with its anti-parallel dimeric ligand.

The extracellular domains of both receptors exhibit a similar pattern of cysteine spacing which putatively subdivides them into five immunoglobulin-like domains (D1-D5) Fig. 1 [11]. Work carried out on PDGFR-α implicated the N-terminal IgG-like domains (D1–D3) as specifying binding of PDGF-AA [12,13] but little is known about binding to the β receptor. Although both the α and β receptors have similar domain arrangements, a specific antagonist (neomycin) for PDGF-BB binding to α did not affect binding to β [14].

In order to understand the nature of PDGF binding to the β receptor, various fragments of the extracellular domain of PDGFR-β were expressed in *E. coli*. Following purification the binding ability of these recombinant receptors was examined in cross-competition experiments with cell-bound receptors. Results obtained indicate areas of importance for ligand binding on the PDGFR-β molecule.

*Corresponding author. *Present address:* Research School of Biosciences, University of Kent at Canterbury, Kent, CT2 7NJ, UK. Fax: (44) 227 763912.

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2. Materials and methods

2.1. Construction of recombinant E. coli *expression vectors*

Fragments of the PDGFR-β cDNA (kindly provided by M. Murray, Zymogenetics, Seattle, USA [15]) were cloned into *Bam*HI site on the expression plasmid pDS78/RBSII [16] which contains an inducible promoter, and generates fusion proteins with six histidine residues at the amino terminal. The PDGFR-β fragments I and II were generated by digesting the cDNA with *Sma*I (which removes codons for the signal sequence at the 5′ end) and *Sph*I and *Fsp*I at the 3′ end respectively (Fig. 1). Construct rβIII was also produced by *Sma*I digestion at the 5′ end but a new *Hin*dIII site was introduced by site directed mutagenesis at nucleotide position 1957 (Zymogenetics, Seattle, US) and resulted in a fragment of DNA coding for the full extracellular domain of the receptor.

2.2. Expression of recombinant PDGFR-β

E. coli M15 cells containing the plasmid pDMI,1 (lac repressor) were transformed with the receptor constructs rβI, rβII and rβIII. The cells were grown to an OD_{600} of O.7, when protein production was induced by IPTG addition (2 mM) and incubated for a further 4 h before harvesting as described by Hochuli et al. [16]. The recovered biomass was lysed overnight at room temperature in 6 M guanidine hydrocloride in 0.1 M sodium phosphate buffer pH 8. Following centrifugation ($4{,}000 \times g$, 10 min, 4°C) the resultant supernatant was applied to a nickel chelate column. The chromatography was performed in 8 M Urea , 0.1 M sodium phosphate buffer and was developed with a pH step gradient (load crude extract – pH 8, wash steps – pH 8 and pH 6, product elution – pH 4). Collected fractions were dialysed against PBS, analysed by SDS-PAGE and tested for activity.

2.3. Biological activity of recombinant receptors (rβ)

The ability of recombinant PDGF receptors rβI, rβII and rβIII to bind PDGF-AA and -BB was measured by cross-competition with native receptors on Swiss 3T3 cells for [^{125}I]PDGF binding using standard protocols [17]. In brief, the cells were incubated simultaneously with *E. coli* derived receptors and [^{125}I]PDGF-AA or -BB, and specific binding was determined after 3 h at 4°C. Non-specific binding was determined using 50 μg/ml of the unlabelled PDGF isoform.

The inhibition of the mitogenic effects of PDGF-BB and PDGF-AA ligands, and epidermal growth factor (EGF) by recombinant receptors was carried out as described previously [18]. Swiss 3T3 cells were incubated with PDGF-AA, -BB or EGF for 20 h at 37°C when DNA synthesis was measured by [^{3}H]thymidine incorporation over 2 h. In parallel rβI or rβII was incubated with the cells and mitogens, and neutralisation of the of mitogenic effect examined.

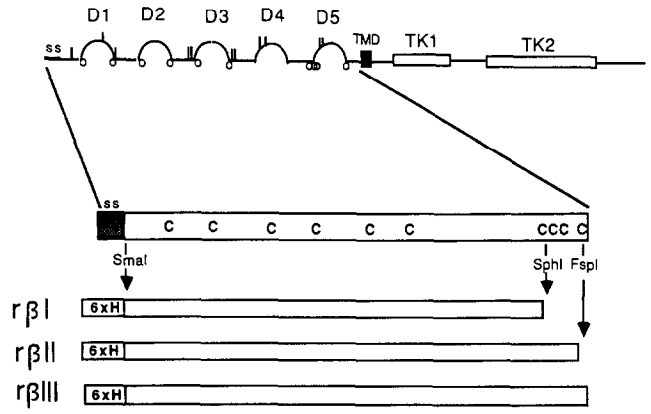

Fig. 1. Domain arrangement of PDGF-receptor β and recombinant constructs rβI-III. ss = signal sequence, D1-D5 = extracellular IgG-like domains [ ], TMD = trans-membrane domain, TK1 + TK2 = split tyrosine kinase domains, o and C = conserved cysteine residues, I = potential sites for N-linked glycosylation, 6 × H = hexa histidine tag, sites of restriction digest indicated by arrows

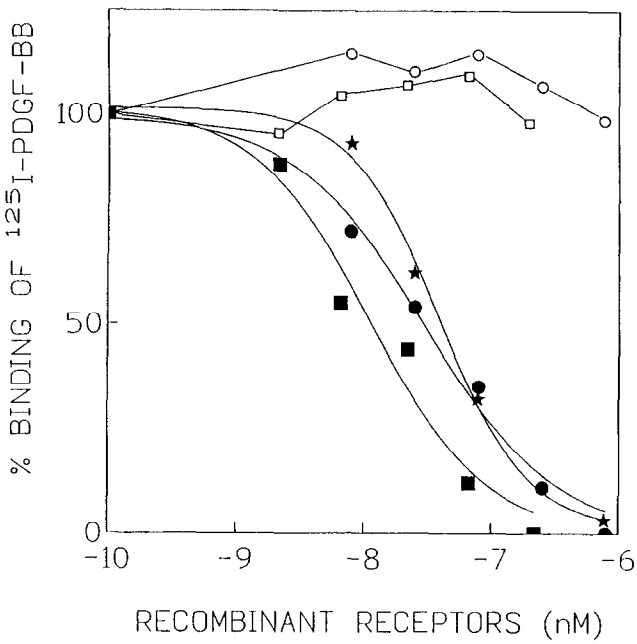

Fig. 2. Cross-competition binding of [^{125}I]PDGF to Swiss 3T3 cells. Binding of [^{125}I]PDGF-BB was competed by increasing amounts of rβI (■), rβII (●), rβIII (★) or [^{125}I]PDGF-AA by increasing amounts of rβI (□), rβII (○) and bound radioactivity was measured in triplicate as described in Section 2. Non-specific binding of [^{125}I]PDGF-AA or -BB was determined by the addition of 50 μg/ml unlablled PDGF-AA or -BB, respectively. Relative specific binding is plotted as percent of specific binding of labelled PDGF-BB in the absence of unlabelled PDGF.

3. Results and discussion

Although much progress has been made in our understanding of the sites of interaction between the activated tyrosine kinase domain of the PDGF receptor and its downstream target signalling molecules [1,19] less is known about the binding of the PDGF ligand to the extracellular domain of the receptor and the mechanism of dimerisation. Work with chimeric receptors indicate that the first three IgG-like domains (D1–3) of PDGFR-α contain PDGF-AA binding determinants and deletion of IgG-like domain 2 implicates this domain in high-affinity binding [12,13]. Indeed recent experiments carried out on recombinant PDGFR-α and PDGFR-β (expressed in insect cells) has suggested the presence of two binding sites for PDGF on the receptors [5]. In combination with recent information on the structure of the PDGF ligand [7] a new model for receptor-ligand interaction was suggested [5]. Rather than one receptor subunit intracting with only one chain of the PDGF dimer [20], the subunit may interact with domains on both chains of the anti-parallel ligand dimer but more detailed structural information is required to determine this. In this study fragments of the extracellular domain of PDGFR-β were expressed in *E. coli* in an attempt to understand more fully its binding to its ligand.

Using convenient restriction sites in the PDGFR-β cDNA (Fig. 1) two truncated (rβI, rβII) and one full-length (rβIII) extracellular portions of the receptor were expressed as fusion proteins to six histidines in *E. coli*, and purified. The apparent molecular masses of 43 kDa for rβI, 50 kDa for rβII, and 53 kDa for rβIII (Fig. 4A) were as predicted from published cDNA sequence [15]. By carrying out cross-competition experiments with native cell bound receptors on Swiss 3T3 cells the ability to bind PDGF-BB ligand specifically and not PDGF-AA was demonstrated (Fig. 2). IC_{50} values of 1, 3 and 4 $\times 10^{-8}$ M for PDGF-BB were calculated for rβI, rβII, and rβIII, respectively.

The ability to inhibit specifically the mitogenic effect of PDGF-BB and not the effect of PDGF-AA or the unrelated mitogen epidermal growth factor (EGF) on Swiss 3T3 cells can be seen in Fig. 3 (rβIII not shown). These results indicate that fragments of PDGFR-β expressed in *E. coli* still retain the same ligand binding specificity as native PDGFR-β and only recognise PDGF-BB [15].

The lower affinity of these recombinant receptors when compared to a construct similar to rβIII expressed in insect cells (4.8×10^{-9}M) [5] may be due to differences in post-translational processing between prokaryotic and eukaryotic expression systems. There are eleven potential glycosylation sites on the extracellular domain of PDGFR-β (nine on rβI) and as oligosaccharides are known to play an important role in many molecular recognition events [21] their absence from the recombinant *E. coli* molecules may lower the affinity. Also at high local concentrations PDGFR extracellular domains have been shown to oligomerise even in the absence of ligand [5]. When rβI, rβII and rβIII are examined under non reducing conditions (Fig. 4B) aggregates were apparent which may be due to oligomerisation and/or improper disulphide bond formation. These aggregates may affect the affinity of the receptor for its ligand, or produce a mixed population of improperly folded inactive aggregates and properly folded high affinty molecules at low concentration, which could also be responsible for the lower affinity of the fragments expressed in *E. coli*.

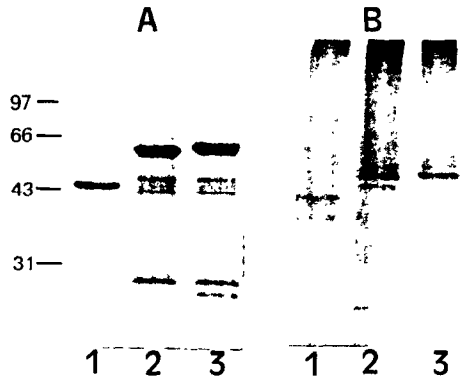

Fig. 4 . SDS/PAGE of recombinant receptors. Recombinant proteins rβI = lane 1, rβII = lane2, rβIII = lane 3 were electrophoresed in 10% polyacrylamide gel in the presence A, or absence B of reducing agent. Molecular weight standards are as indicated.

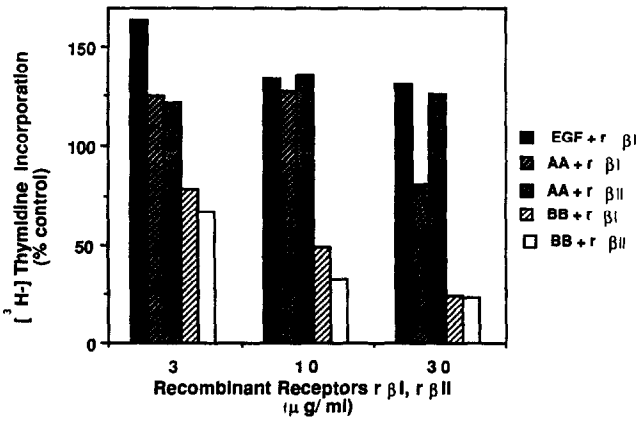

Fig. 3. Effect of recombinant receptors on stimulation of DNA synthesis in Swiss 3T3 cells by PDGF-AA,-BB, or EGF. [^{3}H]Thymidine incorporation was measured following exposure of cells to 1 ng/ml of each growth factor as described in section 2. Increasing amounts of recombinant receptors were added and percent reduction/increase in stimulation was calculated.

It is interesting to note that the truncated extracellular forms of the receptor rβI and rβII, display a similar binding affinity as the complete extracellular form rβIII. In the case of rβI the immunoglobulin like domain D5 is absent as depicted in Fig. 1. This would imply that this domain is not essential for ligand binding and that domains 1–4 play a major role in ligand-receptor interaction and specificity. This has also been indicated in studies using chimeras of the α and β receptors which maps specificity for PDGF-AA to these domains on the a receptor [12]. Interestingly a truncated form of the murine β receptor which lacks domains 1–3 has been reported and is postulated to act in a developmentally regulated manner even in the absence of ligand [22].

This work demonstrates that it is possible to produce soluble forms of the PDGFR-β in *E. coli* which display characteristics of the native cell-bound receptor and which preserve the ligand binding specificity for PDGF-BB as predicted for the full-length receptor [15]. The lower affinity may be due to improper post-translational processing of the recombinant receptors in *E. coli* but these molecules may also lack domains which are required for stable high affinity binding and dimerisation. However this work suggests that this expression system could be further used as a rapid means to produce mutated and defined regions of the extracellular domain of the PDGF receptors and map the sites of ligand interaction.

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