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Identification of individual amino acids in platelet-derived growth factor that contribute to the specificity towards the β-type receptor

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Platelet-derived growth factor constitutes a family of three isoforms (PDGF-AA, -AB, and -BB) composed of two homologous polypeptide chains (A and B). These isoforms interact with two types of receptors termed α or β. Whereas PDGF-AA binds only to the α-receptor, PDGF-BB binds and activates both receptors with high affinity. To map regions that are specific for the β-receptor, we introduced mutations into PDGF-AA located in previously identified epitopes [1991, Biochemistry 30, 3303–3309]. A single amino acid exchange in domain II of PDGF-AA (Ala^{67}→Arg) was sufficient to bring about a reduced but significant activation of the β-receptor. In domain I the exchange of residues Pro^{26}→Arg together with Ser^{28}→Asn switched the specificity towards the β-receptor. These data indicate that parts of the exposed domains are indeed involved in receptor binding. Since these single mutations lead to mutant proteins which are about 100-fold less active than PDGF-BB, it is suggested that other amino acid residues also participate in the binding to the receptors.

Growth factor; Mutagenesis; Receptor; Binding site

1. INTRODUCTION

Platelet-derived growth factor (PDGF) is a major mitogen in serum which promotes the proliferation of fibroblasts and smooth muscle cells in vitro [1–3]. The existence of two homologous PDGF chains termed A and B gives rise to the formation of three different dimeric forms: AA, AB and BB. All these forms exist in nature with variable abundance. In the A- and B-forms, approximately 60% of the amino acid residues are conserved and the highest similarity is found in the center of the molecule comprising the eight cysteine residues which are strictly conserved among the two chains A and B [1–3]. Modification or removal of any of these cysteine residues abolished the formation of dimers [4–7]. The fact that only dimers are biologically active, along with the discovery of two different PDGF-receptors (α and β), led to the hypothesis that one dimeric PDGF may bind two receptor molecules. The model predicts that PDGF-AA binds only α-type receptors, PDGF-AB binds one α-type and one β-type receptor, and PDGF-BB binds mainly β-type receptors. The two receptors share a common structure including an extracellular part that is composed of five immunoglobulin-like domains and a cytoplasmic portion that includes split tyrosine kinase sequences [8–11].

We have recently identified by trypsin treatment two internal surface exposed domains (I and II). Similar segments from both PDGF-AA or -BB were liberated without dissociation of the dimer [6,7]. The remaining resistent 'core' was devoid of biological activity suggesting that domains I and II are involved in receptor binding. Here we describe that mutations in these domains indeed alter the specificity of the PDGF isoforms towards the two receptors.

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2. MATERIALS AND METHODS

PDGF isoforms were prepared as described [12,13]. Growth media were from Gibco; IGF-I was from Bachem. All other chemicals were of highest available purity. Site directed mutagenesis was performed according to [7,13]. To reduce the number of mutation reactions and subcloning steps, the second strand synthesis of U-containing phage DNA was performed with a mixture of primers (22/23 and 26/28, 67, 80 and 92). The numbers refer to the mutated amino acid residues in the sequence shown in Fig. 1. About 20 plaques were chosen from each reaction for DNA sequencing. Phages with the desired mutations were processed as described [7,13]. Proteins were expressed and purified to homogeneity using modified standard protocols. Circular dichroism measurements were done with a Jobin Yvon CD 6 instrument. Growth promoting activity, radioiodination, and PDGF-AA binding to AKR 2B cells were determined as described [7,12,13].

3. RESULTS

Figure 1 describes the mutant proteins chosen for this study. Within the trypsin-sensitive domain I only five amino acids are different between PDGF-AA and -BB. We used two oligonucleotide primers, each of which altered two residues (i.e. residues 22/23 and 26/28). Ap-

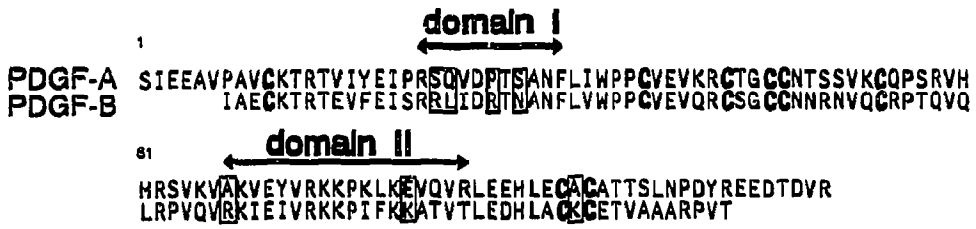

Fig. 1. Comparison of the two PDGF sequences. Bold letters indicate the eight conserved cysteine residues; boxes indicate mutations introduced into PDGF-A. Throughout all experiments residues in PDGF-A were exchanged into the corresponding ones in PDGF-B. The two internal trypsin-sensitive domains are indicated by arrows.

plication of both primers gave a mutant protein 22/23/26/28 in which all residues in domain I except valine[24] were derived from PDGF-B sequences. Valine[24] was left unchanged because of its high structural similarity to isoleucine.

In domain II, neutral or acidic residues in the sequence of PDGF-A were converted into the corresponding basic once of PDGF-BB. By using a mixture of the three oligonucleotide primers, all possible combinations of mutations were obtained.

We have chosen this approach to limit the number of mutant proteins to be prepared, since our aim was to use homogeneous proteins. This should make supplementary analysis possible, e.g. CD-measurements. Furthermore the protein content can be measured accurately and thus a quantitative analysis of the effects can be performed. All mutant proteins were prepared using the standard protocol [13]. In the final purification step of the dimeric protein, in some cases the ionic strength of the buffers had to be adjusted according to the higher isoelectric point of the mutant protein. CD-spectra of all mutant proteins showed the typical curve described for all PDGF isoforms. Spectra of two mutant proteins together with that from PDGF-AA are shown in Fig. 2. In muteine 22/23/26/28 almost the entire domain I of PDGF-AA was exchanged, in muteine 80 only one residue in domain II was altered. Despite the different biological specificities of the proteins (see below) all spectra are very similar excluding major changes in the secondary structure and thus also in the tertiary structure. Therefore the synthesized mutant proteins were properly folded and the measured effects should be related to the properties of the individual amino acid side chains rather than to a conformational change in the protein.

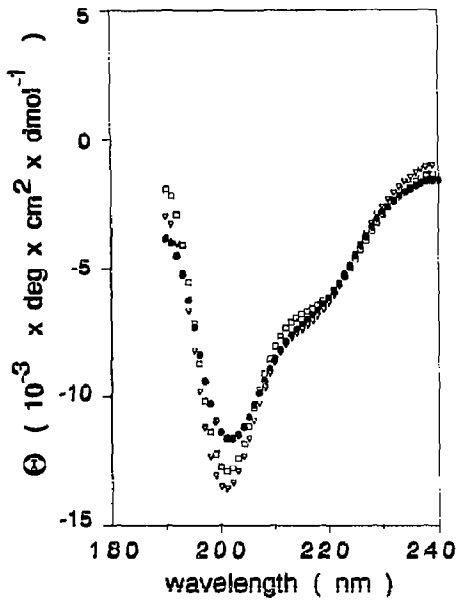

Fig. 2. Circular dichroism spectra of PDGF-AA and two mutant proteins. Circular dichroism spectra were recorded in the range from 190 to 240 nm. ●, PDGF-AA; □, 22/23/26/28; ▽, 80.

AKR-2B cells respond well to all PDGF-isoforms provided insulin at high concentration (3 μg/ml) is present [13]. Without insulin, which can be replaced by low concentrations of insulin-like growth factor I (IGF-I) (15 ng/ml), only PDGF-BB and -AB stimulated DNA-synthesis (Fig. 4). Remarkably the binding of [^{125}I]PDGF-AA was not changed after a preincubation with IGF-I (Fig. 3). The ED_{50} values for PDGF-BB are in the range 2–5 ng/ml, whereas at least 1 μg/ml PDGF-AA is required for a detectable [^{3}H]thymidine incorporation. Our system provides a sensitivity range of about 500-fold, and therefore allows the detection of subtle effects. Furthermore, by inclusion of IGF-I it can be demonstrated that a mutant is still biologically active, i.e. it still activates the α-receptor.

Replacement of residues 22/23 did not alter the specificity. The mutant protein was inactive in the absence of IGF-I and had a similar effect like PDGF-AA in the presence of IGF-I. Changing residues 26/28 resulted in a PDGF-mutant that exhibited significant activity (115 ng/ml) in the absence of IGF-I, indicating that it had acquired PDGF-BB-like properties. The combination of both mutations, 22/23/26/28 did not further increase the specific activity, supporting the previous finding that only residues 26/28 define specificity towards the β-receptor.

Out of the mutant proteins in domain II, only those containing Arg^{67} show significant activity (195 ng/ml or 400 ng/ml). There might be a very weak effect with

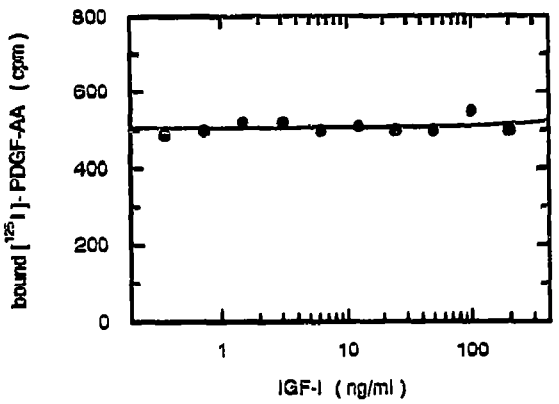

Fig. 3. IGF-I preincubation does not alter the binding of [^{125}I]PDGF-AA binding to AKR 2B cells. Cells were incubated with the indicated concentrations of IGF-I for 90 min at 37°C. [^{125}I]PDGF-AA was then added at 0.2 nM (50% saturation corresponding to its K_D). Bound [^{125}I]PDGF-AA was determined as described [7,13].

mutant proteins 80 or 92. But by extrapolation it was estimated that the ED_{50} values are about 50–100-fold higher than those for mutants 26/28 or 67. The single amino acid exchange Ala^{67}→Arg was sufficient to alter the specificity towards the β-type receptor.

4. DISCUSSION

Numerous attempts have been made to identify those residues in the PDGF molecule that are in contact with its receptor. 'Epitope-sided' peptides exhibited little if any affinities and also peptide-directed antibodies recognized only reduced denatured PDGF. There is only one example where the binding site of a monoclonal antibody was traced back to a peptide segment. Interestingly enough, this antibody partially recognized domain I [14].

Two genetic approaches succeed into identifying regions in the PDGF molecule that are important for biological activity: firstly the construction of chimeric PDGFs, and secondly scanning deletion mutagenesis [15–17]. Both methods identified domain I as a constituent of the binding site. So far no data obtained with pure PDGF mutant proteins have been presented.

Our approach was to express and purify sufficient amounts of mutant proteins to allow biochemical as well as biophysical studies. This approach allows only a limited number of proteins to be prepared and analyzed. Therefore, we first identified the exposed residues in PDGF-AA and -BB. As expected, due to the high similarity of the two chains, similar amino acid stretches of the two isoforms were found to be exposed [6,13]. Two internal domains (I and II) and the C-terminus were accessible to trypsin. Since N-terminal sequences ahead of the first cysteine residue and C-terminal residues behind the last cysteine are not important for biological activity [18] an important role for the two internal domains was suggested. Interestingly, these domains are located in segments with the lowest similarity indicating that specificity might reside in these parts of the sequences.

The corresponding amino acid to Phe^{23} in PDGF-B is a Tyr in PDGF-A. We have constructed a mutant of PDGF-B containing a tyrosine at this position. This mutant can be specifically radioiodinated at this single tyrosine without loss of biological activity [13]. Since the

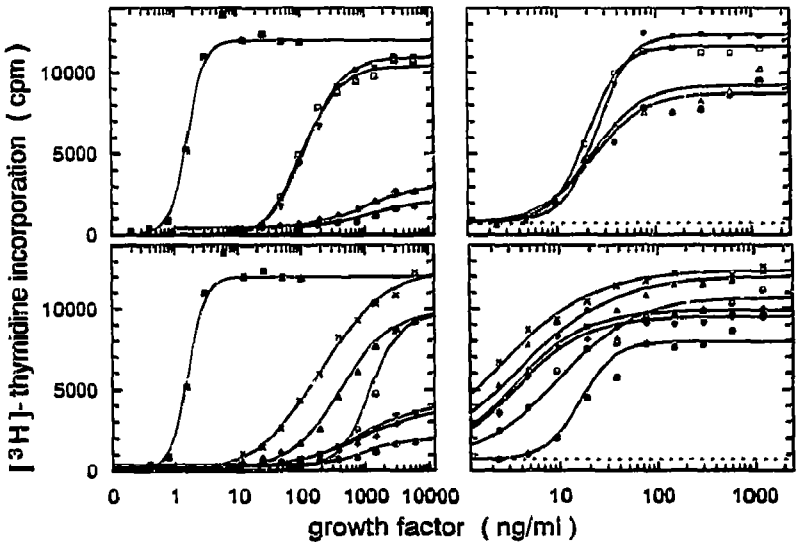

Fig. 4. Biological activities of PDGF isoforms and mutants. The effect of PDGF mutants on DNA synthesis in AKR-2B mouse fibroblasts was measured. In the left diagrams IGF-I was omitted, in the right diagrams IGF-I was present at 15 ng/ml. ED_{50} values (ng/ml) are given in parentheses: first value: without IGF-I, the second one: in the presence of IGF-I. Several proteins did not stimulate DNA synthesis to a significant extent. This is indicated by an ED_{50} value >1000; n.d. = not determined. ●, PDGF-AA (>1,000, 22); ■, PDGF-BB (1.6, n.d.); △, 22/23 (>1,000, 22); ▼, 26/28 (115, 25); □, 22/23/26/28 (100, 20); ▲, 67 (400, 4); ▽, 80 (>1,000, 4); +, 92 (>1,000, 3.5); ×, 67/92 (195, 2.5); ○, 67/80/92 (>1,000, 10).

introduction of a bulky iodine certainly results in steric hindrance, we conclude that this part of the molecule does not interact with the receptor.

Mutations in domain I at position 26/28 brought about a considerable change in specificity. Previous reports by Östman et al. [17] demonstrated that residue 28 (Ser in PDGF-A and Asn in PDGF-B) is the important one. In agreement with all available data residues 22/23 are not important for specificity. Domain I is followed by a segment containing the five central cysteine residues. This segment is neither accessible to proteases nor is there any other indication for a participation in a binding epitope.

Whereas the involvement of domain I seems to be established, there are conflicting results concerning domain II. By using chimeric proteins and scanning mutagenesis Aaronson and coworkers detected binding activity only in domain I [15,16]. By using chimeric PDGF molecules in combination with site-directed mutagenesis of individual amino acids, Heldin and coworkers [17] demonstrated that the exchange of residues 67 (Ala→Arg) and 71 (Tyr→Ile) alter the specificity. The reason for this discrepancy might be related to different assay systems. In Aaronson's system, ligand and receptor are expressed in the same cell. It is thus not possible to extract quantitative data, since the concentrations of ligand and receptors are unknown. Heldin and coworkers used partially purified preparations and binding competition experiments against radioiodinated PDGF-AA or -BB. This approach allowed some quantification though the determination of the concentration of a mutant protein remained difficult. Unfortunately their expression system did not allow the preparation of single mutated proteins, and most conclusions are derived from chimeric constructs into which additional mutations were introduced. In agreement with [17] our results identify identical residues which are important to discriminate between α- and β-type receptors. We believe that these residues directly interact with the receptor, since as demonstrated by CD-spectroscopy the structure of the mutant proteins did not differ from that of PDGF-AA. Other amino acids must also interact with the receptors, since replacement of single amino acid residues as shown here and in [17] does not generate full PDGF-BB-like activity. But almost certainly PDGF exposes a discontinuous epitope explaining the failure to generate peptide-directed antibodies reactive against native PDGF and the extreme low affinities of short peptides derived from the sequence of PDGF. It remains to be identified by X-ray analysis if the two domains are close together, forming one epitope, or if they are located on distinct areas of the PDGF-molecule.

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REFERENCES

[1] Ross, R., Raines, E.W. and Bowen-Pope, D.F. (1986) Cell 46, 155–169.
[2] Deuel, T.F., Tong, B.D. and Huang, J.S. (1985) Curr. Top. Cell. Regul. 26, 51–64.
[3] Heldin, C.-H. and Westermark, B. (1984) Cell 37, 9–20.
[4] Giese, N.A., Robbins, K.C. and Aaronson, S.A. (1987) Science 236, 1315–1318.
[5] Sauer, M.C. and Donoghue, D.J. (1988) Mol. Cell. Biol. 8, 1011–1018.
[6] Vogel, S. and Hoppe, J. (1989) Biochemistry 28, 2961–2966.
[7] Jaumann, M., Hoppe, V., Tatje, D., Eichner, W. and Hoppe, J. (1991) Biochemistry 30, 3303–3309.
[8] 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) Science 240, 1529–1531.
[9] Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M. and Westermark, B. (1988) EMBO J. 7, 1387–1393.
[10] 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.
[11] Heldin, C.H. and Westermark, B. (1989) Trends Genet. 5, 108–111.
[12] Hoppe, J., Weich, H.A. and Eichner, W. (1989) Biochemistry 28, 2956–2960.
[13] Hoppe, J., Weich, H.A., Eichner, W. and Tatje, D. (1990) Eur. J. Biochem. 187, 207–214.
[14] Larochelle, W.J., Giesene, N., Maysiroff, M., Robbins, K.C. and Aaronson, S.A. (1990) Science 248, 1541–1544.
[15] Giese, N., Larochelle, W.J., Maysiroff, M., Robbins, K.C. and Aaronson, S.A. (1990) Mol. Cell. Biol. 10, 5496–5501.
[16] Heidaran, M.A., Pierce, J.H., Jensen, R.A., Matsui, T. and Aaronson, S.A. (1990) J. Biol. Chem. 265, 18741–18744.
[17] Östman, A., Andersson, M., Hellman, U. and Heldin, C.H. (1991) J. Biol. Chem. 266, 10073–10077.
[18] Sauer, M.K., Hannink, M. and Donoghue, D.J. (1986) J. Virol. 59, 292–300.