## Title

Mapping the ligand-binding regions of the platelet-derived growth factor beta-receptor (PDGF[beta]r) and the macrophage colony stimulating factor receptor (M-CSFr)



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# Mapping The Ligand-Binding Regions Of The Platelet-Derived Growth Factor Beta-Receptor (PDGFβr) And The Macrophage-Colony Stimulating Factor Receptor (M-CSFr)

by

Michael Joseph Pazin

**DISSERTATION**

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**in the**

**GRADUATE DIVISION**

**of the**

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**San Francisco**

[redacted]

Mapping the ligand-binding regions of the platelet-derived growth factor β-receptor (PDGFβr) and the macrophage colony stimulating factor receptor (M-CSFr).

Michael Joseph Pazin

6/12/92

This thesis is dedicated to my grandparents:

Michael Pazin

Joseph Ficarra

Marie Pazin

Jenny Ficarra

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Mapping the ligand-binding regions of the platelet-derived growth factor beta-receptor (PDGFr) and the macrophage-colony stimulating factor receptor (M-CSFr).

by Michael Joseph Pazin

Abstract:

The purpose of this dissertation was to characterize the regions of the platelet-derived growth factor beta-receptor (PDGFr) and the macrophage-colony stimulating factor receptor (M-CSFr) which interact with their respective ligands, BB-PDGF and M-CSF. Both of these receptor-tyrosine kinases have extracellular regions composed of five immunoglobulin-like (Ig-like) domains. We made chimeric receptors and measured their ability to bind BB-PDGF. We also measured the ability of BB-PDGF and M-CSF to activate tyrosine autophosphorylation of these chimeras. We determined that the second and third Ig-like domains of the PDGFr are essential for BB-PDGF binding, while domains 1, 4 and 5 are not necessary. We also determined that M-CSFr Ig-like domains 3, 4 and 5 are sufficient for binding M-CSF, while domains 1 and 2 are not necessary. Thus, the location of the ligand binding site is not conserved between these two receptors. We also investigated the cross-phosphorylation of pairs of receptors. BB-PDGF-activated PDGFr was unable to phosphorylate M-CSFr, nor was M-CSF-activated M-CSFr able to phosphorylate PDGFr. Furthermore, BB-PDGF-activated PDGFr was unable to phosphorylate a chimeric receptor containing the PDGFr cytoplasmic region and the M-CSFr extracellular region. However,

the PDGFr was able to phosphorylate a receptor mutant which bound BB-PDGF but was devoid of kinase activity. Thus, receptor cross-phosphorylation required the pairs of receptors to bind the same ligand.

L. T. Williams

LEWIS T. WILLIAMS, M.D., Ph.D.

# Receptor Extracellular Regions

## Introduction

*Receptor tyrosine kinases* The receptor tyrosine kinase family is a family of proteins with conserved functional features. All known members contain a cytoplasmic tyrosine kinase region, an extracellular ligand binding region, and a single transmembrane region separating the kinase and binding regions. Representatives have been characterized in many phyla, including nematodes, arthropods and chordates. Receptor tyrosine kinases participate in regulation of cell growth, cell differentiation, cell survival, cell migration, lineage commitment, and developmental axis determination.

This kinase family can be divided into subfamilies by comparison of their extracellular regions. The epidermal growth factor receptor (EGFr) is the archetype of one subfamily, characterized by extracellular regions with cysteine-rich regions. The insulin receptor (INr) is the archetype for a second subfamily, distinguished by an extracellular region which is proteolytically processed into two subunits ($\alpha$ and $\beta$) which become disulfide linked. The $\beta$ subunits themselves are also disulfide linked, forming a covalent $\alpha\beta_2\alpha$ heterotetramer. The platelet-derived growth factor receptor (PDGFr) subfamily of the receptor tyrosine kinase family contains receptors which have a single transmembrane region, a cytoplasmic tyrosine kinase domain with an insertion at a conserved

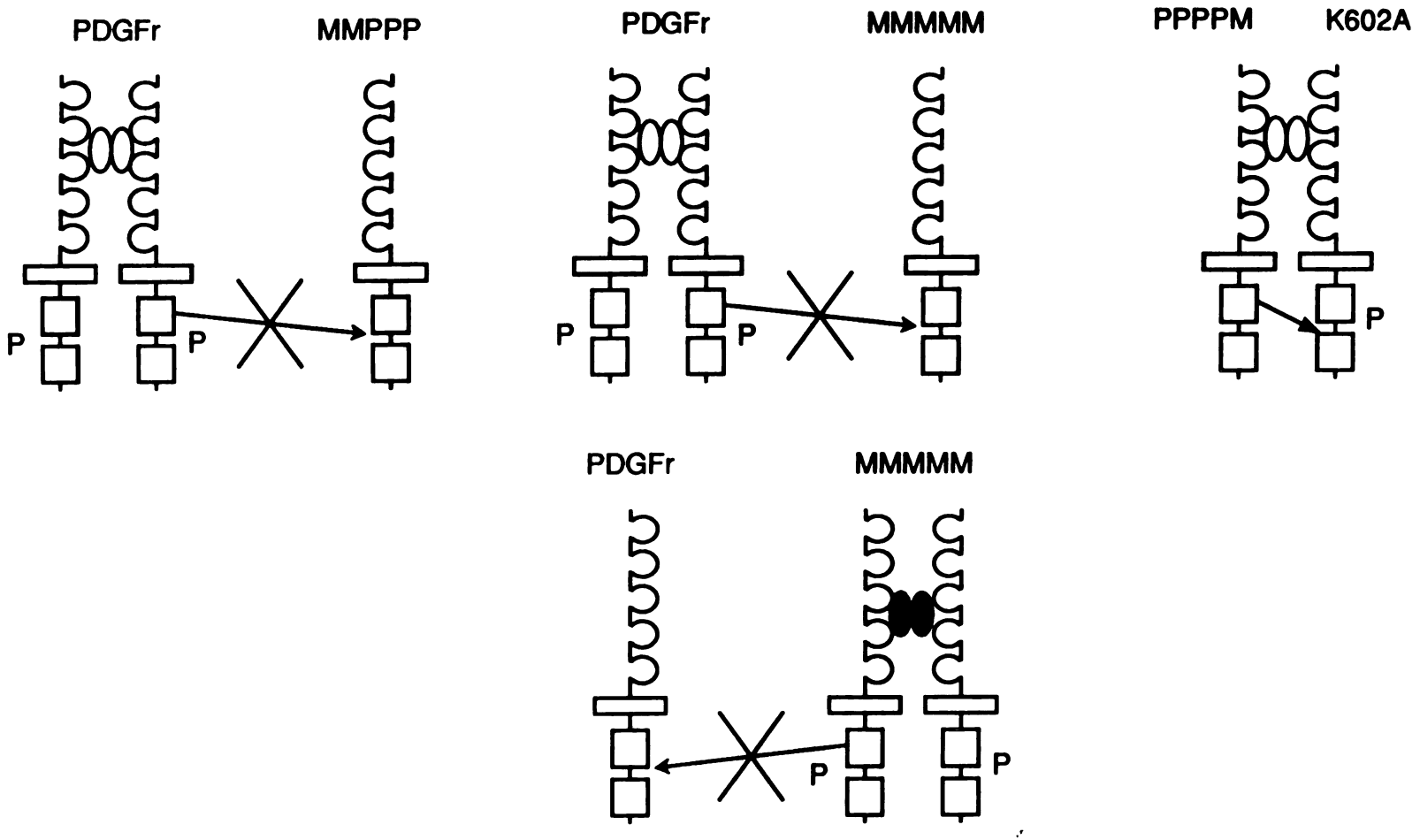
position, and an extracellular region composed of immunoglobulin-like (Ig-like) domains.

The PDGFr subfamily has four members; the platelet-derived growth factor β-receptor (PDGFβr), the macrophage-colony stimulating factor receptor (M-CSFr), the platelet-derived growth factor α-receptor (PDGFαr) and the *steel* or mast cell growth factor receptor (c-kit protein) (Ullrich and Schlessinger, 1990). Ligand binding causes receptor dimerization, autophosphorylation on tyrosine residues, and activation of many signaling pathways (Figure 1). The genes encoding the PDGFβr and the M-CSFr are tightly linked (Roberts et al 1988) as are the genes encoding the PDGFαr and the c-kit protein (Smith et al, 1991). All four of these genes are apparently the result of gene duplication during evolution, and they all have five Ig-like domains. There are two related sets of receptors which are classified in the PDGFr subfamily or a new subfamily (FGFr) by various authors. The fibroblast growth factor receptors (FGFr 1, 2, 3 and 4) have a similar structure to the PDGFr subfamily, except that their extracellular regions consist of two or three Ig-like domains (Dionne *et al.*, 1990; Johnson *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991). The vascular-endothelial growth factor receptor (VEGFr, Flt) (Shibuya *et al.*, 1990; deVries and Williams, 1992) and fetal liver kinase 1 (Flk-1) (Matthews *et al.*, 1991) are also similar to the PDGFr subfamily, except that their extracellular regions are composed of seven Ig-like domains.

*Immunoglobulin-like domains* Immunoglobulin domains were first characterized in immunoglobulins, and high resolution crystal structures have been solved for several antibody-antigen complexes

Fig. 1. **Activation of PDGFβr.** Scheme showing processes activated by BB-PDGF.

# Receptor Cross-phosphorylation



(see Mian *et al.*, 1991 for review). Ig-like domains are found in other immunoglobulin superfamily members, including some cell adhesion molecules (N-CAM and I-CAM), the T-cell receptor complexes (TCRα and TCRβ), the major histocompatibility complex, and growth factor receptors (reviewed in Williams and Barclay, 1988). The Ig domains visualized in the available crystal structures appear to be discrete structural units consisting of two antiparallel β-sheets, which form a β-sandwich or barrel. Studies of antibody-antigen complexes have demonstrated that two Ig domains are sufficient for molecular recognition (reviewed in Mian *et al.*, 1991). Studies on CD-4, which can bind the HIV protein gp120, revealed that gp120 recognizes the first Ig-like domain of CD-4 (reviewed in Sweet *et al.*, 1991). For the PDGFr subfamily, the number of Ig-like domains required for ligand binding is not known. A previous report demonstrated that the first three Ig-like domains of the PDGFαr and PDGFβr discriminate between the A-chain and B-chain of PDGF (Heidaran *et al.*, 1990). The PDGFαr binds AA- and BB-PDGF, while the PDGFβr binds only BB-PDGF (Seifert et al., 1989; Matsui et al., 1989; Kanakaraj et al., 1991).

*Experimental approach* One approach for mapping the PDGFβr ligand binding site is to delete Ig-like domains, and measure binding. However, deletions are often accompanied by processing defects which block receptor transport to the cell surface (Escobedo, J. A., Keating, M. T., and L. T. W.; unpublished). An alternate approach is to substitute various Ig-like domains with the corresponding domains from another PDGFr subfamily member that does not bind BB-PDGF.

The PDGFαr is not suitable because it binds BB-PDGF, the PDGFβr ligand (Matsui *et al.*, 1989; Kanakaraj *et al.*, 1991). However, the M-CSFr and c-kit protein are not known to bind BB-PDGF.

We embarked upon this study to determine which part of the PDGFβr is responsible for recognizing BB-PDGF with high affinity. This is a significant problem for two main reasons. The biology of PDGF in whole animals is not well understood. We reasoned that a detailed knowledge of the PDGFr-PDGF interaction could lead to the design of antagonists. These antagonists would provide reagents for informative experiments about PDGF biology, and possibly therapeutically useful agents. We are also interested in this system as a model for protein-protein interactions. AA-PDGF, BB-PDGF, M-CSF, and mast-cell growth factor are all dimeric proteins which interact with specific receptors, each containing five repeats of an evolutionarily conserved protein fold. We wished to determine which functions, if any, were ascribed to which domains, whether the functions could be exported to heterologous molecules, and whether specific domains had conserved roles between homologous receptors.

In this report, we localized a high affinity binding site for BB-PDGF to the PDGFβr using chimeras between the PDGFβr and the M-CSFr (also referred to as CSF-1r or c-fms). We also localized a high affinity binding site for M-CSF on the M-CSFr.

## Platelet-derived Growth Factor β-Receptor

*Expression of receptor chimeras* In order to map the BB-PDGF binding site on the PDGFβr, we constructed a series of chimeric receptors and stably expressed them in DUKX-B11 cells (Urlaub and Chasin, 1980). All chimeras in this study contained the murine PDGFβr transmembrane and cytoplasmic regions. The receptor chimeras had portions of the human M-CSFr extracellular region to replace PDGFβr sequences. Both the PDGFβr and M-CSFr have extracellular regions composed of five Immunoglobulin-like (Ig-like) domains. The chimeras were all precise substitutions of complete Ig-like domains based upon domain boundaries predicted from the amino acid sequence (Figure 2a and Bazan, F., unpublished). Sequence analysis suggested that each PDGFβr Ig-like domain was more similar to the corresponding M-CSFr Ig-like domain than any other PDGFβr and M-CSFr Ig-like domain (Figure 2b and Bazan, F., unpublished). Like the wild type PDGFβr and M-CSFr, each chimera had 5 Ig-like domains, and the order of the domains was not changed in this study. The chimeras were named for the origin of their Ig-like domains; thus, MMPPM is a mutant PDGFβr in which domains 1, 2 and 5 have been replaced by M-CSFr domains 1, 2 and 5, respectively (see Figure 3). The chimeras were constructed through a combination of site directed mutagenesis, oligonucleotide insertion, and standard cloning techniques. DUKX-B11 cells were transfected with the calcium phosphate method, and selected for the DHFR gene on

Fig. 2. **Boundaries of Ig-like domains of the murine PDGFβr and the human M-CSFr.** a) The PDGFβr sequence is shown above the M-CSFr sequence for each domain. The predicted amino acid sequences of the proteins were aligned. The . represent gaps in the sequences. The / represent portions of the sequence not shown for clarity. b) Alignment matrix showing similarity of PDGFβr and M-CSFr Ig-like domains; higher number indicates higher similarity, >3 is significant.

A)

**DOMAIN 1**

LVITPPGPEFVLNISSTFVLT C / C VYNNSLGPELSERKRIYIFVPDP
PVIEPSVPELVVKPGATVTLR C / C TEPGDPLGG...SAAIHLYVKDP

**DOMAIN 2**

TMGFLPMDSEDLFIFVTDVTETTIP C / C KTTIGDREVDSDTYYVYSLQVSS
ARPWNVLAQE...VVVFEDQDALLP C / C SALMGGRKVMSISIRLKVQKVIP

**DOMAIN 3**

INVSVNAVQT.VVR.QGESITIR C / C NVSVSVNDHGDEKAINISVIENG
GPPALTLVPAELVRIRGEAAQIV C / C VASNVQGKHSTSMFFRVVESAYL

**DOMAIN 4**

YVRLLETLGDVEIAELHRSRTLR V / M RAFHEDDEVQLSFKLQVNVP
..NLSSEQNLIQEVTVGEGLNLK V / F LARNPGGWRALTFELTLRYP

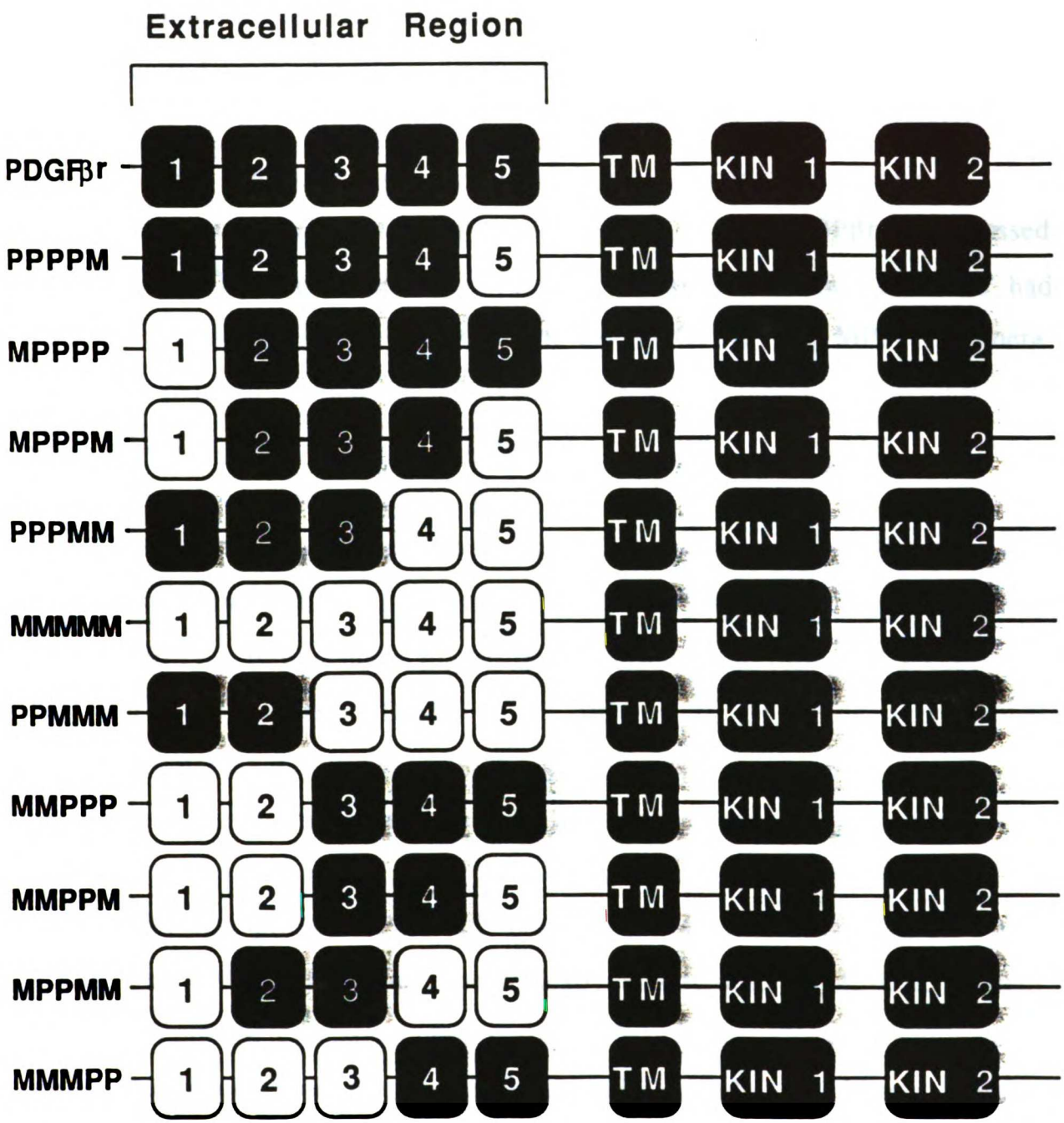
**DOMAIN 5**

VRVLELSESHPANGEQTIR C / C MLQNSMGGDSQEVTVVP.....HSLPFK
PEVSVIW..TFINGSGTLL C / C RAHNSVGSGSWAFIPISAGAHTHPPDEF

B)

| | | muPDGFβr | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| huM-CSFr | 1 | 9.62 | 2.21 | 7.63 | 1.61 | 1.35 |
| muPDGFβr | | * | 3.24 | 4.22 | 2.53 | 1.36 |
| | 2 | 1.90 | 5.82 | 4.16 | 1.20 | 0.02 |
| | | * | * | 1.79 | -0.48 | 0.63 |
| | 3 | 3.37 | 3.47 | 5.77 | 1.55 | 2.58 |
| | | * | * | * | 1.82 | 2.71 |
| | 4 | 0.51 | -1.44 | 4.16 | 5.56 | 2.74 |
| | | * | * | * | * | 3.48 |
| | 5 | 2.17 | 1.22 | 1.85 | 1.95 | 5.26 |
| | | * | * | * | * | * |

Fig. 3. **Representation of chimeric proteins.** cDNAs encoding these chimeras were stably expressed in DUKX-B11 cells. **Black** boxes indicate PDGFβr domains; White boxes are M-CSFr domains. The Ig-like domains are numbered (1-5), and the transmembrane region (TM) and the kinase subdomains (KIN1, KIN2) are labeled. The chimera names indicate identity of Ig-like domains; PPMMM is a PDGFβr with the third, fourth and fifth Ig-like domains replaced by the corresponding M-CSFr domains.


Extracellular Region
PDGFβr
PPPPM
MPPPP
MPPPM
PPPMM
MMMMM
PPMMM
MMPPP
MMPPM
MPPMM
MMMPP
1
2
3
4
5
TM
KIN 1
KIN 2

the expression plasmid by growth in nucleoside-free medium. Individual colonies were screened for chimera expression byimmunoblotting with anti-sera which recognize the PDGFβr cytoplasmic domain. Immunoblotting demonstrated that the PDGFβr and seven mutant chimeras were expressed at approximately similar levels, and that no expression of intact receptor was detected in cells transfected with vector alone (Figure 4). The PDGFβr is expressed as a 180-kDa and 160-kDa doublet. Unexpectedly, two chimeras had an expression pattern distinct from the PDGFβr: the MPPPP chimera had a reduced amount of 180-kDa protein and increased amounts of 160-kDa protein; the MPPPM chimera had little, if any, 180-kDa protein and increased 160-kDa protein.

Previous studies have demonstrated that the 160-kDa protein is an intracellular precursor of the PDGFβr (Keating and Williams, 1987). N-glycanase digestion converted both forms of receptor to a 145-kDa protein (Daniel *et al.*, 1987). To determine whether these chimeras were incompletely glycosylated, immunoprecipitates of receptor from lysate were deglycosylated with N-glycanase. This treatment changed the PDGFβr from a 180-kDa/160-kDa doublet to a 145-kDa product (Figure 5; lanes 3 and 4). As expected, the MPPPP and MPPPM chimeras were also converted to a 145-kDa product (Figure 5; compare lanes 5 and 6, 7 and 8). These properties are consistent with the idea that the MPPPP and MPPPM receptors were expressed primarily as incompletely processed proteins. None of these proteins were detected in immunoprecipitates from vector transfected cells (Figure 5; lanes 1 and 2).

Fig. 4. **Expression of chimeric proteins.** Equal amounts of lysates from transfected cells were analyzed by 6% SDS-PAGE, transferred to nitrocellulose, and blotted with antisera to PDGFβr cytoplasmic region (REF). Mature and precursor sized molecules are indicated with arrows.

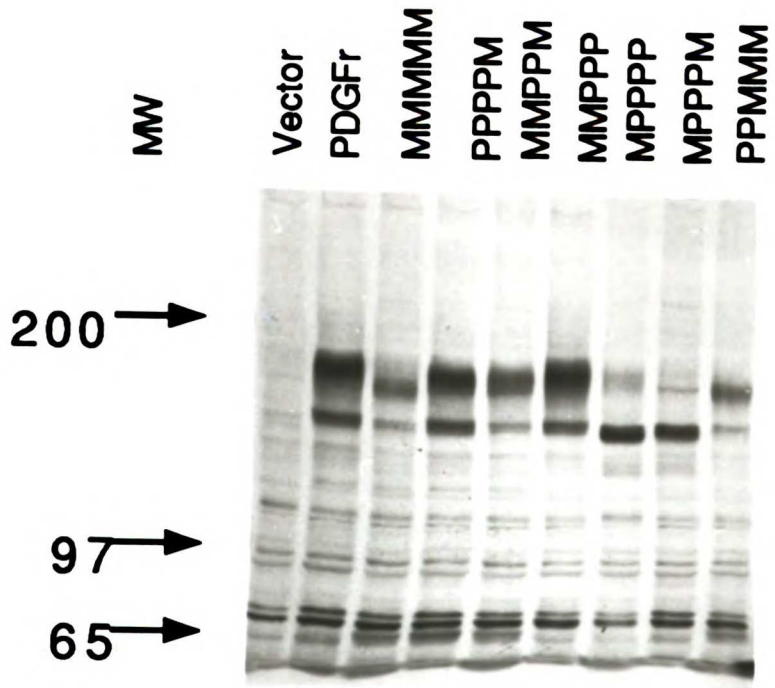
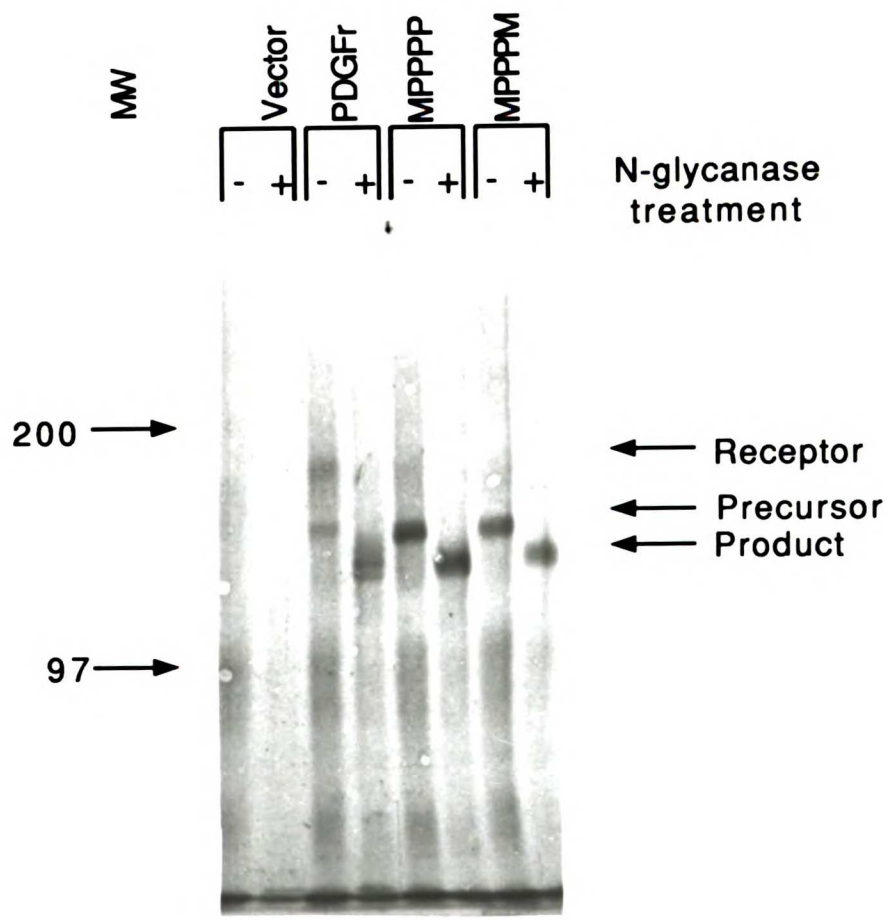

MW
Vector
PDGFr
MMMMM
PPPPM
MMPPM
MMPPP
MPPPP
MPPPM
PPMMM
200
97
65

Fig. 5. **N-Glycanase treatment of chimeras.** Equal amounts of cell lysates were immunoprecipitated with PDGFβr antisera, eluted, treated with N-Glycanase, analyzed by 5% SDS-PAGE, transferred to nitrocellulose, and blotted with PDGFβr antisera. N-Glycanase treated samples indicated with "+" ; mature, precursor and digestion product are indicated with arrows.


MW
Vector
PDGFr
MPPPP
MPPPM
- + - + - + - +
N-glycanase treatment
200
97
Receptor
Precursor
Product

*PDGF binding activity of chimeras* To characterize which chimeras were capable of binding BB-PDGF, the binding of $^{125}$I-BB-PDGF to transfected cells was measured. As expected $^{125}$I-BB-PDGF was bound by the PDGFβr,and the binding was competed by unlabelled BB-PDGF (Figure 6). Two chimeras, PPPPM and MPPPP, also bound $^{125}$I-BB-PDGF. This binding was competed by unlabelled BB-PDGF. We did not test whether this binding could be competed by BB-PDGF. Competition binding curves indicated that the PDGFβr and PPPPM had the same affinity for BB-PDGF (dat not shown). MMMMM, which contained an intact M-CSFr extracellular region, did not bind $^{125}$I-BB-PDGF. Vector transfected cells also had no detectable binding. These results implicated domains 2, 3 and 4 in BB-PDGF binding. However, MPPPM did not bind BB-PDGF. Since the MPPPM chimera was expressed as a 160-kDa protein, we could not exclude the hypothesis that it failed to bind BB-PDGF because it was not on the cell surface.

To test whether the intracellular form of the MPPPM receptor bound PDGF, a soluble competition binding assay for full-length receptors was developed. Briefly, cell lysates were incubated with $^{125}$I-BB-PDGF, with or without competitor, at 4°C. Receptors were then immunoprecipitated and bound $^{125}$I-BB-PDGF was measured. (Experiments in which receptors were first immunoprecipitated, and binding was done on beads gave a much higher background and only a 2:1 PDGFβr/vector ratio.) The MPPPM receptor bound BB-PDGF in this soluble assay (Figure 7), in contrast to its failure to bind BB-PDGF in the whole-cell assay (Figure 6). This is in agreement with the

Fig. 6. **Binding of $^{125}$I-BB-PDGF to cells expressing chimeras.** Cells were removed from plates with PBS-EDTA, incubated with $^{125}$I-BB-PDGF at 4°C with or without unlabeled competitor and centrifuged through a ficoll step-gradient. Cell pellets were lysed, immunoprecipitated, and counted.

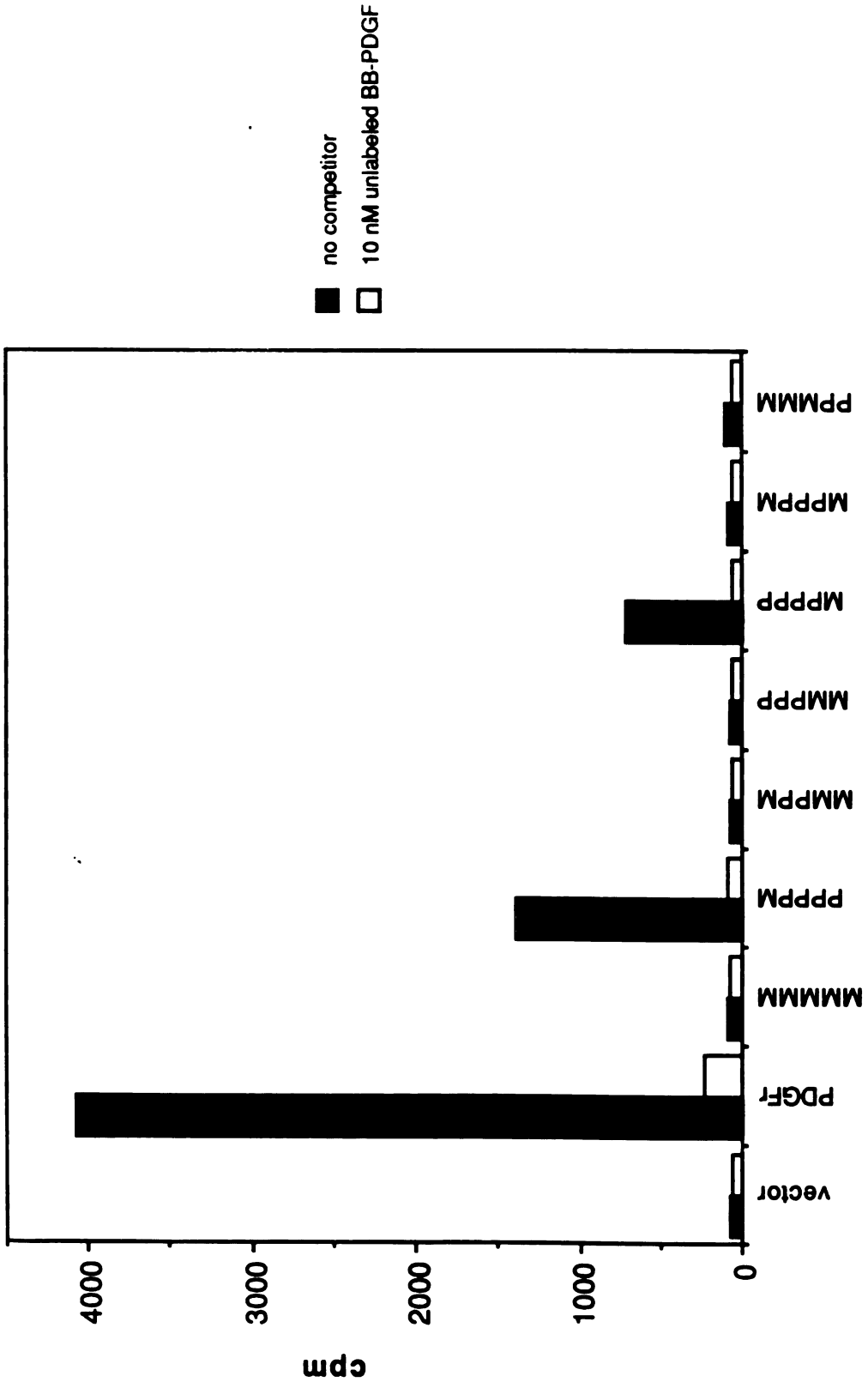
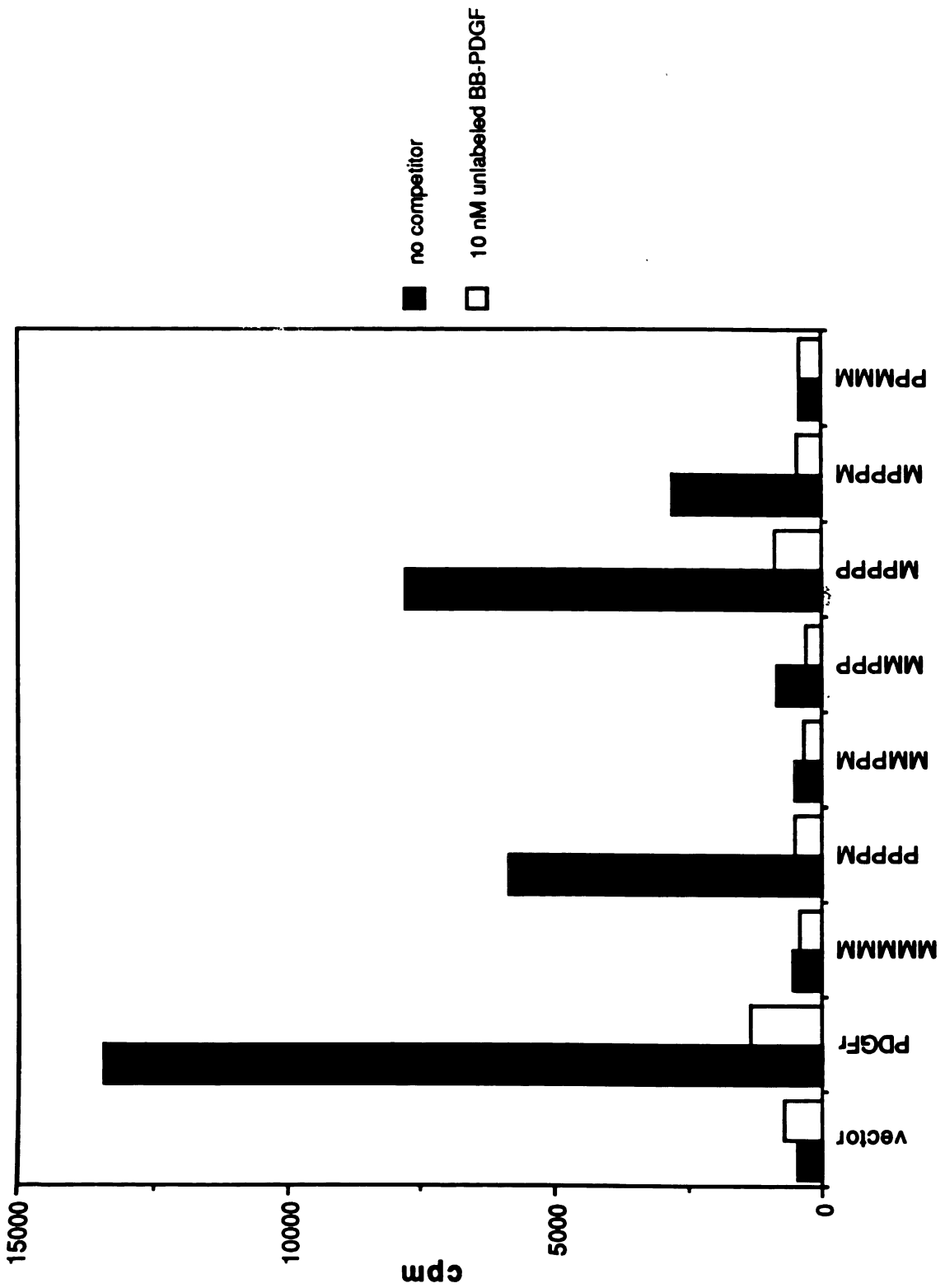

no competitor
10 nM unlabeled BB-PDGF
cpm
0
1000
2000
3000
4000
vector
PDGFr
MMMMM
PPPPM
MMPPM
MMPPP
MPPPP
MPPPM
PPMMM

Fig. 7. **Binding of $^{125}$I-BB-PDGF to solubilized chimeric receptors.** Equal amounts of cell lysates were incubated with $^{125}$I-BB-PDGF at 4°C with or without unlabeled competitor, immunoprecipitated with PDGFβr antisera, and counted.


no competitor
10 nM unlabeled BB-PDGF
cpm
15000
10000
5000
0
vector
PDGFr
MMMMM
pppPM
MMPPM
MMPPP
MPPPP
MPPPM
PPMMM

observation that there are few, if any, MPPPM receptors on the cell surface (Figure 4). The binding activity of PPPPM remained about 40% of the activity of the PDGFβr in both the whole cell and soluble binding assays. In contrast, the amount of binding activity from MPPPP lysates was higher than in whole cells. It is likely that the increase in BB-PDGF binding in lysates occurs for MPPPP and MPPPM because the intracellular form, present in larger amounts than the mature cell surface form is available for binding after lysis of the cells.

To confirm the soluble binding results, a crosslinking experiment was performed. Subsequent to soluble binding, the immunopurified proteins were crosslinked with $BS^3$, separated by 5% SDS-PAGE, transferred to nitrocellulose, and detected by autoradiography. Crosslinking of $^{125}$I-BB-PDGF to solubilized receptors confirmed that PPPPM, MPPPP and MPPPM bound BB-PDGF (Figure 8); furthermore, crosslinking revealed a high molecular weight species for each, which is the receptor dimer (Ueno *et al.*, 1991; Bishayee *et al.*, 1989). The receptor dimer was also detected by immunoblotting (data not shown). These experiments demonstrate that the same sequences sufficient for binding BB-PDGF are sufficient for receptor dimerization.

The amino-terminal portion of the PDGFβr was further investigated with transient transfection assays. Cos-6m cells were transfected with cDNA, solubilized with detergent, and assayed for BB-PDGF binding. Solubilized PDGFβr bound $^{125}$I-BB-PDGF when expressed in COS-6m cells (Figure 9a), which have no detectable binding activity when transfected with vector alone (data not

Fig. 8. **Crosslinking of $^{125}$I-BB-PDGF to solubilized receptors.** Equal amounts of cell lysates were used for binding as in Fig. 5., then crosslinked on beads with 1 mM BS3 at 4°C, 30 minutes. Proteins were analyzed by 5% SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. Receptor dimer, receptor, and uncrosslinked BB-PDGF indicated by arrows.

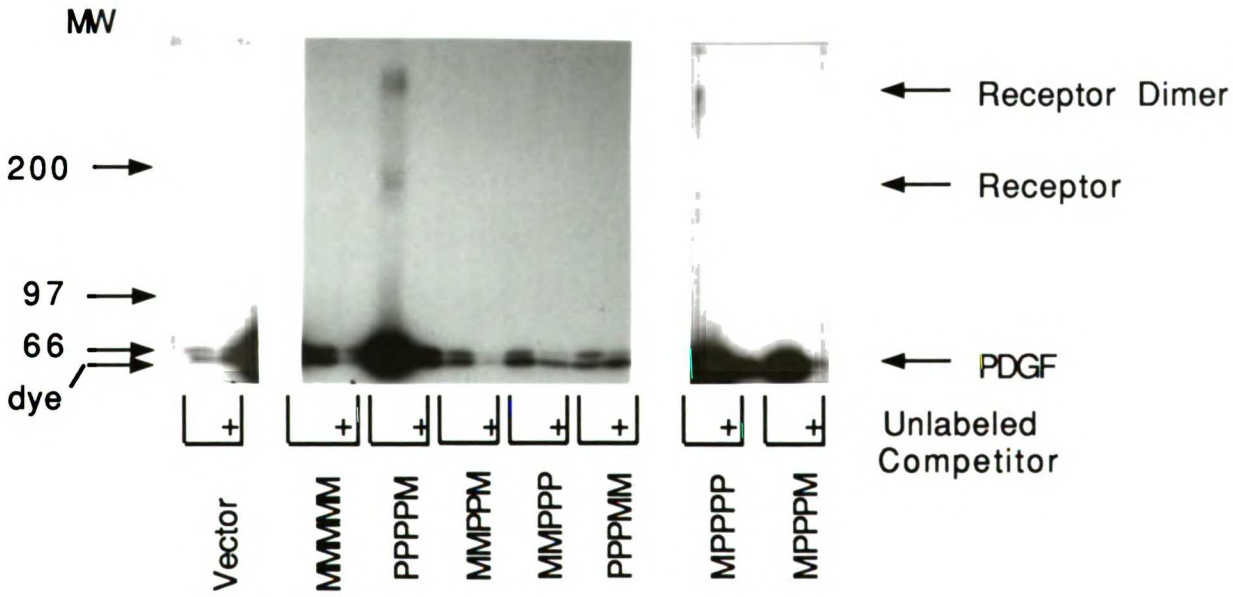
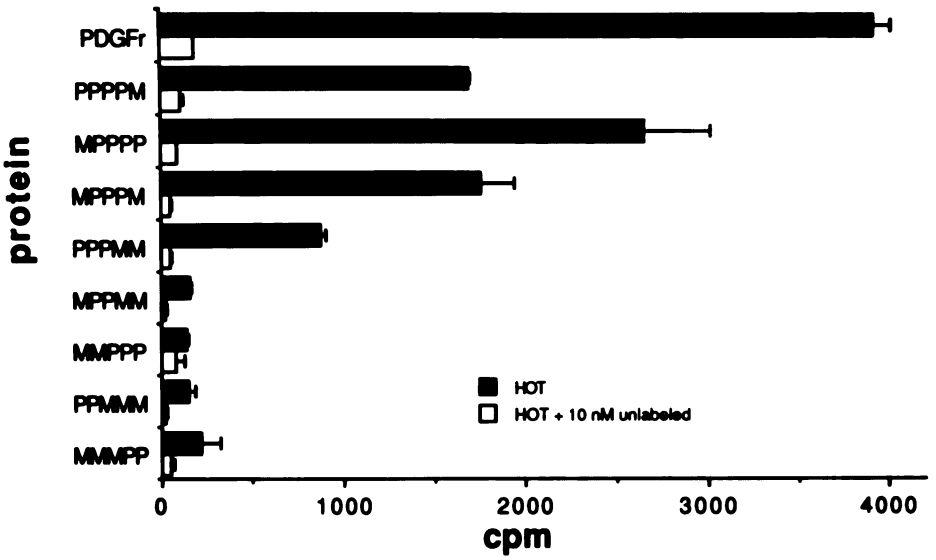
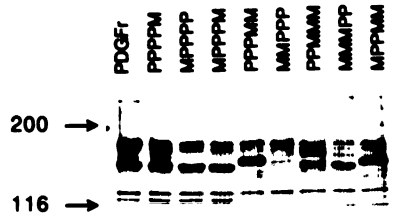
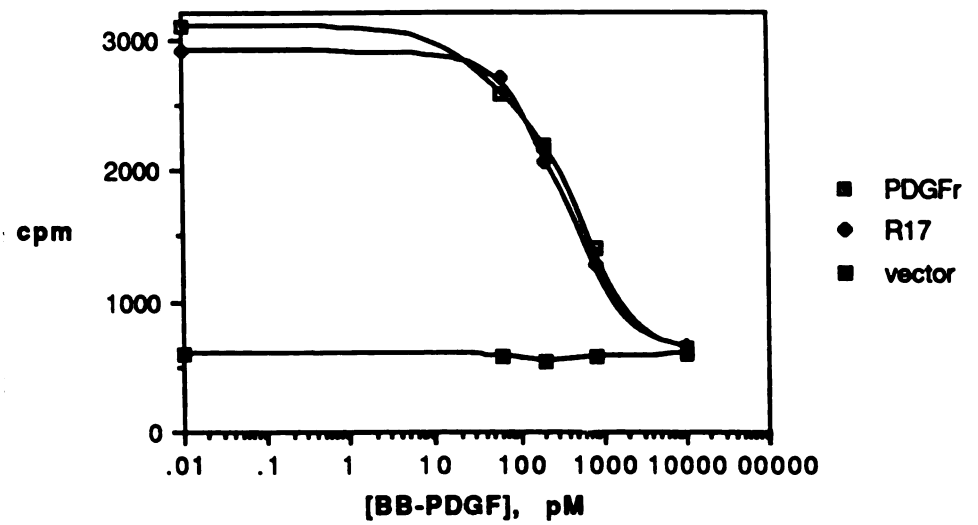

MW
200
97
66
dye
Receptor Dimer
Receptor
PDGF
Unlabeled Competitor
+
Vector
MMMM
PPPPM
MMPPM
MMPPP
PPPMM
MPPPP
MPPPM

Fig. 9. **Binding of $^{125}$I-BB-PDGF to solubilized chimeric receptors expressed in Cos-6m cells.** a) Equal amounts of cell lysates were incubated with $^{125}$I-BB-PDGF at 4°C with or without unlabeled competitor, immunoprecipitated with PDGFβr antisera, and counted. b) binding curve demonstrating that PDGFβr and R17 have same affinity for BB-PDGF.


protein
PDGFr
PPPPM
MPPPP
MPPPM
PPPMM
MPPMM
MMPPP
PPMMM
MMMPP
HOT
HOT + 10 nM unlabeled
0
1000
2000
3000
4000
cpm


PDGFr
PPPPM
MPPPP
MPPPM
PPPMM
MMPPP
PPMMM
MMMPP
MPPMM
200
116


3000
2000
1000
0
cpm
.01
.1
1
10
100
1000
10000
00000
[BB-PDGF], pM
PDGFr
R17
vector

shown). PPPMM was also competent for PDGF binding. However, the MPPMM chimera, which contains the PDGFβr sequences common between MPPPM and PPPMM, was not able to bind BB-PDGF with high affinity. PPPPM, MPPPP and MPPPM receptors bound BB-PDGF when expressed in COS-6m cells, consistent with their activity in stably transfected DUKX-B11 cells (Figure 7). The results of these binding studies, summarized in Figure 11, demonstrate that PDGFβr Ig-like domains 1, 4 and 5 are not essential for BB-PDGF binding. Chimera MMPPP demonstrates that every set of three PDGFβr was not sufficient.

These results suggested that PDGFβr Ig-like domains 2 and 3 were important for BB-PDGF binding. As mentioned in the introduction, several Ig domains in antibodies have been crystallized with their respective ligands. The ligands primarily contact the Ig domains at three turns or loops named complimentarity determining regions (CDRs) ocurring between β-strands. The locations of two of these CDRs are easily predicted with respect to the highly conserved cysteine residues present in most Ig-like domains. We constructed two short substitution mutants: R16, a PDGFβr in which the domain 2/CDR3 sequence (GGRKVNSVT) is replaced with the corresponding M-CSFr sequence (GDREVDSDT); and R17, a PDGFβr in which the domain 3/CDR3 sequence (SVNDHGDE) is replaced with the corresponding M-CSFr sequence (SVQGKHST). These mutants were expressed in Cos 6m cells, and $^{125}$I-BB-PDGF binding to solubilized receptors was measured. PDGFβr, R16, and R17 all bound BB-PDGF with equal affinity (Figure 9b and data not shown).

*Ligand-induced tyrosine autophosphorylation of chimeric receptors* We tested the hypothesis that the domains responsible for binding BB-PDGF and causing receptor dimerization were sufficient for subsequent PDGFβr responses. A salient activity of the PDGFβr is that BB-PDGF binding induces receptor autophosphorylation on tyrosine residues (Figure 1). For each chimeric protein, we measured the tyrosine autophosphorylation activity following stimulation with BB-PDGF. BB-PDGF induced tyrosine autophosphorylation of the PDGFβr (Figure 10, lanes 5 and 6). Additional tyrosine phosphorylated proteins are also visible at 145-kDa, 81-kDa, 65-kDa, and 32-kDa. As expected, PPPPM, which bound BB-PDGF (Figures 6 and 7) and formed receptor dimers (Figure 8), was also autophosphorylated and induced tyrosine phosphorylation of substrates similar to PDGFβr (Figure 10, lanes 8 and 9). MPPPP receptor produced a pattern similar to, but less intense than, the PDGFβr pattern (lanes 14, 15). This is consistent with the lower cell surface expression of MPPPP (Figure 4 and 5). MMPPM failed to respond to BB-PDGF (Figure 10, lanes 11, 12). Thus, both chimeras in this study which bound BB-PDGF at the cell surface (Figure 6) could be induced to autophosphorylate; conversely, all cell surface receptors which failed to bind BB-PDGF failed to autophosphorylate (Figure 10 and data not shown). These results were confirmed in experiments where cells were stimulated, lysed, receptors were immunoprecipitated with PDGFβr antisera, analyzed by SDS-PAGE, and blotted with antiphosphotyrosine antibodies (data not shown), proving that the receptors are indeed tyrosine phosphorylated. These results are summarized in Figure 11.

Fig. 10. **Induction of tyrosine phosphorylation of cellular proteins in cells expressing chimeras.** Equal amounts of cells were stimulated with 0, 2, or 10 nM BB-PDGF, lysed, analyzed by 7.5% SDS-PAGE, transferred to nitrocellulose, and blotted with antiphosphotyrosine antisera. Arrows indicate receptors, as well as tyrosine phosphorylated proteins of 145-kDa, 81-kDa, 65-kDa and 32-kDa.

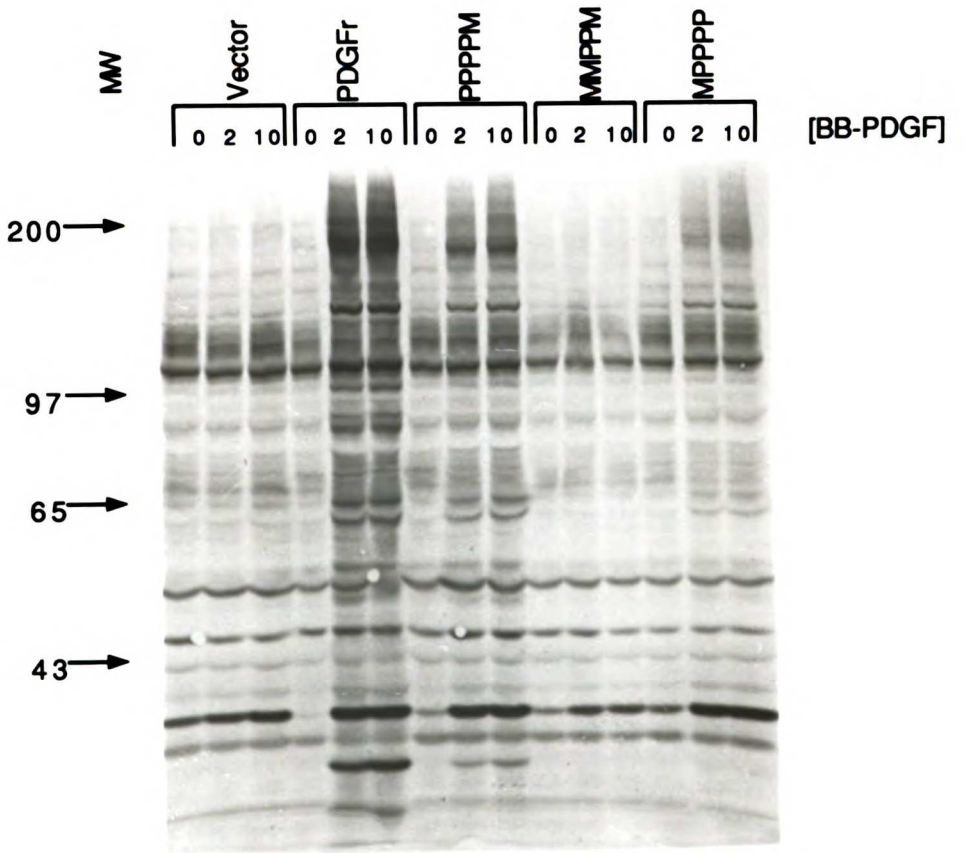
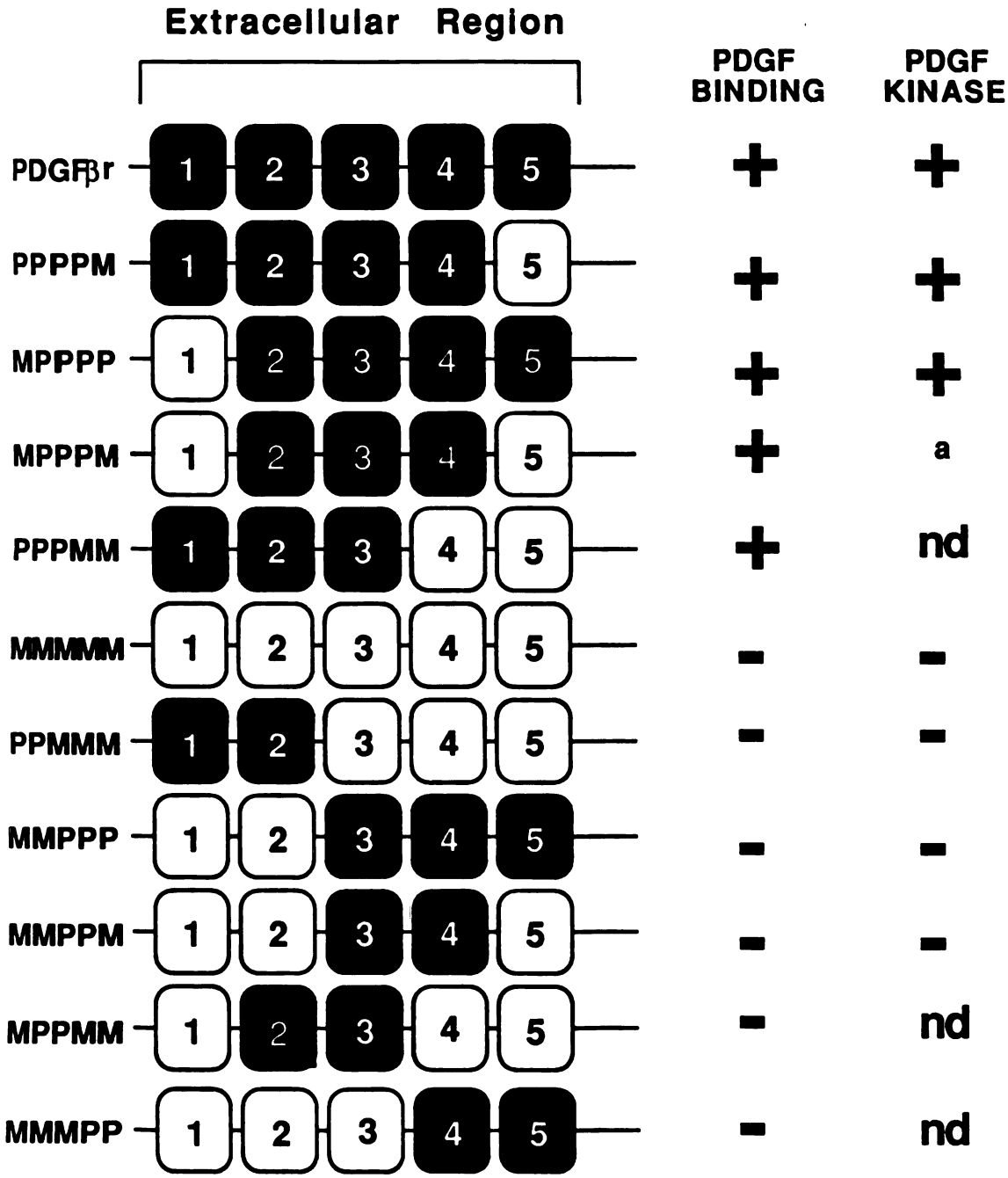

MW
Vector
PDGFr
PPPPM
MMPPM
MPPPP
0 2 10
0 2 10
0 2 10
0 2 10
0 2 10
[BB-PDGF]
200
97
65
43

Fig. 11. **Summary of binding data and kinase activation data.** Receptors represented as in Figure 3, except that only the extracellular regions are shown. PDGF Binding summarizes binding to solubilized receptors (Figures 7, 9); PDGF Kinase summarizes BB-PDGF activation of receptor autophosphorylation (Figure 10 and data not shown). + = binding or ligand activated kinase activity. *a* receptors are not at surface. nd not done.

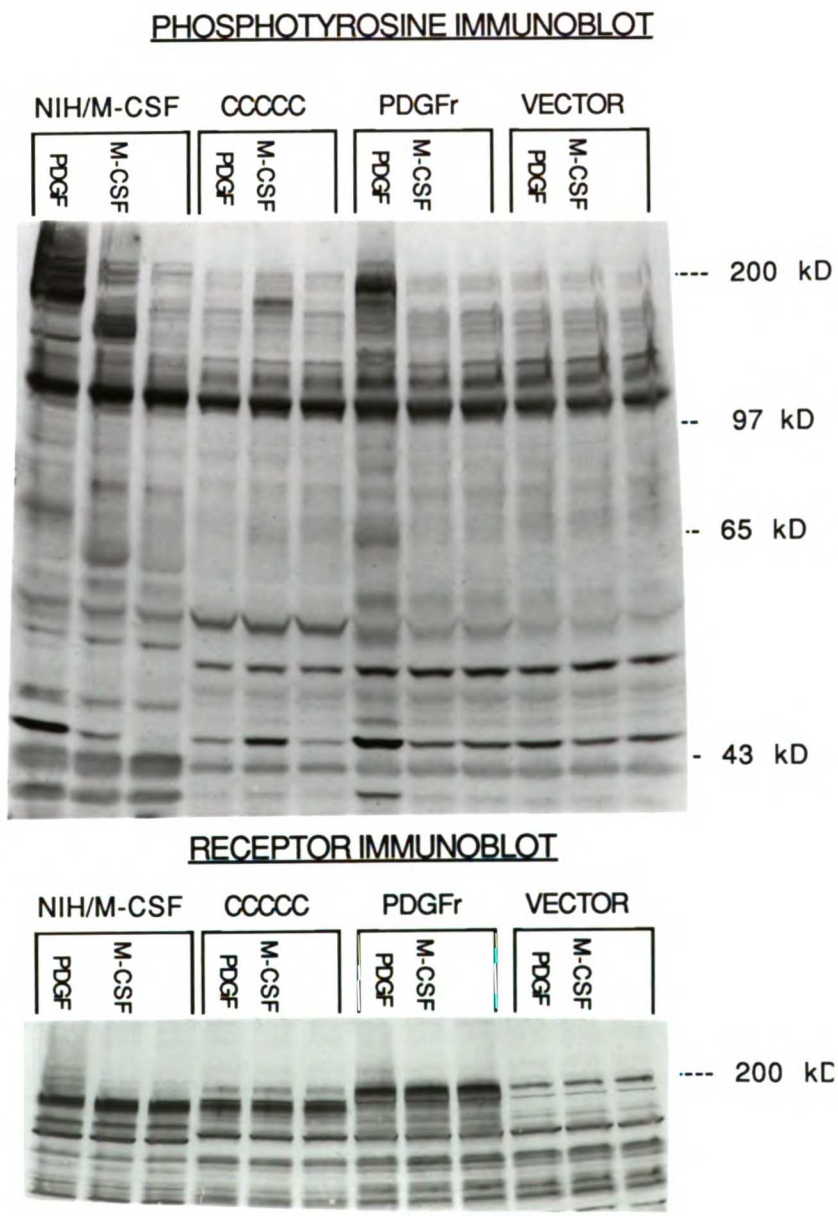

Extracellular Region
PDGF BINDING
PDGF KINASE
PDGFβr 1 2 3 4 5 + +
PPPPM 1 2 3 4 5 + +
MPPPP 1 2 3 4 5 + +
MPPPM 1 2 3 4 5 + a
PPPMM 1 2 3 4 5 + nd
MMMMM 1 2 3 4 5 - -
PPMMM 1 2 3 4 5 - -
MMPPP 1 2 3 4 5 - -
MMPPM 1 2 3 4 5 - -
MPPMM 1 2 3 4 5 - nd
MMMPP 1 2 3 4 5 - nd

## Macrophage-colony Stimulating Factor Receptor

*M-CSF induced tyrosine autophosphorylation of chimeric receptors* As discussed in the introduction, one reason for investigating PDGF binding with chimeric receptors is that many receptor deletion mutants are not expressed at the cell surface. Another advantage is the possibility of mapping functional features in a homologous receptor without making a new set of receptor mutants. This would allow comparison of the ligand-binding regions of the PDGFβr and the M-CSFr, which appear to be paralogs. One could imagine a model where one portion of both receptors, for example Ig-like domains 2 and 3, had diverged after gene duplication to recognize only BB-PDGF or M-CSF. Alternatively, the paralogs might use distinct regions of their extracellular regions for ligand binding.

We sought to test these hypotheses by determining the M-CSFr ligand binding domain. Because of the close correlation observed between BB-PDGF binding and PDGFβr signaling (Figure 11), we used M-CSF stimulated tyrosine autophosphorylation as a measure of M-CSF binding. First, we tested whether the M-CSFr extracellular region would signal through the PDGFβr transmembrane and cytoplasmic regions. Cells stably expressing MMMMM receptor were stimulated with M-CSF, lysed, analyzed by SDS-PAGE, and immunoblotted with antiphosphotyrosine antibodies. Cells expressing MMMMM, but not cells expressing PDGFβr or vector transfected cells, contained a 172 kDa phosphotyrosine protein, which is the MMMMM receptor (Figure 12). Conversely, BB-PDGF stimulation induce tyrosine

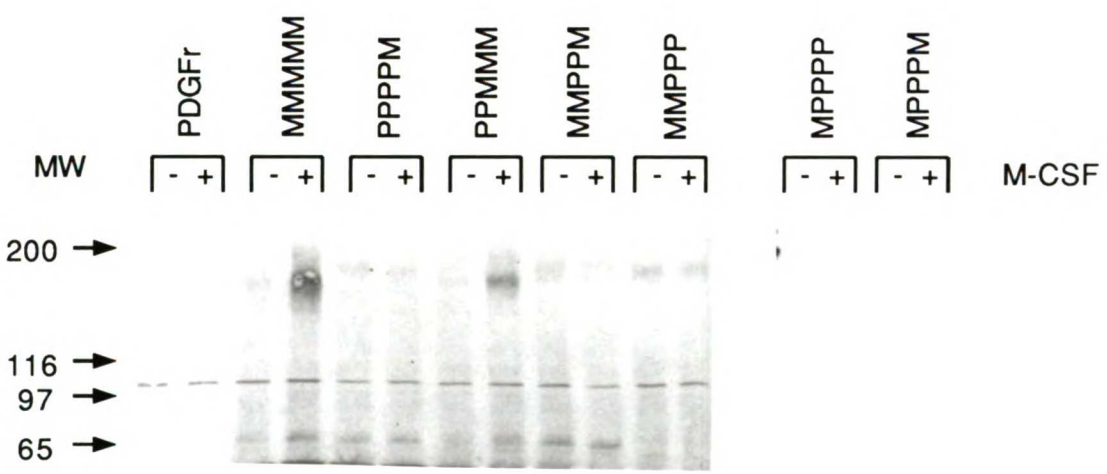
Fig. 12. **M-CSF activation of kinase in intact cells.** Cells expressing chimeras were stimulated with 1.32 nM M-CSF, solubilized, proteins were immunoprecipitated, and immunoblotted with anti-phosphotyrosine antibody.


PHOSPHOTYROSINE IMMUNOBLOT
NIH/M-CSF
CCCCC
PDGFr
VECTOR
PDGF
M-CSF
200 kD
97 kD
65 kD
43 kD
RECEPTOR IMMUNOBLOT
NIH/M-CSF
CCCCC
PDGFr
VECTOR
PDGF
M-CSF
200 kD

phosphorylation of proteins only in PDGFβr expressing cells. As a positive control, a cell line expressing PDGFβr, M-CSFr, and PDGFαr was stimulated with BB-PDGF or M-CSF. (These cells are NIH3T3 fibroblasts, which naturally express PDGFαr and PDGFβr,transfected with M-CSFr; Roussel et al,1987). Stimulation with PDGF and M-CSF induced tyrosine phosphorylation of distinct proteins, especially in the 150 kDa-190 kDa region expected for the receptors. Because the M-CSFr is known to be smaller than the PDGFβr, these results are consistent with each receptor responding to one ligand. Furthermore, if these receptors were capable of cross-phosphorylation, i.e., if the PDGFβr were a substrate for the M-CSFr, stimulation with either ligand might result in phosphorylation of both receptors. Since this was not observed, we conclude that the kinase specificity of ligand induced receptor dimerization precludes cross-phosphorylation in this system.

The demonstration that the M-CSFr extracellular region regulated the PDGFβr kinase in a ligand dependent manner led us to test the other chimeric proteins. Cells were stimulated, lysates were immunoprecipitated with PDGFβr antisera, analyzed by SDS PAGE, and immunoblotted with antiphosphotyrosine antibodies. In addition to MMMMM, the PPMMM chimera also responded to M-CSF (Figure 13). This demonstrates that M-CSFr Ig-like domains 1 and 2 are not essential for binding or signaling. We did not test binding directly with $^{125}$I-M-CSF. As shown previously (see Figure 11) these two chimeras neither bind BB-PDGF nor autophosphorylated in response to BB-PDGF. Surprisingly, MMPPP (the reciprocal chimera to PPMMM) did not respond to BB-PDGF (Figure 11). Thus, some part

Fig. 13. **PPMMM, MMMMM posess M-CSF-activated kinase activity.** Cells expressing chimeras were stimulated with 1.32 nM M-CSF, solubilized, proteins were immunoprecipitated, and immunoblotted with anti-phosphotyrosine antibody.


PDGFr
MMMMM
PPPPM
PPMMM
MMPPM
MMPPP
MPPPP
MPPPM
MW
- +
M-CSF
200
116
97
65

duced receptor

without detectable intern

of both

internalizat

EGF

reduced

of the information required to signal in response to M-CSF is located in a different position (domains 3, 4, 5) the the information required to signal in response to BB-PDGF (domains 1,2,3 or 2,3,4).

Two chimeric proteins, MMPPP and MMPPM, did not respond to either BB-PDGF or M-CSF (Figure 17). A trivial explanation for the failure to respond to M-CSF is that the kinase is inactivated. However, the PDGFβr can be activated *in vitro* in a ligand independent reaction with manganese ions. PDGFβr, MMPPP and MMPPM were immunoprecipitated from lysates of unstimulated cells, incubated with [$^{32}$P] γ ATP and $MnCl_2$, and analyzed by SDS-PAGE. The gel was dried, and phosphoproteins were visualized by autoradiography. For each receptor (but not in extracts prepared from vector-transfected cells) a correctly sized protein was present, suggesting that these receptors have functional tyrosine kinase domains (data not shown). The specific activity of the proteins was approximately equal.

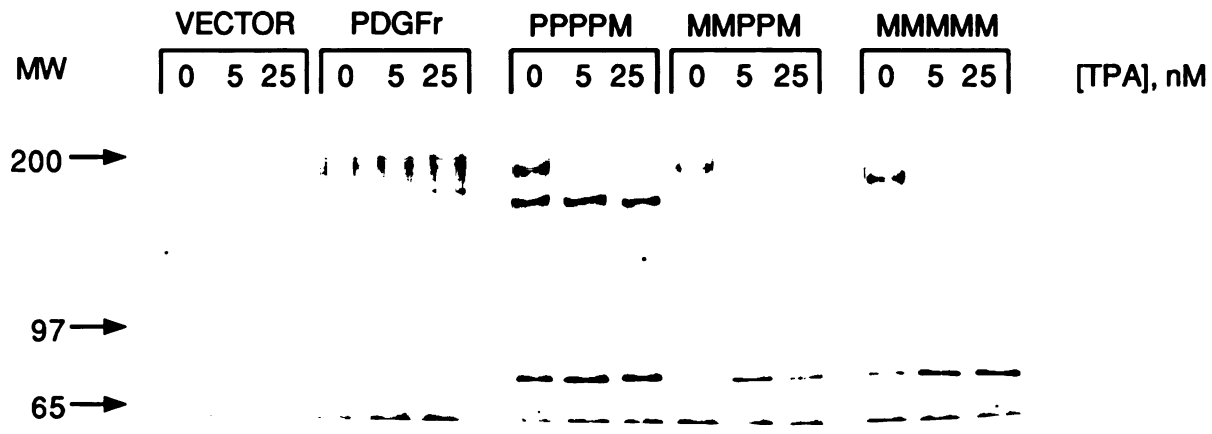
*Phorbol ester-induced receptor degradation* Chronic stimulation of both PDGFβr and M-CSFr with their respective ligands causes receptor internalization (t $^1/_2$=10') and receptor degradation (t $^1/_2$=45') without detectable intermediates. In the case of the M-CSFr, ligand-induced receptor degradation does not require protein kinase C (PK-C; Downing et al 1989). However, stimulating PK-C in the absence of M-CSF is also capable of causing M-CSFr degradation (Downing et al 1989, Roussel et al 1988). 12-tetradecanoyl phorbol 13-acetate (TPA, a phorbol ester which activates PK-C) induces the release of the M-CSFr extracellular region into the medium, while the

cytoplasmic and transmembrane region transiently accumulate as a 50 kDa intermediate prior to degradation.

Previous experiments using v-fms/c-fms chimeras indicated that the determinant for degradation was located within either the cytoplasmic region, transmembrane region, or Ig-like domains 4 and 5 (Roussel et al, 1988). In order to map the M-CSFr region required for TPA-induced receptor degradation, whole cells expressing chimeras were stimulated with TPA one hour at 37°, cell lysates were immunoprecipitated with PDGFβr cytoplasmic antisera, analyzed by 6% SDS-PAGE, and immunoblotted with a PDGFβr cytoplasmic antibody recognizing a second epitope. Upon TPA stimulation, the amount of MMMMM receptor mature form was greatly decreased, while the amount of precursor remained virtually unchanged (Figure 14). In addition, a 76 kDa band, present in unstimulated MMMMM cells, increased in intensity in TPA-stimulated cells. This 76 kDa band, which is not present in vector-transfected cells, is the receptor degradation intermediate. The intermediate was larger (76 kDa *vs.* 50 kDa) than that seen with the M-CSFr, most likely because of the larger size of the PDGFβr cytoplasmic region. The large amount of 76 kDa protein present in unstimulated cells suggests that in these cells, this pathway was constitutively partially activated.

In striking contrast, PDGFβr was unaffected by TPA stimulation (Figure 14). Furthermore, the 76 kDa receptor fragment was not detected in cells expressing the PDGFβr. The failure of PDGFβr to respond to TPA by receptor degradation allowed us to determine that the extracellular region of the M-CSFr contained the information

Fig. 14. **TPA-induced degradation of chimeras.** Intact cells were stimulated with 0, 5, or 25 x $10^{-7}$ M TPA for one hour, 37°C. Cells were solubilized, proteins were immunoprecipitated, and immunoblotted with receptor antibody.


VECTOR
PDGFr
PPPPM
MMPPM
MMMMM
MW
0 5 25
0 5 25
0 5 25
0 5 25
0 5 25
[TPA], nM
200
97
65

receptor and the receptor

required for TPA-induced receptor degradation. The chimeras which were degraded after TPA stimulation (MMMMM, PPPPM, MMPPM and PPMMM) all contained M-CSFr Ig-like domain 5 (Figure 14 and data not shown). The proteins which were insensitive to TPA stimulation (PDGFβr and MMPPP) contained PDGFβr Ig-like domain 5 (Figure 14 and data not shown). Thus, replacement of PDGFβr Ig-like domain 5 with M-CSFr Ig-like domain 5 was sufficient to confer TPA sensitivity upon the PDGFβr (Figure 14) without changing its ability to bind BB-PDGF with high affinity (Figure 5) or autophosphorylate (Figure 10). These results are consistent with the proteolytic cleavage occuring within Ig-like domain 5. Cells treated with excess trypsin at 4°C contain a similarly sized fragment (data not shown). Immunoblotting with antiphosphotyrosine antisera demonstrated that TPA did not induce tyrosine phosphorylation of any of these receptor species (data not shown).

Though the 76 kDa receptor fragment detected in Figure 14 was recognized by two different PDGFβr antisera, we were concerned by its abundance in unstimulated cells. The identity of the 76 kDa receptor fragment was confirmed by phosphotryptic mapping. We reasoned that both the full length receptor and the receptor fragment, which differ only in the extracellular region, should have similar phosphopeptide maps. DUKX-B11 cells stably expressing PDGFβr or PPPPM were stimulated with TPA or vehicle, lysed, and immunoprecipitated with a PDGFβr cytoplasmic antisera. The immune complexes were incubated with [$^{32}$P] γ ATP and $MnCl_2$. As described above, manganese ions activate the PDGFbr kinase in a ligand-independent manner. The reaction products were analyzed

The immu[illegible] (unstimulated) and

PPM fragm[illegible] were cut from the membrane,

[illegible] hydrolyzed [illegible] analyzed by two

[illegible] chromatograph[illegible]

[illegible]

[illegible]

[illegible] result of TPC[illegible]

[illegible] action of the protein. These

[illegible] 17.

by 6% SDS-PAGE, transferred to PVDF membranes (Immobilon; Millipore), and visualized by autoradiography. PPPPM receptor was present as prominent mature, precursor, and 76 kDa bands (Figure 15) in agreement with results obtained by immunoblotting (Figure 14). TPA stimulation substantially increased the 76 kDa signal at the expense of the mature receptor. In striking contrast, the PDGFβr samples contained no 76 kDa protein.

The immobilized PPPPM mature receptor (unstimulated) and PPPPM fragment (TPA stimulated) were cut from the membrane, digested extensively with trypsin, lyophilized, and analyzed by two-dimensional thin-layer electrophoresis/thin-layer chromatography (TLE/TLC). The mature receptor (Figure 16a) and the fragment (Figure 16b) had almost identical phosphotryptic maps, which was confirmed by mixing equal amounts of the two samples prior to TLE/TLC (Figure 16c). The subtle differences between the unstimulated and stimulated PPPPM might be the result of TPA-induced serine/threonine phosphorylation of the protein. These results are summarized in Figure 17.

Fig. 15. ***In vitro*** **kinase of PDGFβr, PPPPM.** Intact cells were stimulated with 0, 25 x $10^{-7}$ M TPA for one hour, 37°C. Cells were solubilized, proteins were immunoprecipitated, and incubated with ATP and Manganese. Proteins were analyzed by SDS-PAGE transferred to PVDF, and deteced by autoradiography.

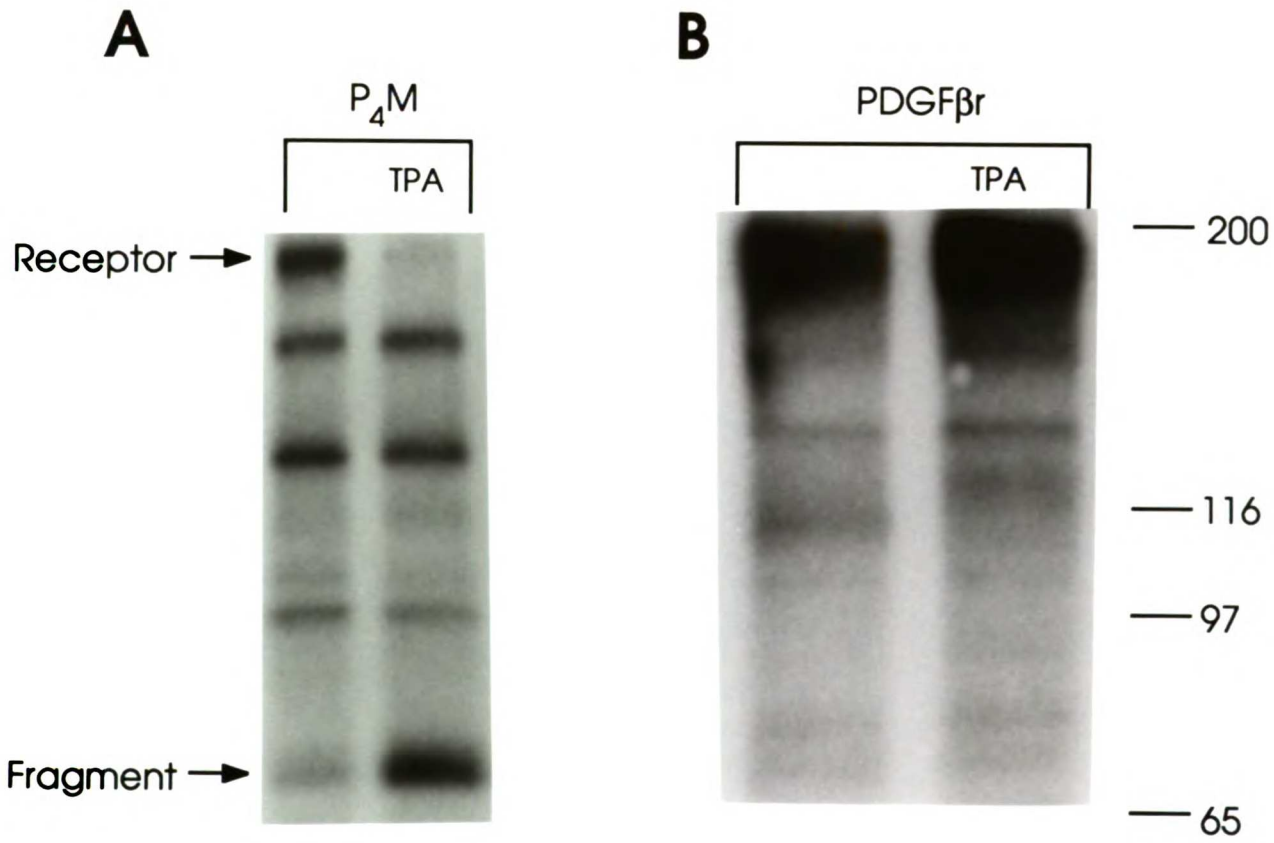

A
P4M
TPA
Receptor
Fragment
B
PDGFβr
TPA
200
116
97
65

Fig. 16. **Phosphotryptic mapping.** Proteins from *in vitro* kinase of PDGFβr, PPPPM were cut from the filter and analyzed as described in methods. a) PPPPM, 180 kDa, unstimulated. b) PPPPM, 76 kDa, TPA-stimulated. c) mix of equal amounts of PPPPM, 180 kDa, unstimulated and PPPPM, 76 kDa, TPA-stimulated.

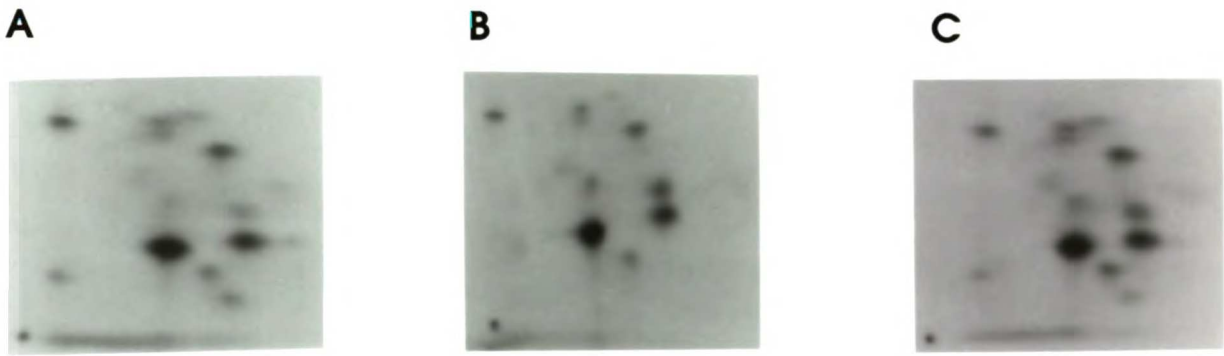
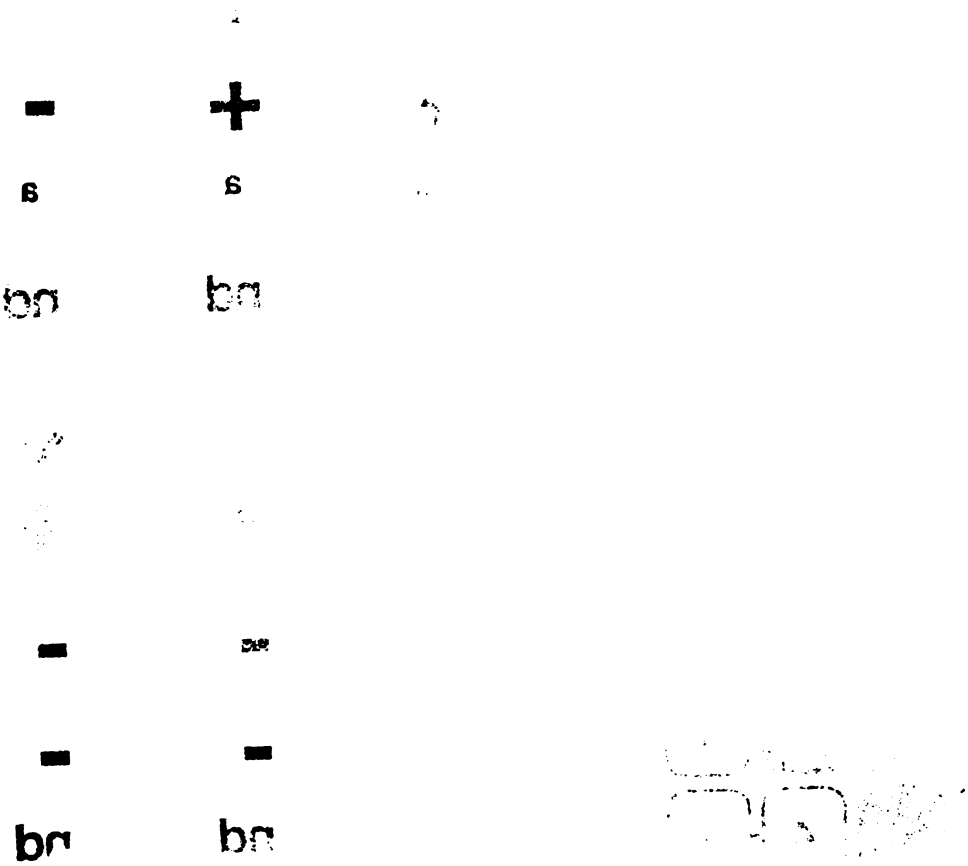
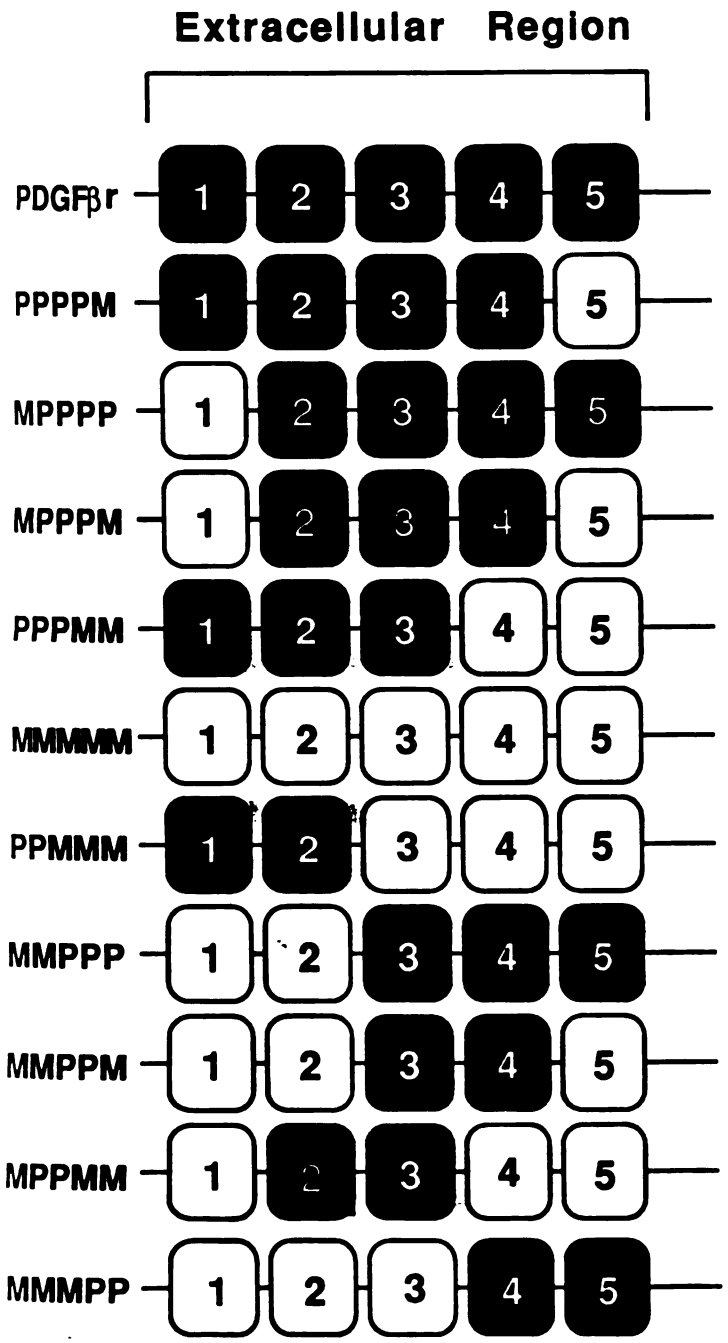

A
B
C

Fig. 17. **Summary of binding data and kinase activation data.** Receptors represented as in Figure 3, except that only the extracellular regions are shown. PDGF Binding summarizes binding to solubilized receptors (Figure 7, 9); PDGF Kinase summarizes BB-PDGF activation of receptor autophosphorylation (Figure 10 and data not shown); M-CSF Kinase summarizes M-CSF activation of receptor autophosphorylation (Figure 12, 13, and data not shown). TPA summarizes down-regulation with phorbol ester (Figure 14 and data not shown)+ = binding or ligand activated kinase activity. *a* receptors are not at surface. nd not done.






| | PDGF BINDING | PDGF KINASE | M-CSF KINASE | TPA |
|---|---|---|---|---|
| PDGFβr | + | + | - | - |
| PPPPM | + | + | - | + |
| MPPPP | + | + | - | nd |
| MPPPM | + | a | a | nd |
| PPPMM | + | nd | nd | nd |
| MMMMM | - | - | + | + |
| PPMMM | - | - | + | + |
| MMPPP | - | - | - | - |
| MMPPM | - | - | - | + |
| MPPMM | - | nd | nd | nd |
| MMMPP | - | nd | nd | nd |

## Cross-phosphorylation of receptors

As mentioned above, when the M-CSFr and PDGFβr are coexpressed in the same cell, stimulation with either M-CSF or BB-PDGF activates tyrosine phosphorylation of only the M-CSFr or PDGFβr, respectively (Figure 12). One explanation of this result is that the M-CSFr cytoplasmic region is not a substrate for PDGFβr. If this were true, then PDGFβr might be able to phosphorylate MMMMM, which responds to M-CSF but not to BB-PDGF. If, however, cross-phosphorylation requires ligand-induced dimerization of kinase and substrate, then neither MMMMM nor MMPPP should be PDGFβr substrates. If the second model is true, then PDGFβr should be able to phosphorylate a kinase-inactive PDGFβr which can bind BB-PDGF. Such a PDGFβr mutant, K602A, has been described (Escobedo et al 1988); a highly conserved lysine residue (lysine 602) in the kinase region was converted to alanine.

In order to distinguish these proteins, specific antisera are necessary. Ab P5 (referred to as Ab 77 in Keating et al 1988) is an anti-peptide antibody which recognizes PDGFβr domain 5 but not M-CSFr (data not shown). Ab M5 is a monoclonal antibody (Mab 2-4A5; kindly provided by Chuck Scher) which recognizes M-CSFr domain 5, but not PDGFβr (data not shown). Ab M1 is a monoclonal antibody (MAb 10-4B2; kindly provided by Chuck Scherr) which, like MAb 14-1C1 and MAb 3-4A4, recognizes M-CSFr domain 1 but not PDGFβr (data not shown; summarized in Figure 20).

PDGFβr and MMPPP (which does not respond to BB-PDGF or M-CSF; Figure 17) were coexpressed in Cos-6m cells. Lysates were

antibodies.

...n diaminylated and

...eptor tyrosine

...ed ...

...ted

...EBr

...n to occur.

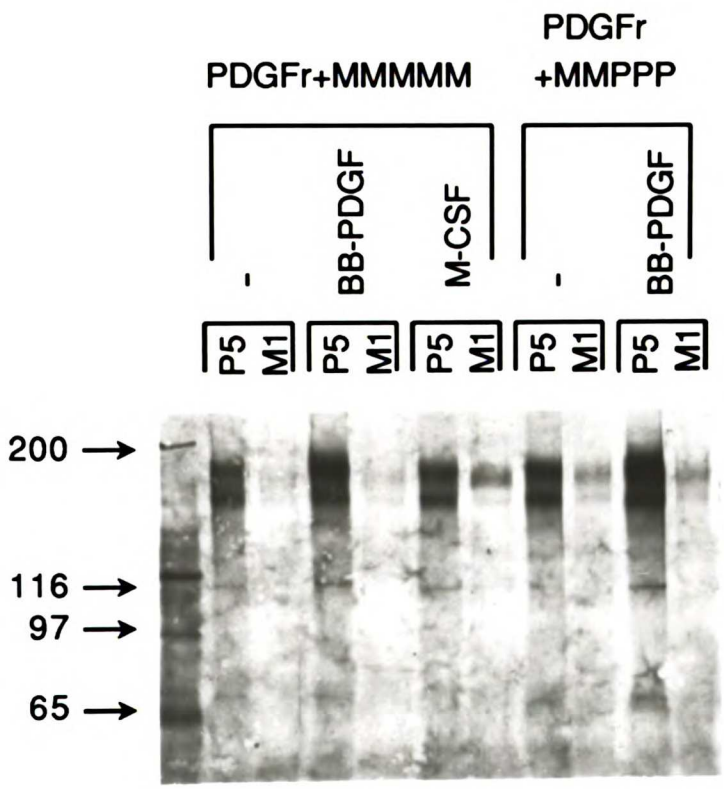
...sponse to

... was folded

prepared from both unstimulated and BB-PDGF stimulated cells, and immunoprecipitated independently with Ab M1 (which recognized only MMPPP) and Ab P5 (which recognized MMPPP and PDGFβr). The incubations of lysates and antibodies were conducted in the presence of 0.1% SDS and 0.5% sodium deoxycholate, which disrupted noncovalent receptor dimers (Ueno et al 1991; Ohtsuka et al 1990). Immunoprecipitates were analyzed by 6% SDS-PAGE, transferred to nitrocellulose, and blotted with antiphosphotyrosine antibodies. Comparison of the Ab P5 immunoprecipitates from unstimulated and BB-PDGF stimulated cells revealed an increase in receptor tyrosine phosphorylation (Figure 18). In contrast, the tyrosine phosphorylation in Ab M1 immunoprecipitates was unchanged with BB-PDGF stimulation. Thus, activated PDGFβr did not cross-phosphorylate MMPPP, though both contained .PDGFβr transmembrane and cytoplasmic regions.

The failure of PDGFβr to phosphorylate MMPPP suggested that coexpression of an activated PDGFβr with an inactive PDGFβr cytoplasmic region is insufficient for cross-phosphorylation to occur. However, although MMPPP had kinase activity in response to manganese (data not shown), it was still possible MMPPP was folded in a manner which precluded cross-phosphorylation by PDGFβr. We coexpressed MMMMM, which was a functional "M-CSFr" (Figure 17), with the PDGFβr. Activation with BB-PDGF again increased tyrosine phosphorylation of PDGFβr (Ab P5) but was not effective with MMMMM (Ab M1) (Figure 18). However, M-CSF was able to increase phosphorylation of MMMMM (Ab M1) but not PDGFβr. Thus, when two proteins containing independently activatable PDGFβr

Fig. 18. **Coexpression of PDGFβr, MMMMM; PDGFβr, MMPPP.** Pairs of receptors were coexpressed in Cos-6m cells, stimulated with 1.32 nM M-CSF or 2 nM BB-PDGF, solubilized, proteins were immunoprecipitated, and immunoblotted with anti-phosphotyrosine antibody.


PDGFr+MMMMM
PDGFr
+MMPPP
-
BB-PDGF
M-CSF
-
BB-PDGF
P5
M1
P5
M1
P5
M1
P5
M1
P5
M1
200
116
97
65

cytoplasmic

bind the same

PDGFβr

PDGF P

PPPM

immunoprecipitate PPPM

taining protein from cells expressing only PPPM (data no

en). We previously demonstrated that kinase activatable

not respon

PDGF (Escobedo et a

and Ab P5 dir

response to

PDGF-

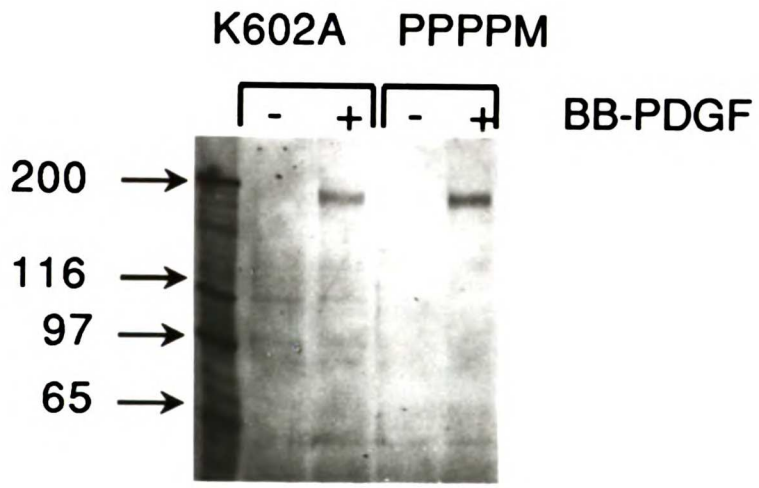
kinase inactive

proteins with

transmembrane region and cytoplasmic region were coexpressed in the same cell, cross-phosphorylation still did not occur. The results with MMMMM and PDGFβr were confirmed with a stable transfectant coexpressing these proteins. These results suggest that the failure of PDGFβr to phosphorylate M-CSFr might be more complex than the first model, which states that the difference in protein sequence alone might account for failure to observe cross-phosphorylation.

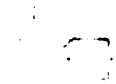
To test the hypothesis that cross-phosphorylation requires cytoplasmic regions connected to extracellular regions which can bind the same ligand, we coexpressed K602A (a kinase inactive PDGFβr) with PPPPM (which autophosphorylated in response to BB-PDGF; Figure 17). PPPPM was chosen, rather than PDGFβr, to allow separation from K602A with available antisera. Stimulation with BB-PDGF caused phosphorylation of PPPPM (Figure 19; Ab M5), consistent with previous results. K602A was also tyrosine phosphorylated (Figure 19; Ab P5), but only in response to BB-PDGF, as seen with PPPPM. Cells expressing K602A alone did not respond to BB-PDGF (Escobedo et al 1988 and data not shown) and Ab P5 did not immunoprecipitate PPPPM or any other phosphotyrosine-containing protein from cells expressing only PPPPM (data not shown). We previously demonstrated that kinase activatable (MMMMM) and non-responsive (MMPPP) chimeras with PDGFβr cytoplasmic regions are not PDGFβr substrates (Figure 18). In striking contrast, a kinase-inactive mutant (K602A) which binds BB-PDGF is a substrate for PDGFβr. This suggests that ligand-induced dimerization or clustering of receptors is required for cross-phosphorylation. In addition, this result suggests that Ig-like domain

Fig. 19. **Coexpression of PPPPM and K602A.** Pairs of receptors were coexpressed in Cos-6m cells, stimulated with 0 or 2 nM BB-PDGF, solubilized, proteins were immunoprecipitated, and immunoblotted with anti-phosphotyrosine antibody.

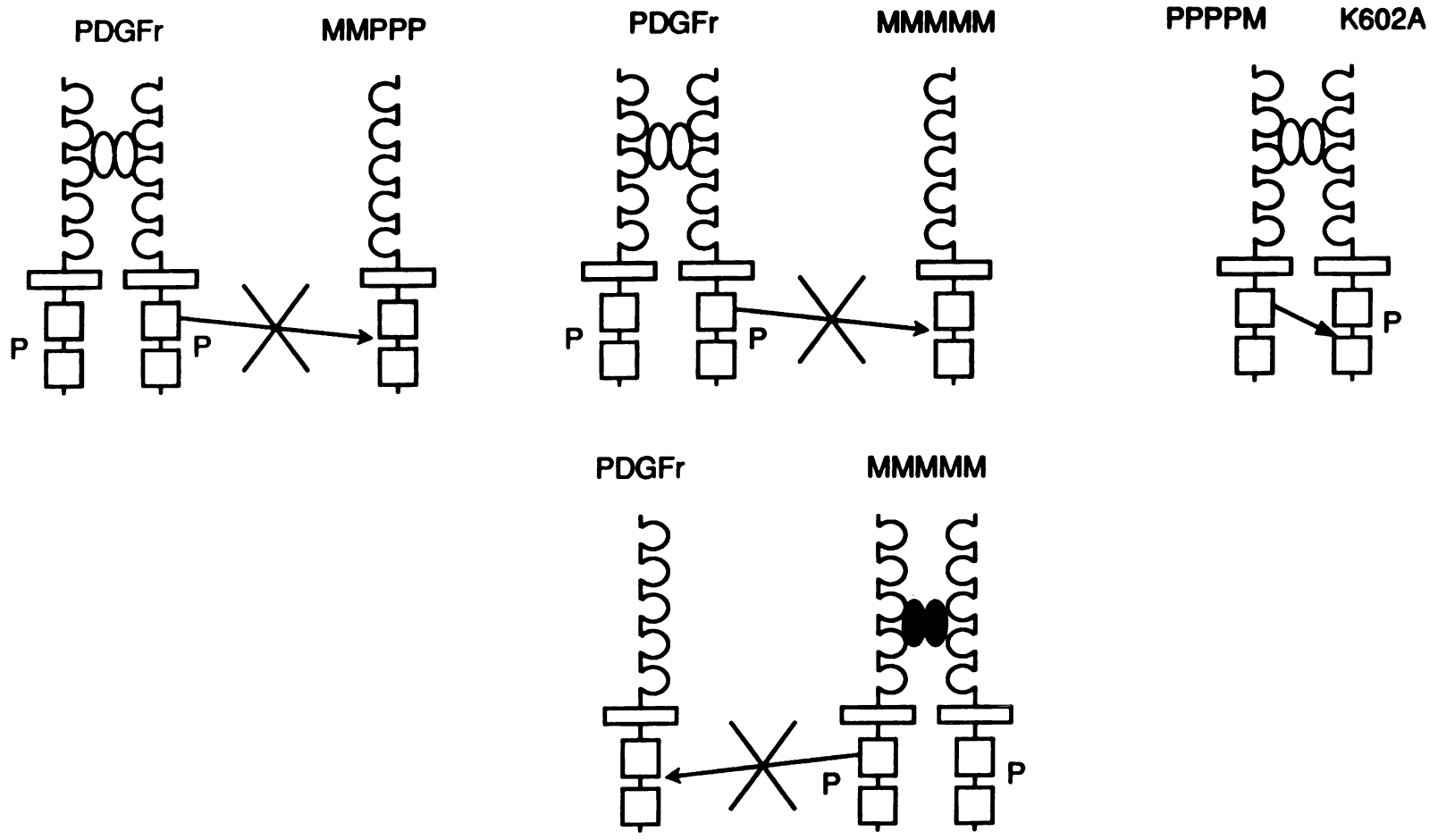

K602A
PPPPM
- + - +
BB-PDGF
200
116
97
65

5 is not important for ligand-induced dimerization, because proteins with distinct fifth domains (PPPPM and K602A) can apparently form heterodimers. These results are summarized in Figure 20.

Fig. 20. **Receptor cross-phosphorylation summary.** Scheme summarizing results in Fig 18, 19. BB-PDGF is represented by paired circles; M-CSF is represented by filled paired circles; tyrosine phosphate residues on receptors are indicated by "P".



# Receptor Cross-phosphorylation



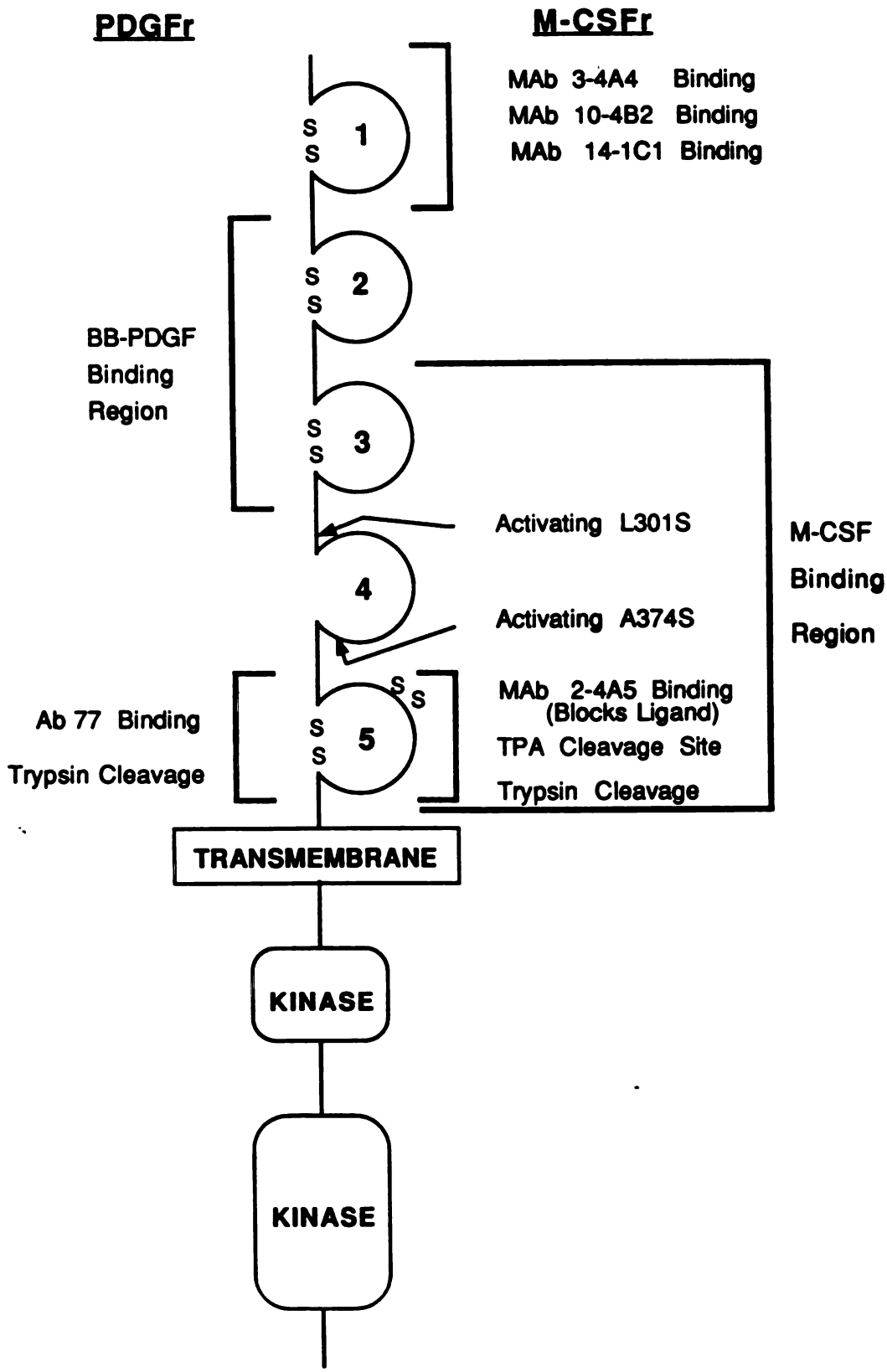
## Discussion

In this section the binding and signaling capacity of several chimeric PDGFβr with various M-CSFr substitutions were characterized. We also examined interaction of several pairs of chimeras. Although the PDGFβr and the M-CSFr bind different ligands (Figure 12), they are both receptor tyrosine kinases with extracellular regions composed of five immunoglobulin-like (Ig-like) domains and have intracellular kinase regions that are interrupted by kinase insert domains. The genes encoding these receptors contain introns at similar locations (Sherr, 1990), are separated by 500 bp in the human genome and are transcribed in the same direction (Roberts *et al.*, 1988). This is consistent with the hypothesis that they are the result of gene duplication during evolution. As previously reported, the PDGFβr could bind BB-PDGF and phosphorylate itself on tyrosine residues Figure 1). Moreover, when the extracellular region of the PDGFβr was replaced with the M-CSFr extracellular region, the resulting chimeric receptor (MMMMM) autophosphorylated in response to M-CSF (Figure 12) but not BB-PDGF (Figure 10), and was no longer able to bind BB-PDGF (Figures 6 and 7; summarized in Figure 17). This suggested that chimeras might be useful in mapping the BB-PDGF binding domain of the PDGFβr.

The results of this study, schematized in Figures 17 and 21, indicate that two groups of Ig-like domains from the PDGFβr are sufficient for binding BB-PDGF with high affinity; both the set 1,2,3 and the set 2,3,4 are functional. Thus, Ig-like domains 2 and 3

Fig. 21. **Stuctural representation of PDGFβr/M-CSFr.** PDGFr results are on the left-hand side of the figure. Antibody binding sites are listed; Ab 77 binding site was known previously (Keating et al, 1988). M-CSFr results are on the right-hand side; Activating mutations are from Woolford et al, 1988 and Roussel et al, 1988. MAb binding sites were mapped by Mike Pazin (data not shown). Data demonstrating that MAb 2-4A5 are in Sherr et al, 1989.

# Structural Map of Receptors



appear to be essential for high affinity BB-PDGF binding, while Ig-like domains 1, 4 and 5 of the PDGFβr were not necessary. The observation that both PPPPM and MPPPPP bound BB-PDGF with high affinity led us to test the overlapping region with chimera MPPPM, as well as the two outer regions PPPMM and MMPPP. Consistent with the model that the Ig-like domains are autonomously folding domains, the chimera MPPPM was also able to bind BB-PDGF. Chimera PPPMM was also able to bind BB-PDGF, unlike chimera MMPPP (Figure 17). The observations that 1) MPPPP and MPPPM were functional BB-PDGF binding proteins without PDGFβr Ig-like domain 1, and 2) MPPMM contains the overlap between PPPMM and MPPPMM, two functional BB-PDGF binding proteins, led us to test MPPMM. Unexpectedly, MPPMM failed to bind BB-PDGF. Thus, Ig-like domains 2 and 3, while essential for BB-PDGF binding, were not sufficient , even in the context of M-CSFr Ig-like domains 1, 4 and 5. There are several explanations for this, and they are not all mutually exclusive.

One possibility is that MPPMM binds BB-PDGF, but with lower affinity than our assay system can detect. $^{125}$I-BB-PDGF binding revealed that PPPMM bound approximately four times more PDGF than background in a Cos-6m cell assay (Figure 9a), an amount which is clearly significant. In this assay, the relatively low concentration of $^{125}$I-BB-PDGF ($< 0.2\ K_D$) ensures that the total binding is proportional to the number of receptors ($\beta_{max}$) and the affinity ($K_D$). A receptor with 4-fold lower affinity than PPPMM expressed at the same level would result in undetectable $^{125}$I-BB-PDGF binding. Moreover, examination of the expression level of MPPMM relative to

PPPMM reveals that MPPMM is expressed at a slightly lower level. Again, a receptor with one-half the affinity and one-half the expression level of PPPMM might not have measurable binding.

A second possibility is that PDGFβr Ig-like domains 2 and 3 do indeed contain the complete ligand-binding region, but require the presence of additional PDGFβr Ig-like domains for proper folding. If PDGFβr Ig-like domain 1 and/or 4 contributed to the conformation of Ig-like domains 2 and/or 3, then in this experimental system, they would appear to be part of the ligand-binding region. Indeed, it is possible that either Ig-like domain 2 or 3 is the BB-PDGF binding region while the other domain is important for folding. An interesting parallel here is the immunoglobulins. Immunoglobulin Fab fragments, which contain four Ig domains ($V_H$, $V_L$, $C_1$ and $C_L$), can recognize epitopes with high affinity. Fv fragments, which contain two Ig domains ($V_H$ and$V_L$) are often unstable, and can have a 10-fold lower affinity than Fab fragments unless stabilized (Slockshuber et al, 1990). Stabilization of Fv fragments is a result of covalently linking the two Ig domains, presumably lowering the entropy of binding. It is possible that PDGFβr Ig-like domains 1 and 4 play a similar role to the Ig domains $C_1$ and $C_L$, in that they correctly position the ligand binding domains for high affinity binding. In the absence of this conformational targeting, Ig-like domains 2 and 3 might still be able to assume the correct binding conformation, but the higher entropic cost would be reflected in a lower binding affinity.

A third possibility is that PDGFβr Ig-like domains 1, 2, 3 and 4 all interact directly with BB-PDGF. From this it follows that PPPMM

and MPPPM are active because they contain 75% of the BB-PDGF binding region, while PPMMM, MPPMM, and MMPPM are all inactive because they contain only 50% of the BB-PDGF binding region, an insufficient amount for high affinity binding. This model presents two problems. First, why does substitution of either PDGFβr Ig-like domain 1 or 4 have little, if any, effect, while the combination has a large effect on binding? Second, it is difficult to imagine how four immunoglobulin-like domains, covalently connected in a head-to-tail array, could interact with a molecule of BB-PDGF, based upon their predicted relative molecular sizes.

In the studies presented in this report, BB-PDGF binding was quantified in the presence of M-CSFr Ig-like domains. It is formally possible that these M-CSFr Ig-like domains can complement the function(s) of the corresponding PDGFβr Ig-like domains by directly binding BB-PDGF. If Ig-like domain 1 (or domain 4 or 5) from both the PDGFβr and M-CSFr bound BB-PDGF (or M-CSF) equally well, the experiments in this study would not measure the importance of those domains in PDGF binding because of complementation.

We have also investigated the M-CSF binding region, and have identified Ig-like domains 3, 4 and 5 as a candidate binding region. This result is in striking contrast to the BB-PDGF binding region, where MMPPP is without activity. All BB-PDGF binding chimera (PPPPM, MPPPP, PPPMM and MPPPM) contain PDGFβr Ig-like domains 2 and 3 (Figure 17). However, M-CSF binding does not require M-CSFr Ig-like domain 2 (PPMMM; Figure 17). For the M-CSFr, unlike the PDGFβr, two naturally occuring point mutations are known in the extracellular region which appear to activate the

receptor in a ligand-independent manner. These mutations, which do not interfere with M-CSF binding, are present at the Ig-like domain 3/4 junction and within Ig-like domain 4 (Woolford et al,1988; Roussel et al, 1988). We did not test whether these mutations would activate the PDGFβr or any of the chimeras. A directed search within Ig-like domains 3, 4 and 5 has uncovered additional activating mutations (van Dalen Wetters et al, 1992). We note here that all of these mutations occur within the proposed M-CSF binding region. Most likely, either these mutations mimic a conformational change caused by bound M-CSF, or they broaden the specificity of the human M-CSFr and allow it to bind murine M-CSF, which is expressed in the assay cells. Furthermore, MAb M5 (MAb 2-4A5, Chuck Scherr) blocks M-CSF binding in a non competitive manner. That is, M-CSF binding does not block subsequent antibody binding, but antibody binding blocks subsequent M-CSF binding. One interpretation is that the antibody epitope is not the M-CSF binding region, but it is near the binding region in tertiary-structure space. This is consistent with our mapping work, because the antibody epitope is in the M-CSF binding region (Figure 21).

Prior to conducting the M-CSFr studies, we considered three possible models for the location of the M-CSF and BB-PDGF binding sites. The first possibility is that the M-CSF and BB-PDGF binding regions do not overlap. Some studies suggest that the ligand binding domains of the insulin and insulin-like growth factor receptors do not overlap (Kjeldsen *et al.*, 1991; but see also Gustafson and Rutter, 1990). It has also been suggested that the AA-PDGF and BB-PDGF

binding sites on the PDGFαr do not overlap (Heidaran *et al.*, 1990, Heidaran *et al.*, 1992).

A second hypothesis is that the M-CSF and BB-PDGF binding sites may overlap completely. Inspection of the chimeras in this study reveals that mutants containing PDGFβr domains 3-5 (MMPPP) or 1 and 2 (PPMMM) of the PDGFβr were unable to bind BB-PDGF or autophosphorylate in response to BB-PDGF. This and our other results imply that the binding region for BB-PDGF includes domain 2 as well as domain 3, suggesting these chimeras are non-functional because they do not contain a complete BB-PDGF binding region. The non-functional chimeras are processed similarly to the PDGFβr, expressed at similar levels (Figure 4) and recognized by four M-CSFr monoclonal antibodies (data not shown; Figure 21) which recognize native, but not denatured, M-CSFr (Sherr *et al.*, 1989). However, we cannot exclude the possibility that these chimeras are inactive because they fail to assume the correct tertiary structure. A precedent for completely overlapping binding sites has been observed in the serine protease family. One serine protease (trypsin) recognizes two sets of ligands, whereas others (thrombin and certain trypsin mutants) recognize only one of these sets; the binding sites are known at the crystallographic level (reviewed in Evnin *et al.*, 1990).

A third model, which we favor, is that the binding regions may partially overlap. We have found that MMPPP does not respond to BB-PDGF or M-CSF; however, PPMMM responds to M-CSF (Figures 6, 7, 13; summarized in Figure 17). Thus, the PDGFβr requires PDGFβr Ig-like domain 2, while the M-CSFr does not require M-CSFr Ig-like

domain 2. However, both receptors seem to require their own Ig-like domain 3. An example of partially overlapping recognition domains has been examined at the crystallographic level. Comparison of antibody D1.3/lysozyme complexes and antibody D1.3/antibody E225 complexes has revealed that antibody D1.3 has both common interfacial residues, and private residues required only for lysozyme or E225 binding (Bentley *et al.*, 1990).

In this study, we have also used chimeric receptors to identify a domain of M-CSFr which is sufficient for PK-C induced receptor degradation. This finding was made possible by our observation that the PDGFβr is not susceptible to TPA induced receptor degradation (Figure 14). Our assignment of this function to the fifth Ig-like domain of the M-CSFr extends the work of Chuck Sherr's lab (Roussel et al, 1988; Downing et al, 1989) which suggested that the determinant(s) lies within Ig-like domains 4, 5, the transmembrane region and the cytoplasmic region.

What is the mechanism of PK-C induced receptor degradation? Two other systems in which TPA desensitizes receptors are mechanistically distinct. In human T-cells activation of PK-C with TPA or T-cell receptor agonists causes downregulation of CD4 (Hurley et al, 1989). This process has been shown to require phosphorylation of CD4 and results in internalization of intact CD4 (Hurley et al, 1989). In cells expressing both the PDGFr and epidermal growth factor (EGF) receptor activation of PK-C with either TPA or PDGF diminishes signaling through EGF (Walker and Burgess, 1991). This process does not result in receptor internalization or proteolysis; rather, the receptor signaling ability is impaired (Walker and

Burgess, 1991). In contrast, it has recently been shown that the extracellular region of the hepatocyte growth factor receptor (c-met) is released from cells, and that TPA treatment accelerates release (Prat et al, 1991). This system seems similar to the M-CSFr.

We considered two models for the M-CSFr heterologous downregulation. One hypothesisis is that PK-C (or a PK-C effector) acts upon the cytoplasmic region of the M-CSFr and causes a conformational change in the extracellular region which makes the receptor labile to a protease. Communication from the cytoplasmic region to the extracellular region is observed in several adrenergic type receptors, which have increased affinity for intracellular G proteins after hormone stimulation (Casey and Gilman, 1988). Binding of GTP to the G protein destabilizes the receptor hormone complex, an example of a negative heterotropic interaction (Casey and Gilman, 1988). Another example is the integrin GPIIb-IIIa ($\alpha_{IIb}\beta_3$); deletion of the cytoplasmic region, or substitution with the cytoplasmic region with that from another integrin ($\alpha_5$) results in increased ligand affinity (O'Toole et al, 1991). However, our data show that the cytoplasmic region of the PDGF$\beta$r, which is not susceptible to PK-C induced degradation, was able to facilitate heterologous downregulation of chimeras containing M-CSFr Ig-like domain 5.

We favor a model in which a protease is the effector of PK-C. Either PK-C activates a transmembrane protease, similar to the yeast vacuolar transmembrane proteases (Jones, E. W., 1991), or a soluble protease. In the former case, phosphorylation of the protease might be the activating event. Activation in the latter case could involve

the induced secretion of a protease, or secretion of a molecule which regulates a constituitively secreted protease.

Removal of the insulin receptor extracellular region by mild proteolysis has been shown to activate autophosphorylation (Shoelson et al, 1988). In contrast, TPA stimulated heterologous downregulation of the M-CSFr does not cause autophosphorylation (Downing et al, 1989). Similarly, we found that PDGFβr/M-CSFr chimeras were not activated by TPA treatment (data not shown). The M-CSFr Ig-like domain 5 does not interfere with BB-PDGF binding or kinase activation in the PPPPM receptor (Figure 17). The M-CSFr Ig-like domain 5 may function as a switch to turn off the PPPPM chimeric receptor in response to TPA. We do not know if this modular switch will function with more distantly related molecules, such as the insulin receptor or transmembrane tyrosine phosphatases.

The biological role of PK-C induced M-CSFr degradation is not well understood. This phenomenon is probably part of the signaling pathway for other receptors. It has been demonstrated in fibroblasts artificially co-expressing the PDGFβr and M-CSFr that PDGF (a potent protein kinase C activator) can cause degradation of the M-CSFr. In addition, lipopolysaccharide (LPS), a potent macrophage activator, activates protein kinase C and causes rapid loss of M-CSF binding sites on macrophages (Guilbert and Stanley, 1984), possibly through receptor degradation.

Our current model for receptor activation is that the extracellular region binds ligand, and that ligand-induced receptor dimerization occurs, either because of the dimeric nature of BB-PDGF,

or because of a conformational change (Figure 1; Duan *et al.*, 1991) Receptor dimerization leads to transmembrane activation of the receptor kinase and an intracellular conformational change (Keating *et al.*, 1988; Bishayee *et al.*, 1988). We have previously demonstrated that the PDGFβr extracellular region will form ligand induced dimers, in the absence of the transmembrane and cytoplasmic regions (Duan *et al.*, 1991). In the case of the M-CSFr, large insertions between the extracellular region and the transmembrane region did not abrogate kinase activation suggesting that signal transduction does not require a unique structure connecting the ligand binding domain with the transmembrane region (Lee *et al.*, 1990). Furthermore, replacement of the M-CSFr extracellular region with the glycophorin extracellular region allowed activation of the M-CSFr kinase in the absence of any M-CSFr extracellular structures, using an antibody which caused receptor multimerization (Lee *et al.*, 1990). Results in this study imply that if PDGFβr Ig-like domains 1, 4 and 5 are involved mediating in ligand-activated receptor phosphorylation, their function is conserved between the PDGFβr and the M-CSFr. These domains may play a role in receptor folding, processing or stability. Preliminary studies suggest that several deletions in the extracellular region of the PDGFβr lead to intracellular expression of receptors (Escobedo, J. A., Keating, M. T., and L. T. W. ; unpublished).

We have further investigated the problem of receptor activation by examining the interactions of pairs of receptors. We first examined the interaction of wild-type PDGFβr and PDGFαr with M-CSFr. Stimulation with M-CSF caused M-CSFr tyrosine

phosphorylation (160 kDa) but not PDGFr tyrosine phosphorylation Figure 12). Stimulation with BB-PDGF did not result in tyrosine phosphorylation of M-CSFr, only PDGFr (180 kDa). We considered two models; 1) either M-CSFr was not a PDGFβr substrate because M-CSFr cytoplasmic region is not recognized by PDGFβr kinase, or 2) only cytoplasmic domains clustered with activated PDGFβr were PDGFβr kinase substrates. Two chimeras which did not bind BB-PDGF were not substrates for activated PDGFβr (Figure 18). While this result does not directly address whether M-CSFr is a potential PDGFβr substrate, this experiment suggests that the explanation is more complex than model 1. In Figure 19 we demonstrate that cross-phosphorylation can occur, if the substrate protein can bind BB-PDGF normally. The experiment which would complete this idea, MMMMM or PPMMM co-expressed with kinase-inactive M-CSFr, has not been tried.

These results, summarized in Figure 20, can be put into context with the emerging receptor cross-phosphorylation story. Previously, it was demonstrated that truncated PDGFβr or FGFr1, lacking kinase sequences, inhibited activation of the respective wild-type receptor responses in an oocyte expression system (Ueno et al 1991; Ueno et al 1992; Amaya et al, 1991). In the case of PDGFβr, the truncated PDGFβr was shown to form heterodimers with the PDGFβr. Remarkably, the heterodimers were not tyrosine phosphorylated. There were two distinct mechanistic explanations for this result. The first possibility was that the truncated receptor, when complexed with PDGFβr, inhibited its ability to autophosphorylate. A second possibility, which we favor, was that the complexes were not

phosphorylated because autophosphorylation occurs in dimers in *trans*. If the two receptors present in a dimer phosphorylate each other, rather than themselves, this would also explain the heterodimer result: the truncated receptor has no phosphorylation sites for the PDGFβr, and has no kinase to phosphorylate the PDGFβr. The demonstration that K602A (PDGFβr with a point mutation which inactivates the kinase) also inhibited PDGFβr signaling in oocytes led us to consider the second model more closely.

While these studies were in progress, Chuck Scher's lab (Ohtsuka et al, 1990) demonstrated that M-CSFr could phosphorylate an epitope-tagged M-CSFr mutant, K621M (an M-CSFr with a point mutation which inactivates the kinase, analogous to K602A). Our results confirmed and extended those findings. We found that not only did cross-phosphorylation occur with the PDGFβr, but it required both the kinase and substrate to be bound to the same ligand. We also found, in the case of Ig-like domain 5, that molecules with different domains (PPPPM and K602A) could interact, further suggesting that Ig-like domain 5 does not play an important role in the PDGFβr.

This leaves an apparent paradox. If K602A does not inhibit PPPPM kinase, than how does it inhibit PDGFβr signaling? We favor the following model. It is known that BB-PDGF binding to PDGFβr leads to receptor dimerization, autophosphorylation, and a cytoplasmic conformational change. We propose that correct autophosphorylation triggers the conformational change, and that this conformational change is required for efficient substrate phosphorylation. In a PPPPM-K602A heterodimer, the K602A

receptor is tyrosine phosphorylated, and we expect has undergone the conformational change. However, its inactivated kinase is unable to phosphorylate substrates, despite its "active" conformation. The PPPPM subunit of a heterodimer remains unphosphorylated by K602A, and is unable to undergo the conformational change required to phosphorylate substrates.

In conclusion, our studies with chimeric proteins have demonstrated that the set of Ig-like domains 2, 3, and 4 of the PDGFβr and the set 1, 2, 3 are sufficient for binding BB-PDGF. PDGFβr Ig-like domains 1, 4 and 5 were not necessary for high affinity binding of BB-PDGF, though they may contribute to the overall structure of the extracellular region. While PDGFβr Ig-like domains 2 and 3 were not sufficient for binding, our studies suggest that they are necessary. We found that, of the receptors tested, every receptor which bound BB-PDGF formed receptor dimers and autophosphorylated on tyrosine residues. We also determined that M-CSFr Ig-likes domains 3, 4 and 5 were sufficient for binding M-CSF. M-CSFr Ig-like domains 1 and 2 were not necessary for binding. Despite the high degree of conservation between the PDGFβr and the M-CSFr, the ligand binding regions were located in distinct sites on the respective receptors. The alignment matrix (Figure 2b) reveals that for each PDGFβr Ig-like domain, the corresponding M-CSFr Ig-like domain is more similar than any other PDGFβr or M-CSFr Ig-like domain (with the exception of PDGFβr Ig-like domain 3, for which M-CSFr Ig-like domain 3 is the second best match). We also determined that the fifth Ig-like domain of the M-CSFr is required for PK-C mediated receptor degradation, a process peculiar to the M-CSFr.

Lastly we characterized the requirements for receptor cross-phosphorylation, and found that cross-phosphorylation only occurs between receptors which can bind the same ligand.

## Bacterial Expression of PDGFβr Extracellular Region Fragments

*Rationale and experimental design* Mapping studies with chimeric receptors provided a great deal of information about the PDGFβr and M-CSFr ligand binding sites and about ligand activation of receptor kinase activity. However, there were two major obstacles to further progress. First, the process of testing chimeric molecules is inherently slow. Stable cell lines require two months to make. While the time could be reduced with transient assays in Cos cells or Xenopus Laevis oocytes, the full range of PDGFβr activities cannot be measured in these systems. A second problem is that while M-CSFr does not bind BB-PDGF and PDGFβr does not bind M-CSF, we cannot exclude the hypothesis that part of the binding energy comes from region(s) which are conserved between the receptors. In this case, an Ig-like domain from one receptor could, in principle, complement the function of an Ig-like domain from the other receptor.

The obvious way to rule out complementation is to make deletions, rather than substitutions. However, when Ig-like domain 5 (J. A. Escobedo, unpublished), domains 1, 2 and 3 (M. T. Keating, unpublished), domains 1, 2, 3 and 4 (M. T. Keating, unpublished), or domain 1 (P. Orchansky, unpublished) were deleted, the mutated receptors were expressed intracellularly. In contrast, expression of a PDGFβr cDNA with a stop codon prior to the transmembrane region led to the secretion of the extracellular region of the PDGFβr (Duan et al., 1991). This soluble protein (XR, for extracellular region) was able to bind BB-PDGF with high affinity, and also functioned as a BB-PDGF

antagonist. Methotrexate amplification of the expression cells has resulted in protein production at 12 mg/ml in protein-free medium (D.-S. Duan, unpublished). We decided to attempt expression of PDGFβr XR deletion mutants in bacteria, so that we could rapidly test deletions and, eventually, point mutations within the smallest active fragment. Similar experiments with the Ig-like molecule CD-4 led to determination of specific resiues within CD-4 Ig-like domain 1 which interact with the HIV coat protein gp120 (Sweet et al., 1991).

## Expression

We chose to express the fragments with a bacterial signal sequence, because reports in the literature indicate that this is most likely to result in an active protein with correct disulfides in the absence of an *in vitro* refolding step. (For the *in vitro* refolding route, it seems that expression at high levels and high temperature (37-42°) leading to inclusion bodies containing 50-80% of the target protein is the best source; *in vitro* refolding requires pure protein). Export of proteins to the periplasmic space and secretion into the medium are both inefficient in *E. coli*, so we chose to use an inducible T7 polymerase system. Briefly, the host strain (BL21DE3) contains a λ lysogen (DE3) encoding the T7 RNA polymerase, under control of a *Lac* repressor (IPTG inducible). Our expression vector (pET-OF), a modified pET-3a vector (Studier et al., 1990) has a T7 promoter upstream from the transcription unit. The advantage is the potential for extremely high-level expression; the disadvantage is the limited availability of host strains, which can be very important in

expression in a manner which depends upon the specific protein (Fuh et al, 1990).

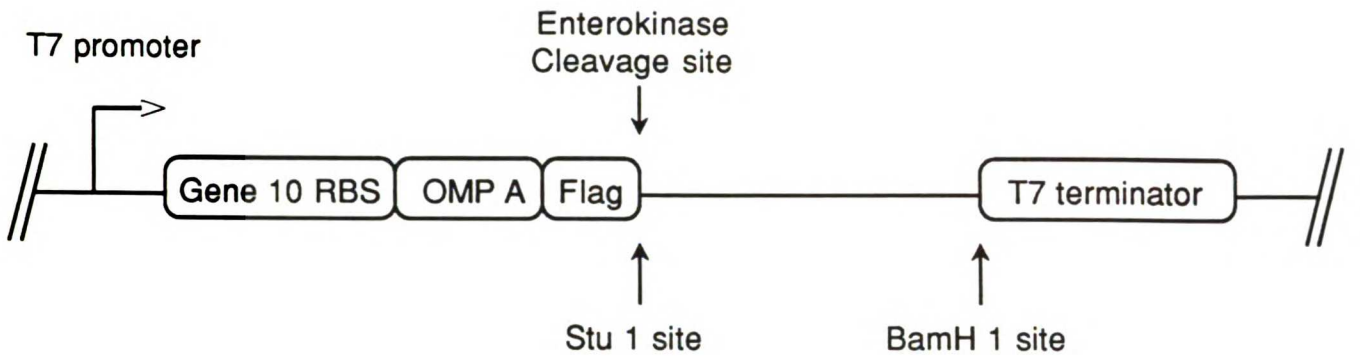
Our expression plasmid, pET-OF, is shown in Figure 22. We inserted a signal sequence from the bacteral protein OMP A and a nine amino-acid epitope tag (Flag; Hopp et al, 1988), followed by a Stu1 (blunt) restriction site, allowing precise joining to engineered inserts. The inserts used in this study are also represented in Figure 22. PDGFβr cDNA was modified by oligo-directed mutagenesis to insert an Nae1 (blunt) site at the 5' end of the N-terminal domain for each insert, and a stop codon followed by a BamH1 site was placed 3' to the C-terminal domain in each insert. In two constructs ($12H_5$ and $123H_5$) five histidine codons were inserted between the stop codon and the BamH1 site, encoding a tag for metal-affinity chromatography.

*Optimization of expression-* Expression of the receptor fragments was optimized with the PRF-1 construct. Several variables were tested; composition of medium, temperature of induction, time of induction, concentration of inducer, and subcellular fractionation. Rich medium (2 x YT) gave highest expression levels (data not shown). Long induction times (24 hours) produced more protein than shorter inductions (1-8 hours; Fig 23). At long induction times, lower temperature (20°C, which has been reported to increase yield of native protein; Browner et al., 1991; Schein, 1989) produced equivalent amounts of protein as 30°C (Fig 23). Induction with sub-maximal IPTG (25 uM) produced more protein than complete induction (Fig 23). It is not clear if this is because a higher fraction of the protein was exported, or if more cell growth occurs (complete

Fig. 22. **pET-OF vector/expression cassettes.** Top drawing illustrates the pET-OF expression vector. Gene 10 RBS is a strong ribosome binding site from the T7 gene 10. OMP A is the signal sequence from the bacterial protein OMP A. Flag is an antibody epitope tag. Enterokinase protease site is indicated. This plasmid is used in bacterial strains which have a T7 RNA polymerase which is inducible by IPTG such as BL21(DE3). pET-OFS has a PDGFβr StuI-BamHI "stuffer" for ease of cloning. Enterokinase cleavage requires that the first amino acid not be proline.

Bottom drawing illustrates expression cassetes used in this study. All have Nae 1 (blunt) sites at 5' end and BamH 1 sites at 3' end. HHHHH is the polyhistidine tag added to some proteins. Stop indicates stop codon.

# pET-OF Bacterial Expression Vector




# Bacterial Expression Inserts

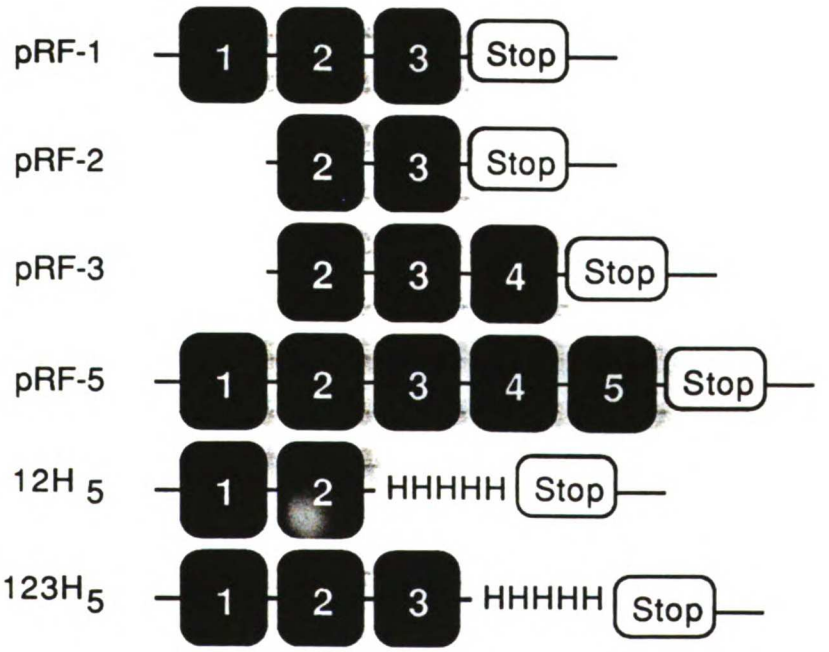
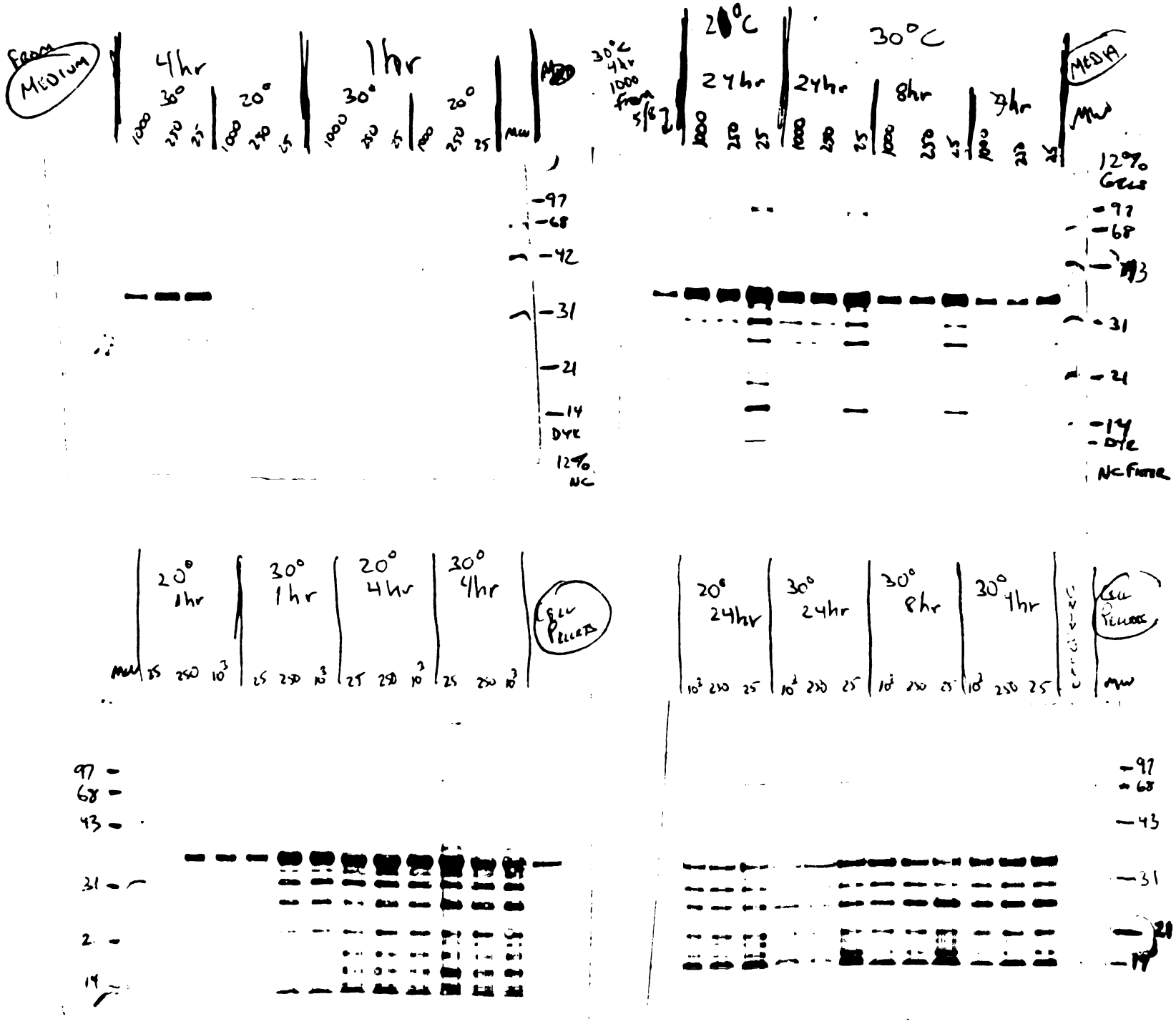


Fig. 23. **Optimization of expression conditions.** Western blots of proteins in Medium and cell pellet. Times indicate time after adding IPTG at O.D. 0.5; [IPTG] is 25, 250, or 1000 uM as indicated. Cells were grown at 30°C until induction.


From MEDIUM
4hr
1hr
30°
20°
1000
250
25
MW
-97
-68
-42
-31
-21
-14
DYE
12% NC
30°C 4hr 1000 from 5/8
20°C
30°C
24hr
8hr
3hr
MEDIA
12% Gels
NC Filter
20° 1hr
30° 1hr
20° 4hr
30° 4hr
Glu Pellets
20° 24hr
30° 24hr
30° 8hr
30° 1hr
Glu Pellets
10³
97
68
43
31
21
14

induction drastically curtails cell growth). Most of the processed protein is apparently exported (Fig 23).

## Activity

*Binding Activity of Fragments-* We attempted to characterize the receptor fragments with a variety of binding assays. Initially, we attempted to use the solubilized receptor binding. While the Flag antibody was able to immunoprecipitate the fragments efficiently, the standard soluble binding protocol gave a very high background (binding to PRF-1 and vector extracts was similar; data not shown). One possible explanation for this unexpected finding is that animal cell lysates contain proteins that efficiently block non-specific $^{125}$I-BB-PDGF binding in this assay. Bacterial proteins were mixed with lysates from vector-transfected CHO cells (D7d) or TX-100 buffer containing 0.3 % gelatin. Though the background was reduced, there was little, if any, binding to any of the bacterial proteins (Figure 24). As expected, lysates from PDGFβr-transfected cells (DR12) bound 10 x background (30 x in this experiment; Figure 24).

We investigated several other binding protocols. One, which we call binding on beads, consists of first immunoprecipitating bacterial proteins, and subsequently incubating them with $^{125}$I-BB-PDGF. Using this protocol, PRF-1, but not PRF-2. or PRF-3, appeared to bind $^{125}$I-BB-PDGF (Figure 25). We examined the dose-dependent competition of $^{125}$I-BB-PDGF binding to PRF-1. The data in Fig 26a demonstrate that the binding is of low affinity, approximately 4 nM, or 40 fold lower than wild type receptor.

Fig. 24. **Soluble binding with bacterial extracts.** Soluble binding on bacterial proteins in the presence of 0.3% Gelatin (Gel) or D7d TX-100 lysates (D7d). Samples with 10 nM unlabelled BB-PDGF competitor indicated with stippled bars.

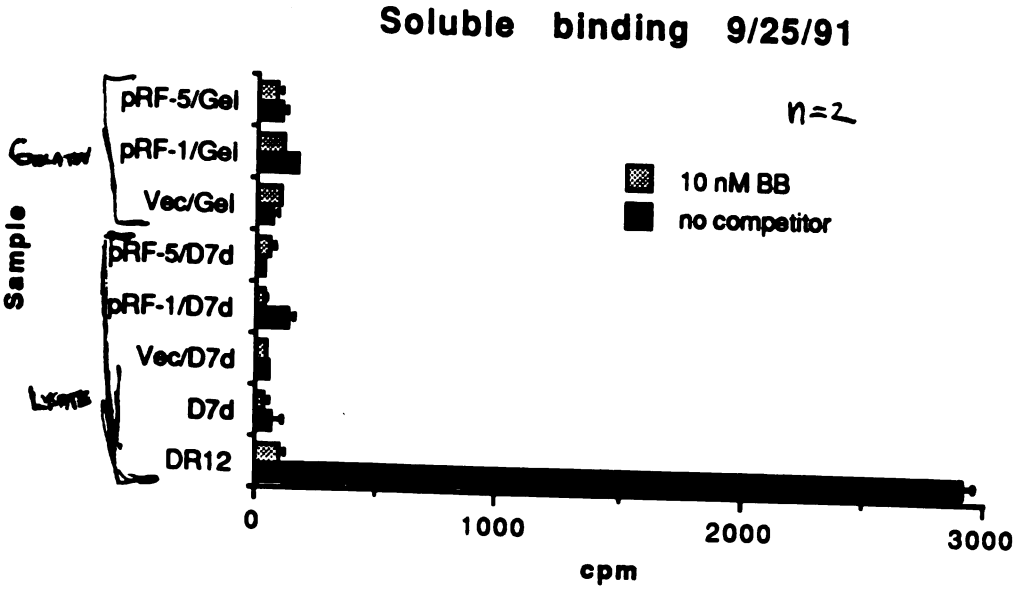

Soluble binding 9/25/91
n=2
10 nM BB
no competitor
Sample
pRF-5/Gel
pRF-1/Gel
Vec/Gel
pRF-5/D7d
pRF-1/D7d
Vec/D7d
D7d
DR12
0
1000
2000
3000
cpm

Fig. 25. **Binding on beads: pRF-1 binds PDGF.** Samples with 8.6 nM unlabelled BB-PDGF competitor inicated with stippled bars. Samples are in triplicate.

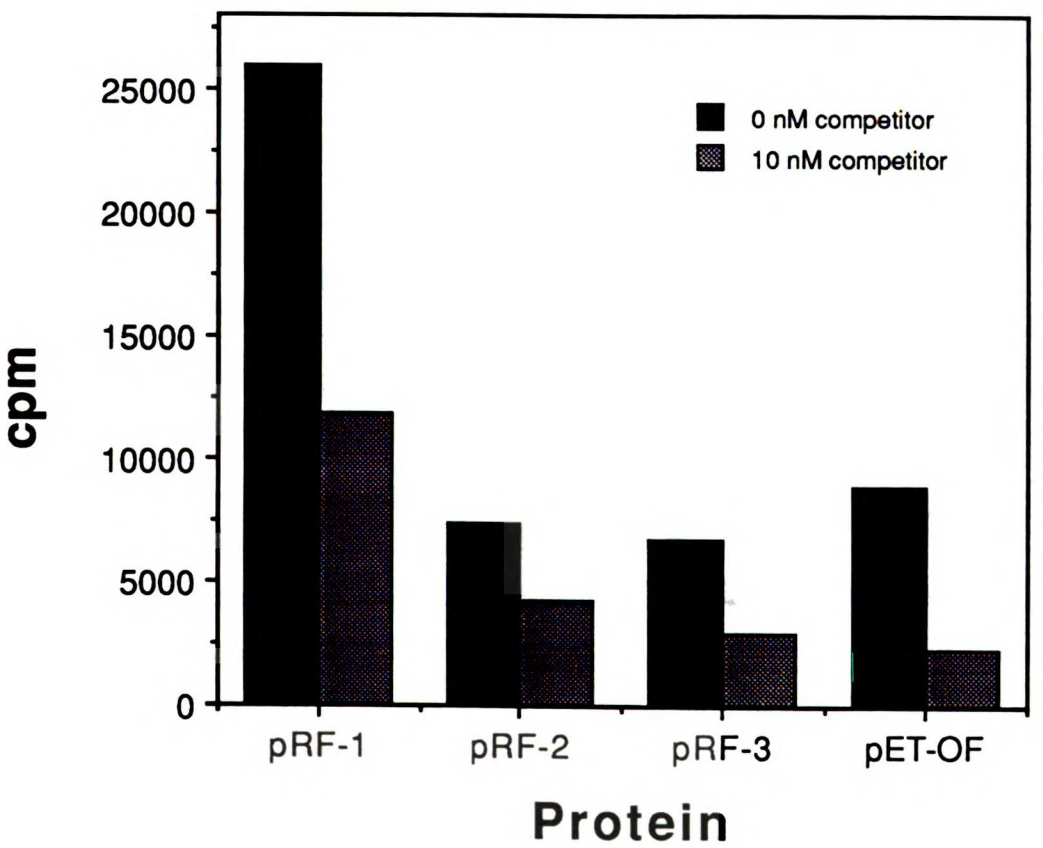
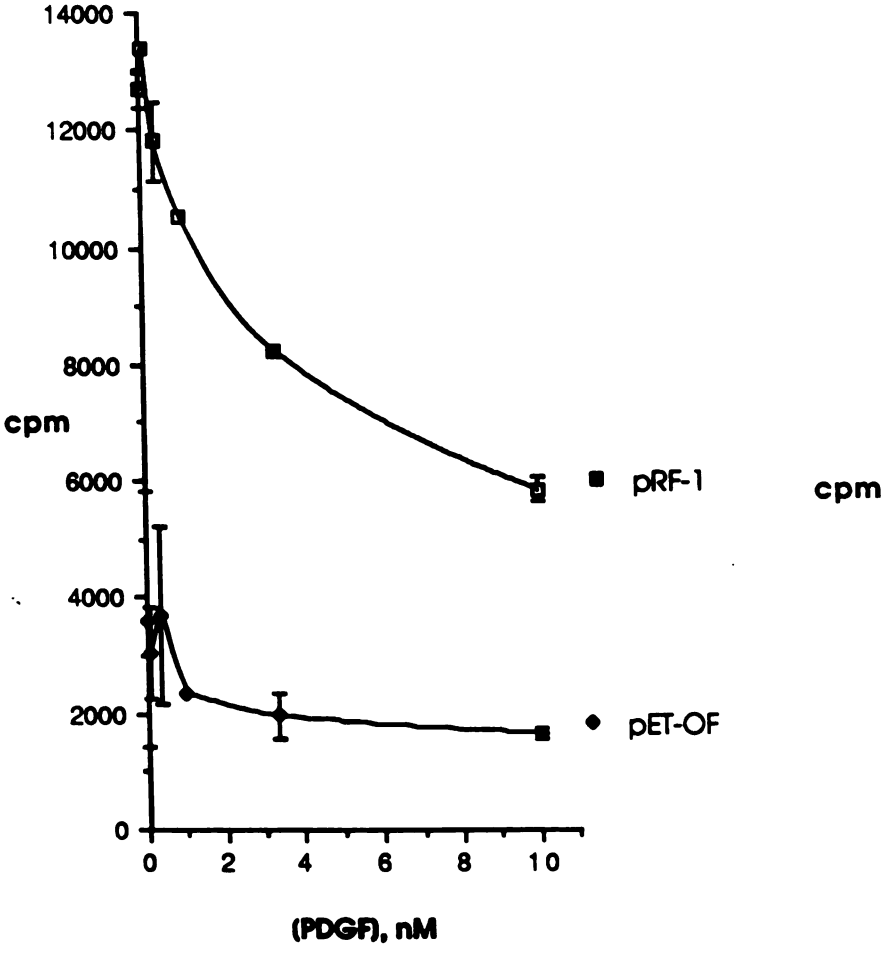
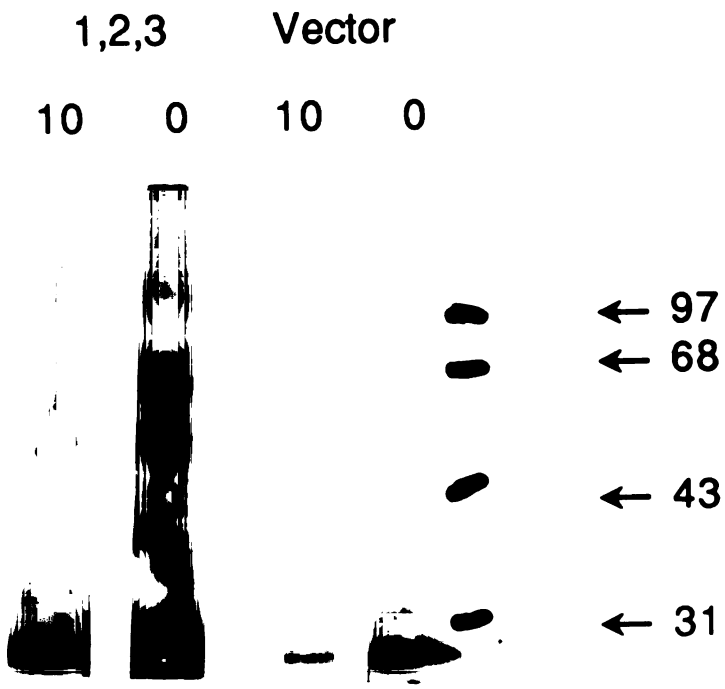

25000
20000
15000
10000
5000
0
cpm
0 nM competitor
10 nM competitor
pRF-1
pRF-2
pRF-3
pET-OF
Protein

Fig. 26. **pRF-1 binds PDGF with low affinity.** a) BB-PDGF binding curve in the presence of increasing concentration of unlabelled BB-PDGF. b) crosslinking pRF-1 to PDGF; Unlabelled BB-PDGF competitor is 0 or 10 nM.




1,2,3
Vector
10
0
10
0
97
68
43
31

Crosslinking revealed that receptor-ligand complexes only formed in the presence of added bacterial proteins, but could only be competed about 50 % with 10 nM unlabelled BB-PDGF (Figure 26b).

Because of the high background in this assay we measured the change in $^{125}$I-BB-PDGF binding with various amounts of bacterial proteins. In Fig 27a, the amount the amount of bound $^{125}$I-BB-PDGF containing extract increases with increasing PRF-1; there is no change in the amount of bound $^{125}$I-BB-PDGF with increasing amounts of vector extracts. In the presence of 5 nM unlabelled BB-PDGF competitor, again the amount of bound $^{125}$I-BB-PDGF increases with increasing PRF-1, albeit more slowly (Fig 27b). Similarly, the binding is independent of the amount of vector extract in the presence of 5 nM unlabelled BB-PDGF competitor. Control experiments prove that the background binding is to the Protein A beads or the reaction tubes (data not shown), and not to proteins in the bacterial extract or the primary antibody.

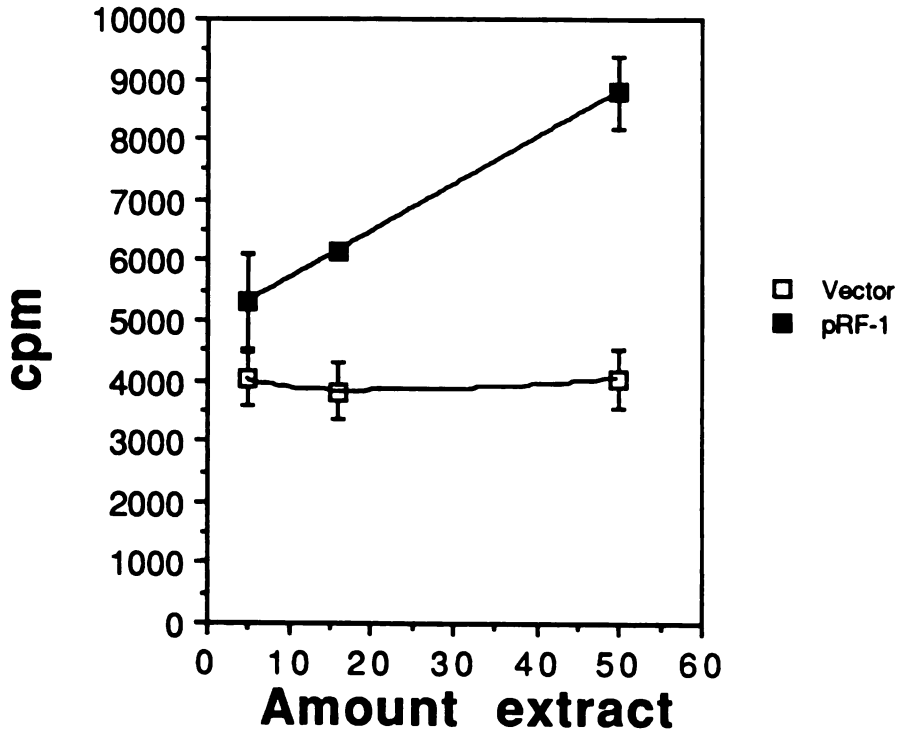
Roxanne Duan purified a small amount of PRF-1 and PRF-2 to homogeneity by batch adsorption to M1 antibody/Protein A, followed by elution with EDTA (M1 antibody is calcium dependent). Roxanne mixed the purified proteins (> 90 % pure) with $^{125}$I-BB-PDGF, plus 0 or 200 nM unlabelled BB-PDGF. Both proteins can be crosslinked to $^{125}$I-BB-PDGF (data not shown); however the binding is apparently low affinity for both proteins. This experiment, which has only been done once, confirms the low affinity binding seen for PRF-1 in crude extracts using highly purified proteins.

*Colony lift binding--* Jim Wells' laboratory demonstrated that $^{125}$I-Growth hormone binding to bacterially expressed growth

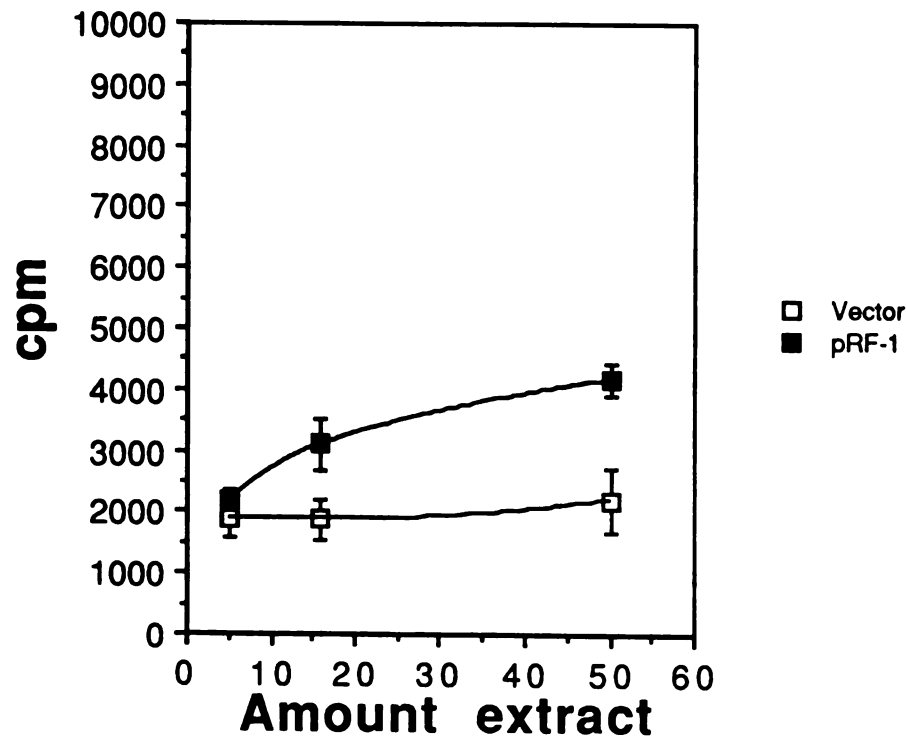
Fig. 27. **pRF-1 binding controls.** Binding dose response with varying amounts of vector or pRF-1 extracts a) no unlabelled BB-PDGF b) 10 nM unlabelled BB-PDGF

☐ Vector
☒ bRF-1

A) Protein dose response




B) Protein dose-response with competitor



hormone receptor could be measured directly in colony filter-lifts (Fuh et al., 1990). We attempted similar experiments with PDGFβr XR fragments, because this techique would allow rapid screening of PDGFβr XR mutants for binding. Briefly, bacteria were patched onto LB plates, grown until patches were visible (4-8 hr 37°) and overlaid with 10 cm nitrocellulose disks (Millipore). Filters were lifted and placed face up on LB/IPTG plates overnight at room temperature. Filters were frozen 10 minutes, -70° and thawed 10 minutes, 37°, to disrupt bacteria. Filters were blocked 2 x 30 minutes RT with binding buffer (0.025 M HEPES, pH 7.5; 0.1 M NaCl; 0.1 % TRITON X-100, 0.02 % $NaN_3$; 30 ug/ml kanamycin; 0.5 % BSA). Filters were washed 3 x 5 minutes, incubated with binding buffer containing 50 pM $^{125}$I-BB-PDGF 1 hr RT, washed 3 x 5 minutes, and air dried. Filters were autoradiographed, and subsequently immunoblotted with Flag antibody. pRF-2, pRF-1, $123H_5$ and $12H_5$ bound 2-4 x as much $^{125}$I-BB-PDGF as bacteria transformed with vector (data not shown). The binding was enhanced by freeze-thaw treatment of the filters. Immunoblotting confirmed protein expression in the appropriate cells, which correlated with binding (data not shown). However, as discussed in *Dot blot binding* we think it is likely that this binding is low-affinity $^{125}$I-BB-PDGF binding to the Flag epitope tag.

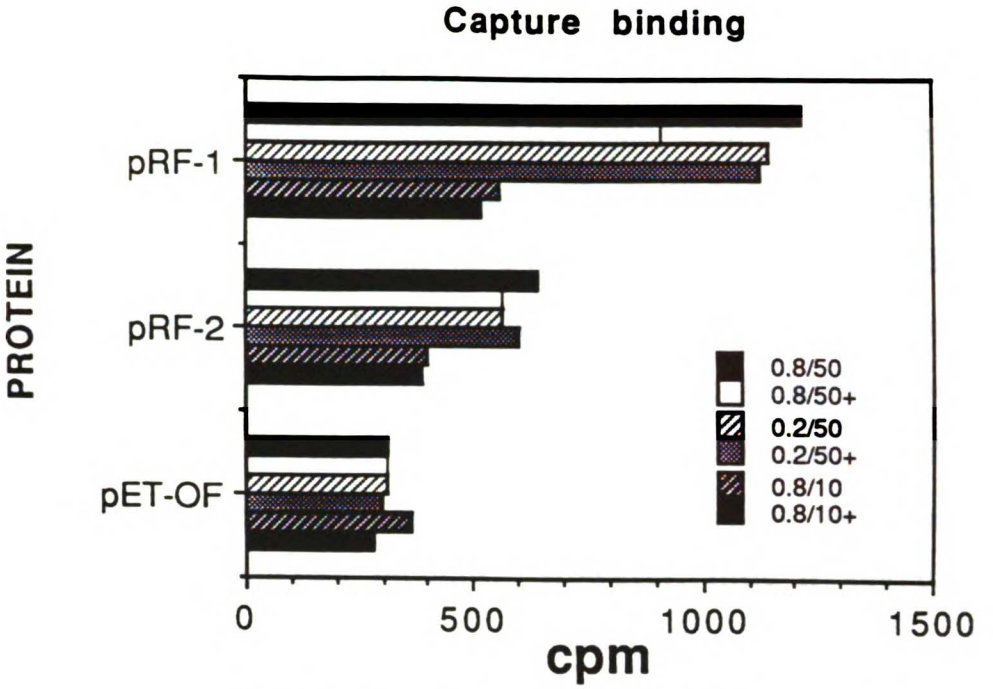
*Dot blot binding--* Though the Colony lift binding initially appeared successful, the high background present in the vector-transformed cells reduced the sensitivity of that technique. We investigated $^{125}$I-BB-PDGF binding to bacterial proteins spotted directly onto nitrocellulose (dot-blots). Briefly, applied to

nitrocellulose and air dried for 2 hr, RT. Filters were processed as for Colony-lift binding. Several observations were made (data not shown): 1) pRF-1, pRF-2, pRF-3, pRF-5, $123H_5$ and $12H_5$ proteins all bound $^{125}$I-BB-PDGF; 2) There was no binding to extracts from vector cells; 3) concentration by 80 % $NH_4SO_4$ precipitation was necessary for $^{125}$I-BB-PDGF binding; 4) binding was dependent upon the amount of protein applied; 5) Inclusion of 10 or 100 nM unlabeled BB-PDGF had little, if any, effect upon binding; 6) Active FGFr1 extracellular region (FGFr1 XR), expressed in E. Coli or SF9 insect cells, did not bind $^{125}$I-BB-PDGF; 7) neither active PDGFβr XR from CHO cells nor extracts from control cells bound $^{125}$I-BB-PDGF; and 8) $^{125}$I-bFGF did not bind to FGFr1 XR expressed in E. Coli (Khoi Le) (data not shown). These results, taken together, are consistent with low-affinity binding of $^{125}$I-BB-PDGF, most likely to the Flag epitope tag.

*Capture binding--* Another rapid screening method we tested is capture binding. This particular protocol was developed by Larry Fretto. Microtiter plate wells were coated with antibodies, and receptor fragments were "captured", and incubated with $^{125}$I-BB-PDGF. Figure 28a provides data from a representative experiment. Wells were coated with 0.8 or 0.2 ug monoclonal antibody, and incubated with 50 or 10 ul bacterial extract. $^{125}$I-BB-PDGF binding to pRF-1 was similar in wells with 0.8 or 0.2 ug antibody, indicating that the amount of antibody incubated with the wells is not limiting. In contrast, reducing the amount of bacterial extract incubated in the well from 50 ul to 10 ul greatly reduced binding, suggesting that the amount of receptor fragment is limiting. Unlabelled BB-PDGF

Fig. 28. **Capture binding.** a) proteins from medium; 0.8 or 0.2 ug of Ab/well, as indicated; 50 or 10 ul of bacterial extract, as indicated; + indicates 10 nM unlabelled BB-PDGF b) proteins from lysates (L), concentrated medium (ppt) c) XR dose response

A)


Capture binding
PROTEIN
pRF-1
pRF-2
pET-OF
0.8/50
0.8/50+
0.2/50
0.2/50+
0.8/10
0.8/10+
0
500
1000
1500
cpm

B)

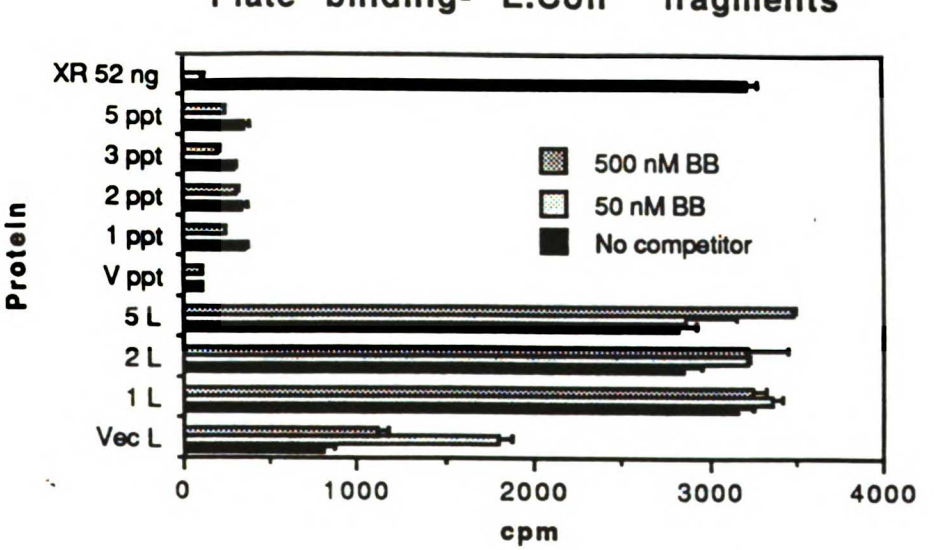
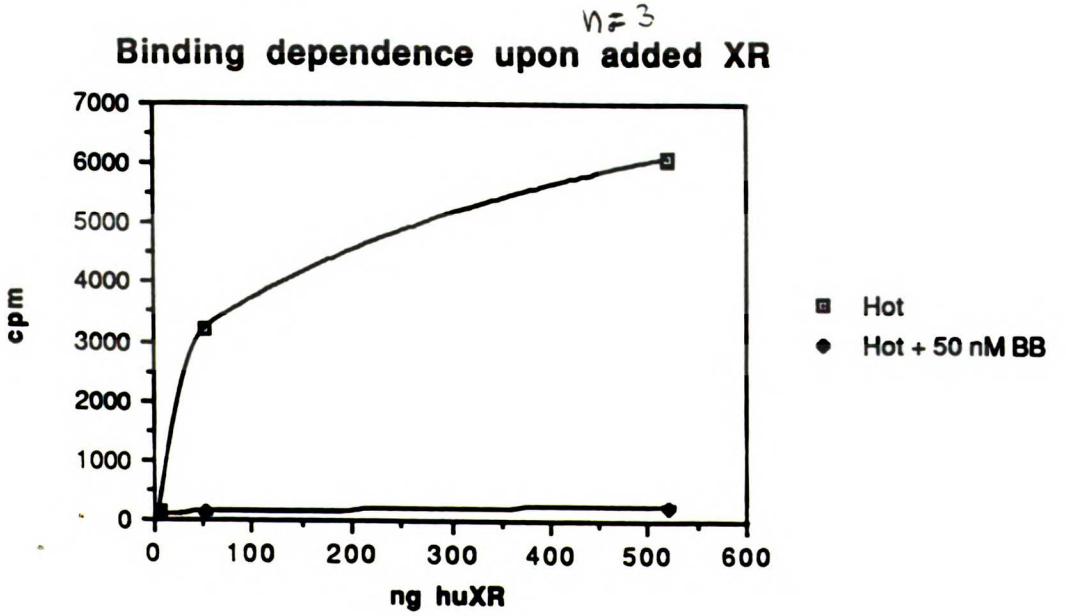


Plate binding- E.Coli fragments
Protein
XR 52 ng
5 ppt
3 ppt
2 ppt
1 ppt
V ppt
5 L
2 L
1 L
Vec L
500 nM BB
50 nM BB
No competitor
0
1000
2000
3000
4000
cpm

n=3

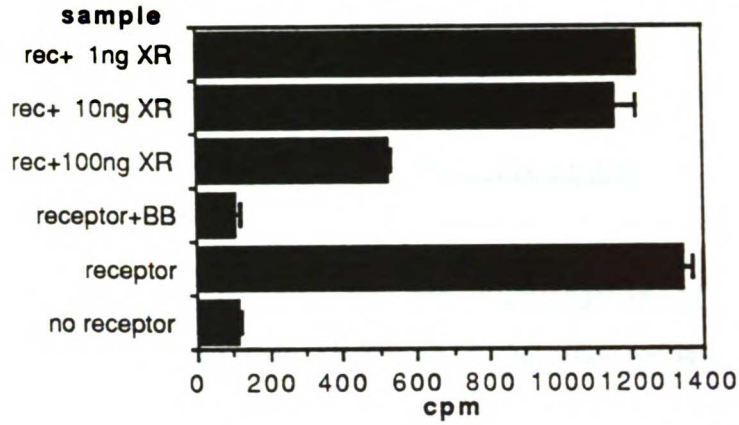

Binding dependence upon added XR
cpm
7000
6000
5000
4000
3000
2000
1000
0
0
100
200
300
400
500
600
ng huXR
Hot
Hot + 50 nM BB

competitor had little, if any, effect upon binding. In contrast, binding to vector extracts was significantly less than to pRF-1, and independent of the amount of extract used. Though pRF-2 bound less than pRF-1, the binding was dependent upon the amount of extract used. This experiment is consistent with the results from binding on beads (Figs 25-27) and direct crosslinking of purified proteins. We tried to increase the sensitivity of the assay by using $NH_4SO_4$-concentrated proteins. However, little if any binding was observed after concentration, and the differences among pRF-1, pRF-2 and pRF-5 vanished after precipitation (Fig 28b). We cannot exclude the hypothesis that $NH_4SO_4$ precipitation inactivates these proteins. Lysates from sonicated bacteria also behaved anomolously. $^{125}$I-BB-PDGF binding to huPDGFβr XR produced in animal cells was strongly dependent upon the amount of protein added (Fig 28c).

One potential problem with the plate binding assay is that immobilization of the receptor could sterically block ligand binding. Indeed, some PDGFβr MAb block binding in the capture assay (L. Fretto, unpublished). We captured huPDGFβr XR (52 ng/well) and measured $^{125}$I-BB-PDGF binding to huPDGFβr XR in the presence of bacterial extracts. Over the concentrations tested, there was no striking difference among vector, pRF-1 and pRF-5 extracts. This was true of bacterial supernatant (Fig 29b), $NH_4SO_4$-concentrated medium (Fig 29b) and bacterial sonicates (Fig 29b). However, muPDGFβr XR, which does not bind to the antibody used to capture the huPDGFβr XR, was able to block binding to the huPDGFβr XR (Fig 29a).

Fig. 29. **Blocking capture binding with bacterial proteins.** a) capture binding controls. 52 ng huPDGFβrXR, except in no-receptor control. 1, 10 or100 ng muPDGFβrXR added as indicated. BB indicates 10 nM unlabelled BB-PDGF. b) blocking capture binding with bacterial proteins

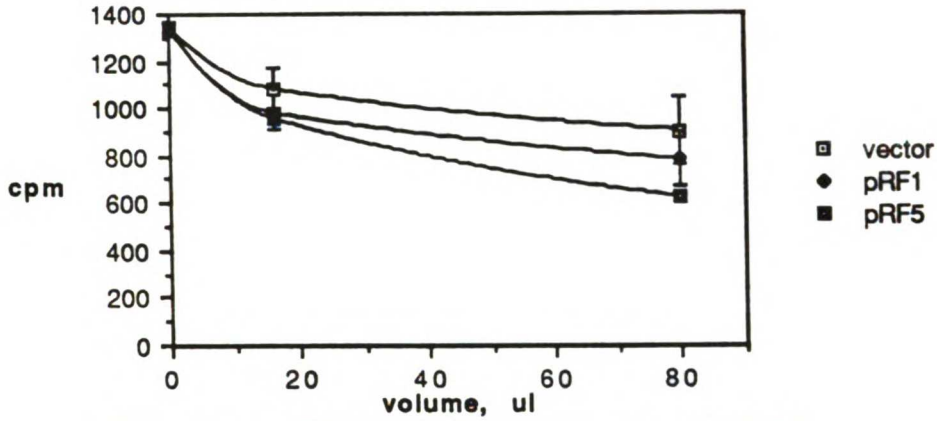
## Controls-10/8/91 Plate Binding




n=2

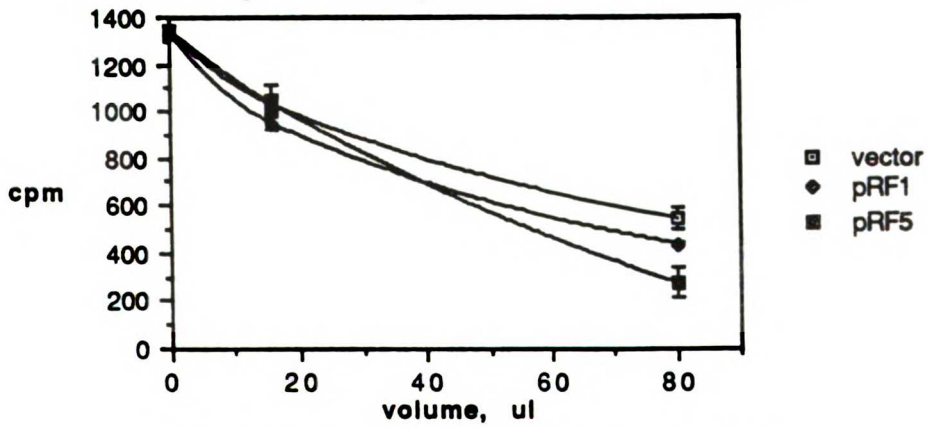
9

## Blocking Binding-Medium 10/8/91

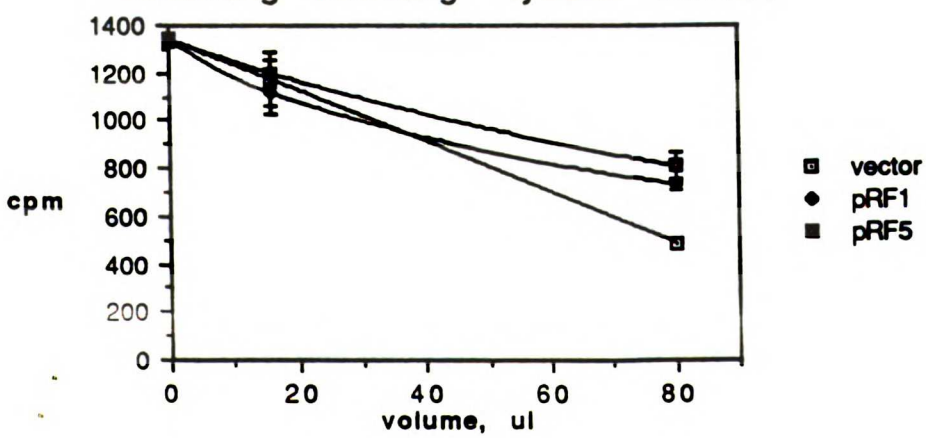



b

## Blocking Binding-Conc med 10/8/91




## Blocking Binding- lysate 10/8/91

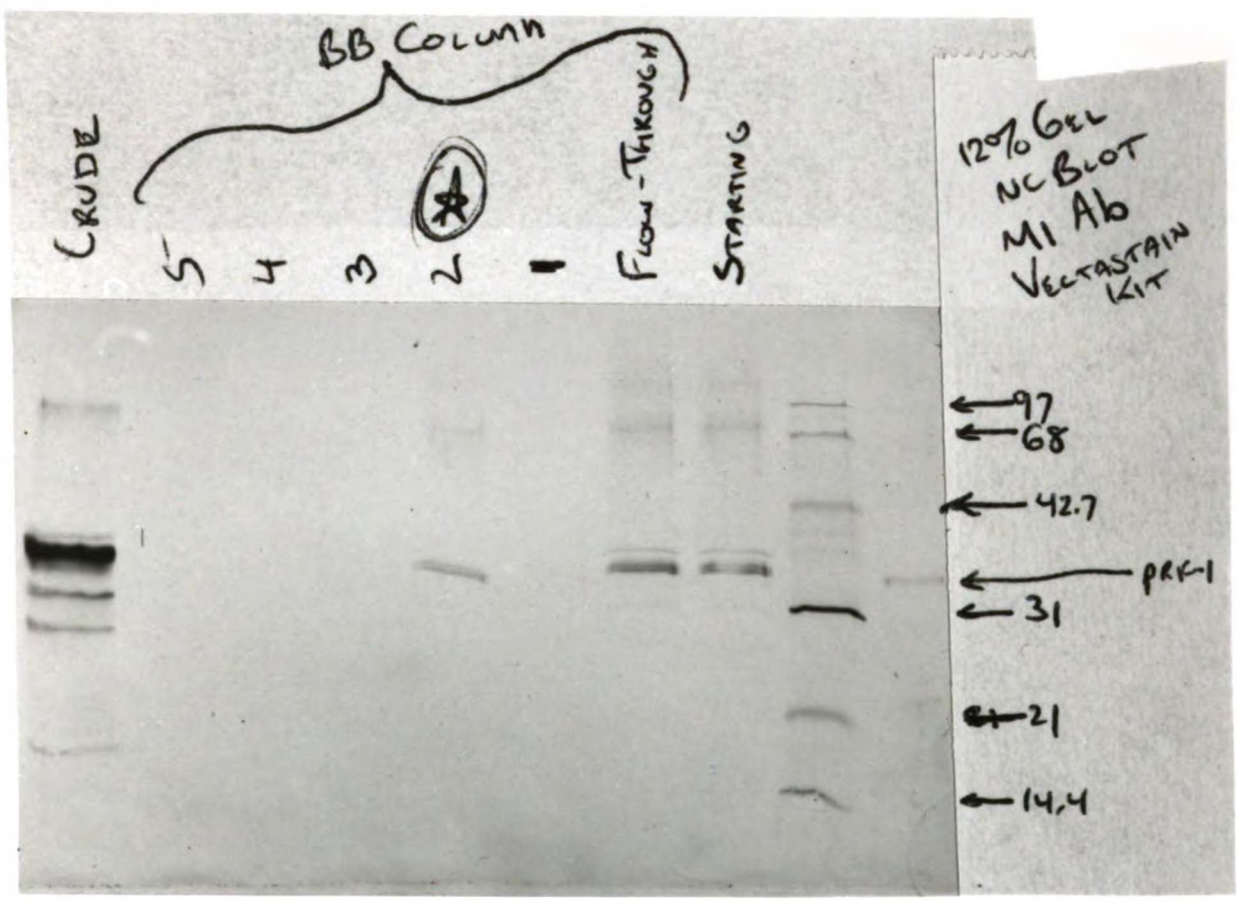


## Purification

*BB-PDGF affinity column purification of pRF-1--* We considered the possibility that the lack of high-affinity BB-PDGF binding activity observed with pRF-1 might be caused by the protein preparation being composed of both active and inactive protein. One way to separate the two is a BB-PDGF affinity column (Duan, R., unpublished). BB-PDGF was coupled to Affigel-10 resin (Biorad), and both muPDGFβr XR and huPDGFβr XR bound to the column. PDGFβr XR could be eluted from the column with Suramin, a poly-anionic small molecule which probably destabilizes ionic interactions. The column could the be regenerated with a high salt and low salt wash, and used several hundred times (Mileas, D and Duan, R., unpublished).

We attempted to purify pRF-1, pRF-5, vector extracts and muPDGFβr XR as a positive control. pRF-1 protein bound to the column (Figure 30), while pRF-5 did not (data not shown). As expected, muPDGFβr XR bound to the column (data not shown). However, less than 5 % of the pRF-1 bound to the column (Figure 30; compare starting material and flow through), whereas approximately 90% of muPDGFβr XR bound to the column (data not shown). The column was not overloaded by pRF-1, because more muPDGFβr XR was loaded on the column and bound. The column was not irreversibly inactivated, because the muPDGFβr XR bound to the column before and after the pRF-1. pRF-1 could be eluted with suramin (Figure 30), like the muPDGFβr XR. While this does not in

Fig. 30. **Purification of pRF-1 with a PDGF affinity column.** Immunoblot of equal amounts of starting material, flow through, and column fractions (elution with suramin).


BB Column
Crude
5
4
3
2
1
Flow-Through
Starting
12% Gel
NC Blot
M1 Ab
Vectastain
Kit
97
68
42.7
PRF-1
31
21
14.4

partition coefficient for p

ld be caused by either a low bin

ent column binding condit

p gradient in

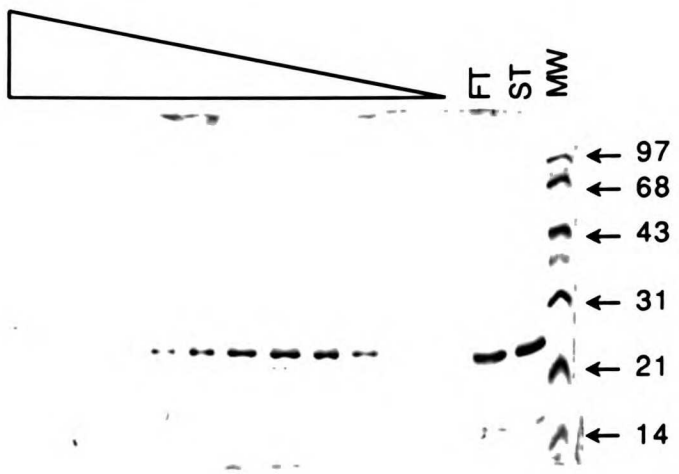
60 mM imidazole

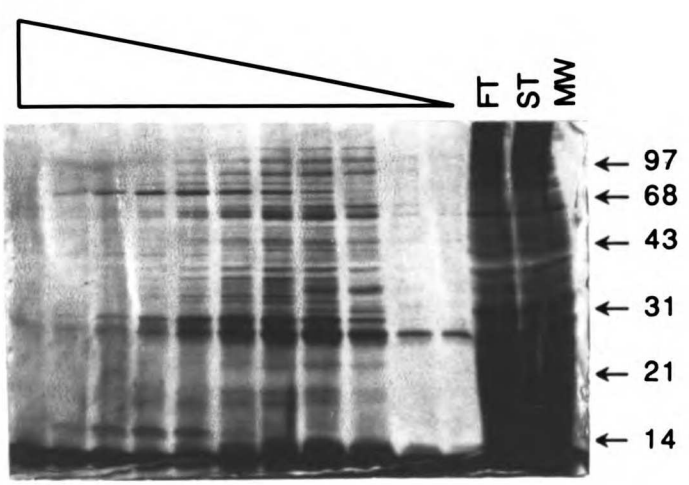
Da protein detected by

itself demonstrate specificity, binding crude muPDGFβr XR to a BB-PDGF affinity coumn and eluting with suramin purifies the protein to greater than 95 % (Duan, R., unpublished). Thus, it is likely that at least some of the pRF-1 could bind BB-PDGF, consistent with binding on beads (Figures 25-27), capture binding (Figure 28a), and direct crosslinking (data not shown). The low partition coefficient for pRF-1 with respect to muPDGFβr XR could be caused by either a low binding affinity, or a requirement for different column binding conditions.

*Metal affinity chromatography--* We considered the possibility that the failure to detect high-affinity binding could be caused by the presence of an inhibitor of binding in the protein preparation or low protein concentration. We partially purified some $12H_5$ on a metal affinity column (IDA resin loaded with $Zn^{++}$; Fast Flow Chelating Sepharose from Pharmacia). The crude protein was made 0.5 M NaCl, 0.02 M Phosphate, pH 7.2, and loaded by gravity flow onto a column charged with $Zn^{++}$according to the manufacturer's instructions. The bound proteins were then eluted with an imidazole step gradient in 0.02 M Phosphate, pH 7.2 containing 0.5 M NaCl. 66 mM imidazole (Figure 31; Fractions 1-4) began eluting a 26 kDa protein detected by immunoblotting (upper panel) and silver staining (lower panel). Elution was completed with 132 mM imidazole (Fractions 5-8) and 200mM imidazole (Fractions 9-12). Comparison of the starting material and flow-through, detected by immunoblotting (lower panel) reveals that the protein bound inefficiently. It was not investigated whether the protein in the flow-through could bind the column if reloaded. Thus, it is possible that the column was overloaded or that sub-optimal binding conditions were used; it is

Fig. 31. **Purification of $12H_5$ on a $Zn^{++}$/IDA affinity column.** Fractions described in text. ST is starting material; FT is flow through. upper panel-immunoblot with Flag antibody lower panel-silver stain


FT
ST
MW
97
68
43
31
21
14


FT
ST
MW
97
68
43
31
21
14

also possible that only a small fraction of the protein is able to bind the resin. However, examination of the silver stain gel (upper panel) demonstrates that the protein has been substantially purified; compare starting material with eluate lanes.

Materials and Methods

BB-PDGF
M-CSF

Goat-anti-Rat Protein A/Sepharose (Rat beads)
$^{125}$I-BB-PDGF (Bolton-Hunter reagent)
Phosphate-Buffered Saline (PBS)
Protein A/Sepharose beads (Protein A)
Quiescing medium (Q-medium)

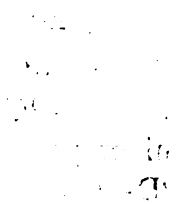
Binding, capture
Binding, immobilized on plate
Binding, whole cell, plate
Binding, whole cell, suspension
Binding, solubilized proteins
Cloning transfectants, limiting dilution
N-Glycanase treatment
Immunoprecipitations
In vitro kinase assay, ligand dependant, 35S
In vitro kinase assay, ligand dependant, 32P
*In vitro* kinase assay
Kinase assay, whole cells, phosphotyrosine blot
Lysates, animal cells
Phosphotryptic analysis
Screening stable transfectants
Silver staining gels
Stable transfections, $CaPO_4$
Surface determination of proteins
Western blotting

BB-PDGF

BB-PDGF was obtained from Chiron (Emeryville, CA). BB-PDGF stock solution was 10 uM in 1M HOAc. Dilutions (1 uM, 100 nM, 10 nM) were made by diluting with PBS. BB-PDGF was stored both at -70°C in small aliquots and at 4°C, for several months. Minimize number of freeze-thaws. Ron Seifert (U. Wash. Seattle) says OK to store in 10 mM HOAc. I collected $^{125}$I-BB-PDGF in 100 mM HOAc to minimize pH problems in binding reactions, etc.

M-CSF

M-CSF was obtained from Genetics Institute (Cambridge, MA). M-CSF was stored both at -70°C in small aliquots and at 4°C, for several months. Minimize number of freeze-thaws.



Goat anti-rat Protein A/Sepharose (Rat beads)

1. Mix 3 ul Goat anti-rat antibody per 40 ul 50% Protein A suspension.
2. Rock overnight 4°C.
3. Wash 3 times as with Protein A.

$^{125}$I-BB-PDGF

$^{125}$I-BB-PDGF can be purchased from Amersham (IM.213).

I have also labelled BB-PDGF as follows:

Reagents:

**[$^{125}$I] Mono-iodo Bolton-Hunter reagent** (Amersham IM.5861, 0.5 mCi)
0.1 M **borate buffer**, pH 8.5 (borax/sodium borate)
**quench solution** (borate buffer with 0.2 M glycine)
**PD-10 column** (Pharmacia , equilibrated with 50 ml 0.1 M HOAc/ 0.1% Pentex BSA (ICN )

Procedure:

1. Speed-vac dry 2.5 ug BB-PDGF (7.58 ul of 10 uM) in a siliconized eppendorf tube
2. Add 10 ul borate buffer; allow to dissolve at 4°C.
3. Dry Bolton-Hunter reagent carefully under gentle $N_2$ stream in hood; use 2 pairs gloves!
4. Add BB-PDGF to Bolton-Hunter reagent, mix
5. Incubate 15 minutes at 4°C.
6. Add 500 ul quench solution
7. Incubate 10 minutes at 4°C.
8. 10 % TCA ppt 1 ul of product with 10 ug BSA in 500 ul
9. Run product on PD-10 column, collect 1 ml fractions (use siliconized tubes for 3, 4, 5).
10. Count 2 ul of each fraction; first peak is protein
11. Calculate Specific activity fom TCA ppt
    [BB-PDGF] from amount of start, vol. of peak, and TCA recovery

Phosphate-buffered saline (PBS)

15.3 mM $Na_2HPO_4$
1.5 mM $KH_2PO_4$
2.7 mM KCl

140 mM NaCl
pH to 7.4 with NaOH

for 1 liter 10x
21.7 g $Na_2HPO_4$ (FW 142)
15 ml 1 M $KH_2PO_4$
13.5 ml 2 M KCl
81.8 g NaCl, and pH

Protein A/Sepharose (Protein A)

1. Protein A powder (Sigma or Pharmacia) is suspended in lysis buffer at 50% (v/v).
2. Rock > 1 hour, 4°C.
3. Let beads settle, or pellet 5 minutes 500 g
4. Wash twice more
5. suspend beads at 50% (v/v) in buffer with 0.2% $NaN_3$

Quiescing medium (Q-medium)

1. Weigh 250 mg BSA into 50 ml tube
2. Pour ~50 ml serum-free medium into tube, dissolve
3. Add 50 ul Transferrin (50 mg/ml in $H_20$)
4. Add 50 ul Insulin (10 mg/ml in 0.02 M HCl)
5. Add 12.5 ml 1 M HEPES pH 7.5
6. Sterile filter; add to 450 ml sterile, serum-free medium

Sodium orthovanadate

1. Make 100 mM Na3VO4 (sodium ortohvanadate) containing 10 mM Tris acid
2. Slowly add HCl to pH8 (pH paper OK)
3. Heat in microwave to boiling; turns yellow
4. Repeat 2 and 3 until pH is already 8; solution will be clear
5. Adjust volume for 100 mM solution. Store dark at4°C (do not freeze!). Do not use yellow solution, must be colorless.

Binding, capture

**Ab binding buffer**

25 mM Tris'HCl, pH 7.8
75 mM NaCl
4 mM $NH_4$ bicarbonate

**Blocking buffer**

0.025 M Hepes pH 7.5
0.1 M NaCl
0.5 % gelatin, EnzymeImmunoAssay grade, Biorad, dissolves at 40°C

**Ligand binding buffer**

0.025 M Hepes pH 7.5
0.1 M NaCl
0.3 % gelatin

1. Dilute MAb 0.5 ug/50 ul in Ab binding buffer
2. Plate 50 ul/well (96 well plates from Dynatech, Immulon II)
3. Incubate 4°C overnight
4. Rinse wells 2 x 100 ul blocking buffer, room temperature
5. Add 200 ul/well blocking buffer
6. Incubate 2 hr, room temperature
7. Add receptor in 100 ul PBS. For hu βXR, use 50 ng/well
8. Incubate 2 hr room temperature
9. Wash 2 x 100 ul binding buffer
10. Add 100 ul binding buffer containing 0.5 ul $^{125}$I-BB-PDGF, 0.25-0.125 nM final, + 0-50 nM unlabeled competitor
11. Incubate 2 hr room temperature
12. Wash 3 x 150 ul PBS
13. Elute with 100 ul 2.5 % SDS (Hot) 20 minutes
14. Count

Binding, protein immobilized on plate

Binding, whole cell, on plates

**Binding buffer**

DME without serum, containing 0.3 % gelatin; heat to 40°C to dissolve 0.5 % BSA or 10 % platelet poor plasma can also be used, but gelatin works best.

**Lysis buffer**

0.025 M Hepes pH 7.2
0.137 M NaCl
1 % Triton X-100
0.1 % SDS

0.5 % Na Deoxycholate

1. Plate cells in 6 well plates, 1 well/point (duplicates or triplicates)
2. Wash cells 1 x 1 ml binding buffer, 4°C
3. Aspirate; add 500 ul binding buffer containing 0.05-0.1 nM $^{125}$I-BB-PDGF + 0, 0.002, 0.02, 0.2, 1, or 10 nM unlabeled BB-PDGF.
4. Rock 4°C, 2-4 hr.
5. Wash 3 x 1 ml PBS, 4°C
6. Lyse with 100 ul lysis buffer (TX-100 ok also)
7. Rock 10 minutes, 4°C
8. Count

For crosslinking: after step 5,

**Quench**

40 ul ).% M EDTA
100 ul 1 M Tris/HCl, pH 7.5
0.15 g glycine
$H_2O$ to 10 ml

1. Add 500 ul PBS/50 mM Hepes pH 7.5, containing 1 mM $BS^3$ (Pierce)
2. Incubate 30 minutes, 4°C
3. Add 50 ul quench
4. Incubate 30 minutes, 4°C
5. Aspirate, make lysates, immunoprecipitate, add sample buffer, and run on 4.5 % gel (3% stack) or 3-8% gradient

Binding, whole cell, in suspension

**Gradient juice**

Dilute Ficol-paque to 28.5 % (v/v) with PBS
add some Congo red for visibility

(If cells are already in suspension start at step 5)

1. Wash cells 2 x PBS
2. Add PBS with 2 mM EDTA
3. Incubate 37°C, 15 minutes
4. Pipet cells off plate
5. Pellet cells 500 g, 5 minutes, 4°C
6. Resuspend cells in PBS at 5 x $10^6$ cells/ml 4°C
7. In 12 x 75 mm borosilicate tubes, on ice, add (in order) 100 ul PBS, 25 ul platelet poor plasma, 100 ul cells, and 25 ul (final reaction concentration) 0.05-0.1 nM $^{125}$I-BB-PDGF + 0, 0.002, 0.02, 0.2, 1, or

10 nM unlabeled BB-PDGF. Can use 0.3 % gelatin or 0.5 % BSA final instead of PPP.

8. Shake 4-24 hours 4°C or 45 minutes 37°C
9. Layer onto 380 ul gradient juice in Eppendorf tube
10. Spin 5 minutes, 4°C
11. Aspirate liquid, cut off pellet with razor, and count

Binding, solubilized proteins

1. Make 450 ul aliquot of lysate (TX-100 buffer!) for each data point. Also, make a 10 ul aliquot for protein assay and a 10 ul aliquot for western blot
2. Add 50 ul TX-100 buffer containing (final reaction concentration) 0.05-1 nM $^{125}$I-BB-PDGF + 0, 0.002, 0.02, 0.2, 1, or 10 nM unlabeled BB-PDGF, and PDGFr Ab (Ab 88 at 1:500 final)
3. Add 15-30 ul Protein A/Sepharose suspension
4. Rock 4-24 hours 4°C
5. Pellet 2k rpm 1 minute in microfuge
6. Wash 1 x 1 ml TX-100, 1 x 1 ml Tris/HCl/LiCl, 1 x 1 ml $H_2O$, as for immunoprecipitations
7. Count pellet

Crosslinking: before or after step 7

**Quench**

40 ul ).% M EDTA
100 ul 1 M Tris/HCl, pH 7.5
0.15 g glycine
$H_2O$ to 10 ml

1. Resuspend in 500 ul PBS/50 mM Hepes pH 7.5, containing 0.1 mM $BS^3$ (Pierce)
2. Incubate 30 minutes, 4°C
3. Add 50 ul quench
4. Incubate 30 minutes, 4°C
5. Aspirate, resuspend in sample buffer, and run on 4.5 % gel (3% stack) or 3-8% gradient

Cloning transfectents, limiting dilution

(If cells are already in suspension start at step 2)

1. Trypsinize cells
2. Resuspend in complete medium at 500 cells/ml
3. Prepare an 8 x 12 96 well plate by adding 100 ul medium to all but one row of 8 wells
4. Add 200 ul cells to each empty well

5. Using multichannel pipettor, transfer 100 ul of cells to next row
6. Using multichannel pipettor, transfer 100 ul of cells to next row, etc.

N-Glycanase treatment

(procedure closely follows Genzyme protocol)
1. Immunoprecipitate receptors (P150/2 points), medium stringency wash
2. Resuspend IP pellets in 40 ul 0.5 % SDS/0.1 M 2-mercaptoethanol
3. Boil 5 minutes
4. Pellet 2k 5 minutes room temperature
5. Make 2 x 10 ul aliquots
6. To each add 10.8 ul 0.5 M NaPhos, pH 8.6, containing 0.5 mM PMSF; 1.8 ul 0.5 M EDTA; 5 ul 7.5 % NP-40; 2.4 or 1.2 ul $H_2O$; and 0 or 1.2 ul N-glycanase (Genzyme).
7. Incubate 37°C, 12-16 hours
8. Add sample buffer to 1 x, run on gel, blot

Immunoprecipitations

**Triton X-100 buffer**
0.02 M Tris/HCl, pH 7.5
0.137 m NaCl
10 % glycerol
1 % Triton X-100
**Denaturing lysis buffer**
0.025 M Hepes pH 7.2
0.137 M NaCl
1 % Triton X-100
0.1 % SDS
0.5 %

1. Add antibody to lysate at correct dilution (often 1:100, 1:500, 1:1000)
2. Rock 3 hrs to overnight, 4°C (If incubation will be more than 4 hours, can add Protein A, step 3, at same time, and skip step 4)
3. Add 15-30 ul Protein A/Sepharose suspension
4. Rock 45 minutes, 4°C
5. Pellet 1 minute 3000 rpm in eppendorf microfuge
6. Carefully aspirate, to avoid suckin' up beads
7. Wash several times:

**Low Stringency**
3 x 1 ml TX-100 buffer, 4°C
**Medium stringency**
1 x 1ml TX-100 buffer
1 x 1ml 0.5 M LiCl/0.1 M Tris pH7.5
1 x 1ml $H_2O$
**High Stringency**
1 x 1ml TX-100 buffer
1 x 1ml 0.5 M LiCl/1 % Triton X-100/0.1 M Tris pH7.5
1 x 1ml 0.5 M LiCl/0.1 M Tris pH7.5
1 x 1ml $H_2O$
**Denaturing**
Make lysate 0.1 % SDS, 0.5 % Na deoxycholate in step 1
Wash 1 x 1ml denaturing lysis buffer
1 x 1ml 0.5 M LiCl/0.1 M Tris pH7.5
1 x 1ml $H_2O$
8. After last wash, carefully remove most of supernatant
9. Resuspend in sample buffer, kinase buffer, elution buffer, etc

<u>In vitro kinase assay, ligand dependant, 35S</u>
From Theinnu Vu

1. For each data point, label 1 well of a 6 well plate with 0.25 mCi/ml 35S methionine in Met- med 2 hours, 37°C
2. Wash cells with PBS, 4°C
3. Lyse cells with 400 ul/well 20 mM Hepes, 7.4, 0.5 % Triton X-100, 10 % glycerol, 1 mg/ml BSA (optional?)
4. Pellet 10 minutes in microfuge, 4°C
5. Add 0 ro 5 nM BB-PDGF to sup
6. Incubate 15 minutes, room temperature
7. Place on ice, add 5 ul 10 mM ATP and 5 ul 1 $MgCl_2$
8. Incubate 15 minutes, 4°C
9. Add 100 ul 20 mM Hepes, 7.4, 3 % Triton X-100, 700 mM NaCl
10. Immunoprecipitate, run on gel, expose to film

<u>In vitro kinase assay, ligand dependant, 32P</u>

**Kinase lysis buffer (KLB)**
25 mM Hepes, pH 7.2
0.5 % Triton X-100
10 % glycerol
Add Aprotinin/Leupeptin/Vanadate/PMSF fresh

**$MgCl_2$ mix**
75 ul 1 M $MgCl_2$
675 ul KLB
**ATP mix**
715 ul KLB
7.5 ul 10 mM ATP
30 ul $\gamma\,^{32}P$ ATP

1. Wash 2 x PBS, 4°C
2. Add 3.7 ml KLB/P150
3. Rock 10 minutes, 4°C
4. Pellet 10 minutes 4°C
5. Make 400 ul aliquots for kinase, 10 ul aliquot for protein assay, and 10 ul aliquot for western
6. Add 0 or 10 nM BB-PDGF (final concentration)
7. Incubate 15 minutes, room temperature
8. Add 50 ul $MgCl_2$ mix
9. Add 50 ul ATP mix
10. Incubate 30 minutes room temperature
11. Make reaction 20 mM EDTA, 0.5 M NaCl
12. Immunoprecipitate, run on gel

<u>In vitro kinase assay, ligand independant</u>

**Kinase buffer**
50 mM Tris/HCl, pH 7.4
10 mM $MnCl_2$ (fresh!) (activates Y kinases)
10-20 uCi $\gamma\,^{32}P$ ATP (hot) or 100 uM ATP (blot or shift expt)
additives, for some kinases
2 mM DTT
10 uM unlabelled ATP
0.2-1% Triton X-100
25 mM β-glycerol phosphate (stabilizes S/T kinases)
substitute 10 $MgCl_2$ for $MnCl_2$

1. Immunoprecipitate kinase from TX-100 lysates
2. Add 50 ul kinase buffer
3. Incubate 20-30 minutes room temperature, flick tube every 5 minutes
4. Stop reaction with 100°C sample buffer, run on gel
5. Dye front of gel very hot! Do not run off, cut and discard

Kinase assay, whole cells, anti-phosphotyrosine blot

1. Start with 1 well/6 well plate/point
2. Reduce volume of medium to 500 ul
3. Add BB-PDGF to 2 nM or M-CSF to 1.3 nM (1:1000 for crude)
4. Place in incubator 5 minutes 37°C
5. Wash 2 x 4°C PBS
6. Lyse with TX-100 buffer, containing vanadate and EDTA
7. Run on gel, or IP for specific protein

Lysates, animal cells

**Triton X-100 lysis buffer**
(nuclei remain intact, can give cleaner results)
0.02 M Tris/HCl, pH 7.5
0.137 m NaCl
10 % glycerol
1 % Triton X-100
**Denaturing lysis buffer**
(disrupts nuclei and many protein-protein interactions)
0.025 M Hepes pH 7.2
0.137 M NaCl
1 % Triton X-100
0.1 % SDS
0.5 %
**Protease phosphatase and kinase inhibitors**
1 mM PMSF (100 mM stock, 2-propanol)
10 uM Leupeptin (10 mM stock, $H_2O$)
1 uM Pepstatin A (1 mM stock, MeOH, store at -20°C)
1 mM sodium orthovanadate (100 mM stock, $H_2O$)(Y Phsphatase inh)
1:100 Aprotinin (Sigma A-6012)
1:50 EDTA (0.5 M, pH 8.0)(kinase inh)

1. Wash cells 2 x 4°C with PBS
2. Add lysis buffer (1 ml/P150, 400 ul/P100, 100 ul/6 well plate)
3. Rock 4°C, 10 minutes
4. Spin 4°C, 10 minutes 14 k in microfuge
5. Supernatant is lysate-transfer to new tube, use for immunoprecipitation, binding, kinase or run at 150 ug/cm well for western etc.

Phosphotryptic analysis

**Blocking solution**
1.5 % Polyvinylpyrrolidone 40 (PVP-40)
in 0.1 m HOAc
**Trypsin solution**
197 mg $NH_4$bicarbonate
2 mg trypsin (TPCK treated, from Worthington biochemicals)
$H_2O$ to 50 ml; can store -20°C overnight
**pH 1.9 buffer**
800 ml $H_2O$
150 ml HOAc
50 ml 88 % formic acid
**TLC buffer**
75 ml N-butanol
60 ml $H_2O$
50 ml pyridine (spermicide!)
15 ml HOAc

1. Immunoprecipitate proteins
2. Label protein with in vitro kinase RXN
3. Run on SDS/PAGE, transfer to PVDF (Immobilon, Milipore)
4. Expose filter to film, cut band (count), place in eppendorf tube
5. Wet PVDF with MeOH, add 1 ml blocking solution
6. Incubate 1 hour, 37°C
7. Wash 5 x 1 ml $H_2O$
8. Add 200 ul trypsin solution
9. Incubate overnight, 37°C
10. Add 200 ul trypsin solution
11. Incubate 4 hours, 37°C
12. Remove supernatant, count (require 1500 cpm/sample).
13. Speed-vac dry (overnight)
14. Resuspend in 1 ml $H_2O$, speed-vac dry, two times
15. Resuspend in 500 ul $H_2O$, speed-vac dry, two times
16. Resuspend in 300 ul $H_2O$, speed-vac dry
17. Resuspend in 100 ul $H_2O$, speed-vac dry, count (cerenkov)
18. Resuspend at 1000 cpm/ul in pH 1.9 buffer
19. Load 1500 cpm/spot on TLC plate, silica?
20. Run 1000 V 27 minutes in pH 1.9 buffer (peptides run to -)
21. Air dry in hood, 1-2 hours
22. Chromatograph in TLC buffer

23. Dry, expose to film with screen 3 days -70°C

Screening stable transfectants

1. Pick colonies after 7-10 days of selection (at 1-3 mm size) with P20 and yellow tips, into 24 well plates.
2. Grow until 50-100 % confluent (3-7 days)
3. Pass 90 % into a 6 well plate well, 10 % 10 cm plate with matching labels
4. When 6 well plates are 50-100 % confluent, immunoprecipitate for protein of interest; run on gel, and immunoblot (can also blot whole cell lysate)
5. Maintain 10 cm plates which correspond to positives. Always keep at least 2 independant positives, and freeze some down immediately.

Silver staining gels

Use Biorad kit according to manufacturer's instructions

Stable transfectants, $CaPO_4$

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