Hut78/B12 in J1-λ phage by Sau3 AI partial digestion, Bam HI ligation, plaque hybridization with a full-length SIV_{mac} probe (*8*), and subsequent restriction enzyme analyses were performed as described (*20*).
22. D. I. Bernstein *et al.*, *J. Gen. Virol.* **67**, 1601 (1986).
23. J. A. Hoxie *et al.*, *Science* **234**, 1123 (1986).
24. P. Maddon *et al.*, *Cell* **47**, 333 (1986).
25. J. D. Lifson *et al.*, *Science* **232**, 1123 (1986).
26. Identical amounts of virus (RT activity 100,000 cpm) were concentrated by PEG precipitation of culture supernatants, applied to 5×10^6 CEM×174 cells, and cultured for 14 days. In situ hybridization was performed (*27*) with comparable 4- to 5-kb *pol*–central region–*env* nick-translated DNA probes derived from the homologous proviral genomes [specific activity 2×10^8 dpm/μg (^{35}S)]. Approximately 500,000 cells per slide were examined for viral-specific RNA production. Controls included HIV-1 and HIV-2 probes on uninfected cells and HTLV-1 probe on HIV-1– and HIV-2–infected cells.
27. H. E. Gendelman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7086 (1985).
28. We thank L. Montagnier for $HIV\text{-}2_{ROD}$ virus and clones; P. Kanki and M. Essex for fresh peripheral blood specimens from Senegal; and B. Haggarty, S. Bonser, and J. Holt for technical and editorial assistance. Supported by grants from NIH, the U.S. Army Medical Research Acquisition Activity, the Life and Health Insurance Medical Research Fund, and the Carter-Wallace Corporation. B.H.H. is a Special Fellow and J.A.H. a Scholar of the Leukemia Society of America and G.M.S. is a PEW Scholar of the Biomedical Sciences.

18 March 1988; accepted 17 May 1988

Two Classes of PDGF Receptor Recognize Different Isoforms of PDGF

CHARLES E. HART, JOHN W. FORSTROM, JAMES D. KELLY, RONALD A. SEIFERT, ROBERT A. SMITH, RUSSELL ROSS, MARK J. MURRAY, DANIEL F. BOWEN-POPE*

Previous studies involving platelet-derived growth factor (PDGF) have been based on the premise that a single cell-surface receptor binds all three isoforms of PDGF (AA, BB, and AB). It is now shown that two populations of PDGF receptor exist and can be distinguished by their ligand binding specificity. The B receptor binds only the BB dimer, whereas the A/B receptor binds AA, BB, and AB dimers. Human dermal fibroblasts appear to express seven times as much B receptor as A/B receptor. The B receptor is responsible for most PDGF receptor phosphorylation.

PLATELET-DERIVED GROWTH FACTOR is a potent mitogen for various types of mesenchymal cells (*1*). In addition to its mitogenic activities, PDGF stimulates a wide variety of processes, including vasoconstriction (*2*), chemotaxis (*3*), activation of intracellular enzymes such as glycogen synthase (*4*), phosphatidylinositol turnover and Ca^{2+} mobilization (*5*), rearrangement of the cytoskeleton (*6*), and stimulation of tyrosine-specific phosphorylation (*7*). Platelet-derived growth factor was originally identified (*8*) and purified (*9*) from human platelets. Pure PDGF from this source consists of dimers of two polypeptides termed A chain and B chain (*10*). The major form of human platelet PDGF appears to be an AB heterodimer. Naturally occurring homodimeric forms of PDGF (AA and BB) have also been isolated, from U2-OS osteosarcoma cell–conditioned media (*11*) and from porcine platelets (*12*), respectively.

In earlier studies involving PDGF, the existence of a single cell-surface receptor for PDGF was assumed. This receptor was described as a 170- to 180-kD membrane glycoprotein with an extracellular ligand-binding domain and an intracellular split tyrosine kinase domain (*13, 14*). At 37°C, binding of PDGF to this cell-surface receptor stimulates receptor-associated tyrosine kinase activity (*14, 15*) and internalization and degradation of the ligand-receptor complex (*16, 17*). Given the multitude of events that are stimulated after PDGF is added to cells in culture, and the recent finding that the A chain and B chain genes often appear to be independently regulated (*18*), it seemed plausible that the three isoforms of PDGF might stimulate effects unique to each isoform, either through interaction with different classes of PDGF receptor or by differential effects on a single receptor. To investigate these possibilities, we examined each of the three isoforms for their receptor-binding characteristics and their ability to stimulate PDGF receptor downregulation and receptor phosphorylation. For these studies we used recombinant AA and BB homodimers that we produced in a *Saccharomyces cerevisiae* expression system (*19*), AB heterodimer purified from platelet-rich plasma (*20*), and PR7212, a monoclonal antibody to a PDGF receptor, which we recently described (*21*).

Binding of PDGF to cells at 37°C leads to depletion of cell-surface PDGF binding sites resulting from receptor occupation and internalization, a phenomenon known as downregulation (*16*). To study ligand-in-

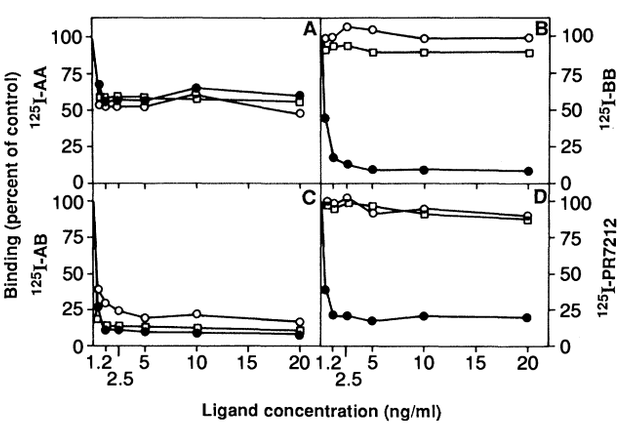

Fig. 1. Receptor downregulation assay. Human dermal fibroblasts grown in 24-well culture dishes (3×10^4 cells per well) were incubated for 2 hours at 37°C with increasing concentrations of the three isoforms of PDGF: (○) AA, (●) BB, or (□) AB. Incubation at 37°C was used to allow ligand-receptor internalization to occur. The cells were washed to remove unbound ligand, then incubated at 4°C for 2 hours with ^{125}I-labeled (**A**) AA, (**B**) BB, (**C**) AB, or (**D**) PR7212. The cells were washed, harvested with Triton X-100 solubilization buffer (*28*), and the total cell-associated ^{125}I was determined. The data were plotted as mean binding as a percent of control binding for determinations made from triplicate wells of a representative experiment. Variation between experiments was less than 10%. At least three experiments were done for each iodinated probe. Nonspecific binding was not subtracted. The inability of any ligand to decrease binding of ^{125}I-labeled AA below 50% reflects the high level of nonspecific binding for this preparation of ^{125}I-labeled AA. AA, AB, and antibody PR7212 were labeled with ^{125}I by use of Iodobeads (Pierce Chemical). BB was labeled with Bolton-Hunter reagent (Du Pont–New England Nuclear).

C. E. Hart and J. D. Kelly, Department of Pathology, University of Washington, Seattle, WA 98195, and ZymoGenetics, Inc., Seattle, WA 98103.
J. W. Forstrom, R. A. Smith, M. J. Murray, ZymoGenetics, Inc., Seattle, WA 98103.
R. A. Seifert, R. Ross, D. F. Bowen-Pope, Department of Pathology, University of Washington, Seattle, WA 98195.

*To whom correspondence should be addressed.

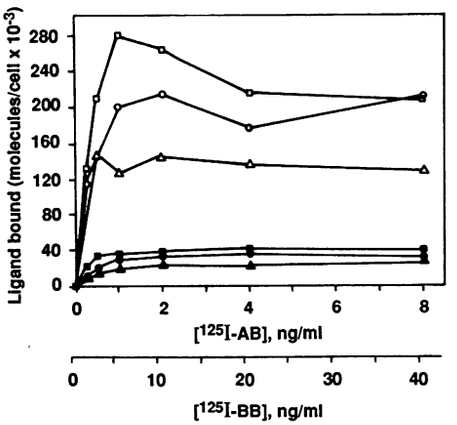

Fig. 2. Saturation binding of ^{125}I-labeled AB and BB was determined as previously described (*28*). Briefly, human dermal fibroblasts grown in 24-well culture dishes were incubated for 3 hours at 4°C with increasing concentrations of either ^{125}I-labeled AB (closed symbols) or BB (open symbols). The cells were washed to remove unbound ligand and harvested with Triton X-100 solubilization buffer (*28*); the total cell-associated ^{125}I was then determined. The data were plotted as mean value of number of ligand molecules bound per cell for determinations made from triplicate wells. Nonspecific binding, determined by the addition of 100-fold excess unlabeled ligand, was subtracted. Results from three individual experiments for each radiolabeled ligand are shown. Like symbols for AB and BB are from matched experiments. The tyrosine mutant form of BB was used for these studies (*27*) and was labeled with ^{125}I by use of Iodobeads. The specific activities of ^{125}I-labeled AB and BB were 945 and 149 cpm/fmol, respectively.

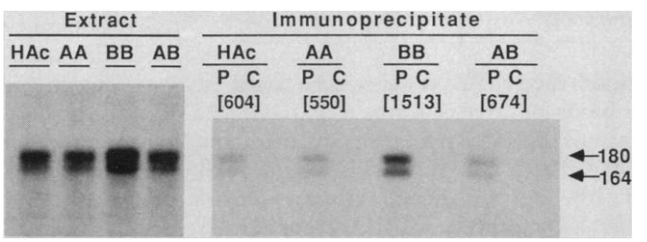

Fig. 3. Analysis of PDGF receptor phosphorylation in membrane extracts. Membrane extracts of human dermal fibroblasts were prepared and incubated with [γ-^{32}P]adenosine triphosphate (3000 Ci/mmol) (Du Pont–New England Nuclear) in the presence of 10 n*M* AA, BB, or AB, or 10 m*M* acetic acid vehicle control (HAc) as described (*21*). The ^{32}P-labeled extracts were analyzed either directly as the starting extract or after immunoprecipitation by antibody PR7212 (P) or a negative control monoclonal antibody (C), as described (*21*). The immunoprecipitated proteins were analyzed by SDS-PAGE on an 8% gel in the presence of 5% β-mercaptoethanol, followed by autoradiography. The regions of the dried gel containing the immunoprecipitated receptor bands (180 and 164 kD) were excised and the ^{32}P in the gel corresponding to each ligand was determined by Cherenkov counting (counts per minute; in square brackets).

duced receptor downregulation by the three isoforms of PDGF, we incubated monolayers of human dermal fibroblasts with AA, BB, or AB at 37°C to allow receptor internalization to occur. The fraction of receptors remaining at the cell surface was then measured by incubation at 4°C with ^{125}I-labeled AA, BB, AB, or PR7212 (Fig. 1). Preincubation with all three isoforms of PDGF at 37°C had equal effects in reducing subsequent binding at 4°C of ^{125}I-labeled AA (Fig. 1A) and AB (Fig. 1C). In contrast, the binding of ^{125}I-labeled BB (Fig. 1B) and PR7212 (Fig. 1D) at 4°C was essentially unaffected by AA or AB, but was reduced to 15% of control by BB (*22*). AA homodimers derived from U2-OS osteosarcoma cell–conditioned medium and BB homodimers purified from platelet-rich plasma were also examined and found to give the same results as the recombinant forms (*23*).

These results suggest that there are two PDGF receptor populations, one of which binds and is downregulated by all three isoforms of PDGF and the other of which binds and is downregulated only by BB homodimers. An alternative interpretation of these results invokes a single class of PDGF receptor that binds all three isoforms of PDGF but is not internalized after AA or AB ligand binding. In this event, the AA or AB ligand–occupied receptor would remain at the cell surface available for PR7212 binding (Fig. 1D). To distinguish between these possibilities, we examined the ability of AA and AB to stimulate receptor internalization and degradation at 37°C. This was done by monitoring the loss of cell-associated ^{125}I-labeled AA and AB and the release into the culture medium of trichloroacetic acid (TCA)–soluble ^{125}I. The results of these experiments indicate that both ^{125}I-labeled AA and AB are internalized and degraded, with a half-time of approximately 45 min (*23*). When methylamine, a lysosomal inhibitor (*16*), was added to the culture medium, the rates of degradation of both ^{125}I-labeled AA and AB were similarly decreased, with the half-time for ligand degradation increasing to more than 3 hours. These results indicate that both ^{125}I-labeled AA and AB are internalized and subsequently degraded by lysosomal action. The finding that AA and AB stimulate receptor internalization along with their inability to significantly decrease binding sites for ^{125}I-labeled BB after a 37°C incubation (Fig. 1C) support the first interpretation presented above. That is, two classes of PDGF receptor exist, one of which binds and is downregulated by all three isoforms of PDGF and the other of which binds and is downregulated only by BB.

To determine the number of receptor sites for the two classes of PDGF receptor, saturation binding studies were done with both ^{125}I-labeled AB and BB on human dermal fibroblasts. The data from three independent experiments are plotted in Fig. 2. The results show saturable binding for both ^{125}I-labeled AB and BB. The mean levels of ligand binding correlate to 31,000 and 210,000 receptor sites for AB and BB, respectively, for a ratio of 7:1 for BB to AB binding.

The results obtained by analyzing ligand-induced PDGF receptor phosphorylation are consistent with the existence of two classes of PDGF receptor that have different ligand binding specificities. Membrane extracts of human dermal fibroblasts were prepared and incubated with adenosine [γ-^{32}P]triphosphate in the presence of each of the isoforms of PDGF. The BB homodimer stimulated phosphorylation of proteins of 180 and 164 kD, detectable in the whole membrane extracts (Fig. 3). No stimulation of protein phosphorylation above basal level was detected in the membrane extracts after addition of either AA or AB. When the phosphorylated membrane extracts were immunoprecipitated with antibody PR7212, bands of 180 and 164 kD were observed in all of the test extracts (Fig. 3). The 180-kD band is the mature cell-surface form of the PDGF receptor recognized by antibody PR7212, and the 164-kD band is an intracellular precursor form of this molecule (*21*). Only the BB-treated extracts showed stimulation of phosphorylation of these two bands above basal level. This stimulation was approximately threefold, as determined by Cherenkov counting of the excised bands. Since the binding of BB to the receptor at 4°C reduces subsequent PR7212 binding by approximately 50% (*21*), the actual level of stimulation of phosphorylation by BB is approximately sixfold.

Stimulation of protein phosphorylation by the three isoforms of PDGF was further studied by examining phosphorylation of the PDGF receptor in intact cells that had been equilibrated with [^{32}P]orthophosphate. BB homodimer stimulated receptor phosphorylation of a 180-kD band (*23*), which represents the mature cell-surface form of the receptor. The stimulation of

phosphorylation was approximately three times the basal level. This again is probably an underestimate of the amount of stimulation because BB binding reduces subsequent PR7212 binding. No stimulation of phosphorylation above basal level was detected after AA or AB treatment (*23*). When the precipitated receptor bands were excised from the dried gel and subjected to phosphoamino acid analysis, basal phosphorylation of the PDGF receptor was observed on both serine and threonine residues with most of the phosphorylation occurring on serine (*23*). After BB was added to the culture medium there was a marked stimulation of tyrosine phosphorylation. Only minimal stimulation of tyrosine phosphorylation was detected after AA or AB was added to the cells (*23*).

Our data suggest that there are two classes of PDGF receptor, as previously reported (*24*) and that they differ in ligand-binding specificity for the three isoforms of PDGF. Similar findings have been described by Heldin and colleagues (*25*). One class of receptor, the B receptor, binds and is downregulated only by the BB form of PDGF, whereas the second class of receptor, the A/B receptor, binds and is downregulated by AA, AB, and BB. Ligand binding competition studies at 4°C have shown the same pattern of ligand binding specificity as inferred from the downregulation studies (*26*). We have also observed the same two binding phenotypes of PDGF receptor in other species and cell types, including mouse 3T3 cells and both human and rat smooth muscle cells (*26*).

The ratio of the two classes of receptor on human dermal fibroblasts is approximately 7:1, with the B receptor being the predominant form. This ratio was calculated from the estimates of ligand binding sites for ^{125}I-labeled BB and AB (210,000 BB binding sites and 31,000 AB binding sites). This ratio is supported by the observation (Fig. 1) that only 10 to 15% of the binding of ^{125}I-labeled BB and PR7212 is eliminated by prior incubation with AA or AB. This large difference in abundance of the two receptor classes correlates with our finding that BB gives a proportionately greater signal for stimulation of phosphorylation than does AA or AB, since only BB would activate the abundant B receptor.

Previous studies of ^{125}I-labeled PDGF binding have been done with PDGF iodinated by procedures that specifically label tyrosine residues, for example, by use of chloramine T, iodine monochloride, and Iodogen. Since only the A chain of PDGF contains tyrosine, only the AA and AB isoforms of PDGF would be labeled in a heterogeneous mixture of PDGF purified from platelets. Thus, when ^{125}I-labeled PDGF was used as a probe for the detection of PDGF receptor, the A/B receptor was being monitored. In our studies, we labeled BB with ^{125}I either by the Bolton-Hunter procedure, which labels primary amines, or by using a mutant form of BB that has a tyrosine residue substituted for phenylalanine at position 23, thereby allowing us to use tyrosine-labeling techniques (*27*). It seems likely that in previous studies in which PDGF receptor phosphorylation was monitored (*15*), only the B receptor was detected.

It is not known whether these two classes of receptors represent two distinct gene products, different post-translational modifications of a single gene product, or functional differences in a single molecule that result from different patterns of association with other cell proteins. The identification of two classes of PDGF receptor that specifically bind and are downregulated in response to different isoforms of PDGF will necessitate the use of purified preparations of each of the three isoforms in order to evaluate the biological activities of PDGF and to determine which isoform of PDGF and which receptor class mediates each of the activities.

REFERENCES AND NOTES

1. R. Ross, E. W. Raines, D. F. Bowen-Pope, *Cell* **46**, 155 (1986).
2. B. C. Berk, R. W. Alexander, T. A. Brock, M. A. Gimbrone, R. C. Webb, *Science* **232**, 87 (1986).
3. H. Seppa, G. Grotendorst, S. Seppa, E. Schiffmann, G. R. Martin, *J. Cell Biol.* **92**, 584 (1982); G. R. Grotendorst, T. Chang, H. E. J. Seppa, H. K. Kleinman, G. R. Martin, *J. Cell. Physiol.* **113**, 261 (1982); T. F. Deuel, R. M. Senior, J. S. Huang, G. L. Griffin, *J. Clin. Invest.* **69**, 1046 (1982).
4. C. P. Chan, D. F. Bowen-Pope, R. Ross, E. G. Krebs, *J. Biol. Chem.* **262**, 276 (1987).
5. M. J. Berridge, J. P. Heslop, R. F. Irvine, K. D. Brown, *Biochem. J.* **222**, 195 (1984).
6. B. J. Bockus and C. D. Stiles, *Exp. Cell Res.* **153**, 186 (1984).
7. J. A. Cooper, D. F. Bowen-Pope, E. Raines, R. Ross, T. Hunter, *Cell* **31**, 263 (1982).
8. R. Ross, J. A. Glomset, B. Kariya, L. Harker, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1207 (1974); N. Kohler and A. Lipton, *Exp. Cell Res.* **87**, 297 (1974).
9. H. N. Antoniades, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7314 (1981); T. F. Deuel *et al.*, *J. Biol. Chem.* **256**, 8896 (1981); C.-H. Heldin, B. Westermark, A. Wasteson, *Biochem. J.* **193**, 907 (1981); E. W. Raines and R. Ross, *J. Biol. Chem.* **257**, 5154 (1982).
10. A. Johnsson, C.-H. Heldin, B. Westermark, A. Wasteson, *Biochem. Biophys. Res. Commun.* **104**, 66 (1982).
11. C.-H. Heldin *et al.*, *Nature* **319**, 511 (1986).
12. P. Stroobant and M. D. Waterfield, *EMBO J.* **3**, 2963 (1984).
13. K. Glenn, D. F. Bowen-Pope, R. Ross, *J. Biol. Chem.* **257**, 5172 (1982); L. T. Williams, P. M. Tremble, M. F. Lavin, M. E. Sunday, *ibid.* **259**, 5287 (1984); R. Gronwald *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
14. C.-H. Heldin, B. Ek, L. Ronnstrand, *J. Biol. Chem.* **258**, 10054 (1983); Y. Yarden *et al.*, *Nature* **323**, 226 (1986).
15. B. Ek and C.-H. Heldin, *J. Biol. Chem.* **259**, 11145 (1984); L. J. Pike, D. F. Bowen-Pope, R. Ross, E. G. Krebs, *ibid.* **258**, 9383 (1983); T. O. Daniel, P. M. Tremble, A. R. Frackelton, L. T. Williams, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2684 (1985).
16. C.-H. Heldin, A. Wasteson, B. Westermark, *J. Biol. Chem.* **257**, 4216 (1982).
17. M. Rosenfeld, D. F. Bowen-Pope, R. Ross, *J. Cell. Physiol.* **121**, 263 (1984).
18. C. Betsholtz *et al.*, *Nature* **320**, 695 (1986).
19. J. D. Kelly, E. W. Raines, R. Ross, M. J. Murray, *EMBO J.* **4**, 3399 (1985). We have developed recombinant expression systems in the yeast *Saccharomyces cerevisiae* that independently express PDGF A chain and B chain homodimers. The A chain and B chain genes were constructed from synthetic oligonucleotides and encode 104 and 109 amino acid polypeptides, respectively. These homodimers have been purified from yeast culture media to greater than 95% purity as determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and amino-terminal sequence analysis. The homodimers have been biologically characterized and shown to have mitogenic activity.
20. The AB heterodimer was purified from platelet-rich plasma by immunoaffinity chromatography by use of monoclonal antibody 121.6.1.1.1, which is specific for the B chain of PDGF. This antibody recognizes both BB homodimer and AB heterodimer. Contaminating BB homodimer was removed from this preparation by immunodepletion, using a second monoclonal antibody, 120.1.2.1.2, that recognizes only BB homodimers. The material isolated by immunoaffinity was further purified by reversed-phase high-performance liquid chromatography to greater than 95% purity as determined by SDS-PAGE and amino-terminal sequence analysis. Amino-terminal sequence analysis showed the A and B chain sequences in equal molar ratio. Both of the monoclonal antibodies used were developed with recombinant BB as the immunizing antigen. Their development and characterization will be described elsewhere.
21. C. E. Hart, R. A. Seifert, R. Ross, D. F. Bowen-Pope, *J. Biol. Chem.* **262**, 10780 (1987). Monoclonal antibody PR7212 recognizes a cell-surface epitope of the PDGF receptor on cultured cells of primate origin, including human, monkey, and baboon, but not mouse or rat. The antibody is useful for both Western blot and radioimmunoprecipitation studies. The antibody does not block ligand binding; however the binding of BB to its receptor at 4°C reduces subsequent PR7212 binding by approximately 50%. AA and AB have no competing activity for antibody binding.
22. This percentage of binding that remains is usually called nonspecific binding. The value of nonspecific binding is characteristic of each iodinated preparation. The nonspecific binding value for the four ^{125}I-labeled probes was 15% for AB, BB, and PR7212 and 50% for AA.
23. C. E. Hart, A. Kaslauskas, J. Cooper, J. W. Forstrom, D. F. Bowen-Pope, unpublished observations.
24. C. E. Hart *et al.*, *J. Cell. Biochem.* **12A**, 63 (1988).
25. C.-H. Heldin, presented data describing the existence of two classes of PDGF receptor at the UCLA symposia on Growth Factors and Their Receptors at Keystone, CO on 25 January 1988.
26. R. A. Seifert, C. E. Hart, J. W. Forstrom, D. F. Bowen-Pope, unpublished observations.
27. The mutant form of BB which has a tyrosine residue at position 23 in place of phenylalanine was produced in a yeast expression system identical to that used to produce the native BB molecule (*19*). The tyrosine mutant was labeled with ^{125}I by use of Iodobeads. The ^{125}I-labeled BB-tyrosine mutant had properties identical to that of the Bolton-Hunter labeled form of BB in both the downregulation experiments and in saturation binding studies.
28. D. F. Bowen-Pope and R. Ross, *Methods Enzymol.* **109**, 69 (1985).

29. We thank S. Osborn, E. Raines, J. Cooper, and A. Kaslauskas for assistance provided during these studies. This research was supported by NIH grants GM35501 and HL18645 and by National Institute of Dental Research grant DE07063-11.

28 September 1987; accepted 5 April 1988