Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10729–10732, November 1992
Biochemistry

Role of fibrinogen α and γ chain sites in platelet aggregation

(expression/hemostasis/mutagenesis/thrombosis/transfection)

DAVID H. FARRELL*, PERUMAL THIAGARAJAN†, DOMINIC W. CHUNG*, AND EARL W. DAVIE*‡

Departments of *Biochemistry and †Medicine, University of Washington, Seattle, WA 98195

Contributed by Earl W. Davie, August 4, 1992

ABSTRACT **Fibrinogen (Fbg) mediates platelet aggregation by its interaction with the platelet glycoprotein IIb-IIIa (integrin $\alpha_{IIb}\beta_3$). Peptides containing the amino acid sequence RGD derived from the α chain (residues α95–97 and residues α572–574) and the sequence HHLGGAKQAGDV derived from the carboxyl terminus of the γ chain of Fbg (residues γ400–411) inhibit these interactions. To determine the role of these sequences in intact Fbg, recombinant human Fbg (rFbg), mutant rFbgs with an RGD → RGE substitution at either position α97 or α574, and a rFbg γ′-containing variant that has a carboxyl-terminal interruption in the HHLGGAKQAGDV sequence have been expressed in transfected BHK cells. Purified rFbg and the two RGE mutant Fbgs were similar to plasma Fbg in platelet aggregation assays. In contrast, the γ′ variant Fbg was markedly defective in platelet aggregation. These data support the proposals that the carboxyl-terminal region of the γ chain of Fbg is essential for optimal platelet aggregation and that the α-chain RGD sequences are neither necessary nor sufficient for platelet aggregation.**

Platelet aggregation is one of the initial hemostatic events that stops bleeding resulting from a vascular injury. Aggregation is mediated by the binding of the plasma protein fibrinogen (Fbg) to a platelet cell surface receptor (1, 2), glycoprotein IIb-IIIa (GPIIb-IIIa). GPIIb-IIIa is a member of the integrin family of receptors that mediate the cellular adhesion of a number of different cell types to a wide variety of ligands (3). Common to many of these ligands is the recognition sequence Arg-Gly-Asp (RGD) that mediates their binding to the integrin (4). In addition, GPIIb-IIIa also recognizes the sequence HHLGGAKQAGDV that is found at the carboxyl terminus of the γ chain of Fbg (5). GPIIb-IIIa is a "promiscuous" receptor and will bind to a number of different RGD-containing ligands, including Fbg (1, 2), fibronectin (6, 7), vitronectin (8), and von Willebrand factor (9). The binding domain for the RGD motif has been localized to GPIIIa on residues 109–171 (10). A second site on GPIIIa including residues 211–222 has also been shown to be important in Fbg binding (11). The binding domain for the γ chain dodecapeptide of Fbg, however, has been localized to residues 296–306 in GPIIb (12, 13). These multiple binding sites have complicated the analysis of the critical domains of Fbg that are involved in binding and aggregation.

Both RGD-containing peptides and the γ dodecapeptide will inhibit aggregation and Fbg binding to platelets, whereas peptides containing the sequence RGE are inactive (3, 6, 14, 15). Yet the relative contribution to binding of the two α RGD sites at positions α95–97 and α572–574 and the single γ dodecapeptide site at position γ400–411 is still not clear. A model has been proposed in which all three sites interact with several GPIIb-IIIa molecules on adjacent platelets (16). However, some studies indicate that the RGD sequence at position α572–574 plays only a minor role in platelet binding, since Fbg plasmin fragment X (which lacks this RGD sequence) can still bind to platelets and mediate aggregation (17, 18). In contrast, other studies show that fragment X is defective in platelet aggregation (19, 20), implying that α572–574 does indeed play a role in aggregation. More recent studies have gone further to suggest that neither RGD sequence is required for platelet binding to Fbg (21), since monoclonal antibodies to either of the RGD sequences fail to inhibit platelet adhesion completely. Platelet aggregation experiments using purified α, β, and γ chains indicate that both the α and γ chains support platelet aggregation, although α chains are only 20–25% as effective as γ chains (22). However, the contribution of RGD sequences in the α chain vs. the dodecapeptide sites in the γ chains of the intact Fbg molecule is still unclear.

About 10% of the normal Fbg circulating in blood contains an altered γ chain of unknown function, the γ′ variant (23–25). The γ′ chain is a nonallelic variant of the γ chain that arises by alternative processing of the mRNA (26, 27). In this variant, the carboxyl-terminal 4 amino acids have been replaced by 20 amino acids that interrupt the dodecapeptide sequence present in the γ chain. A monoclonal antibody to the γ′ carboxyl terminus does not inhibit platelet aggregation, suggesting that the γ′ chain does not support aggregation (28). Furthermore, a peptide corresponding to residues 408–416 in the γ′ chain is only slightly inhibitory toward platelet binding, with an IC_{50} value of 780 μM. Plasmic fragment D of Fbg containing the γ chain also binds to platelets, whereas fragment D containing the γ′ chain does not. In addition, fragment D containing the γ chain inhibits platelet aggregation, whereas fragment D containing the γ′ chain does not (29). Plasma-derived Fbg heterodimers containing one γ chain and one γ′ chain ($\alpha_2\beta_2\gamma\gamma'$) show a 50% reduction in platelet binding and aggregation activity (30). The interpretation of these results regarding the role of the γ chain dodecapeptide in aggregation, however, is complicated by the fact that one functional γ chain still exists in the heterodimer.

In the present experiments, recombinant Fbg (rFbg), mutant rFbgs with an RGD → RGE substitution at either α97 (rFbg-D97E) or α574 (rFbg-D574E), and a recombinant variant containing γ′ chains ($\alpha_2\beta_2\gamma'_2$ or rFbg-γ′) were purified from transfected BHK cells. The rFbg-γ′ was markedly deficient in its ability to mediate platelet aggregation, in contrast to normal rFbg or Fbgs with mutations in the RGD sites. These results directly demonstrate the importance of the carboxyl terminus of the γ chain in platelet aggregation and suggest that the RGD sequences in the α chain are neither necessary nor sufficient for aggregation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Fbg, fibrinogen; GPIIb-IIIa, platelet glycoprotein IIb-IIIa; rFbg, recombinant Fbg; rFbg-γ′, recombinant Fbg containing γ′ chains; rFbg-D97E, recombinant Fbg containing the D97E mutation in the α chain; rFbg-D574E, recombinant Fbg containing the D574E mutation in the α chain.

‡To whom reprint requests should be addressed.

MATERIALS AND METHODS

Fbg Expression Vectors. pAG-1 encoding the α and γ chains, pAG-γ' encoding the α and γ' chains, and pBD-1 encoding the β chain and dihydrofolate reductase selectable marker have been described (31). pAG-D97E encodes a normal γ chain and a modified α chain in which D97 is mutated to E97. It was constructed after mutagenesis of nucleotide 388 [numbered according to Rixon *et al.* (32)] from T to G in pAM-2 (31) by using a single-strand mutagenesis method (Amersham), employing the oligonucleotide 5′-GAG AGG CGA GTT TTC CTC AGC C-3′. The mutated cDNA was subcloned into the *Bam*HI site of pMG-1 (31) to create pAG-D97E. Similarly, pAG-D574E encodes a normal γ chain and a modified α chain in which D574 is mutated to E574. It was constructed after mutagenesis of nucleotide 1819 from C to G by using the oligonucleotide 5′-CAA CAG AGG AGA GTC CAC ATT TGA AAG C-3′ and subcloned into the *Bam*HI site of pMG-1 to create pAG-D574E. The sequences of the modified cDNAs were confirmed using a dideoxynucleotide chain-termination system (United States Biochemical).

Cell Culture. Growth conditions for transfected BHK cell lines BHK-Fbg and BHK-Fbgγ', which secrete rFbg and rFbg-γ', respectively, have been described (31). Cell line BHK-D97E expresses rFbg-D97E, and cell line BHK-D574E expresses rFbg-D574E. These cell lines were constructed by cotransfection of 12.5 μg of pBD-1 with 12.5 μg of pAG-D97E or pAG-D574E, respectively, into a BHK cell line expressing α_2-antiplasmin, by using the calcium phosphate method. Transfected cells were selected in medium containing 20 μM methotrexate (Sigma) and G418 (1 mg/ml; GIBCO) as described (31).

Purification of rFbgs and Plasma Fbg. rFbgs were purified from the serum-free conditioned medium of the transfected BHK cells (D.H.F. and E.W.D., unpublished data). Partially purified human plasma Fbg (Calbiochem) was further purified on a GPRPC-agarose column as described for the rFbgs above. Purified samples of the Fbgs were electrophoresed on 10% polyacrylamide gels by the method of Laemmli (33) and stained with 0.05% Coomassie brilliant blue R.

Isolation of Platelets. Blood (30–40 ml) from healthy volunteers who had not taken any medications for 10 days was drawn into polypropylene syringes containing one-ninth volume of citrate buffer [2.5% (wt/vol) sodium citrate/1.5% (wt/vol) citric acid/2% (wt/vol) dextrose, pH 6.5]. Platelet-rich plasma was obtained by centrifugation at 1000 × *g* for 4 min at room temperature. Apyrase (grade VIII; Sigma) was added to a final concentration of 0.1 unit/ml. The platelet-rich plasma was applied to a 50% (wt/vol) bovine serum albumin (Miles) cushion and centrifuged at 1000 × *g* for 12 min at room temperature as described (34). The plasma was removed and the platelet fraction at the top of the bovine serum albumin cushion was collected. The platelets were applied to a Sepharose 2B column equilibrated in gel-filtration buffer [137 mM NaCl/10 mM Hepes, pH 7.5/5.5 mM glucose/4.3 mM NaH_2PO_4/2.7 mM KCl/1 mM $MgCl_2$/bovine serum albumin (1 mg/ml)]. The platelets were eluted with the same buffer and the peak tubes containing 2 × 10^8 platelets per ml were used for aggregation studies.

Platelet Aggregation Assay. Platelet aggregation was monitored with a four-channel aggregometer (Monitor IV Plus; Helena Laboratories). The reaction mixture consisted of 100 μl of gel-filtered platelet suspension, 1 μl of 250 mM $CaCl_2$, and 125 μl of gel-filtration buffer containing 10 μg of rFbg or plasma-derived Fbg. The reaction mixture was incubated for 1 min at 37°C in the aggregation cuvette with stirring at 1000 rpm. ADP was added in 25 μl to give a final concentration of 10 μM. The aggregation curves were recorded for 5 min.

RESULTS

Expression of Site-Specific Fbg Mutants. rFbgs were expressed that contained mutations in the putative binding domains for the platelet receptor GPIIb-IIIa (16). These sites were the RGD sites on the α chain at positions 95 and 572 that were changed to RGE, and the carboxyl-terminal dodecapeptide on the γ chain that was interrupted by the substitution of the γ' chain (Fig. 1). The rFbgs were purified from serum-free conditioned medium from transfected BHK cells. The purified Fbgs showed the proper complement of α, β, and γ (or γ') chains on reduced polyacrylamide gel electrophoresis (Fig. 2). Only a small degree of proteolysis of the carboxyl end of the α chain was evident in these preparations, and the extent of proteolysis was similar for each preparation. A band slightly above the α chain is also visible in the rFbg preparations and may contain the 15 amino acid carboxyl-terminal extension encoded by the cDNA sequence (32, 35). This extension is absent in plasma Fbg (36, 37). However, the extension did not affect the platelet aggregation activity, since the activity of the wild-type rFbg was essentially identical to that of plasma Fbg, as shown below.

Effect of rFbg-γ' on Platelet Aggregation. The purified Fbgs were used in platelet aggregation assays to assess the contribution of the three putative platelet binding sites to aggregation. In aggregation assays, rFbg-γ' was much less effective than either rFbg or plasma Fbg in mediating platelet aggregation (Fig. 3*A*). To ensure that the assay measured only Fbg-dependent platelet aggregation, a control reaction without Fbg was performed. In the absence of Fbg, very little aggregation was detected, indicating that aggregation was not due to contaminating plasma Fbg or Fbg released from the α granules (Fig. 3*A*). rFbg and plasma Fbg showed virtually identical initial rates of aggregation and extent of aggregation. In contrast, rFbg-γ' was only slightly more active than the negative control without Fbg. These findings were verified using platelets from three donors. In all cases, rFbg-γ' was markedly deficient in its ability to mediate platelet aggregation, whereas rFbg was comparable to plasma Fbg. These results suggest that specific sequences at the γ-chain carboxyl terminus of Fbg are required for optimal platelet aggregation. However, it should be noted that rFbg-γ' was

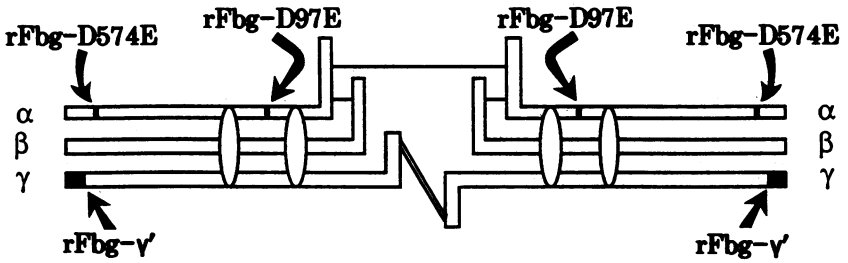

FIG. 1. Location of the mutations in Fbg. The RGE mutations in the α-chain RGD sites at positions 95–97 and 572–574 are indicated by rFbg-D97E and rFbg-D574E, respectively. The replacement of the γ chain with the γ' variant chain is indicated by rFbg-γ'.

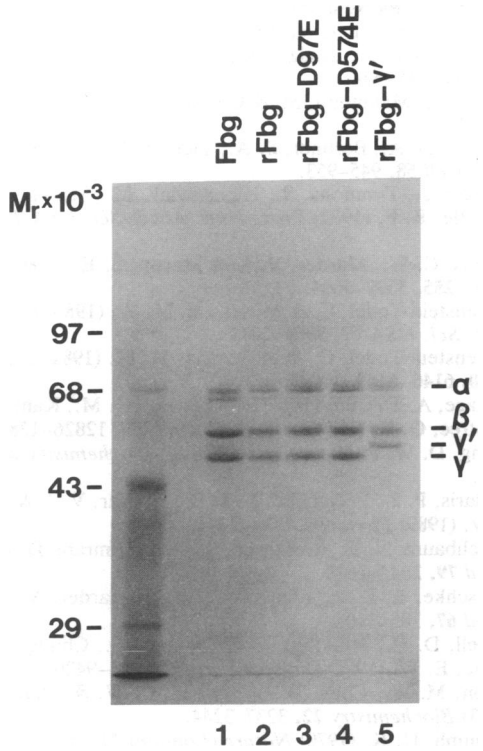

FIG. 2. Purified Fbgs. Purified human plasma Fbg, rFbg, rFbg-γ', rFbg-D97E, and rFbg-D574E were subjected to polyacrylamide gel electrophoresis under reducing conditions and stained with Coomassie brilliant blue R. Lanes: 1, plasma Fbg; 2, rFbg; 3, rFbg-D97E; 4, rFbg-D574E; 5, rFbg-γ'.

capable of mediating some platelet aggregation, albeit to a lesser extent than Fbg or rFbg. This residual activity may arise from the remaining 8 amino acids in the γ chain dodecapeptide sequence common to both the γ and γ' chains. Alternatively, this activity may be due to another site(s), such as the RGD sites in the α chain or some other site in Fbg.

Effect of RGE Mutants on Platelet Aggregation. To assess the contribution of the α chain RGD sites to platelet aggregation, rFbgs with point mutations of D97E or D574E were compared to rFbg and rFbg-γ' in aggregation assays (Fig. 3*B*). There was no appreciable difference between either of the RGD mutants and rFbg. Both the initial rate and the extent of aggregation supported by rFbg-D97E or rFbg-D574E were indistinguishable from those of rFbg. In contrast, rFbg-γ', as shown previously, was markedly deficient in its ability to support platelet aggregation, despite the presence of both RGD sites in its α chain. These results indicate that the RGD sites in Fbg are not essential for platelet aggregation and will not substitute for the absence of the γ chain site in rFbg-γ'.

DISCUSSION

The interaction of RGD-containing ligands with GPIIb-IIIa has been documented extensively. Such ligands as Fbg (4, 5), fibronectin (6, 7), vitronectin (8), and von Willebrand factor (9) contain RGD sequences and all bind to GPIIb-IIIa. The paradigm for RGD-mediated binding of ligands to integrins has been the interaction of fibronectin with the cell surface receptor integrin $\alpha_5\beta_1$ (for reviews, see refs. 38 and 39). In fibronectin, the RGD site is exposed in a β-turn of the protein, which allows it access to its receptor (40). However, in Fbg, the RGD site at position 95 of the α chain is part of a triple helical region called the "coiled coils" domain (41). It is not clear whether this conformation allows access to GPIIb-IIIa.

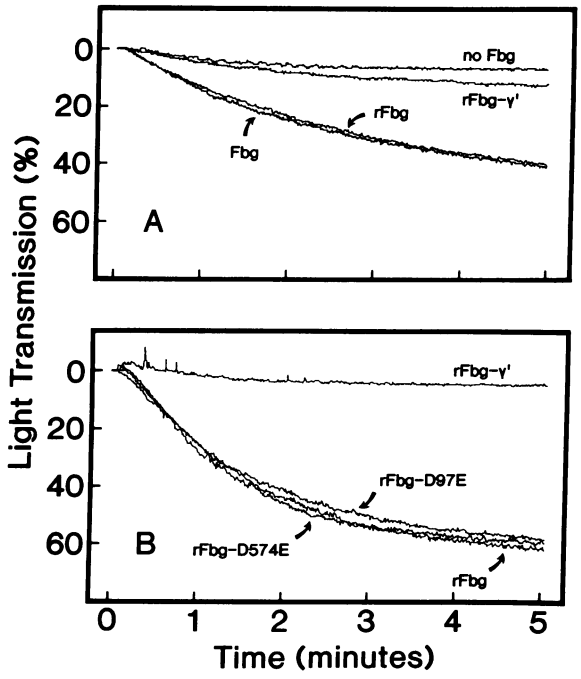

FIG. 3. Platelet aggregation. Platelets were incubated at 37°C with the indicated types of Fbg while stirring at 1000 rpm in a four-channel aggregometer. Aggregation was induced by adding ADP at 10 μM to the reaction mixture, and the extent of aggregation was measured as the increase in light transmission through the cuvette. (*A*) No Fbg, plasma Fbg, rFbg, and rFbg-γ'. (*B*) rFbg, rFbg-D97E, rFbg-D574E, and rFbg-γ'.

In contrast, the second RGD site in the α chain at position 572 is believed to be exposed in a random coil region of Fbg (41, 42); however, this site is not conserved among other species, making its functional significance the subject of controversy. Therefore, the present experiments were undertaken to assess the role of each of the RGD sites and the γ-chain carboxyl terminus in platelet aggregation.

Platelet aggregation assays using rFbgs with RGE mutations in the RGD sites showed little difference in activity compared to rFbg. In contrast, the rFbg-γ' was markedly defective in mediating platelet aggregation. Thus these data suggest that the RGD sites on the α chain do not play a major role in platelet aggregation, whereas the γ-chain carboxyl terminus is essential for aggregation. These results should be compared to those obtained with fibronectin binding to its receptor on BHK cells and von Willebrand factor binding to GPIIb-IIIa on platelets. For fibronectin, an RGD → RGE mutation in a recombinant fibronectin fragment reduced its binding activity by ≥97% (43). For von Willebrand factor, an RGD → RGE mutation selectively abolished its binding to GPIIb-IIIa without affecting its binding to an nonintegrin platelet receptor, glycoprotein Ib/IX (44). These examples suggest that the RGD → RGE mutations in Fbg should have adversely affected platelet aggregation, if indeed these sites were important for aggregation.

The present results suggest that platelet aggregation requires only the bivalent bridging of Fbg between adjacent platelets via the γ-chain carboxyl termini. This model is in accord with recent electron micrographic evidence that shows GPIIb-IIIa binding to Fbg at the distal ends where the γ-chain dodecapeptide is located (45). Binding of GPIIb-IIIa to other sites on Fbg that could correspond to the RGD sites is seen in <15% of the images. This model contrasts with a previous model based on peptide inhibition studies, which suggested that Fbg bridging between platelets was mediated by both RGD sites and the γ carboxyl terminus (16).

The question then remains: Why do RGD-containing peptides inhibit Fbg-dependent platelet aggregation? The explanation may lie in the fact that GPIIb-IIIa is a "promiscuous" receptor and has binding sites for other RGD-containing ligands such as fibronectin, vitronectin, and von Willebrand factor. The fact that RGD peptides bind to GPIIb-IIIa and block Fbg binding does not necessarily imply that Fbg itself binds via its own RGD sites. The RGD peptides can compete for the binding of the γ-chain dodecapeptide to GPIIb-IIIa (46). Therefore, in intact Fbg, the RGD peptides may simply be blocking the γ-chain binding. This would explain their inhibitory activity toward platelet aggregation.

We thank Dr. Shaoming Huang for her illustration of Fbg and for valuable discussions, Laura Stewart of the Puget Sound Blood Center for the use of the platelet aggregometer, Jeff Harris for oligonucleotides, and Zhiyun Cao for excellent technical assistance. This work was supported in part by National Institutes of Health Grants HL-16919 (to E.W.D.) and HL-40860 (to P.T.) and by an American Heart Association Grant-in-Aid (to P.T.).

1. Bennett, J. S. & Vilaire, G. (1979) *J. Clin. Invest.* **64**, 1393–1401.
2. Marguerie, G. A., Plow, E. F. & Edgington, T. S. (1979) *J. Biol. Chem.* **254**, 5357–5363.
3. Hynes, R. O. (1987) *Cell* **48**, 549–554.
4. Ruoslahti, E. & Pierschbacher, M. D. (1986) *Cell* **44**, 517–518.
5. Kloczewiak, M., Timmons, S., Lukas, T. J. & Hawiger, J. (1984) *Biochemistry* **23**, 1767–1774.
6. Haverstick, D. M., Cowan, J. F., Yamada, K. M. & Santoro, S. A. (1985) *Blood* **66**, 946–952.
7. Plow, E. F., McEver, R. P., Coller, B. S., Woods, V. L., Marguerie, G. A. & Ginsberg, M. H. (1985) *Blood* **66**, 724–727.
8. Pytela, R. P., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F. & Ruoslahti, E. (1986) *Science* **231**, 1559–1562.
9. Ruggeri, Z. M., De Marco, L., Gatti, L., Bader, R. & Montgomery, R. R. (1983) *J. Clin. Invest.* **72**, 1–12.
10. D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C.-T. & Plow, E. F. (1988) *Science* **242**, 91–93.
11. Charo, I. F., Nannizzi, L., Phillips, D. R., Hsu, M. A. & Scarborough, R. M. (1991) *J. Biol. Chem.* **266**, 1415–1421.
12. Santoro, S. A. & Lawing, W. J., Jr. (1987) *Cell* **48**, 867–873.
13. D'Souza, S. E., Ginsberg, M. H., Matsueda, G. R. & Plow, E. F. (1991) *Nature (London)* **350**, 66–68.
14. Gartner, T. K. & Bennett, J. S. (1985) *J. Biol. Chem.* **260**, 11891–11894.
15. Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A. & Ginsberg, M. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8057–8061.
16. Hawiger, J., Kloczewiak, M., Bednarek, M. A. & Timmons, S. (1989) *Biochemistry* **28**, 2909–2914.
17. Peerschke, E. I. B. & Galankis, D. K. (1987) *Blood* **69**, 950–952.
18. Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. & Ginsberg, M. H. (1987) *Blood* **70**, 110–115.
19. Niewiarowski, S., Budzynski, A. Z. & Lipinski, B. (1977) *Blood* **49**, 635–644.
20. Holt, J. C., Mahmoud, M. & Gaffney, P. (1979) *Thromb. Res.* **16**, 427–435.
21. Cheresh, D. A., Berliner, S. A., Vicente, V. & Ruggeri, Z. M. (1989) *Cell* **58**, 945–953.
22. Hawiger, J., Timmons, S., Kloczewiak, M., Strong, D. D. & Doolittle, R. F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2068–2071.
23. Francis, C. W., Marder, V. J. & Martin, S. E. (1980) *J. Biol. Chem.* **255**, 5599–5604.
24. Wolfenstein-Todel, C. & Mosesson, M. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5069–5073.
25. Wolfenstein-Todel, C. & Mosesson, M. W. (1981) *Biochemistry* **20**, 6146–6149.
26. Fornace, A. J., Cummings, D., Comeau, C. M., Kant, J. A. & Crabtree, G. R. (1984) *J. Biol. Chem.* **259**, 12826–12830.
27. Chung, D. W. & Davie, E. W. (1984) *Biochemistry* **23**, 4232–4236.
28. Haidaris, P. J., Peerschke, E. I. B., Marder, V. J. & Francis, C. W. (1989) *Blood* **74**, 2437–2444.
29. Kirschbaum, N. E., Mosesson, M. W. & Amrani, D. L. (1992) *Blood* **79**, 2643–2648.
30. Peerschke, E. I. B., Francis, C. W. & Marder, V. J. (1986) *Blood* **67**, 385–390.
31. Farrell, D. H., Mulvihill, E. R., Huang, S., Chung, D. W. & Davie, E. W. (1991) *Biochemistry* **30**, 9414–9420.
32. Rixon, M. W., Chan, W.-Y., Davie, E. W. & Chung, D. W. (1983) *Biochemistry* **22**, 3237–3244.
33. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
34. Timmons, S. & Hawiger, J. (1989) *Methods Enzymol.* **169**, 11–21.
35. Kant, J. A., Lord, S. T. & Crabtree, G. R. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3953–3957.
36. Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D. & Riley, M. (1979) *Nature (London)* **280**, 464–468.
37. Henschen, A., Lottspeich, F., Southan, C. & Topfer-Petersen, E. (1980) *Protides Biol. Fluids* **28**, 51–56.
38. Ruoslahti, E. (1991) *J. Clin. Invest.* **87**, 1–5.
39. Hynes, R. O. (1992) *Cell* **69**, 11–25.
40. Pierschbacher, M. D. & Ruoslahti, E. (1984) *Nature (London)* **309**, 30–33.
41. Doolittle, R. F., Goldbaum, D. M. & Doolittle, L. R. (1978) *J. Mol. Biol.* **120**, 311–325.
42. Weisel, J. W., Stauffacher, C. V., Bullitt, E. & Cohen, C. (1985) *Science* **230**, 1388–1391.
43. Obara, M., Kang, M. S. & Yamada, K. M. (1985) *Cell* **53**, 649–657.
44. Beacham, D. A., Wise, R. J., Turci, S. M. & Handin, R. I. (1992) *J. Biol. Chem.* **267**, 3409–3415.
45. Weisel, J. W., Nagaswami, C., Vilaire, G. & Bennett, J. S. (1992) *J. Biol. Chem.*, in press.
46. Timmons, S., Bednarek, M. A., Kloczewiak, M. & Hawiger, J. (1989) *Biochemistry* **28**, 2919–2923.