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The occupancy of glycoprotein IIb-IIIa complex modulates thrombin activation of human platelets

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Platelet membrane glycoprotein (GP IIb-IIIa), besides its activity as adhesive protein receptor, displays a number of properties supporting its involvement in the mechanisms of transduction of the activation signal. Recently we have observed that GP IIb-IIIa ligands, mostly fibrinogen, inhibit Ca^{2+} movement and cytoskeleton reorganization caused by mild platelet activation. These findings led us to investigate the effect of GP IIb-IIIa ligands on agonist-induced platelet responses, with particular attention to the two major messenger generating systems, involving the activation of phospholipase C and the inhibition of cAMP production. In this paper we demonstrate that the occupancy of the major adhesive protein receptor on the platelet surface modulates the phosphatidylinositol cycle decreasing the amount of IP_3, IP_2 and IP produced after mild platelet activation as well as the pattern of protein phosphorylation. The platelet cAMP content of activated platelets was also affected and kept higher when evaluated under the same experimental conditions. Our data provide evidence for a role of fibrinogen binding in regulating the degree of activation of circulating platelets.

Introduction

It is well known that platelet aggregation is mediated by the activation-dependent binding of several adhesive glycoproteins, including fibrinogen, fibronectin and von Willebrand factor, to the platelet membrane GP IIb-IIIa complex. The interaction of adhesive proteins with GP IIb-IIIa appears to be mediated by an Arg-Gly-Asp(RGD) recognition specificity [1,2]; RGD-containing peptides inhibit binding of fibrinogen, fibronectin and von Willebrand factor to activated platelets with a concomitant inhibition of platelet functions, mainly aggregation [3–5], moreover their binding determines structural modification of the receptor [6]. There is evidence that this membrane component actively participates in the mechanisms of platelet activation: the binding of RGD-containing ligands, including fibrinogen, induces the expression of a neoantigenic site on platelet surface [7], the clustering of platelet associated GP IIb-IIIa [8] and is necessary to mantain Na^+/H^+ exchange in epinephrine-stimulated platelets [9]. Furthermore fibrinogen-receptor associates with cytoskeleton components during platelet aggregation [10] under conditions where fibrinogen is secreted from platelets and bound to their surface. Moreover several observations indicate that GP IIb-IIIa is implicated in Ca^{2+} movements: Brass et al. demonstrated that this membrane component represents a high-affinity binding site for Ca^{2+} on platelet surface [11] and that it is involved in mantaining Ca^{2+} homeostasis in unstimulated platelets [12]. This result is confirmed by the evidence that purified GP IIb-IIIa incorporated onto the surface of liposomes displays Ca^{2+} channel properties when its Ca^{2+}-dependent integrity is mantained [13]. Also the cytoplasmic Ca^{2+} movement in activated platelets is significantly modified in the presence of specific ligands for GP IIb-IIIa complex. Powling and Hardisty [14] hypothesize that the GP IIb-IIIa complex is closely adjacent to a Ca^{2+} channel responsible for Ca^{2+} influx during platelet activation, meanwhile Yamaguchi et al. [15] demonstrated that this membrane component is directly involved in this process. Both these authors observed a decrease of Ca^{2+} influx in activated platelets in the presence of anti-GP IIb-IIIa monoclonal antibody or RGD-containing peptides. Recently we confirmed these observations, and moreover we observed that fibrinogen, the main GP IIb-IIIa

Abbreviations: PRP, platelet-rich plasma; IP, inositol phosphate; IP_2, inositol biphosphate; IP_3 inositol triphosphate; GP, glycoprotein; cAMP, adenosine 3′,5′-monophosphate; BCA, bicinchoninic acid, BSA, bovine serum albumin; PGE_1, prostaglandin E_1; GRGDS, Gly-Arg-Gly-Asp-Ser; PK-C, protein kinase C.

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ligand, displays an inhibitory effect on Ca^{2+} movement and cytoskeleton reorganization caused by mild platelet stimulation [16]. These findings led us to investigate the effect of GP IIb-IIIa ligands on agonist-induced platelet responses, with particular attention to intracellular regulatory processes. The nature of a platelet response initiated by receptor occupancy depends on the selective coupling of a particular receptor to at least two major messenger generating systems. One involves phospholipase C-catalyzed hydrolysis of membrane inositol phospholipids and production of inositol triphosphate (IP_3) and diacylglycerol (DAG), whereas the other is related to the formation of cyclic adenosine 3',5'-monophosphate (cAMP) which is known to inhibit the events characteristic of platelet activation like shape change, adhesion, aggregation and release reaction [17] although the mechanism of this effect is still uncertain. In this article we investigate the effect of GRGDS peptide and fibrinogen on both these messenger-generating systems and demonstrate that the occupancy of the major adhesive protein receptor on the platelet surface modulates the phosphatidyl-inositol cycle affecting the amount of IP_3, IP_2 and IP produced after mild platelet activation as well as the pattern of protein phosphorylation. Moreover the presence of both the GP IIb-IIIa ligands affects platelet cAMP content of activated platelets, evaluated under the same experimental conditions. Our data provide evidence for a role of fibrinogen binding in regulating the degree of activation of circulating platelets.

Materials and Methods

Reagents

Sepharose 2B was from Pharmacia; BCA was obtained from Pierce; $Na^{125}I$, *myo*-[2-^{3}H]inositol (13.8 Ci/mmol) and carrier-free [^{32}P]orthophosphate came from Amersham International. Bovine serum albumin (BSA), human thrombin, apyrase and PGE_1 were purchased from Sigma. GRGDS peptide was from Novabiochem. Insta-Gel was from Packard Instruments. AG 1-X8-formate form (200 mesh) came from Bio-Rad Laboratories. Tosylglycylprolylarginine-4-nitroanilide acetate (Chromozym TH) used for thrombin activity detection was from Boehringer Mannheim. All other chemicals, obtained from various sources, were of analytical grade.

Preparation of platelet-rich plasma

Blood was obtained by clean venipuncture of healthy volunteers who denied having taken any drugs during the previous two weeks. The blood samples were anticoagulated with ACD (acid citrate-dextrose). Platelet-rich plasma was prepared by centrifugation at room temperature for 15 min at $120 \times g$.

GP IIb-IIIa ligands used

In all the reported experiments fibrinogen was used at a concentration near the physiological level (0.4 or 1.2 g/l). The GRGDS concentration (400 μM) was higher with respect to that reported to determine a 50% inhibition of fibrinogen binding [18] and was chosen in order to determine under our experimental conditions a complete inhibition of platelet aggregation. All the experiments were carried out without stirring after thrombin addition to platelet suspension; when fibrinogen was present, no evident formation of insoluble fibrin occurred within 60 s from the addition of the agonist at any concentration used.

Labelling of human thrombin

^{125}I-labelling of thrombin was performed by the Chloramine-T method essentially as previously described [19]. The electrophoretic pattern was not modified by this treatment as verified by SDS-PAGE under reducing and nonreducing conditions and by autoradiography (data not shown). About 95% of the radioactivity precipitated in 10% trichloroacetic acid. After labelling, protein concentration was determined by BCA assay [20]; the enzyme activity was evaluated using tosylglycylprolylarginine-4-nitroanilide acetate as substrate [21].

Thrombin binding to gel-filtered platelets

The binding of labelled enzyme to human platelets was measured in the presence and in the absence of 0.4–1.2 g/l fibrinogen purified from human plasma [16] or 400 μM pentapeptide in the medium, essentially as previously described [19].

Measurement of [^{3}H]inositol phosphates

PRP obtained from 60–80 ml of blood was centrifuged at $800 \times g$ for 15 min to yield a platelet-rich pellet. The pellet was then resuspended in 2 ml of modified Hepes-Tyrode buffer, pH 7.4 (10 mM Hepes, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$) (buffer A) containing 0.5% BSA, 5 mM glucose, apyrase (40 mg/l final concentration) and 1 μM PGE_1. For labelling, the platelet suspension was incubated with *myo*-[2-^{3}H]inositol (0.125 mCi/ml) for 3 h at 37°C. Free *myo*-[2-^{3}H]inositol was removed gel filtering the platelet suspension on Sepharose 2B and eluting with buffer A, containing 5 mM glucose. In some experiments *myo*-[2-^{3}H]inositol-labelled platelets were diluted to 10 ml with the same buffer and pelleted centrifuging at $800 \times g$ for 15 min. The final platelet concentration was adjusted to 10^9 cells/ml with buffer A containing 5 mM glucose. Platelet suspension was rendered 1 mM $CaCl_2$ and 10 mM LiCl and incubated for 15 min at 37°C. Aliquots of 0.5 ml, eventually mixed with fibrinogen (1.2 g/l final concentration) or pentapeptide (400 μM final concentration) were prewarmed at 37°C for 3 min, then

activated with 0.1–0.5 U/ml of thrombin. After a time ranging from 15 to 60 s the reaction was stopped by addition of 1.88 ml of chloroform/methanol/HCl (100 : 200 : 2, v/v). Phase partition was obtained by adding chloroform (0.62 ml) and water (0.62 ml). For determination of zero-time value, a sample was processed as above described without agonist addition. Samples were vortexed and then centrifuged at 800 × g for 10 min. 2 ml of the upper phase containing water-soluble inositol phosphates were neutralized with 2 M CH_3COONH_4 and chromatographyed on 1 ml of AG 1-X8 anion exchange-resin as described by Watson et al. [22]. Briefly, [^{3}H]inositol and [^{3}H]glycerophosphorylinositol were eluted with 16 ml of 60 mM ammonium formate, 5 mM disodium tetraborate; IP with 16 ml of 200 mM ammonium formate, 100 mM formic acid; IP_2 with 16 ml of 400 mM ammonium formate, 100 mM formic acid; IP_3 with 12 ml of 1 M ammonium formate, 100 mM formic acid. Radioactivity in portions (8 ml) of each fraction was determined by scintillation counting in the gel phase using Insta-Gel as scintillation fluid. Results were corrected for all loss factors.

Evaluation of agonist induced protein phosphorylation

Protein phosphorylation in response to stimulation of platelets by thrombin was measured as ^{32}P incorporation into proteins after labelling of the platelet metabolic phosphate pool with carrier-free [^{32}P]orthophosphate.

ACD-anticoagulated PRP obtained from 40–50 ml of blood was applied to a Sepharose 2B column and gel filtered using buffer A containing 0.5% BSA and 5 mM glucose. For labelling, gel-filtered platelets ($2 \cdot 10^9$ cells/ml) were incubated with $^{32}PO_4$ (0.1 mCi/ml) for 90 min at 37°C, then platelets were again gel filtered using buffer A without BSA. Samples containing $2 \cdot 10^9$ cells/ml were prewarmed at 37°C, eventually in the presence of GP IIb-IIIa ligands (1.2 g/l fibrinogen or 400 μM GRGDS, final concentrations). Experiments were started adding 0.1–0.5 U/ml thrombin. At times ranging from 15 s to 60 s aliquots were drawn from the reaction mixture, transferred to vials containing 0.2 vol. of 12% SDS, and boiled for 10 min. For determination of zero-time protein phosphorylation, a sample was processed before the addition of thrombin. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli buffer system [23] and a 5–20% acrylamide gradient under reducing conditions. Gels were stained with Coomassie brilliant blue, dried and autoradiographed. The specific areas corresponding to the 40-kDa and 20-kDa proteins were excised and counted in a liquid scintillation counter.

Measurement of platelet cAMP concentration

PRP obtained from 20–30 ml of blood was gel-filtered as described above, using buffer A, 0.5% BSA as eluent. Platelet count was adjusted to 10^8/ml and rendered 1 mM $CaCl_2$. Samples containing $0.5 \cdot 10^8$ cells were equilibrated at 37°C, eventually in the presence of fibrinogen (0.4 g/l) or 400 μM pentapeptide and activated with 0.1 U/ml thrombin for 30 s. The reaction was stopped adding theophilline (10 mM final concentration) and cooling samples in an ice-bath. Cells were disrupted by sonication. The proteins were precipitated adding 2 vol. of ice-cold ethanol, then pelleted centrifuging for 10 min in a microfuge. The cAMP containing surnatants were dried under vacuum, then dissolved in 1.5 ml of water and cAMP concentration was determined with a radioimmunoassay using cyclic adenosine 3′,5′-mono phosphoric acid 2′-*O*-succinyl-3-[^{125}I]-iodotyrosine methyl ester as tracer.

Results

Effect of GP IIb-IIIa ligands on ^{125}I-thrombin binding to human platelets

In order to verify that platelet–thrombin interaction was not quantitatively modified in the presence of GP IIb-IIIa ligands, we measured the amount of ^{125}I-labelled thrombin bound to gel filtered platelets in the presence of 400 μM GRGDS peptide or fibrinogen (0.4 g/l). Experiments were performed adding the ligands to 150 μl of platelet suspension 1 min before 0.005 U of labelled enzyme. In these experimental conditions, no significant platelet activation occurs. Binding was measured at 30 s from the start of the experiments. The binding of iodinated-thrombin to gel filtered human platelets was not decreased by the presence of GRGDS pentapeptide or fibrinogen (data not shown).

Effect of GRGDS peptide and fibrinogen on thrombin induced inositol phosphate production

Thrombin induced phosphodiesteratic-cleavage of platelet membrane phosphoinositides was monitored measuring the formation of radioactive inositol phosphates in [^{3}H]inositol-labelled platelets. The increase in IP_3 was much smaller than the increment in IP and IP_2, as previously reported by Watson et al. [22]. The formation of IP, IP_2 and IP_3 was time related; moreover, whereas no significant difference in the production of IP and IP_2 was observed treating platelets with 0.1 or 0.5 U/ml thrombin, the IP_3 production appeared to be related to the dose of agonist used (Fig. 1). Platelets activated with thrombin (0.1 U/ml) in the presence of 400 μM GRGDS peptide or fibrinogen (1.2 g/l) showed a lowered production of inositol phosphates, mostly after 15 s of incubation (Fig. 2). Fibrinogen was a more effective inhibitor of inositol-phosphate production than pentapeptide, the effect of which appeared to be counteracted within 30 s from platelet activation. When higher doses of thrombin (0.5 U/ml) were used, the

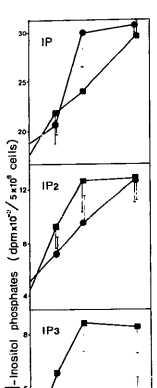

Fig. 1. Time course of inositol phosphate formation induced by different doses of thrombin. [^{3}H]Inositol-labelled platelets were incubated with 0.1 U/ml (●); or 0.5 U/ml (■) thrombin for various times. Each point represents the mean ± S.D. of duplicate determinations obtained in four different experiments.

inositol phosphates production was not modified by the presence of both the ligands (data not shown).

Effect of GRGDS peptide and fibrinogen on thrombin-induced protein phosphorylation

Preliminary time-course experiments revealed that in our experimental conditions 20-kDa and 40-kDa protein were phosphorylated within 15 s from the the addition of the agonist. The amount of ^{32}P incorporated in both these proteins increased up to 60 s, moreover a dependence from the agonist concentration was observed treating platelets with thrombin ranging from 0.1 U/ml to 0.5 U/ml. The effect of GRGDS pentapeptide on 20-kDa and 40-kDa protein phosphorylation was evaluated under the same experimental conditions. Both the GP IIb-IIIa ligands used affected protein phosphorylation induced by the agonist (Fig. 3). Fibrinogen inhibited at the same extent the phosphorylation of 20-kDa and 40-kDa proteins, and it displayed a similar effect at any thrombin concentration used to activate platelets. GRGDS pentapeptide appeared to be a less effective inhibitor of the phosphorylation of 20-kDa and 40-kDa proteins induced by thrombin; anyway, its effect on protein phosphorylation was statistically significant for all the doses of the enzyme used to active platelets.

Effect of GP IIb-IIIa ligands on platelet cAMP levels

Fibrinogen and GRGDS peptide added to resting, gel-filtered platelets, did not show any effect on the cytoplasmic cAMP level. Under our experimental conditions the activation of platelets with thrombin (0.1 U/ml) resulted in a more than 50% decrease of cAMP content. Addition of fibrinogen or GRGDS to platelets before activation promoted a marked increase in cAMP

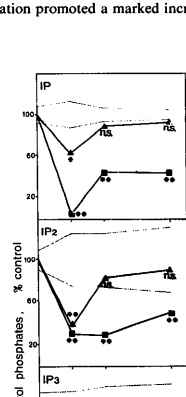

Fig. 2. Effects of GP IIb-IIIa ligands on inositol phosphates production induced by thrombin. [^{3}H]Inositol-labelled platelets were activated with 0.1 U/ml thrombin in the presence of 1.2 g/l fibrinogen (■) or 400 μM GRGDS (▲). Results are expressed as % with respect to the control. Dotted areas are representative of the range of inositol phosphates produced in the absence of GP IIb-IIIa ligands. Each point represents the mean ± S.D. of duplicate determinations obtained in four different experiments. The significance (determined by the *t*-test) of the differences between the results obtained in the presence of fibrinogen or GRGDS and those obtained in their absence is also reported. (*), $P < 0.05$; (**), $P < 0.01$; n.s., not significant.

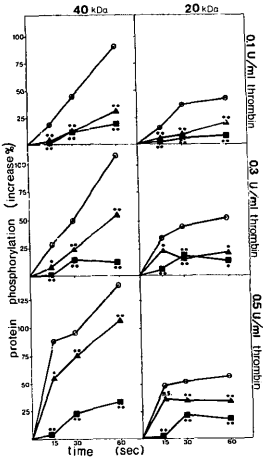

Fig. 3. Time course of 40 kDa and 20 kDa protein phosphorylation induced by different thrombin concentrations. ^{32}P-labelled platelets were activated with 0.1, 0.3 or 0.5 U/ml thrombin for various times in the presence of 1.2 g/l fibrinogen (■) or 400 μM GRGDS (▲) and in their absence (○). Result are expressed as % phosphorylation increase with respect to the basal levels. Each point represents the mean ± S.D. of duplicate determinations obtained in four different experiments. The significance (determined by the *t*-test) of the differences between the results obtained in the absence and in the presence of GP IIb-IIIa ligands is also reported. (*), $P < 0.05$; (**), $P < 0.005$; n.s., not significant.

levels of activated platelets (Table I). Similar results were obtained when 10 μM ADP was used as platelet activator (data not shown).

TABLE I

cAMP concentrations after thrombin activation of platelets in the presence and in the absence of GP IIb-IIIa ligands

Concentrations used: thrombin, 0.1 U/ml; fibrinogen, 0.4 g/l; GRGDS, 400 μM. The significance (determined by *t*-test) of the differences between the results obtained in activated platelets in the presence and in the absence of GP IIb-IIIa ligands is also reported.

Platelet treatment	cAMP (pmol/10^8 cells)
None	3.76 ± 0.22
Thrombin	1.55 ± 0.05
Thrombin + fibrinogen	2.25 ± 0.28 $P < 0.001$
Thrombin + GRGDS	1.95 ± 0.22 $P < 0.001$

Discussion

The GP IIb-IIIa complex beyond any doubt is the platelet membrane component that has been more deeply investigated both in its structural characteristics and functional roles. Its fundamental involvement in platelet aggregation as fibrinogen binding site has been investigated and characterized, as well as its ability to bind other adhesive glycoproteins like von Willebrand factor, fibronectin and vitronectin [24]. Moreover a number of evidences suggest that IIb-IIIa glycoproteins play a role in the mechanism of signal transduction; they are implicated in Ca^{2+} flux across the membrane [12] which is modified, together with the complex structure and distribution on the platelet surface, by the binding of specific agonists [14,15].

We have recently demonstrated that different GP IIIb-IIIa ligands, mostly fibrinogen, determine a lowered cytoplasmic Ca^{2+} movement and decrease the amount of proteins organized in the cytoskeleton of human platelets activated by ADP or low doses of thrombin [16].

In this paper we have investigated the effect of GP IIb-IIIa ligands on other parameter of platelet activation like protein phosphorylation, production of inositol phosphates, and cAMP concentrations. Using low doses of thrombin as agonist, in the presence of physiological fibrinogen concentrations or GRGDS, an inhibition of the events characteristic of platelet activation occurs. The doubt that could be raised is that the presence of fibrinogen in the incubation medium might subtract thrombin from its binding equilibrium with platelets, with a concomitant reduction of the stimulation. However, this does not seem to be the case; in fact similar results were obtained using the pentapeptide GRGDS as GP IIb-IIIa ligand and moreover we had previously demonstrated that both thrombin and ADP stimulation were modulated by fibrinogen with respect to cytoplasmic Ca^{2+} movement and cytoskeleton organization and we have also evidence that the cAMP concentration is influenced quite similarly, both after low thrombin and ADP activation in the presence of fibrinogen or pentapeptide. Furthermore, it must be remarked that thrombin binding to platelet surface is not decreased in the presence of fibrinogen, using experimental conditions in which no significant platelet activation occurs, and therefore the increase of fibrinogen binding to the surface is negligible. Moreover under our experimental conditions no evident formation of insoluble fibrin occurred so that it can be excluded that thrombin is trapped by the insoluble fibrin network. It is therefore unlikely that fibrinogen action is an expression of a reduced availability of agonist. The interpretation of our results can be till now only speculative and it is difficult to hypothesize which are the mechanisms responsible for the evidences reported here. The decreased

production of the inositol phosphates and the lower phosphorylation of 40-kDa protein after low thrombin activation in the presence of fibrinogen and GRGDS could be ascribed to an inhibition of phospholipase C that is responsible for the hydrolysis of phosphatidylinositides to yield DAG and phosphoinositides. It has been reported that 40-kDa protein is the main substrate of DAG dependent PK-C in platelets [25,26] and it is also well documented that IP_3 acts on the mobilization of Ca^{2+} intracellular stores [27]. The decreased cytoplasmic Ca^{2+} concentration, that we have previously demonstrated, could be responsible for the lower phosphorylation of the 20-kDa protein that is considered to be Ca^{2+}-calmodulin dependent [28].

Concerning the lower decrease of cAMP concentration following activation in the presence of GP IIb-IIIa ligands, this could be the consequence of a decreased activity of the cAMP-phosphodiesterase, but it cannot be excluded that a direct effect on adenylyl cyclase occurs. Anyway, cAMP is known to inhibit PK-C and also the production of inositol phosphates [22,29,30], and could be therefore the target of the primary effect of fibrinogen and GRGDS binding, or could cooperate in increasing this effect.

Whatever the sequence of the events which follow the occupancy of GP IIb-IIIa binding sites, our results are indicative of a new role for adhesive proteins and in particular for fibrinogen in platelet function. It is well known that these components are responsible for the adhesion and aggregation of activated platelets; besides this predominant function which is fully operative when a strong activation occurs, it can be supposed that adhesive proteins are able to protect platelets from low intensity and undesired activations. The biochemical events responsible for these mechanisms are till now obscure, but represent evidence that the GP IIb-IIIa complex is involved in the signal transduction in human platelets.

Acknowledgements

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References

1 Lam, S.C.T., Plow, E.F., Smith, M.A., Andieux, A., Ryckwaert, J.S., Marguerie, G. and Ginsberg, M.H. (1987) J. Biol. Chem. 262, 947–950.
2 D'Souza, S.E., Ginsberg, M.M., Lam, S.C.T. and Plow, F. (1988) J. Biol. Chem. 263, 3943–3951.
3 Plow, E.F., Pierschbacher, M.D., Ruoslahti, E., Marguerie, G.A. and Ginsberg, M.H. (1985) Proc. Natl. Acad. Sci. USA 82, 8057–8061.
4 Gartner, T.K. and Bennett, J.S. (1985) J. Biol. Chem. 260, 11891–11894.
5 Haverstick, D.M., Cowan, J.F., Yamada, K.M. and Santoro, S.A. (1985) Blood 66, 946–952.
6 Parise, L.V., Helgerson, S.L., Steiner, B., Nannizzi, L. and Phillips, D.R. (1987) J. Biol. Chem. 262, 12597–12602.
7 Frelinger, A.L. III, Lam, S.C.T., Plow, E.F., Smith, M.A., Loftus, J.C. and Ginsberg, M.H. (1988) J. Biol. Chem. 263, 12397–12402.
8 Isenberg, W.M., McEver, R.P., Phillips, D.R., Schuman, M.A. and Bainton, D.F. (1987) J. Cell Biol. 104, 1655–1663.
9 Banga, H.S., Simons, E.R., Brass, L.F. and Rittenhouse, S.H. (1986) Proc. Natl. Acad. Sci. USA 83, 9197–9201.
10 Phillips, D.R., Jennings, L.K. and Edwards, H.H. (1980) J. Cell. Biol. 86, 77–86.
11 Brass, L.F. and Shattil, S.J. (1984) J. Clin. Invest. 73, 626–632.
12 Brass, L.F. (1985) J. Biol. Chem. 260, 2231–2236.
13 Rybak, M.E., Renzulli, L.A., Bruns, M.J. and Cahaly, D.P. (1985) Blood, 72, 714–720.
14 Powling, M.J. and Hardisty, R.M. (1985) Blood 66, 731–734.
15 Yamaguchi, A., Yamamoto, N., Kitagawa, H., Tanone, K. and Yamazaki, H. (1987) FEBS Lett. 255, 228–232.
16 Sinigaglia, F., Bisio, A., Torti, M., Balduini, C.L., Bertolino, G.P. and Balduini, C. (1988) Biochem. Biophys. Res. Commun. 154, 258–264.
17 Haslam, R.J. (1987) in Thrombosis and Haemostasis (Verstraete, M., Vermylen, J., Lijnen, R., Arnout, J., eds.), pp. 147–174, International Society on Thrombosis and Haemostasis and Leuven University Press, Leuven.
18 Plow, E.F., Pierschbacher, M.D., Ruoslahti, E., Marguerie, G. and Ginsberg, M.H. (1987) Blood 70, 110–115.
19 Sinigaglia, F., Balduini, C.L., Bisio, A. and Balduini, C. (1985) Br. J. Haematol. 59, 587–592.
20 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
21 Collen, D., Lijnen, H.R., De Cock, F., Durieux, J.P. and Loffet, A. (1980) Biochim. Biophys. Acta 165, 158–166.
22 Watson, S.P., McConnell, R.T. and Lapetina, E.G. (1984) J. Biol. Chem. 259, 13199–13203.
23 Laemmli, U.K. (1970) Nature (London) 227, 680–85.
24 Phillips, D.R., Charo, I.F., Parise, L.V. and Fitzgerald, L.A. (1988) Blood 71, 831–843.
25 Kaibuchi, K., Takai, Y., Sawamura, M., Hosijima, M., Fujikura, T. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701–6704.
26 Ieyasu, H., Takai, Y., Kaibuchi, I., Sawamura, M. and Nishizuka, Y. (1982) Biochem. Biophys. Chem. Commun. 108, 1701–1708.
27 Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
28 Daniel, J.L., Purdon, A.D. and Molish, I.R. (1982) Fed. Proc. 41, 1181.
29 Kroll, H.M., Zavoico, G.B. and Scahfer, A.I. (1988) Biochim. Biophys. Acta 970, 61–67.
30 Imai, A., Hattori, H., Takahashi, M. and Nozawa, Y. (1983) Biochem. Biophys. Res. Commun. 112, 693–700.