Protein disulfide isomerase mediates integrin-dependent adhesion

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Abstract Cell adhesion is mediated by the integrin adhesion receptors. Receptor–ligand interaction involves conformational changes in the receptor, but the underlying mechanism remains unclear. Our earlier work implied a role for sulfhydryls in integrin response to ligand binding in the intact blood platelet. We now show that non-penetrating blockers of free sulfhydryls inhibit β_1 and β_3 integrin-mediated platelet adhesion regardless of the affinity state of the integrin. Removal of the inhibitors prior to adhesion fully restores adhesion despite the irreversible nature of inhibitor–thiol interaction, indicating sulfhydryl exposure in response to adhesion. We further show that blocking protein disulfide isomerase (PDI) inhibits adhesion. These data indicate that: (a) ecto-sulfhydryls are necessary for integrin-mediated platelet adhesion; (b) disulfide exchange takes place during this process; (c) surface PDI is involved in integrin-mediated adhesion.

Key words: Integrin; Protein disulfide isomerase; Adhesion; Platelet; Collagen; Fibrinogen

1. Introduction

Adhesion of platelets to components of the exposed subendothelium is a necessary step in blood coagulation. A major adhesion pathway employed by platelets as well as by many other cells is mediated by the heterodimeric adhesion receptors integrins [1,2]. Thus integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ are the platelet adhesion receptors for collagen, fibronectin and fibrinogen, respectively [2]. The affinity of purified integrins, or of integrins in cell lysate, to their ligands is considerably low and the interaction can be disrupted by weak non-ionic detergents [3] or EDTA [4]. However, accumulating evidence indicates that integrin affinity for their ligands is tightly regulated by the cell and that under different conditions, integrins may present different affinity states [5–7]. These different affinity states are manifests of differences in the conformation of the integrin, as accessibility to specific monoclonal antibodies reflects the competence of the integrin to bind to its ligand [7,9]. Ligand-occupied integrins express yet an additional set of epitopes inaccessible on the unoccupied integrin [8,10], implying further changes in integrin conformation upon ligand binding. We have shown that, in contrast to cell lysate, when the integrin $\alpha_2\beta_1$ on intact whole platelets interacts with its collagen ligand, the complex cannot be disrupted by detergents, nor by EDTA. Only a reducing agent in combination with detergent or low pH disrupts the $\alpha_2\beta_1$–collagen complex [11]. This observation suggests involvement of disulfide bridges in the change in conformation upon ligand binding. Little is known about the mechanism leading to changes in conformation. It has been shown that ligand binding results in metal ion displacement [12], and in receptor aggregation [13]. Our observation that platelet adhesion to collagen leads to irreversible binding of the ligand to its integrin receptor suggests that ligand binding evoked disulfide exchange or the involvement of free sulfhydryls on the integrin [11]. Expression of protein disulfide isomerase (PDI) on the surface of several cell types, including the blood platelet, had been demonstrated [14], suggesting an enzymatic mediator for disulfide exchange in the cell-surface receptors and making such a mechanism for ligand-induced change in the conformation of the integrin conceptually plausible. In the work presented here, we demonstrate that indeed surface sulfhydryls are crucial for β_1 and β_3 integrin-mediated adhesion of the platelet and that PDI is involved in this process.

2. Materials and methods

2.1. Materials

N-Ethyl-maleimide (NEM), *para*-chloro-mercuriphenyl sulfonic acid (pCMPS), bacitracin, bovine serum albumin (BSA) (cat. no. A7638) non-immune rabbit serum and control mouse ascites were obtained from Sigma (Israel), thiolyte monobromotrimethyl-ammoniobimane (qBBr) from Calbiochem, acid soluble calf skin collagen from Worthington, human plasma fibrinogen, 97% clottable, from Kabi-Pharmacia. Human plasma fibronectin was purified from fresh plasma according to Engvall and Ruoslahti [15].

Polyclonal rabbit anti-bovine PDI antiserum, which cross reacts with human PDI [16], was a generous gift from Dr. N.J. Bulleid (Department of Biochemistry and Molecular Biology, University of Manchester, Manchester, UK), monoclonal anti-rat PDI (RL-90) which recognizes human PDI [17] was purchased from Alexis Biochemicals (Switzerland). β_1-Activating antibody TS2/16 [5] was kindly donated by Dr. M.A. Schwartz and β_3-activating antibody LIBS6 [10] was kindly donated by Dr. M.H. Ginsberg, both from the Department of Vascular Biology, The Scripps Research Institute, La Jolla, CA, USA. Blocking antibodies 6F1 (for $\alpha_2\beta_1$) [18] and 10E5 (for $\alpha_{IIb}\beta_3$) [19] were a generous gift from Dr. Barry Coller of the Department of Medicine, Mount Sinai Medical Center, New York, USA. Blocking antibody P1D2 (for $\alpha_5\beta_1$) [20] was purchased from Chemicon International Inc., Temecula, CA, USA.

2.2. Platelet preparation and adhesion

Platelet suspensions in buffer (2×10^7 pl/ml) were prepared either by gel filtration [11,21] or by washing [22], and their adhesion to protein-covered plastic, blocked with BSA, was measured according to published methods [11,21]. Inhibitors were added to the platelets 10 min prior to introduction of the platelets to the adhesive surface. When reversibility of the inhibitory effect was tested, the inhibitor was added to 2×10^8 pl/ml, incubated 10 min and the platelet suspension diluted 10 times with either buffer alone or buffer containing the inhibitor. Untreated platelets were always included as controls. When the effect of antibodies (monoclonal β_1-activating antibody TS2/16 [5] and β_3-

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activating antibody LIBS6 [23], polyclonal rabbit anti-bovine PDI [16] and monoclonal anti-PDI clone RL-90 [17], integrin-blocking monoclonal 6F1 anti-$\alpha_2\beta_1$ [18], 10E5 anti-$\alpha_{IIb}\beta_3$ [19] and P1D2 anti-$\alpha_5\beta_1$ [20]) was tested, antiserum or ascites fluid were diluted 1:100 in gel filtration buffer and were incubated with the platelets for 30 min at room temperature before addition to the adhesion surface. Platelets incubated under the same conditions with non-immune rabbit serum or control mouse ascites, diluted 1:100 with the same buffer, were used as control. Adhesion in the presence of 10 μg/ml integrin-blocking antibodies was less than 10% of that of control.

3. Results

3.1. Blocking free sulfhydryls

The effect of sulfhydryl blockers on platelet adhesion to fibrinogen, collagen and fibronectin was measured using one membrane-penetrating reagent (NEM) and two non-penetrating reagents (pCMPS and qBBr). We found that β_1 and β_3 integrin-mediated adhesion was inhibited when free sulfhydryls were blocked (Fig. 1). The very effective inhibition of the non-penetrating reagents indicated that the relevant sulfhydryls were extracellular. The inhibitory effect was concentration-dependent on all substrates studied and by all reagents used (data not shown).

The affinity of integrins to their ligand can be manipulated in vitro by 'activating' agents such as certain antibodies [5,10] and Mn^{2+} ions [24] which bind on the outside and lead to increase in ligand affinity and to induction of epitope expression which correlate with these affinity changes. Conversion to the high affinity state by extracellular Mn^{2+} ions or activating antibodies was demonstrable on all substrates (but not on BSA) (Fig. 2). In order to test whether sulfhydryls are necessary for conversion to high affinity or for ligand binding, we treated resting platelets with pCMPS before or after the addition of Mn^{2+} ions or activating antibodies. We found that blocking the ecto-sulfhydryls either before or after integrin conversion to the high affinity state inhibited both β_1 and β_3 integrin-mediated adhesion (Fig. 2).

3.2. Reversibility of inhibition

All the blockers used in this study interact irreversibly with free sulfhydryls and can be removed only by reducing agents [25,26]. Nonetheless, we observed that if the platelet suspension was exposed to the sulfhydryl blockers and then diluted 10-fold to reduce the concentration of the reagent, adhesion was fully restored (Fig. 3).

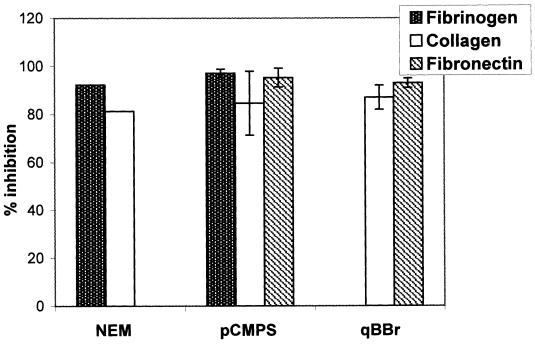

Fig. 1. Inhibition of platelet adhesion by sulfhydryl blockers. Gel-filtered platelets were treated with membrane-penetrating (NEM, 10 μM) and non-penetrating (pCMPS, 125 μM; qBBr, 10 mM) irreversible thiol blockers and their adhesion to immobilized matrix proteins in the absence or presence of the blockers was measured by counting bound platelets. The effect of each thiol blocker was calculated relative to adhesion of untreated platelets to the same substrate. Adhesion to BSA-covered substrates was less than 1% in all experiments.

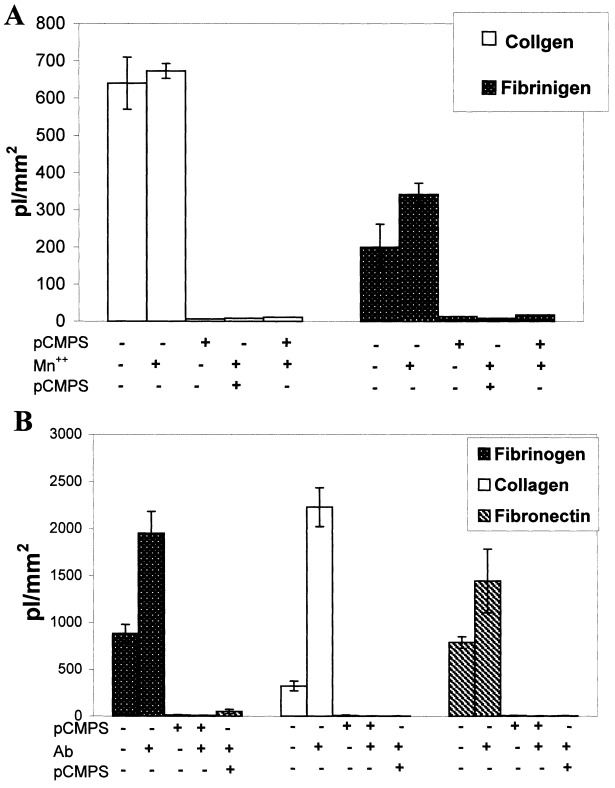

Fig. 2. Integrins in high affinity state are also inhibited by non-penetrating thiol blockers. A: Mn^{2+} (1 mM)-treated platelets adhere in greater numbers than untreated platelets. Non-penetrating pCMPS (125 μM) added either before or after Mn^{2+} blocked adhesion. B: β_1-Activating antibody TS2/16 (in ascites, diluted 1:100) and β_3-activating antibody LIBS6 (Fab fragment) increase platelet adhesion to fibrinogen, collagen and fibronectin. Addition of pCMPS (125 μM) either before or after 30 min incubation with the antibodies blocked platelet adhesion (relative to the effect of ascites control where relevant). Neither Mn^{2+} nor activating antibodies had an effect on adhesion to BSA (not shown).

3.3. Effect of PDI

The mechanism by which free sulfhydryls become available during platelet adhesion was tested by measuring adhesion in the presence of the membrane-impermeable, commonly used PDI inhibitor bacitracin [17,27]. As shown in Fig. 4A, bacitracin at 3 mM was an effective inhibitor of adhesion. The effect of bacitracin was concentration-dependent (data not shown). The specificity of PDI involvement was further corroborated by use of function-blocking antibodies to PDI. Two different antibodies were used, a polyclonal and a monoclonal, and adhesion in their presence was compared with adhesion in the presence of equal dilutions of non-immune rabbit serum and non-immune mouse ascites fluid, respectively. As shown in Fig. 4B, both antibodies inhibited platelet adhesion to all substrates, indicating that PDI is involved in integrin-mediated platelet adhesion.

4. Discussion

We thus show, for the first time, that blocking free sulf-

hydryls on the platelet surface inhibits platelet adhesion to collagen, fibronectin and fibrinogen. Adhesion to these adhesive proteins is mediated by integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$, respectively [28–30]. Inhibition was achieved by three different thiol blockers: NEM, an irreversible, membrane-penetrating blocker; pCMPS, an irreversible, membrane-non-penetrating mercurial agent [25], and qBBr, an irreversible, membrane-impermeable alkylating agent [26]. Thus, both β_1 and β_3 integrin-mediated adhesion of the platelet depends on ecto-sulfhydryls. Adhesion of human skin fibroblasts to collagen and fibronectin, which is mediated by the same β_1 integrins as on the platelets, was also inhibited by pCMPS and bacitracin (Hess and Lahav, unpublished observations), suggesting that involvement of sulfhydryls in integrin-mediated adhesion is shared by several cell types.

In 1968, Aledort et al. [31] showed that the membrane-impermeable thiol blocker pCMPS inhibits adenosine diphosphate-mediated platelet aggregation in platelet-rich plasma. Subsequently, Ando and Steiner [32] reported that the membrane-permeable radioactive *para*-chloromercuribenzoic acid labeled several proteins on isolated platelet membranes, which were not further characterized. These early observations indicated that sulfhydryls of the human platelet membrane play a role in platelet–platelet interaction and that there were several candidates for the role. However, using the weakly fluorescent non-penetrating agent qBBr, Kalomiris and Coller [33] failed to demonstrate the presence of free sulfhydryls on the surface of intact human platelets. Thus, it appeared that while platelet–platelet interaction could be inhibited by impermeable thiol blockers, the relevant proteins could only bind the sulfhydryl reagent if the membrane had been disrupted. An effect of sulfhydryl blockers on cell–cell interaction was also reported for neutrophils, where Schwartz and Harlan have shown that membrane sulfhydryls are involved in adhesion to endothelium [34].

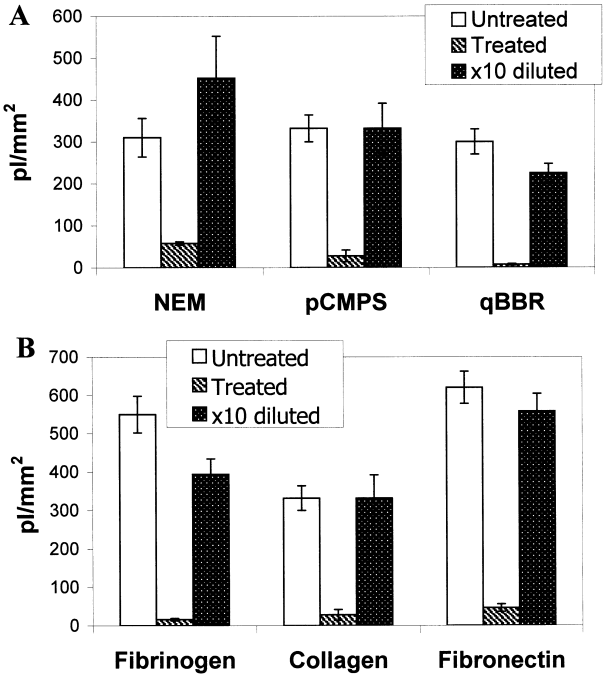

Fig. 3. Presence of the thiol reagent is necessary for adhesion to be inhibited. A: Incubation of platelets with NEM (10 μM), pCMPS (125 μM) or qBBr (10 mM) followed by 10-fold dilution of the blocking agent prior to exposure to the adhesive substrate removed the inhibitory effect of the reagents. Continued presence of the blocking agents at the site of adhesion was necessary for inhibition to take place. B: Continued presence of the thiol reagent as a necessary requisite for inhibition held true for both β_1 and β_3 integrin as manifested by pCMPS (125 μM) effect on adhesion to collagen and fibronectin and to fibrinogen, respectively.

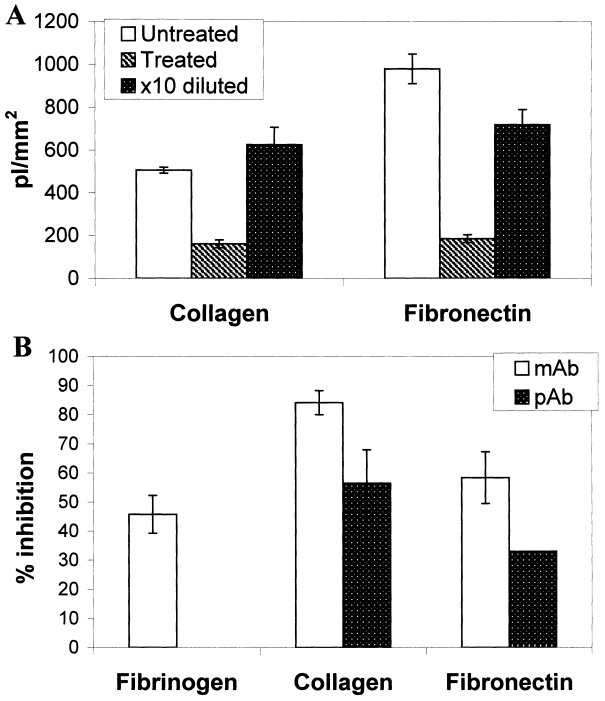

Fig. 4. PDI-specific inhibitors block integrin-mediated adhesion. A: Bacitracin, a non-penetrating, commonly used inhibitor of PDI, reversibly inhibited integrin-mediated platelet adhesion. B: Monoclonal anti-rat PDI ascites that cross react with human PDI and block its function, at 1:100 dilution, and polyclonal anti-PDI antiserum, at 1:100 dilution, inhibit platelet adhesion to the three substrates, relative to adhesion in the presence of control mouse ascites at similar dilutions or non-immune rabbit serum.

In our earlier work, we reported that when intact live platelets interacted with fibrillar collagen, a fraction of the $\alpha_2\beta_1$ integrin could be eluted from its ligand only if the disruption of the ionic interaction was preceded by the disruption of disulfide bonds [11]. This observation suggested that disulfide bonds formed on the intact cell as a consequence of receptor–ligand interaction. The inhibitory effect of pCMPS and qBBr on platelet adhesion to collagen reported here (Fig. 1) corroborates this suggestion, and their inhibitory effect on adhesion to fibronectin and fibrinogen (Fig. 1) implies that this may be a common mechanism for integrins. The 'reversibility' of inhibition for all three reagents (Fig. 3A) and substrates (Fig. 3B) indicates that no toxicity was involved. It also suggests that in the resting state of the platelet, no free sulfhydryls are accessible to the blocking agent and, therefore, their removal prior to exposure to the ligand left no effect. In the process of ligand–receptor interaction, however, either disulfide exchange or exposure of buried sulfhydryl takes place, and sulfhydryl blockers can interact with the forming sulfhydryls thereby blocking any subsequent disulfide-stabilized conformation. This would also account for the lack of labeling of resting intact platelets by qBBr [33], a finding we amply replicated (Gofer-Dadosh and Lahav, unpublished results), and for our observation [11] that dithiothreitol was needed to elute $\alpha_2\beta_1$ from its ligand.

Integrin activation by Mn^{2+} ions or activating antibodies is believed to be a consequence of conformational changes that switch the receptor to a higher affinity state [8,23,24]. Ligand binding causes yet another change in conformation [10]. Indeed, we observed here that, in the presence of Mn^{2+} ions or activating antibodies, platelet adhesion to all three substrates increased, though the extent of the increase differed (Fig. 2). Treatment with pCMPS completely abolished adhesion of Mn^{2+}- or antibody-treated platelets irrespective of the order of addition of the reagents (Fig. 2). Together these data imply that pCMPS acts downstream of conversion to the high affinity state, possibly on the process of ligand binding itself, further supporting our working hypothesis.

For the disulfides to form during the process of ligand binding, a catalyzing agent was likely to be active. In recent years, cell membrane-associated PDI has been reported for several cell types, including platelets [14], and its involvement in integrin $\alpha_{IIb}\beta_3$-mediated platelet–platelet interaction has recently been observed ([27], J. Lahav, unpublished). We therefore tested several membrane-impermeable PDI inhibitors for their effect on platelet adhesion. We found that the commonly used membrane-impermeable PDI inhibitor, bacitracin, abolished more than 80% of platelet adhesion to collagen, fibronectin and fibrinogen (Fig. 4A). The effective bacitracin concentration was similar or lower than that reported to block adhesion of phorbol 12-myristate 13-acetate-activated lymphocytes to fibronectin and collagen [35]. The bacitracin effect was non-toxic, as it could be reversed if the bacitracin was washed away or diluted (Fig. 4A), in agreement with the observation of Mou et al. [35].

Polyclonal antibodies [16] and a function-blocking monoclonal [17] antibody against PDI also inhibited β_1 and β_3 integrin-mediated platelet adhesion (Fig. 4B), strongly supporting our working hypothesis of the common role of PDI-dependent disulfide exchange in integrin-mediated adhesion.

Very few cells have yet been probed for the presence of surface-associated PDI and it is therefore too early to assess the generality of PDI involvement in integrin-mediated adhesion. It is suggested however that at least one other cell system, namely the lymphocyte, depends on the same mechanism, since Mou et al. [35] showed that bacitracin inhibits β_1 and β_7 integrin-mediated adhesion of lymphocytes to fibronectin, collagen and laminin and Ryser et al. had reported that PDI is present on lymphocytes [36]. We observe that bacitracin inhibits adhesion of human skin fibroblasts to the same substrates as it inhibits platelet adhesion, but membrane expression of PDI has not yet been demonstrated on these cells. Correlation between PDI expression on the surface of cells and sulfhydryl involvement in ligand-induced integrin-mediated adhesion of these cells will be necessary for establishing the generality of this mechanism for ligand-induced changes in integrin conformation.

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References

[1] Giancotti, F.G. and Ruoslahti, E. (1999) Science 285, 1028–1032.
[2] Shattil, S.J., Kashiwagi, H. and Pampori, N. (1998) Blood 91, 2645–2657.
[3] Ruoslahti, E. and Pierschbacher, M.D. (1986) Cell 44, 517–518.
[4] Staatz, W.D., Rajpara, S.M., Wayner, E.A., Carter, W.G. and Santoro, S.A. (1989) J. Cell Biol. 108, 1917–1924.
[5] Chan, C. and Hemler, M.E. (1993) J. Cell Biol. 120, 537–543.
[6] Faull, R.J., Kovach, N.L., Harlan, J.M. and Ginsberg, M.H. (1993) J. Cell Biol. 121, 155–162.
[7] O'Toole, T.E., Mandelman, D., Forsyth, J., Shatill, S.J., Plow, E.F. and Ginsberg, M.H. (1991) Science 254, 845–847.
[8] Bazzoni, G., Ma, L., Blue, M.-L. and Hemler, M.E. (1998) J. Biol. Chem. 273, 6670–6678.
[9] Ni, H., Li, A., Simonsen, A. and Wilkins, J.A. (1998) J. Biol. Chem. 273, 7981–7987.
[10] Frelinger III, A.L., Lam, S.C.-T., Plow, E.F., Smith, M.A., Loftus, J.C. and Ginsberg, M.H. (1988) J. Biol. Chem. 263, 12397–12402.
[11] Gofer-Dadosh, N., Klepfish, A., Schmilowitz, H., Shaklai, M. and Lahav, J. (1997) Biochem. Biophys. Res. Commun. 232, 724–727.
[12] D'Souza, S.E., Haas, T.A., Piotrowicz, R.S., Byers-Ward, V., McGrath, D.E., Soule, L.R., Cierniewski, C., Plow, E.F. and Smith, J.W. (1994) Cell 79, 659–667.
[13] Miyamoto, S., Akiyama, S.K. and Yamada, K.M. (1995) Science 267, 883–885.
[14] Essex, D.W., Chen, K. and Swiatkowska, M. (1995) Blood 86, 2168–2173.
[15] Engvall, E. and Ruoslahti, E. (1977) Int. J. Cancer 20, 1–5.
[16] John, D.C.A., Grant, M.R. and Bulleid, N.J. (1993) EMBO J. 12, 1587–1595.
[17] Mandel, R., Ryser, H.J.-P., Ghani, F., Wu, M. and Peak, D. (1993) Proc. Natl. Acad. Sci. USA 90, 4112–4116.
[18] Coller, B.S., Beer, J.H., Scudder, L.E. and Steinber, M.H. (1989) Blood 74, 182–192.
[19] DiFazio, L.T., Stratoulias, C., Greco, R.S. and Haimovich, B. (1994) J. Surg. Res. 57, 133–137.
[20] Wayner, E.A., Carter, W.G., Piotrowicz, R.S. and Kunicki, T.J. (1988) J. Cell Biol. 107, 1881–1891.
[21] Lahav, J. (1988) Blood 71, 1096–1099.
[22] Keely, P.J. and Parise, L.V. (1996) J. Biol. Chem. 271, 26668–26672.
[23] Frelinger III, A.L., Du, K., Plow, E.F. and Ginsberg, M.H. (1991) J. Biol. Chem. 266, 17106–17111.
[24] Gailit, J. and Ruoslahti, E. (1988) J. Biol. Chem. 263, 12927–12932.
[25] Jocelyn, P.C (1987) in: Methods in Enzymology (Jakoby, W.B. and Griffith, O.W., Eds.), Vol. 143, pp. 44–67, Academic Press Inc.
[26] Newton, G.L. and Fahey, R.C (1995) in: Methods in Enzymology (Packer, L., Ed.), Vol. 251, pp. 148–166, Academic Press Inc.
[27] Essex, D.W. and Li, M. (1999) Br. J. Haematol. 104, 448–454.
[28] Sixma, J.J., van Zanten, H., Huizinga, E.G., van der Plas, R.G., Verkley, M., Wu, Y.P., Gros, P. and de Groot, P.G. (1997) Thromb. Haemost. 78, 434–438.
[29] Moroi, M. and Jung, S.M. (1997) Thromb. Haemost. 78, 439–444.
[30] Yamada, K.M. and Geiger, B. (1997) Curr. Opin. Cell. Biol. 9, 76–85.
[31] Aledort, L.M., Troup, S.B. and Weed, R.I. (1968) Blood 31, 471–479.
[32] Ando, Y. and Steiner, M. (1976) Biochim. Biophys. Acta 419, 51–62.
[33] Kalomiris, E.L. and Coller, B.S. (1985) Biochemistry 24, 5430–5436.
[34] Schwartz, B.R. and Harlan, J.M. (1989) J. Leuk. Biol. 45, 177–182.
[35] Mou, Y., Ni, H. and Wilkins, J.A. (1998) J. Immunol. 161, 6323–6329.
[36] Ryser, H.J.-P., Levy, M.E., Mandel, R. and DiSciullo, G.J. (1994) Proc. Natl. Acad. Sci. USA 91, 4559–4563.