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PAR1 activation initiates integrin engagement and outside-in signalling in megakaryoblastic CHRF-288 cells

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Abstract

To better understand the means by which cells such as human platelets regulate the binding of the integrin $\alpha_{IIb}\beta_3$ to fibrinogen, we have examined agonist-initiated inside-out and outside-in signalling in CHRF-288 cells, a megakaryoblastic cell line that expresses $\alpha_{IIb}\beta_3$ and the human thrombin receptor, PAR1. The results show several notable similarities and differences. (1) Activation of PAR1 caused CHRF-288 cells to adhere and spread on immobilized fibrinogen in an $\alpha_{IIb}\beta_3$-dependent manner, but did not support the binding of soluble fibrinogen or PAC-1, an antibody specific for activated $\alpha_{IIb}\beta_3$. (2) Direct activation of protein kinase C with PMA or disruption of the actin cytoskeleton with low concentrations of cytochalasin D also caused CHRF-288 cells to adhere to fibrinogen. (3) Despite the failure to bind soluble fibrinogen, activation of PAR1 in CHRF-288 cells caused phosphoinositide hydrolysis, arachidonate mobilization and the phosphorylation of $p42^{MAPK}$, phospholipase A_2 and the Rac exchange protein, Vav, all of which occur in platelets. PAR1 activation also caused an increase in cytosolic Ca^{2+}, which, when prevented, blocked adhesion to fibrinogen. (4) Finally, as in platelets, adhesion of CHRF-288 cells to fibrinogen was followed by a burst of integrin-dependent ('outside-in') signalling, marked by FAK phosphorylation and a more prolonged phosphorylation of $p42^{MAPK}$. However, in contrast to platelets, adhesion to fibrinogen had no effect on Vav phosphorylation. Collectively, these observations show that signalling initiated through PAR1 in CHRF-288 cells can support $\alpha_{IIb}\beta_3$ binding to immobilized ligand, but not the full integrin activation needed to bind soluble ligand. This would suggest that there has been an increase in integrin avidity without an accompanying increase in affinity. Such increases in avidity are thought to be due to integrin clustering, which would also explain the results obtained with cytochalasin D. The failure of $\alpha_{IIb}\beta_3$ to achieve the high affinity state in CHRF-288 cells was not due to the failure of PAR1 activation to initiate a number of signalling events that normally accompany platelet activation nor did it prevent at least some forms of outside-in signalling. However, at least one marker of outside-in signalling, the augmentation of Vav phosphorylation seen during platelet aggregation, did not occur in CHRF-288 cells. © 1999 Elsevier Science B.V. All rights reserved.

Abbreviations: PKC, protein kinase C; ERK2, $p42^{MAPK}$; $cPLA_2$, cytoplasmic phospholipase A_2; Bis-IM, bisindoylmaleimide-I; PMA, phorbol myristate acetate; BSA, bovine serum albumin; RGDS, Arg-Gly-Asp-Ser

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1. Introduction

The binding of integrins to their cognate ligands is often the outcome of agonist-initiated intracellular events, particularly in cells such as platelets that are normally not adherent. In the case of platelets, the ability of plasma fibrinogen to bind to the integrin $\alpha_{IIb}\beta_3$ following platelet activation is a prerequisite for aggregation, but the signalling pathways that enable $\alpha_{IIb}\beta_3$ to become a high affinity fibrinogen binding site are still under investigation. Pathways leading to integrin activation are commonly referred to as inside-out signalling to distinguish them from the integrin-dependent intracellular events triggered by integrin engagement, termed outside-in signalling. This sequence of inside-out and outside-in signals is not limited to $\alpha_{IIb}\beta_3$, nor is it exclusively hierarchical since outside-in signalling can clearly affect events downstream from activated receptors [1–3].

Although platelets and megakaryocytes are the only normal cells that express $\alpha_{IIb}\beta_3$, several megakaryoblastic and tumor cell lines also express $\alpha_{IIb}\beta_3$ and heterologous expression of the integrin in CHO cells (e.g. [4,5]) and lymphocytes [6,7] has been used to create model systems for studying integrin activation. There are differences, however, between the behavior of the integrin in platelets and in other cells. The addition of an agonist to platelets allows $\alpha_{IIb}\beta_3$ to bind to either soluble or immobilized fibrinogen and is accompanied by conformational changes in the integrin that can be detected by antibodies such as PAC-1 [8]. Megakaryoblastic cell lines on the other hand typically bind neither fibrinogen nor PAC-1 unless the proteins are first immobilized on a solid surface, at least when the cells are activated with phorbol myristate acetate (PMA), which is the stimulus that has been studied most [9,10]. The inability of these cells to bind to soluble fibrinogen when stimulated with PMA suggests that protein kinase C (PKC) activation can produce only an increase in $\alpha_{IIb}\beta_3$ avidity and not an increase in affinity such as occurs in agonist-stimulated platelets [9]. The increase in avidity is thought to be due to the clustering of $\alpha_{IIb}\beta_3$ at points of contact between the cells and the fibrinogen-coated surface, enabling adhesion through the accumulation of low affinity binding sites even though the high affinity state of the integrin needed for binding to soluble ligand has not been achieved (reviewed in [11] and [12]). The response of $\alpha_{IIb}\beta_3$ in the megakaryoblastic cell lines to biologically important agonists such as thrombin is less well-explored, as is the role of PKC when adhesion is initiated by agonists other than PMA.

Potential explanations for the failure of $\alpha_{IIb}\beta_3$ to bind to soluble fibrinogen or PAC-1 in cells other than platelets (and megakaryocytes) include the absence of essential accessory molecules and the failure of one or more key inside-out signalling pathways. In either case, comparisons between platelets and other cells that express $\alpha_{IIb}\beta_3$ continue to provide one of the principal means to understand agonist-induced integrin activation. In the present studies, we have made such a comparison, focusing on the inside-out and outside-in signalling events initiated by activating thrombin receptors in the human megakaryoblastic CHRF-288 cell line. CHRF-288 cells normally express $\alpha_{IIb}\beta_3$ [13] and PAR1 [14], the predominant thrombin receptor expressed on human platelets. PAR1 is a G protein-coupled receptor that is activated by proteolytic cleavage of its N-terminus, exposing a tethered ligand [15]. Thrombin activates PAR1, as do peptides mimicking the exposed ligand (SFLLRN). In contrast to PMA, which is a weak stimulus for platelet aggregation in the absence of other agonists [16,17], thrombin and SFLLRN are full agonists, causing both aggregation and secretion. This difference suggests that the PAR1 agonists are doing more to produce fibrinogen receptor exposure in platelets than just activating PKC.

To better understand the relationship between PAR1 activation and $\alpha_{IIb}\beta_3$ engagement, we asked the following three questions. First, will PAR1 activation in CHRF-288 cells support high affinity $\alpha_{IIb}\beta_3$-dependent binding to soluble ligands or just to immobilized proteins? Second, are the signalling pathways required for each of these events to occur the same in CHRF-288 cells as they are in platelets? Third, is the adhesion of CHRF-288 cells to immobilized fibrinogen sufficient to support outside-in signalling and, if so, are the consequences of that signalling the same as in platelets?

2. Materials and methods

2.1. Materials

Human α-thrombin was a gift from Dr John Fenton (New York State Department of Health, Albany, NY, USA). The thrombin receptor agonist peptide, SFLLRN, was synthesized by the Protein and Nucleic Acid Core Facility of Children's Hospital of Pennsylvania, purified by high pressure liquid chromatography and confirmed by amino acid analysis and protein sequencing. Anti-phosphotyrosine monoclonal antibodies 4G10 and PY20 were obtained from Dr Tom Roberts (Dana Farber Cancer Institute, Boston, MA, USA) and ICN Biochemicals (Irvine, CA, USA), respectively. Bovine serum albumin (BSA) for immunoblots was also obtained from ICN Biochemicals. Human fibrinogen was obtained from Kabi (distributed by Helena Laboratories, Beaumont, TX, USA). Fab fragments of the anti-$\alpha_{IIb}\beta_3$ monoclonal antibody 7E3 were provided by Dr Barry Coller (Mt. Sinai Hospital, New York, NY, USA) and Dr Robert Jordan (Centacor). The anti-$\alpha_{IIb}\beta_3$ monoclonal antibodies A2A9 and PAC-1 were supplied by Drs Joel Bennett (University of Pennsylvania) and Sanford Shattil (Scripps Institute, La Jolla, CA, USA). Fab fragments of the LIBS6 antibody were provided by Dr Mark Ginsberg (Scripps Institute). A rabbit polyclonal antiserum directed against residues 577–590 of Vav was provided by Drs Xosé Bustelo and Mariano Barbacid (Bristol-Meyers Squibb). The anti-FAK antibody, BC3, was obtained from Dr Tom Parsons (University of Virginia). CHRF-288 cells were a gift from Dr M. Lieberman (University of Cincinnati College of Medicine, Cincinnati, OH, USA). Bisindolylmaleimide-I (Bis-IM) was obtained from CalBiochem (San Diego). Cytochalasin D was obtained from Sigma.

2.2. Adhesion

CHRF-288 cells were washed and resuspended in serum-free RPMI 1640 medium with 1% BSA for 4 h or overnight at a concentration of 5×10^6 cells/ml. Incubations were performed in untreated 35 mm dishes (Falcon or Nunc) that were coated overnight with 100 μg/ml fibrinogen and then washed with phosphate-buffered saline (PBS), blocked with 5 mg/ml BSA for 30 min and washed four times with PBS containing Ca^{2+} and Mg^{2+}. Non-adherent cells were removed by washing with PBS containing Ca^{2+} and Mg^{2+}. To quantitate adhesion, CHRF-288 cells were labelled overnight with [^{35}S]methionine and then serum-starved for 4 h prior to the start of the experiment. After removing non-adherent cells, the remaining adherent cells were lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris (pH 7.2), 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 1.0 mM Na_3VO_4 and aprotinin at 100 U/ml) and ^{35}S was measured. Adhesion was also measured colorimetrically by lysing the cells in 0.6 ml 0.1 M citrate buffer, pH 5.4, with 0.1% Triton X-100 and 2.33 mg/ml *p*-nitrophenyl phosphate. The reaction was stopped after 30 min by adding 0.3 ml 2 M NaOH and the OD_{405} was recorded.

2.3. Flow cytometry

CHRF-288 cells were resuspended in RPMI 1640 medium with 10% fetal calf serum at a final concentration of 3×10^6 cells/ml and then incubated for 15 min at room temperature or 37°C with thrombin or PMA and a FITC-conjugated antibody. Analysis was performed on a Becton Dickinson (Mountain View, CA, USA) FACScan.

2.4. Lysate preparation and immunoprecipitation

Adherent CHRF-288 cells were lysed as described above. Non-adherent cells present in the media were also sedimented at $3000\times g$ for 1 min, lysed with 500 μl RIPA buffer and combined with the lysate from the adherent cells in order to examine proteins from the entire cell population and to maintain consistency with samples prepared from cells plated on BSA. Lysates were normalized for protein levels before immunoprecipitation or electrophoresis. FAK [18] and Vav [19] were precipitated as previously described.

2.5. Immunoblotting

Standard 7.5% acrylamide gels were used for electrophoresis. When examining a shift in electrophoretic mobility, an acrylamide stock containing an

acrylamide:bis ratio of 35:0.6% was used. Anti-phosphotyrosine immunoblots were performed using a mixture of 4G10 and PY20 antibodies. MAP kinase immunoblots were performed using a monoclonal antibody specific for $p42^{MAPK}$ (ERK2), UBI. Cytoplasmic phospholipase A_2 ($cPLA_2$) immunoblots were probed with a polyclonal anti-$cPLA_2$ antibody (Genetics Institute). Goat anti-mouse or anti-rabbit immunoglobulin-horseradish peroxidase conjugated antibodies (Bio-Rad Laboratories, Richmond, CA, USA) and a chemiluminescence detection kit (Amersham) were used for detection [20].

2.6. Cytosolic calcium

CHRF-288 cells were loaded with 5 μM Fura-2/AM (Molecular Probes) in RPMI 1640 without Phenol red for 1 h at 37°C. The detached cells were then washed, resuspended in RPMI 1640 without phenol red, allowed to equilibrate 30 min at room temperature, washed again and used at 1×10^6 cells/ml. Changes in the cytosolic free Ca^{2+} concentration were measured with an SLM/Aminco model AB2 fluorescence spectrophotometer [21].

2.7. Arachidonate release

CHRF-288 cells were serum-starved overnight in the presence of 0.5 μCi/ml [^{3}H]arachidonic acid, washed three times and resuspended at a final density of 1.25×10^6 cells/ml. Cells were plated (0.8 ml/plate) and lipids were extracted using Bligh/Dyer solvent (2:1 methanol:chloroform). To ensure maximal recovery, media from plates were added to 2 ml of a 1:1 solution of methanol:chloroform. The adherent cells were scraped on ice into 1 ml of cold methanol and the solutions were combined. Free [^{3}H]arachidonic acid was quantitated by thin layer chromatography [22], followed by scintillation counting of the regions that co-migrated with arachidonic acid standards.

3. Results

CHRF-288 cells normally grow in suspension, so to start, we asked whether activation of PAR1 with thrombin or the agonist peptide, SFLLRN, would cause CHRF-288 cells to either bind soluble fibrinogen and PAC-1 or adhere to immobilized fibrinogen. In Fig. 1, cells that were exposed to thrombin or PMA were incubated with either A2A9 [23], a monoclonal antibody that binds to the resting as well as the activated conformation of $\alpha_{IIb}\beta_3$, or PAC-1 [8], a monoclonal antibody that binds selectively to activated $\alpha_{IIb}\beta_3$. In the absence of an agonist, the CHRF-288 cells bound A2A9, but not PAC-1. There was no increase in PAC-1 binding when the cells were activated. The ability of CHRF-288 cells to bind to soluble fibrinogen was tested in two ways. First, using flow cytometry to detect the binding of an anti-fibrinogen antibody to CHRF-288 cells after they were incubated with fibrinogen and, second, by observing for visible aggregation when CHRF-288 cells were stirred in the presence of Ca^{2+} and fibrinogen. In neither case did thrombin receptor activation appear to cause fibrinogen binding (data not shown).

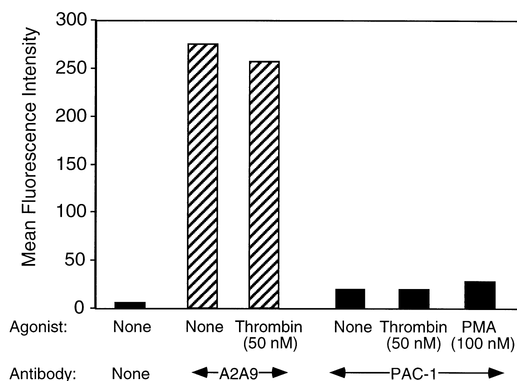


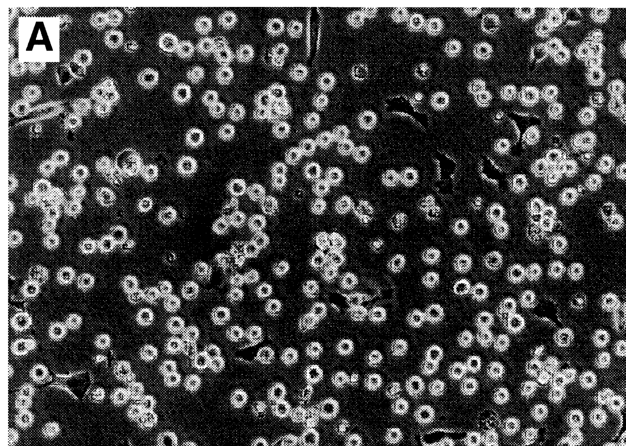
Fig. 1. Binding of anti-$\alpha_{IIb}\beta_3$ antibodies to CHRF-288 cells. CHRF-288 cells were incubated with PMA or thrombin as indicated plus FITC-conjugated A2A9 or PAC-1. A2A9 is a complex-specific anti-$\alpha_{IIb}\beta_3$ antibody that recognizes resting and activated $\alpha_{IIb}\beta_3$. PAC-1 is specific for the activated form of $\alpha_{IIb}\beta_3$. The results shown are representatives of those obtained in two experiments.

To test for adhesion, serum-depleted CHRF-288 cells were incubated in dishes coated with either fibrinogen or BSA. There was little adhesion to fibrinogen in the absence of an agonist or to BSA under any circumstances, but cells stimulated with either SFLLRN (Fig. 2) or PMA (Fig. 3A) rapidly adhered to and spread on fibrinogen (note that most of the cells seen in Fig. 2A are neither adherent nor spread, while those seen in Fig. 2B have done both). Adhesion was half-maximal within 5 min (Fig. 2C) and

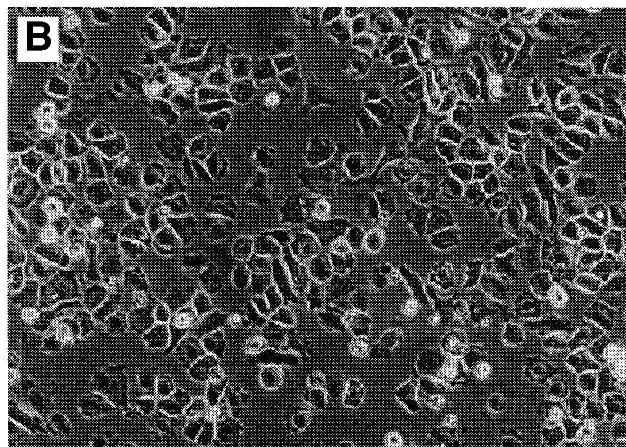
could be inhibited by the anti-$\alpha_{IIb}\beta_3$ antibody, 7E3, and by an RGD-containing peptide (data not shown).

Collectively, these results show that CHRF-288 cells will adhere to immobilized fibrinogen in an $\alpha_{IIb}\beta_3$-dependent manner when activated via a G protein-coupled receptor agonist. However, as has been observed when other megakaryoblastic cell lines were stimulated with PMA, the increase in integrin avidity that is implied by adhesion to immobilized fibrinogen is apparently not accompanied by the increase in affinity that is needed to bind soluble fibrinogen. Increased avidity is thought to be due to an accumulation of relatively low affinity interactions between integrin and ligand, perhaps facilitated by an agonist-induced increase in lateral mobility of the integrin [9,24]. In keeping with that hypothesis, we found that cytochalasin D, which disrupts the actin cytoskeleton, was as effective as SFLLRN and PMA in causing the CHRF-288 cells to adhere to fibrinogen (Fig. 4). A similar result has been obtained for $\alpha_{IIb}\beta_3$ in platelets (J. Bennett et al., unpublished observation) and LFA-1 in EBV-transformed B-cells [24]. In the latter case, it was shown that concentrations similar to those that caused the adhesion of CHRF-288 cells to fibrinogen in the present study caused an increased adhesion of the lymphocytes to ICAM-1 and increased the membrane mobility of LFA-1, while higher concentrations inhibited adhesion [24]. We observed the same concentration-dependent adhesion in CHRF-288 cells incubated with cytochalasin D.

SFLLRN, BSA-coated dish



SFLLRN, fibrinogen-coated dish



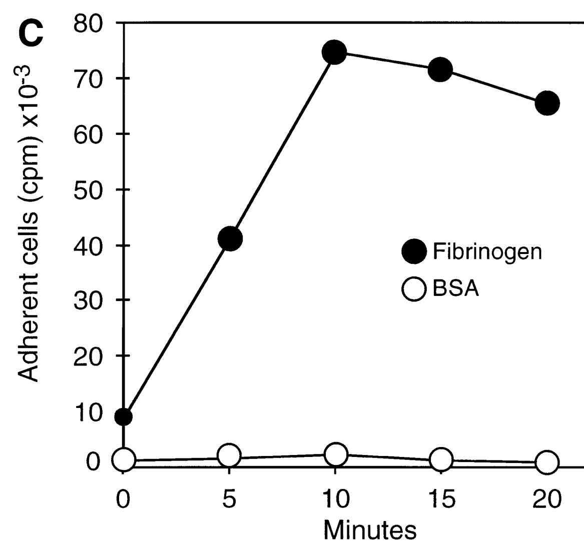


Fig. 2. Adhesion of CHRF-288 cells to a fibrinogen-coated surface. CHRF-288 cells that were serum-starved overnight were incubated for 25 min with 10 μM SFLLRN in plastic dishes that were coated with either (A) BSA or (B) fibrinogen. Note that most of the cells in (A) have not adhered to the dish, while those in (B) have both adhered and spread. (C) CHRF-288 cells that were serum-starved overnight and radiolabelled with [^{35}S]methionine were incubated with 10 μM SFLLRN in a plastic dish coated with either BSA or fibrinogen. At each of the times shown, non-adherent cells were removed and the adherent cells were quantitated by scintillation counting. The results shown are representatives of two such studies with each data point determined in triplicate.

3.1. Inside-out signalling pathways

Given that PAR1 activation in platelets allows $\alpha_{IIb}\beta_3$ to bind soluble ligands, while in CHRF-288 cells PAR1 activation supports only binding to immobilized ligands, we next compared some of the signalling events initiated by PAR1 agonists in CHRF-288 cells with those that occur in platelets. PAR1 activation in platelets stimulates phospholipase C, leading to PKC activation and an increase in cytosolic Ca^{2+}. This is also true in CHRF-288 cells where thrombin and SFLLRN are known to stimulate inositol phosphate formation and cause a transient increase in the cytosolic Ca^{2+} concentration to 0.5–1.0 μM [14]. In permeabilized platelets, pertussis toxin inhibits phosphoinositide hydrolysis [25] and PAC-1 binding [26]. In contrast, pre-incubating CHRF-288 cells overnight with a high concentration of pertussis toxin inhibited SFLLRN-induced phosphoinositide hydrolysis by 50% (not shown), but had little effect on adhesion in response to SFLLRN and no effect on adhesion in response to PMA (Fig. 3B). This suggests that there may be differences in the repertoire of G proteins used for PAR1 signalling

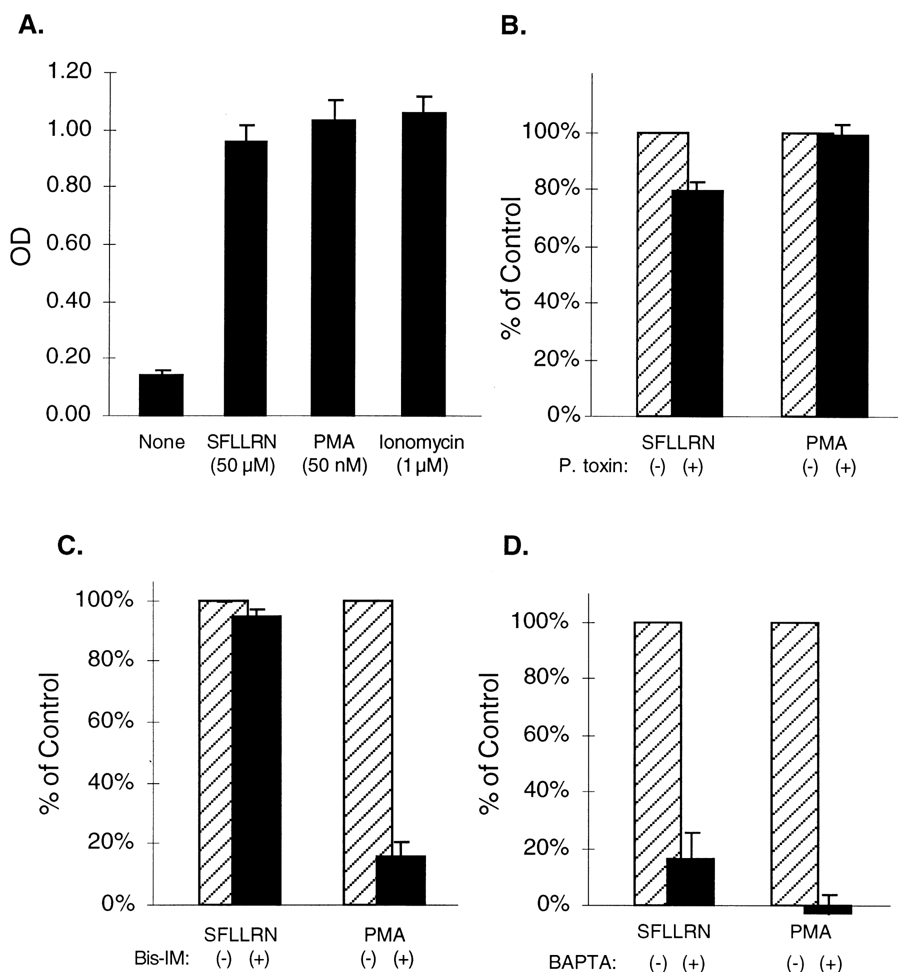


Fig. 3. Adhesion of CHRF-288 cells: inside-out signaling. (A) CHRF-288 cells were allowed to adhere to a fibrinogen-coated surface either without stimulation or in the presence of SFLLRN, PMA or ionomycin at the concentrations shown. Non-adherent cells were removed by washing and adherent cells were quantitated colorimetrically. The results are the mean ± S.E.M. from six studies expressed as a % of the increase in optical density (OD) in the absence of the inhibitor after subtracting the baseline OD in the absence of agonist. (B) The cells were pre-incubated overnight with 200 ng/ml pertussis toxin ($n = 3$). (C) The cells were pre-incubated with 10 μM Bis-IM for 30 min before the addition of SFLLRN or PMA ($n = 5$). (D) CHRF-288 cells loaded with 25 μM BAPTA were incubated with SFLLRN or PMA ($n = 4$).

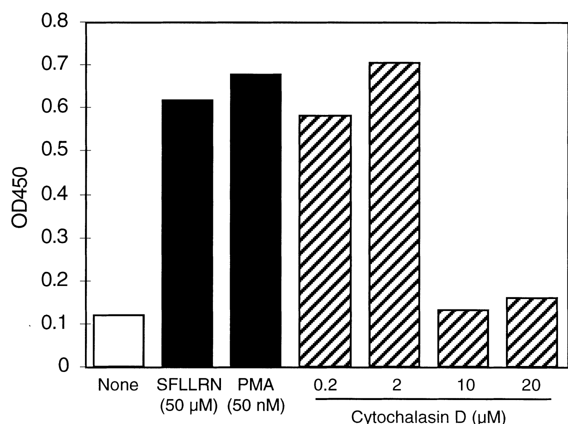


Fig. 4. Cytochalasin D causes CHRF-288 cells to adhere to fibrinogen. CHRF-288 cells were incubated in a fibrinogen-coated dish for 30 min with SFLLRN, PMA or cytochalasin D at the concentrations indicated. The results shown are representatives of two similar experiments.

in the two types of cells, with pertussis toxin-insensitive members of the G_q family playing a larger role in coupling PAR1 to phospholipase C in CHRF-288 cells than in platelets.

The observation that PMA causes CHRF-288 cells to adhere to fibrinogen suggests that PKC could be one of the mediators of PAR1-initiated adhesion. To test this, CHRF-288 cells were pre-incubated with the PKC inhibitor Bis-IM and then stimulated with either SFLLRN or PMA. Bis-IM inhibited adhesion in response to PMA by 90%, but had little effect on adhesion in response to SFLLRN (Fig. 3C), suggesting that the PMA-sensitive, Bis-IM-inhibitable isoforms of PKC are not essential for adhesion following PAR1 activation. This contrasts with results obtained looking at the role of cytosolic Ca^{2+}. Increasing the cytosolic Ca^{2+} concentration with the Ca^{2+} ionophore ionomycin mimicked the response to SFLLRN. Loading the cells with the intracellular Ca^{2+} chelator BAPTA decreased adhesion in response to both SFLLRN and PMA by $>90\%$ (Fig. 3A and D).

Collectively, these results suggest that an increase in cytosolic Ca^{2+} mediated by a pertussis toxin-insensitive G protein is needed for PAR1 agonists to cause adhesion. One of the better-described consequences of the rise in cytosolic Ca^{2+} in platelets is the activation of phospholipase A_2 and the formation of thromboxane A_2 (TxA_2) from arachidonic acid. The released TxA_2 contributes to platelet aggregation by amplifying the effects of the original agonist. To determine whether phospholipase A_2 is turned on in CHRF-288 cells when PAR1 is activated, we measured the phosphorylation of $cPLA_2$ [27,28] and release of [^{3}H]arachidonate from membrane phospholipids. $cPLA_2$ phosphorylation was detectable within 5 min of the addition of SFLLRN and was sustained for at least 20 min (Fig. 5). Arachidonate release from radiolabelled CHRF-288 cells was also maximal within 5 min and, like $cPLA_2$ phosphorylation, occurred to the same extent whether the cells were in suspension or were allowed to adhere to fibrinogen, a point that will be returned to latter.

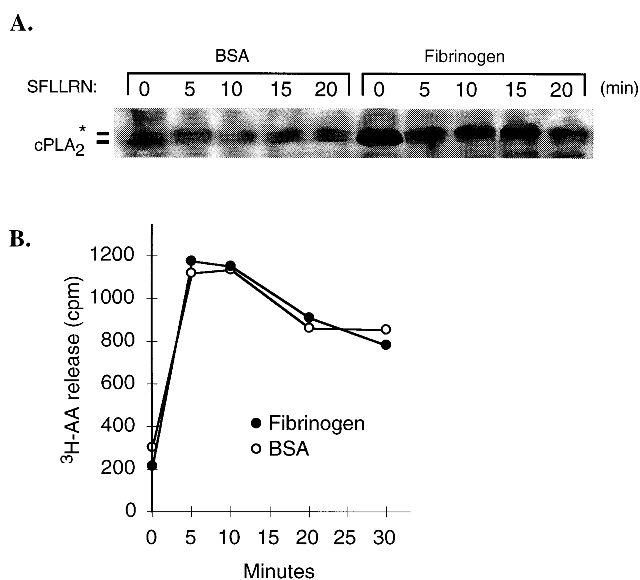


Fig. 5. Phosphorylation of phospholipase A_2 and release of arachidonate. (A) CHRF-288 cells that were serum-starved overnight were incubated with 10 μM SFLLRN in a dish coated with either BSA or fibrinogen. At each of the times shown, lysates were prepared from all of the cells in the dish (adherent and non-adherent) and equal amounts of protein were subjected to gel electrophoresis and Western blotting with antibodies that recognize $cPLA_2$. The asterisk indicates the position of phosphorylated $cPLA_2$. (B) CHRF-288 cells were labelled overnight with [^{3}H]arachidonate and serum-starved the following day for 4 h. Afterwards, the cells were incubated with 10 μM SFLLRN in dishes coated with either BSA or fibrinogen. At the times indicated, phospholipids were extracted and free [^{3}H]arachidonate was measured. The results shown are typical of three such experiments performed in triplicate.

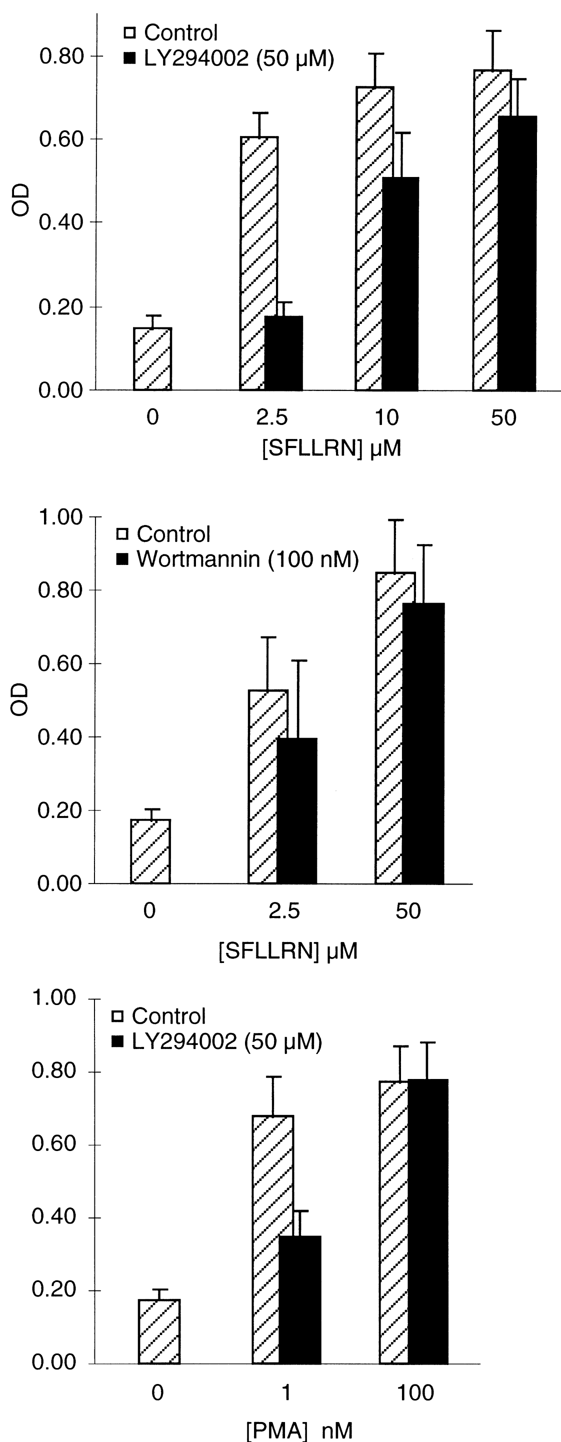


Fig. 6. Inhibition of PI 3-kinase. CHRF-288 cells were stimulated with SFLLRN or PMA after pre-incubating the cells for 30 min with either wortmannin or LY294002. Adhesion to a fibrinogen-coated surface was measured 30 min later ($n = 2$–4).

3.2. PI 3-kinase activation and Vav phosphorylation

In platelets, PAR1 activation by thrombin or SFLLRN also causes an increase in PI 3-kinase activity and the accumulation of 3-phosphorylated phosphoinositides [29]. CHRF-288 cells, like platelets, contain the p85/p110 (growth factor-activated) and p101/p110γ ($G_{\beta\gamma}$-activated) forms of PI 3-kinase [30]. Activation of these enzymes in platelets [31] and CHRF-288 [30] cells has been shown to be sensitive to wortmannin with an IC_{50} of 1–10 nM. At low concentrations, neither wortmannin nor a second PI 3-kinase inhibitor, LY294006, affected the adhesion of CHRF-288 cells to fibrinogen in response to SFLLRN (data not shown). Only when wortmannin and LY294002 were added at much higher concentrations (100 nM and 50 μM), adhesion was inhibited and even this inhibition could be overcome by increasing the concentration of SFLLRN or PMA (Fig. 6). This suggests that PAR1-initiated adhesion of the CHRF-288 cells is not absolutely dependent on PI 3-kinase activity. Notably, we obtained a similar result with platelets. At 5 μM SFLLRN, wortmannin and LY294002 inhibited platelet aggregation, but at higher SFLLRN concentrations, aggregation occurred despite the inhibitors (not shown).

Finally, an additional response to PAR1 activation in platelets is the phosphorylation of the 95 kDa protein, Vav [19]. Vav is an exchange factor for RhoA and Rac1 [32,33] and a substrate for the tyrosine kinases Lck [33], Syk [34] and ZAP-70 [34]. Vav phosphorylation in thrombin-stimulated platelets is not inhibited by antagonists of platelet aggregation such as the synthetic peptide Arg-Gly-Asp-Ser (RGDS), but since vigorous Vav phosphorylation also occurs when platelet adhesion to fibrinogen is forced by the anti-$\alpha_{IIb}\beta_3$ antibody LIBS6, Vav is potentially an intermediate in both outside-in and inside-out signalling [19]. Vav is expressed in CHRF-288 cells and became phosphorylated when the cells were stimulated with SFLLRN (Fig. 7). Phosphorylation was detectable within 5 min and, like $cPLA_2$ phosphorylation (Fig. 5), occurred to the same extent whether or not the CHRF-288 cells were allowed to adhere to fibrinogen, an issue that will be discussed further in the next section.

3.3. Outside-in signalling

In platelets, the inside-out signalling that supports $\alpha_{IIb}\beta_3$ activation leads to integrin-dependent outside-in signalling when signalling complexes form around the integrin cytoplasmic domains [11]. Since CHRF-288 cell $\alpha_{IIb}\beta_3$ does not form high affinity fibrinogen binding sites, it was unclear whether outside-in signalling would occur and would be the same or different than in platelets. Two recognized markers were used to detect outside-in signalling in PAR1-activated CHRF-288 cells: phosphorylation of the 125 kDa focal adhesion kinase, FAK, and sustained phosphorylation of the serine/threonine kinase, ERK2. CHRF-288 cells were incubated with SFLLRN in dishes coated with fibrinogen or BSA and then lysed. FAK was precipitated and then probed with an anti-phosphotyrosine antibody. There was no detectable phosphorylation of FAK in unstimulated cells or in cells incubated on BSA (not shown), but in the fibrinogen-coated dishes, FAK became phosphorylated within 10 min, somewhat slower than the kinetics of adhesion (Fig. 8).

In the second approach, outside-in signalling was detected using ERK2 phosphorylation as an endpoint. In cells that are chronically adherent, such as fibroblasts, integrin engagement enhances growth factor-induced MAPK phosphorylation [1–3,35]. To test whether this also occurs in CHRF-288 cells following PAR1 activation, the cells were incubated with SFLLRN, lysed and then immunoblotted with an anti-ERK2 antibody. Phosphorylation was detected as a decrease in electrophoretic mobility and occurred in both the adherent and the non-adherent cells. However, while ERK2 phosphorylation was undetectable after 5 min in cells plated on BSA, it was sustained for at least 20 min in cells allowed to adhere to fibrinogen (Fig. 9A, right, and B, left). Notably, although this result resembles those obtained with chronically adherent cells, it is different

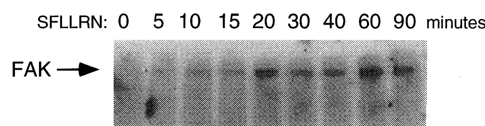


Fig. 8. Phosphorylation of FAK. CHRF-288 cells that were serum-starved overnight were incubated with 10 μM SFLLRN in a dish coated with fibrinogen. At each of the times shown, lysates were prepared, FAK was immunoprecipitated and an anti-phosphotyrosine blot was performed. The results shown are representatives of two such studies.

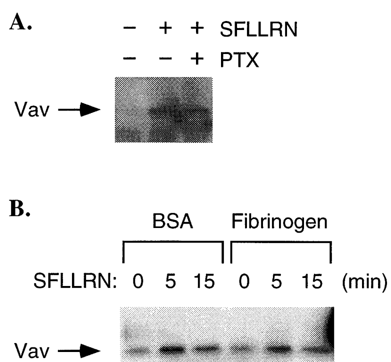


Fig. 7. Phosphorylation of Vav in CHRF-288 cells. (A) Serum-starved CHRF-288 cells were incubated with or without 10 μM SFLLRN for 10 min in dishes coated with fibrinogen. After lysing the cells, Vav was immunoprecipitated with an anti-Vav antibody and then immunoblotted with a phosphotyrosine antibody. Where indicated, the cells were pre-incubated with pertussis toxin before stimulation with SFLLRN. (B) The same as (A) except that the cells were incubated with SFLLRN in dishes coated with fibrinogen or BSA for the times indicated. The results shown in this figure are representatives of at least two similar studies.

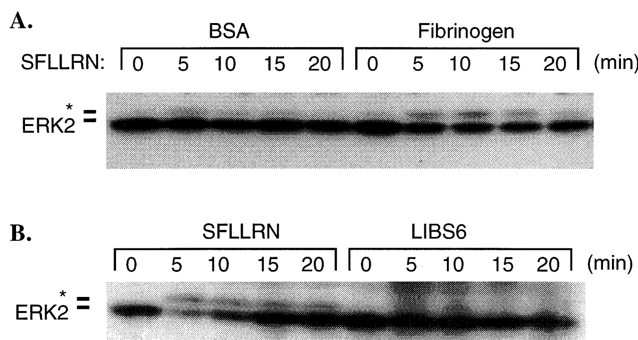


Fig. 9. MAP kinase activation in adherent and non-adherent CHRF-288 cells. (A) CHRF-288 cells that were serum-starved overnight were incubated with 10 μM SFLLRN in a dish coated with either BSA or fibrinogen. At each of the times shown, lysates were prepared from all of the cells in the dish (adherent and non-adherent) and equal amounts of protein were subjected to gel electrophoresis and Western blotting with antibodies that recognize ERK2. The asterisk indicates the position of phosphorylated ERK2. (B) CHRF-288 cells that were serum-starved overnight were incubated with 10 μM SFLLRN or 100 μg/ml LIBS6 Fab fragments in a dish coated with fibrinogen. At each of the times shown, lysates were prepared and subjected to gel electrophoresis and Western blotting with antibodies that recognize ERK2. The results shown are representatives of at least two studies of each type.

than what has been reported in human platelets. In platelets, activation of PAR1 causes ERK2 phosphorylation [36,37], but the extent of phosphorylation has been shown to decrease, rather than increase, when the platelets are allowed to aggregate [35].

Finally, to determine whether integrin engagement and adhesion alone are sufficient to cause outside-in signalling in CHRF-288 cells, the cells were incubated with Fab fragments of the $\alpha_{IIb}\beta_3$ antibody LIBS6, in the absence of SFLLRN. LIBS6 binds to the integrin β_3 subunit, forcing a conformational change that allows fibrinogen to bind in the absence of an agonist [38]. As already described, addition of LIBS6 to platelets causes adhesion to fibrinogen and phosphorylation of Vav, even when the autocrine and paracrine effects of secreted agonists are eliminated [19]. When added to CHRF-288 cells, LIBS6 caused most of the cells to adhere to fibrinogen. However, in contrast to SFLLRN, there was little ERK2 phosphorylation during LIBS6-induced adhesion (Fig. 9B, right). Therefore, these results suggest that adhesion alone is insufficient to trigger outside-in signalling in CHRF-288 cells and that the enhancement in ERK2 phosphorylation observed when SFLLRN-activated CHRF-288 cells adhere to fibrinogen is not due to simple additivity.

4. Discussion

Integrins such as $\alpha_{IIb}\beta_3$ appear to exist in at least three states, an inactive ligand-free state, a fully activated state in which high affinity binding to soluble ligands is possible and a low affinity/high avidity state which may be more attributable to integrin clustering than to integrin activation in the usual sense. The agonist-induced switch from an inactive to an active conformation is particularly relevant for platelets since fibrinogen binding to $\alpha_{IIb}\beta_3$ is essential for platelet aggregation at sites of vascular injury. Recent studies have focused on the identification of signalling pathways and molecules that can interact with integrin cytoplasmic domains and regulate integrin conformation from within. A number of such molecules have been identified [39–42], but a complete picture has yet to emerge. In the present studies, we examined the interaction of $\alpha_{IIb}\beta_3$ with fibrinogen following thrombin receptor activation in CHRF-288 cells. Previous work by other investigators has shown that other megakaryoblastic cell lines will adhere to immobilized fibrinogen or PAC-1, but not bind to soluble fibrinogen when they are stimulated with PMA [9,10]. In platelets, activation of PAR1 has effects that go beyond activation of PKC alone (reviewed in [43]) and there was reason to believe that this is also the case in CHRF-288 cells, where thrombin and SFLLRN were shown to increase the cytosolic Ca^{2+} concentration [14] and activate PI 3-kinase [30]. Therefore, we asked first whether PAR1 activation would cause CHRF-288 cells to bind to either soluble or immobilized fibrinogen. Second, we asked whether the signalling pathways initiated by thrombin and SFLLRN in CHRF-288 cells were similar to those evoked during platelet activation and, finally, we asked whether the consequences of outside-in signalling would be the same in CHRF-288 cells as in platelets, particularly if the CHRF-288 cells proved to be incapable of binding soluble fibrinogen.

The results show that $\alpha_{IIb}\beta_3$ does not undergo the conformational change needed to bind to soluble fibrinogen or PAC-1 when CHRF-288 cells are activated by thrombin or SFLLRN. The cells will, however, adhere to immobilized fibrinogen in an $\alpha_{IIb}\beta_3$-dependent manner. Adhesion was followed by cell spreading. These responses apparently require an increase in the cytosolic Ca^{2+} concentration since raising intracellular Ca^{2+} with an ionophore was sufficient to cause adhesion and suppressing the SFLLRN-induced increase in Ca^{2+} with BAPTA inhibited adhesion. Notably, although PMA was also effective in causing the CHRF-288 cells to adhere to fibrinogen, an inhibitor of PKC activity had no apparent effect on adhesion in response to SFLLRN, even though it inhibited the same response to PMA. By extension, this suggests that the Bis-IM-sensitive isoforms of PKC are not required for the adhesion caused by PAR1 activation. Other responses to thrombin and SFLLRN that are known to occur in platelets and support platelet aggregation, including activation of phospholipase C, phospholipase A_2 and PI 3-kinase, also occur in CHRF-288 cells ([14,30] and the present studies) and therefore, do not readily account for the failure of CHRF-288 cells to bind soluble fibrinogen.

Present theories suggest that the binding of integrins to immobilized ligands do not need to be due to an increase in affinity, but requires only an increase in avidity due to the accumulation of weaker interactions between the ligand and multiple copies of the integrin [11]. This implies an increase in the lateral mobility of the integrin, allowing it to accumulate at the interface between the cell and the ligand-coated surface. Such a redistribution of $\alpha_{IIb}\beta_3$ has been observed in platelets when they adhere to fibrinogen [44]. Studies in lymphocytes have shown that exposure to cytochalasin D increases LFA-1 mobility and causes the cells to adhere to immobilized ICAM-1 as effectively as when the cells are incubated with PMA [24]. We found that cytochalasin D causes CHRF-288 cells to adhere to fibrinogen. The extent of adhesion was similar to that seen after incubation with thrombin or PMA. This is also true in platelets, where cytochalasin D also causes $\alpha_{IIb}\beta_3$-mediated adhesion to fibrinogen. In addition, Shattil and co-workers [5] have recently shown that CHO cells expressing a chimera of $\alpha_{IIb}\beta_3$ fused to FKBP will adhere to immobilized fibrinogen when exposed to a cytoplasmic FKBP dimerizing agent. The cells also bound soluble PAC-1 and fibrinogen, although with less efficiency than normally seen in agonist-activated platelets. This suggests that more than just integrin clustering is needed for the binding of soluble ligands.

In platelets, integrin engagement with soluble fibrinogen is associated with outside-in signalling. Some molecules, such as the non-receptor tyrosine kinase Syk, are activated to a greater extent when platelets are allowed to aggregate than when they are not [45,46]. Others, such as FAK, are activated only when integrin clustering has occurred [11]. Agonist-independent clustering of $\alpha_{IIb}\beta_3$ can cause FAK phosphorylation and Syk activation even in the absence of adhesion [5]. We finished by asking whether adhesion of the CHRF-288 cells to fibrinogen would initiate integrin-dependent outside-in signalling and, if so, whether the consequences would be the same as seen during platelet aggregation. Two established markers of outside-in signalling were measured, phosphorylation of FAK and the sustained phosphorylation of ERK2. FAK became phosphorylated when the CHRF-288 cells were incubated with SFLLRN, but only when they were allowed to adhere to fibrinogen. ERK2, on the other hand, became phosphorylated even in the absence of adhesion, but adhesion extended the duration of phosphorylation. This was in contrast to the phosphorylation of $cPLA_2$ and release of arachidonate from the cells, which were unaffected by adhesion. ERK2 was also phosphorylated, although minimally, when adhesion of the CHRF-288 cells was forced by LIBS6 in the absence of an added agonist.

The findings with ERK2 resemble those reported in adherent cells, but contrast with those obtained when platelets aggregate. Nadal and co-workers [35] have recently shown that the engagement of $\alpha_{IIb}\beta_3$ suppresses rather than promotes ERK2 phosphorylation in platelets. They found that ERK2 becomes phosphorylated to a greater extent in thrombasthenic platelets, which lack $\alpha_{IIb}\beta_3$, than in normal platelets. From this, they concluded that outside-in signalling through $\alpha_{IIb}\beta_3$ in platelets has a negative effect on MAPK regulation. This would be consistent with an earlier finding by Papkoff et al. [36] who observed an increased activity of a MAPK substrate, ribosomal S6 kinase, in response to thrombin in the presence of RGDS.

In summary, the present studies on CHRF-288 cells extend previous work on the interaction of $\alpha_{IIb}\beta_3$ with fibrinogen on megakaryoblastic cell lines and transfected cells by showing that (1) activation of CHRF-288 cells via a G protein-coupled receptor can initiate $\alpha_{IIb}\beta_3$-dependent adhesion, even if it does not promote the conformational switch required to bind soluble fibrinogen, (2) this response depends on an increase in the cytosolic Ca^{2+} concentration, but may not absolutely require PKC or PI 3-kinase, (3) the interaction of CHRF-288 cells with immobilized fibrinogen is sufficient to support outside-in signalling within the cells, although the pattern of outside-in signalling events that was observed is different than that observed during platelet aggregation, and (4) crosstalk from outside-in signalling pathways can reinforce downstream events from activated PAR1 in CHRF-288 cells, including the activation of at least one form of MAPK. However, it appears to do so in a manner that is distinct from that in platelets, which suggests that the failure to form high affinity $\alpha_{IIb}\beta_3$-fibrinogen complexes may influence the repertoire of outside-in signals that can occur.

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