Differential effect of the inhibition of Grb2–SH3 interactions in platelet activation induced by thrombin and by Fc receptor engagement

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The adaptor protein Grb2 (growth factor receptor-bound protein 2) is involved in cell proliferation via the Ras signalling pathway. In order to study the role of Grb2 in blood platelet responses, we used a peptide containing two proline-rich sequences derived from Sos (peptidimer), which binds to Grb2–Src homology 3 domain (SH3) with a high affinity, and hence inhibits Grb2–SH3-mediated protein interactions. Platelet aggregation and 5-hydroxytryptamine (serotonin) release measured in the presence of the peptidimer were: (i) significantly decreased when induced by thrombin; and (ii) potentiated when induced by the engagement of the Fc receptor. In thrombin-activated platelets, the Grb2–SH2 domain formed an association with the β3 subunit of the αIIb–β3 integrin (GPIIb–IIIa), Shc, Syk, Src and SHP1 (SH2-containing phosphotyrosine phosphatase 1), whereas these associations did not occur after the engagement of the receptor for the Fc domain of IgG (FcγRIIa) or in resting platelets. Grb2–SH3 domains formed an association with the proline-rich sequences of Sos and Cbl in both resting and activated platelets, since the peptidimer abolished these associations. Inhibition of both fibrinogen binding and platelet aggregation by the peptide RGDS (Arg-Gly-Asp-Ser) had no effect on thrombin-induced Grb2–SH2 domain association with the aforementioned signalling molecules, indicating that these associations occurred during thrombin-induced ‘inside-out’ signalling. Platelet aggregation induced by direct activation via αIIb–β3 (‘outside-in’ signalling) was potentiated by the peptidimer. The results show that inhibition of Grb2–SH3 interactions with signal-transduction proteins down-regulates thrombin-induced platelet activation, but also potentiates Fc receptor- and αIIb–β3-mediated platelet activation.

Key words: adaptor protein, extracellular-signal-regulated protein kinase (ERK), integrin αIIb–β3, peptidimer, SH2/SH3.

INTRODUCTION

The adaptor protein Grb2 (growth factor receptor-bound protein 2) is composed of two Src homology 3 (SH3) and one SH2 domains [1]. The SH3 domains of Grb2 bind proline-rich sequences of a number of proteins, among which are the Ras guanine-nucleotide-exchange factor, Sos, dynamin, SLP-76 and Wiskott–Aldrich syndrome protein (WASP)[2–8]. Grb2 is known to link membrane receptors, upon their stimulation, to the Ras-signalling pathway by the association of its SH2 domain with specific phosphotyrosine-containing sequences on receptors and non-receptor signalling molecules, leading to cell proliferation or differentiation [1,3,9,10]. Inhibition of Grb2–SH2 domain interactions with phosphotyrosine-containing motifs on membrane receptors, or inhibition of its SH3 domains with downstream signalling molecules, blocks the Ras pathway and hence cell proliferation, thereby proving attractive for the development of anti-cancer therapeutics [11–13]. A role for Grb2 has also been ascribed in the regulation of actin cytoskeleton re-organization, as well as in cell motility downstream of tyrosine kinase receptors [14–16].

In platelets, anucleated and terminally differentiated cells, which have a key role in haemostasis, the role of Grb2 protein remains undefined, although its interaction with signalling molecules has been reported. Grb2 is constitutively associated with Vav and WASP, and associates with Shc in thrombopoietin-activated platelets [17–20]. In platelets activated via the receptor for the Fc domain of IgG (FcγRIIa), Grb2 associates via its SH2 domain with proteins of 36–38 and 63 kDa, and also via its SH3 domains with Sos1, SLP-76 and a protein of 120 kDa [21]. In vitro, Grb2 has been shown to associate with a peptide corresponding to the residues 740–762 of the β3 cytoplasmic domain of the integrin αIIb–β3, provided that the two tyrosine residues are phosphorylated [22]. Recently, it has been shown that the N-terminal SH3 domain of Grb2 is constitutively associated with focal adhesion kinase (‘FAK’) and Pyk2, whereas the SH2 domain of Grb2 is associated with Shc in an activation-dependent manner, and independently of the ‘outside-in’ signalling mediated by fibrinogen binding to activated αIIb–β3 integrin [23].

The aim of the present work was to characterize and compare the role of Grb2 in platelet activation induced by thrombin with that after FcγRIIa or αIIb–β3 engagement. For this purpose, we used a cell-permeant peptide, composed of two proline-rich sequences derived from Sos and called peptidimer, which specifically binds to Grb2–SH3 domains with a high affinity and inhibits Grb2–Sos association [13]. Using this specific cell-penetrating peptide, we have shown that platelet responses induced by thrombin are inhibited, whereas those induced by Fc receptor engagement or by direct activation via αIIb–β3 are potentiated. During thrombin-induced inside-out signalling, Grb2 associates with αIIb–β3, Shc, Syk, Src and SHP1 (SH2-containing phosphotyrosine phosphatase 1), whereas these interactions do not occur after Fc receptor engagement. Our results...
show that interactions mediated by Grb2 are involved differently in the regulation of platelet activation induced by thrombin or by Fc7RIIa engagement.

**EXPERIMENTAL**

**Antibodies and reagents**

A Sos-derived peptidimer [VPPPVP<BR>RRR]-K linked to the third helix of antennapedia homeodomain was prepared as described previously [13].

Anti-Grb2, anti-Cbl and anti-Syk polyclonal antibodies, goat anti-SH2 (where SH2 is SH2-containing inositol phosphatase) and anti-SLP-76 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-<BR>p85/phosphoinositide 3-kinase (PI 3-kinase) sera and anti-Grb2 monoclonal antibody (mAb) were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Rabbit polyclonal anti-Shc, anti-Sos and anti-SHIP1 were from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-<BR>(xIIb/IIIc integrin) mAb, P256, was kindly given by Dr N. Hogg (Imperial Cancer Research Fund, London, U.K.). Anti-(phospho-p38) and anti-[phospho-p42/44 extracellular-signal-regulated kinase (ERK)1/2] were from Promega (Madison, WI, U.S.A.) and BioLabs (Beverly, MA, U.S.A.). Goat anti-rabbit horseradish-peroxidase-labelled antibody was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Goat anti-rabbit horseradish-peroxidase-labelled antibody was from Bio-Rad (Ivry Sur Seine, France). The anti-phosphotyrosine mAbs PY20 and 4G10 were peroxidase-labelled antibody was from Bio-Rad (Ivry Sur Seine, France), metrizamide was from Eurobio (Les Ulis, France). Anti-<BR>Grb2, anti-Cbl and anti-Syk polyclonal antibodies, goat anti-SH2 (where SH2 is SH2-containing inositol phosphatase) and anti-SLP-76 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-<BR>p85/phosphoinositide 3-kinase (PI 3-kinase) sera and anti-Grb2 monoclonal antibody (mAb) were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Rabbit polyclonal anti-Shc, anti-Sos and anti-SHIP1 were from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-<BR>(xIIb/IIIc integrin) mAb, P256, was kindly given by Dr N. Hogg (Imperial Cancer Research Fund, London, U.K.). Anti-(phospho-p38) and anti-[phospho-p42/44 extracellular-signal-regulated kinase (ERK)1/2] were from Promega (Madison, WI, U.S.A.) and BioLabs (Beverly, MA, U.S.A.).

**Platelet preparation**

Human platelets were isolated from fresh platelet concentrates obtained from healthy donors who had not taken aspirin for at least 1 week. Platelet concentrates were centrifuged at room temperature for 15 min at 130 <BR>g to eliminate other cell types, and then subjected to a washing process, as described previously [25]. Briefly, platelets were isolated on a metrizamide gradient and re-suspended in isolating buffer [140 mM NaCl/5 mM KCl/12 mM trisodium citrate/10 mM glucose/12.5 mM sucrose (pH 6)]. After washing on a second metrizamide gradient, platelets were collected and re-suspended in 10 mM Hepes buffer, pH 7.4, containing 140 mM NaCl, 5 mM NaHCO3, 0.5 mM MgCl2, 3 mM KCl and 10 mM glucose. Platelet concentration was adjusted to 10<sup>10</sup> cells/ml for immunoprecipitation studies, or to 5 x 10<sup>9</sup> cells/ml for studying the total platelet lysates. CaCl<sub>2</sub> (1 mM) was added 10 min before platelet stimulation.

**Platelet activation, aggregation and release**

Platelets were stimulated over a period of 2 min with either 2.5 µg/ml Syb antibody or 0.25 unit/ml human thrombin at 37 °C in an aggregometer (Coulter, Havertown, PA, U.S.A.) with constant stirring (1100 rev./min). For direct activation via aIIb/IIIc integrin, platelets were pre-incubated for 1 min at 37 °C with 1V.3 mAb IgG (10 µg/ml) to inhibit the Fc receptor pathway, before addition of 10 µg/ml P256 mAb for 2 min. To study the total platelet proteins, the reactions were stopped by the addition of a 1:4 (v/v) dilution of a solution containing 10% (w/v) SDS and 5 mM EDTA, and the samples were transferred to ice for complete lysis. After 30 min, a 1:4 (v/v) dilution of 4 x concentrated SDS sample buffer and 5% (v/v) 2-mercaptoethanol were added, and samples were boiled for 5 min before SDS/PAGE and Western blot analysis.

To study the platelet aggregation and release, the platelet-rich plasma was adjusted to a density of 5 x 10<sup>10</sup> platelets/ml, and incubated with 0.6 mM 12<sup>C</sup>-labelled 5-hydroxytryptamine (Amersham, Les Ulis, France) for 30 min at room temperature, before isolation of the platelets, as described above. Imipramine was added to the platelet suspension 10 min before the agonist in order to prevent re-uptake of the 5-hydroxytryptamine during the experiment. Inhibition of fibrinogen binding to aIIb/IIIc integrin and platelet aggregation were performed by the pre-incubation of platelets for 1 min with RGDS peptide (Arg-Gly-<BR>Asp-Ser; 400 µg/ml). For the in vivo blockade of Grb2 SH3 domains, platelet suspensions were incubated for 15 min at 37 °C with 5 µM peptidimer conjugate. The extent of aggregation was determined by measuring changes in the light transmission of platelet suspensions stirred at 37 °C in a cuvette in the aggregometer (Coulter). The aggregation was monitored for 2 min, and the reaction was stopped by transfer of the solution into 0.2 vol. of ice-cold 0.1 M EDTA and centrifugation for 1 min at 12000 g immediately afterwards. The 5-hydroxy[<sup>12</sup>C]-tryptamine was counted for radioactivity in the supernatant by liquid-scintillation counting. Release was expressed as the percentage of 5-hydroxy[<sup>12</sup>C]tryptamine liberated compared with the total unstimulated platelet content.

**Immunoprecipitation**

For immunoprecipitation studies, platelet stimulation was stopped by the addition of one-third the volume of a solution of cold 3 x concentrated Nonidet P40 (NP-40) lysis buffer, containing 3% (v/v) NP-40, 150 mM Tris, 450 mM NaCl, 15 mg/l leupeptin, 15 mg/l aprotinin, 3 mM EGTA, 3 mM Na<sub>2</sub>VO<sub>3</sub> and 3 mM PMSF. The mixture was transferred on to ice for 30 min for complete lysis. Insoluble material was removed by centrifugation for 10 min at 16000 g at 4 °C, and the supernatant was incubated with antibodies raised against the relevant proteins of interest (5 µg/ml) for 2 h at 4 °C. Immune complexes were incubated with Protein A-Sepharose beads (40 µl of 50%, slurry) for 1 h at 4 °C. The different protein complexes were isolated by brief centrifugation. After washing three times with 1 x concentrated cold NP-40 lysis buffer (described above), immunoprecipitates were re-suspended in SDS sample buffer containing 5% 2-mercaptoethanol and then analysed by Western blotting.

The peptidimer, when coupled with CNBr-activated Sepharose beads, is able to precipitate Grb2 by means of its SH3 domains from the cell lysates [13]. A phosphotyrosine peptide derived from the Shc sequence PSY(PO<sub>2</sub>H<sub>2</sub>)VNVPD, previously coupled with CNBr-activated Sepharose beads, was also used to precipitate Grb2 protein by means of its SH2 domain [13]. Under these conditions, Grb2 was precipitated by the Sepharose beads-linked peptides overnight at 4 °C. Protein complexes were isolated by brief centrifugation, washed and then analysed as described above for the immunoprecipitate samples.

**Western blot analysis**

Samples were boiled for 5 min and subjected to SDS/PAGE (12% gels). Separated proteins were transferred on to a nitrocellulose membrane (Bio-Rad system). The membrane was incubated for 1 h in a blocking buffer containing 5% low-fat
skimmed milk powder, 2% (v/v) Tween 20, 100 mM NaCl and 20 mM Tris at pH 7.4. Specific antibodies raised against the protein of interest were added over a period of 2 h, before a 1 h incubation with horseradish-peroxidase-conjugated secondary antibody. Enhanced chemiluminescence was used for signal detection.

In order to study other proteins of interest on the same samples, the nitrocellulose membranes were stripped to remove the bound antibody by incubation in a solution containing 2% SDS, 62.5 mM Tris, pH 6.8, and 100 mM 2-mercaptoethanol for 40 min at 60 °C. After extensive washing, the membranes were re-probed with the other antibodies, as described above.

Statistics
Results are expressed as the means ± S.E.M. for at least three independent experiments. Statistical significance was determined by Student’s t test (paired-data analysis). P values < 0.05 were considered to be statistically significant.

RESULTS
Blockade of the tandem Grb2–SH3 domains differently modifies the platelet responses induced by thrombin and Syb antibody
To study the role of Grb2 protein in platelet signalling, we used a peptidimer that specifically associates with Grb2–SH3 domains, and inhibits Grb2 interaction with the proline-rich sequences of Sos [13]. In order to allow the cellular penetration of the peptidimer, the latter was linked via its C-terminal residue to the third helix of antennapedia homeodomain. It was thus referred to as peptidimer-conjugate. The peptidimer-conjugate was incubated with platelets 15 min before activation by two different agonists: thrombin, which activates platelets via a G-protein-coupled, seven-transmembrane domain receptor, and Syb, which is an anti-CD9 mAb. Platelet activation induced by the latter occurs via the Fc receptor, since blockade of FcγRIIa was found to totally inhibit the anti-CD9-induced platelet activation [26]. Platelet aggregation induced by 0.25 unit/ml thrombin was inhibited dose-dependently by the peptidimer-conjugate (Figure 1a). In contrast, the aggregation induced by Syb was increased by the peptidimer-conjugate (Figure 1b). For all further experiments, we chose a low concentration of the peptidimer-conjugate (5 μM), which led to a significant level of inhibition of the platelet aggregation induced by thrombin (66 ± 3.18%) and a significant increase (26 ± 3.60%) in that induced by Syb, as shown in the histograms (Figure 1). The control peptide (antennapedia) affected neither aggregation induced by thrombin nor that induced by Syb (Figure 1). The peptidimer-conjugate did not induce aggregation of resting platelets. At higher concentrations of thrombin (1–5 unit/ml) or Syb (10–20 μg/ml), the aggregation was, however, not significantly modified by the peptidimer-conjugate (results not shown).

Figure 1 Differential effect of the peptidimer-conjugate on platelet aggregation induced by thrombin (a) or Syb (b)
Washed platelets were incubated for 15 min at 37 °C with different concentrations of peptidimer-conjugate (1, 5 and 10 μM) or 10 μM antennapedia (control), before the addition of the agonist. Platelets were then activated for 2 min by addition of either 0.25 unit/ml thrombin or 2.5 μg/ml Syb while stirring. The aggregation is expressed as percentages of light transmission. Results are means ± S.E.M. for three independent experiments. (*P < 0.05; ***P < 0.001).
We next studied the 5-hydroxytryptamine release from platelet-dense granules. As observed for platelet aggregation in the presence of the peptidimer-conjugate, thrombin-induced 5-hydroxytryptamine release was significantly inhibited by 59±5%, and Syb-induced 5-hydroxytryptamine release was increased by 13±6%, (Figure 2). Using higher concentrations of these agonists, 5-hydroxytryptamine release was not significantly modified by the peptidimer-conjugate (results not shown).

To verify that the peptidimer-conjugate was influencing the outside-in signalling mediated via αIIbβ3, we used the P256 mAb to activate platelets directly through αIIbβ3, also in the presence of specific anti-Fc receptor mAb, IV.3, to inhibit the binding of the P256 Fc domain to FcγRIIa. P256-induced platelet aggregation was significantly increased in the presence of the peptidimer-conjugate by 40±5%, but not in the presence of the control peptide, antennapedia (Figure 3). This result indicated that inhibition of the Grb2–SH3 interaction positively regulates the outside-in signalling via the αIIbβ3 integrin. Taken together, these results suggest that signalling downstream of Grb2 protein contributes to a positive regulation of thrombin-mediated platelet activation, but to a negative regulation of platelet activation induced by the engagement of either αIIbβ3 integrin or FcγRIIa.

With an aim to study the modifications in signal transduction induced by the peptidimer-conjugate, we first studied the protein tyrosine phosphorylation pattern. All further studies were performed with agonist concentrations (i.e. 0.25 unit/ml thrombin and 2.5 μg/ml Syb) that induced platelet responses with which the peptidimer-conjugate had a maximal effect (see Figure 1). Under such conditions, only slight differences were observed between the tyrosine phosphorylation patterns in platelets activated 2 min by either thrombin or Syb (Figure 4, upper panel). The peptidimer-conjugate had no significant effect, except for a slight increase in the overall tyrosine phosphorylation pattern observed in Syb-activated platelets. Phosphotyrosine immunoprecipitation was also performed. Among the proteins of interest, we examined Shc (known to form a complex with Grb2) and Syk, a tyrosine kinase involved in signal transduction in platelets mediated by both thrombin and Syb, and especially after FcγRIIa engagement. As shown in Figure 4 (lower panel), we did not observe any modification of tyrosine phosphorylation of Syk and Shc in the presence of the peptidimer.

Study of Grb2 interactions with other proteins

In order to elucidate further the mechanism by which the peptidimer-conjugate could differently affect the platelet responses to thrombin or Syb, we studied Grb2 interactions with different signalling molecules. First, we immunoprecipitated Grb2 from lysates of resting platelets, and those activated by thrombin or Syb, using a rabbit polyclonal antibody. Experiments were performed in the presence or absence of peptide RGDS to inhibit both fibrinogen binding to αIIbβ3 integrin and platelet aggregation, and to dissociate the outside-in signalling from thrombin- and Syb-induced signalling. A control anti-Grb2 immunoblot assay was performed in all the experiments to verify that equal amounts of Grb2 protein were precipitated under each condition, and to localize Grb2 on the nitrocellulose membranes (Figure 5, lower panel). Analysis by anti-phosphotyrosine immunoblotting showed that Grb2 was not phosphorylated in unstimulated platelets (Figure 5, lane 2). Platelet activation by thrombin or Syb increased Grb2 tyrosine phosphorylation very slightly (lanes 3–6). Interestingly, a number of tyrosine-phosphorylated proteins, including p200,
p120, p90–100, p72, p60–64, p52–55 and p36–38, were co-immunoprecipitated with Grb2 in activated platelets (lanes 3–6). The amounts of Grb2-associated phosphorylated proteins were more important in thrombin-activated than in Syb-activated platelets, especially that of p36–38 kDa (Figure 5, compare lane 3 with lane 5). In the presence of RGDS, the amounts of Grb2-associated phosphoproteins were slightly increased in Syb-activated platelets (compare lane 5 with lane 6). These results suggest that Grb2 involvement in tyrosine kinase-dependent events could be more important in the thrombin-receptor-signalling pathway than in that of FcγRIIa.

**Characterization of the molecular interactions of Grb2–SH3 domains with signalling proteins**

Since Grb2 is composed entirely of the SH3/SH2/SH3 motif, we precipitated Grb2 using a sequence with a high affinity for the Grb2–SH2 domain that corresponded to the phosphoryrosine-containing peptide of its partner, Shc, linked to Sepharose beads. Under these conditions, the co-precipitated proteins could be associated with Grb2–SH3 domains, and possibly with Grb2 phosphotyrosine residues. Sepharose beads alone were used as a control (Figure 6, lanes 1), and a sample of a whole platelet lysate (WL) was loaded. Results are representative of three experiments. WB, Western blotting.
These associations increased after activation by thrombin and, to a lesser extent, by Syb (lanes 3 and 6). Grb2 association with Sos and Cbl were inhibited by the peptidimer-conjugate (lanes 4 and 7), but were not affected by the control peptide, antennapedia (lanes 5 and 8). We wanted to ensure that the inhibitory effect of the peptidimer-conjugate was not due to an in vitro effect upon cell lysis. To meet this aim, the peptidimer-conjugate was incubated with platelets in the isolating buffer before the final washing procedure (see the Experimental section). The results indicated that Grb2–SH3 domains formed an association with the proline-rich sequences of Sos and Cbl. Moreover, in unstimulated platelets, Grb2 associated with p85α SH2 subunit of αIIb–β3, anti-Shc, anti-Syk, anti-Src, anti-SHP1 and monoclonal anti-Grb2 antibodies. A control of Sepharose beads alone was also performed and a sample of a whole platelet lysate (WL) was loaded. Results are representative of three experiments. WB, Western blotting.

**Characterization of the molecular interactions mediated by the Grb2–SH2 domain**

Since interactions of Grb2–SH3 domains with other proteins could not account for the differential involvement of Grb2 in platelet responses to thrombin or Syb, we therefore investigated the interactions of the Grb2–SH2 domain with other signalling molecules. For this purpose, we used the peptidimer linked to Sepharose beads to precipitate Grb2 and Grb2–SH2-domain-associated proteins in platelets activated by thrombin or Syb. Sepharose beads without the peptidimer were used as a control (Figure 7, lane 1), and a sample of a whole-platelet lysate was loaded under the same conditions (lane 7). The presence of a band of 100–115 kDa in Grb2 immunoprecipitates led us to search for a possible interaction of αIIb–β3 integrin with Grb2. Figure 7 shows that Grb2 was associated with the β3 subunit of αIIb–β3 in thrombin-activated, but not in Syb-activated, platelets (lanes 3 and 5). It is known that Grb2 associates with Shc in proliferative cells, and since a protein of approx. 52–55 kDa was co-immunoprecipitated with Grb2 (see Figure 5), we searched for the presence of Shc in the Grb2 immunoprecipitate. An anti-Shc immunoblot showed that Shc was associated with Grb2 in thrombin-activated, but not in Syb-activated, platelets (Figure 7). Since Src, Syk and SHP1 are known to be tyrosine-phosphorylated in activated platelets, and since their molecular masses (60, 72 and 64 kDa respectively) correspond to bands associated with Grb2 (see Figure 5), we tested whether these proteins were associated with Grb2. Immunoblots confirmed that Grb2 was associated with Syk, Src and SHP1 after activation by thrombin, but not by Syb (Figure 7). In thrombin-activated platelets, the most important Grb2-associated phosphorytrosine protein was that of approx. 36–38 kDa in size. Since LAT (linker for activator of T cells) is a 36–38 kDa protein present in platelets and is phosphorylated on tyrosine after activation by collagen [27], we tested whether LAT could be the p36–38 protein associated with Grb2 in our conditions. We were unable to detect the presence of LAT in Grb2 immunoprecipitates, and the p36–38 protein remains unidentified. This negative result did not, however, exclude the possibility that p36–38 corresponded to LAT, considering that antibodies raised against LAT are poorly suited to Western blotting experiments.

The presence of RGDS to prevent fibrinogen binding to αIIb–β3 and platelet aggregation did not modify any of the Grb2 interactions that occurred in thrombin-activated platelets in the absence of RGDS (Figure 7, lane 4). In contrast, in platelets activated by Syb, the presence of RGDS increased the association of Grb2 with the above proteins, i.e. Syk, Src, Shc, SHP1 and β3 (lane 6). Similar results to those obtained with Syb were obtained in platelets activated by cross-linking FcγRIIa (results not shown). The present results suggest the formation of a multi-molecular complex associated with Grb2, downstream of thrombin-receptor activation, but not after FcγRIIa engagement. Such associations could explain, at least partly, the differential effect of the peptidimer-conjugate on platelet responses to the two agonists.

**Effect of the peptidimer-conjugate on ERK2 activation**

We investigated the effect of the inhibition of Grb2–SH3 interactions in the regulation of ERK2 [p42 mitogen-activated protein kinase (MAPK)] and p38 MAPK activation. For this purpose, we studied the phosphorylation of these proteins in platelets activated by thrombin or Syb in the presence or absence of the peptidimer-conjugate and/or RGDS. Quantification of the phosphorylated ERK2 protein was obtained by densitometric scanning of the autoradiographs from three different experiments. Since the maximal effect of the peptidimer-conjugate on platelet responses was observed at low doses of thrombin and Syb, we used the same concentrations for the study of ERK2 phosphorylation. In resting platelets, ERK2 was not phosphorylated. ERK2 phosphorylation occurred only slightly in thrombin-activated platelets, but was significantly enhanced by the peptidimer-conjugate (3.4-fold increase) and by RGDS, although not significantly (2-fold increase; Figure 8a, lanes 2-4, and Figure 8b). The effects of RGDS and peptidimer-conjugate were not additive (3.4-fold increase). In Syb-activated platelets, ERK2 was weakly phosphorylated (lane 6), although this phosphorylation was enhanced significantly in the presence of either RGDS (2.4-fold increase) or the peptidimer-conjugate
actions between Grb2–SH3 domains and other signalling proteins suggest a differential regulatory role of Grb2, or its associated proteins, for platelet responses, depending on the agonist.

In thrombin-activated platelets, aggregation and release of granule content were strongly inhibited by the peptidimer-conjugate. The thrombin receptor belongs to the superfamily of G-protein-coupled receptors. The signals that link these receptors to Grb2 and Ras signalling, albeit poorly characterized, are initiated by tyrosine-phosphorylation events [31,32]. In platelets, no direct involvement of Grb2 has yet been demonstrated, although Grb2 is associated with different signalling proteins. In the present study, the fact that: (i) platelet responses to thrombin were inhibited by the peptidimer-conjugate, and (ii) the outside-in signalling via αIIb-β3 was potentiated by the peptidimer-conjugate, suggest an important role for Grb2 in the inside-out signalling after activation by thrombin. Moreover, this strong positive role of Grb2 could override its role in down-regulating the transduction of the signal during the outside-in signalling. Alternatively, the prevention of interactions of signalling proteins with Grb2 caused by the peptidimer-conjugate might result in new protein interactions, and hence modifications of the platelet response, and not to inhibition of functions mediated by the Grb2-SH3 interaction.

Unlike platelet responses to thrombin, those induced by the FcγRIIa engagement were potentiated in the presence of the peptidimer-conjugate. Two hypotheses could explain this potentiation. The potentiation of platelet responses by the peptidimer-conjugate could result as a consequence of the enhanced αIIb-β3-mediated outside-in signalling. This hypothesis is supported by the fact that platelet aggregation induced by direct activation via αIIb-β3 integrin was potentiated by the peptidimer-conjugate. Alternatively, Grb2 could be involved in a down-regulation of the FcγRIIa pathway, and hence its inhibition by the peptidimer-conjugate would result in enhanced platelet responses. The latter hypothesis is supported by the fact that Grb2 associates with signalling molecules further downstream after FcγRIIa engagement, as shown by the present results and those of Robinson et al. [21].

In order to understand better the differential involvement of Grb2 in the agonist-dependent signal transduction, it is important to identify Grb2 interactions with signalling molecules. To meet this end, the use of a phosphotyrosine peptide coupled with Sepharose beads to precipitate Grb2 by means of its SH2 domain allowed us to investigate the proteins associated with Grb2 by other domains. The association of Grb2 with Sos and Cbl occurred in both thrombin- and Syb-activated platelets. The fact that: (i) Grb2/Cbl and Grb2/Sos exist only in separate complexes [33,34], and (ii) the peptidimer-conjugate, which has a high affinity for Grb2, led to the dissociation of these protein complexes in platelets in vivo, is consistent with an interaction occurring between Cbl and Sos and the tandem SH3 domains of Grb2, probably through a proline-rich motif [35]. Unlike Cbl and Sos, the p85/PI 3-kinase associates with Grb2 independently of the presence or the absence of the peptidimer-conjugate, indicating that a phosphotyrosine of Grb2 could associate directly with PI 3-kinase-SH2 domains after platelet activation. Indeed, by using glutathione S-transferase fusion proteins, we found that, in thrombin-activated platelets, Grb2 association with the p85 C-terminal SH2 domain was slightly enhanced (results not shown).

We have shown previously an association of Cbl with PI 3-kinase that was dependent on FcγRIIa [36] and αIIb-β3 engagement [37], and which could lead to PI 3-kinase activation. Such an association was not observed in the inside-out signalling of platelets activated by thrombin. Thus it is tempting to speculate

**Figure 8** Effect of the peptidimer on ERK2 and p38 MAPK phosphorylation

Washed platelets were incubated with saline solution, or either 400 μg/ml RGDS or 5 μM peptidimer-conjugate, or both. Platelets were then activated with either 0.25 unit/ml thrombin or 2.5 μg/ml Syb, and lysed in an SDS-containing buffer (10% SDS and 5 mM EDTA). Samples were resolved on SDS/PAGE (10% w/v gel), transferred to a nitrocellulose membrane, and blotted with anti-phospho-p38 MAPK, anti-phospho-p42/44 MAPK and anti-p42/44 MAPK (a). Results are representative of three experiments. WL, whole platelet lysate. (b) Densitometric scanning of the phosphorylated ERK2 obtained from three independent experiments (**P < 0.01; ***P < 0.001). AU, arbitrary unit; WB, Western blotting.

(2.9-fold increase) (Figure 8a, lanes 7–8, and Figure 8b). Moreover, the potentiating effects of the peptidimer-conjugate and of RGDS on ERK2 phosphorylation were additive (5.8-fold increase) in Syb-activated platelets (Figure 8a, lane 9, and Figure 8b). The use of anti-MAPK antibodies ensured that equivalent amounts of MAPK were present in all conditions (lower panels). In unstimulated platelets, the phosphorylation of ERK2 was not affected by the presence of either the peptidimer-conjugate or antennapedia (results not shown). Moreover, the results showed that p38 MAPK was weakly phosphorylated in resting platelets (Figure 8a). The phosphorylation of p38 MAPK increased in platelets activated by either thrombin or Syb, and no significant changes were observed in the presence of either the peptidimer-conjugate or RGDS (Figure 8a). Taken together, these results suggest that the maintenance of Grb2–SH3 interactions favours the down-regulation of ERK2 phosphorylation, especially in platelets activated by Syb and, to a lesser extent, in those activated by thrombin.

**DISCUSSION**

The adaptor protein Grb2 is especially known to be involved in the activation of the Ras signalling pathway, leading to cell proliferation [28]. In unstimulated cells, Grb2/Sos exists as a cytoplasmic pre-formed complex [3]. Upon activation, this complex translocates to the plasma membrane, where it serves as a scaffold for the recruitment of other proteins, and propagates the signal to downstream transducers, such as Ras and MAPKs [29,30]. In blood platelets, anucleated and non-proliferative cells, the role of Grb2 is still unknown. In the present work, the results obtained with a peptidimer-conjugate to inhibit molecular inter-
that the different effects of inhibition of the Grb2–SH3 domain interaction could be due to a modification of the balance between Cbl/Grb2 and Cbl/PI 3-kinase associations in platelets activated via FcγRIIA or αIIb/β3. Indeed, inhibition of the Cbl/Grb2 association by the peptidimer could lead to an increase in Cbl/PI 3-kinase association, as mentioned above. This hypothesis is unlikely, however, since, in activated platelets, Cbl/PI 3-kinase association was not modified by the presence of the peptidimer-conjugate; nor was that of the PI 3-kinase activity associated with Cbl (results not shown). More to the point, the fact that both constitutive and activation-induced associations of Grb2 with Sos, Cbl and p85/PI 3-kinase were at a higher level in thrombin-activated platelets than after activation via FcγRIIA could not explain the opposed effects of the peptidimer-conjugate in the two activation processes.

We thus examined whether the different involvement of Grb2 in Syb- or thrombin-activated platelets could be explained by different protein interactions of the Grb2–SH2 domain, using the peptidimer coupled with Sepharose beads to precipitate Grb2. Platelet activation by thrombin induced the association of Grb2–SH2 domain with the β3 subunit of the αIIb–β3 integrin, Shc, Syk, Src and protein tyrosine phosphatase 1C (PTP1C). In contrast, in Syb-activated platelets, Grb2 was not associated with these signalling molecules. Given that there is competition between SHIP and Grb2 in terms of associating with Shc in B cells and fibroblasts [38,39], we suggest that, in Syb-activated platelets, SHIP could similarly compete with Grb2 to associate with Shc and other molecules. Indeed, we found that SHIP associated with Grb2 in Syb-activated platelets, and that picatannol (an inhibitor of Syk) inhibited this association (A. Saci, F. Rendu and C. Bachelot-Loza, unpublished work). Thus, in Syb-activated platelets, tyrosine phosphorylation of SHIP or Grb2 could be necessary for the association of the two proteins, and could prevent any interaction forming between Grb2 and other proteins, such as Shc and Syk (i.e., Shc, Syk, Src, PTP1C and the β3 subunit of αIIb–β3 integrin).

Thrombin-induced interactions with β3, Shc, Syk, Src and PTP1C were unaffected by pre-incubation of platelets with either RGDS or the peptidimer-conjugate. These results indicate that such interactions occurred during the inside-out signalling induced by thrombin independently of the outside-in signalling induced by fibrinogen binding to αIIb/β3. The latter is consistent with the observation that, in platelets activated directly via αIIb/β3, Grb2 did not associate with the aforementioned signalling molecules (results not shown). Moreover, in Syb-activated platelets, a slight increase of these Grb2 associations was observed. An association between Shc and the Grb2–SH2 domain that occurred independently of platelet aggregation during thrombin-induced platelet aggregation has already been reported by Ohmori et al. [23]. However, our results are in contrast with those of Law et al. [22]. The latter paper describes an association of Grb2 with β3 cytoplasmic peptide, probably mediated via an unidentified protein, but only when the two tyrosine residues (Tyr171 and Tyr395) of the β3 peptide are phosphorylated. In vivo, phosphorylation of the two β3 tyrosine residues was only observed in aggregating platelets. This apparent discrepancy could be due to the different experimental conditions used in our study. Indeed, the associations that we observed were found under the in vivo conditions employed, i.e. directly in the platelet lysates. The usual technical difficulty in co-immunoprecipitating β3 with its associated proteins was probably counterbalanced by the potency of the peptidimer coupled with Sepharose beads that we used to precipitate Grb2. It still holds that, since no consensus binding sequence to Grb2 has been described in the β3 cytoplasmic domain, the interaction that we observed between these two proteins might be bridged by other unidentified proteins.

The role of an adaptor protein is to regulate protein interactions in cells and to bring enzymes into close proximity with their substrates. Therefore inhibition in vivo of Grb2–SH3 domain interactions by the peptidimer-conjugate could disrupt the downstream signalling cascade involving the Grb2–SH2 domain. The fact that, in thrombin-activated platelets, Grb2 was implicated in specific associations not involved in Syb-activated platelets lends support to the notion of a different involvement of Grb2 in the two signalling pathways. Altogether, the results suggest that Grb2–SH3 interactions are required for signal transduction mediated by the thrombin receptor to activate αIIb–β3 integrin, i.e. in the platelet’s inside-out signalling. In contrast, the inside-out signalling downstream of FcγRIIA engagement, and/or the outside-in signalling downstream of αIIb/β3 integrin engagement, appear to be negatively regulated when Grb2–SH3 domains are able to interact with signalling proteins.

Earlier data have indicated that platelet MAPKs are activated by thrombin and down-regulated as a result of the engagement of αIIb–β3 integrin by linking with fibrinogen [40,41]. Here, we have shown that thrombin-induced phosphorylation of ERK2 increased when Grb2 interactions were inhibited by the peptidimer-conjugate and, to a lesser extent, in the presence of RGDS. The fact that the combination of RGDS and the peptidimer-conjugate had no additive effect on ERK2 phosphorylation suggests that inhibition of Grb2 interactions down-regulates the phosphorylation of ERK2 downstream of αIIb–β3 engagement. In Syb-activated platelets, the phosphorylation of ERK2 increased significantly in the presence of either the peptidimer-conjugate or RGDS. Moreover, the combination of the two peptides (RGDS and peptidimer-conjugate) resulted in additive effects, suggesting that the up-regulation of ERK2 induced by inhibition of the recruitment of signalling proteins by Grb2, or, in contrast, by new interactions formed after the liberation of proteins associated with Grb2–SH3 domains, is involved after the engagement of both Fc receptor and αIIb–β3 integrin. That inhibition of Grb2–β3 interactions alters steps downstream of αIIb–β3 engagement is supported further by the fact that, in platelets activated directly through αIIb–β3, the phosphorylation of ERK2 was very weak, whereas in the presence of the peptidimer-conjugate this phosphorylation was increased (results not shown). Given that activated ERK2 is involved in the phosphorylation of phosphophatase A2, after Fc receptor engagement [42], we suggest that the potentiation of platelet responses by the peptidimer-conjugate after activation via FcγRIIA is, at least in part, the result of increased ERK2 activation.

In conclusion, Grb2 and its associated proteins could be involved in different signal transduction mechanisms, leading to opposite platelet responses by recruiting various signalling molecules.

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REFERENCES


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