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The phytotoxin fusicoccin promotes platelet aggregation via 14-3-3/Glycoprotein Ib-IX-V interaction[§]

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[§]This paper is dedicated to Professor Alessandro Ballio on the occasion of his 90th birthday. ¹To whom correspondence should be addressed (e-mail: camoni@uniroma2.it). ^{*}These authors contributed equally to this work.

SYNOPSIS

The fungal toxin fusicoccin induces plant wilting by affecting ion transport across the plasma membrane of plant cell. The activity of this toxin is so far unknown in humans. Here we show that fusicoccin is able to affect the platelet aggregation process.

The toxin associates to platelet intracellular binding sites and induces aggregation in platelet-rich plasma in a dose-dependent manner. We identified the adhesion receptor glycoprotein Ib-IX-V as fusicoccin target. The toxin promotes the binding of the regulatory 14-3-3 proteins to glycoprotein Ib α and hampers that to glycoprotein Ib β subunit. As a result, platelet adhesion to von Willebrand Factor is stimulated, leading to platelet spreading and integrin $\alpha_{IIb}\beta_3$ activation.

We anticipate our study to be a starting point for future therapeutic use of fusicoccin in genetic bleeding diseases characterized by qualitative or quantitative abnormalities of the platelet membrane adhesion receptors. Furthermore, the present study also sets the stage for future work to determine the potential pharmacological application of fusicoccin as a drug directed to other 14-3-3/target complexes.

Heading title

A fungal phytotoxin induces platelet aggregation.

Keywords

Fusicoccin, 14-3-3 proteins, platelet aggregation, protein-protein interactions, receptors, drug, toxin.

INTRODUCTION

Fusicoccin (FC) (Figure 1A), a diterpene glucoside produced by fungus *Phomopsis amygdali*, is the causative agent in the wilting of peach and almond trees infected by the pathogen [1]. Despite the fact that *P. amygdali* is host specific, FC is active on any higher plant, and its effects have been attributed to its ability to stimulate the plasma membrane H⁺-ATPase, a key enzyme in the control of ion transport across the plasma membrane of plant cells. FC promotes the permanent association of regulatory 14-3-3 proteins to the proton pump cytosolic C-terminal domain [2,3], which plays an autoinhibitory function, thereby leading to its displacement and enzyme activation [4].

14-3-3 is a family of highly homologous proteins that form both homo- and heterodimers. This family consists of two distinct gene products in yeast, seven in human cells and twelve in *Arabidopsis thaliana* [5,6].

14-3-3 proteins are involved in the coordination of a diverse array of cellular processes, such as cell cycle progression, apoptosis, protein trafficking, signal transduction and gene expression [5,6]. 14-3-3 proteins exert their effects through binding to a variety of protein targets in a sequence-specific and phosphorylation dependent manner. The analysis of 14-3-3 targets and structural studies have initially established two binding consensus motifs, RSX(pS/pT)XP and RXY/FX(pS/pT)XP (where X is any amino acid and pS/pT represents phosphorylated Ser or Thr), which have been defined as mode I and II recognition motifs, respectively [7-9]. These motifs are recognized by a conserved amphipathic groove present in each 14-3-3 monomer. The vast majority of binding sites matches either mode I or II binding consensus, but unrelated binding sequences have also been identified. A limited number of targets binds 14-3-3 through a C-terminal binding site. Although structurally unrelated, these C-terminal binding motifs have been proposed as mode III [10]. The H⁺-ATPase binds 14-3-3 proteins via a mode III motif (XXYpTV), located at the very end of the C terminus [3].

FC action has been clarified at the molecular level: the crystal structure of the ternary complex between 14-3-3, a phosphorylated pentapeptide of H⁺-ATPase and FC revealed that the toxin accommodates into a cavity in the 14-3-3 groove and strictly contacts the C-terminal residue of the peptide, leading to mutual stabilization of both ligands [11].

Although a report shows its effects on an animal system [12], the activity of this toxin is completely unknown in humans where FC binding proteins have not yet been identified. According to FC mechanism of action in plants, potential targets of the toxin are proteins which bind 14-3-3 via a C-terminal motif, even though FC ability to regulate 14-3-3 association to targets with internal motifs cannot be excluded.

In humans, a well-characterized 14-3-3 target containing a mode III motif is the Ib α subunit of glycoprotein Ib-IX-V (GPIb-IX-V) [13], a membrane protein complex expressed on the platelet plasma membrane which mediates the initial adhesion of circulating platelets to vessel wall matrix under high shear conditions [14]. All the six 14-3-3 isoforms expressed in platelets bind to GPIb-IX-V [15]. In particular, binding of 14-3-3 ζ to the C-terminal sequence RYSGHpSL_{COOH} of GPIb α is essential for the ability of GPIb-IX-V to bind subendothelial von Willebrand Factor (vWF) [16].

In the present paper we explored the possibility that FC action could not be restricted to the H⁺-ATPase/14-3-3 interaction, but it may be rather extended to other 14-3-3 targets sharing with the H⁺-ATPase the mode III binding motif. Therefore, we investigated the FC effect on the interaction between 14-3-3 proteins and GPIb α and the functional consequence of FC action on the platelet aggregation process.

EXPERIMENTAL

Chemicals

FC was prepared according to Ballio *et al.* [17]. [³H]FC, specific activity 8.75 Ci/mmol, was prepared as described by Ballio *et al.* [18]. The aglycone of FC was prepared as described by Ballio *et al.* [19]. BSA-FC (BSA-dideacetyl fusicoccin, FC:BSA ratio 10:1) was prepared according to Aducci *et al.* [20].

The following biotinylated peptides: H⁺-ATPase (LKGLDIDTIQQNYpTV_{COOH}), Raf-1 (EGSLSQRQRSTpSTPN_{COOH}), Raf-1 ^{Δ PN} (EGSLSQRQRSTpST_{COOH}), GPIb α ^{term} (DLLSTVSIRYSGHpSL_{COOH}) and the corresponding unphosphorylated peptides were synthesized by Neosystem (Strasbourg, France). GPIb α ^{int} (VPRAWLLFLRGpSLPT_{COOH}), GPIb β (RARARAAARLpSLTDP_{COOH}), the corresponding unphosphorylated peptides and MP α C myristoylated peptide (C₁₃H₂₇CONH-SIRYSGHpSL_{COOH}), were synthesized by JPT Peptide

Technologies (Berlin, Germany). Human von Willebrand Factor was purchased by Haematologic Technologies, Inc. (VT, USA). IgG from goat serum, anti-GPIb α (CD42b-C20 and CD42b-N19) and anti-GPIb β (CD42c-C20) antibodies were from Santa Cruz Biotechnology (CA, USA). RGDS peptide, ristocetin, protein kinase A and the other chemicals were from Sigma-Aldrich (MO, USA).

Binding of 14-3-3 ζ to phosphopeptides

0.5 nmol of biotinylated peptides were immobilized onto 20 μ l of streptavidin-agarose magnetic beads (Sigma-Aldrich, MO, USA). Beads were incubated for 60 min at RT in 50 μ l of buffer HT (25 mM Hepes-OH, 75 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.04% Tween-20, pH 7.5) containing 10 μ M of ³²P-labelled 14-3-3 ζ in the presence of various FC concentrations. Beads-bound radioactivity was measured in a liquid scintillation β -counter. Dissociation constants (K_D) were estimated with homologous competitive binding experiments, using GraphPad Prism5 (GraphPad Software Inc., CA, USA) for data analysis.

Platelets preparation

Washed platelets were prepared from platelet-rich plasma (PRP) obtained from healthy donors who had not taken medication for at least 9 days before bleeding. PRP was obtained by differential centrifugation at 1100 g for 5 min as described by Jin *et al.* [21].

Expression of 14-3-3 ζ

Human 14-3-3 ζ was expressed in *Escherichia coli* as fusion protein with the glutathione-S-transferase (GST) using pGEX-2TK vector, following the procedure described by Sottocornola *et al.* [22].

Immunoprecipitation and overlay assay

Immunoprecipitation was performed using the anti-GPIb α antibody CD42b-C20 and the Protein G Immunoprecipitation kit IP50 (Sigma-Aldrich) according to the manufacturer's instructions. SDS-PAGE was performed as described by Laemmli [23]. Non-reducing gel electrophoresis was performed omitting β -mercaptoethanol from the loading buffer. Overlay experiments were performed according to Camoni *et al.* [24] using 0.3 μ M of ³²P-labelled 14-3-3 ζ (8.3 kBq/ml) as probe and, where indicated, 40 μ M FC. Densitometric analysis was performed using the ImageJ image processing program [25], developed by the National Institutes of Health and freely available at <http://rsbweb.nih.gov/ij>. Densitometric data are expressed as % of the maximum Integrated Densitometric Value (the product of Area and Mean Grey Value).

Binding of FC to isolated platelets

To measure FC binding activity of platelets, 30 μ l of platelet suspension (2.0 x 10⁸/ml) were incubated for 30 min at 37°C with 1 μ M [³H]FC in 100 μ l PBS containing 5 mM MgCl₂, in the presence of different concentrations of FC or FC derivatives. Platelets were centrifuged at 2000 g and, after the addition of 500 μ l 7% perchloric acid, the radioactivity was measured in a scintillation β -counter. Dissociation constant (K_D) and maximal binding capacity (B_{max}) were estimated with homologous competitive binding experiments, using GraphPad Prism5 (GraphPad Software Inc., CA, USA) for data analysis.

Platelet adhesion

Platelet adhesion to immobilized vWF was performed in polystyrene microtitre plate wells as described by Lu *et al.* [26], using vWF concentrations ranging from 0.1 to 20 μ g/ml. Control experiments were carried out replacing vWF with BSA. Where indicated, platelets were pre-incubated for 10 min with 40 μ M FC or 2 μ g/ml anti-GPIb α (CD42b-N19) antibody. The number of vWF-bound platelets was calculated by measurement of endogenous acid phosphatase as described by Lu *et al.* using an automated plate reader [26].

Platelet aggregation

PRPs were preincubated at 37°C with different FC concentrations and, when indicated, 1 mg/ml ristocetin, 0.75 mM RGDS or 0.1 mM MP α C were added. Aggregation was measured using a turbidometric platelet aggregometer Chrono-log 560 CA (Chrono-log Corporation, PA, USA). 500 μ l PRP were placed in a cuvette at 37°C under continuous stirring at 1000 rpm. Aggregation was expressed as percentage of change in light transmission, with the values of the blank sample (PPP, Platelet-Poor Plasma) and PRP set at 100% and 0%, respectively.

Statistical analysis

Statistical significance was assessed by unpaired Student's *t*-test. All values are expressed as means \pm s.e.m.

RESULTS

FC action on mode I and mode III targets

In order to ascertain the potential ability of FC to be a general modulator of 14-3-3 interactions with client proteins, we analyzed the structural requisites of 14-3-3 targets to allow the FC regulatory function. To this purpose, the effect of FC on 14-3-3 interaction with different phosphopeptides, reproducing internal and C-terminal binding sequences, was tested. For these studies, the human 14-3-3 ζ isoform was used.

H⁺-ATPase peptide mimics the C-terminal mode III recognition site of the plasma membrane H⁺-ATPase. As expected, 10 and 100 μ M FC significantly stimulates the association of 14-3-3 ζ (Figure 2A).

Raf-1 peptide mimics the mode I recognition site surrounding Ser259 of Raf-1 kinase, while the shorter Raf-1 ^{Δ PN} peptide, which reproduces the first 13 amino acids of Raf-1 peptide, has the pSer as penultimate amino acid and therefore represents a mode III binding sequence. As shown in Figure 2A, 14-3-3 binding to Raf-1 peptide is unaffected by 10 μ M FC. This result is in agreement with structural data showing that the amphipathic groove of 14-3-3 is totally occupied by mode I peptide, thus not allowing FC binding [11]. 14-3-3 binding to Raf-1 peptide is instead significantly inhibited at higher FC concentrations (100 μ M). It is known that FC can directly bind 14-3-3 proteins even though at low affinity ($K_D = 66 \mu$ M) [11]. At high FC concentrations, the 14-3-3 binding groove involved in peptide binding is occupied by the toxin and therefore peptide association is hampered.

Raf-1 ^{Δ PN} peptide does not bind 14-3-3 ζ but FC addition can significantly promote the interaction. This result reveals that the mode III Raf-1 ^{Δ PN} peptide allows FC to accommodate into the 14-3-3 binding groove (Figure 2B), thus leading to the formation of a ternary complex. Hence, this result demonstrates that FC action can be more generally extended to 14-3-3 targets with a C-terminal recognition motif.

GPIb-IX-V is formed by four different transmembrane proteins: GPIb, which consists of disulfide-linked GPIb α and GPIb β , GPIX and GPV. In this complex, three 14-3-3 binding sites have been proposed: the mode III motif -SGHpS⁶⁰⁹L-COOH, located at the C terminus of GPIb α , and the internal motifs -LRGpS⁵⁵⁷LPT and -ARLpS¹⁶⁶LTDP, located on the cytoplasmic region of GPIb α and GPIb β , respectively [16].

To ascertain whether the interaction between 14-3-3 proteins and the mode III binding motif of GPIb α can be stabilized by FC similarly to the interaction with the H⁺-ATPase, an *in vitro* assay using a synthetic phosphopeptide reproducing the 14-3-3 binding sequence of GPIb α was carried out. Interestingly, 14-3-3 binding to GPIb α ^{term} phosphopeptide is strongly stimulated by both 10 and 100 μ M FC (Figure 2A). In this interaction a $K_D = 1.33 \pm 0.07 \mu$ M for 14-3-3 in the presence of 10 μ M FC and a $K_D = 13.61 \pm 0.33 \mu$ M in the absence of the toxin was calculated from homologous

competitive binding curves (data not shown). Remarkably, FC produces a ten-fold decrease of K_D , according to the effect observed in the H^+ -ATPase/14-3-3 interaction [3,11].

FC effect on 14-3-3 interaction was studied also with peptides reproducing the internal motifs of GPIb α and GPIb β . Surprisingly, GPIb α^{int} peptide does not bind 14-3-3 ζ both in the absence and in the presence of FC. On the other hand, the effect of FC on GPIb β peptide resembles that obtained with mode I Raf-1 peptide: GPIb β peptide binds 14-3-3 ζ and the interaction is significantly hampered by 100 μ M FC.

The effect of FC on 14-3-3 interaction with GPIb α^{term} and GPIb β phosphopeptides was further investigated using different FC concentrations, ranging from 0.1 to 150 μ M. As shown in Figure 2C, 14-3-3 association to GPIb α^{term} is stimulated by FC in a dose-dependent manner. The EC_{50} is 20 μ M, and 90% of maximal activation (EC_{90}) is reached at around 70 μ M. Conversely, FC-mediated inhibition of 14-3-3 interaction with the mode I GPIb β peptide is statistically significant from FC concentrations higher than 50 μ M ($P < 0.01$ by Student's *t*-test). These data propose the potential ability of FC to affect 14-3-3 binding to GPIb-IX-V when administrated in the micromolar range, thus altering glycoprotein biochemical properties.

FC effect on 14-3-3 interaction with GPIb α and GPIb β

In order to verify whether FC can affect the interaction between GPIb and 14-3-3 ζ , the protein was purified from isolated platelets by immunoprecipitation using a polyclonal antibody anti-GPIb α and the interaction was tested by overlay assay. As shown in Figure 3A, upper panels, 14-3-3 ζ recognizes the bands corresponding to GPIb α (reducing conditions, lane R.) and GPIb complex (non-reducing conditions, lane N.R.). Interestingly, FC greatly increases 14-3-3 association to the proteins, with a more pronounced effect observed with GPIb α subunit.

The interaction of 14-3-3 with the β subunit of GPIb was also studied. As shown in Figure 3B, upper panels, 14-3-3 ζ associates to a band corresponding to GPIb β which dissociates from the α subunit in reducing conditions. Notably, a reduced interaction was observed in the presence of the toxin. The opposed effects of FC on 14-3-3 ζ interaction with GPIb α and GPIb β in the overlay assay resemble the effects of the toxin on 14-3-3 binding to GPIb α^{term} and GPIb β phosphopeptides (Figure 2A) and therefore are very likely ascribable to the presence of a 14-3-3 mode III motif in GPIb α and a mode I-like internal motif in GPIb β .

These data, summarized in Figure 3C, propose the potential ability of FC to bind platelet *in vivo* and consequently to affect the physiological process where GPIb-IX-V takes part. This hypothesis require FC ability to cross the platelet plasma membrane. As shown in Figure 3D, [3 H]FC associates to isolated platelet. Binding specificity was demonstrated by observed competition with increasing cold FC concentrations (open triangle), but not with the inactive aglycone of FC (open square).

Similarly, [3 H]FC binding is not reversed by BSA-FC (filled circle), a membrane-impermeable macromolecular derivative of FC, which is instead able to stimulate 14-3-3-binding to the GPIb α^{term} peptide similarly to the toxin (data not shown).

This finding reveals the ability of FC to gain access to the platelet cytosol and thus to associate to intracellular binding sites. The K_D , as estimated by homologous competition curve fitting (data not shown), is 16.1 ± 0.59 μ M. This value is fully consistent with FC activity on 14-3-3/GPIb peptides binding assay, thus further suggesting FC capability to affect GPIb-IX-V-mediated functional processes. Maximal FC binding capacity (B_{max}) is $2.0 \pm 0.2 \times 10^5$ sites/platelet. This value is four times higher than the presumed number of GPIb α molecules/platelet, which is around 50000 [14], thus suggesting that also other platelet proteins are responsible for FC binding. Among these, 14-3-3 proteins can significantly contribute to total FC binding, since it is known their ability to directly bind FC [11].

FC stimulates platelet adhesion to vWF

Under high shear rate flow conditions, such as in arteries and capillaries, platelet adhesion is mediated by the interaction between subendothelial vWF and its receptor GPIb-IX-V [14]. Platelet binding activates intracellular signalling events leading to activation of the ligand binding function of the integrin $\alpha_{IIb}\beta_3$ and the consequent platelet spreading and aggregation. Since 14-3-3 ζ association to GPIb α subunit is essential for the vWF binding activity of GPIb-IX-V, it is conceivable that FC-induced stabilization of 14-3-3 binding could promote platelet adhesion to vWF.

FC effect on adhesion was therefore studied by measuring purified platelet binding to different concentrations of immobilized vWF. As reported in Figure 4A, 40 μ M FC strongly stimulates platelet adhesion to vWF both when resting (-ADP) or activated (+ADP) platelets were used. The observed FC stimulatory effect even in maximal activation condition (+ADP) indicates toxin ability to increase the number of platelets bound to vWF via GPIb α , resulting in platelet spreading and integrin activation. Moreover, FC stimulatory effect on adhesion is considerably higher when lower concentrations of vWF were used (Figure 4B). Thus, this finding provides a quantitative correlation between the FC effect and the functional response mediated by the GPIb-IX-V receptor.

In order to further confirm the involvement of GPIb-IX-V in the FC-mediated stimulation of adhesion, the effect of a polyclonal antibody, recognizing an extracellular epitope of GPIb α , was tested. As shown in Figure 4C, the antibody partially inhibits adhesion of resting platelets. Instead, adhesion of ADP-activated platelets, which is mainly mediated by binding of integrin $\alpha_{IIb}\beta_3$ to vWF [27,28], is unaffected. Interestingly, anti-GPIb α antibody partly hampers FC-mediated platelet adhesion to vWF both when resting or activated platelets were used, thus demonstrating that FC effect involves stimulation of GPIb α binding to vWF.

FC induces platelet aggregation

The effect of different FC concentrations on platelet aggregation was tested in PRP by using an optical aggregometer. As shown in Figure 5A, FC triggers platelet aggregation. The effect is detectable at 10 μ M and reaches its maximum at 40 μ M FC. The slight and dose-dependent aggregation induced by FC does not resemble the well-known effect induced by ristocetin, which causes massive platelet agglutination [29]. RGDS peptide, which inhibits aggregation blocking fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ [30], completely abolishes FC-induced aggregation (Figure 5B), thus demonstrating the involvement of integrin $\alpha_{IIb}\beta_3$ activation in this process. The full inhibitory effect of RGDS peptide also indicates that in PRP the toxin does not promote a detectable platelet agglutination resulting from the interaction of GPIb-IX-V and blood plasma vWF.

The involvement of 14-3-3 proteins in FC-induced platelet aggregation was demonstrated by testing the myristoylated phosphopeptide MP α C, which reproduces the 14-3-3 binding site of GPIb α . It is well ascertained that this peptide is able to cross the platelet membrane and to inhibit platelet aggregation by competition with GPIb-IX-V for 14-3-3 binding [16]. As shown in Figure 5C, MP α C peptide completely abolishes the aggregation induced by the toxin, demonstrating that 14-3-3 proteins are critical in the FC-promoted process. Following addition of 1.0 mg/ml ristocetin induces a faster aggregation in FC-activated platelets, thus revealing an additive effect of FC and ristocetin. As already shown by Dai *et al.* [16], ristocetin effect is strongly hampered in platelets treated with 0.1 mM MP α C peptide. Notably, FC effect in MP α C-treated platelets is completely inhibited also after ristocetin addition, confirming the strict requirement of 14-3-3 proteins for the effect of the toxin on platelet aggregation.

The effect on platelet aggregation of simultaneous addition of FC and ristocetin was also tested. As shown in Figure 5D, no effect of FC on ristocetin-induced aggregation is detectable in the early phase of aggregation, while only a slight effect can be measured after around 3 min, confirming that the aggregation process promoted by the toxin is slower than that induced by ristocetin.

DISCUSSION

The discovery of small molecules capable to selectively modulate 14-3-3 binding with client protein represents a promising strategy to control physiological processes where 14-3-3 proteins play a role. In this study we show that FC, a cell-permeable fungal toxin whose activity has been so far deeply investigated only in plants, can also affect an essential physiological process in humans. We demonstrated that platelets possess specific intracellular FC binding sites, and we identified the platelet adhesion receptor GPIb-IX-V as FC target. The toxin stabilizes 14-3-3 interaction to Iba subunit, while association to Ib β subunit is hampered. Results obtained suggest also the rationale for the FC effect. In fact, the presence of a C-terminal 14-3-3 binding motif in GPIb α allows FC insertion in the 14-3-3 binding groove. As a result, the toxin stabilizes the association of GPIb α to 14-3-3 similarly to the H⁺-ATPase/14-3-3 interaction (Figure 2B). The FC binding pocket is instead occupied in the 14-3-3/GPIb β complex, and consequently the toxin cannot bind to the complex. Since FC can form a low-affinity binary complex with 14-3-3, high FC concentration can inhibit 14-3-3 binding to GPIb β by competition.

It is well ascertained that 14-3-3 binding to the Iba subunit and dissociation from the Ib β subunit contribute to activate vWF binding functions of GPIb-IX-V [16]. Accordingly, we found that FC is able to stimulate GPIb α -dependent platelet association to vWF and to promote platelet aggregation via integrin $\alpha_{IIb}\beta_3$ activation. FC effect on aggregation is partial and dose-dependent, thus suggesting toxin ability to partly activate an intracellular pathway. We also found that the MP α C peptide, a cell-permeable competitor of 14-3-3 intracellular targets, completely abolishes FC-induced aggregation. Despite we cannot rule out the possibility that FC effect on aggregation involves other 14-3-3 client proteins, our data strongly indicate that GPIb-IX-V complex is a fundamental target of FC action.

The elucidation of the molecular basis of FC-induced platelet aggregation opens new opportunities for a therapeutic use of the toxin. Possible pharmaceutical applications are genetic bleeding disorders as Bernard-Soulier syndrome and Glanzmann's thrombasthenia, characterized by qualitative or quantitative abnormalities of the platelet membrane proteins GPIb-IX-V and integrin $\alpha_{IIb}\beta_3$, respectively [31]. Moreover, since the interaction between vWF and GPIb-IX-V is of pivotal importance in the initial adhesion of platelets to the subendothelium under high shear conditions, such as those found in atherosclerotic vessels, inhibition of GPIb-IX-V activity is one of the major targets of anti-thrombotic drug research [32]. Our study sets the stage for future work to design FC-derived molecules which could specifically reduce aggregation by inhibiting 14-3-3 binding to GPIb α .

Moreover, a number of 14-3-3 clients with mode III binding motifs have been identified in mammals [10]. Our data suggest that 14-3-3 association with these targets could be stabilized by FC similarly to 14-3-3/ GPIb α interaction. Future work will address the structural pre-requisites for FC action and the potential pharmacological application of FC as a drug directed to 14-3-3/target complexes.

ABBREVIATIONS FOOTNOTE

Abbreviations used: FC, fusicoccin; GPIb-IX-V, glycoprotein Ib-IX-V; GPIb α , glycoprotein Iba; GPIb β , glycoprotein Ib β ; vWF, von Willebrand Factor; PRP, Platelet-Rich Plasma; PPP, Platelet-Poor Plasma.

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FIGURE LEGENDS

Figure 1. Chemical structure of the fungal toxin fusicoccin.

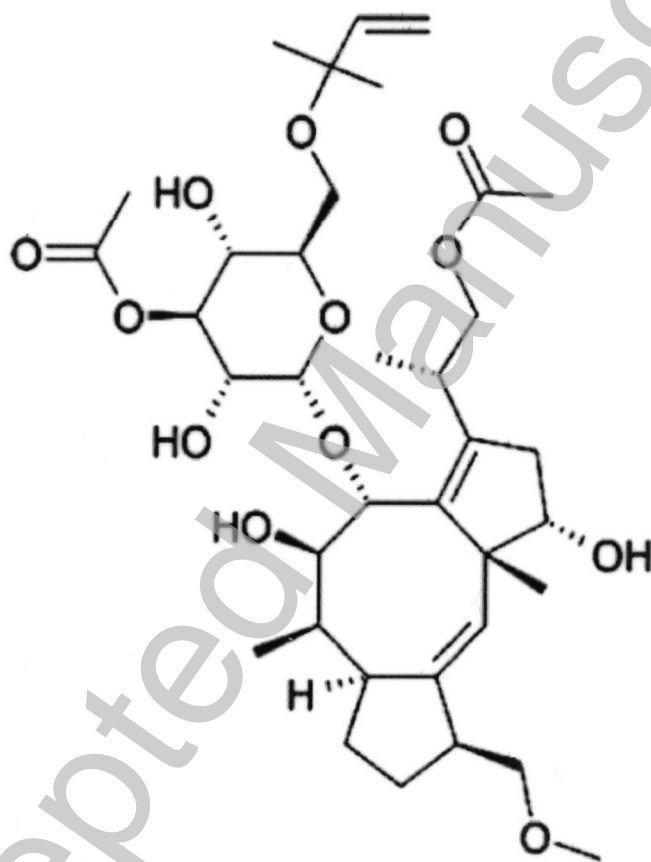
Figure 2. FC differentially regulates binding of 14-3-3 proteins to mode I and III peptides. (A) 14-3-3 ζ binding assay to phosphopeptides reproducing the binding sites of H⁺-ATPase, Raf-1, GPIb α , and GPIb β . Raf-1 ^{Δ PN} peptide replicates Raf-1 peptide, lacking the last two amino acids. GPIb α ^{term} reproduces the C-terminal binding sequence (D596-L610), GPIb α ^{int} reproduces the putative binding sequence located between residues V156-T170. Immobilized peptides were incubated with ³²P-14-3-3 ζ in the absence (white bars) or in the presence of 10 μ M (grey bars) or 100 μ M (black bars) FC. After washing, resin-associated radioactivity was measured. **P* < 0.01 by Student's *t*-test (*n* = 6). The experiment was carried out also using the corresponding unphosphorylated peptides and no binding to 14-3-3 was detected (data not shown). (B) 3-D models, based on existing crystal structure of 14-3-3 complexes, of the mode I and mode III binding motifs. (C) Effect of different FC concentrations on the association of GPIb α ^{term} and GPIb β phosphopeptides with 14-3-3 ζ . □, GPIb α ^{term} peptide; ●, GPIb β peptide. Error bars are s.e.m. of six independent experiments.

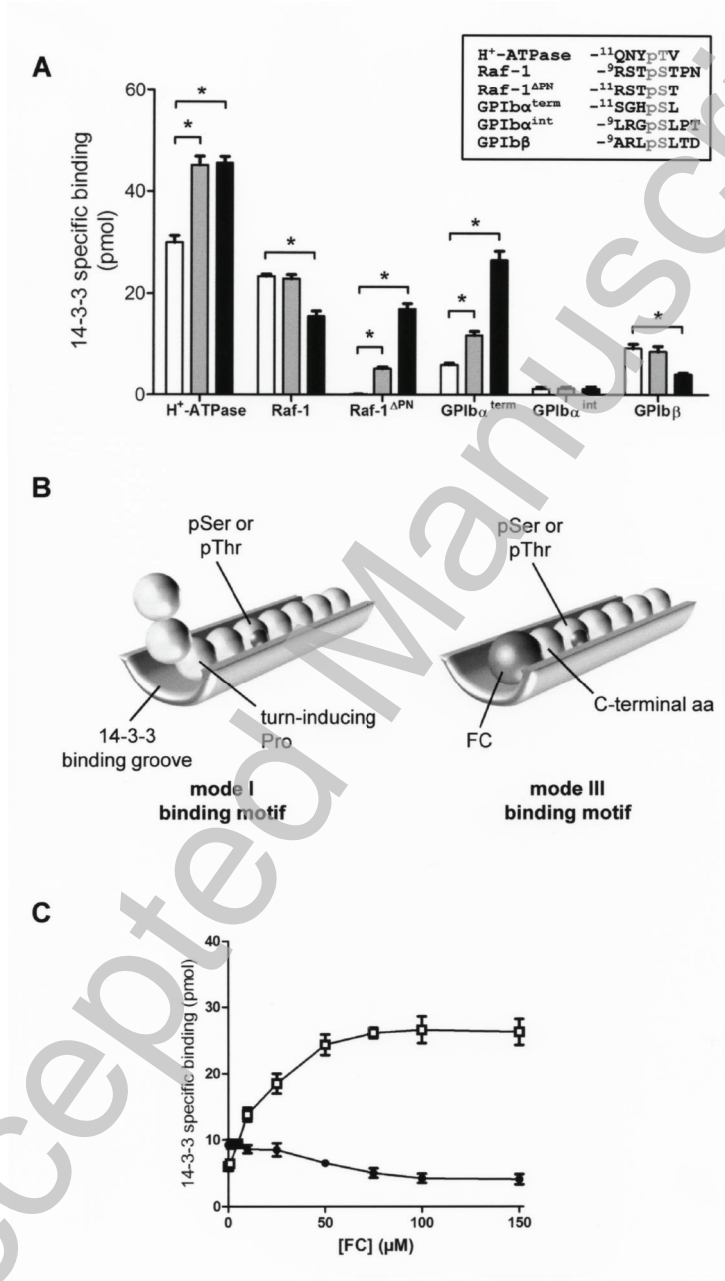
Figure 3. FC effect on the interaction between 14-3-3 ζ and GPIb. GPIb was immunopurified from platelet protein extract using an anti-GPIb α polyclonal antibody and subjected to SDS-PAGE (lanes anti-GPIb α) on 7% (panel A) or 12% (panel B) gels. After transfer onto a nitrocellulose membrane, the protein was incubated with 0.3 μ M ³²P-14-3-3 ζ in the absence (-FC) or in the presence of 40 μ M FC (+FC). The bands corresponding to GPIb α (lane R., reducing conditions), to GPIb complex (lane N.R., non-reducing conditions) and to GPIb β were identified by western blotting with anti-GPIb α and anti-GPIb β antibody. Lane IgG, negative control with IgG from goat serum; Lane Ctrl, negative control with anti-GPIb α not incubated with platelet protein extract. A typical experiment of six performed is shown. (C) Relative amount of 14-3-3 proteins bound to GPIb α , GPIb complex and GPIb β estimated from overlay assays by densitometric analysis using ImageJ software. Data are expressed as % of the maximum Integrated Densitometric Value (the product of Area and Mean Grey Value).

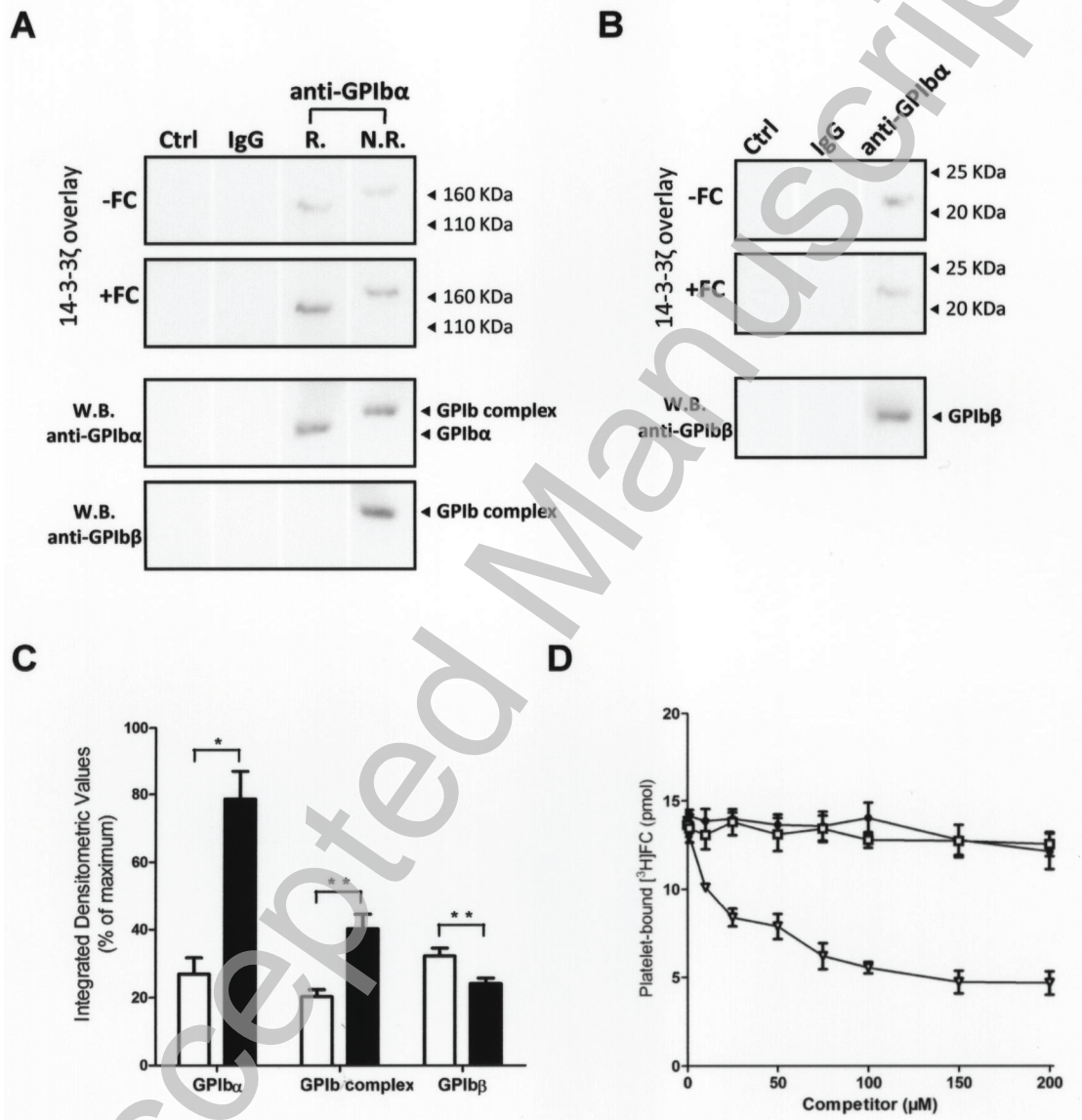
Error bars are s.e.m. of six independent experiments. **P* < 0.01; ***P* < 0.05 by Student's *t*-test. (D) Binding of [³H]FC to isolated human platelets. Platelets were incubated with 0.42 μ M [³H]FC in the presence of increasing concentrations of FC (▽), of the inactive aglycone of FC (□) and of the membrane-impermeable derivative BSA-FC (●). Platelets were sedimented by centrifugation and radioactivity was measured by scintillation counting. Error bars are s.e.m. of three independent experiments.

Figure 4. FC induces platelet adhesion to vWF. (A) Effect of FC on platelet adhesion to immobilized vWF. Purified platelets were incubated onto polystyrene wells coated with different concentrations of purified vWF. After washings, adherent platelets were determined measuring the endogenous acid phosphatase activity. +ADP, incubation in the presence of 50 μ M ADP. Black bars, platelets previously treated with 40 μ M FC; white bars, control platelets. **P* < 0.01 by Student's *t*-test (*n* = 9). (B) Data in Figure 4A are expressed as % of FC stimulation versus vWF coating concentration. ●, -ADP; ▼, +ADP. (C) Effect of anti-GPIb α antibody on platelet adhesion to vWF. 2 μ g/ml of a polyclonal anti-GPIb α recognizing the extracellular portion of the protein were added to resting platelet (Ctrl) or in the presence of 40 μ M FC and 50 μ M ADP. **P* < 0.01, by Student's *t*-test (*n* = 6).

Figure 5. Effect of FC on platelet aggregation. (A) Effect on the aggregation upon the addition (arrow) of various FC concentrations or 1.0 mg/ml ristocetin to PRP. (B) Effect of 40 μ M FC in the presence of 0.75 mM RGDS peptide. (C) Effect of 40 μ M FC in the presence of 0.1 mM MP α C peptide. Where indicated, 1.0 mg/ml ristocetin was added to each sample. (D) Effect of simultaneous administration of 40 μ M FC and 1.0 mg/ml ristocetin. Aggregation traces represent a typical experiment of at least three performed.

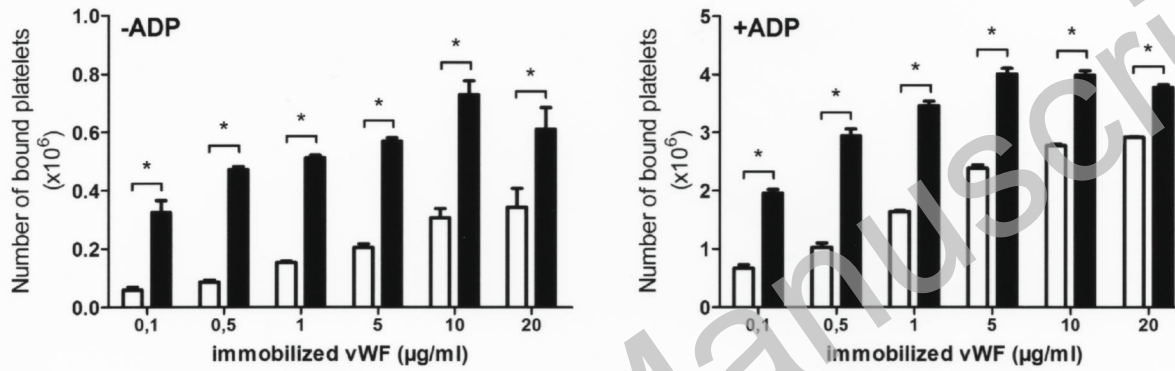




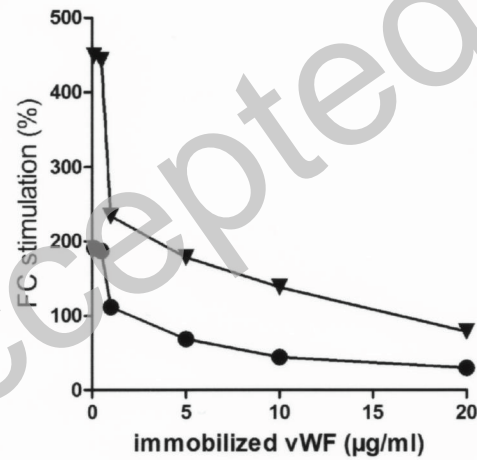


THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20102037

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