Fgr but not Syk tyrosine kinase is a target for β_2 integrin-induced c-Cbl-mediated ubiquitination in adherent human neutrophils

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An early and critical event in β_2 integrin signalling during neutrophil adhesion is activation of Src tyrosine kinases and Syk. In the present study, we report Src kinase-dependent β_2 integrin-induced tyrosine phosphorylation of Cbl occurring in parallel with increased Cbl-associated tyrosine kinase activity. These events concurred with activation of Fgr and, surprisingly, also with dissociation of this Src tyrosine kinase from Cbl. Moreover, the presence of the Src kinase inhibitor PP1 in an *in vitro* assay had only a limited effect on the Cbl-associated kinase activity. These results suggest that an additional active Src-dependent tyrosine kinase associates with Cbl. The following observations imply that Syk is such a kinase: (i) β_2 integrins activated Syk in a Src-dependent manner, (ii) Syk was associated with Cbl much longer than Fgr was, and (iii) the Syk inhibitor piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) abolished the Cbl-associ-

ated kinase activity in an *in vitro* assay. Effects of the mentioned interactions between these two kinases and Cbl may be related to the finding that Cbl is a ubiquitin E3 ligase. Indeed, we detected β_2 integrin-induced ubiquitination of Fgr that, similar to the phosphorylation of Cbl, was abolished in cells pretreated with PP1. However, the ubiquitination of Fgr did not cause any apparent degradation of the protein. In contrast with Fgr, Syk was not modified by the E3 ligase. Thus Cbl appears to be essential in β_2 integrin signalling, first by serving as a matrix for a subsequent agonist-induced signalling interaction between Fgr and Syk, and then by mediating ubiquitination of Fgr which possibly affects its interaction with Cbl.

Key words: adhesion molecule, inflammation, leucocytes.

INTRODUCTION

It is now well established that many cytokine-regulated functions of polymorphonuclear neutrophils (PMNs) are modulated by adhesion of the cells and ligation of β_2 integrins [1]. β_2 Integrins constitute a major group of adhesion molecules expressed on PMNs. These heterodimeric transmembrane receptors are designated CD11/CD18, because they are composed of a common β -chain, CD18, and one of four unique α chains, CD11 (a–d). CD11b/CD18 is the prominent β_2 integrin expressed on the surface of PMNs [2]. In human PMNs, one of the initial events in β_2 integrin-mediated signalling is activation of Src tyrosine kinases [p58^{c-fgr} (Fgr), p 59/61^{hck} (Hck) and p53/56^{lyn} (Lyn)] as well as the non-Src tyrosine kinase p72^{syk} (Syk) [3–6]. There is genetic evidence that the highly homologous Src tyrosine kinases Fgr, Hck and Lyn are, to a large extent, redundant [7] and necessary for β_2 integrin-dependent modulation of various PMNs functions. This is exemplified by experiments showing that adhesion-induced activation of the respiratory burst and degranulation were impaired in PMNs from *fgr*^{−/−} *hck*^{−/−} mice, because these cells displayed defective adhesion and spreading on various types of biological surface [8]. These deficiencies have also been observed *in vivo* as reduced inflammation-dependent tissue injury [9]. Recently, Mócsai et al. [10] reported that the non-receptor tyrosine kinase Syk, activation of which often depends on a Src kinase signal, is also an essential component of β_2 integrin signalling in PMNs. More specifically, these workers [10] demonstrated that ligation of β_2 integrins on PMNs from *syk*^{−/−} mice did not induce spreading, degranulation and activation of the respiratory burst in these cells.

Src tyrosine kinase and Syk activities are repressed in most resting cells, including human PMNs. This can be readily ascribed

to interaction of the Src homology (SH)2 domain of Src with phosphorylated Tyr⁵²⁷ in the C-terminal part of the molecule and/or to binding of the Src SH3 domain to an intramolecularly formed type II polyproline helix formed by the linker between the SH2 domain and the kinase domain. It is assumed that dephosphorylation of Tyr⁵²⁷ and/or binding of other molecules to the SH2 or SH3 domains of Src kinases results in exposure and phosphorylation of Tyr⁴¹⁶ in the catalytic site and, thereby, activates these tyrosine kinases [11]. Such a mechanism would also explain the suppressed activity of Syk in resting cells, since Syk has been reported to be stimulated by activation of Src tyrosine kinases [10,12]. Little is known about the mechanisms by which β_2 integrins regulate Src-like tyrosine kinases and Syk in PMNs. A plausible regulatory mechanism in this context is that these tyrosine kinases interact with specific adapter/docking proteins (e.g. c-Cbl) in PMNs.

The proto-oncogene product c-Cbl is a multi-domain protein that is expressed in haematopoietic and other types of cells and is now known to be a key regulator of various intracellular signalling molecules and pathways [13]. It has been shown [13] that the N-terminal portion of c-Cbl [the tyrosine kinase-binding domain ('TKB')] can interact directly with a number of tyrosine kinases, including Syk/ZAP-70 and Src family members. In addition, c-Cbl can recruit proteins that contain an SH3 domain via its proline-rich domain and/or it can recruit proteins comprising an SH2 domain through its ability to be phosphorylated on tyrosine residues under the influence of a large number of receptors [13]. It appears that c-Cbl plays a complex functional role, in particular with regard to its involvement in regulation of non-receptor tyrosine kinases. On one hand, it has been demonstrated that binding of the SH3 domain of Src to the proline-rich domain of c-Cbl causes partial activation of Src, resulting in

Abbreviations used: mAb, monoclonal antibody; PBST, PBS containing 0.2% Tween 20; piceatannol, 3,4,3',5'-tetrahydroxy-*trans*-stilbene; PMN, polymorphonuclear neutrophil; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; SH, Src homology; TNF- α , tumour necrosis factor- α .

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tyrosine phosphorylation of c-Cbl [14] and recruitment of proteins containing an SH2 domain such as phosphoinositide 3-kinase ('PI 3-kinase') and Vav [13]. On the other hand, binding of Tyr⁴¹⁶ of Src kinase to the tyrosine-kinase-binding domain of c-Cbl down-regulates the activity of the kinase [14]. Furthermore, it has been established that c-Cbl can bring about such negative regulation by promoting intracellular ubiquitination of various activated non-receptor tyrosine kinases, such as c-Src [15] and Syk [16], as well as the platelet-derived growth factor ('PDGF') [17] and epidermal growth factor ('EGF') [18] receptor tyrosine kinases. The basis of this type of a regulatory mechanism is the ability of the RING domain of c-Cbl to bind ubiquitin-conjugating enzymes (E2s) and the fact that c-Cbl is a E3 ubiquitin ligase [13,19]. Ubiquitination often triggers rapid degradation of tyrosine kinases by a proteasomal or lysosomal pathway [20]. Thus ubiquitination of signalling molecules can terminate a receptor-induced signal and also prevent excessive intracellular signalling. However, additional functions for ubiquitin have been discovered. For instance, monoubiquitin can regulate the recycling of receptor tyrosine kinases, transcriptional activities and cellular localization of proteins [20,21].

In the present study, we carried out experiments to elucidate further the signalling mechanism(s) of β_2 integrins in regulation of PMN adhesion and motility. We focused our work on a potential role for c-Cbl in governing the activities of Fgr and Syk during the initial signalling of β_2 integrins in these cells.

EXPERIMENTAL

Antibodies

The antibodies and their sources were as follows: monoclonal antibody (mAb) IB4 [mouse anti-(human CD18), IgG_{2a} isotype] was obtained from Dr S. Wright (Department of Microbiology, Rockefeller University, NY, U.S.A.) [22]; mAb 4G10 (mouse anti-phosphotyrosine) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.); and anti-Syk and anti-ubiquitin mAbs and anti-c-Cbl and anti-Fgr polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-c-Cbl and anti-Fgr mAbs were from Transduction Laboratories (Lexington, KY, U.S.A.) and Wako Bioproducts (Richmond, VA, U.S.A.) respectively. Horseradish peroxidase-conjugated immunoglobulins were from Dakopatts (Glostrup, Denmark).

Chemicals

Protein A-Sepharose, dextran and Ficoll-Hypaque were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The protease inhibitors pefabloc, pepstatin, leupeptin, aprotinin and antipain were from Boehringer-Mannheim (Mannheim, Germany). Benzamidin and the Syk inhibitor piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) [23] were from Sigma-Aldrich (Stockholm, Sweden). PP1 {4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine}, the selective inhibitor of Src family members [24], was from Alexis Biochemicals (Läufeligen, Switzerland). All electrophoresis reagents were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). [γ -³²P]ATP was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich.

Isolation of human PMNs

Blood from healthy donors was collected and isolated under endotoxin-free conditions as described previously [25]. Briefly, the blood was subjected to dextran sedimentation, followed by a brief hypotonic lysis of erythrocytes. The lysis was stopped by

adding 3 ml of buffer containing 565 mM NaCl/2.7 mM KCl/6.7 mM Na₂HPO₄ · 2H₂O/1.5 mM KH₂PO₄ (pH 7.3) and 3 ml of Ringer's modified phosphate buffer [120 mM NaCl/4.9 mM KCl/1.7 mM KH₂PO₄/1.2 mM MgSO₄ · 7 H₂O/8.3 mM Na₂HPO₄ · 2H₂O/10 mM glucose (pH 7.3)]. The cell suspension was centrifuged on Ficoll-Hypaque (15 ml) and washed twice with Ringer's modified phosphate buffer. Finally, the cells were resuspended in calcium-containing medium [136 mM NaCl/4.7 mM KCl/1.2 mM KH₂PO₄/1.2 mM MgSO₄/5.0 mM NaHCO₃/1.0 mM CaCl₂/5.5 mM glucose/20 mM Hepes (pH 7.4)]. The cell suspension consisted of approx. 97% PMNs.

Engagement of β_2 integrins

The β_2 integrins were ligated by allowing the cells adhere either to fibrinogen-coated surfaces in the presence of tumour necrosis- α (TNF- α), which selectively activates the β_2 integrins [3-4], or to plates coated with the anti-(β_2 integrin) antibody (anti-CD18) [3,26]. The subsequent incubations were performed at 37 °C for the indicated periods of time. Resting suspended cells were taken as control cells (time 0). The reactions were terminated by placing the plates on ice, after which the cells were scraped off and lysed in buffer containing 100 mM Tris/HCl (pH 7.5)/1% (v/v) Nonidet P40/5 mM EDTA/5 mM EGTA/50 mM NaCl/5 mM NaF/1 mM Na₃VO₄/protease inhibitors (20 μ g/ml aprotinin, 2.5 mM benzamidin, 2 mM pefabloc and 1 μ g/ml each of pepstatin, leupeptin and antipain).

Immunoprecipitation and Western blotting

Cell lysates were clarified by centrifugation (15000 *g*, 10 min), and proteins in the supernatants were immunoprecipitated by adding the appropriate antibodies (2-3 μ g) for 2 h and Protein A-Sepharose [40 μ l of a 50% (w/v) slurry] for 1 h. The beads were subsequently collected by centrifugation, washed three times in 50 mM Hepes (pH 7.4)/1% (v/v) Nonidet P40/150 mM NaCl/1 mM Na₃VO₄, re-suspended in 2 × Laemmli sample buffer and then boiled under reducing conditions for 5 min. The immunoprecipitated proteins were subjected to SDS/PAGE [8% (w/v) gels] and transferred on to polyscreen PVDF transfer membranes. The membranes were blocked with PBS containing 0.2% Tween 20 (PBST) and 3% (w/v) BSA and were incubated for 1 h with a primary antibody (0.5 μ g/ml) as indicated in the Figure legends. Thereafter, the membranes were washed three times (15 min each) in PBST and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse immunoglobulins (1:10000) in PBST and 3% (w/v) BSA. After this treatment, the blots were washed and antibody binding was visualized by enhanced chemiluminescence.

Protein kinase assays of anti-c-Cbl immunoprecipitates

Kinase assays were performed *in vitro* essentially as reported previously [27]. Briefly, c-Cbl was immunoprecipitated from Nonidet P40 lysates of PMNs as described in the subsection above. The immunoprecipitated proteins were incubated at 25 °C for 30 min in kinase assay buffer [20 mM Hepes (pH 7.0)/0.5% Triton X-100/100 mM MnCl₂/10 mM MgCl₂/1 mM dithiothreitol/0.1 mM Na₃VO₄] containing 0.25 μ Ci [γ -³²P]ATP in the absence or presence of PP1 (3 μ M) or piceatannol (10 μ M) and then put on ice to terminate the reactions. The precipitates were washed and then boiled in 2 × Laemmli sample buffer containing 50 mM dithiothreitol, subjected to SDS/PAGE [8% (w/v) gels] and subsequently transferred on to PVDF membranes. Phosphorylated proteins were detected using a Phosphor-Imager

system (Bio-Rad Laboratories) and by blotting the PVDF membranes with an anti-phosphotyrosine antibody as described above. Alternatively, the kinase-assay gels or PVDF membranes were treated with 1 M KOH at 55 °C for 1 h to ensure that phosphorylation of c-Cbl on serine and/or threonine residues [27] did not occur.

RESULTS

Engagement of β_2 integrins induces Src-dependent tyrosine phosphorylation of c-Cbl

To engage β_2 integrins on PMNs, the cells were allowed to adhere to a surface coated with fibrinogen, a ligand for β_2 integrins in the presence of TNF- α [28]. The c-Cbl proteins were immunoprecipitated from PMN lysates and the tyrosine phosphorylation status of this adapter protein was investigated by Western blotting with an anti-phosphotyrosine antibody. We found a low basal level of tyrosine-phosphorylated c-Cbl in resting suspended cells (Figure 1), which increased in a time-dependent manner in PMNs plated on a surface coated with fibrinogen in the presence of TNF- α (Figure 1A, upper panel). Maximal tyrosine phosphorylation of c-Cbl occurred 20–30 min after adding PMNs to the coated surface. To confirm further that tyrosine phosphorylation of c-Cbl was due to engagement of β_2 integrins, we performed experiments in which the cells were plated on to a surface coated with anti-CD18 antibodies (Figure 1B, upper panel). A similar increase in the phosphoryl-

Figure 1 β_2 integrins induce tyrosine phosphorylation of c-Cbl

PMNs (10^7) pretreated in the absence (–) or presence (+) of PP1 (3 μ M) were plated for different periods of time on fibrinogen in the presence of TNF- α (20 ng/ml) (**A**) or on a surface coated with anti-CD18 antibodies (**B**). PMNs were lysed, and c-Cbl was immunoprecipitated (IP) from clarified lysates with an anti-c-Cbl polyclonal antibody as described in the Experimental section. The immunoprecipitated proteins were separated by SDS/PAGE [8% (w/v) gels] and analysed by immunoblotting (Blot) with an anti-phosphotyrosine antibody (PY; upper panels). The same blots were stripped and reprobed with an anti-c-Cbl antibody to confirm that equal amounts of c-Cbl had been immunoprecipitated at each time point (lower panels). Arrows indicate the position of c-Cbl. The blots shown are representative of eight (**A**) or three (**B**) separate experiments.

Figure 2 Adhesion increases tyrosine kinase activity in anti-c-Cbl immunoprecipitates

PMNs were plated on to fibrinogen in the presence of TNF- α (20 ng/ml) for the indicated time periods without inhibitor (**A**), or for 20 min in the absence (–) or presence (+; lane 3) of PP1 (3 μ M) (**B**). PMNs were lysed, and c-Cbl was immunoprecipitated (IP) from clarified lysates with an anti-c-Cbl polyclonal antibody. The immunoprecipitated proteins were resuspended in a kinase assay buffer containing [γ - ^{32} P]ATP as described in the Experimental section. The *in vitro* assay was incubated for 30 min, after which the anti-c-Cbl immunoprecipitates were washed. (**B**, lane 4) PP1 (3 μ M) was present only during this *in vitro* assay. The precipitated proteins were separated by SDS/PAGE [8% (w/v) gels], transferred on to PVDF membranes and subjected to Phosphor-Imager analysis. (**A**, right-hand panel) As a control for the left-hand panel in (**A**), the anti-c-Cbl immunoprecipitates were treated with SDS and boiled to dissociate c-Cbl bound to antibodies. c-Cbl was subsequently re-immunoprecipitated (Re-IP) with the anti-c-Cbl antibody and precipitates were analysed by autoradiography. (**B**, right-hand panel) PVDF membrane that had been subjected to Phosphor-Imager analysis was subjected to Western blotting (Blot) with an anti-phosphotyrosine antibody (PY). (**C**) PMNs were treated as in (**B**), after which Fgr was immunoprecipitated and the *in vitro* kinase assay was performed as in (**B**) in the absence (–) or presence (+) of PP1 (3 μ M) in the assay. The positions of c-Cbl and Fgr are indicated by arrows. The autoradiographs and the blots shown are representative of six (**A**), three (**B**) and two (**C**) separate experiments.

Figure 3 c-Cbl-associated kinase activity is impaired by the Syk inhibitor piceatannol

PMNs (10^7) were plated on to fibrinogen in the presence of $\text{TNF-}\alpha$ (20 ng/ml) for 20 min. PMNs were lysed, and c-Cbl (left-hand panel) or Syk (right-hand panel) were immunoprecipitated (IP) from clarified lysates. The immunoprecipitated proteins were then resuspended in kinase assay buffer containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Experimental section. The *in vitro* assay was performed for 30 min in the absence (—) or presence (+) of the Syk inhibitor piceatannol (10 μM), after which the anti-c-Cbl or anti-Syk immunoprecipitates were washed. The precipitated proteins were separated by SDS/PAGE [8% (w/v) gels], transferred on to PVDF membranes and subjected to Phosphor-Imager analysis. The positions of c-Cbl and Syk are indicated by arrows. The effects of piceatannol have been reproduced in five separate experiments.

ation of c-Cbl was observed upon antibody-induced β_2 integrin engagement on PMNs. In both types of experiments, equal loading of the lanes was verified by reprobing the blots with an anti-c-Cbl antibody (Figures 1A and 1B, lower panels). Studies using other cell models [29–31] have shown that c-Cbl is a substrate for Src tyrosine kinases directly as well as indirectly, via activation of Syk. Therefore we investigated the effect of PP1, a selective inhibitor of Src-family tyrosine kinases [24], on tyrosine phosphorylation of c-Cbl induced by engagement of β_2 integrins in PMNs. We found that pretreatment with this agent completely abrogated the c-Cbl phosphorylation (Figure 1).

Ligation of β_2 integrins increases tyrosine kinase activity in anti-c-Cbl immunoprecipitates

Other investigators [27,29,32] have shown that c-Cbl-bound tyrosine kinase activity is necessary for phosphorylation of c-Cbl in T-cells and macrophages. Accordingly, we performed experiments to ascertain whether engagement of β_2 integrins on PMNs leads to increased kinase activity in anti-c-Cbl immunoprecipitates. PMNs were allowed to adhere to fibrinogen, and c-Cbl was immunoprecipitated from cells lysates. The anti-c-Cbl immunoprecipitates were incubated in a kinase assay buffer containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, after which phosphorylation of c-Cbl and possibly c-Cbl-associated proteins was analysed. These *in vitro* assays revealed a transient β_2 integrin-induced rise in phosphorylation of a distinct band at 120 kDa, and this increase peaked at 20 min and had nearly disappeared after 30 min (Figure 2A, left-hand panel). To confirm that this 120 kDa protein was c-Cbl, samples of resting cells and cells with ligated β_2 integrins were dissociated from the anti-c-Cbl antibodies after the *in vitro* assay and then re-precipitated with the same antibody and the radioactivity was determined. These experiments verified that the 120 kDa protein phosphorylated in the *in vitro* assay was c-Cbl (Figure 2A, right-hand panel). To determine whether the overall kinase activity recovered in anti-c-Cbl immunoprecipitates

Figure 4 β_2 integrins induce tyrosine phosphorylation of Fgr and Syk and cause these proteins to dissociate from c-Cbl

PMNs (10^7) were plated for different periods of time on fibrinogen-coated surfaces in the presence of $\text{TNF-}\alpha$ (20 ng/ml) without inhibitor (A), or in the absence (—) or presence (+) of PP1 (3 μM) (B and C). The cells were lysed, and Fgr (A and B) or Syk (C) was immunoprecipitated (IP) with an anti-Fgr or anti-Syk antibody respectively. The immunoprecipitated proteins were separated by SDS/PAGE [8% (w/v) gels], transferred on to a PVDF membrane and then immunoblotted (Blot) with an anti-phosphotyrosine (PY) antibody (A–C, top panels). The same blots were subsequently stripped, reprobed with an anti-c-Cbl antibody (A–C, middle panels) and then probed with either an anti-Fgr (A and B, bottom panels) or an anti-Syk (C, bottom panel) antibody. The arrows indicate the positions of Fgr, c-Cbl or Syk. The blots shown are representative of four (A), five (B) and five (C) separate experiments.

corresponded to the activity of serine/threonine or tyrosine kinases, we treated the PVDF membranes (containing the proteins phosphorylated *in vitro*) with KOH, which cleaves phospho-serine and phospho-threonine bonds but not phospho-

Figure 5 β_2 integrins induce ubiquitination of Fgr but not Syk

PMNs (10^7) were incubated for the indicated time periods on surfaces coated with fibrinogen in the presence of TNF- α (20 ng/ml) without inhibitors (**A** and **C**), or in the absence or presence of PP1 (3 μ M) or lactacystin (10 μ M) (**B**). Cells were lysed, and Fgr (**A** and **B**) or Syk (**C**) was immunoprecipitated (IP) with an anti-Fgr or anti-Syk antibody respectively.

tyrosine bonds [27,32]. This alkaline treatment had no effect on the *in vitro* phosphorylation of c-Cbl (results not shown), demonstrating that it was mainly tyrosine kinase activity that was recovered in the anti-c-Cbl immunoprecipitates. This conclusion is supported by our findings that the kinase activity was completely inhibited in c-Cbl immunoprecipitates from PMNs that had been pretreated with PP1 (Figure 2B, left-hand panel, lane 3) and by Western-blot analysis of tyrosine-phosphorylated proteins on the same membrane (Figure 2B, right-hand panel). Although Fgr is associated with c-Cbl in resting PMNs, autophosphorylation of Fgr-bound c-Cbl was not detected. This has also been observed in resting macrophages [27,32], indicating that the activity of Fgr associated with c-Cbl was repressed in resting PMNs. However, in contrast with other investigators studying adherent macrophages [27,32], we did not detect a phospho-protein band in the range of 60 kDa (the location of Src-like tyrosine kinases) that co-precipitated with c-Cbl upon integrin-mediated PMN adhesion. This suggests that it was not a Src tyrosine kinase that gave rise to the tyrosine kinase activity recovered in the anti-c-Cbl immunoprecipitates from PMNs adhering to a surface coated with an agent capable of ligating β_2 integrins. To ensure that a Src-family tyrosine kinase had not contributed to the phosphorylation of c-Cbl *in vitro*, we performed experiments in which PP1 was added solely during the kinase assay conducted *in vitro*. This showed that PP1 had only a limited effect on the tyrosine kinase activity in the anti-c-Cbl immunoprecipitates, as analysed using the Phosphor-Imager (Figure 2B, left-hand panel, lane 4) or by Western blotting with an anti-phosphotyrosine antibody (Figure 2B, right-hand panel, lane 4). To determine whether PP1 inhibited the activity of Src tyrosine kinases in such experiments, we performed control assays *in vitro* using anti-Fgr immunoprecipitates from PMNs adhering under identical conditions. PP1 completely prevented the β_2 integrin-induced autophosphorylation of Fgr *in vitro* (Figure 2C), which supports the finding that PP1 has a substantial impact on this Src tyrosine kinase. By comparison, other investigators have performed this type of assay and found that PP1 blocked the autophosphorylation of Lyn but not Syk [33].

Next, we investigated whether the *in vitro* phosphorylation of c-Cbl was attributed to the Syk tyrosine kinase. To this end, we tested the effect of the Syk inhibitor piceatannol on the kinase activity in the anti-c-Cbl immunoprecipitates. The addition of piceatannol during the *in vitro* kinase assay blunted phosphorylation of c-Cbl and Syk (Figure 3).

Ligation of β_2 integrins decreases levels of Fgr and Syk bound to c-Cbl

In an attempt to elucidate the involvement of Fgr and Syk in regulation of c-Cbl, we conducted experiments to determine whether Fgr and Syk associated with c-Cbl in human PMNs. We immunoprecipitated Fgr and Syk from PMN lysates and then performed Western-blot analysis using an anti-c-Cbl antibody to assess the extent to which c-Cbl was bound to these immunoprecipitates. There was substantial binding of Fgr and Syk to c-Cbl in resting cells (Figure 4). The SH3 domain of Fgr was

The immunoprecipitated proteins were separated by SDS/PAGE [8% (w/v) gels], transferred on to PVDF membranes and immunoblotted (Blot) with an anti-ubiquitin mAb (**A–C**, upper panels). For loading control, the blots were subsequently stripped and reblotted with the anti-Fgr (**A** and **B**, bottom panels) or anti-Syk (**C**, bottom panel) antibody. The positions of Fgr (58 kDa; **A** and **B**), monoubiquitinated Fgr (75 kDa; **A** and **B**) and Syk (72 kDa; **C**) are indicated by arrows. The blots shown are representative of three (**A** and **B**) and nine (**C**) separate experiments.

probably bound to the proline-rich domain of c-Cbl, because abrogation of the basal tyrosine phosphorylation of c-Cbl (Figure 1) did not change the basal amount of Fgr associated with c-Cbl (Figure 4B, lanes 1 and 2). Surprisingly, after β_2 integrin-mediated adhesion, levels of c-Cbl-associated Fgr (Figures 4A and 4B) and Syk (Figure 4C) in the PMNs were decreased in a time-dependent manner. Notably, we found that the Fgr–c-Cbl complex dissociated more rapidly than the Syk–c-Cbl complex. More precisely, after 10 min, most of the Fgr had been released, whereas no dissociation of the Syk–c-Cbl complex was exhibited at all. Interestingly, pretreatment of PMNs with PP1 partially reversed the adhesion-induced dissociation of both the Fgr–c-Cbl (Figure 4B) and Syk–c-Cbl (Figure 4C) complexes.

We also found that β_2 integrin ligation caused a time-dependent increase in phosphorylation/activation of Fgr (Figure 4A, upper panel) and Syk (Figure 4C, upper panel), which confirms results reported previously [3,4]. Nevertheless, we observed a delay in adhesion-induced phosphorylation of Syk compared with Fgr, which supports the concept that activation of Src-like kinases is a prerequisite for activation of Syk. Further evidence for this is the finding that pretreatment of PMNs with PP1 completely blocked the basal as well as the adhesion-induced tyrosine phosphorylation of both Fgr and Syk (Figures 4B and 4C).

Ligation of β_2 integrins induces ubiquitination of Fgr but not Syk

The recent discovery that c-Cbl can function as a ubiquitin E3 ligase for several signalling proteins, including Syk [16] and Src [15], prompted us to investigate whether engagement of β_2 integrins leads to ubiquitination of Fgr and Syk, an issue that has not yet been addressed. We immunoprecipitated Fgr and Syk from PMN lysates, the immunoprecipitates were separated by SDS/PAGE and then immunoblotted with an anti-ubiquitin antibody. This procedure did not reveal a covalent modification of Syk and Fgr in suspended control cells. However, plating PMNs on immobilized fibrinogen in the presence of TNF- α induced a time-dependent ubiquitination of Fgr (Figures 5A and 5B) but not Syk (Figure 5C). This was shown by detection of a 75 kDa protein band, which corresponds to the addition of two molecules of ubiquitin per molecule of Fgr. If ubiquitination is involved in modulating the Fgr signal, then, logically, such modification of Fgr should occur only after activation of this kinase. Indeed, we found that PP1 completely inhibited the adhesion-induced ubiquitination of Fgr (Figure 5B), which is in accordance with *in vitro* results reported by Yokouchi et al. [34]. In addition, pretreatment of PMNs with the proteasome inhibitor lactacystin had no effect on adhesion-induced ubiquitination of Fgr (Figure 5B, upper panel, lane 4).

Ligation of β_2 integrins does not induce degradation of Fgr or Syk

Since a role of c-Cbl-induced ubiquitination is well established in protein degradation, we tested whether the amounts of Fgr and Syk were changed upon β_2 integrin-mediated PMN adhesion. PMNs were plated on to a surface coated with fibrinogen in the presence of TNF- α for up to 6 h, after which Fgr and Syk were immunoprecipitated. The amounts of Fgr and Syk in these immunoprecipitates were determined by Western blotting. No alterations were found in the level of either Fgr or Syk in adherent PMNs (Figure 6). These findings are in accordance with our observation that the cell-permeant proteasome inhibitor lactacystin did not lead to accumulation of ubiquitinated Fgr (Figure 5B).

Figure 6 β_2 integrin-mediated adhesion does not induce degradation of Fgr or Syk

PMNs (10^7) were incubated for the indicated time periods on surfaces coated with fibrinogen in the presence of TNF- α (20 ng/ml). Cells were lysed and Fgr (A) or Syk (B) were immunoprecipitated (IP). The immunoprecipitated proteins were separated by SDS/PAGE [8% (w/v) gels], transferred on to PVDF membranes and immunoblotted (Blot) with the antibodies indicated in the Figure. The positions of Fgr (58 kDa) and Syk (72 kDa) are shown on the left. The blots shown are representative of three separate experiments.

DISCUSSION

The activation of Src-family members and Syk by β_2 integrins in PMNs is well established; however, details on their regulation is limited. In many cell systems, the proto-oncogene product c-Cbl has been shown to play a pivotal role in regulating various non-receptor tyrosine kinases, including Src and Syk [13,15,16,19]. Therefore, in the present study, we investigated the potential involvement of c-Cbl in β_2 integrin signalling in PMNs, an issue that has not been addressed previously. Other investigators have observed that β_2 integrin-mediated cell adhesion causes tyrosine phosphorylation of c-Cbl [35], and our present results show that this occurs via a Src tyrosine kinase-dependent pathway. Although important, these findings do not imply that c-Cbl participates in regulating non-receptor tyrosine kinases in PMNs. However, we detected c-Cbl in Fgr and Syk immunoprecipitates from resting PMNs, which suggests that c-Cbl can serve as a matrix for a subsequent agonist-induced signalling interaction between Fgr and Syk. We found that β_2 integrin-induced activation of Syk was entirely dependent on activation of a Src tyrosine kinase, and that it occurred after activation of Fgr. These results also indicate that Src-like tyrosine kinases can phosphorylate c-Cbl either directly or indirectly via activation of a Src-dependent tyrosine kinase such as Syk.

It is presumed that Src tyrosine kinases must be associated with c-Cbl to be able to phosphorylate this proto-oncogene product. For instance, Feshchenko et al. [29] observed that, even though Lck had higher enzymic activity than Yes in Cos cells, the latter protein had a greater ability to phosphorylate c-Cbl, and the reason for this was that Yes became associated with c-Cbl, whereas Lck did not. Similarly, engagement of β_1 integrins in

mice macrophages has been found to increase the activities of c-Cbl-associated c-Src [32], Fgr and Lyn [27]. Studies of T- and 32Dcl3 cells have shown that T-cell-receptor-CD3 cross-linking or interleukin-3 stimulation respectively, induced increases in the kinase activity of Fyn co-precipitated with c-Cbl [36,37]. In contrast, although the overall c-Cbl-bound tyrosine kinase activity was increased by β_2 integrin-mediated adhesion, we did not detect any phosphorylated Fgr or other phosphorylated Src-family members in association with anti-c-Cbl immunoprecipitates. This was due to the fact that adhesion of these cells led to rapid (within 10 min) and significant dissociation of the Fgr-c-Cbl complex. Our data, indicating that the inhibitor PP1 had only a limited effect on c-Cbl-bound tyrosine kinase activity *in vitro*, further supports the view that Src-like tyrosine kinases do not make a major contribution to the c-Cbl-associated kinase activity that probably leads to phosphorylation of c-Cbl.

Syk is another tyrosine kinase that might phosphorylate c-Cbl. We found that, similar to Fgr, Syk co-immunoprecipitated with c-Cbl from resting PMNs, but, in contrast with Fgr, only part of the Syk was removed from c-Cbl in PMNs undergoing integrin-mediated cell adhesion, and this was not detectable until 20 min after the onset of adhesion. Moreover, the Syk inhibitor piceatannol abolished the kinase activity associated with c-Cbl, suggesting further that Syk is the main tyrosine kinase implicated in the *in vitro* phosphorylation of c-Cbl. Therefore we propose that Src-family members (such as Fgr) may initiate rapid activation of c-Cbl-bound Syk, which will lead to the prolonged tyrosine kinase activity associated with c-Cbl. That assumption is well supported by the finding that engagement of integrins on PMNs from Syk^{-/-} mice does not result in phosphorylation of c-Cbl [10]. Furthermore, it has been suggested [38] that the phosphorylation of c-Cbl occurring following ligation of the Fc γ receptor in PMNs is induced by Syk-bound c-Cbl, but not by Src-like tyrosine kinases. Moreover, Saki et al. [30] have found that engagement of the α IIb- β_3 integrin on platelets increased the amount of Syk associated with c-Cbl and also caused phosphorylation of tyrosine residues in c-Cbl. By comparison, overexpression of Syk in Cos cells has also been observed to increase phosphorylation of c-Cbl [29]. Furthermore, our finding that piceatannol blocks the *in vitro* phosphorylation of c-Cbl strengthens the possibility that c-Cbl-bound Src-dependent Syk participates in the prolonged β_2 integrin-induced phosphorylation of c-Cbl.

To understand better the differences in β_2 integrin-induced regulation of Fgr and Syk tyrosine kinases and to elucidate further results showing that c-Cbl can function as a ubiquitin E3 ligase for several signalling proteins, we investigated the ability of β_2 integrins to induce ubiquitination of Fgr and Syk in PMNs. We found that β_2 integrin-mediated adhesion caused ubiquitination of Fgr but, surprisingly, not Syk. Ubiquitination often triggers rapid degradation of tyrosine kinases [20]; however, we did not find any increased degradation of Fgr upon β_2 integrin-mediated cell adhesion. Thus, in our cell model, Fgr ubiquitination might rather regulate the Fgr-c-Cbl interaction, cellular localization and/or the activity of Fgr.

Our results show that only two ubiquitin moieties were added to each Fgr, which reflects monoubiquitination of this protein [20]. This is in contrast with studies showing that c-Cbl-mediated ubiquitination of Syk/ZAP-70 in natural killer and B-cells [16,39] and Lck in T-cells [40] resulted in high-molecular-mass ubiquitinated tyrosine kinases, indicating that multi-ubiquitination had occurred. We found a clear link between adhesion-induced activation and tyrosine phosphorylation of Fgr and the subsequent ubiquitination of this kinase. This agrees with findings by Yokouchi et al. [34] showing that Src kinase activity and

tyrosine phosphorylation of c-Cbl were required for ubiquitination of Src and c-Cbl *in vitro*. However, in contrast with these authors, we did not detect any ubiquitination of c-Cbl in PMNs undergoing β_2 integrin-mediated adhesion (results not shown). Moreover, β_2 integrin-induced ubiquitination of Fgr was abolished in PMNs pretreated with PP1, which may explain why the dissociation of Fgr from c-Cbl was partly reversed. This partial reversal implies that additional mechanisms are involved in the dissociation of Fgr from c-Cbl, which is supported by our finding that β_2 integrins eventually cause dissociation of Syk from c-Cbl, even if Syk is not ubiquitinated. We also found that pretreatment of PMNs with the membrane-permeant proteasome inhibitor lactacystin did not result in accumulation of ubiquitinated Fgr, which further strengthens our findings that Fgr is not degraded upon adhesion of the cells. This is also compatible with the monoubiquitination of Fgr we observed, a covalent modification that, according to other studies [20,21,41], can also regulate receptor recycling and protein localization.

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