The EMBO Journal Vol.17 No.15 pp.4391–4403, 1998

A β_1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration

Fanying Meng and Clifford A.Lowell[1]

Department of Laboratory Medicine, University of California at San Francisco, San Francisco, CA 94143-0100, USA

[1]Corresponding author
e-mail: clowell@cgl.ucsf.edu

We have used mutant macrophages which are deficient in expression of Src-family kinases to define an integrin signaling pathway that is required for macrophage adhesion and migration. Following ligation of surface integrins by fibronectin, the p120$^{c\text{-}cbl}$ (Cbl) protein rapidly becomes tyrosine phosphorylated and associated with the Src-family kinases Fgr and Lyn. In *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ triple mutant cells, which are defective in spreading on fibronectin-coated surfaces *in vitro* and show impaired migration *in vivo*, Cbl tyrosine phosphorylation is blocked, Cbl protein levels are low, adhesion-dependent translocation of Cbl to the membrane is impaired and Cbl-associated, membrane-localized phosphatidylinositol 3 (PI-3)-kinase activity is dramatically reduced. In contrast, adhesion-dependent activation of total cellular PI-3 kinase activity is normal in mutant cells, demonstrating that it is the membrane-associated fraction of PI-3 kinase which is most critical in regulating actin cytoskeletal rearrangements that lead to cell spreading. Treatment of wild-type cells with the Src-family-specific inhibitor PP1, Cbl antisense oligonucleotides or pharmacological inhibitors of PI-3 kinase blocks cell spreading on fibronectin surfaces. These data provide a molecular description for the role of Src-family kinases Hck, Fgr and Lyn in β_1-integrin signal transduction in macrophages.

Keywords: Cbl/integrin/macrophages/MAP kinases/PI-3 kinase/Src-family kinases

Introduction

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits that functionally serve as both adhesive and signaling receptors. Clustering of integrins at the cell surface produces intracellular signaling responses which lead to rearrangement in the actin cytoskeleton, cell spreading and cell adhesion. Among the earliest events in integrin-mediated signal transduction is increased tyrosine phosphorylation of multiple cytoskeletal-associated proteins, such as paxillin (Burridge *et al*., 1992), tensin (Bockholt and Burridge, 1993), cortactin (Vuori and Ruoslahti, 1995) and p130^{cas} (Vuori *et al*., 1996). Integrins depend on associated tyrosine kinases to carry out their signaling functions. It is well known that integrin engagement can trigger rapid tyrosine phosphorylation and enzymatic activation of focal adhesion kinase (FAK) (Schaller and Parsons, 1994) and its related family member (PYK2) (Li *et al*., 1996). Increasing evidence suggests that Src-family kinases are also involved in integrin-mediated cell adhesion and migration. Integrin-dependent adhesion of neutrophils to extracellular matrix (ECM) proteins leads to activation of the Src-family kinases in these cells, Hck, Fgr and Lyn (Berton *et al*., 1994, 1996). During adhesion to ECM and subsequent cell spreading, these kinases migrate to a detergent-insoluble cytoskeletal-associated cell fraction (Yan *et al*., 1996). In *hck*$^{-/-}$*fgr*$^{-/-}$ knockout mice, neutrophils show impaired integrin-dependent spreading and respiratory burst (Lowell *et al*., 1996) and macrophages manifest disorganized cytoskeletal structures and reduced *in vitro* migration on fibronectin (Fn) or through matrigel (P.W.Suen and C.A.Lowell, unpublished). Examination of *hck*$^{-/-}$*fgr*$^{-/-}$ double mutant macrophages has revealed reduced phosphorylation and an altered subcellular localization of focal adhesion-associated proteins such as paxillin, cortactin and tensin. Similarly, fibroblasts and neural cells derived from *src*- and *fyn*-deficient mice show defects in adhesion, migration and cytoskeleton organization (Beggs *et al*., 1994; Thomas *et al*., 1995). Taken together, these data suggest that Src-family kinases play critical roles in integrin-initiated biological responses.

Great efforts have been made to identify new molecules involved in integrin signal transduction. Recently, a cytoplasmic proto-oncogene product, c-Cbl, has been described as a newly tyrosine-phosphorylated protein in adhering cells (Manie *et al*., 1997; Ojaniemi *et al*., 1997). Cbl has been identified as a potential tyrosine kinase substrate in many other signaling pathways as it is tyrosine phosphorylated rapidly upon receptor activation by epidermal growth factor (EGF), platelet-derived growth factor (PDGF) (Bowtell and Langdon, 1995; Galisteo *et al*., 1995), the lymphocyte antigen (Donovan *et al*., 1994; Panchamoorthy *et al*., 1996), Fcγ (Marcilla *et al*., 1995; Matsuo *et al*., 1996), interferon-α (IFN-α) (Uddin *et al*., 1996), granulocyte–macrophage colony-stimulating factor (GM-CSF) (Odai *et al*., 1995) and interleukin-3 (IL-3) (Anderson *et al*., 1997). In the antigen receptor signaling pathway, Src-family kinases are believed to be responsible for Cbl tyrosine phosphorylation; impaired Cbl phosphorylation is seen in Lyn-deficient chicken DT40 B-cells or in splenic T-cells from *fyn*$^{-/-}$ mice but not in Syk-deficient DT40 B-cells (Tezuka *et al*., 1996). Similarly, Cbl phosphorylation is reduced dramatically in osteoclasts derived from *src*$^{-/-}$ mice (Tanaka *et al*., 1996). In some signaling pathways, Cbl may function as a negative regulator. Mammalian Cbl has high structural similarity with the *Caenorhabditis elegans* gene *Sli-1*, which is a negative regulator of *Let-23*-mediated signal transduction (Jongeward *et al*., 1995; Yoon *et al*., 1995). Moreover, treatment of fibroblasts with Cbl antisense

oligonucleotides results in enhanced EGF receptor phosphorylation and hyperactivation of JAK–STAT signaling following EGF stimulation (Ueno *et al*., 1997), while overexpression of Cbl downregulates FcεR signaling in mast cells (Ota and Samelson, 1997). In contrast to these negative regulatory functions, Cbl serves in a positive role in osteoclasts. Treatment of osteoclasts with antisense Cbl oligonucleotides results in impaired bone resorption capability (Tanaka *et al*., 1996). This phenotype is very similar to what is seen in *src*$^{-/-}$ osteoclasts or in wild-type osteoclasts treated with RGD peptides (van der Pluijm *et al*., 1994). These observations suggest that impaired Cbl phosphorylation or reduced Cbl protein levels lead to defects in integrin signaling pathways that are required for normal osteoclast function.

Cbl associates with many proteins *in vivo*, including other adaptor proteins such as CrkL or p130^{cas} (Salgia *et al*., 1996; Smit *et al*., 1996) as well as with the Src-family kinases themselves (Tezuka *et al*., 1996; Anderson *et al*., 1997). The most commonly observed Cbl-associated protein is phosphatidylinositol 3-kinase (PI-3 kinase) (Matsuo *et al*., 1996; Panchamoorthy *et al*., 1996). PI-3 kinase has also been implicated in integrin signal transduction. Clustering of surface integrins following plating of fibroblasts on Fn-coated surfaces leads to dramatically enhanced PI-3 kinase activity; pharmacologic inhibition of PI-3 kinase activity blocks cell spreading (King *et al*., 1997). Similarly, treatment of platelets with PI-3 kinase inhibitors results in inhibition of the normal actin cytoskeletal rearrangements, membrane ruffling and filopodia extensions that occur in response to $\alpha_{IIb}\beta_3$ cross-linking with thrombin (Hartwig *et al*., 1996). In carcinoma cells, PI-3 kinase has been shown to be involved in the signaling pathways induced by ligation of integrin $\alpha_4\beta_6$ which are required for cell invasion in *in vitro* assays (Shaw *et al*., 1997). In this system, the D_3 phosphoinositides produced by PI-3 kinase activation act on the small GTPase Rac to promote membrane ruffling, filopodia extension and cell migration.

In the present study, we use macrophages from knockout mice, antisense oligonucleotides and pharmacologic inhibitors to define a β_1 integrin signaling pathway leading from Src-family kinases to Cbl to membrane localization of PI-3 kinase which is required for cell spreading on Fn-coated surfaces. In the absence of the predominate Src-family kinases in myeloid cells, integrin signaling is blocked and macrophages manifest impaired migration in an *in vivo* peritonitis model. Interestingly, these mutations do not affect integrin-mediated MAP kinase activation or NF-κB translocation, suggesting that the integrin signaling pathways which regulate cytoskeletal changes and cell migration are different from the pathways which activate MAP kinases and NF-κB.

Results

Deficiency of Src-family kinases impairs macrophage spreading on fibronectin in vitro and inflammatory macrophage migration in vivo

Previous examination of neutrophils from double mutant *hck*$^{-/-}$*fgr*$^{-/-}$ mice revealed reduced cell spreading on ECM-coated surfaces, which resulted in impaired adhesion-dependent neutrophil activation (Lowell *et al*., 1996).

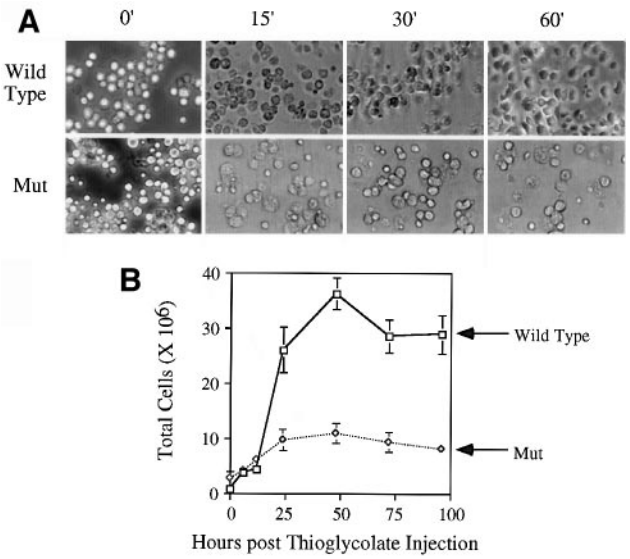

Fig. 1. Triple mutant macrophages demonstrate defective adhesion *in vitro* and reduced migration *in vivo*. (**A**) Wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$ *lyn*$^{-/-}$ (labeled as Mut) PEMs were plated on Fn-coated dishes and photographed (40×) under phase microscopy at the indicated times. (**B**) Wild-type and mutant mice (n = 21; three animals per time point) were injected with 3% thioglycolate, and peritoneal exudate cells were collected at the indicated times. Total cells were counted and analyzed by flow cytometry using cell surface markers to distinguish macrophages. Total macrophages were averaged from animals at each time point. This experiment is representative of four separate experiments.

Peritoneal macrophages (PEMs) isolated from triple mutant *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice have an even more profound defect in cell spreading over ECM-coated surfaces (Figure 1A). While wild-type cells are fully spread on Fn-coated surfaces after 60 min of plating, triple mutant cells remain round and refractile. Loose attachment of cells to Fn (defined as the number of cells that remain on the surface following gentle removal of the media) is equivalent in mutant and wild-type PEMs. However, mutant cells are easily detached by shaking, indicating that the processes of firm adhesion by cell spreading have not occurred. No spreading of wild-type or mutant PEMs was observed on polylysine-coated surfaces (data not shown). Single mutant *hck*$^{-/-}$, *fgr*$^{-/-}$ and *lyn*$^{-/-}$ PEMs spread and adhere normally to Fn-coated dishes (data not shown). It is important to note that this appears to be the major signaling event affected in these cells, as triple mutant macrophages show normal responses to lipopolysaccharide (LPS) activation (Meng and Lowell, 1997), CSF-1 or cytokine stimulation (unpublished data), and are able to phagocytose red blood cells (Crowley *et al*., 1997).

To test the physiological consequences of impaired macrophage adhesion directly, we examined the migratory capacity of *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs in an *in vivo* peritonitis model. In this model, an inflammatory stimulus (thioglycolate) is injected intraperitoneally and the number of cells invading the peritoneal cavity is enumerated by counting and flow cytometry. In this model, triple mutant mice showed a gross defect in the kinetics of inflammatory macrophage migration into the peritoneum (Figure 1B). Wild-type mice had large numbers of intraperitoneal macrophages while mutant mice showed a 2- to 3-fold reduction in the number of these cells at all times beyond

Table I. Peripheral blood leukocyte counts in wild-type and *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice

	Wild-type (n = 8)	*hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ (n = 11)
Neutrophils per mm^3	867 ± 136	4582 ± 560
Monocytes per mm^3	110 ± 37	617 ± 133
Lymphocytes per mm^3	6793 ± 419	5505 ± 613
Eosinophils per mm^3	243 ± 43	191 ± 61

Errors are standard errors of the mean.

24 h after thioglycolate injection. In contrast, single mutant *lyn*$^{-/-}$ (data not shown), *hck*$^{-/-}$ and *fgr*$^{-/-}$, as well as double mutant *hck*$^{-/-}$*fgr*$^{-/-}$ animals do not manifest impaired macrophage recruitment in this peritonitis model (Lowell *et al*., 1994), demonstrating that deficiency of all the major Src-family kinases in these cells is required for migratory defects to become readily apparent. No differences between wild-type or mutant macrophages were found in the expression of integrin subunits CD11a, CD11b, CD11c, CD18, CD29, CD49d, CD49e, CD49f, CD51 and CD61, macrophage markers Mac2, Mac3, F4/80 and CD115, or co-stimulatory molecules CD80 and CD86, demonstrating that the loss of Src-family kinases does not affect macrophage maturation or expression of adhesion receptors *in vivo*. The reduced migration of macrophages into the peritoneal cavity is not due to reduced numbers of peripheral blood monocytes. Indeed, both neutrophil and monocyte numbers are significantly higher in triple mutant mice compared with wild-type animals (Table I). Peripheral neutrophilia is also seen in other knockout mice with deficiencies in cell adhesion molecules (ICAM-1 or the endothelial selectins; Xu *et al*., 1994; Frenette *et al*., 1996).

Rapid and transient tyrosine phosphorylation of Cbl following adhesion of primary macrophages

In order to define the altered integrin-mediated signaling pathways which give rise to the functional defects in triple mutant cells, we began a search for novel tyrosine kinase substrates that become phosphorylated following adhesion to fibronectin. We found a previously uncharacterized 120 kDa, Grb-2-associated, molecule that was tyrosine phosphorylated rapidly following plating of PEMs on Fn-coated surfaces. Using specific antisera, we defined this protein as Cbl. Subsequently, Cbl was reported to be the primary tyrosine-phosphorylated product seen in both B-lymphocytes and macrophage cell lines adherent to ECM proteins (Manie *et al*., 1997; Ojaniemi *et al*., 1997; Sattler *et al*., 1997). In wild-type PEMs plated on Fn, Cbl became tyrosine phosphorylated rapidly as early 1 min in response to cell adhesion (Figure 2A). The level of Cbl tyrosine phosphorylation was constant during the first 15 min of adhesion and decreased from 30 min to become barely detectable at ~60 min, and remained undetectable up to 8 h later (data not shown). Cbl phosphorylation was also observed in PEMs plated on dishes coated with vitronectin or anti-integrin antibodies recognizing the α_4, α_5 and β_1 subunits, but not in cells plated on collagen or laminin (to which cells did not attach as PEMs do not express the principle integrins that recognize these ECM proteins) or in cells attached non-specifically to polylysine

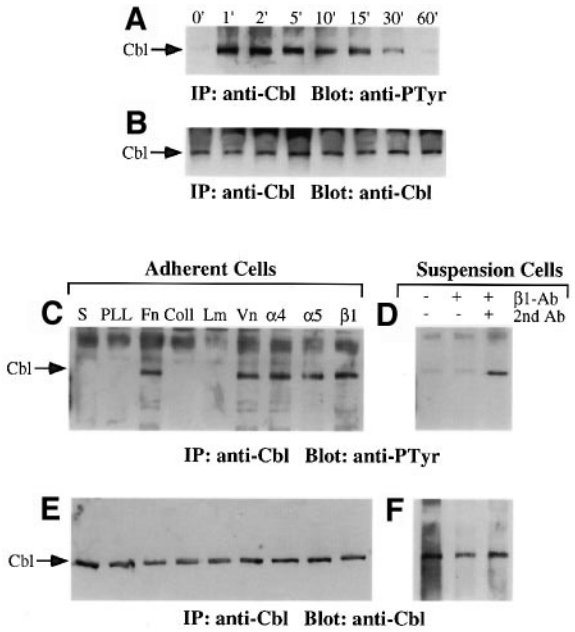

Fig. 2. Cbl is rapidly and transiently tyrosine phosphorylated following integrin cross-linking. (**A**) PEMs were plated on Fn-coated plates (10 μg/ml) and cell lysates were prepared at the indicated times. A 250 μg aliquot of each lysate was immunoprecipitated with anti-Cbl polyclonal antibody, and the immunoprecipitated proteins were resolved on an 8% SDS–PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10. (**B**) The same blot as in (A) was stripped and re-probed with anti-Cbl antibody to confirm equal precipitation of Cbl in all lanes. (**C**) PEMs were plated on dishes pre-coated with the indicated proteins for 15 min, and tyrosine-phosphorylated Cbl was detected with 4G10 antibody. S = cells in suspension prior to plating, PLL = polylysine-coated, Fn = fibronectin, Coll = collagen, Lm = laminin, Vn = vitronectin, α_4 = pre-coating with anti-α_4 mAb, α_5 = pre-coating with anti-α_5 mAb and β_1 = pre-coating with hamster anti-β_1 mAb. Plating on non-integrin-specific mAbs (such as L-selectin) did not lead to Cbl tyrosine phosphorylation (data not shown). (**D**) PEMs were maintained in suspension and treated with anti-β_1 mAb (10 μg/ml) for 10 min followed by addition of F(ab′)$_2$ anti-hamster Ig (80 μg/ml; labeled as 2nd Ab) for 10 min. Cell lysates were prepared and used for Cbl immunoprecipitation following by immunoblotting with anti-phosphotyrosine mAb. (**E** and **F**) The same filters as in (C) and (D) were re-probed with anti-Cbl antibody to confirm equal precipitation.

(Figure 2C). Cbl tyrosine phosphorylation could also be induced in cells in suspension by cross-linking the β_1 surface integrins with monoclonal antibodies (mAbs) (Figure 2D). Taken together, this evidence demonstrates that Cbl tyrosine phosphorylation occurs in a rapid and transient fashion following integrin-mediated adhesion of primary PEMs.

In vivo association of Cbl with Fgr and Lyn

In other signaling pathways, Cbl tyrosine phosphorylation is dependent on association with the SH2 and SH3 domains of many signaling molecules, including Src-family kinases (Donovan *et al*., 1994; Panchamoorthy *et al*., 1996; Tezuka *et al*., 1996; Anderson *et al*., 1997). To determine whether Cbl was associated with Hck, Fgr or Lyn during integrin-induced signaling events, we assayed for kinase activity in Cbl immunoprecipitations from either cells in suspension or cells adherent to Fn. Abundant tyrosine kinase activity co-immunoprecipitated with Cbl from adherent cells but little activity was seen in Cbl immunoprecipitates from cells in suspension (Figure 3A). The

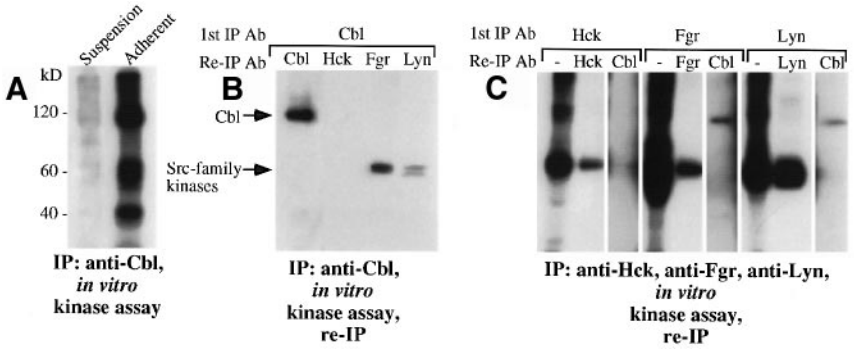

Fig. 3. Cbl associates *in vivo* with Fgr and Lyn but not Hck. (**A**) PEMs were either held in suspension or plated on Fn-coated plates at 37°C for 15 min and lysed in NP-40 buffer. A 250 μg aliquot of cell lysate was immunoprecipitated with anti-Cbl antibody and immunoprecipitates were used for *in vitro* kinase assays. Phosphorylated proteins were resolved on an 8% SDS–PAGE and revealed by autoradiography. Treatment of this gel with 1 M KOH did not alter the pattern of phosphorylated proteins observed, confirming that the majority of phosphorylation events were on tyrosine residues. (**B**) Anti-Cbl immunoprecipitates made from Fn-adherent cells were subjected to *in vitro* kinase assay and followed by re-immunoprecipitation with anti-Cbl, Hck, Fgr and Lyn. (**C**) A 250 μg aliquot of cell lysate from Fn-adherent cells was immunoprecipitated with Hck, Fgr and Lyn, followed by *in vitro* kinase assay. The products of the kinase assay were then re-immunoprecipitated with the same antibody or with anti-Cbl. Autoradiographs of Src-family kinase immunoprecipitates/*in vitro* kinase assays were exposed for 30 min while Cbl re-immunoprecipitations were exposed for 12 h.

predominant bands labeled in the Cbl precipitates were ~120, 60 and 35–40 kDa. When these labeled proteins were dissociated from the anti-Cbl antibodies, then re-immunoprecipitated with anti-Cbl, Hck, Fgr or Lyn, we found that the 120 kDa labeled protein corresponded to Cbl itself, while the ~60 kDa proteins corresponded to Fgr and Lyn (Figure 3B). To confirm that only Fgr and Lyn, but not Hck, were associated with Cbl, cell lysates from adherent cells were immunoprecipitated with antibodies against Hck, Fgr and Lyn, subjected *in vitro* kinase assay, then re-immunoprecipitated with either the same antibodies or with anti-Cbl. As shown in Figure 3C, huge amounts of kinase activity were detected in the Hck, Fgr and Lyn immunoprecipitates from Fn adherent cells; re-immunoprecipitation confirmed that phosphorylated Cbl was present in the immunoprecipitates of Fgr and Lyn but not of Hck. These data demonstrate that the Src-family kinases Fgr and Lyn become associated with Cbl in primary macrophages upon integrin-mediated cell adhesion. The identity of the 35–40 kDa protein labeled in the Cbl immunoprecipitations is likely to be CrkL, an SH2-SH3-SH3 adaptor molecule which associates with Cbl following β_1 integrin ligation in megakaryoblastic cell lines (Sattler *et al*., 1997), although we have not proved this formally in primary PEMs.

Impaired Cbl tyrosine phosphorylation and reduced Cbl expression in hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ macrophages but not in single mutant cells

To investigate the effects of loss of Hck, Fgr and Lyn kinases on β_1 integrin-induced Cbl tyrosine phosphorylation directly, we examined Cbl immunoprecipitates obtained from wild-type and mutant PEMs following adhesion to Fn-coated surfaces. Using equal amounts of lysates, Cbl tyrosine phosphorylation was completely absent in mutant cells attached to Fn (Figure 4A). When the same membrane was re-probed with anti-Cbl antibody after stripping, we observed a lower level of Cbl expression in *hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$* cells compared with wild-type cells (Figure 4D). To normalize the amount of Cbl in the immuoprecipitations, we used 3-fold more lysate from mutant cells compared with wild-type cells. Despite equal amounts of Cbl protein in each immunoprecipitation, we were unable to observe tyrosine-phosphorylated Cbl in triple mutant PEMs adherent to Fn (Figure 4B and E). In contrast, Cbl tyrosine phosphorylation and expression were normal in single mutant *hck$^{-/-}$*, *fgr$^{-/-}$* and *lyn$^{-/-}$* macrophages plated on Fn (Figure 4C and F). These data indicate that primary Src-family kinases in macrophages function in a redundant role to phosphorylate Cbl during integrin-dependent adhesion and cell spreading. The lower Cbl protein level in *hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$* cells was confirmed further by direct immunoblotting (Figure 5A). In contrast, the expression levels of Erk2 (Figure 5B), Syk, Pyk2, the JAK-family kinases, Grb2 and Shc (data not shown) were equivalent in wild-type and triple mutant PEMs. In all subsequent experiments comparing Cbl immunoprecipitation, we used more *hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$* cell lysate and validated equal levels of Cbl in immunoprecipitations in order to make comparisons more direct. Experiments in which Cbl levels could not be balanced between mutant and wild-type were discarded.

In contrast to Cbl protein levels, triple mutant PEMs expressed higher levels of *cbl* mRNA as determined by Northern blotting (Figure 5C). This observation suggests that triple mutant cells are attempting to compensate for the low Cbl protein levels by overexpressing the *cbl* gene. Hence, it is likely that Cbl protein is less stable in triple mutant cells. Unfortunately, we were unable to demonstrate this directly using *in vitro* pulse–chase labeling in mutant cells due to the low metabolic labeling observed in both wild-type and mutant PEMs. We conclude that *hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$* macrophages have reduced levels of Cbl protein and the remaining Cbl is not tyrosine phosphorylated following adhesion to Fn.

Cbl isolated from triple mutant PEMs lacks associated tyrosine kinase activity

To determine whether the absence of Hck, Fgr and Lyn in PEMs affected the integrin-induced *in vivo* association of Cbl with tyrosine kinase activity, we carried out Cbl immunoprecipitation and *in vitro* kinase assays with wild-type, triple mutant and single mutant PEMs. As shown in Figure 6A, there was no Cbl-associated tyrosine kinase

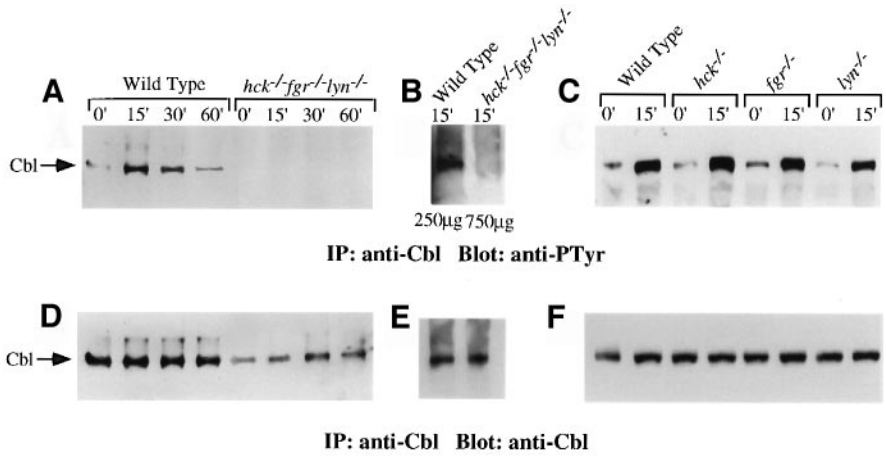

Fig. 4. Tyrosine phosphorylation of Cbl is impaired in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ but not single mutant PEMs following integrin-mediated adhesion. (**A**) PEMs from wild-type and triple mutant mice were seeded on Fn-coated dishes and cell lysates were prepared at the indicated times. A 250 µg aliquot of each lysate was immunoprecipitated with anti-Cbl antibody, resolved on 8% SDS–PAGE and immunoblotted with 4G10. (**B**) A 250 µg aliquot of lysate from wild-type and a 750 µg aliquot of lysate from *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs adherent to Fn (15 min) were immunoprecipitated with anti-Cbl antibody and immunoblotted with anti-phosphotyrosine antibody. (**C**) A 250 µg aliquot of cell lysate was prepared from non-adherent (0′) or Fn-adherent (15′) PEMs isolated from wild-type or single mutant animals, then immunoprecipitated with anti-Cbl antibody and immunoblotted with 4G10. (**D**, **E** and **F**) Filters corresponding to those in (A), (B) and (C) were re-probed with anti-Cbl antibody after stripping.

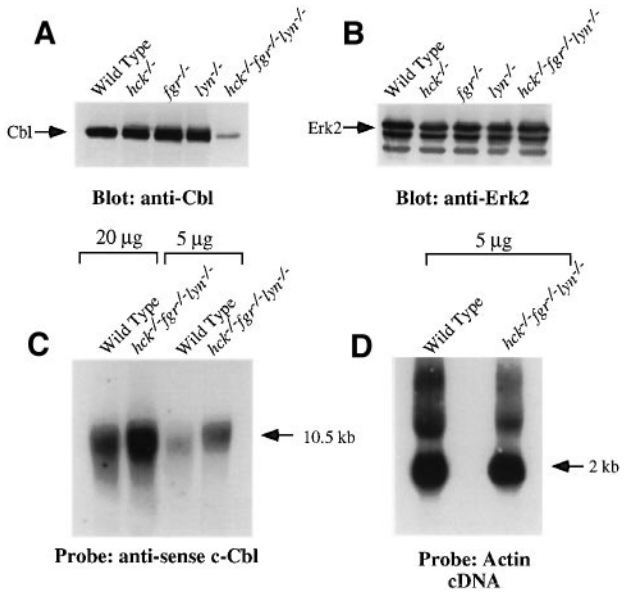

Fig. 5. Cbl protein levels are low in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs while mRNA levels are high. (**A**) A 30 µg aliquot of total cellular protein from freshly isolated wild-type and mutant macrophages was resolved on 8% SDS–PAGE and immunoblotted with anti-Cbl antibody. (**B**) The filter in (A) was re-probed with anti-Erk2 antibody to confirm equal loading. (**C**) Total RNA (20 or 5 µg) from either wild-type or triple mutant PEMs was electrophoresed on a 1% agarose/6% formaldehyde gel, then transferred to nylon. The membrane was hybridized with [α-^{32}P]CTP-labeled antisense Cbl RNA probe. (**D**) Equal loading was confirmed by hybridization with [α-^{32}P]dCTP-labeled human β-actin cDNA probe.

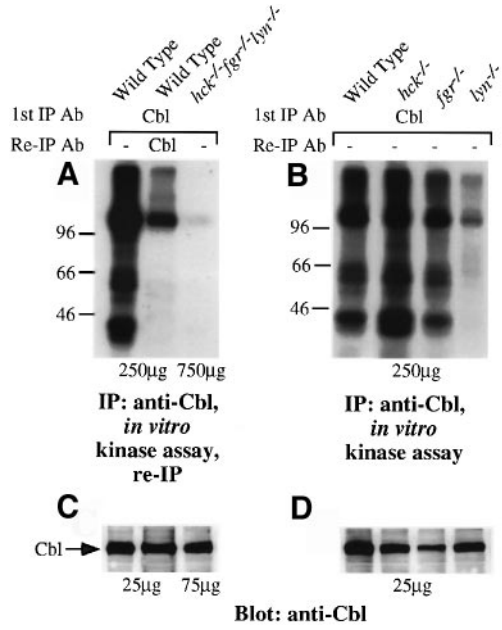

Fig. 6. Cbl isolated from adherent *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs lacks associated tyrosine kinase activity. PEMs isolated from wild-type, single mutant and triple mutant animals were plated on Fn-coated dishes for 15 min and cell lysates were prepared. (**A**) Equal amounts of Cbl from wild-type (250 µg) and triple mutant (750 µg) lysate were immunoprecipitated with anti-Cbl antibody followed by *in vitro* kinase assays. Labeled proteins were then re-immunoprecipitated with anti-Cbl to demonstrate labeling of Cbl. (**B**) A 250 µg aliquot of lysate from Fn-adherent single mutant PEMs was immunoprecipitated with anti-Cbl followed by *in vitro* kinase assay. (**C** and **D**) Separate aliquots of each lysate (wild-type/single mutant 25 µg; triple mutant 75 µg) were electrophoresed and immunoblotted with anti-Cbl to demonstrate equal Cbl levels in all samples.

activity and tyrosine-phosphorylated Cbl in the immunoprecipitates derived from adherent *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ macrophages. In contrast, Cbl-associated tyrosine kinase activity and tyrosine-phosphorylated Cbl was seen readily in immunoprecipitates from single mutant *hck*$^{-/-}$, *fgr*$^{-/-}$ and *lyn*$^{-/-}$ cells (Figure 6B). The significance of the reduced Cbl-associated tyrosine kinase activity in *lyn*$^{-/-}$ cells is unclear since *lyn*$^{-/-}$ PEMs show normal adhesion-dependent Cbl tyrosine phosphorylation (Figure 4C), normal Cbl expression (Figure 4F), normal cell spreading on Fn *in vitro* (data not shown) and normal migration *in vivo* (data not shown). This evidence suggests that Src-family kinases become associated with Cbl in Fn-adherent PEMs (Figure 3) and are responsible for the tyrosine phosphorylation of Cbl seen both *in vivo* (Figure 4) and

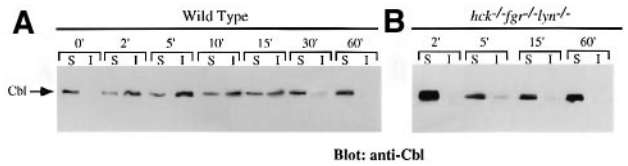

Fig. 7. Cbl remains in the detergent-soluble fraction in *hck*$^{-/-}$*fgr*$^{-/-}$ *lyn*$^{-/-}$ PEMs following adhesion to Fn. PEMs from wild-type and triple mutant mice were plated on Fn for the indicated times. Subsequently cells were chilled on ice for 6 min, then lysed in 400 µl of Triton X-100 lysis buffer for 3 min to obtain the soluble fraction. After a brief wash (<5 s), the remaining cells were lysed sequentially in a modified RIPA buffer. (**A**) A 25 µg aliquot of total protein extracts from the Triton X100-soluble (S) and -insoluble (I) fractions was electrophoresed on 8% SDS–PAGE, transferred to nitrocellulose and blotted with anti-Cbl polyclonal antibody. (**B**) A 75 µg aliquot of each fraction from triple mutant PEMs was used for anti-Cbl immunoblotting. Fewer points were examined because of the requirement for large amounts of triple mutant lysate in order to normalize Cbl proteins levels between mutant and wild-type.

in vitro (Figure 6A). It is most likely that Fgr and Lyn act in a redundant fashion to phosphorylate Cbl in PEMs adherent to Fn.

Defective Cbl redistribution following adhesion to Fn in hck$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ *macrophages*

Both in CSF-1 receptor signaling pathways and following integrin ligation, a portion of the newly tyrosine-phosphorylated Cbl moves from the cytosol to the cytoskeletal-associated subcellular fraction (Wang *et al*., 1996; Ojaniemi *et al*., 1997). We investigated this process in wild-type and mutant PEMs using differential detergent solubilization as described by Kaplan *et al.* (1994) following cell adhesion. In non-adherent wild-type cells, Cbl is in the Triton X-100-soluble cytosolic fraction; however, within 2 min after plating on Fn-coated plates, a portion of Cbl was translocated rapidly to the Triton X-100-insoluble cytoskeletal fraction and remained there for up to 15 min following plating (Figure 7A). After 30 min of adhesion, the portion of Cbl in the Triton X-100-insoluble fraction decreased and became undetectable at 60 min. This profile of Cbl subcellular distribution coincides very well with the pattern of Cbl tyrosine phosphorylation following macrophage adhesion. In contrast to wild-type cells, Cbl translocation to the Triton X-100 fraction was barely observed in triple mutant PEMs adherent to Fn (Figure 7B); the majority of Cbl remained in the cytoplasmic fraction throughout the time course. Given the absence of Cbl tyrosine phosphorylation in *hck*$^{-/-}$*fgr*$^{-/-}$ *lyn*$^{-/-}$ PEMs, these data demonstrate a correlation between phosphorylation and subcellular localization.

Adherent triple mutant macrophages have reduced Cbl- and cytoskeletal-associated PI-3 kinase activity

As one of the primary proteins associated with Cbl is PI-3 kinase (Matsuo *et al*., 1996), we investigated the activation of PI-3 kinase and its association with Cbl in *hck*$^{-/-}$*fgr*$^{-/-}$ *lyn*$^{-/-}$ macrophages plated on Fn-coated surfaces. Cell lysates were immunoprecipitated with antibodies against the p85 subunit of PI-3 kinase or with anti-Cbl, and the immunoprecipitates were assayed for the ability to phosphorylate inositol. There was no difference between wild-type and mutants in the amount of adhesion-induced

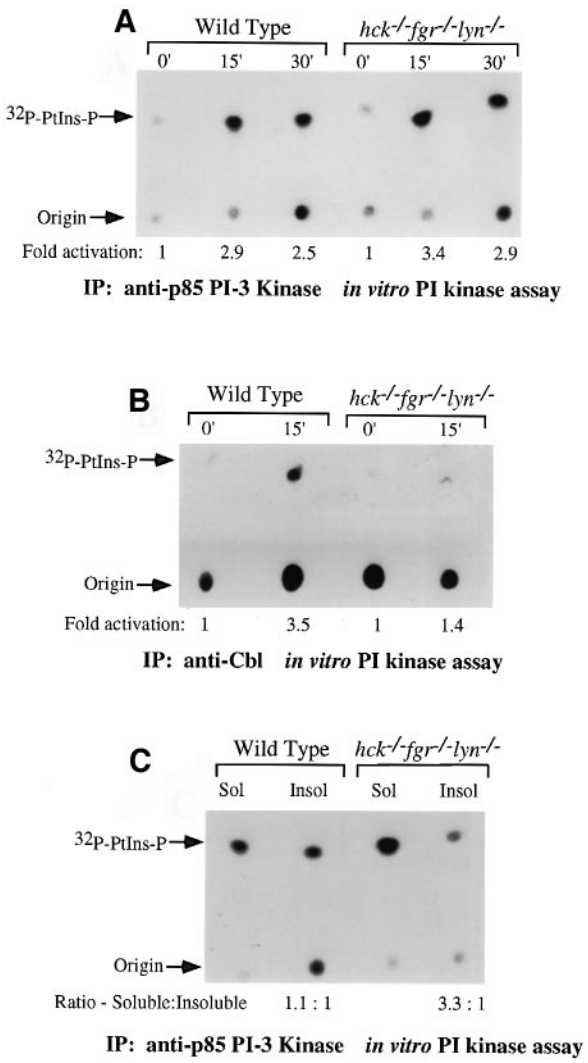

Fig. 8. Formation of a Cbl–PI-3 kinase complex and translocation of PI-3 kinase to the cytoskeleton is defective in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs. PEMs from wild-type and triple mutant mice were plated on Fn-coated dishes for the indicated times. (**A**) A 200 µg aliquot of cell lysate from wild-type and triple mutant macrophages was immunoprecipitated with an mAb against the p85 subunit of PI-3 kinase. A phospholipid kinase assay was performed on the immunoprecipitates and the products were resolved by thin-layer chromatography then visualized by autoradiography. The amount of [^{32}P]phosphoinositol was quantitated by phosphoimager analysis and the fold activation of PI-3 kinase activity relative to non-adherent cells (time 0′) was calculated. Immunoblotting confirmed equal amounts of p85 in all precipitates (data not shown). (**B**) Cell lysates from wild-type (200 µg) or triple mutant (600 µg) PEMs either non-adherent (0′) or Fn-adherent (15′) were immunoprecipitated with anti-Cbl antibody and the immunoprecipitates were used for phospholipid kinase assays. The amount of lipid kinase activity associated with Cbl was quantitated relative to wild-type non-adherent cells. Immunoblotting confirmed equal amounts of Cbl in all precipitates (data not shown). (**C**) Wild-type and triple mutant PEMs were plated on Fn for 15 min and cell lysates were prepared by fractionation into Triton X-100-soluble (Sol) or -insoluble (Insol) as described in Materials and methods. A 500 µg aliquot of these cell fractions was used for immunoprecipitation with anti-p85 and the precipitates subjected to a lipid kinase assay. The ratio of [^{32}P]phosphoinositol in the soluble over insoluble cell fractions was calculated. The results shown are representative of three independent experiments.

stimulation of total PI-3 kinase activity (Figure 8A). However, the fraction of PI-3 kinase that was associated with Cbl was dramatically lower in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ cells

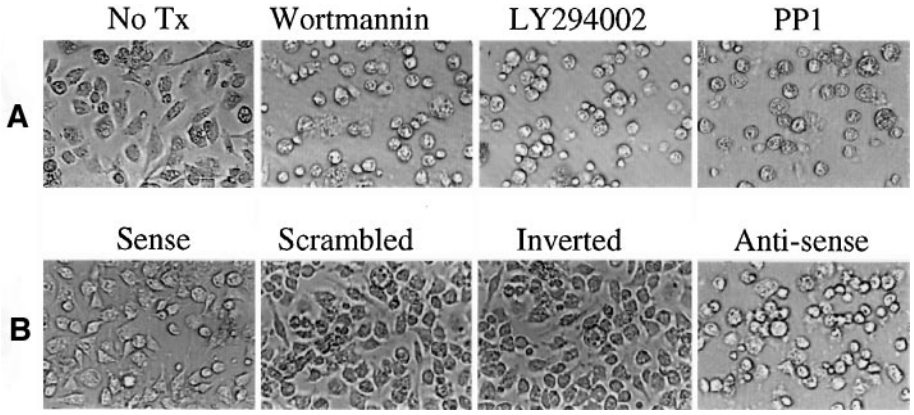

Fig. 9. Blockade of wild-type macrophage spreading by PI-3 kinase inhibitors, anti-Src family kinase blocker and antisense oligonucleotides directed against Cbl. (**A**) Wild-type PEMs were pre-treated with the indicated inhibitors for 1 h and then plated on Fn-coated dishes for 60 min in the presence of the corresponding inhibitors, following which photographs were taken. For the PI-3 kinase inhibitors, wortmannin was used at 100 nM while LY294002 was used at 10 μM; the anti-Src family kinase inhibitor PP1 was used at 20 μM. (**B**) Wild-type PEMs were maintained in suspension and treated with the indicated Cbl oligonucleotides for 2 h prior to plating on Fn, then allowed to spread for 60 min, in the presence of the appropriate oligonucleotides and photographed. Sequences of the indicated oligonucleotides are described in Materials and methods.

than in wild-type cells (Figure 8B). Since adhesion to Fn induces translocation of Cbl to the cytoskeletal subcellular compartment, we examined the translocation of PI-3 kinase to this fraction as well using the differential Triton X-100 solubilization procedure. In wild-type cells, total PI-3 kinase activity was roughly equal (1.1:1) in both the cytosolic and cytoskeletal fractions following 15 min of Fn adhesion (Figure 8C). In contrast, the amount of cytoskeletal-associated PI-3 kinase activity was dramatically lower in mutant cells (3.3 cytosolic to 1 cytoskeletal). We conclude that Src-family kinases are not required for activation of PI-3 kinase following cell adhesion, but are required for association of PI-3 kinase with Cbl and for the translocation of PI-3 kinase to the cytoskeletal fraction. Thus it is likely that in the absence of Cbl tyrosine phosphorylation, the formation of the Cbl–PI-3 kinase complex is blocked and translocation of the Cbl–PI-3 kinase complex to the cytoskeleton is impaired. The presence of low levels of PI-3 kinase activity in the Triton X-100 fraction of mutant cells may represent contamination of protein from the cytosolic fraction during isolation. Interestingly, the level of CSF-1-induced Cbl tyrosine phosphorylation and Cbl-associated PI-3 kinase activity was equal in bone marrow-derived macrophages from wild-type and mutant mice (C.Fitzer-Attas *et al*., unpublished), demonstrating that other signaling pathways involving Cbl do not appear to be affected in triple mutant cells.

Inhibition of PI-3 kinase activity or treatment of cells with antisense Cbl oligonucleotides inhibits macrophage spreading.

To confirm further the role of Cbl and PI-3 kinase in integrin-mediated spreading, we examined the effects of two types of inhibitors on wild-type macrophages. As seen in tumor cells and platelets (Hartwig *et al*., 1996; Shaw *et al*., 1997), treatment of primary PEMs with the PI-3 kinase-specific inhibitors, wortmannin or LY294002, blocks the ability of these cells to spread and firmly adhere to Fn-coated surfaces (Figure 9A). The Src-family kinase-specific tyrosine kinase inhibitor PP1 (Hanke *et al*., 1996) also had a similar effect. Pre-treatment of cells with Cbl antisense oligonucleotides (the same oligonucleotides which inhibit osteoclastic bone resportion, Tanaka *et al*., 1996) also completely inhibited cell spreading (Figure 9B). As seen with triple mutant PEMs, inhibitor- or antisense-treated wild-type cells were able to attach loosely to the Fn but were unable to adhere firmly due to impaired cell spreading. In contrast, treatment with oligonucleotides representing the sense strand of *cbl* mRNA, a scrambled oligonucleotide or an oligonucleotide containing an inverted sequence relative to the antisense oligonucleotide had no effect on firm adhesion and cell spreading. These data indicate that in primary macrophages knockout of the major Src-family kinases, inhibition of Src-kinase activity, loss of Cbl through use of antisense oligonucleotides or treatment with PI-3 kinase inhibitors all produce the same phenotype—blockade of β_1 integrin-dependent macrophage spreading.

Normal stimulation of MAP kinases and NF-κB DNA binding activity in Fn-adherent $hck^{-/-}fgr^{-/-}lyn^{-/-}$ macrophages

Activation of downstream signaling events, such as enhancement of MAP kinase (Erk1/2) activity or stimulation of NF-κB DNA binding activity, is a major component of integrin signaling (Haskill *et al*., 1991; Chen *et al*., 1994; Morino *et al*., 1995). To determine whether these signaling pathways were affected in mutant cells, we assessed the activation level of three members of the MAP kinase family, Erk2, JNK and p38, as well as NF-κB activation in Fn-adherent $hck^{-/-}fgr^{-/-}lyn^{-/-}$ macrophages. We found no major impairment in adhesion-dependent activation of Erk2 and JNK kinases in triple mutant PEMs (Figure 10A and B); indeed, activation of Erk2 and JNK was slightly higher in mutant cells compared with wild-type cells, similar to what is observed in single mutant $lyn^{-/-}$ B-cells (Chan *et al*., 1997, 1998). However, the mechanism and the physiological significance of adhesion-induced hyperactivation of MAP kinases in mutant cells remains to be elucidated further. No detectable integrin-induced activation of p38 kinase was observed in either cell type (data not shown). Similarly, adhesion-dependent activation of NF-κB DNA binding activity

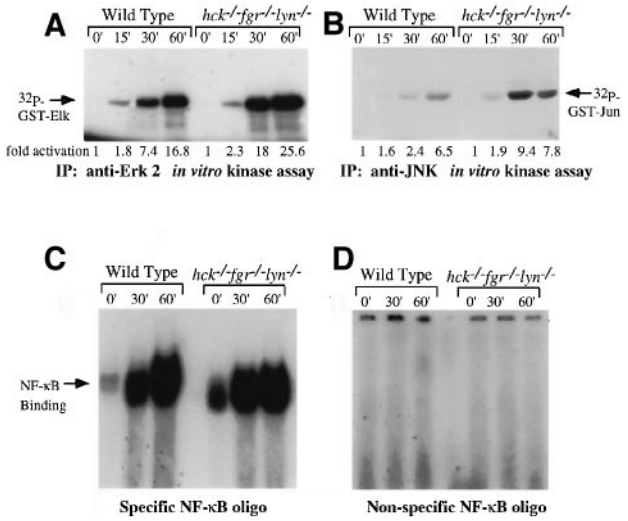

Fig. 10. Normal activation of MAP kinases and NF-κB in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs following integrin-dependent adhesion. PEMs from wild-type and mutant mice were plated on Fn for the indicated times and cell lysates were prepared. A 100 μg aliquot of each cell lysate was used for immunoprecipitation with polyclonal antibodies against Erk2 (**A**) and JNK (**B**) kinase, respectively. Immunoprecipitates were used for *in vitro* kinase assays using Elk-1 as a substrate for Erk2 and c-Jun fusion protein as a substrate for JNK kinase. Phosphorylated substrates were resolved by 12% PAGE and quantitated by phosphoimager analysis. The fold induction of kinase activity was determined relative to wild-type non-adherent (0′) cells. There was no detectable p38 activation in response to adhesion (data not shown). (**C**) Wild-type and mutant PEMs were plated on Fn for the indicated times, following which nuclear extracts were prepared as described in Materials and methods. Nuclear extracts (5 μg) were incubated with a [α-^{32}P]dCTP-labeled oligonucleotide corresponding to the murine immunoglobulin κ enhancer site and NF-κB binding activity was determined by electrophoretic mobility shift assay (EMSA). (**D**) Nuclear extracts were incubated with an [α-^{32}P]dCTP-labeled oligonucleotide containing a mutation in the NF-κB recognition site to confirm that binding was NF-κB specific.

was normal in triple mutant macrophages (Figure 10C). Activation of NF-κB plays a primary role in adhesion-dependent induction of pro-inflammatory cytokine expression in blood monocytes (Haskill *et al*., 1991). We also found no impairment of adhesion-induced secretion of IL-1β in mutant cells (data not shown). Hence, we conclude that Src-family kinases are not required for the induction of downstream MAP kinase activation or stimulation of NF-κB following adhesion to Fn.

Discussion

Cell adhesion mediated by the binding of surface integrins to ECM proteins leads to signaling events which control cytoskeletal organization, gene expression and cell growth/survival. Along with the advances in identifying the tyrosine kinases which regulate these signaling pathways, the list of potential substrates of these kinases has increased dramatically. Recently, the Cbl protein has been shown to become tyrosine phosphorylated in several hematopoietic cell lines following integrin-mediated cell adhesion (Manie *et al*., 1997; Ojaniemi *et al*., 1997). Consistent with these reports, we too find that Cbl is tyrosine phosphorylated in an adhesion-dependent fashion using primary murine macrophages. Moreover, we show that Cbl associates with two of the major Src-family kinases expressed in primary cells, Fgr and Lyn. In macrophages derived from knockout mice lacking Hck, Fgr and Lyn, we find that integrin-mediated tyrosine phosphorylation of Cbl, its translocation to the cytoskeletal-associated subcellular fraction and its association with PI-3 kinase activity are impaired. The cytoskeletal localization of PI-3 kinase is also reduced in these cells, although activation of total cellular PI-3 kinase activity is unaffected. Defects in this signaling pathway (induced either pharmacologically, with antisense oligonucleotides or in the mutant cells) produce impaired cell spreading *in vitro* and in the knockout mice reduced macrophage recruitment *in vivo*.

These observations allow formulation of a model describing this novel integrin signaling pathway which regulates the initial events leading to actin cytoskeletal rearrangement (Figure 11). Following clustering of integrins by binding to ECM proteins, tyrosine kinases become activated. The mechanism by which Src-family kinases are activated involves dephosphorylation of their regulatory tail by the tyrosine phosphatase CD45 (Roach *et al*., 1997). Following activation, a number of substrates become tyrosine phosphorylated; among them is Cbl. Based on our observations, it is most likely that Fgr and Lyn are directly responsible for Cbl phosphorylation since these are the two kinases which we find associated with Cbl in Fn-adherent PEMs. An exclusive role for Fgr and Lyn in Cbl phosphorylation cannot be proven without generation of *fgr*$^{-/-}$*lyn*$^{-/-}$ double mutant animals, which we have not yet done, hence we cannot rule out that Hck may also contribute to Cbl phosphorylation following spreading on Fn. If Hck is not required for β_1 integrin-induced Cbl tyrosine phosphorylation, it would be predicted that *fgr*$^{-/-}$*lyn*$^{-/-}$ PEMs would show the same spreading defects as seen in triple mutant cells.

The phosphorylation of Cbl correlates with, and is probably required for, mobilization to the cytoskeletal-associated subcellular fraction. The mechanism by which Cbl becomes associated with the Triton X-100-insoluble subcellular fraction is unknown, though the association of Cbl with Src-family kinases may be responsible. Indeed, association of Cbl with Src (Tanaka *et al*., 1995; Ojaniemi *et al*., 1997), Fyn (Panchamoorthy *et al*., 1996), Lck (Donovan *et al*., 1994), Lyn (Tezuka *et al*., 1996) and Hck (Anderson *et al*., 1997) has been observed in other signaling pathways. Alternatively, Cbl may be brought to the cytoskeleton via association with other newly tyrosine-phosphorylated proteins, such as p130^{cas} (Khwaja *et al*., 1996), CrkL (Sattler *et al*., 1997), paxillin or tensin. In this process, Cbl also associates with PI-3 kinase. The Cbl–PI-3 kinase association is mediated by the SH2 domain of the p85 subunit of PI-3 kinase (Ojaniemi *et al*., 1997), which explains the requirement for Cbl phosphorylation to occur prior to association with PI-3 kinase. During this process, PI-3 kinase is translocated from the cytosol to the membrane/cytoskeleton; it is likely that Cbl–PI-3 kinase association is responsible for bringing PI-3 kinase to the cytoskeletal-associated subcellular fraction, since the impaired phosphorylation and translocation of Cbl in triple mutant macrophages correlate with the loss of PI-3 kinase in the Triton X-100-insoluble fraction. However, it is also possible that other adaptor proteins may facilitate translocation of PI-3 kinase (especially in non-hematopoietic cells which do not express Cbl). At

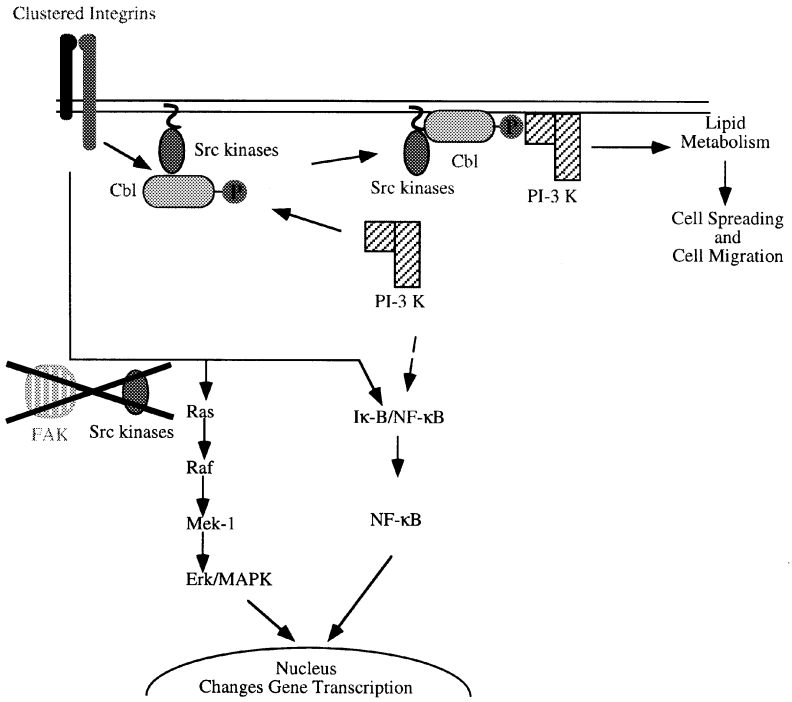

Fig. 11. Model for the role of Src-family kinases, Cbl and PI-3 kinase in integrin-mediated signaling. While the Src-family kinases, Cbl and PI-3 kinase are required for integrin-induced cell spreading and migration, neither the Src-family kinases nor FAK appear to be required for integrin signaling leading to MAPK or NF-κB activation.

the membrane the products of PI-3 kinase, the D_3 phosphoinositides, could then act on other cytoskeletal-associated molecules such as the small GTPase Rac or the actin regulatory proteins such as gelsolin, profilin and α-actinin (Toker and Cantley, 1997) to transmit further signaling reactions leading to cell spreading and migration.

The central role of PI-3 kinase in initiating actin cytoskeletal rearrangements, membrane ruffling and cell migration following integrin clustering has been demonstrated in several cell systems, including neutrophils, fibroblasts, platelets and, most recently, carcinoma cells (Eberle *et al*., 1990; Hartwig *et al*., 1996; King *et al*., 1997; Shaw *et al*., 1997). In all these systems, different integrin subunits are involved; however, in each case, integrin-mediated adhesion leads to activation of PI-3 kinase and accumulation of D_3 phosphoinositides. Treatment of cells with PI-3 kinase-specific inhibitors leads to blockade of cell spreading. In carcinoma cells, activation of PI-3 kinase has been associated with a specific integrin-dependent function: promotion of invasion *in vitro*. However, in none of the previous studies was the recognition that association with Cbl (or other adaptor proteins expressed in non-hematopoietic cells) and cytoskeletal translocation of PI-3 kinase are critical events leading to actin cytoskeletal rearrangement. In this regard, it is the fortuitous observation in the Src-family kinase knockout macrophages that total cellular activation of PI-3 kinase is normal yet the association of PI-3 kinase with Cbl and translocation of PI-3 kinase activity are impaired, which allows us to infer that the cytoskeletal localization of the PI-3 kinase–Cbl complex (not just overall activation of lipid kinase activity) is the critical event in regulating integrin-induced cytoskeletal rearrangements. We believe that the abnormal subcellular localization of PI-3 kinase in adherent *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ macrophages is a cause of the abnormal cytoskeletal actin in these cells rather than an effect of it. This is based on the rationale that treatment of wild-type cells with Cbl antisense oligonucleotides or PI-3 kinase inhibitors produces the same cellular phenotype of impaired Fn-induced cell spreading observed in the mutant macrophages. It is likely that the non-Cbl-associated PI-3 kinase is involved in other signaling processes. Recently, PI-3 kinase has been identified as a potential transducer in IL-1-induced NF-κB activation (Reddy *et al*., 1997). Based on this observation, it is plausible to speculate that integrin-induced activation of non-Cbl-associated PI-3 kinase may be responsible for the normal NF-κB activation seen in *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ macrophages.

The experiments presented in this study were done using primary macrophages. Therefore, the model of Hck/Fgr/Lyn phosphorylation of Cbl, formation of the Cbl–PI-3 kinase complex and translocation of the complex to the membrane can only be applied directly to β_1 integrin signaling in macrophages. Since the Hck, Fgr and Lyn kinases as well as the adaptor protein Cbl are found primarily in hematopoietic cells, it is possible that in non-hematopoietic cells different Src-family kinases may phosphorylate other adaptor proteins which in turn bind to PI-3 kinase and translocate it to the membrane. Further study of the general applicability of this model to β_1 integrin signaling in all cell types is important.

Our data also demonstrate that in macrophages the downstream signaling pathways such as MAP kinase or NF-κB activation that follow integrin clustering do not require the major Src-family kinases expressed in these cells. A similar conclusion on the role of FAK in integrin

signaling has also been reported, i.e. that FAK is not required for integrin-induced MAP kinase activation in fibroblasts (Lin *et al*., 1997). Hence the possibility that dual and separable integrin signaling pathways lead to actin cytoskeletal rearrangements versus activation of the MAP kinase cascade may be a generalizable conclusion. It should be noted that FAK is expressed very poorly in monocytes/macrophages (Lin *et al*., 1994; De Nichilo and Yamada, 1996; Roach *et al*., 1997; our results), hence it is likely that the Src-kinases play the predominant role in adhesion signaling in these cells. Similarly, the FAK-related protein Pyk2, while being highly expressed in macrophages, is not activated following adhesion to Fn (data not shown).

The definition of this β_1 integrin-signaling pathway may provide a molecular understanding of the osteopetrotic phenotype seen in *src*$^{-/-}$ mice. These animals develop a bony overgrowth due to the inability of their osteoclasts to form ruffled border membranes required for bone resorption (Boyce *et al*., 1992; Lowe *et al*., 1993). This is similar to what is seen when integrin-mediated osteoclast adhesion is blocked with RGD peptides (Ross *et al*., 1993). Tanaka *et al*. have demonstrated that Cbl and Src associate in osteoclasts and that Cbl is poorly phosphorylated in *src*$^{-/-}$ osteoclasts (Tanaka *et al*., 1995). Treatment of wild-type osteoclasts with Cbl antisense oligonucleotides blocked bone-resorbing activity, thus establishing a Src–Cbl signaling pathway required for functional activity in these cells. It is possible that the impaired Cbl phosphorylation seen in *src*$^{-/-}$ osteoclasts results in inefficient transfer of PI-3 kinase to the membrane, thus preventing the proper cytoskeletal rearrangements needed for adhesion to bone matrix and ruffled-border formation.

The physiological consequences of impaired integrin signaling have been difficult to study in *in vivo* models; most analyses have depended on *in vitro* cell functional studies. The use of the triple mutant mice allows us to perform these *in vivo* experiments. As reported here, mutant mice show a dramatic block in *in vivo* macrophage migration in a peritonitis model. We have also found that double mutant *hck*$^{-/-}$*fgr*$^{-/-}$ mice have defects in neutrophil migration in an endotoxin model (Lowell and Berton, 1998) despite the fact that macrophage adhesion from these mice is not as profoundly affected as in triple mutant cells. Additionally, by breeding the *hck, fgr* and *lyn* mutations into *mev/mev* autoimmune animals, we have found that a reduction in leukocyte adhesion and extravasation is sufficient to rescue the mice from progressive arthritis and lethal pneumonitis (R.Holmes and C.A.Lowell, unpublished). Hence, in all these *in vivo* models, it appears that loss of integrin signaling has many of the same consequences on inflammatory processes as blockade of leukocyte integrins. This opens up the potential that therapeutics directed at molecules involved in regulation of integrin signaling leading to actin cytoskeletal rearrangements may be a novel approach to treatment for inflammatory diseases.

Materials and methods

Mice and thioglycolate-elicited peritoneal macrophages

hck$^{-/-}$, *fgr*$^{-/-}$, *lyn*$^{-/-}$ and *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice were generated as described by Lowell *et al.* (1994), Chan *et al.* (1997) and Meng and Lowell (1997), respectively. Isolation of thioglycolate-elicited PEMs was performed as described by Lowell *et al.* (1994). In brief, PEMs were flushed from the peritoneum 4 days after thioglycolate injection and starved for 2 h in Dulbecco's modified Eagle's medium (DMEM) without serum on a rotator before plating on substrates. Flow cytometry confirmed that, on average, >90% of the peritoneal exudate cells were F4/80-positive macrophages, 1% were Gr-1-positive neutrophils and <10% were lymphocytes (B220- or CD3-positive B- and T-cells). After plating on Fn, non-adherent cells (lymphocytes) were washed away with phosphate-buffered saline (PBS) and attached cells were 99% F4/80-positive macrophages.

Complete peripheral blood leukocyte counts were obtained by collection of 200–300 μl of blood by retroorbital sinus bleeding into EDTA-containing microtubes (Becton-Dickinson, San Jose, CA). Samples were then sent to Consolidated Veterinary Diagnostics (CVD; W. Sacramento, CA) for automated blood counts and differentials. There was no difference in the peripheral blood leukocyte counts in resting or thioglycolate-treated mice.

Photomicroscopy, inhibitors and antisense oligonucleotides

Wild-type and mutant PEMs were plated on Fn-coated 6-well dishes then photographed under phase microscopy using a Zeiss inverted microscope. To test the effect of metabolic inhibitors on cell spreading, cells were treated in suspension with 100 nM wortmannin (Sigma Chemical Co.), 10 μM of the more specific PI-3 kinase inhibitor LY294002 (Sigma), or with 20 μM of the Src-family-specific tyrosine kinase inhibitor PP1 (Hanke *et al*., 1996) (Calbiochem, Foster City, CA) for 1 h prior to plating. To examine the effects of oligonucleotides directed against Cbl on macrophage spreading, cells were treated with 10 μM phosphorothioate-modified oligonucleotides for 2 h prior to plating. The sequence of the sense, antisense and control scrambled and inverted oligonucleotides are exactly as described (Tanaka *et al*., 1996). The sense, scrambled and inverted oligonucleotides were used as controls to rule out non-specific chemical effects of antisense oligomers and to demonstrate specificity for Cbl inhibition. Cell spreading was monitored over the course of several hours in the presence of all inhibitors.

In vivo migration assay

Mice were injected with 3% thioglycolate (2 ml/mouse) and the peritoneum was flushed with PBS/0.2% bovine serum albumin (BSA) at the indicated times post-thioglycolate injection. Total cell numbers were counted and analyzed by flow cytometry on a Becton-Dickinson FACScan using CellQuest software. Cells were stained with anti-Gr-1 for neutrophils, anti-F4/80 for macrophages, anti-B220 for B-cells and CD3 for T-cells (Pharmingen). Total cell numbers corresponding to each cell type were calculated based on percentages determined by flow cytometry. This experiment was performed on four separate cohorts of animals.

Adhesion of PEMs to protein-coated plates

Six-well tissue culture plates (Falcon) were coated with Fn (10 μg/ml, Sigma Co, St Louis, MO), vitronectin (10 μg/ml, Sigma Co.), polylysine (50 μg/ml, Collaborative Biomedical Products, Bedford, MA) and anti-integrin antibodies (Abs) (20 μg/ml, Pharmingen, San Diego, CA) against the α_4, α_5 and β_1 subunits overnight at 4°C and washed with cold PBS before use. Isolated PEMs were starved in serum-free media for 1 h in polypropylene tubes on a rotator prior to plating on ECM- or Ab-coated 6-well dishes. A total of 5×10^6 cells were plated per well, plates were centrifuged for 10 s, then incubated at 37°C for the indicated times. Following incubation, adherent cells were washed with cold PBS to remove all non-adherent cells, and then lysed in lysis buffer as described by Meng and Lowell (1997). For Ab cross-linking experiments, cells in suspension were incubated with hamster anti-rat integrin β_1 (10 μg/ml) for 10 min at 4°C followed by addition of F(ab′)$_2$ goat anti-hamster IgG (80 μg/ml, Boehringer Mannheim, Indianapolis, IN) for 10 min at 37°C. Cells were then washed with cold PBS and lysed in lysis buffer. For experiments involving immune complex kinase assays, adherent cells were lysed in an NP-40 lysis buffer as described (Ojaniemi *et al*., 1997).

Immunoprecipitation and immunoblotting

Protein extracts were pre-cleared with protein A–Sepharose/rabbit Ig conjugates at 4°C for 1 h and then incubated with specific polyclonal antisera followed by protein A–Sepharose (Sigma Chemical Co., St Louis, MO) at 4°C for 1 h each. The immunocomplexes were washed three times with lysis buffer and eluted by boiling in 2× Laemmli sample buffer [2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT), 60 mM Tris–HCl pH 6.8 and 0.001% bromophenol blue] for 5 min. The immunoprecipitated proteins were resolved on 8% SDS–PAGE,

transferred to nitrocellulose and immunoblotted with appropriate Abs. Nitrocellulose membranes were blocked either with 5% milk or 10% goat serum (in TBS + 0.2% Tween-20) for polyclonal antibodies, or 2% BSA (in TBS + 0.2% Tween-20) for monoclonal antibody (mAb) blotting. The anti-phosphotyrosine mAb used was 4G10 (UBI, Lake Placid, NY), while the anti-Hck, anti-Fgr and anti-Lyn polyclonal Abs used were as described (Chan *et al*., 1997; Meng and Lowell, 1997). Anti-Cbl antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Immunoblots were incubated for several hours at room temperature, washed with TBS, then incubated with perioxidase-labeled secondary Abs (anti-mouse for mAbs, anti-rabbit for polyclonal Abs; all from Boehringer) for 1 h at room temperature. Membranes were re-washed then developed with ECL reagents (Amersham Co, Arlington Heights, IL). For re-probing, membranes were stripped in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl pH 6.7) at 50°C for 30 min and re-blocked with appropriate blocking solution before incubation with appropriate antibodies.

In vitro kinase assays

Adherent cells were lysed in NP-40 buffer and immunoprecipitated with appropriate antibodies and protein A–Sepharose beads for 1 h each at 4°C. After washing twice with NP-40 buffer and twice with kinase buffer (20 mM HEPES pH 7.0, 10 mM $MgCl_2$, 100 mM $MnCl_2$, 0.5% Triton X-100, 0.1 mM $NaVO_3$), an *in vitro* kinase assay was performed using kinase buffer containing 0.5 μCi of [γ-^{32}P]ATP for each reaction. Reactions were carried out for 15 min at room temperature. For Cbl/Src-family kinase co-immunoprecipitation experiments, the ^{32}P-labeled proteins were released from immunoprecipitates by boiling in releasing buffer (Ojaniemi *et al*., 1997) followed by 10-fold dilution with RIPA buffer. A second round of immunoprecipitation was carried out by addition of the appropriate antibodies and protein A–Sepharose beads. The immunoprecipitated proteins were electrophoresed in 10% SDS–PAGE, gels were fixed in 30% methanol and 10% glacial acetic acid and subjected to autoradiography. Some gels were subjected to alkaline hydrolysis in 1 M KOH to validate that proteins were phosphorylated on tyrosine residues.

For MAP kinase assays, cell lysates (100 μg) were immunoprecipitated with polyclonal antibodies against Erk2, JNK1 and p38 (Santa Cruz Biotechnologies) at 4°C for 1 h followed by incubation with protein A–Sepharose beads for another hour. The beads were washed twice with kinase buffer and *in vitro* kinase assays were performed as described by Meng and Lowell (1997). Elk-1 was used as a substrate for Erk2 and p38 while c-Jun–GST fusion protein was used as a substrate for JNK kinase.

For PI-3 kinase assays, cells in suspension and adherent cells were lysed and immunoprecipitated with anti-p85 monoclonal antibody (UBI, Lake Placid, NY) or anti-Cbl polyclonal antibody. The PI-3 kinase activity assay was carried out exactly as described (Gold *et al*., 1992). To increase the specificity of the lipid kinase assays, adenosine was added to all reactions to inhibit PI-4 kinase activity. The fold activation of PI-3 kinase activity was quantitated by phosphoimager analysis.

Northern blots

Total RNA was isolated from PEMs using Ultraspec™ RNA (Bioteck, Houston, TX). Aliquots of 20 and 5 μg of total RNA per lane were electrophoresed on a 1% agarose/6% formaldehyde gel (Sambrook *et al*., 1989) then transferred to an N^+ nylon (Amersham) membrane. Membranes were hybridized with an antisense *cbl* RNA probe of 600 bp. To synthesize the antisense *cbl* RNA probe, a *Pst*I–*Eco*RI fragment from the 5′ region of the murine *cbl* cDNA clone (gift of Dr W.Langdon) was subcloned into a pGEM3 vector (Promega, Madison, WI). This subclone was linearized with *Bgl*I and used as a template for *in vitro* transcription using an SP6 RNA polymerase kit from Promega. For the human β-actin probe, human actin cDNA was labeled randomly *in vitro* with [α-^{32}P]dCTP. The membrane was hybridized at 42°C overnight under high stringency conditions (50% formamide, 5× SSPE, 2× Denhart's and 0.1% SDS). Following the first wash in 1× SSPE and 0.5% SDS at room temperature, the membrane was washed continuously in 0.1× SSPE and 0.1% SDS at 65°C until no radioactivity could be detected in washing buffer. The Northern blot was exposed to hyperfilm (Amersham) for 6 h at –80°C before developing.

Triton X-100 fractionation

PEMs were plated on Fn-coated plates, incubated at 37°C for the indicated times, then chilled on ice for 6 min, washed with cold PBS and lysed in Triton X-100 lysis buffer [0.3 M sucrose, 0.5% Triton X-100, 10 mM PIPES, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM $Na_3(VO)_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of leupeptin, pepstatin and aprotinin] for 3 min. After a brief wash with cold Triton X-100 lysis buffer (<5 s), the remaining cells were lysed further in modified RIPA buffer [50 mM HEPES, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM $Na_3(VO)_4$ and 1 μg/ml of leupeptin, pepstatin and aprotinin] then scraped off the dish. Cell debris was removed by centrifugation at 14 000 r.p.m. at 4°C for 5 min and clear supernatants were transferred to fresh tubes. Equal amounts of total protein from the Triton X-100-soluble and -insoluble fractions from each time point of adhesion were used for Cbl immunoblotting or PI-3 kinase analysis. To achieve a comparable level of Cbl between wild-type and triple mutant macrophages, a 3-fold excess of triple mutant total cellular protein was used for Cbl immunoblotting and Cbl-associated PI-3 kinase assays.

NF-κB translocation assay

PEMs were plated on Fn-coated plates for the indicated times and nuclear extracts were prepared exactly as described (Meng and Lowell, 1997). Wild-type oligonucleotides corresponding to the NF-κB-binding site were used for specific binding while mutant oligonucleotides were used as control to assess for non-specific binding. The oligonucleotides were synthesized, annealed and labeled as described by Hambleton *et al*. (1995).

Acknowledgements

This work was supported by NIH grants DK50267 and HL54476 to C.A.L. F.M. is supported by NIH training grant DK07636. C.A.L. also acknowledges the Howard Hughes Medical Institute RRP program for support of laboratory renovation.

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Received April 3, 1998; revised June 3, 1998; accepted June 4, 1998