Role of phosphoinositide 3-kinase and the Cbl adaptor protein in coupling the $\alpha4\beta1$ integrin to mitogen-activated protein kinase signalling

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Cell adhesion mediated by $\beta1$ integrin receptors leads to the initiation of intracellular signals that affect cell differentiation and survival. Here we have analysed the mechanism by which the $\alpha4\beta1$ integrin activates the mitogen-activated protein kinase pathway in HL60 cells, a myelomonocytic cell line that lacks the expression of focal adhesion kinase. A role for phosphoinositide 3-kinase (PI-3K) in $\alpha4$ integrin-mediated activation of extracellular signal-regulated protein kinase 2 (ERK2) is suggested by the ability of PI-3K inhibitors and a dominant-negative form of the p85 subunit of PI-3K to block the activation of ERK2 by integrin. Stimulation of $\alpha4\beta1$ integrins on HL60 cells also leads to increased tyrosine phosphorylation of the 120 kDa adaptor protein Cbl. PI-3K activity associated with Cbl also increases on the stimulation of $\alpha4\beta1$ integrins, although immunodepletion experiments suggest that Cbl-associated PI-3K does not account for all of the PI-3K activity induced on the stimulation of integrins in these cells. The expression of wild-type Cbl or the 70Z/3 Cbl mutant enhances basal ERK2 activity in transfectants with a minimal effect on $\alpha4$ integrin-mediated ERK2 activity. In contrast, overexpression of the Hut Cbl truncation mutant, which does not associate with p85, has no effect on the ERK2 pathway. These results suggest that PI-3K has a major role in coupling $\alpha4\beta1$ integrins to ERK2 activation in myeloid cells and that the Cbl adaptor protein has a role in basal, but not $\alpha4\beta1$ integrin-mediated, activation of ERK2.

Key words: adhesion, extracellular signal-regulated protein kinase 2, tyrosine phosphorylation.

INTRODUCTION

Cells use $\beta1$ integrin receptors to initiate and maintain adhesive interactions with extracellular matrix proteins and cellular counter-receptors [1]. In addition, ligand engagement of integrins results in the initiation of intracellular signalling pathways that affect cell survival and differentiation [2]. One critical signalling event that occurs on the stimulation of $\beta1$ integrin is activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated protein kinase (ERK) isoforms ERK1 and ERK2 [3–5]. In the THP-1 monocytic cell line, $\alpha4\beta1$ integrin-mediated increases in ERK1/ERK2 activity have been specifically implicated in tissue factor expression [5].

In addition to the activation of MAPK, the stimulation of $\beta1$ integrins also activates the lipid kinase phosphoinositide 3-kinase (PI-3K), which is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. PI-3K phosphorylates the D-3 position of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 to generate PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [6]. The attachment of Cos-7 cells to fibronectin results in the accumulation of the PI-3K products PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 as well as the activation of the PI-3K-dependent Akt kinase [7]. PI-3K is also activated on $\beta1$ integrin-mediated cell adhesion of mouse macrophages [8,9]. Taken together, these studies indicate that PI-3K might be a key molecule in $\beta1$ integrin-mediated signalling pathways. Interestingly, cross-talk between the PI-3K pathway and ERK signalling has been suggested by the ability of the constitutively active form of the p110γ subunit of PI-3K to activate MAPK [10].

Tyrosine phosphorylation is likely to have a critical role in integrin signalling, given the growing number of proteins that have been reported to be tyrosine-phosphorylated in various cell systems on the activation of integrins [2]. Recent studies have shown that the stimulation of $\beta1$ integrins in mouse macrophages, fibroblasts and human B cells leads to increased tyrosine phosphorylation of the multi-domain docking protein Cbl [8,9,11,12]. Cbl is the cellular counterpart of the Cas NS-1 murine leukaemia retroviral oncogene v-*cbl* and is primarily expressed in haemopoietic cells [13]. The product of the c-*cbl* proto-oncogene contains many tyrosine residues and a proline-rich region that allows the protein to interact *in vitro* with several Src homology 2 (SH2) and Src homology 3 (SH3) domain-containing proteins such as Fyn, Lck, Grb2, Nck, Crk-L and the p85 subunit of PI-3K [14–18]. Analyses of Cbl function in several species indicate a role for Cbl as a negative regulator of tyrosine kinase and MAPK signalling [19–23]. However, PI-3K and phospholipase Cγ activity is decreased in Cbl-deficient thymocytes, suggesting that Cbl might also positively regulate certain signalling pathways [22].

In this report we have examined membrane-proximal $\alpha4\beta1$-stimulated signalling events in HL60 cells. We have focused in particular on the upstream requirements for PI-3K in $\beta1$ integrin-mediated activation of ERK2, and the role of Cbl in this process. Activation of $\alpha4$ integrins in HL60 cells results in the activation of ERK2 and PI-3K. Pharmacological and genetic inhibition of PI-3K signalling demonstrates a role for PI-3K in $\alpha4$ integrin-mediated activation of ERK2. Furthermore, the stimulation of $\alpha4$ integrin results in tyrosine phosphorylation of Cbl and

Abbreviations used: ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PI-3K, phosphoinositide 3-kinase; H2, Src homology 2; WT, wild-type.

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recruitment of PI-3K activity to Cbl. Although the stimulation of α4 integrin results in activation of PI-3K-associated Cbl, the expression of wild-type (WT) and mutant forms of Cbl primarily results in increased basal activity of ERK2, with a minimal effect on α4 integrin-mediated enhancement of ERK2 activity.

EXPERIMENTAL

Antibodies

The anti-Cbl and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-glycophorin monoclonal antibody (mAb) 10F7 was purchased from the American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.). The anti-α4 mAb NIH49d-1 was kindly provided by Dr. S. Shaw (National Institutes of Health, Bethesda, MD, U.S.A.). The anti-phosphotyrosine mAb PY20 was kindly provided by Dr. M. Kamps (University of California, San Diego, CA, U.S.A.). The anti-phosphotyrosine mAb 4G10 and anti-p85 antibody were purchased from Upstate Biotechnologies (Lake Placid, NY, U.S.A.). The anti-(green fluorescent protein) (anti-GFP) antibody was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The anti-haemagglutinin (anti-HA) rat mAb was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The glutathione S-transferase (GST) vector and GST fusion protein expressing the SH2 domain of Crk were kindly provided by Dr. R. Herrera and Dr. S. Hubbell (Warner-Lambert/Parke-Davis, Ann Arbor, MI, U.S.A.).

Cells

The cell line HL60 was maintained in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum (FCS) (Atlanta Biologicals, Atlanta, GA, U.S.A.), L-glutamine and penicillin/streptomycin. Cells were serum-starved for 12–16 h before each assay. Cells were washed once in serum-free RPMI and resuspended at 10^6 cells/ml in serum-free medium or 0.1% (v/v) FCS medium.

DNA constructs

The SRα-HAERK2 construct was kindly provided by C. Rosette/M. Karin (University of California, San Diego, CA, U.S.A.). The pEGFP–C2 vector was purchased from Clontech Laboratories. The SRα-WT p85 and SRα-Δp85 vectors were kindly provided by W. Ogawa and M. Kasuga (Kobe University School of Medicine, Kobe, Japan). The pEGFP–Δp85 vector was constructed as described previously [24]. In brief, a 2.1 kb *Kpn*I fragment containing Δp85 was inserted in-frame into the *Kpn*I site of pEGFP–C2. The resulting vector encoded a 115 kDa fusion protein consisting of GFP fused to the N-terminal end of Δp85. To create the pEGFP–WT p85 vector, SRα-WT p85 was digested with *Eco*RI, overhanging ends were filled in, and phosphorylated *Bam*HI linkers were added. Subsequently, the DNA was digested with *Kpn*I to release a 2.1 kb *Kpn*I/*Bam*HI fragment, which was then subcloned into the *Kpn*I/*Bam*HI site of pEGFP–C2. The original Cbl, 70Z/3 Cbl and HUT Cbl vectors were kindly provided by Dr. W. Langdon (University of Western Australia, Nedlands, Western Australia). To create the pEGFP–c-Cbl and pEGFP–70Z/3-Cbl vectors, a 2.7 kb fragment was removed from the original vectors by complete digestion with *Sal*I and partial digestion with *Sac*I. The fragment was then cloned into the *Sac*I/*Sal*I site of the pEGFP–C2 vector. For the pEGFP–HUT-Cbl vector, a 2.4 kb fragment was prepared in the same manner.

Anti-phosphotyrosine assay

HL60 cells (15×10^6) were serum-starved overnight in RPMI containing 0.1% (v/v) FCS, washed once with Leibovitz's medium (L15) (Life Technologies, Grand Island, NY, U.S.A.) and resuspended in 1 ml of L15 medium. Cells were incubated with 15 μg/ml human IgG (Organon Teknika, Durham, NC, U.S.A.) for 15 min on ice and then with the indicated mAb at 15 μg/ml for 30 min on ice. Cells were washed once with L15 medium and resuspended in 1 ml of L15 containing 7.5 μg of goat anti-mouse IgG (Organon Teknika). Cells were incubated for 30 min on ice, divided into aliquots in 1.5 ml centrifuge tubes, then placed in a water bath at 37 °C for the indicated durations. Cells were pelleted by centrifugation at 11000 ***g*** for 1 min and then lysed by the addition of 1 ml of RIPA buffer [1% (v/v) Triton/1% (w/v) deoxycholic acid/158 mM NaCl/10 mM Tris/HCl (pH 7.2)/5 mM EDTA] containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium vanadate and 1 mM PMSF, followed by incubation on ice for 15 min. Lysates were microcentrifuged at 11000 ***g*** at 4 °C for 25 min. Protein content was quantified with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.); equivalent amounts of protein from each lysate supernatant were immunoprecipitated with Protein A–Sepharose beads (Zymed, South San Francisco, CA, U.S.A.) precoated with the anti-phosphotyrosine mAb PY20 or with anti-Cbl. Anti-glycophorin immunoprecipitations were performed with goat anti-mouse Sepharose beads. GST and GST–SH2-Crk precipitations were performed with glutathione beads conjugated with GST alone or with the GST fusion protein. Beads were washed twice in RIPA buffer. Samples were boiled for 5 min in 1 × SDS sample buffer and separated by SDS/PAGE [10% (w/v) gel]. After transfer to a PVDF membrane, the blot was blocked in PBS/5% (v/v) milk overnight at 4 °C and then immunoblotted with a 1:2000 dilution of the anti-phosphotyrosine mAb 4G10 in PBS/5% (v/v) milk for 2 h at room temperature. The blot was washed three times (10 min each) with PBS/0.1% (v/v) Tween and then twice with PBS, followed by incubation with a 1:10000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Caltag, South San Francisco, CA, U.S.A.) in PBS/5% (v/v) milk for 1.5 h at room temperature. The blot was washed six times (10 min each) with PBS/0.1% (v/v) Tween followed by detection by enhanced chemiluminescence (Pierce). The membrane was then stripped by incubation at 50 °C for 30 min in stripping buffer [62.5 mM Tris/HCl (pH 6.8)/2% SDS/0.1 M 2-mercaptoethanol] and reprobed with anti-Cbl [0.5 μg/ml in PBS/5% (v/v) milk] followed by a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL, U.S.A.). Further reprobing involved immunoblotting with anti-p85 [1:1000 dilution in PBS/5% (v/v) milk] followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG.

Lysates for sequential precipitation experiments were prepared as described above. These lysates were incubated sequentially with four aliquots of Protein A–Sepharose beads coated with anti-Cbl antibody, followed by incubation with an aliquot of Protein A–Sepharose beads coated with the anti-phosphotyrosine antibody PY20. Each immunoprecipitate was separated by SDS/PAGE [7.5% (w/v) gel] and transferred to PVDF membrane. The membrane was blotted as described above with the anti-phosphotyrosine mAb 4G10. The membrane was then

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stripped as described above and reprobed with anti-Cbl or anti-p85.

PI-3K activity assay

Cells (20×10^6) were stimulated for the indicated durations as described above for anti-phosphotyrosine assays. Lysates were immunoprecipitated with rotation at 4 °C with Protein A–Sepharose beads coated with the anti-phosphotyrosine mAb PY20 or with an anti-Cbl antibody. PI-3K activity in immunoprecipitates was determined as described previously [25]. The labelled PtdIns was detected with a Molecular Dynamics PhosphorImager (Sunnyvale, CA, U.S.A.).

Lysates for the sequential PI-3K activity were prepared as described above for the sequential anti-phosphotyrosine assay. After lysate had been removed from beads, they were washed as described above and frozen at −70 °C until the entire sequential procedure was complete. Only the first and fourth Cbl immunoprecipitates were subjected to the PI-3K activity assay along with the fifth-step anti-phosphotyrosine immunoprecipitates and the uncleared anti-phosphotyrosine immunoprecipitates.

MAPK activity assay

Cells (10^6) in 0.5 ml of L15 medium were stimulated by mAb cross-linking as described above for anti-phosphotyrosine assays. For assays involving inhibitors, the indicated concentration of wortmannin (Sigma, St. Louis, MO, U.S.A.), LY294002 (Alexis Corporation, San Diego, CA, U.S.A.) or DMSO at the level of the highest inhibitor concentration was added during the 30 min incubation with goat anti-mouse IgG. Kinase activity in anti-ERK2 or anti-HA immunoprecipitates was assessed as described previously [25] by using myelin basic protein as an exogenous substrate. Labelled myelin basic protein was detected with a PhosphorImager. Statistical analysis of densitometric values from multiple experiments was performed with Student's t test.

Aliquots from each sample were separated by SDS/PAGE [10 % (w/v) gel] on a minigel, transferred to PVDF and immunoblotted with an anti-ERK2 antibody [0.5 μg/ml in PBS/5 % (v/v) milk] followed by a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG to confirm equivalent amounts of ERK2 or HA-ERK2 protein in each sample.

Transient transfection

Cells were maintained at approx. 0.5×10^6 cells/ml before transfection; 10^7 cells were washed twice in Opti MEM medium (Life Technologies) and resuspended in 200 μl of Opti MEM. SRα-HAERK2 DNA (25 μg) and 75 μg of pEGFP–C2 (or pEGFP–Δp85, pEGFP–WT p85, pEGFP–c-Cbl, pEGFP–70Z/3-Cbl or pEGFP–HUT-Cbl) were added to cells and left for 10 min at room temperature. Cells were electroporated in cuvettes with a 4 mm gap by using a BTX (San Diego, CA, U.S.A.) square-wave electroporator set at 230 V for one 15 ms pulse and then left to recover for 30 min at room temperature before resuspension in RPMI containing 0.1 % (v/v) FCS at 10^6 cells/ml. Cells were harvested 16–20 h after electroporation and purified on a Ficoll (Sigma) gradient to remove dead cells and debris.

Approx. 2×10^5 transiently transfected cells were analysed for GFP expression in the FL1 channel of a FACScan (Becton-Dickinson, Mountain View, CA, U.S.A.). The percentage of GFP$^+$ cells was determined in comparison with untransfected HL60 cells.

GFP fusion protein immunoblotting

Cells were transiently transfected as described above with 75 μg of pEGFP–C2, pEGFP–c-Cbl, pEGFP–70Z/3-Cbl or pEGFP–HUT-Cbl per 10^7 cells. The number of GFP$^+$ cells was calculated from the percentage of GFP$^+$ determined by flow cytometry. Equal numbers of GFP$^+$ cells from each transfectant were stimulated for 2 min by mAb cross-linking as described above for the anti-phosphotyrosine assays. Cell lysates were then immunoprecipitated with anti-GFP–coated Protein A–Sepharose beads as described above. The samples were separated by SDS/PAGE [7.5 % (w/v) gel] on a mini-gel, transferred to PVDF and blotted as described above with the anti-phosphotyrosine mAb 4G10. The membrane was then stripped as described above and reprobed with anti-p85 polyclonal antibody or with anti-Cbl or anti-Cbl-b polyclonal antibody.

RESULTS

Effects of inhibiting PI-3K on the α4 integrin-mediated activation of MAPK

The HL60 myelomonocytic cell line expresses the $\alpha4\beta1$ integrin [26] and activation of $\alpha4\beta1$ with an $\alpha4$-specific antibody resulted in the activation of the ERK2 isoform of MAPK that peaked at 10 min of stimulation (Figures 1A and 1B, and results not shown). To determine whether PI-3K has a role in the $\alpha4$-stimulated activation of ERK2, we performed the MAPK activity assay in the presence of two chemically distinct PI-3K inhibitors, wortmannin and LY294002 [27,28]. Wortmannin inhibits $\alpha4$-stimulated ERK2 activity in a dose-dependent manner but has little effect on the control-stimulated activity (Figure 1A). At 100 nM, wortmannin completely inhibited the $\alpha4$-stimulated increase in ERK2 activity (Figure 1A). Consistent with the wortmannin results was the observation that treatment of HL60

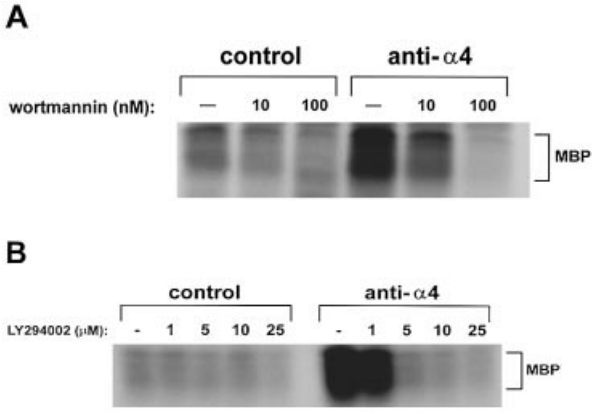

Figure 1 Wortmannin and LY294002 inhibit α4 integrin-mediated ERK2 activity

HL60 cells were stimulated by mAb cross-linking for 10 min with the anti-glycophorin mAb 10F7 as a negative control (control) or with the anti-$\alpha4$ mAb NIH49d-1 (anti-$\alpha4$) and GAM IgG in the presence of DMSO or 10 nM or 100 nM wortmannin (**A**) or in the presence of DMSO or 1, 5, 10 or 25 μM LY294002 (**B**). Lysates were immunoprecipitated with an anti-ERK2 polyclonal antibody, incubated with ^{32}P and the substrate myelin basic protein (MBP), then separated by SDS/PAGE as described in the Experimental section. Radiolabelled myelin basic protein was detected by analysis with a Molecular Dynamics PhosphorImager. Results shown are from one representative experiment of a minimum of three.

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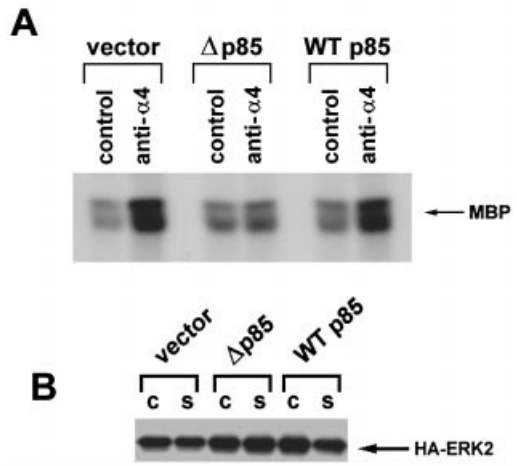

Figure 2 Effect of Δp85 and WT p85 on α4-stimulated ERK2 activity

(**A**) HL60 cells were transiently transfected with pEGFP–C2 (vector), pEGFP–Δp85 (Δp85) or pEGFP–WT p85 (WT p85) along with SRα-HAERK2 as described in the Experimental section. Transfectants were stimulated for 10 min by mAb cross-linking as described in the legend to Figure 1. Lysates were immunoprecipitated with an anti-HA mAb and analysed for ERK2 activity as described in the Experimental section. Results shown are from one representative experiment of a minimum of four. (**B**) Equivalent amounts of protein were used in the ERK2 kinase activity assay. Aliquots of control-stimulated (c) and α4-stimulated (s) samples from (**A**) were removed before the activity assay, separated by SDS/PAGE and immunoblotted with an anti-ERK2 polyclonal antibody as described in the Experimental section. Abbreviation: MBP, myelin basic protein.

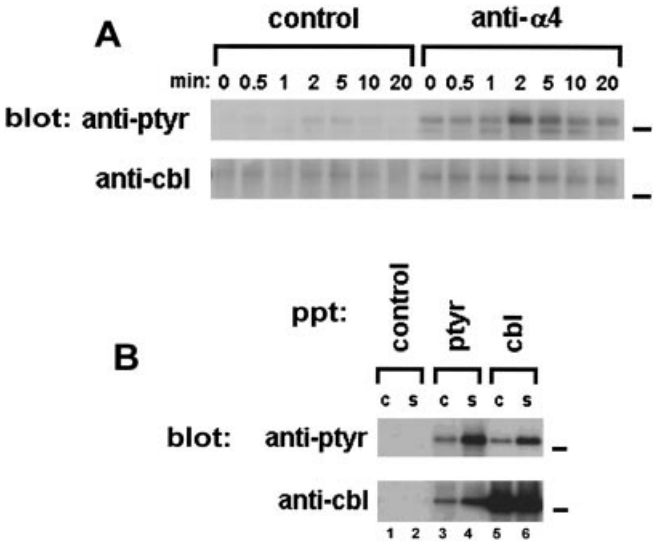

Figure 3 α4 stimulation leads to Cbl tyrosine phosphorylation

(**A**) HL60 cells were stimulated for the indicated durations as described in the legend to Figure 1. Cell lysates were immunoprecipitated with the anti-phosphotyrosine mAb PY20, separated by SDS/PAGE and immunoblotted with an anti-phosphotyrosine mAb as described in the Experimental section. The blot was stripped and reprobed with anti-Cbl. Results shown are from one representative experiment of a minimum of three. The 97 kDa band is indicated at the right. (**B**) HL60 cells were stimulated for 2 min as described in the legend to Figure 1. Control-stimulated (c) and α4-stimulated (s) cell lysates were immunoprecipitated with anti-glycophorin mAb (control; lanes 1 and 2), anti-phosphotyrosine mAb (ptyr; lanes 3 and 4), or anti-Cbl antibody (cbl; lanes 5 and 6), separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibody. The blot was stripped and reprobed with anti-Cbl. Results shown are from one representative experiment of a minimum of four. The 97 kDa band is indicated at the right.

cells with LY294002 also resulted in a dose-dependent inhibitory effect on α4-stimulated ERK2 activation, with the 5 μM dose almost completely abolishing the α4-mediated increase in ERK2 activity (Figure 1B).

We also assessed the effect of a transiently expressed dominant-negative p85 (Δp85) subunit of PI-3K on α4-stimulated activation of ERK2. The Δp85 construct that we used in this analysis contains a deletion of the binding site for the p110 catalytic subunit [29] and has been used extensively to block PI-3K activity in cells [24,25,29,30]. Both Δp85 and WT p85 were expressed as fusion proteins with GFP, permitting the efficient detection of transfected cells expressing the p85 constructs by flow cytometry. HL60 cells expressing GFP alone and untransfected cells were used as controls. After electroporation with GFP, GFP–Δp85 or GFP–WT p85, GFP expression was detected in 15–25% of the electroporated cells (results not shown).

The GFP–Δp85 and GFP–WT p85 fusion constructs were co-transfected with an HA-tagged ERK2 vector into HL60 cells. Anti-HA immunoprecipitates from unstimulated and α4-stimulated transfectants were then analysed for ERK2 activity. Cells transfected with the GFP control vector exhibited an increase in ERK2 activity on cross-linking of the α4β1 integrin that was similar to that observed for endogenous ERK2 activity after the stimulation of α4 integrins (Figure 2A). This result suggested that the expression of GFP alone did not affect α4-stimulated increases in ERK2 activity. In contrast, expression of the GFP–Δp85 construct markedly inhibited α4 integrin-stimulated increases in ERK2 activity (Figure 2A). In multiple experiments, activation of the GFP–Δp85 transfectant resulted in only an average 1.2-fold increase (S.D. 0.2) in ERK2 activity in comparison with control-stimulated cells from the same transfectant (Figure 2A). To determine whether the effects observed with Δp85 were due to deletion of the p110-binding site, we analysed cells transfected with the WT p85 GFP fusion protein. When compared with transfectants expressing GFP alone, there was partial inhibition of α4 integrin-mediated activation of ERK2 activity with expression of WT p85. In the experiment shown in Figure 2(A), there was a 3.1-fold increase in ERK2 activity after the stimulation of α4 integrins in GFP transfectants, whereas the α4 stimulation of transfectants expressing GFP–WT p85 resulted in a 1.9-fold increase in ERK2 activity. In multiple experiments, the stimulation of α4 integrins resulted in a 2.9-fold increase (S.D. 0.6) in ERK2 activity in the presence of co-transfected GFP, and a 2.7-fold increase (S.D. 0.9) in ERK2 activity in the presence of co-transfected GFP–WT p85 (results not shown). Thus the expression of WT p85 did not cause the marked inhibition of α4-stimulated ERK2 activity that we observed consistently with Δp85 expression (Figure 2A), despite comparable levels of expression. ERK2 immunoblotting of sample aliquots removed before the kinase assay revealed equivalent amounts of HA-tagged ERK2 in each of the samples (Figure 2B).

α4β1 Integrin cross-linking increases Cbl tyrosine phosphorylation

Although our results are consistent with studies in other cell systems in which the inhibition of PI-3K leads to the inhibition of integrin-mediated stimulation of MAPK activity [7], the mechanism by which β1 integrins are coupled to PI-3K remains unclear. Although many PI-3K-coupled cell surface receptors associate directly with the p85 subunit of PI-3K, we were unable to detect co-precipitated p85 or PI-3K activity in anti-β1 immunoprecipitates from either unstimulated or α4-stimulated HL60 cells (results not shown). Because the increased

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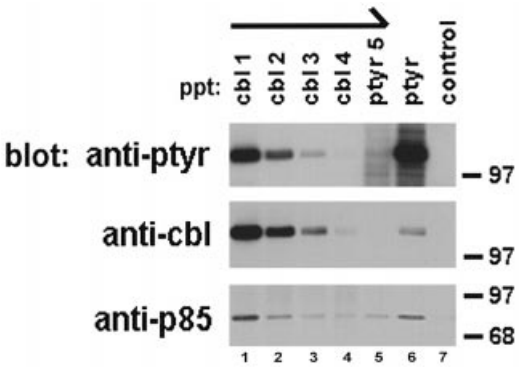

Figure 4 The majority of the 120 kDa tyrosine-phosphorylated protein is Cbl

HL60 cells were stimulated for 2 min as described in the legend to Figure 1. Lysates were immunoprecipitated (ppt) sequentially with four aliquots of Cbl beads (lanes 1–4) followed by immunoprecipitation with anti-phosphotyrosine (ptyr) beads (lane 5). Uncleared lysates were immunoprecipitated with anti-phosphotyrosine beads (lane 6) or anti-glycophorin beads (control; lane 7). Each immunoprecipitate was separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine, anti-Cbl or anti-p85 as described in the Experimental section. Results shown are from one representative experiment of a minimum of three. The molecular masses of bands are indicated (in kDa) at the right.

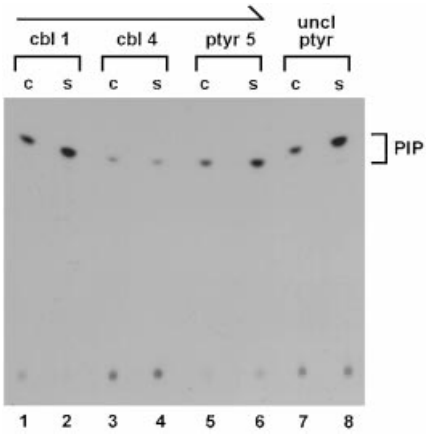

Figure 5 α4-stimulated PI-3K activity remains in phosphotyrosine immunoprecipitates after depletion of Cbl

HL60 cells were stimulated for 10 min as described in the legend to Figure 1. Lysates were immunoprecipitated with four aliquots of Cbl beads, followed by immunoprecipitation with anti-phosphotyrosine beads (ptyr). Uncleared lysates were immunoprecipitated with anti-phosphotyrosine beads (uncl ptyr). The first and fourth Cbl immunoprecipitates and the cleared and uncleared anti-phosphotyrosine immunoprecipitates were analysed for PI-3K activity as described in the Experimental section. Radiolabelled PtdIns (PIP) was detected by analysis with a Molecular Dynamics PhosphorImager. Results shown are from one representative experiment of a minimum of two.

tyrosine phosphorylation of intracellular proteins after receptor stimulation led to the assembly of intracellular signalling complexes containing PI-3K, we tested whether the stimulation of α4 integrin resulted in increased tyrosine phosphorylation. Figure 3(A) shows that the stimulation of α4 integrin led to a rapid increase in tyrosine phosphorylation of a 120 kDa substrate, with maximal tyrosine phosphorylation occurring at 2 min of α4 stimulation. No other tyrosine-phosphorylated substrates were seen after stimulation of α4 integrin (results not shown).

To determine the identity of this tyrosine-phosphorylated protein, we examined proteins in the 120 kDa range. The 125 kDa FAK protein was not detectable in anti-phosphotyrosine immunoprecipitates from α4-stimulated HL60 lysates and there was no detectable FAK protein in anti-FAK immunoprecipitates from these cells (results not shown). However, reprobing of the anti-phosphotyrosine immunoprecipitates from Figure 3(A) with an anti-Cbl antibody suggested that the 120 kDa band represented the adaptor protein Cbl. The identity of the 120 kDa protein was confirmed by anti-phosphotyrosine immunoblotting of anti-Cbl immunoprecipitates (Figure 3B).

Most of the 120 kDa tyrosine-phosphorylated protein was Cbl

To determine whether the entire 120 kDa tyrosine-phosphorylated band seen in an anti-phosphotyrosine immunoprecipitate consisted of Cbl, we used a sequential immunoprecipitation procedure in which α4-stimulated lysate was passed sequentially over four aliquots of anti-Cbl beads and then the Cbl-depleted lysate was immunoprecipitated with anti-phosphotyrosine beads. In addition, uncleared α4-stimulated lysates were immunoprecipitated with anti-phosphotyrosine beads or with anti-glycophorin beads as a negative control. The stimulated lysate was cleared of Cbl protein by the fourth immunoprecipitation step, as seen by anti-Cbl immunoblotting (Figure 4, lane 4). Accordingly, on removal of the Cbl protein there was progressively less tyrosine-phosphorylated Cbl (Figure 4, lanes 1–4). However, a small amount of tyrosine-phosphorylated material at 120 kDa could still be immunoprecipitated by an anti-phosphotyrosine mAb after immunodepletion of Cbl (Figure 4, lane 5). These results suggested that Cbl was the predominant substrate that became tyrosine-phosphorylated after the stimulation of α4 integrins in HL60 cells.

Because the Cbl protein associates with the 85 kDa subunit of PI-3K in a variety of systems [14,16,18], we investigated the ability of α4 integrin stimulation to increase the association of p85 and Cbl. When the blot from Figure 4 was reprobed with an anti-p85 antibody, the amount of associated p85 detected in anti-Cbl immunoprecipitates decreased with each sequential Cbl immunoprecipitation (lanes 1–4). However, p85 was still faintly detectable in the anti-phosphotyrosine immunoprecipitation of the Cbl-depleted lysate, suggesting an association of some p85 with other tyrosine-phosphorylated proteins in α4-stimulated cells (Figure 4, lane 5).

Phosphotyrosine-associated α4-stimulated PI-3K activity remained after the removal of Cbl-associated activity

Because the stimulation of β1 integrins has been linked to the activation of PI-3K in several cell types [7,8], we next examined the ability of the stimulation of α4 integrins to increase PI-3K activity co-precipitated by an anti-phosphotyrosine mAb and by an anti-Cbl antibody. The stimulation of HL60 cells by α4 mAb cross-linking resulted in a 4.9-fold increase in phosphotyrosine-associated PI-3K activity when compared with PI-3K activity in control-stimulated cells (results not shown). In addition, the stimulation of α4 integrins resulted in a 2.3-fold increase in the PI-3K activity found in anti-Cbl immunoprecipitates (results not shown). We used the immunodepletion strategy outlined for Figure 4 to characterize further the α4 integrin-stimulated PI-3K activity. Control-stimulated and α4-stimulated lysates were sequentially passed over four aliquots of anti-Cbl beads; the cleared lysates were then immunoprecipitated with anti-phosphotyrosine beads. In addition, uncleared lysates were immunoprecipitated with anti-phosphotyrosine beads. The first anti-Cbl immunoprecipitation demonstrated that the stimulation of α4

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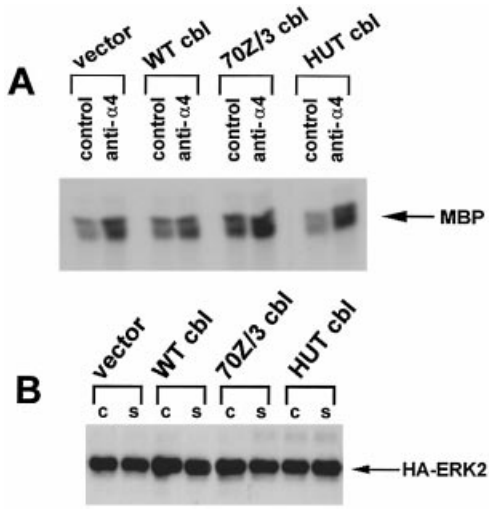

Figure 6 Effect of WT Cbl, 70Z/3 Cbl and HUT Cbl on α4-stimulated ERK2 activity

(**A**) HL60 cells were transiently transfected with pEGFP–C2, pEGFP–c-Cbl, pEGFP–70Z/3-Cbl or pEGFP–HUT-Cbl along with SRα-HAERK2 as described in the Experimental section. Transfectants were stimulated for 10 min by mAb cross-linking as described in the legend to Figure 1. Lysates were analysed for ERK2 activity as described in the legend to Figure 2. Results shown are from one representative experiment of a minimum of three. (**B**) Equivalent amounts of protein were used in the ERK2 kinase activity assay. Aliquots of each sample from (**A**) were removed before the activity assay, separated by SDS/PAGE and immunoblotted with an anti-ERK2 polyclonal antibody as described in the Experimental section. Abbreviation: MBP, myelin basic protein.

integrins led to increased Cbl-associated PI-3K activity (Figure 5, lanes 1 and 2). Analysis of PI-3K activity in the fourth sequential anti-Cbl immunoprecipitate demonstrated minimal PI-3K activity in either unstimulated or α4-stimulated lysates (Figure 5, lanes 3 and 4). This demonstrated the effectiveness of this procedure for immunodepleting Cbl and its associated PI-3K activity. Although Cbl had been depleted from these lysates, a final immunoprecipitation with an anti-phosphotyrosine mAb revealed that α4 integrin-mediated increases in PI-3K activity could still be observed in anti-phosphotyrosine immunoprecipitates (Figure 5, lanes 5 and 6). The α4 integrin-mediated increase in PI-3K activity in Cbl-depleted lysates was less than that in anti-phosphotyrosine immunoprecipitates that had not been depleted of Cbl (2.1-fold compared with 2.8-fold respectively). These results suggested that two separate pools of α4β1 integrin-stimulated PI-3K activity could be identified in HL60 cells: Cbl-associated PI-3K activity and Cbl-depleted, phosphotyrosine-associated PI-3K activity.

Effect of GFP–Cbl fusion protein expression on α4-stimulated ERK2 activity

To explore the potential role of Cbl in the α4β1 integrin-mediated activation of MAPK, we examined the effect of the transient expression of three different Cbl constructs on α4-stimulated ERK2 activity. The three Cbl constructs that were studied included WT Cbl, an internal deletion mutant lacking a 17-residue region of the RING finger motif (70Z/3 Cbl) and a truncation mutant lacking the C-terminal 259 residues of the Cbl protein (HUT Cbl). The 70Z/3 Cbl mutant can inhibit integrin-mediated cell adhesion in HL60 cells [30] and the HUT Cbl mutant lacks putative protein-binding motifs that might have a role in integrin-mediated signalling. Each Cbl protein was expressed as a GFP fusion construct that was co-transfected into HL60 cells together with an HA-tagged ERK2 vector. Anti-HA immunoprecipitates were subsequently assayed for ERK2 activity.

For each GFP–Cbl construct, a transfection efficiency of 20–25% was achieved routinely (results not shown). Although the transfection efficiency for the GFP–Cbl fusion constructs was comparable to that obtained with the GFP vector alone, the level of green fluorescence typically reached the fourth decade in transfectants expressing GFP only, whereas fluorescence for the GFP–Cbl transfectants consistently extended into the third decade (results not shown).

Transient expression of WT Cbl or 70Z/3 Cbl led to increased ERK2 activity in anti-HA immunoprecipitates for control-stimulated cells in comparison with control-stimulated cells from the GFP vector transfectant (Figure 6A). WT Cbl increased basal ERK2 activity by an average of 1.5-fold (S.D. 0.2) in multiple experiments (results not shown). The enhanced basal ERK2 activity was the most marked for the 70Z/3 Cbl transfectant (Figure 6A), in which there was an average 2.0-fold (S.D. 0.4) increase in basal ERK2 activity in multiple experiments when compared with control-stimulated cells expressing GFP only (results not shown). In contrast, the expression of HUT Cbl did not produce a significant increase in control-stimulated ERK2 activity (Figure 6A).

Analysis of ERK2 activity in anti-HA immunoprecipitates from α4-stimulated cells demonstrated increased ERK2 activity when compared with control-stimulated cells expressing the same construct (Figure 6A). However, the overall level of increase in ERK2 activity in α4 integrin-stimulated cells expressing WT Cbl was comparable to the increase in ERK2 activity in α4 integrin-stimulated cells expressing the GFP control vector. When compared with basal levels of ERK2 activity in cells expressing GFP only, there was an average 3.2-fold (S.D. 0.6) increase in ERK2 activity in α4 integrin-stimulated cells expressing 70Z/3 Cbl. Although this was higher than the 2.5-fold (S.D. 0.1) increase in ERK2 activity observed on the stimulation of α4 integrin in HL60 cells expressing GFP alone, this difference was not statistically significant (results not shown). In contrast, expression of the HUT Cbl truncation mutant had no effect on α4 integrin-mediated increases in ERK2 activity (Figure 6A). Figure 6(B) demonstrates that equivalent amounts of HA-tagged ERK2 protein were present in each sample.

Association of p85 with WT Cbl and 70Z/3 Cbl but not HUT Cbl

We next assessed the biochemical status of the GFP–Cbl fusion proteins that were used in this study. HL60 cells were transiently transfected with each of the three Cbl constructs, stimulated by α4 mAb cross-linking, and then the fusion proteins were immunoprecipitated with an anti-GFP antibody. As with endogenous Cbl, tyrosine phosphorylation of the GFP–WT Cbl fusion protein increased on α4β1 integrin engagement (Figure 7). Analysis of the tyrosine phosphorylation state of 70Z/3 Cbl revealed a striking level of hyperphosphorylation in control-stimulated cells; the level of tyrosine phosphorylation was further enhanced after the stimulation of α4 integrins (Figure 7). Tyrosine phosphorylation of the GFP–HUT Cbl fusion protein was not observed, even after stimulation of α4 integrins (Figure 7).

Because α4 integrin-mediated increases in p85 association with endogenous Cbl in HL60 cells were observed (Figure 4), we also assessed the association of p85 with each of the GFP–Cbl fusion proteins. For the WT Cbl and 70Z/3 Cbl fusion proteins we observed an association with endogenous p85 that was enhanced

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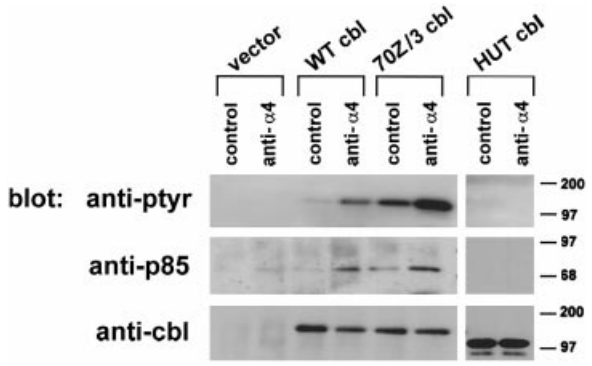

Figure 7 Tyrosine phosphorylation and p85 association with GFP–Cbl fusion proteins

HL60 cells were transiently transfected with pEGFP–C2, pEGFP–c-Cbl, pEGFP–70Z/3-Cbl or pEGFP–HUT-Cbl as described in the Experimental section. Transfectants were stimulated as described in the legend to Figure 1. Cell lysates were immunoprecipitated with an anti-GFP antibody, separated by SDS/PAGE and immunoblotted with the anti-phosphotyrosine mAb 4G10 as described in the Experimental section. The blot was stripped and reprobed with an anti-p85 antibody or an anti-Cbl antibody. Results shown are from one representative experiment of a minimum of two. The molecular masses of bands are indicated (in kDa) at the right.

on the stimulation of α4 (Figure 7). In accordance with the degree of tyrosine phosphorylation, the 70Z/3 Cbl protein was associated with more p85 in the control-stimulated lane than was the control-stimulated WT Cbl (Figure 7). In contrast, we could not detect p85 in immunoprecipitates containing the GFP–HUT Cbl fusion protein, even after the stimulation of α4 integrins (Figure 7). Immunoblotting with an anti-Cbl antibody demonstrated that the expected 150 kDa WT Cbl and 70Z/3 Cbl and the 102 kDa HUT Cbl fusion proteins were expressed and equivalent amounts of protein were loaded for each sample (Figure 7).

DISCUSSION

Studies in a variety of cell types have established that β1 integrin engagement leads to increased MAPK activity [3–5]. In the present study we investigated the role for PI-3K and the adaptor protein Cbl in coupling the $\alpha4\beta1$ integrin to ERK2 activation in the human myelomonocytic cell line HL60. Although the stimulation of α4 integrins leads to the tyrosine phosphorylation of Cbl and an increased association of PI-3K with Cbl, the effects of expression of WT Cbl and two Cbl mutants on MAPK activation were most consistent with a role for Cbl in regulating the basal activity of MAPK in HL60 cells.

The ability of wortmannin, LY294002 and a dominant-negative form of the p85 subunit of PI-3K to inhibit α4 integrin-mediated activation of ERK2 in our studies suggests a central role for PI-3K in coupling $\alpha4\beta1$ to MAPK. A requirement for PI-3K in the activation of MAPK has also been observed in other signal transduction systems with different cell types. For example, in the Jurkat T-cell line, the PI-3K inhibitor wortmannin interferes with T-cell-receptor-mediated ERK2 activation [31]. In addition, wortmannin blocks the middle-T antigen-stimulated activation of ERK1 in fibroblast cell lines [32], as well as the vasopressin-induced activation of MAPK [33]. Thus, in non-integrin signalling systems, results with inhibitors have suggested that PI-3K lies upstream of MAPK.

A report with the use of Cos-7 cells has also established that PI-3K is required for ERK2 activity initiated by adhesion to fibronectin [7]. Results with the use of wortmannin and LY294002 as inhibitors were the first indication of this requirement; it was subsequently confirmed by transient expression of a dominant-negative p85 subunit, which led to the inhibition of β1 integrin-stimulated ERK2 activity. Although these results are similar to our findings, it should be noted that, unlike HL60 cells, FAK is expressed in Cos-7 cells and becomes tyrosine-phosphorylated on the attachment of Cos-7 to fibronectin [7]. A role for FAK in linking the integrin receptor to MAPK has been proposed on the basis of the activation of MAPK on the adhesion of cells overexpressing FAK [34]. Furthermore, tyrosine-phosphorylated FAK associates with the SH2 domain of the Grb2 adaptor molecule, which is involved in initiating signals through the Ras pathway [35]. Activated FAK might also recruit Src family kinases [36], which are capable of tyrosine-phosphorylating FAK as well as FAK-associated proteins such as $p130^{cas}$, eventually leading to the formation of a signalling complex that might be able to regulate signal transduction to MAPK [37,38].

However, there is evidence that integrin signalling to MAPK might also be independent of FAK. Expression of a dominant-negative form of FAK does not disrupt β1 integrin-mediated ERK2 activation [4] and the expression of a constitutively active form of FAK does not enhance adhesion-stimulated MAPK activity [39]. ERK2 activation by a subset of integrins also involves the adaptor protein Shc, not FAK [40]. Because we were unable to detect FAK in HL60 cells, our studies suggest that PI-3K has a role in FAK-independent mechanisms for coupling β1 integrins to MAPK signalling.

Our results demonstrating $\alpha4\beta1$ integrin-mediated tyrosine phosphorylation of the Cbl adaptor protein are consistent with previous studies with mouse macrophage cell lines [8]. However, one notable difference is in the kinetics of β1 integrin-mediated tyrosine phosphorylation of Cbl, which we find to be rapid and transient, whereas others have reported gradual and persistent phosphorylation of Cbl on the stimulation of integrins. This difference in the kinetics of integrin-mediated phosphorylation of Cbl might be related to differences between human myeloid cells and mouse macrophages, the nature of the integrin activating signal and the specific type of integrin being engaged ($\alpha4\beta1$ compared with $\alpha5\beta1$).

We explored the relationship between Cbl, p85 and MAPK activation by determining the effect of overexpression of WT and mutant forms of Cbl on basal and integrin-mediated ERK2 activation. In our system, the 70Z/3 Cbl mutant exhibited (1) hyperphosphorylation under both unstimulated and integrin-stimulated conditions, and (2) enhanced basal and integrin-mediated association with p85. This suggests that perturbing the association between Cbl and p85 via the expression of 70Z/3 Cbl can modulate downstream ERK2 signalling. Our results are consistent with this hypothesis. However, the primary effect of expression of 70Z/3 Cbl was the enhancement of the basal ERK2 activity. In cells expressing 70Z/3 Cbl, ERK2 activity was enhanced after the stimulation of α4 integrins. However, the level of enhanced ERK2 activity was comparable to the increased ERK2 activity observed in α4 integrin-stimulated cells expressing control vector. In other systems, the hyperphosphorylation of 70Z/3 Cbl has also been described [41]. In addition, transient expression of 70Z/3 Cbl in Jurkat T-cells increases the Ras-dependent activation of nuclear factor of activated T-cells ('NFAT') and synergizes with ionomycin stimulation to enhance activation further [42]. Thus, in our system and in the Jurkat system, the expression of 70Z/3 Cbl results in enhanced downstream signalling.

Our studies with WT Cbl transfectants also demonstrate enhanced basal ERK2 activity when compared with control transfectants, although this effect is not as pronounced as that

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observed with 70Z/3 Cbl. When compared with unstimulated cells expressing WT Cbl, there seems to be a decrease in α4 integrin-stimulated ERK2 activity. This would be consistent with other studies suggesting a negative regulatory role for WT Cbl [19,23]. However, there is a minimal effect of WT Cbl when compared with the α4 integrin-mediated activation of ERK2 in HL60 cells expressing control vector, suggesting that the predominant effect of WT Cbl is enhancing the basal ERK2 activity in HL60 cells. Our studies with the HUT Cbl mutant are also consistent with this model, because HUT Cbl does not associate with p85, even after the stimulation of α4 integrins, and does not affect either basal or α4 integrin-stimulated ERK2 activity.

Although Cbl is the major phosphoprotein associated with p85 in activated T-cells [43,44], our studies with HL60 cells show that phosphotyrosine-associated PI-3K activity remains in integrin-stimulated cells even after the immunodepletion of Cbl. This suggests the existence of at least two biochemically distinct pools of $\alpha4\beta1$ integrin-stimulated PI-3K activity. Differences in the amount of PI-3K activity detected in anti-phosphotyrosine immunoprecipitates compared with anti-Cbl immunoprecipitates have also been noted after stimulation with interleukin 3 in a murine myeloid cell line [14]. In addition, separate Cbl-associated and CD28-associated pools of CD28-stimulated PI-3K activity have been observed [30]. Although these pools of PI-3K activity might initiate similar downstream effects, it is interesting to note that Cbl has been implicated in regulating functionally divergent signalling pathways involved in T-cell anergy by balancing the activation of the GTP-binding proteins Ras and Rap1 [45]. Thus perturbing the amount of p85 that associates with Cbl might have a critical role in regulating signalling events that are dependent on PI-3K.

In summary, we propose that engagement of the $\alpha4\beta1$ integrin leads to increased ERK2 activity via the activation of PI-3K. Although the engagement of $\alpha4\beta1$ integrin leads to Cbl tyrosine phosphorylation and association with the p85 subunit of PI-3K, the predominant effect of Cbl-associated PI-3K is associated with the regulation of basal, rather than $\beta1$ integrin-stimulated, ERK2 activity. Thus these studies provide evidence for PI-3K-dependent activation of MAPK signalling by $\beta1$ integrins and a distinct role for Cbl in regulating MAPK activity in myeloid cells.

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