β1 and β2 integrins activate different signalling pathways in monocytes

Merit REYES-REYES, Nancy MORA, Gerardo GONZALEZ and Carlos ROSALES[1]

Departamento de Inmunología, Instituto de Investigaciones Biomédicas UNAM, Apto. Postal 70228, Cd. Universitaria, México D.F. 04510, Mexico

Integrin-mediated signals play an important but poorly understood role in regulating many leucocyte functions. In monocytes and macrophages, integrins of the β2 subfamily are involved in cell–cell interactions that are important for migration of the cells through the endothelium and also for phagocytosis. On the other hand, in the same cells, β1 integrin-mediated adhesion to extracellular matrix proteins results in a strong induction of immediate early genes that are important in inflammation. To investigate the signalling pathways from these two types of integrin in monocytic cells, THP-1 cells were selectively stimulated via β1 or β2 integrins by cross-linking each type of receptor with specific monoclonal antibodies or their natural ligands. The involvement of extracellular signal-regulated kinase (ERK), Syk and phosphoinositide 3-kinase (PI-3K) was then analysed. Nuclear factor κB (NF-κB) activation was also detected in THP-1 cells transiently transfected with an NF-κB-driven luciferase reporter gene. We found that binding of both types of integrin to their natural ligands activated ERK in a Syk- and PI-3K-dependent manner. Yet, cross-linking of integrins by anti-β1 antibodies caused activation of ERK while that by anti-β2 antibodies did not. Also both types of integrin activated NF-κB. However, PI-3K was required for β1 integrin-, but not β2 integrin-, mediated NF-κB activation. In addition, inhibition of PI-3K with wortmannin and LY294002 blocked β1 integrin-mediated NF-κB activation, but did not affect that mediated by β2 integrin. These data suggest that distinct integrins activate different signalling pathways in monocytic cells.

Key words: ERK, NF-κB, phosphoinositide 3-kinase, PI-3K, signal transduction.

INTRODUCTION

Integrins are membrane receptors composed of two gene products, α and β chains, which are linked in a non-covalent, though very stable, heterodimeric structure. The various integrins have been classified into subfamilies according to the β subunit they share [1,2]. The β1 integrin subfamily is comprised mainly of the receptors for extracellular matrix (ECM) proteins, such as fibronectin, collagen, laminin and vitronectin [3]. β2 Integrin subfamily expression is limited to white blood cells. Within this subfamily there are four β2 integrins: leucocyte function antigen (LFA)-1, Mac-1, gp150/95 and αd/β2 [4–6]. These integrins are mainly receptors for adhesion molecules on the surfaces of other cells, such as intercellular cell-adhesion molecule (ICAM)-1 and ICAM-2. The β2 integrins are very important for leucocyte function, as demonstrated by the congenital leucocyte-adhesion deficiency (LAD) syndrome [7,8]. In LAD, white blood cells do not express normal levels of β2 integrins, and patients present recurring infections and die very young [7].

During inflammation, the different types of leucocyte leave the bloodstream in an orderly fashion [9]. Adhesion molecules act sequentially to allow leucocytes to leave the circulation [10]. Selectins provide the first interactions with endothelial cells, while integrins of the β2 subfamily mediate subsequent strong cell attachment and migration. Once out of the blood vessel, leucocytes interact with ECM proteins through integrins of the β1 subfamily. It thus becomes evident that β1 and β2 integrins regulate different cell processes during inflammation.

Integrins were first described as surface receptors interacting with the cytoskeleton, thus integrating the extracellular environment with the cell interior [3,11]. However, it is now clear that integrins can induce functional changes in the cell by activating intracellular biochemical signalling cascades, just as other receptors do [12–14]. In monocytes, β2 integrins are essential for cell migration and phagocytosis [8,15], whereas β1 integrins lead to activation of immediate early genes that are characteristic of monocytic differentiation. These genes contain consensus nuclear factor κB (NF-κB) elements in their 5′ regulatory regions. Several of these genes code for important inflammatory mediators, such as interleukin-1 (IL)-1, IL-8 and tumour necrosis factor α (TNFα) [16,17]. The signalling cascades used by each type of integrin to promote the various leucocyte responses are still poorly understood [17–19]. Monocyte binding to fibronectin through β1 integrins induces NF-κB activation [20,21], but this signalling pathway is independent of extracellular signal-regulated kinase (ERK) [22]. Very little is known about signalling by integrins that do not bind ECM proteins, such as β2 integrins. There is therefore great interest in elucidating the various signalling pathways that distinct integrins use in the various cell types [17–19].

In the present study, we explored the similarities between the signalling cascades activated by two types of integrin in the same cell type. Adhesion of human blood monocytes [23,24] or monocytic cells [20,21] to ECM proteins, as well as ligation of their β1 integrins with antibodies, results in activation of the nuclear transcription factor NF-κB. Activation of this nuclear factor is required for transcription of the inflammatory genes [25,26]. To test the possibility that β2 integrins activate the same signalling elements as β1 integrins, THP-1 cells were stimulated via different integrins. We then measured activation of NF-κB, ERK and phosphoinositide 3-kinase (PI-3K). We found that both β1 and β2 integrins caused activation of ERK and PI-3K in a Syk-dependent manner. In addition, both types of integrin caused activation of NF-κB. However, only β1 integrins used

Abbreviations used: ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; pERK, activated phospho-ERK; MEK, ERK kinase; PI-3K, phosphoinositide 3-kinase; NF-κB, nuclear factor κB; ICAM, intercellular cell-adhesion molecule; IL, interleukin; TNFα, tumour necrosis factor α.

[1] To whom correspondence should be addressed (e-mail carosal@servidor.unam.mx).

PI-3K for NF-κB activation. These data indicate that, in effect, different signalling pathways are used by distinct integrins on the same cell, leading to different cell responses.

MATERIALS AND METHODS

Cells and reagents

THP-1 cells, from a human monocytic leukaemia cell line, were maintained as described in [27]. TS2/16, an anti-β1 integrin monoclonal antibody, was provided by Dr Martin Hemler (Dana Farber Cancer Research Institute, Boston, MA, U.S.A.). IB4, an anti-β2 integrin monoclonal antibody was donated by Dr Eric J. Brown (University of California in San Francisco, San Francisco, CA, U.S.A.). W6/32, an anti-MHC class I monoclonal antibody, AP3, an anti-β3 integrin monoclonal antibody, and AIIB2, an inhibiting anti-β1 integrin monoclonal antibody, were all from A.T.C.C. (Manassas, VA, U.S.A.). AFT-8, an anti-phosphotyrosine monoclonal antibody, was described previously [27]. The anti-ERK rabbit polyclonal IgG (catalogue no. sc-94), the anti-activated phospho-ERK (pERK) rabbit polyclonal IgG (catalogue no. sc-7383) and the anti-PI-3K (p110β subunit) rabbit polyclonal IgG (catalogue no. sc-7189) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The specific PI-3K inhibitors wortmannin and LY294002, and the selective Syk tyrosine kinase inhibitor piceatannol, were from Calbiochem (San Diego, CA, U.S.A.). The specific ERK kinase (MEK) inhibitor PD98059 was from New England Biolabs (Beverly, MA, U.S.A.). The NF-κB-responsive reporter plasmid 3XMHC-luc, containing NF-κB elements upstream of the luciferase (*luc*) gene, was provided by Dr John Westwick and Dr David A. Brenner (University of North Carolina, Chapel Hill, NC, U.S.A.). Human fibronectin was from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) and recombinant human ICAM-1, consisting of 455 amino acids from the external domain minus the transmembrane and cytoplasmic domains, was from R&D Systems (Minneapolis, MN, U.S.A.). All other chemicals were from Sigma (St Louis, MO, U.S.A.).

Cell lysates

Tissue-culture dishes (30 mm) were coated overnight at 4 °C with 20 μg/ml fibronectin or 5 μg/ml ICAM-1, and blocked with 1 % BSA as described in [20]. THP-1 cells were washed and re-suspended in cold serum-free RPMI 1640 medium. Cells were plated on fibronectin- or ICAM-1-coated dishes or left untreated in suspension and then incubated at 37 °C for 15 min. Plates were then put on ice and 5 ml of cold PBS was added. Cells were scraped and centrifuged at 290 ***g*** for 5 min at 4 °C. Cell pellets were immediately lysed in 100 μl of cold RIPA buffer as described previously [27]. For stimulation with antibodies, THP-1 cells were incubated in medium alone or medium containing 5 μg/ml antibody for 1 h at 4 °C. Cells were then washed with cold medium and incubated at 37 °C in RPMI 1640 medium containing 5 μg/ml F(ab′)$_2$ goat anti-mouse IgG (Cappel, Aurora, OH, U.S.A.) for 3 min. Next, cells were diluted with 5 ml of cold PBS, centrifuged and lysed as described above. In assays involving inhibition of Syk, PI-3K or ERK, THP-1 cells were preincubated with the corresponding inhibitor at the following concentrations: 30 μg/ml piceatannol, 50 nM wortmannin, 50 μM LY294002 or 30 μM PD98059.

Western blotting

Total cell lysates from equivalent cell numbers or immunoprecipitates of ERK or PI-3K were resolved by SDS/PAGE (12 and 8 % gels, respectively). Proteins were then electrotransferred on to PVDF membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.). Membranes were incubated in blocking buffer [1 % BSA, 5 % non-fat dry milk (Carnation; Nestle Food Co., Glendale, CA, U.S.A.) and 0.1 % Tween 20 in PBS] overnight at room temperature. Membranes were subsequently probed with the corresponding antibody in blocking buffer, for 1 h at room temperature: anti-phosphotyrosine (AFT8) at 1 μg/ml, anti-ERK at 75 ng/ml, anti-pERK at 75 ng/ml and anti-PI-3K at 100 ng/ml. Membranes were washed with PBS six times, for 5 min each time, and incubated with a 1/3000 dilution of horseradish peroxidase-conjugated F(ab′)$_2$ goat anti-mouse IgG for 1 h at room temperature. After washing six more times with PBS, antibody-reactive proteins were detected using a chemiluminescence substrate (SuperSignal; Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions.

Flow cytometry

THP-1 cells (1×10^6) in 100 μl of FACS buffer (PBS containing 0.5 % BSA and 1 % sucrose) were incubated with the corresponding monoclonal antibody at 1 μg/ml for 45 min at 4 °C. Cells were then washed three times with FACS buffer, resuspended in 100 μl of FACS buffer plus 20 μl of a 1/10 dilution of FITC-conjugated F(ab′)$_2$ goat anti-mouse IgG, and incubated at 4 °C for 30 min. After three more washes cells were resuspended in 500 μl of cold 1 % paraformaldehyde in PBS, and analysed using a FACScan (Becton Dickinson, San Jose, CA, U.S.A.).

Adhesion assay

THP-1 cells were loaded with calcein-AM (where AM is acetoxymethyl ester; Molecular Probes, Eugene, OR, U.S.A.) by incubating at 1×10^7 cells/ml in serum-free RPMI 1640 medium with 1 μM calcein-AM at 37 °C for 30 min. After three washes with medium, 5×10^4 cells in 50 μl of medium were added to each well of a 96-well plate coated with 5 μg/ml fibronectin, and incubated at 37 °C and 5 % CO_2 for 1 h. Plates were then washed three times with 250 μl of PBS each time. Fluorescence of adhered cells was finally determined in a microplate Fluoroskan AF reader (LabSystems, Franklin, MA, U.S.A.) at an excitation wavelength of 485 nm, and an emission wavelength of 538 nm.

Immunoprecipitation

ERK or PI-3K was immunoprecipitated from THP-1 cell lysates (equivalent to 1.5×10^7 cells) with 1 μg of anti-ERK or anti-PI-3K (p110β subunit) antibody, respectively. The antibody was first incubated with 20 μl of Protein G–Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 2 h at 4 °C, and then mixed with the cell lysate for another 2 h at 4 °C. Sepharose beads were then washed once with cold RIPA buffer and five more times with cold washing buffer (0.25 M Tris/HCl, pH 7.5/0.1 M NaCl). Immunoprecipitates were resuspended in 30 μl of electrophoresis sample buffer and resolved by SDS/PAGE.

PI-3K activity assay

PI-3K was immunoprecipitated from THP-1 cell lysates (equivalent to 1.5×10^7 cells) and its activity determined as described previously [22].

Reporter-gene assays

The NF-κB-responsive reporter plasmid 3XMHC-luc was transfected into THP-1 cells by the DEAE–dextran method [28] with minor modifications as described in [22]. Luciferase enzymic activity was determined in cell lysates using a luminometer

(Monolight 2010 luminometer; Ann Harbor, MI, U.S.A.) for 20 s as described in [22]. Protein concentration in cell lysates was determined using the bicinchoninic acid method (Pierce). Reporter activity data were normalized according to protein concentration in the cell lysates, and transfection efficiency as described in [21,22,27].

RESULTS

Cross-linking of β1 and β2 integrins induces different patterns of tyrosine-phosphorylated proteins

It is well known that integrins activate several tyrosine kinases after these receptors are cross-linked on the cell membrane by their cognate ligands [14]. When THP-1 cells were stimulated with anti-β1 integrin or anti-β2 integrin antibodies, a strong increase in tyrosine phosphorylation of several proteins was detected (Figure 1). The pattern of protein phosphorylation observed after stimulation of β1 and β2 integrins was, however, different. Cross-linking of MHC class I molecules, an unrelated surface receptor, did not induce activation of tyrosine kinases in these cells (Figure 1). Proteins of approx. 40, 120, 150 and 160 kDa were strongly phosphorylated in response to β1 integrin cross-linking. In contrast, β2 integrins showed phosphorylation of only the high-molecular-mass proteins (Figure 1). These results suggested that the different types of integrin might signal through different target proteins in monocytic cells.

β1 Integrin cross-linking activates ERK

The protein of approx. 40 kDa phosphorylated after treatment with anti-β1 integrins antibodies could be ERK, since this kinase has been shown to be activated after β1 integrin engagement in fibroblasts [13]. In order to determine whether ERK was also activated by integrins in monocytic cells, we analysed ERK phosphorylation in THP-1 cells after treatment with anti-β1 integrin and anti-β2 integrin antibodies. It is known that ERK phosphorylation is required for its activation [29]. Cross-linking of β1 integrins with monoclonal antibody TS2/16 indeed resulted in activation of ERK, indicated by Western blotting with an anti-phosphotyrosine antibody, and also with an anti-pERK antibody

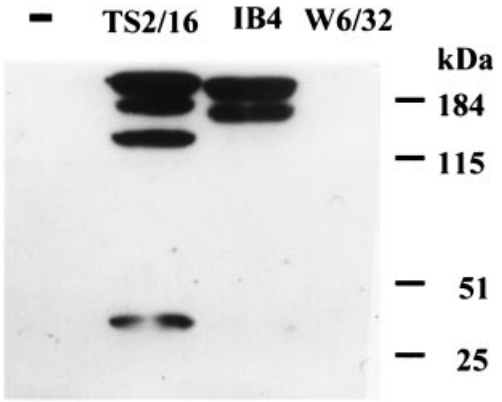

Figure 1 Cross-linking of β1 and β2 integrins induces different patterns of tyrosine-phosphorylated proteins

THP-1 cells (1×10^6) in 3 ml of serum-free RPMI 1640 medium were stimulated for 3 min in the absence (–) or presence (5 μg/ml) of anti-β1 integrin antibody (TS2/16), anti-β2 integrin antibody (IB4) or anti-MHC class I antibody (W6/32). Cell lysates were prepared as described in the text and proteins resolved by SDS/PAGE. Anti-phosphotyrosine Western blotting was then performed using 1 μg/ml of the monoclonal antibody AFT8.

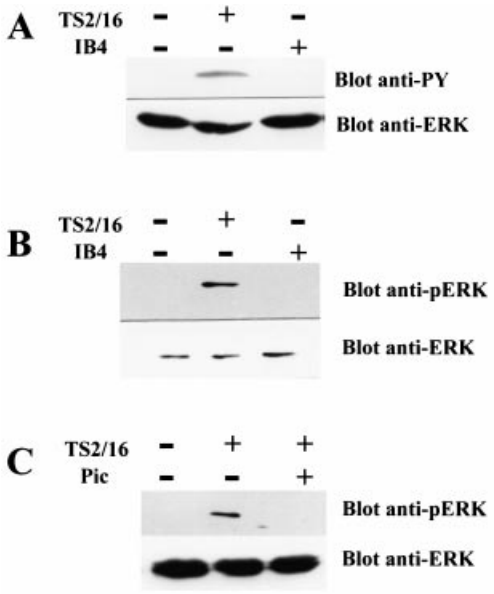

Figure 2 β1 Integrins activate ERK

THP-1 cells (1.5×10^7) in 1 ml of serum-free RPMI 1640 medium were stimulated for 3 min in the absence (–) or presence (5 μg/ml) of anti-β1 integrin antibody (TS2/16) or anti-β2 integrin antibody (IB4). Cell lysates were prepared and ERK was immunoprecipitated as described. Western blotting of immunoprecipitates was performed with (**A**) an anti-phosphotyrosine (anti-PY) monoclonal antibody or (**B**) an anti-pERK antibody. (**C**) Cells were treated with 30 μg/ml piceatannol (Pic) before stimulation with TS2/16, and cell lysates were Western blotted for pERK. Lower panels are anti-ERK Western blots of the same membranes after stripping them of bound antibodies. Data are representative of three different experiments.

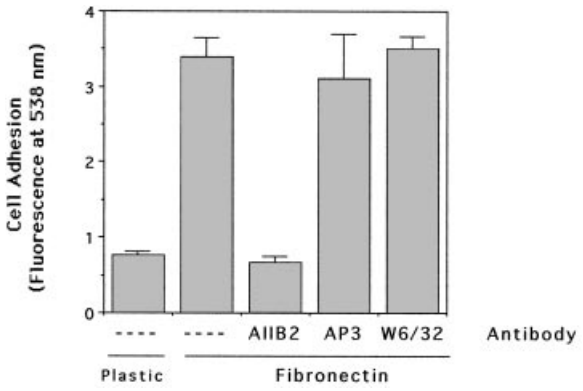

Figure 3 Adhesion to fibronectin does not involve β3 integrins

Calcein-loaded THP-1 cells (5×10^4) in 50 μl of serum-free RPMI 1640 medium were plated over fibronectin or plastic and incubated for 60 min at 37 °C. Some cells were also incubated with 20 μg/ml anti-β1 integrin (AIIB2), anti-β3 integrin (AP3) or anti MHC class I (W6/32) monoclonal antibodies during the adhesion assay. Fluorescence of adhered cells was determined at 538 nm. Data are means $\pm$ S.E.M. from three different determinations.

(Figure 2). In contrast, cross-linking of β2 integrins with the monoclonal antibody IB4 on the same cells did not induce activation of ERK (Figure 2). Moreover, activation of ERK by β1 integrin stimulation of these cells was dependent on the tyrosine kinase Syk, as indicated by inhibition of ERK phosphorylation by the specific Syk inhibitor piceatannol (Figure 2C). These results supported the idea that these two types of integrin use different signalling pathways in monocytes.

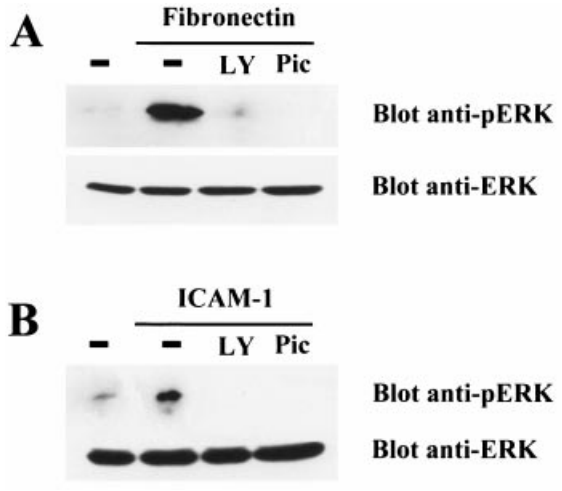

Figure 4 Adhesion to fibronectin and ICAM-1 causes ERK activation

THP-1 cells (1×10^7) in 1 ml of serum-free RPMI 1640 medium were plated over fibronectin (**A**) or ICAM-1 (**B**) and incubated for 15 min at 37 °C. Some cells were treated with 50 μM LY294002 (LY) or 30 μg/ml piceatannol (Pic) before stimulation. Cell lysates were prepared, and proteins were resolved by SDS/PAGE and Western blotted with an anti-pERK antibody. Lower panels are anti-ERK Western blots showing equivalent amounts of protein in each lane. Data are representative of three different experiments.

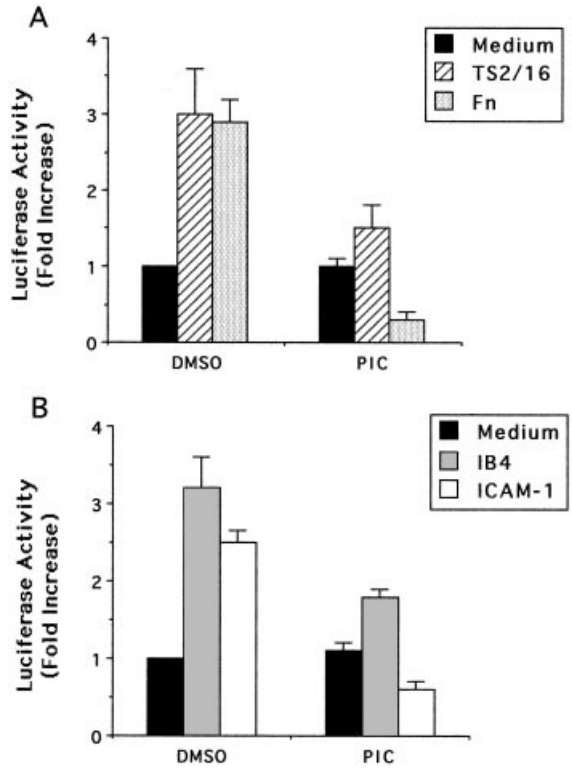

Figure 5 Integrin stimulation induces Syk-dependent activation of NF-κB

THP-1 cells (3×10^6) transiently transfected with the NF-κB reporter plasmid 3XMHC-luc were placed in 4 ml of serum-free medium and (**A** and **B**) left untreated (Medium), (**A**) stimulated by 5 μg/ml TS2/16 antibody or by adhesion to fibronectin (Fn) or (**B**) stimulated by 5 μg/ml IB4, the anti-β2 integrin antibody, or by adhesion to ICAM-1. Cells were also pretreated with 30 μg/ml piceatannol (PIC) or just the solvent DMSO. Later (4 h), cell lysates were prepared and luciferase activity, representing NF-κB activation, was determined. Data are means $\pm$ S.E.M. from four different determinations.

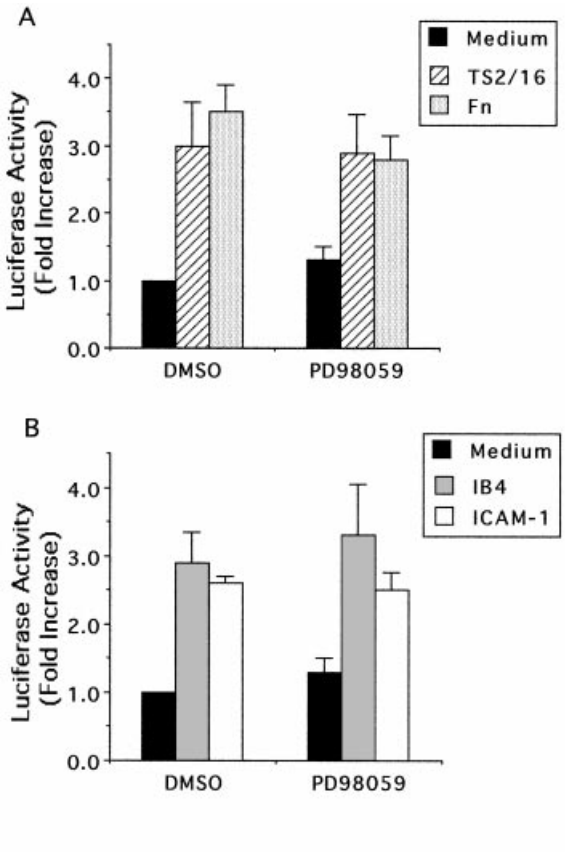

Figure 6 ERK is not required for integrin-mediated NF-κB activation

THP-1 cells (3×10^6) transiently transfected with the NF-κB reporter plasmid, 3XMHC-luc, were placed in 4 ml of serum-free medium and (**A** and **B**) left untreated (Medium), (**A**) stimulated by 5 μg/ml TS2/16 antibody or by adhesion to fibronectin (Fn) or (**B**) stimulated by 5 μg/ml IB4 antibody or by adhesion to ICAM-1. Cells were also pretreated with 30 μM PD98059, the MEK inhibitor, or the solvent DMSO. Later (4 h), cell lysates were prepared and luciferase activity, representing NF-κB activation, was determined. Data are means $\pm$ S.E.M. from six different determinations.

Adhesion to fibronectin does not involve β3 integrins

THP-1 cells also express the integrin $\alpha v\beta 3$, which is also a receptor for fibronectin. Although the level of expression of β3 integrins is 6-fold lower than that of β1 integrins (mean fluorescence intensity, 46.9 for β3 integrins compared with 306.5 for β1 integrins), we wished to confirm that THP-1 cell adhesion to fibronectin does not involve both types of integrin. THP-1 cells adhered efficiently to fibronectin. In the presence of 20 μg/ml AIIB2, the inhibiting anti-β1 integrin monoclonal antibody, cell adhesion to fibronectin was completely blocked (Figure 3). Neither the anti-β3 integrin monoclonal antibody AP3 nor the anti-MHC class I monoclonal antibody W6/32 affected adhesion to fibronectin (Figure 3). These results indicate that THP-1 cell adhesion to fibronectin depends entirely on β1 integrins and is independent of the $\alpha v\beta 3$ integrin.

β2 Integrins also activate ERK

Our results with anti-β2 integrin antibodies indicated that ERK was not used by β2 integrins in monocytic cells. In order to confirm this result, the monocytic THP-1 cells were also stimulated by adhesion to the natural ligands for β1 and β2 integrins. Cells plated over fibronectin showed, as expected, activation of

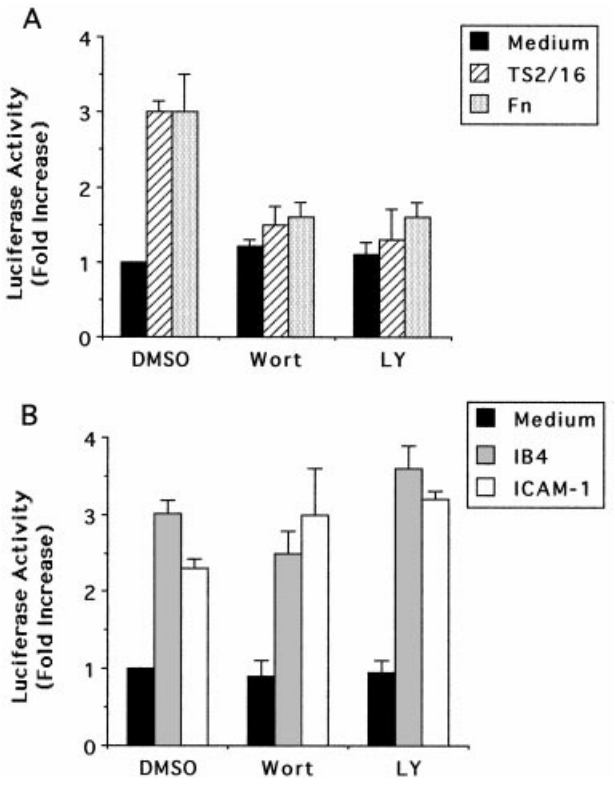

Figure 7 β1 Integrin-, but not β2 integrin-, dependent activation of NF-κB requires PI-3K

THP-1 cells (3×10^6) transiently transfected with the NF-κB reporter plasmid 3XMHC-luc, were placed in 4 ml of serum-free medium and left untreated (Medium) or (**A**) stimulated by 5 μg/ml TS2/16 or by adhesion to fibronectin (Fn) or (**B**) stimulated by 5 μg/ml IB4 antibody or by adhesion to ICAM-1. Cells were also pretreated with the PI-3K inhibitors 50 nM wortmannin (Wort) or 50 μM LY294002 (LY), or the solvent DMSO. Later (4 h), cell lysates were prepared and luciferase activity, representing NF-κB activation, was determined. Data are means $\pm$ S.E.M. from six different determinations.

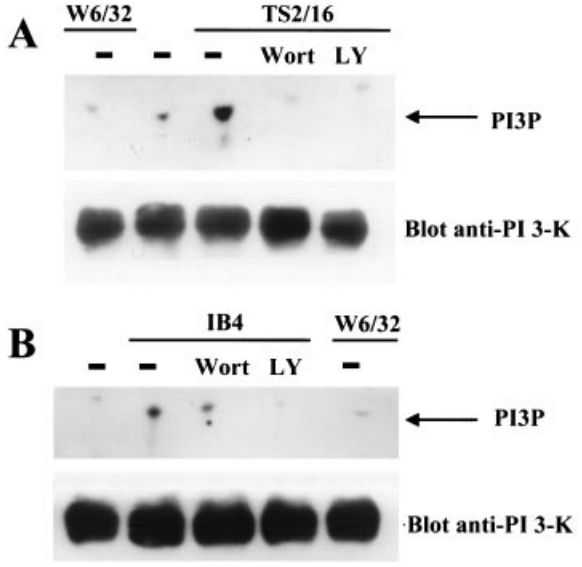

Figure 8 Activation of PI-3K after integrin cross-linking

THP-1 cells (1.5×10^7) in 4 ml of serum-free medium were stimulated with (**A**) 5 μg/ml TS2/16 antibody or 5 μg/ml W6/32 antibody, or (**B**) 5 μg/ml IB4 antibody or 5 μg/ml W6/32 antibody. PI-3K activity was then measured by immune-complex kinase assays from cell lysates. Some cells were pretreated with 50 nM wortmannin (Wort) or 50 μM LY294002 (LY). Lower panels in each figure are Western blots of PI-3K showing that the same amount of protein immunoprecipitated in each determination. PI3P, PtdIns3*P*. Data are representative of three separate experiments.

ERK (Figure 4). This activation was also inhibited by piceatannol and LY294002, a specific inhibitor of PI-3K (Figure 4A), indicating that Syk and PI-3K are both required for β1 integrin-mediated ERK activation. In contrast with the effects of anti-β2 integrin antibodies, adhesion of THP-1 cells to ICAM-1 efficiently activated ERK (Figure 4B). This response was also blocked by piceatannol and by LY294002 (Figure 4B), indicating that β2 integrins also utilize Syk and PI-3K to activate ERK. Thus both β1 and β2 integrins seem to activate a similar signalling cascade when engaged by their respective natural ligands. Cross-linking with monoclonal antibodies did not seem to induce the same response from β2 integrins.

Integrin stimulation induces activation of NF-κB

Because it has been reported that β1 integrins can activate gene induction of inflammatory cytokines in monocytes and that NF-κB is required for this induction [23,24], we evaluated the activation of this nuclear factor upon stimulation of both types of integrin in THP-1 cells. Treatment of cells with the anti-β1 monoclonal antibody TS2/16 or adhesion of cells to fibronectin induced activation of NF-κB, as indicated by an increase (approx. 3-fold) in luciferase activity (Figure 5A). Similarly, activation of β2 integrins by the monoclonal antibody IB4 and by adhesion to ICAM-1 resulted in activation of NF-κB (Figure 5B). In addition, piceatannol blocked activation of NF-κB induced by TS2/16 or fibronectin (Figure 5A), and also by IB4 or ICAM-1 (Figure 5B). Thus both types of integrin clearly have the potential for signalling into the cell by activating nuclear transcription factors in a Syk-dependent manner.

ERK is not required for integrin-mediated NF-κB activation

Because MEK is known to be required for activation of ERK, we investigated whether ERK activation was also MEK-dependent in integrin signalling, and whether ERK activation was necessary for NF-κB activation. Treatment of THP-1 cells with the specific MEK inhibitor PD98059 resulted in clear inhibition of integrin-mediated ERK activation (results not shown) [22]. In contrast to the PD98059 inhibition of ERK, the same treatment, with this MEK inhibitor, did not have any effect on NF-κB activation induced by either type of integrin (Figure 6). All together, these data indicated not only that both β1 integrins and β2 integrins use the MEK/ERK signalling pathway, but also that this signalling pathway does not lead to activation of the nuclear factor NF-κB.

NF-κB activation by β1 integrins, but not by β2 integrins, is dependent on PI-3K

Since the ERK pathway was not required for the activation of NF-κB by integrins, we tried to determine whether other signalling molecules, such as PI-3K, could be upstream of NF-κB in this integrin pathway. To address this idea, we used the PI-3K-specific inhibitors wortmannin and LY294002. Treatment of THP-1 cells with 50 nM wortmannin or 30 μM LY294002 for 60 min before stimulation via β1 integrins resulted in complete inhibition of NF-κB activation (Figure 7A). In contrast, the

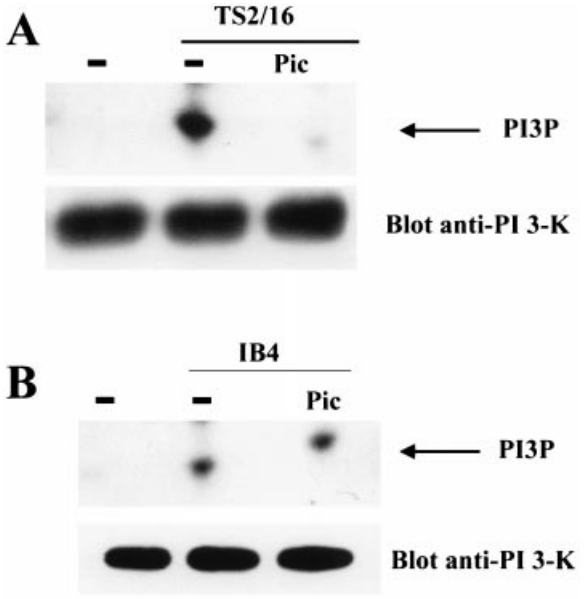

Figure 9 Activation of PI-3K with anti-β2 integrin antibodies is independent of Syk

THP-1 cells (1.5×10^7) in 4 ml of serum-free medium were stimulated with (**A**) 5 μg/ml TS2/16 antibody or (**B**) 5 μg/ml IB4 antibody. PI-3K activity was then measured by immune-complex kinase assays from cell lysates. Some cells were pretreated with 30 μg/ml piceatannol (Pic). Lower panels are Western blots of PI-3K showing that the same amount of protein immunoprecipitated in each determination. PI3P, PtdIns3*P*. Data are representative of three separate experiments.

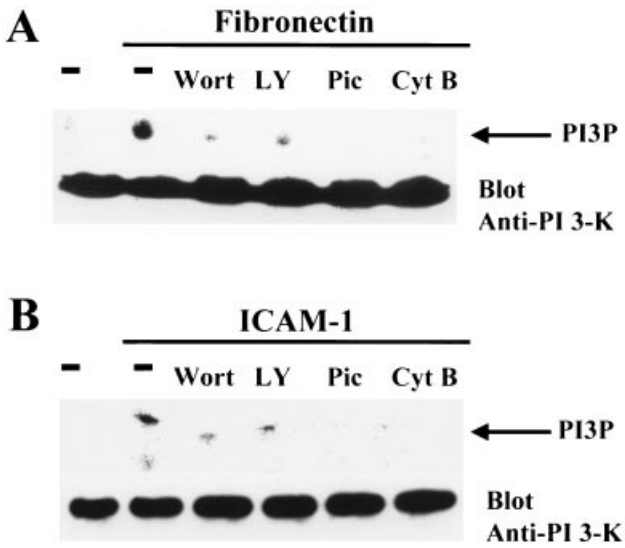

Figure 10 Activation of PI-3K after adhesion to fibronectin or ICAM-1

THP-1 cells (1×10^6) in 1 ml of serum-free RPMI 1640 medium were plated over fibronectin (**A**) or ICAM-1 (**B**) and incubated for 15 min at 37 °C. Some cells were treated with 50 nM wortmannin (Wort), 50 μM LY294002 (LY), 30 μg/ml piceatannol (Pic) or 10 μg/ml cytochalasin B (Cyt B) before stimulation. PI-3K activity was then measured by immune-complex kinase assays from cell lysates. Lower panels are Western blots of PI-3K showing that the same amount of protein immunoprecipitated in each determination. PI3P, PtdIns3*P*. Data are representative of three separate experiments.

same treatment before stimulation via β2 integrins did not inhibit NF-κB activation (Figure 7B). These results supported the hypothesis that different integrins are signalling via different pathways in monocytic cells. β1 Integrins seem to require PI-3K for efficient activation of NF-κB, whereas β2 integrin-dependent activation of this nuclear factor seems to be independent of the same PI-3K enzyme.

Syk is required for integrin-mediated PI-3K activation

To further explore the signalling pathways initiated by both types of integrin, we looked directly at PI-3K activity after cross-linking of integrins by specific antibodies and by their natural ligands. Anti-β1 integrin antibodies clearly activated PI-3K in these cells (Figure 8A). This activity was blocked completely by the specific PI-3K inhibitors wortmannin and LY294002 (Figure 8A). Similarly, anti-β2 integrin antibodies also caused activation of PI-3K, although at a somewhat lower level (Figure 8B). This activity was also blocked completely by wortmannin and LY294002 (Figure 8B). Because Syk activation is known to be the initial signalling step for β1 integrins in monocytic cells [20,22], we then looked at the relationship between Syk and PI-3K after integrin cross-linking by specific antibodies. Anti-β1 integrin antibody-induced PI-3K activation was blocked completely by piceatannol (Figure 9A). However, piceatannol did not have any effect on the PI-3K activation caused by anti-β2 integrin antibodies (Figure 9B). These results indicated that although both antibodies can induce PI-3K activation, the anti-β2 antibodies can do so by bypassing the tyrosine kinase Syk.

In contrast to the results with antibodies, when the natural integrin ligands were used, both types of integrin were capable of inducing PI-3K activation in a Syk-dependent manner (Figure 10). Piceatannol completely blocked PI-3K activity induced by adhesion of THP-1 cells to fibronectin (Figure 10A) and also to ICAM-1 (Figure 10B). In addition, treatment with 10 μg/ml cytochalasin B (which disrupts the actin filaments) for 15 min at 37 °C before stimulation of integrins resulted in complete inhibition of PI-3K activation induced by both fibronectin (Figure 10A) and ICAM-1 (Figure 10B). These results indicate that both β1 and β2 integrins initiate similar signal-transduction pathways involving Syk, PI-3K and ERK. However, the downstream effectors of these pathways are clearly different. While β1 integrins used Syk and PI-3K for NF-κB activation, β2 integrins did not use PI-3K for NF-κB activation. Both types of integrin also activated ERK, but this enzyme was not required for NF-κB activation. These data support the hypothesis that different signalling pathways are used by distinct integrins in the same cell to initiate different cell responses.

DISCUSSION

Several examples have shown that various integrins lead to different cell responses [30,31], suggesting that each type of integrin activates a particular signalling pathway. In this work, we have examined the signal-transduction pathways initiated by two types of integrin on the same cell type. We have found that in monocytic cells cross-linking of β1 integrins activates a signalling pathway that is different from the one activated by cross-linking of β2 integrins. Ligation of both types of integrin to their natural ligands caused activation of ERK and also of PI-3K. In addition, both types of integrin induced activation of the nuclear transcription factor NF-κB. However, each type of integrin utilizes a different signalling pathway for NF-κB activation. β1 integrins require Syk and PI-3K for this activation, whereas β2 integrins activate NF-κB independently of PI-3K.

Fibronectin signalling in monocytes is known to activate NF-κB [20,21]. Previously, β2 integrins had not been considered to participate in this response, so it had been assumed that they did not activate nuclear factors. In contrast, our own data, using the anti-β2 integrin monoclonal antibody IB4 and ligation to ICAM-1, show that cross-linking of β2 integrins can indeed activate NF-κB. In addition, a very recent report shows that β2 integrins on neutrophils are also capable of inducing gene activation and cytokine production [32]. These results go along with our finding of a strong activation of NF-κB after cross-linking of β2 integrins in monocytic cells. Our paper is the first report of activation of this nuclear factor by β2 integrins. In neutrophils β2 integrins induced production of IL-8 and IL-1 but not of TNFα or IL-6 [32], whereas in monocytes β1 integrins activate IL-1 and TNFα genes [16,33]. This underscores the fact that various integrins can activate different signalling pathways involving different downstream molecules [34].

Because both types of integrin activate NF-κB to similar levels, but the results of tyrosine phosphorylation suggested that different signalling pathways were being used, we looked at the activation of other molecules said to participate in integrin signalling [13,14]. The best described, although still incomplete, integrin-signalling pathway involves activation of the MEK signalling cascade [35,36]. In order to confirm that the ERK pathway was also used by β1 integrins in monocytes and to compare this with β2 integrin signalling we looked at activation of ERK after cross-linking these integrins with monoclonal antibodies. β1 Integrin cross-linking resulted in activation of ERK, whereas β2 integrin cross-linking did not cause activation of ERK. This result is in agreement with earlier studies indicating that ERK was not used by β2 integrins in monocytic cells [23,37]. However, it was reported recently that cross-linking of the α chains of β2 integrins in these cell types resulted in ERK activation [38]. Thus it seemed that cross-linking of β2 integrins by distinct antibodies was capable of inducing different signals. When the natural ligand ICAM-1 was used to ligate β2 integrins, they could clearly activate ERK (Figure 4). In the older reports only anti-β2 integrin antibodies were used. Thus investigators concluded that β2 integrins did not use the ERK signalling pathway. Our own data, using monoclonal antibodies, support this conclusion. However, when these integrins are cross-linked via their α chains [38] or are engaged by ICAM-1 (Figure 4), they turn on ERK efficiently. This indicates that various anti-β2 integrin antibodies are capable of inducing different responses, probably by causing a particular activating conformation on β2 integrins that results in altered signalling. Because many studies on integrins are based on the use of these monoclonal antibodies, we should be very careful when using antibodies to induce integrin activation.

Because both types of integrin activate NF-κB, and also the ERK pathway, it was interesting to determine the relationship between ERK activation and NF-κB activation. The specific inhibitor of MEK, PD98059 [39,40], was able to efficiently block ERK activation, but it did not affect integrin-mediated NF-κB activation (Figure 6). These data are very interesting because they point out not only that various integrins use different signalling pathways, but also that the same types of integrin initiate different signalling cascades.

A candidate signalling molecule used by integrins in the activation of NF-κB was the lipid kinase PI-3K. This enzyme has been shown to be involved in signalling by several types of receptor [41,42], including integrins [14,43]. Using wortmannin and LY294002, the specific inhibitors of PI-3K [44,45], we found that only β1 integrin-mediated activation of NF-κB was inhibited. This indicates that activation of this nuclear factor by β2 integrins is independent of PI-3K. Moreover, the fact that piceatannol, the Syk inhibitor, blocks both PI-3K activation and NF-κB activation further supports the existence of separate signalling pathways for each type of integrin. In these pathways Syk is an upstream common element, but then the pathways separate downstream of Syk. β1 Integrins use PI-3K in a cytoskeleton-dependent manner to arrive at NF-κB. In contrast, for β2 integrins PI-3K is clearly not part of this pathway, although the cytoskeleton is also required for activation of NF-κB. Recently, the GTPase Rac has been involved as a possible intermediate between PI-3K and NF-κB [22]. Whether the dependence on the cytoskeleton of β2 integrin-dependent NF-kB activation is also mediated by Rac remains to be determined.

All together, these data suggest strongly that distinct integrins activate different signalling pathways: β1 and β2 integrins turn on the ERK pathway and also another pathway that leads, independently of ERK, to activation of NF-κB. This alternative signalling pathway is also different between β1 and β2 integrins. β1 Integrins activate NF-κB in a PI-3K-dependent manner. In contrast, β2 integrins, although they efficiently turn on PI-3K, do not use this enzyme in the pathway that leads to NF-κB.

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