Phosphatidylinositol-3-kinase activation mediates proline-rich tyrosine kinase 2 phosphorylation and recruitment to β_1 -integrins in human CD34⁺ cells

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Objective. β_1 -integrins mediate hematopoietic stem and progenitor cell homing and retention in the bone marrow (BM) and inhibit hematopoietic proliferation and differentiation. Having no intrinsic kinase activity, integrins recruit intracellular kinases, such as the focal adhesion kinase (FAK) or the related proline-rich tyrosine kinase 2 (PYK2), to initiate signal transduction. Phosphatidylinositol-3-kinase (PI3K), which is involved in β_1 -integrin signaling in many cell types, is physically and functionally associated with FAK in anchorage-dependent cells. Because PYK2 is the principal focal adhesion kinase expressed in primary human CD34⁺ cells, we assessed its functional relationship with PI3K in CD34⁺ cells in response to integrin engagement.

Methods. β_1 -integrins on primary mobilized peripheral blood CD34⁺ cells and CD34⁺ KG1A cells were engaged by adhesion to fibronectin (FN) or by cross-linking with an anti- β_1 integrin antibody, respectively. PI3K activity and PYK2 phosphorylation were then assessed in the presence or absence of the PI3K inhibitor, wortmannin. Association between PI3K, PYK2, and the β_1 -integrin subunit were also evaluated in co-immunoprecipitation experiments.

Results. β_1 -integrin engagement induced PI3K activation, which was required for, and temporally preceded, PYK2 phosphorylation, indicating that PI3K lies upstream of PYK2 in CD34⁺ cells. Furthermore, although PYK2 and PI3K were constitutively associated, interaction of the PYK2/PI3K complex with β_1 -integrins required prior integrin engagement and PI3K activation.

Conclusion. Activation of PI3K following β_1 -integrin engagement on human CD34⁺ cells results in subsequent phosphorylation of PYK2, and is required for the recruitment of the PI3K/PYK2 complex to β_1 -integrins at the cell surface. © 2004 International Society for Experimental Hematology. Published by Elsevier Inc.

Introduction

Integrins are heterodimeric transmembrane receptors consisting of an α and β subunit that associate noncovalently [1,2]. Although no intrinsic enzymatic activity is conferred by the small intracellular cytoplasmic domain of integrins, this region is responsible for recruiting and activating signaling molecules that possess such activity. Hematopoiesis normally takes place in the bone marrow (BM), where proliferation and differentiation of CD34⁺ hematopoietic progenitor cells (HPC) is regulated by cytokines, chemokines, and extracellular matrix (ECM) molecules (e.g.,

fibronectin [FN] and proteoglycans) [3]. Adhesion receptors are responsible for cell-cell and cell-ECM interactions that not only retain cells in a specific location, but are involved in the activation of many signaling pathways that determine cell fate [4–6]. β_1 -integrins play a vital role in the homing and release of HPC to and from the BM microenvironment [7–12], and also regulate proliferation depending on the cytokine milieu [13,14]. Furthermore, β_1 -integrin engagement preserves the engrafting ability of HPC during ex vivo culture, possibly by preventing cell death or terminal differentiation [15,16]. Yet, the exact cellular mechanisms underlying integrin-mediated effects on adhesion, proliferation, and survival of CD34⁺ cells are not yet known.

In anchorage-dependent cells, the nonreceptor tyrosine kinase, focal adhesion kinase (FAK), localizes to the intracellular domain of integrins via its focal adhesion targeting

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domain (FAT) and is autophosphorylated upon integrin engagement, resulting in recruitment of Src-family kinases, p130^{Cas}, Hef, Grb2, and PI3K [17]. In primary human CD34⁺ cells, FAK is expressed at a low level, whereas the focal adhesion kinase family member, PYK2 (65% homology to FAK), is abundantly present [18]. Specifically, an alternatively spliced isoform of full-length Pyk2 (i.e., Pyk2H) is predominant in normal HPC, and is both increased in expression and spliced with less regularity in p210^{BCR/ABL} cells [18,19]. Unlike FAK, PYK2 gene products are predominantly localized to the cytosol in hematopoietic cells, but like FAK, stimulation of cytokine, chemokine, or adhesion receptors results in PYK2 (i.e., Pyk2 and/or Pyk2H) phosphorylation, potential recruitment of Src-family kinases, and activation of other signaling pathways [20].

Another kinase intimately involved in β_1 -integrin signaling is PI3K [21–23], which phosphorylates phosphatidylinositol (PI) and its derivatives at the D-3 position of the inositol ring and is a key signaling molecule in the activation of protein kinase B and the RAS/RAF/ERK pathway [24–26]. In many cell types, phosphorylated FAK serves as a substrate for the SH2 domains of the p85 regulatory subunit of PI3K, resulting in association between these two proteins and subsequent activation of PI3K [17]. Similarly, various differentiated hematopoietic progeny demonstrate PI3K and PYK2 activation interdependence in response to signals that stimulate adhesion [27–30]. We previously demonstrated that PYK2 mediates integrin-induced signaling cascades that result in inhibition of HPC proliferation and differentiation [31]. Here we evaluated whether β_1 -integrin-induced signaling via PYK2 in human CD34⁺ cells involves PI3K activation, and whether this activation is subsequent to, or precedes, PYK2 activation.

Materials and methods

Cells

Mobilized peripheral blood was obtained from normal donors that were selected according to standard criteria of the American Association of Blood Banks. Mononuclear cells were obtained by apheresis from donors who received 5 μ g/kg/day G-CSF administered subcutaneously for 5 days in accordance with the Guidelines from the Committee for the Protection of Human Subjects at the University of Minnesota.

CD34⁺ cells were isolated by sequential selection with Cephate SC (CellPro, Bothell, WA, USA) followed by further purification using the MACS CD34⁺ cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). CD34⁺ populations were greater than 95% pure. CD34⁺ cells were cultured overnight in BIT-9500 media (Stem Cell Technologies; Vancouver, BC, Canada) containing 50 mM 2-mercaptoethanol (BioRad, Hercules, CA, USA), 40 mg/mL low-density lipoprotein (Sigma Chemical Co, St. Louis, MO, USA), and the following cytokines: 10 pg/mL GM-CSF (Immunex, Seattle, WA, USA), 200 pg/mL G-CSF (Amgen, Thousand Oaks, CA, USA), 200 pg/mL SCF (a kind gift from Amgen), 50 pg/mL LIF,

200 pg/mL MIP-1 α , and 1 ng/mL IL-6 (R&D Systems, Minneapolis, MN, USA).

The KG1A and K562 cell lines (ATCC, Manassas, VA, USA) were maintained in IMDM/10% serum media with L-glutamine (GibcoBRL, Rockville, MD, USA) and Pen/Strep (Sigma, St. Louis, MO, USA).

Engagement of integrins

Prior to adhesion, CD34⁺ cells were incubated with the β_1 -integrin activating antibody, 8A2 (a gift from Dr. Harlan, University of Washington, Seattle, WA, USA), for 30 minutes at 37°C at a 1:10⁵ dilution [32]. Cells were then plated into wells precoated with either FN (100 μ g/mL; Gibco BRL) or poly-L-lysine (PLL; 10 μ g/mL; Sigma), and blocked with 5 μ g/mL bovine serum albumin (BSA; Sigma) as described previously [14]. Adhesion experiments were performed in a humidified atmosphere at 37°C/5% CO₂, and the nonadherent fraction was removed by 3 sequential washes with IMDM (Gibco BRL). Adherent cells were lysed in the wells, after which adherent and nonadherent fractions were combined. Cells maintained in suspension served as a negative control, whereas K562 cells were a positive control due to their constitutive PI3K activity [33]. In some studies, cells were pretreated with 50 to 100 nM wortmannin (Sigma).

Prior to β_1 -integrin engagement with cross-linking antibodies, KG1A cells were maintained in IMDM/1% fetal calf serum for 48 hours as previously described [34]. Engagement of integrins on KG1A cells was achieved by incubating the cells with a saturating dose of the blocking anti- β_1 integrin 4B4 antibody (Coulter, Hialeah, FL, USA) at 4°C for 30 minutes and subsequent incubation with a goat-anti-mouse (GAM) antibody (4 μ L/10⁶ cells; BD Pharmingen, Los Angeles, CA, USA) at 37°C for 1 to 5 minutes. Control KG1A cells were incubated with mouse IgG₁, followed by the secondary GAM antibody (BD Pharmingen).

Immunoprecipitation

Cells were lysed in immunoprecipitation buffer, containing 50 mM Tris [pH 7.4], 250 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM NaF, 2 mM NaVO₄, 1 mM NaPO₄ (all purchased from Sigma, St. Louis, MO, USA), and 1 pellet of Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) per 10 mL of buffer. Protein lysates were precleared with 30 μ L of protein-G-agarose beads (Boehringer Mannheim, Indianapolis, IN, USA) for 1 hour at 4°C on a rotating platform. The supernatant was then transferred to a new set of tubes, wherein 4 μ g of polyclonal anti-PI3K-p85 or anti-PYK2 antibodies (BD Pharmingen) were added to the lysates and rotated overnight at 4°C. Thirty-five μ L of protein-G-agarose beads were added to the immune complexes and gently rotated for 1 hour. Beads were then washed 3 times with phosphate-buffered saline (PBS)/1% NP-40 and bound material recovered by boiling in Laemmli buffer for 5 minutes.

Western blotting

Denatured PI3K-p85 or PYK2 immune complexes were resolved by SDS-PAGE and transferred to PVDF membrane (BioRad, Hercules, CA, USA), which was blocked with PBS containing 5% nonfat dry milk and probed with anti-PI3K-p85, -PYK2, or -phosphotyrosine antibodies (4G10; Upstate Biotech). Anti-phosphotyrosine blots were blocked with 5% BSA in PBS-T. Following 5 consecutive washes with PBS-T, blots were incubated with secondary HRP-conjugated antibodies and developed using the Hyper-ECL detection system (Amersham Pharmacia, Piscataway, NJ, USA).

PI3K assay

Twenty μ L of PI3K-p85 immune complexes collected on protein G-agarose beads were washed 3 times with PBS/1% NP-40; 3 times with buffer containing 0.1M Tris-HCl (pH 7.4), 5 mM LiCl, and 0.1 mM sodium orthovanadate; and 2 times with TNE (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA) containing 0.1 mM sodium orthovanadate. Following complete removal of TNE buffer, the following were added sequentially: 50 μ L of TNE, 10 μ L (20 mg) of PI, 10 μ L of 100 mM $MgCl_2$. Reactions were begun by adding 30 μ Ci ^{32}P - γ -ATP (Amersham Pharmacia) and then incubated at 37°C on a shaking platform for 10 minutes. Twenty μ L of 6N HCl was added to stop the reaction. Afterwards, 160 μ L of $CHCl_3$:MeOH (1:1) was added and the mixture was vortexed briefly. Organic and aqueous phases were separated by centrifugation at 15,000 rpm for 10 minutes and 50 μ L of each sample was spotted onto a silicon TLC potassium oxalate pretreated plate. The lipids were separated in chromatography solution ($CHCl_3$:MeOH: H_2O : NH_4OH (60:47:11.3:2)) and developed by autoradiography at $-80^\circ C$. Reagents used in the assay were purchased from Sigma, unless otherwise indicated.

Densitometry

The intensity of anti-phosphotyrosine and PYK2 bands observed in Western blots were determined using AlphaImager 2200 system densitometer and AlphaImager v5.5 software (Alpha Innotech, San Leandro, CA, USA). The density of the anti-phosphotyrosine band was normalized according to the density of the corresponding PYK2 bands for each of 4 independent experiments.

Statistical analysis

Results were expressed as mean $\pm$ SEM. Comparisons among experiments were assessed using the Student's *t*-test.

Results

To determine whether PI3K was activated in primary human CD34⁺ cells following integrin engagement, we plated CD34⁺ cells on FN or the nonspecific adhesive substrate, PLL, and allowed the cells to adhere for varying lengths of time prior to lysis for kinase activity experiments. PI3K was activated in CD34⁺ cells following adhesion to FN, reaching maximum activity by 5 minutes, whereas no PI3K activation was seen in control unstimulated cells or those adherent to PLL (Fig. 1A). To verify that the activity measured indeed resulted from immunoprecipitated PI3K, we assessed its activity in CD34⁺ cells following exposure to wortmannin, an inhibitor that, when used at concentrations of 100 nM or less, efficiently targets PI3K activity [35]. When cells were preincubated with 100 nM wortmannin, PI3K activity was significantly inhibited (Fig. 1B). In some studies, minimal PI3K activation was observed in cells maintained in suspension or cultured on PLL, possibly resulting from preincubation with the β_1 -integrin activating antibody, 8A2 (See Materials and methods).

We have previously shown in primary CD34⁺ cells that Pyk2 and its alternatively spliced isoform, Pyk2H, are phosphorylated upon β_1 -integrin engagement [31]. To test whether PI3K lies upstream or downstream of integrin-

Figure 1. PI3K is activated in primary human CD34⁺ cells following adhesion to fibronectin. (A): Primary CD34⁺ cells were either maintained in suspension or allowed to adhere onto PLL or FN for 5, 10, or 20 minutes. Lysates were immunoprecipitated with polyclonal anti-PI3K-p85 antibody. Immunoprecipitated material was split, such that 75% was used in PI3K activity assays and the remaining 25% was probed with monoclonal anti-PI3K-p85 antibody to demonstrate equal protein loading. (B): CD34⁺ cells were either maintained in suspension or allowed to adhere to PLL or FN for 2 minutes in the presence or absence of wortmannin (100 nM). Lysates were subjected to PI3K activity assays and Western blots as described above. K562 cell lysates were included as a positive control. Blots are representative of $n \geq 3$ independent experiments.

dependent PYK2 activation, phosphorylation of PYK2 was assessed in wortmannin-treated cells adherent to FN or PLL. Upon integrin engagement, PYK2 was maximally phosphorylated following 5 minutes of adhesion to FN (Fig. 2A), as we have demonstrated previously [31], whereas no phosphorylation was observed when cells were plated on PLL. Surprisingly, PYK2 phosphorylation was significantly inhibited when FN-adherent cells were precultured in the presence of wortmannin (Fig. 2A & B), suggesting that PI3K activation is required for PYK2 activation in response to β_1 -integrin engagement in CD34⁺ cells.

Direct physical interactions between the FAT domain of FAK and the intracellular domain of the β_1 -integrin subunit mediates FAK activation upon integrin engagement in anchorage-dependent cells [17]. Moreover, propagation of FAK-mediated signals depends, at least in part, on this physical interaction. Because sufficient primary CD34⁺ cells needed to study such protein-protein interactions are difficult to obtain, we utilized the CD34⁺ KG1A cell line to assess the interaction between β_1 -integrins, PI3K, and PYK2. Like primary CD34⁺ cells, engagement of β_1 -integrins in KG1A cells using blocking anti- β_1 -integrin antibodies followed by a secondary GAM cross-linking, resulted in activation of

Figure 2. PYK2 phosphorylation is PI3K-dependent. (A): Primary CD34⁺ cells were either maintained in suspension or allowed to adhere onto PLL or FN for 2 or 5 minutes. Lysates were immunoprecipitated with polyclonal anti-PYK2 antibodies and probed for tyrosine phosphorylation (pY). Membranes were stripped and reprobed with monoclonal anti-PYK2 antibody. Blots are representative of *n* = 4 independent experiments. (B): Graphical representation of relative PYK2 phosphorylation was done by normalizing phosphorylated PYK2 with total PYK2 protein and is presented as the mean ± SEM of 4 independent experiments. **p* = 0.044 ***p* = 0.043.

PI3K (Fig. 3A) and phosphorylation of PYK2 (Fig. 3B). Also like primary CD34⁺ cells, wortmannin inhibited PI3K activation and decreased PYK2 phosphorylation by more than 80% (Fig. 3A & B).

We next evaluated whether β_1 -integrins, PYK2, and/or PI3K are physically associated in KG1A cells by performing co-immunoprecipitation assays. Unlike FAK, which requires phosphorylation to recruit PI3K [17], PYK2 and PI3K were constitutively associated, regardless of integrin engagement (Fig. 4A) or PYK2 phosphorylation status (Fig. 4A vs Fig. 3B). This association between PYK2 and PI3K was also independent of PI3K activity, as wortmannin had no effect on the interaction (Fig. 4A). Furthermore, β_1 -integrin association with either PYK2 or PI3K was not observed in the absence of integrin engagement (time = 0; Fig. 4B). Upon integrin cross-linking, however, association of β_1 -integrins with PYK2 and PI3K occurred rapidly, as β_1 -integrins co-immunoprecipitated with either kinase (Fig. 4B). Finally, interaction between the PYK2/PI3K-containing complex and β_1 -integrins was inhibited when cells were pretreated with wortmannin, suggesting that PYK2 association with the β_1 -integrin complex is PI3K dependent (Fig. 4B).

Figure 3. PI3K activation and PYK2 phosphorylation in response to β_1 -integrin engagement in CD34⁺ KG1A cells mimics primary CD34⁺ cells. β_1 -integrins on CD34⁺ KG1A cells were cross-linked for 1 or 2 minutes using the anti- β_1 -4B4 antibody (β_1). Cell lysates were then immunoprecipitated with (A) anti-PI3K-p85 antibodies to assess PI3K activity, or (B) anti-PYK2 antibodies to assess its phosphorylation as described above. Blots are representative of *n* = 3 independent experiments.

Discussion

In this study, we demonstrate that β_1 -integrin engagement leads to PI3K activation in primary human CD34⁺ HPC, as well as CD34⁺ KG1A cells. Treatment with the PI3K inhibitor, wortmannin, inhibits β_1 -integrin-mediated activation of not only PI3K, but also PYK2, suggesting that PI3K activation is required for PYK2 phosphorylation and activation in response to β_1 -integrin signaling. We further demonstrate that PYK2 and PI3K constitutively associate with one another independent of β_1 -integrin engagement or the status of PI3K activation. Moreover, interaction between the β_1 -integrins and the PI3K/PYK2 complex is stimulated by β_1 -integrin engagement and subsequent PI3K activation. This data collectively suggests that β_1 -integrin-induced stimulation of PYK2 phosphorylation, and subsequent recruitment to the integrin complex, is mediated by prior induction of PI3K activity.

FAK and PI3K mediate β_1 -integrin-dependent effects on proliferation, apoptosis, and/or migration in a wide variety of cell types [17]. In anchorage-dependent cells, FAK is the major kinase responsible for initiating the β_1 -integrin signaling cascade, including activation of PI3K. Although activation of FAK and PI3K is modified by cytokine and/or cell-ECM interactions, FAK is expressed at low levels in primary CD34⁺ cells wherein its phosphorylation is undetectable following integrin engagement [31]. In contrast, PYK2 (particularly its alternatively spliced isoform Pyk2H) is expressed abundantly in CD34⁺ cells and appears to replace FAK for integrin-mediated signaling events in HPC

Figure 4. PI3K-p85 and PYK2 constitutively interact, but their association with β_1 -integrins is dependent on PI3K activity. β_1 -integrins on CD34⁺ KG1A cells were cross-linked for 1 or 2 minutes using the anti- β_1 -4B4 antibody (β_1) and cell lysates were immunoprecipitated with polyclonal antibodies against the p85 subunit of PI3K or PYK2, respectively. (A): Association between the p85 subunit of PI3K and PYK2 was determined by probing PI3K-p85 immunoprecipitates with monoclonal antibodies against PYK2, or vice versa. Blots were then stripped and reprobed with monoclonal antibodies against the primary target of the initial immunocomplex. (B): PI3K-p85 subunit and PYK2 immunocomplexes were also immunoblotted with anti- β_1 -integrin antibodies. Blots were stripped and reprobed with monoclonal antibodies against the PI3K-p85 subunit or PYK2, respectively.

and their progeny [28,31,36,37]. Like FAK, PYK2 can physically interact with PI3K [27,38], and in a number of cell types PYK2 activates PI3K in response to various stimuli [30,39]. There is also evidence to the contrary, suggesting that activation of PI3K is required for phosphorylation and activation of PYK2 [28,40]. Therefore, in contrast to FAK, interactions between PYK2 and PI3K appear to be stimulus and cell type dependent.

Using primary human CD34⁺ cells, we demonstrate here that activation of PI3K in response to β_1 -integrin stimulation is required for subsequent robust activation of PYK2, indicating that PI3K mediates the intracellular signaling cascade initiated by β_1 -integrin engagement. Similar results were also observed in the CD34⁺ myeloid leukemic cell line, KG1A. Because β_1 -integrin signaling appears similar between primary CD34⁺ cells and the CD34⁺ KG1A cell line, we used the latter model to dissect molecular events involved in integrin-induced activation of PI3K and PYK2. PI3K and

PYK2 are constitutively associated in CD34⁺ KG1A cells, irrespective of integrin signaling or PI3K activation status. However, neither PYK2 nor PI3K associate with β_1 -integrins when the integrins are not engaged.

Recent work demonstrating increased myeloproliferation upon inhibition of PI3K supports the placement of PI3K and PYK2 within the same signalling cascade [41]. Because recruitment of the PI3K/PYK2 complex to the intracellular tail of β_1 -integrins is not observed when PI3K activity is suppressed by wortmannin, PI3K-mediated phosphorylation of PYK2 appears required for stable association of PYK2 with the β_1 -integrin complex. Like FAK, integrin engagement may result in minimal phosphorylation and recruitment of PYK2 to the integrin complex via its FAT domain, resulting in a weak interaction between PYK2 and the β_1 -integrin. Moreover, this nominal phosphorylation of PYK2 may be PI3K independent. Nevertheless, subsequent activation of PI3K results in robust phosphorylation of PYK2 and firm binding between the PYK2/PI3K complex and β_1 -integrins. This hypothesis is consistent with the observation that while robust phosphorylation of PYK2, and its strong interaction with β_1 -integrins, is PI3K activity dependent, minimal phosphorylation of PYK2 and its association with integrins can still be observed in lieu of wortmannin (Fig. 4). Differences in signaling conferred by minimally phosphorylated PYK2, vs PYK2 robustly phosphorylated in a PI3K-dependent manner, have yet to be determined.

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