Involvement of Hic-5 in platelet activation: integrin α IIb β 3-dependent tyrosine phosphorylation and association with proline-rich tyrosine kinase 2

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Hic-5 and paxillin, members of the LIM protein family, have been shown to be localized in focal adhesion and to have a role in integrin-mediated signalling. In the present study we examined the involvement of Hic-5 in human platelet activation: platelets express Hic-5 but not paxillin, whereas human umbilical-vein vascular endothelial cells and MEG-01 cells express mainly paxillin. When platelets were stimulated with thrombin, collagen or the stable thromboxane A₂ analogue U46619, Hic-5 was markedly tyrosine-phosphorylated, in a manner dependent on integrin α IIb β 3-mediated aggregation. In addition, direct activation of protein kinase C with PMA resulted in tyrosine phosphorylation of Hic-5 only when platelets were fully aggregated with the exogenous addition of fibrinogen. Furthermore, PMA-induced Hic-5 tyrosine phosphorylation was also observed when platelets adhered to immobilized fibrinogen.

In studies on immunoprecipitation and immunodepletion, Hic-5 seemed to associate with proline-rich tyrosine kinase 2 (Pyk2) but only marginally with focal adhesion kinase. When platelets were stimulated with thrombin, both Hic-5 and Pyk2 translocated to the cytoskeleton from the cytosol and membrane fractions in a manner dependent on α IIb β 3-mediated aggregation. Finally, on stimulation with PMA, Hic-5, as well as Pyk2, translocated to the cell periphery, where a meshwork of actin filaments assembled after adhesion to immobilized fibrinogen. Our results suggest that Hic-5 might be important in platelet aggregation and adhesion, in a manner dependent on α IIb β 3-mediated outside-in signalling, through association with Pyk2.

Key words: adhesion, focal adhesion kinase, protein kinase C.

INTRODUCTION

Protein tyrosine phosphorylation is one of the most important events constituting signal transduction pathways from the cell surface in response to various extracellular stimuli; in this, platelets are no exception [1,2]. It has been well established that agonist-induced protein tyrosine phosphorylation in platelets is divided into integrin α IIb β 3-dependent and α IIb β 3-independent processes [2,3]. Whereas the early phase of platelet protein tyrosine phosphorylation, including that of the Src family tyrosine kinases and Syk, is independent of the binding of fibrinogen to the activated integrin α IIb β 3, some of the tyrosine-phosphorylated proteins that appear in the later phase are closely related to the integrin-mediated response and subsequent platelet aggregation [2–5]. We showed previously that tyrosine phosphorylation of proline-rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK) belongs to the later phase of platelet activation and is dependent on integrin α IIb β 3 outside-in signalling and protein kinase C (PKC) activation [6,7].

The *hic-5* gene was originally isolated, as one of the clones inducible by transforming growth factor β 1 and by H₂O₂ from mouse osteoblastic cells by using a differential display method [8]. One of the unique characteristics of Hic-5 is its similarity to paxillin [9–11]. Hic-5 and paxillin share structural features, notably four C-terminal LIM domains and LD domains at the N-terminus [10–12]. Immunocytochemical studies have shown that, as well as paxillin, Hic-5 is localized in focal adhesions and associates with Pyk2 in rat fibroblasts [9]. The roles of Hic-5 in

platelet activation are unknown, although (1) Hic-5 is a member of the focal adhesion complex and has some roles in integrin signalling [9,10,12,13], and (2) Hic-5 is highly expressed in platelets [14]. In the present study we characterized the mode of Hic-5 tyrosine phosphorylation in platelet activation.

MATERIALS AND METHODS

Materials

The anti-(Hic-5) polyclonal antibody was kindly provided by Dr T. Sasaki (Sapporo Medical University School of Medicine, Sapporo, Japan) [9]. Botrocetin and von Willebrand factor were donated by Dr Y. Fujimura (Nara Medical University, Nara, Japan). Recombinant human thrombopoietin was a gift from Kirin Brewery Co. (Gunma, Japan). Ro 31-8220, a specific PKC inhibitor, was provided by Roche Products (Welwyn Garden City, Herts., U.K.).

The following materials were obtained from the indicated suppliers: thrombin (Green Cross, Osaka, Japan); collagen (Hormon-Chemie, Munich, Germany); U46619 (Calbiochem-Novabiochem Co., CA, U.S.A.); GRGDS peptide (Peptide Institute, Osaka, Japan); anti-Pyk2 polyclonal antibody and anti-phosphotyrosine monoclonal antibody (mAb) (4G10) (Upstate Biotechnology, Lake Placid, NY, U.S.A.); anti-Pyk2 mAb, anti-paxillin mAb, anti-(Hic-5) mAb and anti-phosphotyrosine mAb (PY20) (Transduction Laboratories,

Abbreviations used: FAK, focal adhesion kinase; HUVEC, human umbilical-vein endothelial cells; mAb, monoclonal antibody; PKC, protein kinase C; Pyk2, proline-rich tyrosine kinase 2.

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Lexington, KY, U.S.A.); anti-FAK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); anti-Syk mAb (Wako Pure Chemical Industries, Tokyo, Japan); PMA, FITC-conjugated phalloidin, and fibrinogen (Sigma Chemical Co., St Louis, MO, U.S.A.); Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.).

Cell preparation

Platelet-rich plasma was prepared as described previously [15] and incubated with 1 mM acetylsalicylic acid for 30 min to exclude any secondary effects of thromboxane A_2 . The platelets were washed and resuspended as described previously [15]. The final platelet suspensions were adjusted to 10^9 cells/ml and supplemented with 1 mM $CaCl_2$ unless stated otherwise. MEG-01 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and maintained at 37 °C in RPMI 1640 medium containing 10% (v/v) fetal calf serum, penicillin G (100 i.u./ml) and streptomycin sulphate (100 mg/ml) in an atmosphere of air/ CO_2 (19:1). Human umbilical vein endothelial cells (HUVEC) were prepared as described previously [16].

Platelet aggregation monitoring

Platelet aggregation was monitored by measuring light transmission with an AA-100 platelet aggregation analyser (Sysmex, Kobe, Japan). The instrument was calibrated with a platelet suspension for zero light transmission and with a buffer for 100% transmission.

Unless stated otherwise, platelets were stimulated at 37 °C under continuous stirring at 1000 rev./min. To inhibit platelet aggregation induced by thrombin or the stable thromboxane A_2 analogue U46619, platelets were preincubated with 500 μ M GRGDS (a competitive antagonist to inhibit fibrinogen- α IIb β 3 interaction; single-letter amino acid codes) [4] for 5 min and not stirred; platelet aggregates were not formed under these conditions (results not shown). In contrast, when platelets were stimulated with PMA, 200 μ g/ml fibrinogen was added 5 min before stimulation to induce platelet aggregation fully, whereas 200 μ M GRGDS was added to block it completely.

Immunoprecipitation

Washed platelet reactions were terminated with an equal volume of 2 $\times$ ice-cold lysis buffer [2% (v/v) Triton X-100/100 mM Tris/HCl (pH 7.4)/6 mM EDTA/2 mM Na_3VO_4 /100 mM NaCl/25 mM NaF/1 mM PMSF/50 μ g/ml leupeptin]. All subsequent immunoprecipitation steps were performed at 4 °C. The lysates were centrifuged at 15000 *g* for 5 min, then precleared with Protein A-Sepharose CL-4B. The resultant supernatants were incubated for 2 h with the antibody indicated. Protein A-Sepharose CL-4B was then added and it was incubated for a further 1 h. The Sepharose beads were washed three times with 1 $\times$ lysis buffer. The samples were then solubilized with an SDS sample buffer and used for immunoblotting.

Immunoblotting

The proteins were resolved by SDS/PAGE [8% (w/v) gel] under reducing or non-reducing conditions, then transferred electrophoretically to PVDF membranes. When Hic-5 was detected in immunoprecipitates, samples without boiling were resolved under non-reducing conditions. This is because the band of IgG heavy chain (50–55 kDa) interfered with the detection of Hic-5 (55 kDa) under reducing conditions (see Figure 6A). Accordingly, Hic-5

immunoblotting studies were performed with the use of unreduced samples except for that shown in Figure 6(A). The membranes were blocked with 1% (w/v) BSA in PBS. After extensive washing with PBS containing 0.4% (v/v) Tween 80, the immunoblots were incubated for 2 h with anti-Pyk2 mAb (0.25 μ g/ml), anti-FAK polyclonal antibody (0.1 μ g/ml), anti-(Hic-5) polyclonal antibody (0.3 μ g/ml), anti-paxillin mAb (0.05 μ g/ml) or anti-phosphotyrosine mAb (1 μ g/ml PY20 plus 1 μ g/ml 4G10). Antibody binding was detected with peroxidase-conjugated goat anti-mouse IgG, or anti-rabbit IgG, and detected with ECL[®] chemiluminescence reaction reagents (Amersham Pharmacia Biotech). When indicated, quantitative analysis was performed with a PDI400oe Scanner together with Quantity One 2.5a software for Macintosh.

Platelet adhesion assay

The adhesion of platelets to fibrinogen was assayed as described previously [7]. Non-adherent platelets from BSA-coated dishes were used as a control. The protein concentrations of the resultant supernatant were determined by dye binding [17]; 0.5 mg of protein was used for immunoprecipitation.

Fractionation of platelets

Subcellular fractionation of platelets was performed essentially as described [18]. The proteins in these fractions, after being boiled for 3 min, were separated by SDS/PAGE and detected by immunoblotting as described above.

Actin staining

For actin staining, cells were fixed with 3% (w/v) paraformaldehyde in PBS for 40 min, then permeabilized with 0.2% (v/v) Triton X-100 for 8 min. Actin filaments were detected by staining with 5 μ g/ml FITC-conjugated phalloidin. Actin staining was observed and photographed with a confocal microscope.

Immunofluorescent staining

Cells fixed and permeabilized as described above were incubated for 2 h with an anti-paxillin mAb (2.5 μ g/ml) or anti-Pyk2 mAb (25 μ g/ml) at room temperature, washed in PBS, then incubated for 1 h with FluoroLink[™] Cy3[™]-labelled goat anti-mouse IgG (Amersham Pharmacia Biotech). Cells were finally washed three times with PBS. Immunofluorescent staining was observed and photographed with a confocal microscope.

Presentation of data

Unless stated otherwise, the results shown are from a single experiment representative of at least four separate experiments.

RESULTS

Identification of Hic-5, but not paxillin, in human platelets

We first checked the expression of Hic-5 and paxillin in platelets. The whole-cell lysates obtained from HUVEC, MEG-01 (a human megakaryoblastic cell line) cells [19] and platelets were immunoblotted with anti-paxillin mAb, which detects both paxillin and Hic-5 [14]. Whereas HUVEC and MEG-01 cells were found to express paxillin (68 kDa), only a 55 kDa protein was detected in platelets (Figure 1). Hic-5, a relatively new member of the LIM protein family, possesses four LIM domains at its C-terminus and is similar to paxillin in structure; its

Figure 1 Human platelets express Hic-5 but not paxillin

The lysates obtained from HUVEC, MEG-01 cells or platelets were immunoprecipitated (IP) with anti-paxillin mAb or anti-(Hic-5) polyclonal antibody. The immunoprecipitates, along with whole cell lysates, were resolved by SDS/PAGE, then immunoblotted with anti-paxillin mAb or anti-(Hic-5) polyclonal antibody. Arrows indicate the locations of paxillin and Hic-5. The locations of molecular mass markers are indicated (in kDa) at the right. When the MEG-01 lysates were immunoprecipitated with anti-paxillin mAb and immunoblotted with anti-(Hic-5) polyclonal antibody, a protein of approx. 60 kDa was often detected; this protein could not be identified.

molecular mass is 55 kDa [8–10]. To determine whether or not this 55 kDa protein specifically expressed in platelets was Hic-5, the cell lysates were immunoprecipitated with anti-paxillin mAb and anti-(Hic-5) polyclonal antibody, then immunoblotted with anti-(Hic-5) polyclonal antibody and anti-paxillin mAb respectively. As reported previously [14], platelets specifically expressed Hic-5 but not paxillin (Figure 1). Platelet Hic-5 was immunoprecipitated efficiently with either anti-paxillin mAb or anti-(Hic-5) polyclonal antibody (Figure 1). Accordingly, we used a commercially available anti-paxillin mAb to detect platelet Hic-5 in subsequent studies except for that shown in Figure 2(B), where specific anti-(Hic-5) mAb was used. Platelet Hic-5 was usually recognized as a 55 kDa doublet when analysed with both anti-paxillin mAb (Figure 1) and anti-(Hic-5) mAb (see Figure 2B). A similar observation has been already reported with the use of other cells [10,14,20]. There is a possibility that Hic-5 is not a single molecule but consists of at least two isoforms, as with paxillin [21].

Hic-5 tyrosine phosphorylation in activated platelets

We next examined Hic-5 tyrosine phosphorylation in human platelets stimulated with several activators. Thrombin, collagen or U46619 elicited Hic-5 tyrosine phosphorylation when immunoprecipitates with anti-paxillin mAb were analysed (Figure 2A). Similar results were obtained when platelets stimulated with thrombin or U46619 were immunoprecipitated with anti-(Hic-5) mAb (Figure 2B). The Hic-5 phosphorylation occurred gradually as platelet aggregation proceeded; maximum tyrosine phosphorylation was attained 2–5 min after stimulation (Figure 2A). Under the conditions described above, platelets were stirred continuously and therefore underwent aggregation (results not shown).

In contrast, adrenaline (epinephrine), which activates Gi [22], did not induce Hic-5 phosphorylation (Figure 2). Although thrombopoietin reportedly induces the tyrosine phosphorylation of several proteins (including JAK2, Cbl and Vav) in platelets [23,24], this cytokine failed to induce the tyrosine phosphorylation of Hic-5 (Figure 2). We reported recently that interactions between glycoprotein Ib and von Willebrand factor activate tyrosine kinases in platelets [25]. However, this mode of platelet activation, elicited by the combined addition of botrocetin plus von Willebrand factor [25], failed to induce the tyrosine phosphorylation of Hic-5 (Figure 2).

Integrin α IIb β 3- and PKC-dependent Hic-5 tyrosine phosphorylation in platelets

Integrin α IIb β 3 is one of the most important and abundant platelet integrins; it has an important role in adhesion and aggregation by binding mainly to fibrinogen [4]. Because Hic-5 is reportedly localized to focal adhesions and involved in cytoskeletal reorganization [9,10,12,13], we examined the involvement of integrin α IIb β 3 in Hic-5 tyrosine phosphorylation. When platelet aggregation was suppressed by GRGDS (a competitive inhibitor of fibrinogen binding to α IIb β 3), the tyrosine phosphorylation of Hic-5 induced by thrombin or U46619 was mostly abolished (Figures 3A and 3B); Hic-5 tyrosine

Figure 2 Platelet Hic-5 becomes tyrosine-phosphorylated on stimulation with thrombin, collagen or U46619

(A) Platelets were stimulated with 0.1 i.u./ml thrombin, 50 μ g/ml collagen, 1 μ M U46619, 100 ng/ml thrombopoietin (TPO), 50 μ M adrenaline (epinephrine) or 10 μ g/ml von Willebrand factor plus 6 μ g/ml botrocetin (vWF + Bo) for the indicated durations (', minutes; '', seconds). Platelet lysates were immunoprecipitated with anti-paxillin mAb and resolved by SDS/PAGE, then immunoblotted with anti-phosphotyrosine (PY) mAb (upper panel) or anti-paxillin mAb (lower panel). (B) Platelets were stimulated with 0.1 i.u./ml thrombin or 1 μ M U46619 for the indicated durations. Immunoprecipitates obtained with anti-(Hic-5) mAb were resolved by SDS/PAGE, then immunoblotted with anti-phosphotyrosine (PY) mAb (upper panels) or anti-(Hic-5) mAb (lower panels).

Figure 3 Hic-5 tyrosine phosphorylation is dependent on α IIB β -mediated aggregation and PKC activation

Platelets were stimulated with 0.1 i.u./ml thrombin (A), 1 μ M U46619 (B) or 1 μ M PMA (C) for various durations (' , minutes; '' , seconds); in conditions under which platelets formed aggregates (+) or not (—) (see the Materials and methods section). Tyrosine phosphorylation of Hic-5 was determined as described in the legend to Figure 2.

Figure 4 Ro 31-8220 or staurosporine inhibits thrombin-elicited Hic-5 tyrosine phosphorylation

Platelets pretreated without (control) or with 10 μ M Ro 31-8220 (Ro) or 1 μ M staurosporine (Ssp) for 5 min were stimulated with 1 i.u./ml thrombin for 2 min. Tyrosine phosphorylation of Hic-5 was determined as described in the legend to Figure 2.

phosphorylation induced by thrombin and U46619 was inhibited to $9.4 \pm 0.9\%$ and $9.4 \pm 0.6\%$ (means $\pm$ S.D., $n = 3$) of the control respectively by pretreatment with GRGDS. Furthermore, the active phorbol ester PMA, which directly activates PKC [26], induced Hic-5 tyrosine phosphorylation (Figure 3C) but only when platelets had been aggregated by the exogenous addition of fibrinogen (see the Materials and methods section). These results suggest that the tyrosine phosphorylation of Hic-5 is dependent on α IIB β -mediated platelet aggregation. Because platelet stimuli capable of activating PKC were found to induce Hic-5 tyrosine phosphorylation, we further examined the involvement of PKC in Hic-5 tyrosine phosphorylation with the PKC inhibitors Ro 31-8220 and staurosporine [27,28]. Pretreatment with these PKC inhibitors resulted in a marked inhibition of the thrombin-elicited phosphorylation of Hic-5 (Figure 4).

We next attempted to determine whether the fibrinogen- α IIB β interaction was sufficient to phosphorylate Hic-5 (in the absence of PKC activation) by using a platelet adhesion assay. The adhesion of platelets to immobilized fibrinogen did not by itself lead to tyrosine phosphorylation of Hic-5 (Figure 5A); Syk tyrosine phosphorylation was observed under the same conditions (results not shown), as described previously [29]. However, Hic-5 was markedly phosphorylated when platelets were treated with PMA (Figure 5A). Similar results were obtained with the tyrosine phosphorylation of Pyk2 (Figure 5B) and FAK (Figure 5C), as reported previously [7]. Accordingly, in platelets both α IIB β -mediated signalling and PKC activation are necessary for the tyrosine phosphorylation of Hic-5, as with that of Pyk2 and FAK.

Association of Hic-5 with Pyk2 in platelets

Hic-5 is a constituent of focal adhesion and has been shown to associate with Pyk2 through its N-terminal domain [9]. Furthermore, Pyk2, but not FAK, specifically phosphorylates Hic-5 in transfected COS-7 cells [20]. Accordingly, we examined whether Hic-5 interacted with Pyk2 in platelets. In Hic-5 immunoprecipitation studies, thrombin was found to induce the stimulation-dependent association of a tyrosine-phosphorylated 110 kDa protein with Hic-5 (Figure 6A). Reprobing of the same membrane with anti-Pyk2 mAb revealed that this 110 kDa tyrosine-phosphorylated protein was Pyk2, which was found to associate constitutively with Hic-5 (Figure 6B). The Pyk2-Hic-5 interaction was also confirmed by Pyk2 immunoprecipitation studies (Figure 6C). We next performed Hic-5 immunodepletion studies with the use of resting platelets. Immunodepletion of Hic-5 resulted in the removal of most Hic-5 and Pyk2 from the

Figure 5 Tyrosine phosphorylation of Hic-5 (A), Pyk2 (B) and FAK (C) is dependent on α IIB β -mediated adhesion (to immobilized fibrinogen) and PKC activation

Platelets treated with or without 10 nM PMA were placed on immobilized fibrinogen for 1 h as described in the Materials and methods section. Platelets recovered from BSA-coated dishes were used as a control (left lanes). Platelet lysates were immunoprecipitated with anti-paxillin mAb (A), anti-Pyk2 polyclonal antibody (B) or anti-FAK polyclonal antibody (C) and resolved by SDS/PAGE, then immunoblotted with anti-phosphotyrosine (PY) mAb (upper panels) or the antibody used for immunoprecipitation (lower panels).

Figure 6 Hic-5 associates with Pyk2 in platelets

Platelets were treated without or with 0.1 i.u./ml thrombin (THR) for 2 min. **(A)** The immunoprecipitates obtained with anti-paxillin mAb were resolved by SDS/PAGE, then immunoblotted with anti-phosphotyrosine (PY) mAb. Arrows indicate the locations of a 110 kDa tyrosine-phosphorylated protein associated with anti-paxillin mAb (p110) and IgG (heavy chain). The locations of molecular mass markers are indicated (in kDa) at the right. **(B, C)** The immunoprecipitates obtained with anti-paxillin mAb **(B)** and anti-Pyk2 mAb **(C)** were immunoblotted with anti-Pyk2 mAb and anti-paxillin mAb respectively. Whole-cell lysates **(C)** were also analysed. **(D)** The cell lysates were immunodepleted with anti-paxillin mAb or control mouse IgG. Left panel: the platelet lysates immunodepleted with anti-paxillin mAb (1st ID, immunodepleted once; 2nd ID, immunodepleted twice) or not (—) were subjected to SDS/PAGE, then immunoblotted with anti-FAK polyclonal antibody, anti-Pyk2 mAb or anti-paxillin mAb. Right panel: the levels of FAK, Pyk2 and Hic-5 in cell lysates after the second immunodepletion were quantified as described in the Materials and methods section. Immunodepletion with control mouse IgG was also performed (control). Data are expressed as a percentage of control values obtained from the cell lysates without immunodepletion. Results are means $\pm$ S.D. ($n = 3$).

Figure 7 Hic-5 translocates into the cytoskeletal fraction in an aggregation-dependent manner

(A) Platelets were stimulated with 0.1 i.u./ml thrombin for the indicated durations (' minutes; '' seconds). The platelet lysates were fractionated into cytoskeleton (CS), membrane skeleton (MS) and cytosol (CY) as described in the Materials and methods section. Each fraction was resolved by SDS/PAGE and immunoblotted with anti-Pyk2 mAb (upper panel) or anti-paxillin mAb (lower panel). **(B)** Platelets were stimulated with 0.1 i.u./ml thrombin for various durations in conditions under which platelets formed aggregates (+) or not (—) (see the Materials and methods section). The cytoskeletal samples were subjected to SDS/PAGE and probed with anti-Pyk2 mAb (upper panel) or anti-paxillin mAb (lower panel).

lysates but not that of FAK (Figure 6D). These findings suggest that Pyk2 associates constitutively with Hic-5 in platelets.

Integrin-dependent cytoskeletal translocation of Hic-5 and Pyk2 in aggregating platelets

We next examined the subcellular distribution of Hic-5 and Pyk2, with special emphasis on cytoskeletal reorganization. Most Pyk2 and Hic-5 was present in the cytosol and membrane skeleton fractions in unstimulated platelets (Figure 7A). When platelets were stimulated with thrombin (and aggregated), not only Pyk2, as described previously [7], but also Hic-5 translocated to the cytoskeleton in a time-dependent manner (Figure 7A). The translocation of Hic-5 (and Pyk2) to the cytoskeleton was mostly abolished by the inhibition of α IIB β 3-mediated platelet aggregation (Figure 7B).

Immunofluorescence studies of Hic-5 and Pyk2 in adhering platelets

Targeting of signalling proteins to specific regions of the cell is also important for control of the adhesion response. Previous studies demonstrated that Hic-5 was localized to focal adhesions in rat fibroblasts [9]. We finally investigated the intracellular localization of Hic-5 and Pyk2 after platelet adhesion. Under the conditions used, platelets adhered to immobilized fibrinogen, as examined by immunofluorescent staining and confocal microscopy (Figure 8A). In the presence of PMA, adhering platelets extended lamellipodia and spread; submembrane networks of actin were formed in the periphery of the spreading cells (Figure 8D). Both Hic-5 and Pyk2 translocated to the cell periphery, where the formation of stress fibres occurred after spreading on immobilized fibrinogen only when platelets were stimulated (with PMA) (Figures 8E and 8F).

Figure 8 Subcellular localization of Hic-5 and Pyk2 in adherent platelets

Platelets treated without (A–C) or with (D–F) 10 nM PMA were placed on immobilized fibrinogen for 10 min as described in the Materials and methods section. The cells were fixed, then incubated with FITC-conjugated phalloidin (A, D), anti-paxillin mAb (B, E) or anti-Pyk2 mAb (C, F). Actin staining and immunofluorescent staining were performed as described in the Materials and methods section.

DISCUSSION

The proteins Hic-5 and paxillin are closely related in structure [9–11], suggesting that they have overlapping or redundant functions. However, previous reports have revealed that these cytoskeletal proteins are involved in differential biological responses; paxillin is implicated in cell growth [30], whereas Hic-5 is implicated in cell differentiation [31,32]. Stimulation of rat calvarial cell differentiation by retinoic acid resulted in a 4-fold increase in *hic-5* expression [32]. In addition, *in situ* hybridization studies demonstrated that *hic-5* was expressed in certain terminally differentiated cells, such as neurons [32], in adult mice. In the present study we have shown that platelets express Hic-5, but not paxillin, whereas MEG-01 cells express mainly paxillin. This is consistent with the idea proposed by Hagmann et al. [14] that a switch from paxillin to Hic-5 expression must occur late in the maturation of megakaryocytes into platelets. In fact, we have confirmed that Hic-5 expression is enhanced during the differentiation of CD34⁺ cells (from mouse bone marrow) into megakaryocytes forming pro-platelets (results not shown).

In the present study we have shown that the tyrosine phosphorylation of Hic-5 is dependent on both PKC and α IIB β 3-mediated outside-in signalling: PKC activation or α IIB β 3 binding to fibrinogen on their own failed to induce Hic-5 tyrosine phosphorylation in platelets. In addition, Hic-5 translocated to the cytoskeleton in a manner dependent on α IIB β 3-mediated signalling. Because Hic-5 seems to be closely involved in α IIB β 3 outside-in signalling, it is likely that Hic-5 is important in

eliciting platelet cytoskeletal responses resulting from the ligation of α IIB β 3 integrin. Although paxillin and its tyrosine phosphorylation seem to be crucial in regulating both focal adhesion assemblies and integrin-mediated signal transduction in other cells [30,33], it is possible that Hic-5, rather than paxillin, has a role in integrin-mediated function in platelets. Furthermore, it should be noted that blood stem cells gain expression of the platelet-specific integrin α IIB β 3 during late-stage differentiation into platelets [34] and that Hic-5 is expressed in platelets but not in MEG-01 cells. These facts further suggest the involvement of Hic-5 in α IIB β 3 signalling and in differentiated platelet functional responses.

Another important finding was that platelet Hic-5 associates with Pyk2. Our finding is in accord with a previous report that Pyk2 has a higher affinity to Hic-5 than to paxillin [9]. Paxillin seems to associate with FAK and is tyrosine-phosphorylated after integrin stimulation in a FAK-dependent manner in many cell types [33,35,36]. In contrast, Hic-5 seems to be a specific substrate for Pyk2 [20]. Because platelet Hic-5 becomes tyrosine-phosphorylated in a manner dependent on α IIB β 3-mediated signalling and selectively associates with Pyk2, it is possible that Pyk2 phosphorylates Hic-5, after the ligation of integrin in platelet activation. Furthermore, both Hic-5 and Pyk2 translocated to the cell periphery after spreading on immobilized fibrinogen and were tyrosine-phosphorylated under identical conditions, suggesting that Hic-5 and Pyk2 become localized to focal contacts via a mechanism involving tyrosine phosphorylation in platelets. We showed previously that the profile of Pyk2 is similar to that of FAK, in terms of α IIB β 3 integrin-dependent tyrosine phosphorylation [7]. It is possible that the association of Hic-5 with Pyk2 specifically transduces Pyk2 functions after α IIB β 3-mediated outside-in signalling.

In cells other than platelets, Pyk2 (but not FAK) activation is independent of integrin-mediated signalling [33,37]. Although the C-terminal domains of Pyk2 are known to mediate its targeting to focal adhesion, N-terminal domains are thought to interfere with it [33,38]. This interference has been suggested to explain why the differential mode of Pyk2 is distinct from that of FAK (even though that they belong to the same gene family) [33,38]. As described earlier, the platelet is an exception in that both Pyk2 and FAK are activated similarly in a manner dependent on integrin α IIB β 3-mediated signalling [7]. Here we have shown that Pyk2 associates specifically with Hic-5, which is specifically expressed in platelets. Because Hic-5 is localized almost exclusively to focal adhesions, there is a possibility that this paxillin homologue might lead and keep Pyk2 to focal contacts in platelets, explaining the mode of Pyk2 activation in these anucleate cells.

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