has been well characterized as an integrin activator [3,4]. The talin head domain (THD) contains four subdomains: F0, F1, F2, and F3. The F3 domain itself can bind to the β3 cytoplasmic domain and exert αIIbβ3 activation [5]. Other subdomains also have important roles in the activation [6-8]. The kindlin family members (kindlin-1, -2, and -3), which are focal adhesion proteins, have recently been proven to be critical for integrin activation [9,10]. Kindlin-1 and -2 are widely expressed and kindlin-3 expression is restricted mainly to hematopoietic cells [11]. Several studies suggest that the binding of talin and kindlins to the integrin β3 cytoplasmic domain is pivotal for the final step in the inside-out activation of αIIbβ3. Moreover, since kindlins synergistically augment talin-dependent αIIbβ3 activation, they act as a co-activator of talin [12,13]. However, regulatory molecules other than talin and kindlins necessary to αIIbβ3 activation remain to be fully clarified.

Since platelets are inadequate for gene manipulation, the CHO cell system has been used to study essential regulators of integrin αIIbβ3 function. For example, αIIbβ3-expressing CHO cells contributed to the elucidation of the functional importance of kindlin-1 and -2 as co-activators and of THD as a direct activator of integrin [10,12]. It was also shown that the Rap1-GTP-interacting adaptor molecule promotes talin-dependent integrin activation in the CHO cell system [14]. A chimeric integrin, αIIbα6Aβ3β1 or αIIbα5β3, expressed on CHO cells having the extracellular and transmembrane domains of αIIbβ3 connected to the cytoplasmic domains of α6Aβ1or α5β3 has been constitutively active on CHO cells but susceptible to integrin regulatory proteins [15]. Several integrin regulatory proteins including H-ras, PEA-15, CD98, and talin were characterized in this cell system [15-18]. Thus, the CHO cell system has been utilized to analyze the mechanisms by which integrin function is regulated.

Integrin-linked kinase (ILK) was originally identified as a serine/threonine kinase associated with integrin β1 and β3 cytoplasmic domains. It consists of three domains: an N-terminal ankyrin repeat domain, a putative pleckstrin homology domain, and a C-terminal kinase domain [19]. Many studies have shown that ILK is widely expressed and involved in interactions between integrins, cytoskeletal proteins, and signaling molecules. A deficiency or aberrant function of ILK resulted in the impairment of adhesion, spreading, migration, proliferation, and survival of the cells [20]. ILK seems to have two functions: that of a scaffold protein and that of a protein kinase, whereas the kinase activity is controversial [21,22]. At focal adhesion sites, ILK forms a heterotrimeric complex composed of the adaptor proteins PINCH and parvin [23-28]. PINCH consists of two members, PINCH-1 and -2, each of which consists of five LIM domains. PINCH-1 and -2 are ubiquitously expressed in mammalian tissues and show overlapping expression in many tissues. Parvin comprises three members, α-, β-, and γ-parvin, and contains N-terminal nuclear localization sequences and two calponin homology domains. In mammalian tissues, α- and β-parvin are ubiquitously expressed but γ-parvin is expressed mainly in hematopoietic tissues. These adaptor proteins are known to directly bind to several cytoplasmic proteins including Nck2 for PINCH and filamentous actin for parvin [25,29]. The ankyrin repeat domain of ILK binds to PINCH and the kinase domain binds to parvin. ILK interacts directly or indirectly with several other cytoskeletal and signaling proteins and exerts diverse roles in different tissues [30].

In our previous study, we identified ILK as a molecule important for integrin activation, using an expression cloning system as follows. First, we established CHO cells expressing constitutively active integrin αIIbα6Bβ3 whose αIIb cytoplasmic domain we replaced by that of integrin α6B (parental cells). Next we obtained mutant cells with inactive integrin using genome-wide mutagenesis, and finally isolated an ILK cDNA was isolated as a factor that complements the function of inactive αIIbα6Bβ3 in mutant cells by expression cloning [31]. Although the role of ILK at focal adhesion sites has been well studied, there are only a few reports on the involvement of ILK in integrin activation [32,33]. In the present study, we further investigated the mechanisms by which ILK regulates integrin activation in the CHO cell system.

Materials and Methods

Plasmids

Human wild-type (WT) αIIb and β3 subcloned into expression plasmid pcDNA3 (Invitrogen, San Diego, CA) were provided by Drs P. Newman and G. White (Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI). pRKHA, including full-length mouse talin-1 was a gift from Dr K. Yamada (NIH, Bethesda, MD). The N-terminal head region (residues 1-433) of talin-1 was constructed by polymerase chain reaction (PCR) and subcloned into green fluorescence protein (GFP) containing vector pEGFP-N1 to make a fusion protein of THD with GFP (THD-GFP) (Clontech, Mountain View, CA). Mouse α-parvin cDNA and mouse PINCH-1 cDNA (Thermo Scientific Open Biosystems, Lafayette, CO) were amplified by PCR and then were subcloned into expression plasmids, pcDNA3.1 (Invitrogen) for α-parvin and pBApo-CMV Pur DNA (Takara Bio, Shiga, Japan) for PINCH-1. pcDNA3-αIIbα6B was created using PCR-based mutagenesis as previously described [31]. Nucleotide and amino acid numbers begin with the start codon (ATG) and the first Met residue, respectively. The full length of rat ILK cDNA was amplified by PCR then subcloned into pcDNA3 and GFP-encoding plasmid pAcGFP1-Hyg-C1 to make a fusion protein of ILK with GFP (GFPILK-WT) (Clontech). Three point mutations (H99D/F109A/W110A) in the ankyrin repeat domain of ILK were introduced into pAcGFP1-Hyg-C1 to make a fusion protein of the ILK mutant with GFP (GFPILK-H99D/F109A/W110A). Two point mutations (M402A/K403A) in the ILK kinase domain were introduced in pAcGFP1-Hyg-C1 to make a fusion protein of the ILK mutant with GFP (GFPILK-M402/K403A). The ILK mutant (H99D/F109A/W110A) was designed to disrupt the PINCH binding based on the crystal structure of a complex of the ankyrin repeat domain of ILK with the LIM1 domain of PINCH, PDB 3F6Q [34]. The ILK mutant (M402A/K403A) was designed to disrupt the parvin binding as previously reported [35]. Expression plasmid pCMV-SPORT6, containing full-length mouse kindlin-2 (Thermo Scientific Open Biosystems) was obtained. All PCR-generated DNA inserts were verified by

sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Cell Cultures

CHO-K1 cells from ATCC were cultured in DMEM supplemented with 10% fetal bovine serum and 1% non-essential amino acids (Sigma-Aldrich, St. Louis, MO). CHO cells stably expressing constitutively active αIIbα6Bβ3 (parental cells) were previously established [31]. CHO-K1 cells were cotransfected with pcDNA3-αIIbα6B and pcDNA3-β3 using Lipofectamine 2000 (Invitrogen) and selected with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). G418-resistant cells expressing αIIbα6Bβ3 were cloned to isolate parental cells by a limiting dilution method. ILK-deficient mutant cells, which result in its inactive form from active αIIbα6Bβ3 (mutant cells), were previously established by the introduction of random mutations into the parental cells using a chemical mutagen, ethyl methane sulfonate (EMS) [31]. For αIIbβ3-expressing CHO cells, pBApo-CMV Pur DNA-αIIb and pcDNA3-β3 were cotransfected into CHO-K1 cells by Lipofectamine 2000. After selection with 12 μg/ml puromycin (Clontech) and 1 mg/ml G418, clones expressing αIIbβ3 were established by the limiting dilution method.

Flow cytometry

Flow cytometry analyses were performed as previously described [31]. Cells suspended in Tyrode's buffer containing 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 1% bovine serum albumin were incubated with the primary antibody of 5 μg/ml of a mouse monoclonal antibody (mAb) specific for αIIbβ3, HIP8 (BD Biosciences) for 30 minutes at 4°C. After washing, the cells were incubated with the secondary Ab of ~1 μg/ml phycoerythrin (PE)-conjugated rat anti-mouse IgG (BD Biosciences) for 30 minutes at 4°C, washed once, stained with 1 μg/ml 7-aminoactinomycin D (7AAD) (Sigma-Aldrich) to discriminate dead cells, and then analyzed on a flow cytometer (FACSCalibur; BD Biosciences). As a negative control, cells were incubated with the secondary Ab alone. For the binding of a ligand-mimetic, activation-specific anti-αIIbβ3 mAb, PAC-1 (BD Biosciences), cells were incubated with 10 μg/ml PAC-1 for 30 minutes at room temperature in the absence or presence of 10 μM of a peptidomimetic antagonist of αIIbβ3, Ro44-9883 (a gift from Astellas Pharma, Tokyo, Japan), washed once, and then incubated with 10 μg/ml PE-conjugated anti-mouse IgM (eBioscience, San Diego, CA) for 30 minutes at 4°C. After washing, cells were stained with 7AAD and then analyzed. As a positive control for PAC-1 binding, cells were incubated with 15 mM dithiothreitol (DTT) for 10 minutes at 37°C to activate integrin αIIbα6Bβ3 and incubated with PAC-1 as mentioned above. Integrin activation was quantified as an activation index calculated using the following formula: 100 x (F − Fo) / (Fmax − Fo), where F is the median fluorescence intensity (MFI) of PAC-1 binding, Fo is the PAC-1 binding in the presence of Ro44-9883, and Fmax is the maximal PAC-1 binding in the cells treated with DTT. For fibrinogen binding, cells were incubated with 150 μg/ml Alexa-Fluor 647-conjugated fibrinogen (Molecular Probes, Eugene, OR) under similar conditions to the above assay. In some experiments using αIIbβ3-expressing CHO cells, the activation indexes were normalized by αIIbβ3 expression, as shown by the following formula: 100 x (F − Fo) / (F_1 - F_2), where F and Fo are the same as mentioned above, F_1 is the HIP8 binding, and F_2 is the binding of the secondary Ab alone.

Immunoblotting

Immunoblotting was performed using procedures previously described [31]. In brief, cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated with either one of the following primary Abs: 0.125 μg/ml mouse mAb specific for ILK, 3/ILK (BD Biosciences), 0.25 μg/ml mouse mAb specific for PINCH, 49/PINCH (BD Biosciences), rabbit polyclonal Ab specific for α-parvin (IgG fraction; 1:3,000) (Sigma-Aldrich), 1 μg/ml mouse mAb specific for β-parvin, 11A5 (Millipore, Temecula, CA), mouse mAb specific for talin, 8D4 (ascites fluid; 1:2,000) (Sigma-Aldrich), rabbit polyclonal Ab specific for kindlin-2 (IgG fraction; 1:1,000) (ProteinTech Group, Chicago, IL), 0.5 μg/ml rabbit polyclonal Ab specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FL-335 (Santa Cruz Biotechnology), horseradish peroxidase (HRP)-conjugated rabbit polyclonal Ab specific for β-actin (IgG fraction; 1:2000) (MBL, Woburn, MA), or 1 μg/ml mouse mAb specific for GFP, B-2 (Santa Cruz Biotechnology) for 90 minutes at room temperature. After washing, bound Abs except for the HRP-conjugated Abs were incubated with peroxidase-conjugated secondary Abs (Kirkegaard & Perry Labs, Gaithersburg, MD) Detection was performed using a chemiluminescence kit (Immobilon Western; Millipore, Bedford, MA). Chemiluminescence was visualized by an image analyzer, LAS-3000PLUS (Fuji Photo Film, Kanagawa, Japan).

Immunoprecipitation

Parental cells were solubilized at concentrations of 2 x 10^7 cells/ml in a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 1% Triton X-100) containing proteinase inhibitors. After centrifugation at 15,000 x g for 12 min, the supernatant (200 μl) was subjected to immunoprecipitation using protein A/G agarose (Santa Cruz Biotechnology) and the following Abs: 1 μg mouse mAb specific for ILK, 3/ILK, 1 μg mouse mAb specific for PINCH, 49/PINCH, 1 μg mouse IgG_1 isotype control, MOPC 21 (Sigma-Aldrich), 1 μg mouse IgG_{2a} isotype control, UPC-10 (Sigma-Aldrich), 1 μg rabbit polyclonal Ab specific for α-parvin, and 1 μg pooled rabbit IgG (Invitrogen). The immunoprecipitants were analyzed by immunoblotting as described above. As a positive control, cell lysates (15 μl) were loaded onto a lane.

Short interfering RNA (siRNA) and transfection

Total RNA from parental cells was extracted with Trizol reagent (Invitrogen). PINCH-1, α- and β-parvin, and kindlin-2 mRNA were amplified by a one-step RT-PCR kit (Qiagen, Valencia, CA) using primers specific to both mouse and rat homologues according to the manufacturer's instructions. RT-PCR products were directly sequenced using specific primers.

siRNAs against RNA targets were designed and synthesized by Invitrogen (Stealth RNAi). The siRNA target sequences of hamster mRNA are as follows: PINCH-1 (p) 157 sense 5'-CGGGUUAUUAAAGCCAUGAACAACA-3'; PINCH-1 (p) 755 sense 5'-CCTGCAATACCAAATTAACACTCAA-3'; α-pavin (pa) 503 sense 5'-CCAGGAGCATCAAGTGGAATGTAGA-3'; α-pavin (pa) 761 sense 5'-CAGACAAGCTCAACGTGGTAAAGAA-3'; β-pavin (pb) 900 sense 5'-UCCACAACUUCUACCUGACACCUGA-3'; β-pavin (pb) 1011 sense 5'-AAGAUGUGGUAAACUUGGACCUCAA-3'; kindlin-2 (k) 770 sense 5'-GAUCGCUAAUGGAACAAGAUGUGUGAA-3'; kindlin-2 (k) 770 scrambled control sense 5'-GAUAUCGUAAAGAACUAGUGCGGAA-3'; kindlin-2 (k) 1733 sense 5'-AAGGCGGCAAGAGAGAAGAACUUAU-3'; kindlin-2 (k) 1733 scrambled control sense 5'-AAGCGGGAAAGAAAGAAGUCGCUAU-3'. The sequences of ILK siRNA (Ilk1255) and its scrambled control were previously described [31]. Stealth RNAi-negative control duplexes (Invitrogen) were used as controls in knockdown experiments targeting PINCH-1 and parvins. Cells cultured in six-well plates were transfected with 12.5-50 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) or cotransfected with 30 nM siRNA and the indicated amounts of plasmid (0.5 μg pEGFP-N1 encoding THD-fused GFP or 0.015 μg pEGFP-N1 plus 0.485 μg pcDNA3 as a negative control) using Lipofectamine 2000. Transfected cells were usually analyzed at 72 hours after transfection. For transfections with plasmid DNA alone, Lipofectamine 2000 was employed.

Statistical analysis

The statistical significance of observed data was determined using one-way analysis of variance followed by Bonferroni's post hoc test using PRISM 5 software (GraphPad Software; La Jolla, CA, USA). P values of 0.05 or less were considered statistically significant.

Results

Evaluation of PINCH, α-parvin, talin, and kindlin-2 in ILK-deficient αIIbα6Bβ3- inactive mutant CHO cells and αIIbα6Bβ3-active parental CHO cells

We previously obtained ILK-deficient mutant cells by treating parental cells expressing constitutively active αIIbα6Bβ3 with the chemical mutagen EMS [31]. In the mutant cells, ILK mRNAs contained two nonsense mutations, R317X and W383X, in a compound heterozygous state, resulting in a complete lack of ILK expression. It has been shown that ILK forms a ternary complex with PINCH and parvins to make an IPP complex [25]. To assess the role of ILK-binding proteins in ILK-deficient mutant cells with the inactive state of αIIbα6Bβ3, we examined the protein expression of ILK-binding adaptor proteins, PINCH and α-parvin. In addition to a lack of ILK expression, mutant cells showed severe reductions in the protein expression of PINCH and α-parvin as compared to parental cells. In contrast, talin and kindlin-2, which play critical roles in integrin activation, were present at normal levels of protein expression (Figure 1A). Transfection of a plasmid encoding ILK cDNA into mutant cells showed the increased expression of ILK and concomitant increases in PINCH and α-parvin expression levels but did not affect talin and kindlin-2 expression levels. Moreover, flow cytometry using an activation-specific anti-αIIbβ3 mAb, PAC-1, showed that ILK-plasmid transfection increased PAC-1 binding compared to empty-plasmid transfection (Figure 1B). These data suggest that ILK, PINCH, and α-parvin are necessary to restore the active state of αIIbα6Bβ3 in mutant cells.

Detection and assessment of ILK, PINCH, and parvin (IPP) complex in αIIbα6Bβ3-active parental cells

Since ILK, PINCH, and parvins form the IPP complex, we assessed IPP complex formation in αIIbα6Bβ3-active parental cells, which show constitutively active αIIbα6Bβ3. Immunoprecipitation experiments revealed that ILK is coprecipitated with PINCH and α-parvin, indicating the presence of the IPP complex in those cells (Figure 2). To evaluate the importance of these proteins comprising the complex on the active state of αIIbα6Bβ3, we performed RNA interference (RNAi) experiments targeting PINCH or parvins, and we analyzed the active state of αIIbα6Bβ3 by flow cytometry using PAC-1. For PINCH siRNA, we targeted PINCH-1, one of the two PINCH isoforms, because we failed to find sequences of PINCH-2 mRNA in CHO cells. Each of the two PINCH-1 siRNAs (p157 and p755) decreased PINCH expression and concomitantly decreased ILK and α-parvin expression compared to nontargeting negative control siRNA in parental cells (Figure 3A), leading to a decreased integrin activation index, which was determined by flow cytometry analysis of PAC-1 binding (Figure 3B). For parvin siRNA, α- and β-parvins were knocked down since both parvins are thought to bind to ILK. A mixture of two α-parvin siRNAs (pa503 and pa761) or two β-parvin siRNAs (pb900 and pb1011) reduced α-parvin or β-parvin expression, respectively; however, ILK and PINCH expression levels were less significantly affected (Figure 3C). When a mixture of α-parvin and β-parvin siRNAs was transfected into parental cells, the expression levels of α-parvin and β-parvin were decreased and concomitant decreases in ILK and PINCH expression were observed. Flow cytometry analysis evaluating the activation state exhibited that the transfection of both α- and β-parvin siRNAs, but not that of either α- or β-parvin siRNA significantly decreased PAC-1 binding (Figure 3D). These data suggest that the IPP complex formation of ILK, PINCH, and parvins is necessary for αIIbα6Bβ3 activation in a CHO cell system.

Knockdown of kindlin-2 in αIIbα6Bβ3-active parental cells

In our previous work, talin siRNA decreased PAC-1 binding to αIIbα6Bβ3-active parental cells [31]. To confirm that kindlin-2 plays an important role in integrin activation in the CHO cell system, we performed the kindlin-2 siRNA experiment in parental cells (Figure 3E, F). Each of two different siRNAs (k770 and k1733) reduced kindlin-2 expression and decreased PAC-1 binding in association without significantly affecting ILK or talin expression. In addition, when an ILK cDNA was cotransfected with kindlin-2 siRNA into ILK-deficient mutant

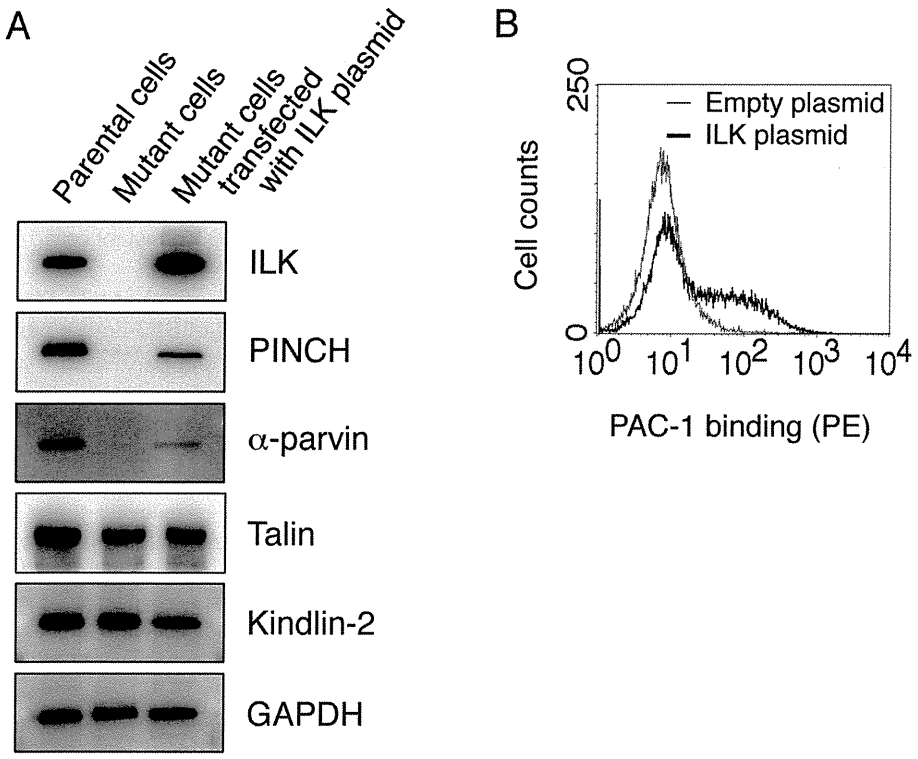

Fig. 1

Figure 1. Characterization of ILK-deficient mutant cells expressing inactive αIIbα6Bβ3. (A) Immunoblotting for ILK, PINCH, α-parvin, talin, and kindlin-2. Cell lysates obtained from parental cells with constitutively active αIIbα6Bβ3, ILK-deficient mutant cells with inactive αIIbα6Bβ3, and mutant cells transiently transfected with rat ILK cDNA were electrophoresed on SDS-PAGE gels and immunoblotted with indicated Abs. GAPDH shows an internal loading control. (B) Flow cytometry analysis showing PAC-1 (an activation-specific mAb for αIIbβ3) binding to mutant cells transiently transfected with either ILK plasmid or empty plasmid. Bound PAC-1 was detected with a PE-conjugated secondary mAb.

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cells, PAC-1 binding was decreased as compared to those of the cotransfection of both the ILK cDNA and scrambled kindlin-2 siRNA (data not shown). These data indicate that kindlin-2 is required for αIIbα6Bβ3 activation in parental cells.

Role in binding of ILK to PINCH and parvin for integrin activation

To examine the significance of ILK-PINCH binding for integrin activation, we generated a GFP-fused ILK mutant (GFPILK-H99D/F109A/W110A) in which mutations were introduced into the binding sites for the LIM1 domain of PINCH in the ankyrin repeat domain of ILK. This ILK mutant is designed to disrupt ILK-PINCH binding but not ILK-α-parvin binding. When GFPILK-WT cDNA was transfected into mutant cells, PAC-1 binding was increased (Figure 4A). Transfection of the GFPILK-H99D/F109A/W110A cDNA into mutant cells failed to recover PAC-1 binding and did not induce an obvious upregulation of PINCH expression, whereas the ILK mutant protein was well expressed and α-parvin was similarly increased compared to the case with GFPILK-WT cDNA transfection, indicating the ILK-α-parvin complex (Figure 4B). These data suggest that ILK-PINCH binding is required for stable PINCH expression even in the presence of ILK, as well as for αIIbα6Bβ3 activation in the CHO cell system. When cell lysates of the mutant cells transfected with the GFPILK-H99D/F109A/W110A cDNA was subjected to immunoprecipitation with anti-α-parvin Ab, the ILK mutant was coprecipitated (data not shown). In addition, we generated a GFP-fused ILK mutant (GFPILK-M402A/K403A) that disrupts the parvin binding and that impairs the localization of ILK to focal adhesions as shown in the previous report [35]. Transfection of GFPILK-M402A/K403A cDNA into mutant cells showed strongly impaired PAC-1 binding and did not induce an overt upregulation of α-parvin expression (Figure 4C, D). These data suggest that ILK-parvin binding is necessary for stable parvin expression, as well as for αIIbα6Bβ3 activation in the CHO cell system.

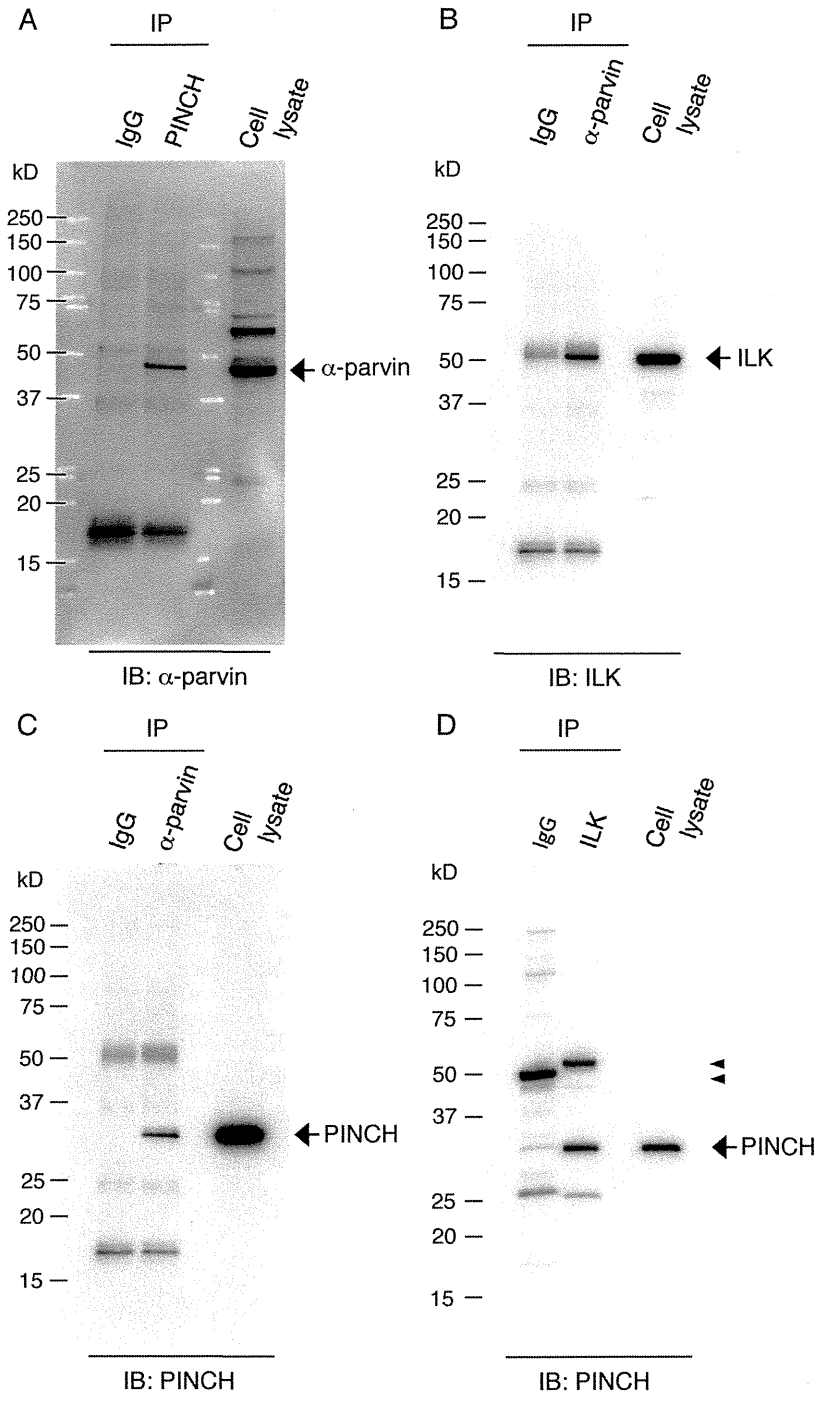

Fig. 2

Figure 2. Detection of IPP complex proteins in αIIbα6Bβ3-active parental cells. Cell lysates obtained from αIIbα6Bβ3-active parental cells were immunoprecipitated with Abs against PINCH (A), α-parvin (B, C), and ILK (D). The co-precipitates were detected by Abs for α-parvin (A), ILK (B), and PINCH (C, D). IgG means immunoprecipitation (IP) using non-immune control IgG. IB stands for immunoblotting. Arrows indicate the predicted sizes of the indicated proteins. Arrowheads (D) indicate the antibody heavy chains used in the IP. Different mobilities between those of the two IgG antibodies are probably caused by differences in the amino acid compositions of them.

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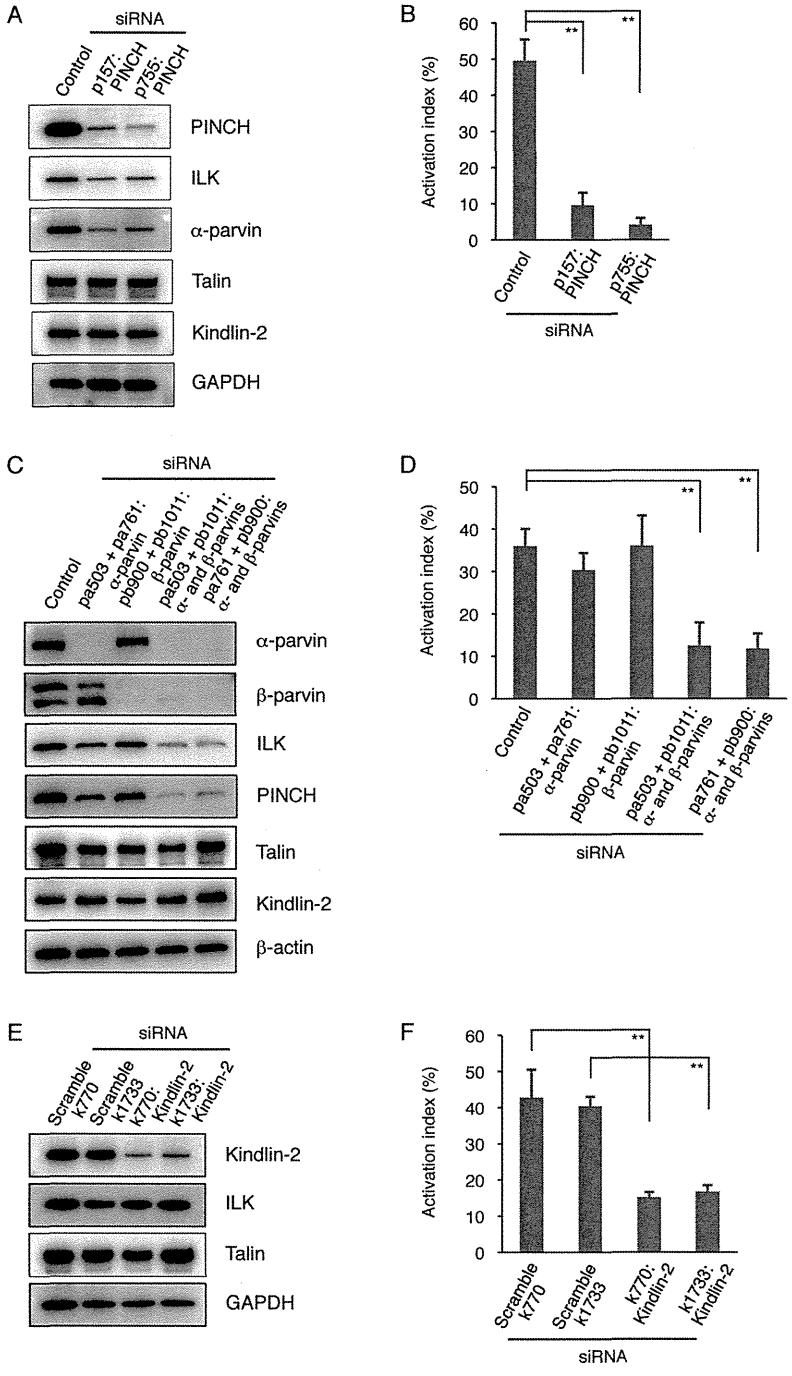

Fig. 3

Figure 3. Knockdown effects of PINCH, parvins, and kindlin-2 in αIIbα6Bβ3-active parental cells. αIIbα6Bβ3-active parental cells were transiently transfected with PINCH siRNAs (p157 and p755) (A), α-parvin siRNAs (pa503 and pa761) (C), β-parvin siRNAs (pb900 and pb1011) (C), kindlin-2 siRNAs (k770 and k1733) (E), negative control siRNAs, and scrambled siRNAs. Cell lysates were electrophoresed on SDS-PAGE gels, and the separated proteins were immunoblotted with the indicated Abs. GAPDH and β-actin are shown as internal loading controls. The activation indexes of transfected cells (B, D, F) were calculated using the formula shown in Materials and Methods. A value of 100% implies the maximum PAC-1 binding to the cells treated with dithiothreitol (DTT). Data represent means ± standard deviation (SD) of three (B, F) or four (D) independent experiments. ** indicates $P < 0.01$.

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Moreover, when fibrinogen, a natural ligand for αIIbβ3, was used instead of PAC-1 in these experiments, similar results were obtained (Figure 4E).

Analysis of ILK in inactive αIIbβ3-expressing CHO cells

αIIbβ3 is present in an inactive state on CHO cells, and overexpression of the THD into those cells can induce αIIbβ3 activation [36]. The THD directly binds the integrin β3 cytoplasmic domain and causes integrin activation. To examine ILK's contribution to THD-mediated αIIbβ3 activation, we performed knockdown experiments targeting ILK under transient THD expression (Figure 5). For the cotransfection of THD-GFP cDNA with scrambled ILK siRNA (scramble Ilk1255), the THD-GFP highly expressing cells exhibited a significant increase in the PAC-1 binding as compared to the case with GFP cDNA cotransfection (Figure 5A, B). In contrast, the cotransfection of THD-GFP cDNA with ILK siRNA (Ilk1255) showed decreased PAC-1 binding in the cells with high expression of THD-GFP (Figure 5A, B). The protein expression levels of ILK, PINCH, and α-parvin were suppressed by the cotransfection of ILK siRNA and THD-GFP cDNA, whereas the expression level of THD-GFP was not changed in the presence of ILK siRNA (Figure 5C). These results suggest that THD is sufficient to restore partially the integrin activation upon elimination of ILK by its siRNA (Ilk1255) and that ILK may assist THD in regulating the integrin activation state by assembling the IPP complex. In addition, these findings obtained from αIIbβ3-expressing CHO cells support the importance of the IPP complex observed in the αIIbα6Bβ3-expressing CHO cells. □

Overexpression of the IPP complex in inactive αIIbβ3-expressing CHO cells

To examine the IPP complex's role in THD-mediated αIIbβ3 activation, we performed overexpression experiments of ILK, PINCH-1, and α-parvin in inactive αIIbβ3-expressing CHO cells. As expected, THD-GFP overexpression into αIIbβ3-expressing CHO cells induced PAC-1 binding in the cells with high expression of THD-GFP, as compared to the case with GFP cDNA transfection (Figure 6A, B). Interestingly, although quadruple overexpression of GFP and IPP did not significantly increase PAC-1 binding, quadruple overexpression of THD-GFP and IPP caused higher PAC-1 binding compared to the case with THD-GFP overexpression, suggesting a supportive effect of IPP on THD-mediated αIIbβ3 activation (Figure 6A, B). Kindlin-2 binds to the integrin β3 cytoplasmic domain and functions as a co-activator of talin [12,37]. As expected, double overexpression of THD-GFP and kindlin-2 cooperatively increased PAC-1 binding (Figure 6A, B), suggesting that both THD and kindlin-2 are required for the full activation of αIIbβ3. Regarding protein expression, THD-GFP was adequately expressed in each transfection, and the expression levels of ILK, PINCH, α-parvin, and kindlin-2 were higher than their endogenous expression levels in the cells with indicated transfection (Figure 6C). Thus, these data suggest that the IPP complex supports the THD for integrin αIIbβ3 activation.

Discussion

ILK is a multidomain scaffold protein that interacts with several cytoplasmic proteins [38,39]. In integrin adhesion sites, ILK exists in a ternary complex composed of the two other proteins PINCH and parvin. The ternary complex formation can stabilize each component and exert its function. In fact, ILK-deficient mutant CHO cells exhibited profoundly reduced PINCH and α-parvin expression levels, leading to inactive αIIbα6Bβ3 (Figure 1). The introduction of ILK expression into ILK-deficient mutant cells increased the expression levels of PINCH and α-parvin, accompanied by αIIbα6Bβ3 activation (Figure 1). The involvement of the IPP complex formation in integrin activation was confirmed in the knockdown experiments of PINCH and parvins in αIIbα6Bβ3-active parental cells (Figure 3). The ILK mutants with defects in either PINCH or parvin binding did not activate αIIbα6Bβ3 in ILK-deficient mutant cells (Figure 4). Since it has been reported that the parvin-binding defective ILK mutant (M402A/K403A) fails to localize to focal adhesions [35], these two ILK mutants are probably not recruited to the αIIbα6Bβ3 sites in a process of integrin activation. Thus, our data support a previous report that the proper complex formation of ILK, PINCH, and parvin is necessary for ILK recruitment to the integrin adhesion sites [35,40].

Recent studies of integrin regulatory proteins have shown that both talin and kindlins directly bind to different regions in the integrin β cytoplasmic domain and cooperate in a final step of integrin activation [9,12]. In our experiments, kindlin-2 knockdown in αIIbα6Bβ3-active parental cells reduced the activation state of αIIbα6Bβ3, and talin knockdown exhibited similar results in our previous work [31]. The knockdown of PINCH and of parvins in the IPP complex decreased the activation state of αIIbα6Bβ3 in parental cells to a similar extent as did the knockdown of either kindlin-2 or talin. In inactive αIIbβ3-expressing CHO cells, overexpression of THD-GFP induced αIIbβ3 activation, and ILK knockdown reduced THD-GFP-mediated αIIbβ3 activation (Figure 5). Moreover, overexpression of the IPP complex further augmented the activation state of αIIbβ3 induced by the THD in inactive αIIbβ3-expressing CHO cells (Figure 6). These data suggest that the IPP complex participates in the cooperation of talin and kindlin-2 during the activation processes of not only αIIbα6Bβ3 but also αIIbβ3. The precise binding sites of ILK in the integrin β cytoplasmic domain remain to be determined, although their interaction has been reported [19]. There seem to be two possible direct and indirect manners of ILK binding to the integrin cytoplasmic domain. It was recently reported that the binding of PAT-4 (ILK) to UNC-112 (kindlin) in *C. elegans* is necessary for UNC-112 recruitment to adhesion sites [41]. While kindlin alone appears to bind to integrin in mammalian cells, the IPP complex would contribute to effective binding of kindlin to the β integrin cytoplasmic domain to fully induce conformational changes of integrin.

Adaptor proteins, PINCH-1 and -2, share high amino acid sequence identity [27]. Those are ubiquitously expressed in different tissues and show overlapping expression in many tissues. Both isoforms bind equally well to ILK, but its binding is

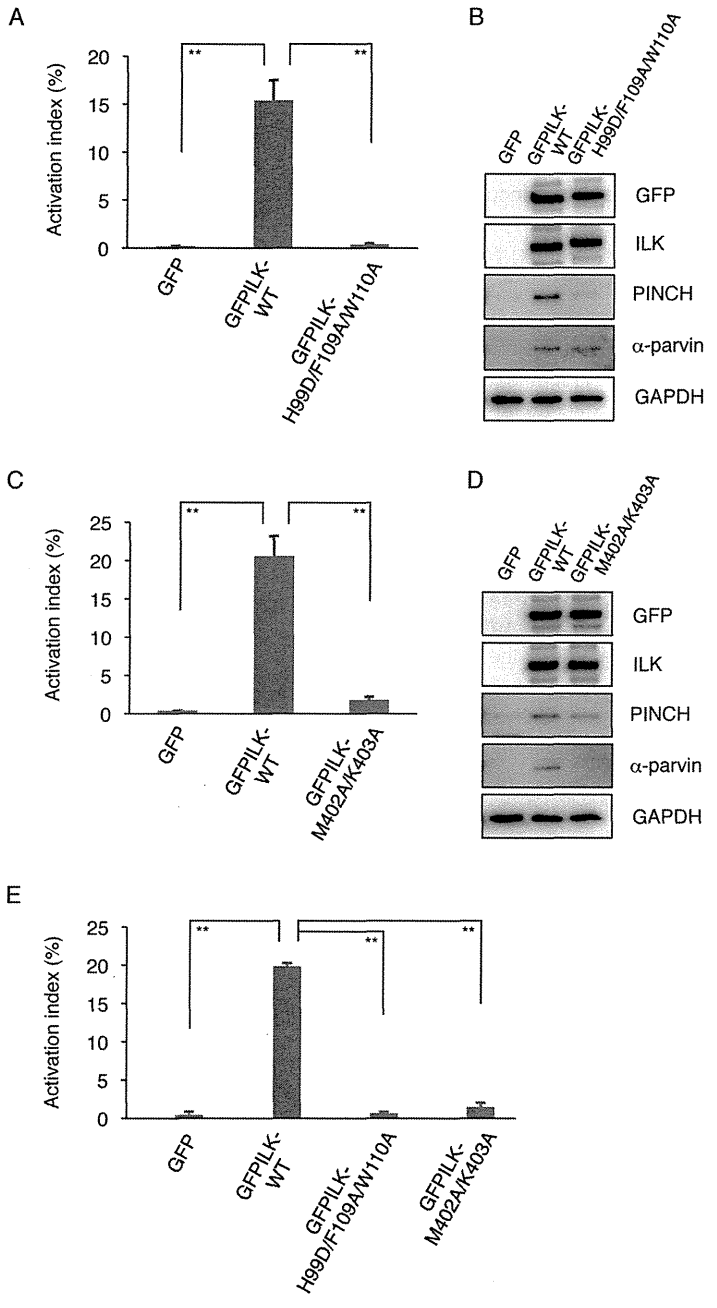

Fig.4

Figure 4. Effects of ILK mutants with defects in either PINCH or parvin binding. The activation indexes of transfected cells (A, C, E). ILK-deficient mutant cells were transiently transfected with GFP cDNA, GFP-fused wild-type ILK (GFPILK-WT) cDNA, GFP-fused ILK mutant with defective PINCH binding (GFPILK-H99D/F109A/W110A) cDNA (A, E), or GFP-fused ILK mutant with defective parvin binding (GFPILK-M402A/K403A) cDNA (C, E). After transfection, the binding of either PAC-1 (A, C) or fibrinogen (E) to the cells was analyzed by flow cytometry. The activation index was determined by the formula shown in Materials and Methods. A value of 100% represents the maximal binding of PAC-1 or fibrinogen to the cells treated with dithiothreitol. Data represent means ± SD of three independent experiments. ** indicates $P < 0.01$. Immunoblotting showing protein expression of GFP (B, D), GFP-fused wild-type ILK (GFPILK-WT) (B, D), GFP-fused ILK mutant with defective PINCH binding (GFPILK-H99D/F109A/W110A) (B), and GFP-fused ILK mutant with defective parvin binding (GFPILK-M402A/K403A) (D) in ILK-deficient mutant cells. Cell lysates were electrophoresed and immunoblotted with indicated Abs.

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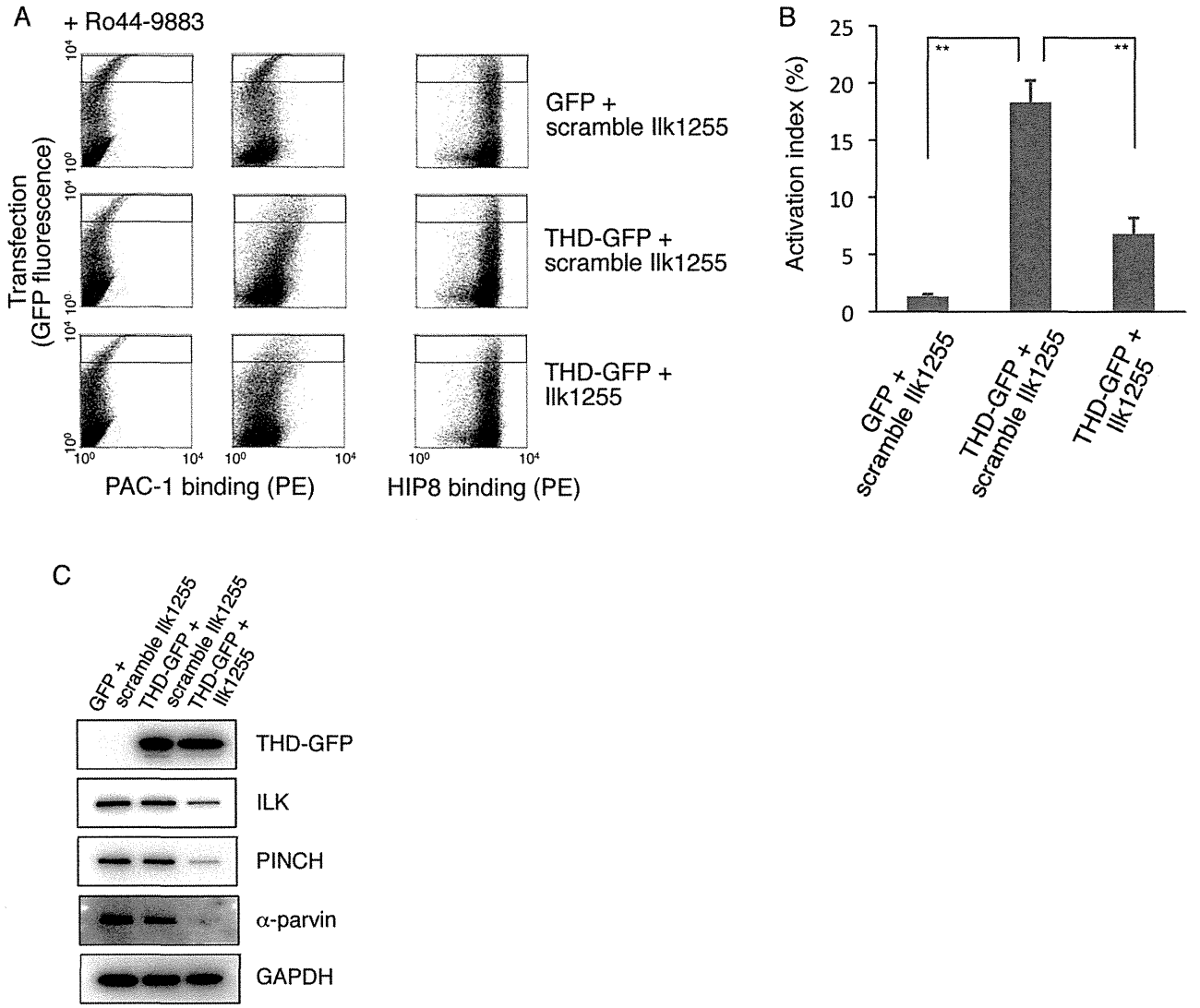

Fig. 5

Figure 5. Knockdown effects of ILK on THD-mediated αIIbβ3 activation. (A) Dot plot detecting PAC-1 binding to cotransfected cells. Inactive αIIbβ3-expressing CHO cells were transiently cotransfected with GFP cDNA plus scrambled ILK siRNA (scramble Ilk1255), with THD-GFP cDNA plus scrambled ILK siRNA (scramble Ilk1255), or with THD-GFP cDNA plus ILK siRNA (Ilk1255). Highly transfected cells (cells in gated regions) were analyzed for PAC-1 binding or HIP8 (an αIIbβ3-specific mAb) binding. (B) The activation indexes of transfected cells. The activation index was determined by the formula shown in Materials and Methods. A value of 100% implies the median fluorescence intensity of HIP8 binding to the cells in gated regions. Data represent means ± SD of three independent experiments. ** indicates $P < 0.01$. (C) Immunoblotting to evaluate expression levels of IPP and THD-GFP. Cell lysates were electrophoresed and immunoblotted with indicated Abs.

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mutually exclusive [34,42]. PINCH-1 is expressed in hematopoietic systems, and strong expression of it has been observed in megakaryocytes during fetal liver hematopoiesis [27]. PINCH-2 also joins in the IPP complex and contributes to the stabilization of individual proteins. We examined only PINCH-1 expression in CHO cells since we were unable to find PINCH-2 mRNA in parental CHO cells. Our knockdown experiment using PINCH-1-specific siRNA revealed the reduction of both ILK and α-parvin expression levels. In addition, published amino acid sequences of hamster PINCH-2

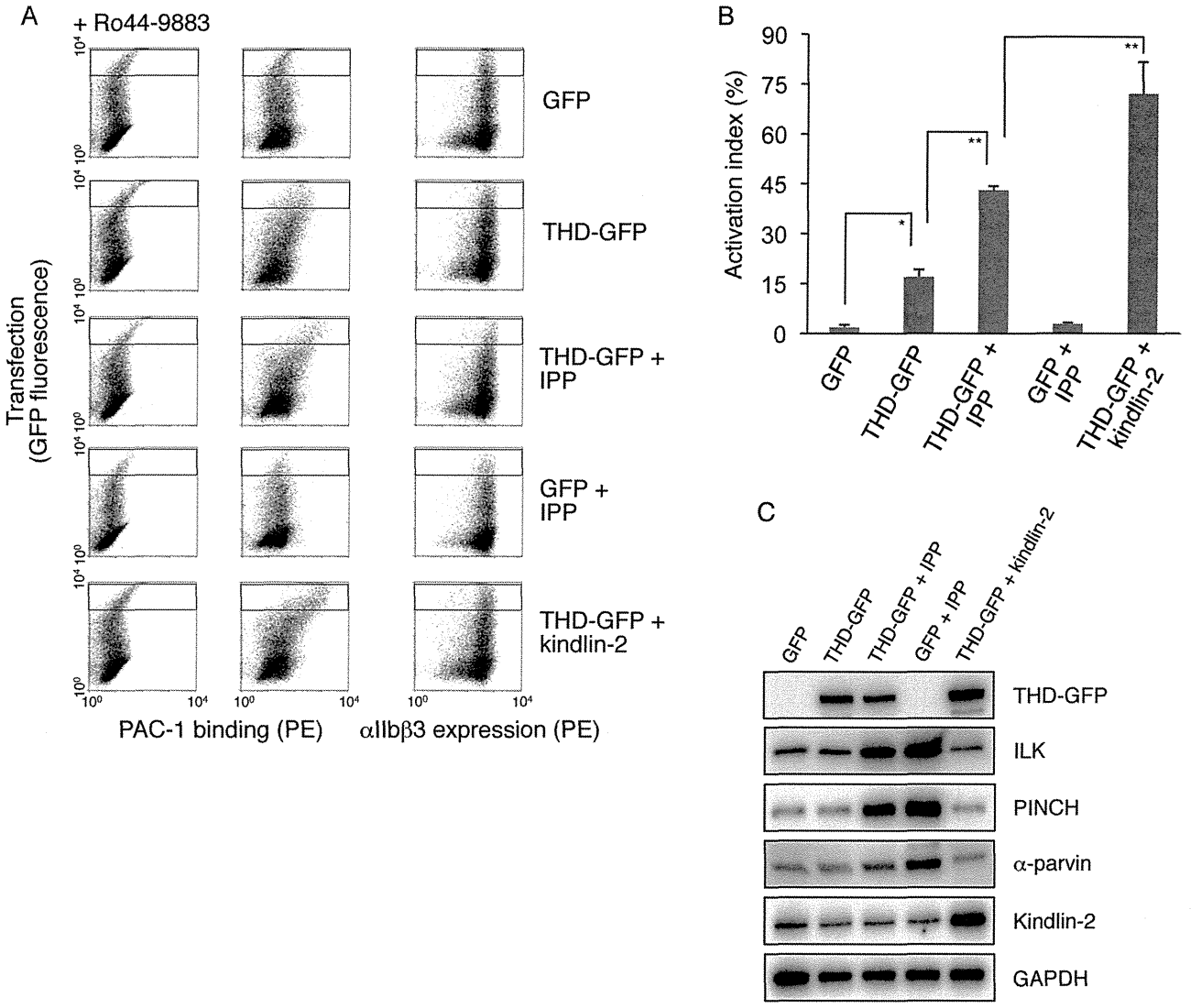

Fig. 6

Figure 6. Effects of IPP overexpression on THD-mediated integrin activation in inactive αIIbβ3-expressing CHO cells. (A) Dot plot detecting PAC-1 binding to transfected cells. Inactive αIIbβ3-expressing CHO cells were transiently transfected with GFP cDNA, with THD-GFP cDNA, with THD-GFP cDNA plus IPP (ILK, PINCH, and α-parvin) cDNAs, with GFP cDNA plus IPP cDNAs, or with THD-GFP cDNA plus kindling-2 cDNA. Highly transfected cells in the gated regions were analyzed for PAC-1 binding or HIP8 (an αIIbβ3-specific mAb) binding. (B) The activation indexes of transfected cells. The index was determined by the formula shown in Materials and Methods. A value of 100% implies the median fluorescence intensity of HIP8 binding to the cells in gated regions. Data represent means ± SD of three independent experiments. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. (C) Immunoblotting to evaluate expression levels of IPP, THD-GFP, and kindlin-2. Cell lysates obtained from transfected cells were electrophoresed and immunoblotted with indicated Abs.

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(GenBank accession number EGW10997) showed an amino acid length composed of 144 residues, shorter than that of mouse PINCH-2 (accession number NP659111) composed of 341 residues. This suggested that proper PINCH-2 may not be expressed in CHO cells. Unlike PINCH, α- and β-parvins were expressed in CHO cells and the knockdown of both parvins but

not either α- or β-parvin decreased ILK and PINCH expression to a similar extent as the parvins. Thus, the parvins are complemented with each other in the formation of the IPP complex, and either one seems to support integrin activation by maintaining the IPP complex.

Platelets are likely to have α- and β-parvins, and both parvins contribute to the formation of the IPP complex [43,44]. The functional importance of the IPP complex for platelet integrin regulation has not been fully elucidated. There are only a few reports in which the IPP complex stably exists to a similar extent between resting and stimulated platelets [43,44]. It has been shown in human platelets that ILK is activated and binds to the β subunit of αIIbβ3 and the integrin collagen receptor α2β1 after stimulation with thrombin, phorbol 12-myristrate 13-acetate, and collagen [45,46]. These processes seem to aggregation-dependently occur in αIIbβ3 or aggregation-independently arise in α2β1. In a recent study using an ILK-conditional knockout mouse, ILK-deficient platelets exhibited reduced abilities of aggregation, fibrinogen binding, and α-granule secretion [33]. The ILK-deficient platelets also showed decreased expression levels of PINCH and α-parvin, suggesting that the IPP complex is involved in the regulation of integrin affinity. In platelets, the IPP complex may be translocated from the cytoplasm to the integrin β cytoplasmic domain in response to agonist stimulation and may participate in the control of integrin affinity.

The important role of ILK as a signal regulatory protein has been well investigated. Most works demonstrated that ILK functions in outside-in signaling through integrins [20,25,26,47-50]. In the present study, we showed that ILK supports integrin activation by assembling the IPP complex in the CHO cells. This effect may occur through a process in which kindlin-2 recruits ILK to integrins [37,51]. The IPP complex may participate in the cooperation of talin and kindlins in a final step of integrin activation and stabilize the active conformation of integrin.

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Author Contributions

Conceived and designed the experiments: SH ST YT TM. Performed the experiments: SH HSI. Analyzed the data: SH HSI ST YT TM. Wrote the manuscript: SH YT TM.

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Dysfunction of protein C anticoagulant system, main genetic risk factor for venous thromboembolism in Northeast Asians

Tong Yin · Toshiyuki Miyata

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Abstract Venous thromboembolism (VTE) is a life-threatening medical disorder worldwide. A great deal of evidence suggests that prevalence of VTE varies significantly among ethnic populations, with consistently lower incidence found in Asians. While the distribution of genetic risk factors may vary among races, genetic risk factors can play a major role among individuals with different genetic backgrounds. Two clinically evaluated low-frequency genetic mutations that predispose to VTE—the factor V Leiden mutation and prothrombin G20210A mutation—are found predominantly in Caucasians, and virtually never in Asians. The findings of a recent genetic study of VTE in northeast Asians, which greatly advanced our knowledge in this area, indicate that the most frequent genetic risk factors for VTE in northeast Asians can be attributed to a dysfunction of the protein C anticoagulant system. Several low-frequency genetic mutations, *PROS1* p.Lys196Glu in Japanese and *PROC* p.Arg189Trp and p.Lys193del in Chinese, are significantly associated with increased risk for VTE, with odds ratio more than 2 through the reduced protein C anticoagulant activity. Construction of a multifactorial model based on the genetic risk factors in the protein C anticoagulant system could facilitate genetic counseling for VTE risk in these populations. The influence of prevalent genetic mutations on the risk of VTE should be further investigated in Asian countries.

Keywords Asian thrombophilia · Genetic risk factor · Protein C anticoagulant system · Venous thromboembolism

Introduction

Venous thromboembolism (VTE), a multifactorial disorder consisting of deep venous thrombosis (DVT) and pulmonary embolism (PE), represents a major thrombotic medical disorder worldwide. Despite acknowledged problems with different criteria and misclassification in determining VTE, there is strong evidence that the prevalence of VTE varies significantly among different ethnic/racial populations. Among the few studies with sufficiently diverse ethnic population samples to make direct comparisons [1–4] (Table 1), the most notable findings were from epidemiological studies based on ethnically diverse populations in California [1.3.4]. They suggested that the annual incidence of idiopathic DVT in persons over 18 years is higher among African Americans (29 per 100,000 individuals per year) than among Caucasians (23 per 100,000 individuals per year), is significantly lower among Hispanic populations (14 per 100,000 individuals per year), and is strikingly lower among Asian-Pacific Islanders (6 per 100,000 individuals per year) [1]. Population-based epidemiological studies of VTE are relatively rare in Asians. Recently, Sakuma et al. [5] reported the annual estimated incidence of PE and DVT in Japanese to be 6.19 and 11.55 patients per 100,000 individuals per year, respectively. Lee et al. [6] analyzed the incidence of symptomatic VTE in almost the entire population of

T. Yin
Institute of Geriatric Cardiology, General Hospital of Chinese People's Liberation Army, Beijing 100853, China
e-mail: yintong2000@yahoo.com

T. Miyata (✉)
Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 5658565, Japan
e-mail: miyata@ncvc.go.jp; miyata@ri.ncvc.go.jp

Table 1 Prevalence of VTE in diverse ethnic populations

Population origin	Diagnosis	Incidence rate per 100,000 individuals/year					Year	Reference
		African	Caucasian	Hispanics	Asian	Others		
Hospital patients	Idiopathic DVT	29	23	14	6	–	1991–1994	White et al. [1]
General population	PE or DVT	22	21	9	2	15[a]	1978–1985	Klatsky et al. [2]
Hospital patients	Total VTE	141	104	55	21	64	1996	White et al. [3]
Hospital patients	Idiopathic VTE	32	28	15	6	–	1996	White et al. [3]
General population	First-time VTE	141	103	62	29	23	1996	White and Keenan [4]

VTE venous thromboembolism; *DVT* deep vein thrombosis; *PE* pulmonary embolism

[a] In mixed or other ethnic populations

Taiwan. The incidence of PE and DVT among Taiwanese adults was 4.8 and 16.5 per 100,000, respectively, which is lower than among Caucasians and African-Americans, and similar to that of other Asians. Nevertheless, prevalence of PE and DVT in Asia may be increasing with improvements in diagnosis and access to healthcare [7].

VTE is recognized to be a multifactorial, complex disorder, which results from an interaction between environmental, clinical, and genetic risk factors. While racial differences in the incidence of VTE have been well established, interactive risk factors also vary by race [8]. Generally accepted environmental and clinical risk factors for VTE—such as obesity and access to complex surgery and cancer treatments—as well as prevalence of VTE risk factors such as human immunodeficiency virus and the sickle cell trait, are likely to emerge as important mediators of the racial difference in VTE [7]. Data from studies in Asian patients indicate a lower incidence of symptomatic VTE complicating trauma, immobilization, surgery, and/or use of thalidomide [7]. In addition to these well-established risk factors for VTE, it is conceivable that genetic risk factors may vary and play a major role in the different distribution of VTE among people from different genetic backgrounds [8].

The involvement of genetic factors in increased risk for VTE was confirmed in family-based studies in Caucasians, where genetics were thought to account for up to 60 % of risk [9]. Well-established genetic risks for VTE include increased pro-coagulant activities and hereditary deficiencies of natural anticoagulants. Two well-known and clinically evaluated genetic mutations associated with VTE are factor V Leiden mutation (factor V p.Arg506Gln mutation) and prothrombin G20210A mutation, which are found predominantly in populations with European ancestry, and are virtually non-existent in Asians without European admixture [8]. The relatively lower incidence of VTE in Asians compared to Caucasians may partly be due to the lower prevalence of these predisposing genetic factors.

Recently, in northeast Asian populations, where VTE incidence appears low, the most represented genetic risk factors are congenital deficiency of natural anticoagulants, especially genetic deficiency concerning the dysfunction of the protein C anticoagulant system [10]. The purpose of this review is to discuss the prevalence of this deficiency in Asians, and to evaluate the influence of genetic mutations in the protein C anticoagulant system on the risk of VTE in these populations. In this review, the A of the ATG initiator Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1 [11].

Protein C anticoagulant system

Natural anticoagulation in healthy individuals is primarily achieved through the actions of the anticoagulant systems, which include antithrombin, tissue factor pathway inhibitor, protein C, and protein S. Antithrombin plays a major role through the inhibition of thrombin and factor Xa. Tissue factor pathway inhibitor performs its physiological and pathological roles through the inhibition of factor Xa and factor VIIa-tissue factor complex. Unlike these protease inhibitors, the inactive serine-protease zymogen, protein C, must first be activated by thombomodulin (TM)-bound thrombin on the endothelial surface, with the help of the endothelial cell protein C receptor (EPCR); this produces activated protein C (APC) that then proteolytically inactivates factors Va and VIIIa in the presence of protein S. Protein S also stimulates factor Xa inhibition by tissue factor pathway inhibitor, resulting in the down-regulation of the extrinsic coagulation pathway. Thus, the protein C anticoagulant system, consisting of protein C, protein S, TM, and EPCR, regulates the balance between procoagulant and anticoagulant activities. Thrombus formation occurs when this balance is disturbed.

Deficiency of natural anticoagulants and prevalence in Asians

Deficiencies of proteins C and S result in the dysfunction of the protein C anticoagulant system. Hereditary protein C deficiency is usually inherited as an autosomal dominant trait. It is associated with an increased risk of VTE, and is thus considered hereditary thrombophilia. Protein C deficiency is classified into type I (low plasma concentration of both functional and immunological protein C) and type II (low concentration of functional protein C with normal antigen concentration). The inheritance pattern of protein S deficiency is usually autosomal-dominant. Protein S deficiency is classified as type I (quantitative deficiency of both activity and antigen concentration), type II (qualitative deficiency characterized by decreased activity with normal antigen concentration), or type III (normal concentration of total protein S and low concentration of free protein S).

The frequency of deficiencies of protein C, protein S, and antithrombin in VTE patients of Western ethnicity was reported to be 1.4–8.6, 1.4–7.5, and 0.5–4.9 %, respectively [12]. Compared with Caucasians, deficiencies of protein C and protein S in Asians were higher in both the general population and in VTE patients [13–23]. As shown in Table 2, the most prevalent deficiencies in Asian VTE patients were protein S deficiency, followed by protein C deficiency. A report from Hong Kong claimed that as many as 42 % of Chinese VTE patients have reduced activity of the protein C anticoagulant system [13]. In the Taiwanese population, about 50 % of VTE patients showed reduced activity of protein C and protein S [24]. In the Japanese population, the frequency of mutations of the protein C gene was almost three times higher than in Caucasian patients, and protein S deficiency was approximately 5–10 times more prevalent in Japanese VTE patients [20]. These studies suggested that Asian individuals have thrombophilias that differ from those of Caucasians, with a high likelihood of thrombophilia being due to an abnormality of protein C or protein S. They also indicate that there may be an overall higher prevalence of abnormality in protein C or protein S in Asian populations in general, and that a higher occurrence of this class of genetic risk factors may be expected in patients with VTE from the same genetic background [8].

Genetic mutations in the protein C anticoagulant system with VTE in Asians

Recently, several genetic mutations that are associated with a reduction of protein C anticoagulant activity and increased risk for VTE have been confirmed in the protein C anticoagulant system in Japanese and Chinese populations. While differences in VTE by race due to genetic predisposition will probably always be present, understanding the racially specific genetic risk factors for VTE can provide important information about etiological mechanisms, as well as novel therapeutic targets.

PROS1 p.Lys196Glu as a genetic risk of VTE in Japanese

Approximately 8–47 % of Japanese and Chinese individuals who develop VTE have reduced activities of protein S (Table 2). At present, more than 200 mutations have been described in the protein S gene (*PROS1*), and large deletions/duplications can also be identified as causes of protein S deficiency [25, 26]. The most common *PROS1* mutation is a p.Lys196Glu mutation (rs121918474, c.586A>G, protein S Tokushima, p.Lys155Glu in the mature protein numbering), which accounts for 9–30 % of protein S molecule abnormalities in people of Japanese decent [10, 20, 27–29].

An abnormal protein S molecule with the p.Lys196Glu mutation was identified in thrombophilic Japanese patients almost simultaneously by two independent groups in 1993 [30, 31]. It is a missense mutation that causes Lys196 to be replaced by Glu, formerly known as protein S Tokushima. This mutation is present in the second EGF-like domain of the protein S molecule. The allele frequency is approximately 0.9 % in the Japanese population, which means that 1 out of 55 Japanese carries the mutation as a heterozygote [29, 32, 33]. The frequency is much higher, approximately 6–10 % among DVT patients (Table 3) [20, 28, 32]. While homozygotes for this mutation have been identified in VTE patients, with a prevalence of one homozygote out of approximately 85 patients [20, 32], they have thus far not been identified in the general population [29]. The protein S p.Lys196Glu mutation can also be found in VTE patients with congenital protein C deficiency, thereby facilitating the development of VTE [34], and is frequently seen in VTE patients who are pregnant [35]. So far, 3 independent case–control studies, all performed in Japan, have reached the conclusion that the protein S p.Lys196Glu mutation is a risk factor for VTE, with odds ratio between 3.74 and 8.56 [20, 28, 32] (Table 3).

A genotype–phenotype study of the general Japanese population showed that individuals heterozygous for the mutant Glu-allele had a 16 % mean reduction in plasma protein S anticoagulant activity compared to wild-type individuals [27]. A patient with DVT who is a homozygote of protein S p.Lys196Glu mutation showed 35 % protein S anticoagulant activity and 37 % specific anticoagulant activity (activity/amount of protein S) [20]. In vitro studies using the recombinant proteins have shown that mutant protein S with Glu196 had impaired APC cofactor function

Table 2 Prevalence of protein S, protein C, and antithrombin deficiency in Asians

Population	Number of deficiency/total (%)						Reference
	Protein S deficiency		Protein C deficiency		Antithrombin deficiency		
	VTE patients	General population	VTE patients	General population	VTE patients	General population	
Japanese	20/113 (17.70 %)	8/392 (2.02 %)	9/113 (7.96 %)	2/392 (0.51 %)	2/113 (1.7 %)	0/392 (0 %)	Suehisa et al. [16]
Japanese	–	–	7/108 (6.48 %)	6/4,517 (0.13 %)	6/108 (5.56 %)	7/4,517 (0.15 %)	Sakata et al. [19]
Japanese	–	Male: 14/1,252 (1.12 %) Female: 23/1,438 (1.60 %)	–	–	–	–	Sakata et al. [18]
Japanese	40/85 (47.06 %)	1/126 (0.79 %)	27/85 (31.76 %)	1/95 (1.05 %)	6/85 (7.06 %)	0/95 (0 %)	Kinoshita et al. [20]
Chinese	10/52 (19.23 %)	–	9/52 (17.31 %)	–	5/52 (9.62 %)	–	Liu et al. [13]
Chinese	28/85 (32.94 %)	–	16/85 (18.82 %)	–	3/85 (3.53 %)	–	Shen et al. [24]
Chinese	39/116 (33.62 %)	8/125 (6.40 %)	20/116 (17.24 %)	5/125 (40.00 %)	6/116 (5.17 %)	8/125 (6.40 %)	Shen et al. [14]
Chinese	4/50 (8.00 %)	–	2/50 (4.00 %)	–	2/50 (4.00 %)	–	Ho et al. [15]
Chinese	6/56 (10.71 %)	–	6/56 (10.71 %)	–	4/56 (7.14 %)	–	Chen et al. [17]
Chinese	–	2/3,493 (0.06 %)	–	10/3,493 (0.29 %)	–	3/3,493 (0.09 %)	Zhu et al. [22]
Chinese	11/32 (34.40 %) (protein S or protein C deficiency)						Tang et al. [23]
Thai	10/85 (11.76 %)	–	8/85 (9.41 %)	–	4/85 (4.71 %)	–	Angchaisuksiri et al. [21]

– Data unavailable

Table 3 Influence of genetic variants in the protein C anticoagulant system on VTE in Asians

Gene	Nucleotide change	Amino acid change(in mature protein)	rs number	Risk allele	No. of deficiency/total (%)		Odds ratio (95 % CI)	*p* value	Population	Reference
					Cases	Controls				
PROS1	c.586A>G	p.Lys196Glu (p.Lys155Glu)	rs121918474	G	5/85 (5.88)	5/304 (1.64)	3.74 (1.06–13.2)	–	Japanese	Kinoshita et al. [20]
PROS1	c.586A>G	p.Lys196Glu (p.Lys155Glu)	rs121918474	G	15/161 (9.32)	66/3,651 (1.81)	5.58 (3.11–10.01) adjusted: 4.72 (2.39–9.31)	<0.001	Japanese	Kimura et al. [32]
PROS1	c.586A>G	p.Lys196Glu (p.Lys155Glu)	rs121918474	G	6/60 (10.00)	3/234 (1.28)	8.56 (2.07–35.30)	<0.05	Japanese	Ikejiri et al. [28]
PROC	c.565C>T	p.Arg189Trp (p.Arg147Trp)	rs146922325	T	5/116 (4.31)	11/1,292 (0.85)	5.10 (1.7–14.8)	–	Chinese	Tsay et al. [38]
PROC	c.565C>T	p.Arg189Trp (p.Arg147Trp)	rs146922325	T	59/1,003 (5.88)	9/1,031 (0.87)	7.10 (3.50–14.39) adjusted: 7.34 (3.61–14.94)[a] or 7.13 (3.49–14.56)[b]	3.31×10^{-10} adjusted: 3.88×10^{-8} [a] or 6.88×10^{-8} [b]	Chinese	Tang et al. [23], [39]
PROC	c.565C>T	p.Arg189Trp (p.Arg147Trp)	rs146922325	T	68/1,304 (5.21)	12/1,334 (0.90)	6.06 (3.26–11.25)	1.03×10^{-10}	Chinese	Tang et al. [48]
PROC	c.574_576del	p.Lys193del (p.Lys151del)	rs199469469	Del	68/1,003 (6.78)	25/1,031 (2.42)	2.93 (1.84–4.67) adjusted: 2.71 (1.68–4.36)	2.59×10^{-6} adjusted: 4.59×10^{-5}	Chinese	Tang et al. [23]
PROC	c.574_576del	p.Lys193del (p.Lys151del)	rs199469469	Del	85/1,304 (6.52)	32/1,334 (2.40)	2.84 (1.88–4. 29)	2.77×10^{-7}	Chinese	Tang et al. [48]
THBD	c.2729A>C in tight LD with c.1418C>T	In tight LD with p.Ala473Val	rs3176123	C	33/55[c] (60.00)	462/1,032[c] (44.77)	2.76[c] (1.14–6.67)	0.02[c]	Japanese	Sugiyama et al. [47]
THBD	c.−151G>T	–	rs16984852	T	35/1,304 (2.68)	13/1,334 (0.97)	2.80 (1.48–5.32)	1.02×10^{-3}	Chinese	Tang et al. [48]
PROCR	c.4600A>G	p.Ser219Gly	rs867186	G	15/65[d] (23.07)	7/71[d] (9.86)	2.75 (1.04–7.30)	<0.05	Chinese	Chen et al. [52]
PROCR	c.4600A>G	p.Ser219Gly	rs867186	G	41/112 (36.61)	23/112 (20.54)	1.78 (1.11–2.89)	<0.05	Chinese	Yin et al. [53]

CI confidence interval, *LD* linkage disequilibrium, – Data unavailable

[a] Data were analyzed by logistic regression adjusted for age, gender, smoking status, alcohol abuse, malignant tumor, type 2 diabetes, sedentariness/immobilization, and pregnancy/puerperium

[b] Data were calculated by unconditional logistic regression adjusted for age, gender, smoking status, malignant tumor, sedentariness/immobilization, and pregnancy/puerperium

[c] Male patients

[d] Patients with one G allele

[36]. Plasma protein S activities in carriers of the p.Lys196Glu mutation showed reduced activity as described, but antigen levels were within normal limits [27, 30, 31].

The protein S p.Lys196Glu mutation is race-specific; so far this mutation has not been identified in any population other than Japanese. Chinese and Koreans populations, despite being geographically and genetically close to Japanese, did not carry this mutation [37]. Thus, the protein S p.Lys196Glu mutation must be a recent occurrence and fixed within the Japanese population.

PROC p.Arg189Trp and p.Lys193del as genetic risks of VTE in Chinese

At least 161 different protein C gene (*PROC*) mutations have been reported, and most of them are missense mutations. The predominant genetic defects in the *PROC* gene may be different for different races. Recently, the p.Arg189Trp mutation of protein C (rs146922325, c.565C>T, p.Arg147Trp in the mature protein numbering) was reported by two independent studies to be not only the most frequent variant for protein C deficiency but also a significant risk factor for VTE in Chinese populations [38, 39]. This missense mutation was initially described in an American patient with symptomatic protein C deficiency [40], and was later reported in an asymptomatic individual [41]. Although a rare mutation in Western populations, the p.Arg189Trp mutation was present in approximately 0.9 % of the general Chinese population (Table 3) [38, 39]. The heterozygous state of the p.Arg189Trp mutation is associated with decreased plasma functional activity and a relatively normal protein C antigen level, indicating type II protein C deficiency. This mutation was identified in almost half of the probands with hereditary protein C deficiency [38, 39]. First-degree relatives bearing this variant had an 8.8-fold increased risk of VTE [39]. Two independent population-based case–control studies showed the odds ratio of VTE in carriers of the variant ranged from 5 to 7 (Table 3) [38, 39]. The p.Arg189Trp mutation is located at the C-terminal region of the light chain adjacent to the EGF-2 like domain, and may impair the interaction of protein C with other molecules suggesting that Arg189 may constitute an exosite for the binding of factor Va and/or the thrombin–thrombomodulin complex. Further functional studies are needed to elucidate the deleterious effect of this mutation on the activation of protein C and the inactivation of factor Va by APC. Data on the prevalence of this mutation and the thrombotic risk associated with it in other populations (especially other Asian populations) are still quite limited, and should be further evaluated.

Recently, using coagulation screening tests, resequencing, and a case–control study, Tang et al. revealed that the *PROC* p.Lys193del mutation (rs199469469, c.574_576del, p.Lys151del in the mature protein numbering) was associated with both decreased protein C anticoagulant activity and an increased risk of VTE in Chinese, with an odds ratio of 2.7 (Table 3) [23]. The nomenclature of one amino acid deletion in this case is somewhat complicated, as positions 192 and 193 of protein C are both Lys and one of the Lys residues is deleted in this case. We call the mutation the "p.Lys193del" according to the recommendation of the Human Genome Variation Society [11]. This variant was first described in three Japanese patients who suffered from protein C deficiency [42]. In other studies on protein C and protein S deficiencies in Japanese individuals, this mutation was identified in 2 of 85 VTE patients, as well as in 1 of 30 healthy individuals in one study [20], and in 4 of 173 VTE patients in another [34]. Despite being identified as a rare genetic mutation in Japanese, the contribution of the variant to the risk for VTE was not further evaluated in the general Japanese population. Another recent study found that the prevalence of p.Lys193del mutation was 2.36 % in the general Chinese population [23]. It was identified in 68 of 1,003 VTE patients (6.78 %) and in 25 of 1,031 healthy individuals (2.42 %), therefore, it conferred an increased risk of VTE with an adjusted odds ratio of 2.7 (Table 3) [23]. Patients with the p.Lys193del mutation showed lower anticoagulant activity of protein C, but relatively normal amidolytic activity compared to the wild-type carriers [23, 34, 42]. The anticoagulant activity of the recombinant mutant protein C showed about 40 % of the wild-type, consistent with the value of plasma from the homozygous patient [23]. Although this mutation has been reported previously in Japanese populations, further studies are needed to evaluate its prevalence in other Asians, and to determine whether this polymorphism is a risk factor for VTE in other Asian populations.

Some other *PROC* mutations were also reported in VTE patients from Asia [39, 40, 43–45]. Both protein C p.Arg211Trp and p.Met406Ile (p.Arg169Trp and p.Met364Ile in the mature protein numbering), which are related to type I protein C deficiency, were first reported in Japanese patients with VTE [43, 44]. Protein C p.Arg211Trp is a recurrent mutation occurring at a CpG mutation hotspot at the thrombin cleavage site in the heavy chain; it has also been described in Caucasian patients with VTE. It was reported to account for about 10 % of *PROC* mutations in Japanese [45]. In contrast, p.Met406Ile, which occurs at a non-CpG site of the serine protease domain, has been described exclusively in Japan, accounting for ~8 % of *PROC* mutations in Japanese VTE patients [45]. In resequencing the *PROC* gene in probands of protein C deficiency, 8 novel coding sequence mutations contributed to 7 amino acid exchanges; 3 evidently detrimental novel null mutations were also supposed to contribute to the development of VTE in Chinese [39].

THBD mutations as VTE risk in Asians

Thrombomodulin (TM encoded by *THBD*), another critical component of the protein C anticoagulant system, is a transmembrane glycoprotein of 557 amino acids, and is expressed mainly on the endothelial cells. TM binds thrombin and alters its substrate specificity. The resulting TM–thrombin complex efficiently catalyzes protein C activation. The intron-less human *THBD* gene is 3.6 kb in length. Based on the important anticoagulant role of TM, mutations within *THBD* could predispose individuals to VTE. In addition, *THBD* mutations may affect the plasma-soluble TM level. Several studies have focused on the influence of genetic polymorphisms in *THBD* on soluble TM level and VTE. One study conducted in the USA found mutations—including c.127G>A (p.Ala43Thr), c.1418C>T (p.Ala473Val), c.1752C deletion, and c.3645A>G—were not associated with VTE [46]. An association study of the Japanese population that included 2,247 individuals showed that c.2729A>C in tight linkage disequilibrium with c.1418C>T (p.Ala473Val) was associated with the soluble TM level [47]. This mutation also showed a marginal association with VTE, but only in males (Table 3).

A recent large study of the Chinese population showed an association of the soluble TM levels with c.−151G>T in *THBD* (Table 3) [48]. Furthermore, this genetic mutation increased risk of VTE. The study enrolled 1,304 individuals with VTE and 1,334 age- and sex-matched controls. By resequencing and genotyping of the *THBD* gene, the study showed that c.−151G>T in *THBD* could cause a predisposition to VTE, with a 2.8-fold increased risk of developing VTE in the population and a 3.42-fold increased risk of VTE in the family [48]. The prevalence of this variant in the Chinese population was 0.97 %, indicating an allele frequency of 0.49 %. Compared with the wild-type allele, the c.−151G>T mutation significantly reduced the reporter gene-expression level in cultured cells [48].

In addition, rare nonsynonymous mutations, p.Ser190Trp, p.Ser212Ter, p.Leu220Ter, and p.Asp126Tyr in *THBD* were also identified in 108 thrombophilic individuals with VTE [48]. The prevalence and relative risk of VTE with these mutations in other populations, especially in Asians, will require further evaluation.

The extensive resequencing studies on *THBD* in VTE patients revealed yet another aspect of the *THBD* mutations, that is, a possible link between the nonsynonymous mutations and atypical hemolytic uremic syndrome (aHUS), a type of microangiopathy characterized by uncontrolled complement activation. One of the causative genes for aHUS is *THBD* [49]. In vitro, TM binds to C3b and complement factor H and negatively regulates the complement by accelerating complement factor I-mediated inactivation of C3b. The TM mutations were less effective than wild-type TM in enhancing factor I-mediated inactivation of C3b. Thus, some missense mutations of TM are characterized as causative for the development of aHUS [49]. A missense mutation, p.Asp486Tyr, in the Ser/Thr rich domain of TM, which has been identified in both VTE patients and controls [46–48], was characterized as a causative mutation for aHUS. Missense mutations in the lectin-like domain of TM are also reportedly causative for aHUS. Thus, nonsynonymous mutations in *THBD* would affect not only VTE but also aHUS to a certain degree.

PROCR mutations as VTE risk in Asians

On the pathway of the protein C anticoagulant system, protein C is activated on the endothelial surface by the membrane-bound TM–thrombin complex. Protein C activation is enhanced approximately 20-fold when protein C binds to the endothelial protein C receptor (EPCR) encoded by *PROCR*. EPCR also serves as a cellular binding site for factor VII and factor VIIa. A soluble form of this receptor (sEPCR) in plasma inhibits both APC activity and protein C activation by competing for protein C with membrane-bound EPCR. These findings suggest an important role for EPCR in VTE.

Several studies have reported that the *PROCR* p.Ser219Gly mutation (rs867186, c.655A>G) present within the membrane-spanning region reduced plasma sEPCR levels to 56–87 % [50]. Significantly higher levels of factor VII, factor VIIa, and downstream markers of activated coagulation in the extrinsic pathway (factor IX activation peptide, factor X activation peptide), and prothrombin F1 + 2 were also identified in Gly carriers, compared to Ser/Ser [51]. Evidence for the association between the p.Ser219Gly mutation and VTE is conflicting in ethnically diverse populations. A recent meta-analysis in 4,821 VTE patients and 6,070 controls found a significant association of this mutation with VTE [50]. Under an additive genetic model, the odds of VTE increased by a factor of 1.22 for every additional copy of the G allele in all ethnic populations, suggesting a moderate effect for VTE. The reported frequency of the G allele in northeast Asians is approximately 10 % [52, 53]. Thus far, only two independent, small-scale studies of Chinese populations have reported a significant association between the p.Ser219Gly mutation and VTE in Asian populations (Table 3) [52, 53]. Further studies restricted to idiopathic VTE patients in Asian might facilitate the positive association of this variant.

Perspectives

The genetic mutations in the protein C anticoagulant system (*PROS1* p.Lys196Glu, *PROC* p.Arg189Trp, *PROC*

p.Lys193del, and *THBD* c.−151G>T) associated with risk of VTE in Asians are all classified into low-frequency variations with allele frequencies of less than 5 %. Three genetic mutations in the protein C anticoagulant system (*PROC* p.Arg189Trp, *PROC* p.Lys193del, and *THBD* c.−151G>T) were detected concurrently in the Chinese population, with a respective frequency of 0.90, 2.40, and 0.97 %, and a respective odds ratio for VTE of 6.06, 2.84, and 2.80. Their estimated population-attributable risks were therefore calculated to be 4.67, 4.14, and 1.48 %, respectively [48]. Taken together, about 10 % of VTE events in the general Chinese population could be explained by these mild to moderate thrombophilic risk factors. Hence, as we have described [29], these low-frequency genetic variations could play an important role in the development of VTE. The risk loci may act in concert with each mutation adding or detracting a small amount from the phenotype; the environment also interacts with the genotype to produce the final phenotype [8].

Recent genome-wide association studies have found additional genetic polymorphisms that are potentially related to VTE risk, but most have been detected predominately in European-ancestry populations [54, 55]. Genome-wide association studies do serve an important role in identifying new loci of interest, as well as confirming previously suggested loci for VTE. However, their main potential is for identifying common mutations (>5 %) with relatively lower risk (odds ratio <1.5). The candidate gene resequencing in the protein C anticoagulant system or the exome sequencing would facilitate the discovering of low-frequency variations with high risk for VTE in Asians. An accumulating body of evidence strongly suggests that genetic studies should be carried out in ethnically diverse populations, and that studies of common variations, as well as low-frequency variations, are warranted [29].

As VTE is a complex disease with genetic factors accounting for part of the risk, a multifactorial non-Mendelian inheritance model that includes the influence of genetic and environmental factors should be proposed for genetic counseling of VTE risk. Recently, a multiple single-nucleotide polymorphism test based on 31 VTE-associated polymorphisms or the 5 most strongly associated polymorphisms was found to improve risk prediction of first venous thrombosis in Caucasians [56]. Future studies should consider the construction of a multifactorial model based on the genetic risk factors in the protein C anticoagulant system, which is specific for Asian populations.

In summary, the genetic mutations leading to dysfunction of the protein C anticoagulant system could be a major risk factor for VTE in northeast Asian populations, especially in Japanese and Chinese. Conditions where the procoagulant activity surpasses the anticoagulant activity, including the protein C anticoagulant system, could trigger the development of thrombosis in individuals with risk genetic variants. Genetic analysis for VTE is highly restricted in Japanese and Chinese populations, and other Asian populations are not yet well studied. Even in geographically close populations, such as Japanese and Chinese, low-frequency mutations are not evenly distributed. The *PROS1* p.Lys196Glu mutation, for example, is exclusively identified in Japanese populations. Whether dysfunction of the protein C anticoagulant system occurs in other Asian countries is an important unresolved issue of the thrombophilia study among Asians, and an international survey is warranted to disclose it.

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