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Mitotic dissociation of IQGAP1 from Rac-bound β1-integrin is mediated by protein phosphatase 2A☆

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Abstract

Assembly of F-actin that links with β1-integrin during the G1 phase of cell cycle is released from β1-integrin and disrupted at mitosis. However, it remains unclear how F-actin assembly to which β1-integrin anchors is cell cycle-dependently regulated. We show that β1-integrin was co-immunoprecipitated and co-localized with a small GTPase Rac and its effector IQGAP1, along with PP2A-AC, in HME cells during G1. When the cells were accumulated to G2/M, the co-immunoprecipitation or co-localization of IQGAP1 and PP2A-AC with β1-integrin was lost, leaving Rac bound to β1-integrin. The dissociated IQGAP1 was co-immunoprecipitated with the concomitantly dissociated PP2A-A and -C, indicating the complex formation among the proteins in G2/M cells. Falling ball viscometric assays revealed that only IQGAP1-bound β1-integrin-Rac in G1 cells exhibited an enhanced F-actin cross-linking activity. The results suggest that the mitotic loss of F-actin assembly to which β1-integrin anchors is due to PP2A-mediated dissociation of IQGAP1 from Rac-bound β1-integrin.

Keywords: Protein phosphatase 2A; β1-integrin; Rac; IQGAP1; F-actin assembly; Cell adhesion

Cell adhesion and spreading is mediated by the integrin family of the cell surface receptor [1,2] that intracellularly links with the actin cytoskeleton through the β subunit of integrin [3]. The cell adhesive function of integrin is modulated by phosphorylation with integrin-linked protein kinase (ILK) [4,5] or Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II [6], or dephosphorylation with PP2A [4–6] of the amino acid residues within the cytoplasmic domain of β1-integrin, probably through regulation of the ability of β1-integrin to link with F-actin [6–8]. Quiescent HME cells during G1 have dephosphorylated β1-integrin that binds to PP2A-AC and F-actin, but the cells at G2/M have phosphorylated β1-integrin that lacks PP2A-AC and F-actin assembly [6]. Although the β1-integrin linking with F-actin may require dephosphorylation of β1-integrin by PP2A [4–6], it is unlikely that F-actin assembly to which β1-integrin anchors is directly regulated by PP2A.

Assembly of F-actin is primarily regulated by the Rho family of small GTPases, including Rho, Rac, and Cdc42 [9–11]. As downstream effectors of Rac or Cdc42, the Arp 2/3 complex mediates F-actin polymerization [12,13] through the WASP/WAVE family proteins [14–16], and a Ras GTP-association protein (GAP)-related protein IQGAP [17–19] has F-actin cross-linking activity [20,21]. We demonstrate here that β1-integrin in HME cells during G1 is associated with

☆ *Abbreviations:* PP, serine/threonine protein phosphatase; PP2A-AC, the core enzyme of PP2A; PP2A-A, the regulatory subunit A of PP2A; PP2A-C, the catalytic subunit C of PP2A; HME cells, nonmalignant human mammary epithelial cells; F-actin, actin filaments; EGF, epidermal growth factor; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BSA, bovine serum albumin; DTT, dithiothreitol; EGTA, ethylene glycol bis(1-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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Rac, IQGAP1, and PP2A-AC, exhibiting an enhanced F-actin cross-linking activity. In the cells at G2/M, IQGAP1 and PP2A-AC, but not Rac, are dissociated from β1-integrin and form a complex, thereby leading to the lack of F-actin cross-linking activity of β1-integrin-Rac. The results suggest that F-actin assembly that links with Rac-bound β1-integrin is regulated by PP2A-mediated recruitment and dissociation of IQGAP1.

Materials and methods

Cell culture and flow cytometric analysis. HME cells were obtained from BioWhittaker and maintained in serum-free defined medium MCDB 170 [22] supplemented with EGF, insulin, hydrocortisone, ethanolamine, phosphoethanolamine, and prostaglandin E2 as described [6]. Quiescent HME cells adhering to laminin-coated plastic dishes (Becton–Dickinson) were harvested with 0.25% (w/v) trypsin–2.65 mM EDTA solution (Invitrogen) after incubation in an EGF-deprived medium for 3 days. For accumulation of cells to G2/M, asynchronous cultures were incubated with 0.1 μg/ml demecolcine (*N*-deacetyl-*N*-methylcolchicine) (Sigma) for 16 h [6]. After incubation, rounded cells were collected by a brief treatment with trypsin–EDTA solution. The cells thus harvested were analyzed with a Cycle Test Plus kit (Becton–Dickinson) using a flow cytometer (Beckman–Coulter).

Western blot analysis. Cells were lysed in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM PMSF, and 0.2 mM sodium orthovanadate) on ice at 5 W for 20 s using a cell disruptor (Misonix). The cell lysates were incubated with monoclonal antibody to β1-integrin (BD Pharmingen) or rabbit polyclonal antibody to IQGAP1 (Santa Cruz Biotechnology) for 45 min at room temperature, followed by the slurry of protein A–Sepharose beads (Amersham Biosciences) for 45 min more at room temperature. For preparation of whole cell lysates, the cells were lysed in 1% SDS, 20 mM Tris–HCl, pH 7.4, 1 mM PMSF, and 1 mM sodium orthovanadate, and boiled for 2 min, before sonicating briefly. The protein content was determined using a protein reagent kit (Pierce) with BSA as the standards. After SDS–PAGE, the proteins were transferred onto membranes (Millipore), and the membranes were blocked in 5% (w/v) non-fat milk in TBS at 4 °C overnight. The proteins on a membrane were immunoblotted with antibody to β1-integrin, Rac (Upstate Biotechnology), PP2A-A (Santa Cruz Biotechnology), PP2A-C (BD Bioscience), IQGAP1, Arp 2, Arp 3, or WAVE2 (Santa Cruz Biotechnology). After incubation of the membrane in horseradish peroxidase-conjugated secondary antibodies, the reactivity was visualized using a chemiluminescence kit (Amersham Biosciences). The band intensity was quantified using an Edas 290 system equipped with a digital camera (Kodak).

Immunofluorescence. Cells on laminin-coated glass slides (Nalge Nunc) were fixed in 3.7% buffered-formaldehyde solution, permeabilized in 0.2% Triton X-100 in TBS, and blocked in 3% BSA in TBS. The fixed cells were incubated with antibody to β1-integrin, Rac, or IQGAP1. After incubation with fluorescein- or rhodamine-conjugated secondary antibody (Molecular Probes), cells were examined using a confocal laser scanning microscope (Carl Zeiss).

Falling ball viscometric assays. The β1-integrin immunoprecipitates with protein A–Sepharose beads were incubated with 0.5 mg/ml rabbit skeletal muscle actin (Cytoskeleton) in 10 mM Tris–HCl, pH 7.4, 1 mM DTT, 2 mM $MgCl_2$, 0.1 mM EGTA, 100 mM KCl, and 0.1 mM ATP at 25 °C for 1 h. Falling ball viscometric assays [23] were performed at an angle of 50° at 25 °C using a 0.6-mm steel ball in 0.2-ml glass pipette. The viscosity was calculated using 85% glycerol solution (81.5 centi-Poise at 25 °C) as standard.

Results and discussion

Dissociation of IQGAP1 and PP2A from Rac-bound β1-integrin at G2/M

After incubation in an EGF-deprived medium for 3 days, HME cells were tightly adhering to laminin. Flow cytometric analysis revealed that approximately 80% of the cells were in the G1 phase of the cell cycle (Fig. 1A). Therefore, these cells will be hereafter referred to as G1 cells. In contrast, cells that were collected by brief trypsinization after incubation with demecolcine for 16 h adopted round morphology, and more than 90% of them were accumulated to the G2/M phase of the cell cycle (Fig. 1B). These HME cells thus collected will be hereafter referred to as G2/M cells.

As F-actin assembly is regulated by the Rho family of small GTPases, such as Rho, Rac, or Cdc42 [9–11], we examined first whether β1-integrin binds to a small GTPase. Immunoprecipitation of β1-integrin and Western blot analysis revealed that Rac was co-immunopre-

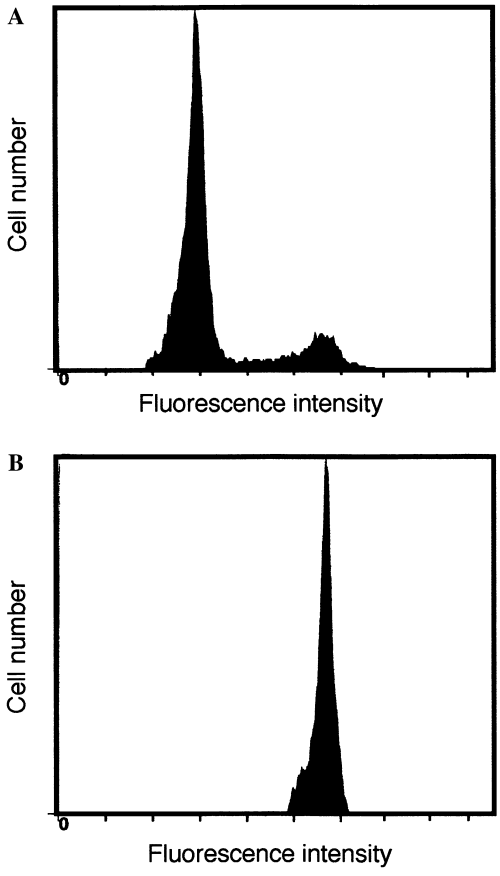

Fig. 1. Flow cytometric analysis. (A) After incubation of HME cells on laminin-coated dishes in an EGF-deprived medium for 3 days, the cells were harvested with trypsin–EDTA and examined by a flow cytometer. (B) Asynchronous cultures of HME cells were incubated with demecolcine for 16 h and collected by a gentle flow of trypsin–EDTA for flow cytometry.

cipitated with β1-integrin in both G1 and G2/M cells (Fig. 2). Quantitative measurement of the band intensity of β1-integrin and Rac indicated that the relative amount of Rac to β1-integrin in G2/M cells did not differ significantly from that in G1 cells (Fig. 2). The result suggests that a small GTPase Rac is involved in the regulation of F-actin assembly that links with β1-integrin and that there is no significant difference between G1 and G2/M cells with respect of the amount of Rac that bound to β1-integrin.

To determine the effector molecules downstream of Rac, the β1-integrin immunoprecipitates were blotted with antibody to IQGAP1, Arp 2/3, or WAVE2. Among these proteins, IQGAP1 was co-immunoprecipitated with β1-integrin in G1 cells (Fig. 2). However, the relative amount of IQGAP1 that was co-immunoprecipitated with β1-integrin was significantly smaller in G2/M cells than in G1 cells (Fig. 2). On the other hand, neither Arp 2/3 nor WAVE2 was co-immunoprecipitated with β1-integrin in both cells (data not shown). As well as Rac and IQGAP1, Western blot analysis revealed that PP2A-AC, consisting of PP2A-A and PP2A-C [24–26], was co-immunoprecipitated with β1-integrin in G1 cells (Fig. 2). This agrees with the previous result [6]. The relative amount of either PP2A-A or -C that was co-immunoprecipitated with β1-integrin was significantly smaller in G2/M cells than in G1 cells (Fig. 2). The result suggests that most of IQGAP1 and PP2A-AC that are associated with β1-integrin during G1 are not at G2/M, contrary to Rac that remains bound to β1-integrin during both the phases of cell cycle.

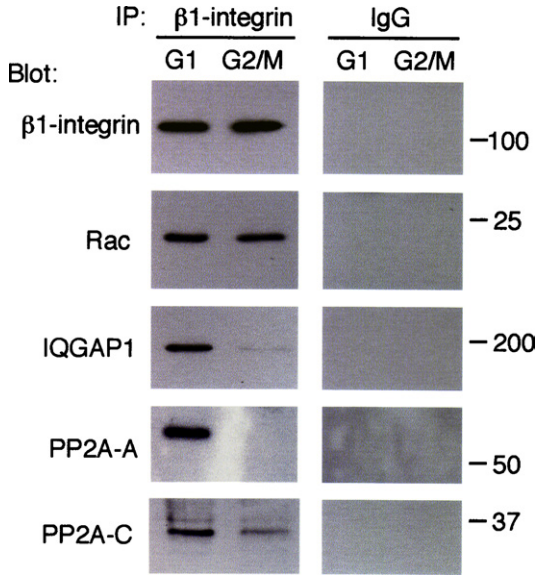

Fig. 2. Western blot analysis of the β1-integrin immunoprecipitates. HME cells during G1 or G2/M were lysed and incubated with antibody to β1-integrin or control IgG (IP). The β1-integrin immunoprecipitates or IgG precipitates were resolved in SDS–PAGE and blotted with antibody to β1-integrin, Rac, IQGAP1, PP2A-A, or PP2A-C (blot). The representative data of three independent experiments are shown. Molecular size markers are indicated on the right in kiloDalton.

Loss of co-localization of IQGAP1 with β1-integrin or Rac at G2/M

To confirm the cell cycle-specific association of IQGAP1 with β1-integrin and Rac, cells were doubly immunostained to examine the localization of the proteins. When G1 cells were immunostained with

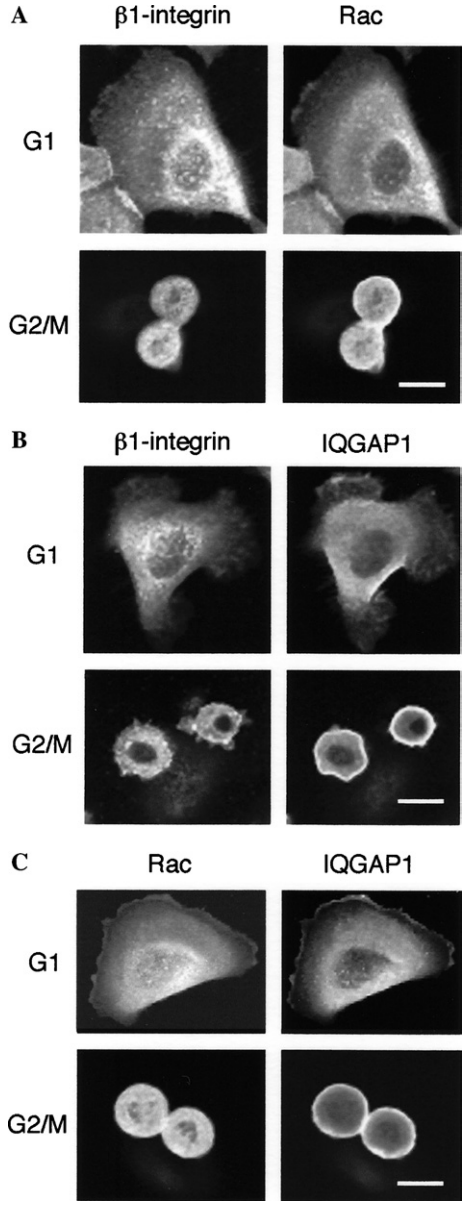

Fig. 3. Localization of β1-integrin, Rac, and IQGAP1 in HME cells. HME cells on laminin-coated glass slides during G1 or G2/M were fixed and doubly immunostained with antibody to β1-integrin and Rac (A), β1-integrin and IQGAP1 (B), or Rac and IQGAP1 (C). Then the cells were incubated with fluorescin- or rhodamine-conjugated secondary antibody and examined under a confocal laser scanning microscope. Bars, 10 μm.

antibodies to β1-integrin and Rac, most of β1-integrin staining on the basal side of the cell was co-localized with Rac staining (Fig. 3A). In G2/M cells, most of Rac staining overlapped with β1-integrin staining on the basal side of the cell (Fig. 3A). Contrary to Rac, most of IQGAP1 staining that was co-localized with β1-integrin staining during G1 exhibited a distinct localization from β1-integrin staining in the peripheral region of cell at G2/M (Fig. 3B). The G1-specific co-localization and the G2/M-specific loss of co-localization were also observed between Rac and IQGAP1 stainings (Fig. 3C). These results suggest that most of β1-integrin, Rac, and IQGAP1 are co-localized to each other on the basal side of G1 cells where the cells adhere to the substratum dependent on β1-integrin, and that most of IQGAP1 translocate to the peripheral region of the rounded cell at G2/M, leaving β1-integrin and Rac on the basal side of the cells.

The complex formation of IQGAP1 and PP2A free from Rac-bound β1-integrin at G2/M

Western blot analysis and immunofluorescence suggested that IQGAP1 and PP2A-AC were not associated with β1-integrin and Rac in G2/M cells (Figs. 2 and 3). As the total amounts of IQGAP1, PP2A-A, and PP2A-C were comparable between G1 and G2/M cells (Fig. 4A), the absence of co-immunoprecipitation of these proteins with β1-integrin in G2/M cells (Fig. 2) is due to their dissociation from Rac-bound β1-integrin. To examine the interaction between IQGAP1 and PP2A-AC that were concomitantly dissociated from Rac-β1-integrin in G2/M cells, IQGAP1 was immunoprecipitated from G2/M cells. Western blot analysis revealed that both PP2A-A and -C were co-immunoprecipitated with IQGAP1 in G2/M cells (Fig. 4B). The results suggest that PP2A-AC that is dissociated from β1-integrin at G2/M forms a complex with the concomitantly dissociated IQGAP1, free from β1-integrin and Rac. IQGAP1 has the binding ability to small GTPases, such as Rac and Cdc42 as their effector, and calmodulin or calmodulin-related proteins [17–19]. In addition to these proteins, the present result suggests that IQGAP1 can bind directly or indirectly to PP2A-AC. As PP2A-AC, but not PP2A-C alone, has the binding ability to β1-integrin [6,8] and PP2A-A has the binding domain for PP2A-C as the scaffold subunit [24,25], the interaction between IQGAP1 and Rac-bound β1-integrin is mediated through PP2A-AC, especially PP2A-A.

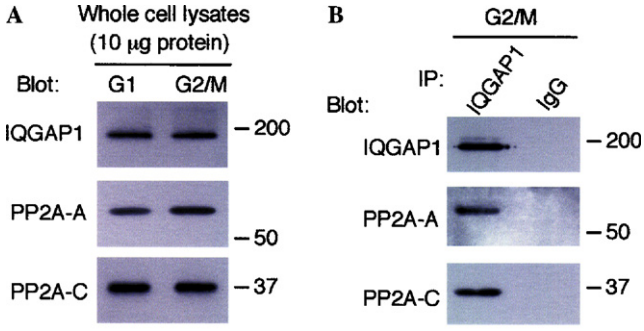

Fig. 4. The complex formation of IQGAP1 with PP2A-AC in G2/M cells. (A) Whole cell lysates (10 μg protein) from G1 or G2/M cells were probed with antibody to IQGAP1, PP2A-A, or PP2A-C, after SDS–PAGE (blot). The representative data of two independent experiments are shown. Molecular markers are indicated on the right in kiloDalton. (B) After SDS–PAGE of the IQGAP1 immunoprecipitates or IgG precipitates from G2/M cells, the samples were blotted with antibody to IQGAP1, PP2A-A, or PP2A-C (blot). The representative data of three independent experiments are shown. Molecular markers are indicated on the right in kiloDalton.

F-actin cross-linking activity of β1-integrin

IQGAP1 exhibits F-actin cross-linking activity [20,21] when bound to Rac or Cdc42. To examine whether IQGAP1-bound β1-integrin in G1 cells has F-actin cross-linking activity and whether β1-integrin that lacks IQGAP1 in G2/M cells does not, the β1-integrin immunoprecipitates from G1 or G2/M cells with the protein A–Sepharose beads were incubated with exogenous actin. Falling ball viscometric assays revealed that the viscosity of the actin solution after incubation

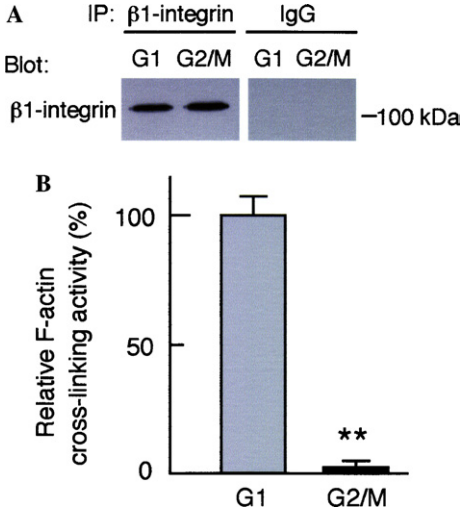

Fig. 5. F-actin cross-linking activity of the β1-integrin immunoprecipitates. (A) The β1-integrin immunoprecipitates or control IgG precipitates from G1 or G2/M cells were directly resolved in SDS–PAGE and probed for β1-integrin (blot). (B) The β1-integrin immunoprecipitates or control IgG precipitates from G1 or G2/M cells with the protein A–Sepharose beads were incubated with rabbit skeletal muscle actin for 1 h. After incubation, the viscosity of actin solution was determined by falling ball viscometric assays using 85% glycerol solution as standard. After subtraction of the background using the control IgG precipitates, F-actin cross-linking activity was normalized to the amount of β1-integrin from G1 cells that was quantified by measurement of the band intensity of β1-integrin on SDS–PAGE gels shown in (A). Results represent means ± SD of triplicate assays. $^{**}P < 0.01$, by Student's t test.

with the β1-integrin immunoprecipitates from G1 cells was 56.3 centi-Poise (cP). In contrast, the viscosity after incubation of the β1-integrin immunoprecipitates from G2/M cells with actin was nearly the background level. After subtraction of the background and normalization of the viscosity to the arbitrary amount of β1-integrin by measurement of the band intensity of β1-integrin used in the assays (Fig. 5A), the relative F-actin cross-linking activity of β1-integrin in G2/M cells was less than 2% ($P < 0.01$, Student's t test) of that in G1 cells (Fig. 5B). The result suggests that IQGAP1-bound β1-integrin-Rac in G1 cells exhibits an enhanced F-actin cross-linking activity, but Rac-bound β1-integrin that lacks IQGAP1 in G2/M cells has no remarkable F-actin cross-linking activity.

The primary role of PP2A in cell adhesion and spreading is to associate with β1-integrin and dephosphorylate it [4–6] to link with F-actin during the G1 phase of cell cycle [6–8]. Along with such role as the protein phosphatase, the present results suggest that PP2A may play a role in regulation of F-actin assembly to which β1-integrin anchors by recruitment of IQGAP1 to Rac-bound β1-integrin during G1 and by dissociation of IQGAP1 from Rac-bound β1-integrin at G2/M.

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