β_3 integrin phosphorylation is essential for Arp3 organization into leukocyte $\alpha_V\beta_3$-vitronectin adhesion contacts

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Summary

Integrins play a pivotal role in self-regulated hematopoietic adhesion and migration. Leukocyte $\alpha_V\beta_3$ integrin-mediated adhesion to vitronectin requires protein kinase C activation and phosphorylation on tyrosine 747 of the β_3 cytoplasmic tail. We have previously shown that β_3 phosphorylation is required for Rho activation. In this study, an antibody specific to phosphorylated β_3 tyrosine 747 was used to localize phosphorylated $\alpha_V\beta_3$ in vitronectin adhesive structures. Early adhesion contacts containing phosphorylated β_3 preceded actin stress fiber formation. β_3 phosphorylation decreased progressively throughout the course of adhesion coincident with the appearance of actin stress fibers. Time-dependent increases in colocalization of β_3 with tyrosine 402 phosphorylated Pyk2 in similar adhesive structures was observed, providing evidence for downstream signaling complex formation. Surprisingly, Arp3 organized into similar adhesion contacts in cells expressing wild-type β_3 but not in those expressing a nonphosphorylatable mutant of β_3, suggesting that β_3 phosphorylation is required for sequestration of Arp3 to adhesion complexes. Suppression of actin stress fiber formation by an inhibitor to Rho kinase disrupted Arp3 organization while prolonging β_3 phosphorylation throughout the adhesion time course. These data confirm a requirement for β_3 phosphorylation in $\alpha_V\beta_3$-mediated adhesion to vitronectin and suggest that β_3 phosphorylation permits signaling complex assembly at the adhesion site necessary for actin stress fiber formation in leukocytes.

Key words: Cytoskeleton, PSSA, Pyk2, Actin

Introduction

Integrin activity in hematopoietic cells is constitutively repressed and requires stimulation by mediators of the inflammatory response. This regulation confines leukocyte adhesion and migration to sites of infection, protecting healthy tissue from unwarranted damage. A unique feature of hematopoietic β_3 integrins is that they require phosphorylation on the β_3 cytoplasmic tail for optimal receptor function (Blystone et al., 1997). Two tyrosine residues are present within the cytoplasmic tail at positions 747 and 759 of the β_3 sequence. Through mutational analysis and peptide mapping, it has been resolved that tyrosine 747 (Y747) is required for $\alpha_V\beta_3$-mediated adhesion to the ligand vitronectin and that this is the major site of phosphate incorporation in β_3 expressed as $\alpha_V\beta_3$ in hematopoietic cell types (Blystone et al., 1997).

β_3 integrins play important functional roles in hematopoietic cell types including platelets, monocytes and osteoclasts. $\alpha_{IIb}\beta_3$ integrins mediate platelet aggregation and clot retraction with receptor dysfunction resulting in Glanzmann's thrombasthenia (Bajt et al., 1992). $\alpha_V\beta_3$ is the major adhesion receptor in osteoclasts and mediates adhesion to various bone matrix proteins. During osteoclast-mediated bone resorption, $\alpha_V\beta_3$ regulates cytoskeletal organization required for cell migration and formation of the sealing zone (McHugh et al., 2000). $\alpha_V\beta_3$ also modulates $\alpha_L\beta_2$-dependent migration along ICAM-1, suggesting an important role for β_3 in transendothelial migration of macrophages (Weerasinghe et al., 1998).

Adhesive structures resulting from changes in actin dynamics have been well characterized in numerous cell types (Nobes and Hall, 1995). Actin stress fiber formation in fibroblast cell lines occurs as a result of Rho activation, whereas Rac and Cdc42 induce the formation of lamellipodia and filopodia, respectively (Hall, 1998). Cells of hematopoietic lineage form neither focal contacts nor adhesions, but rather exhibit smaller, biochemically similar adhesive structures, termed podosomes, that contain the appropriate signaling and adaptor proteins to achieve membrane extension (Evans et al., 2003; Worthylake and Burridge, 2001). In addition, leukocytes need an additional stimulus to achieve firm adhesion through actin-dependent behaviors. Ligand-induced phosphorylation on β_3 Y747 and activation of protein kinase C (PKC) are required for Rho activation and firm adhesion of leukocytes to vitronectin (Butler et al., 2003). This phosphorylation event results in receptor clustering and subsequent direct and indirect recruitment of a signaling and adaptor protein complex to the cytoplasmic tail, including Pyk2, Src, Syk, phosphoinositide 3-kinase, vinculin, talin and α-actinin proteins (van der Flier and Sonnenberg, 2001). The interactions among these signaling molecules, in conjunction with the Arp2/3 complex of proteins and the cellular actin machinery, result in coordinated changes in actin dynamics necessary to achieve inflammatory phenotypes.

Proline-rich tyrosine kinase 2 (Pyk2) is a nonreceptor protein tyrosine kinase that is closely related to focal adhesion kinase and serves many of the same functions in hematopoietic cells (Sasaki et al., 1995). It has been shown to be a proximal link between integrin and chemokine signaling in natural killer cells and has been implicated in the regulation of the Rac pathway leading to lamellipodial formation and transendothelial migration (Gismondi et al., 2003). In osteoclasts, Pyk2 plays an important role in the cell adhesion and spreading that is imperative for active bone resorption. Pyk2 phosphorylation at tyrosine 402 (Y402), believed to result in Pyk2 activation, has been shown to play a crucial regulatory role in adhesion-dependent cytoskeletal organization in osteoclasts, notably in the formation of the sealing zone, an $\alpha_V\beta_3$-dependent structure (Lakkakorpi et al., 2003).

Integrin-mediated adhesion, phagocytosis and migration require a coordinated rearrangement of the actin cytoskeleton. Actin nucleation and polymerization occur as a result of active signaling complexes recruiting the appropriate cellular machinery for cytoskeletal rearrangement. The Arp2/3 complex of proteins mediates initiation and growth of actin filaments. Activation of the complex results in an open conformation, permitting the Arp2 and Arp3 subunits to form a template actin filament for nucleation (Robinson et al., 2001). The Arp3 subunit contains residues that interact with ATP on actin. It has been shown that the C-terminal VCA domains of WASp/Scar proteins, which are downstream of Rho GTPases, interact with the Arp2/3 complex to activate actin nucleation, regulating filament formation and extension (Machesky et al., 1999). DeMali et al. have recently shown that the Arp2/3 complex localizes to adhesion contacts that contain active integrin complexes, presenting a functional link between integrin engagement and actin polymerization (DeMali et al., 2002). In these studies, vinculin was shown to mediate the link between Arp2/3 and the integrin.

Rho is a small GTP-binding protein in the signaling pathway that leads to leukocyte adhesion and stress fiber formation. Rho cycles between a GDP-bound inactive state and GTP-bound active state, and when in its active conformation, it activates downstream signaling pathways. In leukocytes, activation of Rho is required for $\alpha_V\beta_3$-mediated adhesion to vitronectin. This Rho activation is dependent on the phosphorylation of β_3 Y747 (Butler et al., 2003). Rho kinase (ROCK) is a downstream Rho effector implicated in numerous cellular processes. In NIH 3T3 cells, activation of the Rho-ROCK pathway results in myosin light chain (MLC) phosphorylation from suppression of MLC phosphatase, initiating changes in actin dynamics (Kimura et al., 1996). In leukocytes, inhibition of ROCK prevents retraction of trailing edge adhesive contacts (Alblas et al., 2001).

To better understand early adhesion events preceding actin stress fiber formation, we visualized the localization and behavior of β_3 Y747 phosphorylation using a phosphorylated state-specific antibody (PSSA) specific to the phosphorylated form of β_3 Y747. $K\alpha_V\beta_3$ WT cells adherent to vitronectin presented progressively decreasing β_3 Y747 PSSA staining along with increasing Arp3 organization as adhesion sites matured and actin stress fiber formation occurred. Importantly, Arp3 failed to organize in $K\alpha_V\beta_3$ Y747,759F cells, suggesting that phosphorylation on β_3 Y747 is important for the downstream Arp2/3 organization that leads to actin assembly. To disrupt the normal progression of adhesion, we inhibited ROCK. This resulted in sustained β_3 Y747 PSSA staining throughout the adhesion time course in $K\alpha_V\beta_3$ WT cells, suggesting the presence of a feedback regulation of β_3 Y747 phosphorylation. Arp3 failed to organize in $K\alpha_V\beta_3$ WT cells when ROCK was inhibited, introducing a rate-limiting step for actin stress fiber formation. In addition, competitive inhibition of phosphorylated β_3 with integrin tail phosphomimetic peptides disrupted Arp3 organization in $K\alpha_V\beta_3$ WT cells. Our results confirm the requirement of β_3 Y747 phosphorylation for $\alpha_V\beta_3$-mediated adhesion to vitronectin. Furthermore, our data suggest that β_3 Y747 phosphorylation is required for the appropriate localization of the Arp2/3 complex and its subsequent assembly of actin.

Materials and Methods

Cells and materials

K562 cells were transfected with cDNA encoding wild-type ($K\alpha_V\beta_3$ WT) or Y747,759F ($K\alpha_V\beta_3$ Y747,759F) β_3 and maintained as previously described (Blystone et al., 1997). Equivalent expression between $K\alpha_V\beta_3$ WT and $K\alpha_V\beta_3$ Y747,759F cells was rigorously maintained by fluorescence-activated cell sorting. Murine bone marrow-derived macrophages (murine BMM) and human monocyte-derived macrophages (human MDM) were isolated as previously described (Blystone et al., 1997). CS-1 melanoma cells expressing β_3 ($CS\beta_3$) were produced and maintained as previously described (Blystone et al., 1997). Anti-β_3 monoclonal antibody, 1A2, and vitronectin were prepared as previously described (Blystone et al., 1994). Anti-β_3 Y747 phosphorylated state-specific antibody (β_3 Y747 PSSA) and anti-Pyk2 Y402 rabbit polyclonal PSSA were from Biosource International (Camarillo, CA). Anti-Arp3 rabbit polyclonal, anti-Pyk2 and anti-vinculin monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and rhodamine phalloidin from Cytoskeleton (Denver, CO). All reagents unless otherwise indicated were purchased from Sigma (St Louis, MO).

Cell adhesion assays

96 well-plates (Immulon II, Dynatech, Chantilly, VA) were coated with vitronectin (1.5 μg/ml) in PBS overnight at 4°C. Plates were washed twice with PBS, followed by the addition of 100 μl casein blocker per well and incubation at room temperature for 30 minutes. Overlapping peptides corresponding to the tyrosine-phosphorylated β_3 cytoplasmic tail were constructed as cell permeant fusions with the HIV-TAT leader (TAT-Yp: YGRKKRRQRRR G DTANNPL **Yp** KEATSTFT-COOH and TAT-Y/F: YGRKKRRQRRR G DTANNPL **F** KEATSTFT-COOH, where the leader sequence is underlined and the position of Y747 of β_3 is in bold).

Phorbol myristate acetate (PMA; 10 ng/ml), TAT-fusion proteins (10 mM) and/or anti-β_3 antibodies (5 μg/ml) were added to empty wells, followed by cells (1×10^5 in 100 μl of Hanks' Balanced Salt Solution (HBSS) with 1.0 mM Ca^{2+} and 1 mM Mg^{2+} added ($HBSS^{++}$)). Plates were incubated for 1 hour at 37°C. Following two washes with $HBSS^{-}$, cells were fixed with 5% formaldehyde in PBS, washed, stained with 0.5% crystal violet, and washed and lysed with 200 μl methanol. Adhesion was quantitated by absorbance at 570 nm using a Molecular Devices microplate reader (Sunnyvale, CA).

Immunoprecipitation

K562 cells expressing wild-type, Y747F, Y759F or Y747,759F mutant $\alpha_V\beta_3$ were incubated either with or without (untreated) MnCl

(2 mM) and sodium pervanadate (100 μM) for 10 minutes at 37°C. Cells were lysed in PBS buffer containing 1% Nonidet P-40, phenylmethylsulfonyl fluoride (1 mM), sodium orthovanadate (100 μM) and iodoacetimide (1.85 mg/ml). β_3 was immunoprecipitated from cleared lysates using mAb, 7G2, as previously described (Butler et al., 2003). Whole cell lysates and IP samples were separated on 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blotted with β_3 Y747 PSSA antibodies and HRP-conjugated anti-rabbit secondary antibody (Pierce Biotechnology, Rockford, IL). Reactivity was visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ).

Fluorescent microscopy

12-mm glass coverslips were coated overnight at 4°C with vitronectin (1 μg/ml) in PBS. 8×10^4 K562 cells expressing wild-type or mutant (Y747,759F) $\alpha_V\beta_3$ were adhered to coverslips in the presence of either PMA (10 ng/ml), Y27632 (ROCK inhibitor; 10 μM) or both for indicated times at 37°C in Iscove's Modified Dulbecco's Media (IMDM). For TAT-peptide experiments, cells were pre-incubated with TAT-Yp or TAT-Y/F peptide (100 μM) for 15 minutes at 37°C, centrifuged at low speed and resuspended in IMDM for adhesion to vitronectin in the presence of PMA (10 ng/ml). Cells were washed twice with PBS. Following fixation with 3.7% formaldehyde for 1 hour, cells were permeabilized with 0.005% NP-40 for 15 seconds at 4°C followed by two washes in PBS. For β_3 Y747 PSSA staining, cells were incubated with phosphorylated state-specific antibody (0.387 μg/ml) in PBS. For total β_3 staining, 400 μl of 1A2 tissue culture supernatant was used per well. Other antibodies were diluted 1:500 in PBS. Primary antibody incubation was for 30 minutes at 37°C. Cells were washed three times in PBS. Anti-rabbit IgG, FITC-conjugated secondary antibody (1:1000), anti-mouse IgG, TRITC-conjugated secondary antibody (1:1000) and/or rhodamine-phalloidin (1:1200) were diluted in PBS containing 0.3% goat serum. Secondary antibody incubation was for 30 minutes at 37°C, followed by five washes in PBS. Coverslips were reversed onto *o*-phenylenediamine in glycerol. Fluorescence was visualized on a Nikon Eclipse E800 fluorescent microscope (Nikon, Melville, NY). Images were digitally captured with a Hamamatsu ORCA-ER digital camera (Bridgewater, NJ) and processed with Simple PCI and Adobe PhotoShop 5.5 software.

Results

Tyrosine-phosphorylated β_3 tail peptides block PMA-mediated adhesion to vitronectin

Overlapping peptides corresponding to the tyrosine-phosphorylated β_3 cytoplasmic tail were constructed as cell permeant fusions with the HIV-TAT leader. To investigate β_3 tyrosine phosphorylation, TAT-fusion peptides were tested for efficacy on the basis of their blocking abilities during cell adhesion to vitronectin. Four cell types: CSβ_3, K$\alpha_V\beta_3$ WT, murine BMM and human MDM, and a matched set of functional β_3 cytoplasmic tail peptide mimetics were selected in pilot studies. With the addition of PMA, K$\alpha_V\beta_3$ WT cells achieved maximum adhesion to vitronectin (Fig. 1A). In K$\alpha_V\beta_3$ WT cells, β_3 phosphorylation on Y747 is required for firm adhesion to vitronectin. As a result, TAT-peptides containing the Y747 phosphorylated form of the β_3 cytoplasmic tail (TAT-Yp) competitively inhibited K$\alpha_V\beta_3$ WT cell adhesion. The TAT-Yp peptide did not affect $\alpha_V\beta_3$-mediated adhesion to vitronectin by CSβ_3 cells. These cells exhibit a PKC-independent constitutive adhesion representative of nonhematopoietic cell types. Murine BMMs and human MDMs also displayed enhanced adhesion to vitronectin in the presence of PMA and this adhesion was reduced in both cell types with introduction of TAT-Yp (Fig. 1B). Adhesion of all four cell types to vitronectin was reduced by co-incubation with β_3 antibody. In support of a role for β_3 Y747 phosphorylation in hematopoietic $\alpha_V\beta_3$-mediated cell adhesion, the TAT-Y/F peptide, containing a Phe substitution for Tyr at position 747, had no effect on cell adhesion. The ability of TAT-Yp peptide to disrupt adhesion suggests that the phosphorylated β_3 cytoplasmic tail specifically interacts with other molecules within the cell to achieve adhesion. TAT-Yp peptide sequences were used to design an immunogen to create rabbit polyclonal antibodies against phosphorylated Y747 of β_3. Specificity of the antibodies generated was verified by western blotting cell extracts expressing either WT β_3 under nonphosphorylating (untreated) or phosphorylating conditions, or β_3 in which cytoplasmic tyrosines at positions 747 and 759 were mutated to Phe either alone or in combination under phosphorylating conditions. The resulting antibody, β_3 Y747 PSSA, is specifically reactive with β_3 phosphorylated at Y747 (Fig. 1C). β_3 Y747 PSSA blots of β_3 immunoprecipitations specifically recognized the Y747 phosphorylated form of β_3. In whole-cell lysates, phosphorylated β_3 was the predominant band. Although other bands were present, it was determined that this was due to nonspecific reactivity with the HRP-conjugated rabbit secondary antibody. It must be noted that this secondary antibody was different from the FITC-conjugated antibody used for immunostaining, which did not present this cross-reactivity.

β_3 Y747 phosphorylation is an early adhesion event

The β_3 cytoplasmic tail contains a tyrosine at position 747 that is required for $\alpha_V\beta_3$-mediated adhesion. To determine when β_3 Y747 phosphorylation is required, K$\alpha_V\beta_3$ WT (Fig. 2A-C,E-G) and K$\alpha_V\beta_3$ Y747,759F (Fig. 2D,H) cells were adhered to vitronectin in the presence of PMA for 10 (Fig. 2A,D,E,H), 30 (Fig. 2B,F) or 70 (Fig. 2C,G) minutes and stained for rhodamine phalloidin (Fig. 2A-D) and β_3 Y747 PSSA (Fig. 2E-H) as described in Materials and Methods. At the 10 minute adhesion time point, K$\alpha_V\beta_3$ WT cells exhibited β_3 Y747 PSSA staining in punctate form, indicative of podosomes (Fig. 2E). This staining was absent in K$\alpha_V\beta_3$ Y747,759F cells (Fig. 2H). Organization of actin into stress fibers was detected with rhodamine phalloidin; as stress fiber formation progressed, β_3 Y747 PSSA levels in K$\alpha_V\beta_3$ WT cells decreased (Fig. 2G) to amounts comparable to those in β_3 Y747,759F mutant cells (Fig. 2H). More interestingly, at the 70 minute time point, K$\alpha_V\beta_3$ WT cells containing mature stress fibers were completely devoid of β_3 Y747 PSSA staining, compared with those cells in an early adhesive state (Fig. 2I,J).

Increased β_3 Y747 phosphorylation with ROCK inhibition

We have previously shown that in K$\alpha_V\beta_3$ WT cells, Rho is needed for actin assembly and that its activity is dependent on β_3 Y747 phosphorylation (Butler et al., 2003). In the Rho-ROCK signaling pathway leading to actin assembly and stress fiber formation, ROCK has been shown to lie upstream of actin

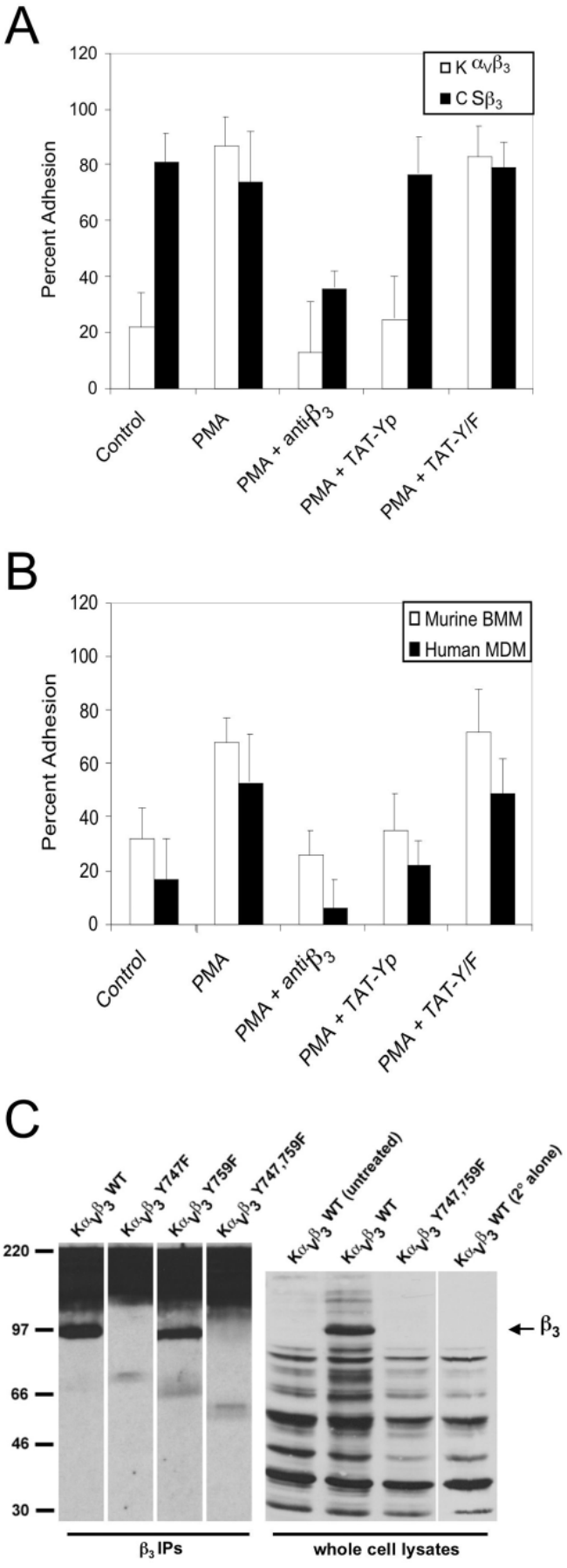

Fig. 1. Inhibition of adhesion by β_3 cytoplasmic tail peptidomimetics. K$\alpha_V\beta_3$ WT or CSβ_3 (A) and murine BMM or human MDM (B) cells were adhered to vitronectin in the presence (PMA) or absence (control) of PMA (10 ng/ml), and either anti-β_3 antibody (5 μg/mL), TAT-Yp (10 mM) or TAT-Y/F (10 mM) fusion peptides for 1 hour at 37°C as described in Materials and Methods. Data represent the mean±s.d. from triplicate wells from three separate experiments, presented as percent adhesion. (C) K562 cells expressing wild-type, Y747F, Y759F or Y747,759F mutant $\alpha_V\beta_3$ were incubated without (untreated) or with MnCl (2 mM) and sodium pervanadate (100 μM) for 10 minutes at 37°C. Whole cell lysates and β_3 immunoprecipitates were separated by SDS-PAGE and western blotted with anti-β_3 Y747 PSSA and HRP-conjugated secondary antibody or secondary alone (2° alone).

reorganization (Maekawa et al., 1999). We utilized the ROCK inhibitor, Y27632, to disrupt actin related events downstream of β_3 Y747 phosphorylation. Interestingly, ROCK inhibition resulted in sustained levels of β_3 Y747 phosphorylation at late adhesion time points (Fig. 3A-C). This suggests a feedback mechanism of β_3 Y747 phosphorylation involving either ROCK or molecules downstream of ROCK.

As the human and murine β_3 cytoplasmic tail sequences are identical, wild-type murine BMMs were adhered to vitronectin under equivalent conditions to compare β_3 Y747 PSSA staining with our K562 cell model. β_3 Y747 PSSA staining was present in murine BMMs adhering to vitronectin in the presence of PMA (Fig. 4A) in similarly appearing adhesion contacts as those observed in K$\alpha_V\beta_3$ WT cells. As seen in K$\alpha_V\beta_3$ WT cells, the presence of phosphorylated β_3 Y747 decreased over the duration of murine BMM adhesion to vitronectin in the presence of PMA (Fig. 4B) and was sustained during ROCK inhibition (Fig. 4C,D).

Arp3 organizes in adherent K$\alpha_V\beta_3$ WT cells

Actin cytoskeletal rearrangement has been observed following leukocyte adhesion to a vitronectin matrix in the presence of PMA. On β_3 Y747 phosphorylation in the presence of PMA, K$\alpha_V\beta_3$ WT cells show evidence of actin organization over time of adhesion to vitronectin (Fig. 2A-C). The Arp2/3 complex is a docking site involved in actin polymerization upon which free actin monomers bind to form filaments and fibers. In K$\alpha_V\beta_3$ WT cells, we observed an increase in Arp3 detection over adhesion time (Fig. 5E-G). Arp3 organized into adhesion contacts analogous to those containing β_3 Y747 PSSA, although we did not observe direct colocalization of Arp3 with β_3. Arp3 detection was minimal in K$\alpha_V\beta_3$ Y747,759F cells (Fig. 5A-C). These data suggest a requirement of β_3 Y747 phosphorylation for Arp3 recruitment to early adhesion contacts.

To further explore this event, we studied the effect of ROCK inhibition on Arp3 organization. In adherent K$\alpha_V\beta_3$ WT cells, inhibition of ROCK with Y27632 resulted in a sustained presence of phosphorylated β_3 (Fig. 3). Arp3 failed to organize in K$\alpha_V\beta_3$ WT cells at the 70 minute adhesion time point in the presence of Y27632 (Fig. 5H), illustrating a requirement for ROCK in Arp3 organization. This indicates that phosphorylation of β_3 is necessary, but not sufficient, for Arp2/3 sequestration to podosomes.

We have previously shown that Rho activation is required

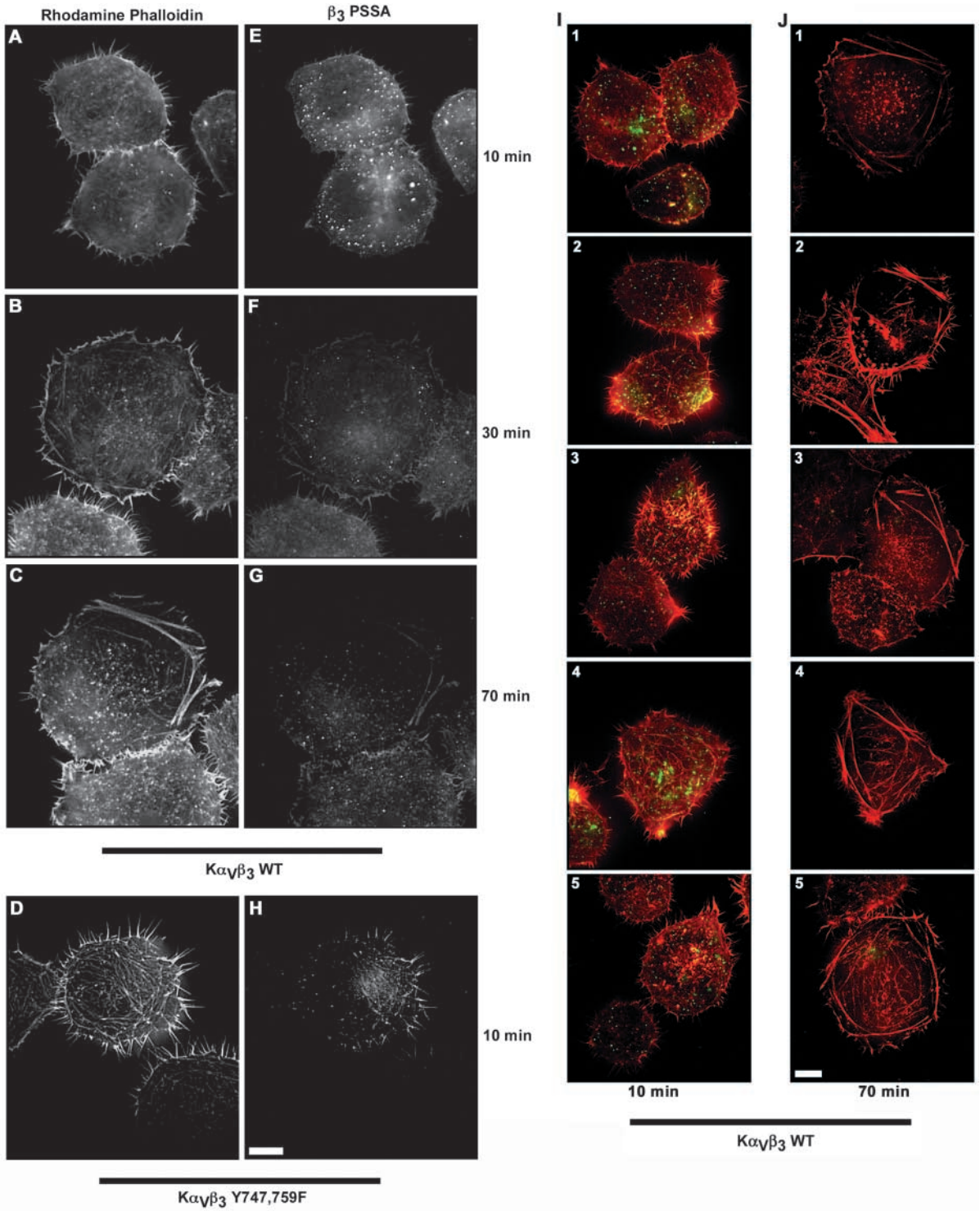

Fig. 2. Diminishing β_3 Y747 PSSA staining during adhesion. K$\alpha_V\beta_3$ WT (A-C,E-G,I,J) and K$\alpha_V\beta_3$ Y747,759F (D,H) cells were adhered to vitronectin-coated coverslips in the presence of PMA (10 ng/ml) for 10 (A,D,E,H,I) 30 (B,F) or 70 (C,G,J) minutes. Cells were stained with rhodamine phalloidin to detect actin organization (A-D) and also β_3 Y747 PSSA with FITC-conjugated secondary antibody (E-H) as described in Materials and Methods. I(1-5) and J(1-5) show replicate merged images of K$\alpha_V\beta_3$ WT cells following adhesion for 10 (I) or 70 (J) minutes followed by costaining with rhodamine phalloidin (red) and β_3 Y747 PSSA (green). Bar, 5 μm.

for adhesion to both fibronectin and vitronectin (Butler et al., 2003). The striking difference between $\alpha_V\beta_3$-mediated adhesion to these two ligands is that adhesion to vitronectin requires phosphorylation on Y747, and activation of both PKC and phosphoinositide 3-kinase, whereas these events are not required for adhesion to fibronectin. Taking this into account, we adhered K$\alpha_V\beta_3$ Y747,759F cells to fibronectin and stained for Arp3. Arp2/3 complex formation and organization is

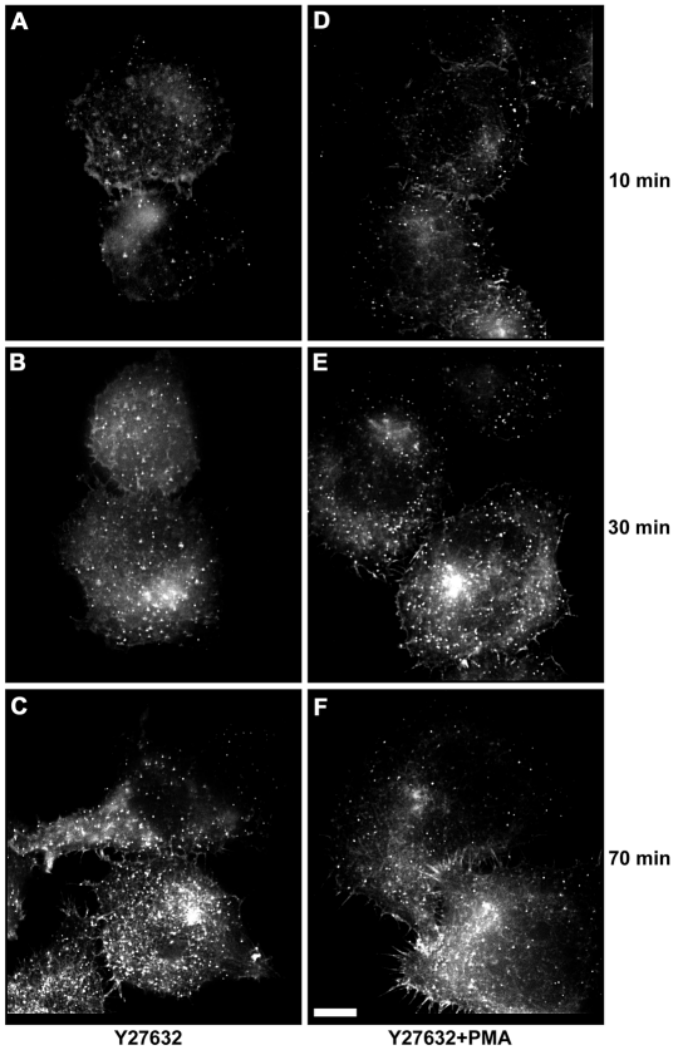

Fig. 3. Sustained β_3 Y747 phosphorylation during ROCK inhibition. K$\alpha_V\beta_3$ WT cells were adhered to vitronectin-coated coverslips in the presence of Rho kinase (ROCK) inhibitor, Y27632 (10 μM) (A-C) or Y27632 and PMA (10 ng/ml) (D-F) for 10 (A,D), 30 (B,E) or 70 (C,F) minutes. Cells were stained for β_3 Y747 PSSA and FITC-conjugated secondary antibody as described in Materials and Methods. Bar, 5 μm.

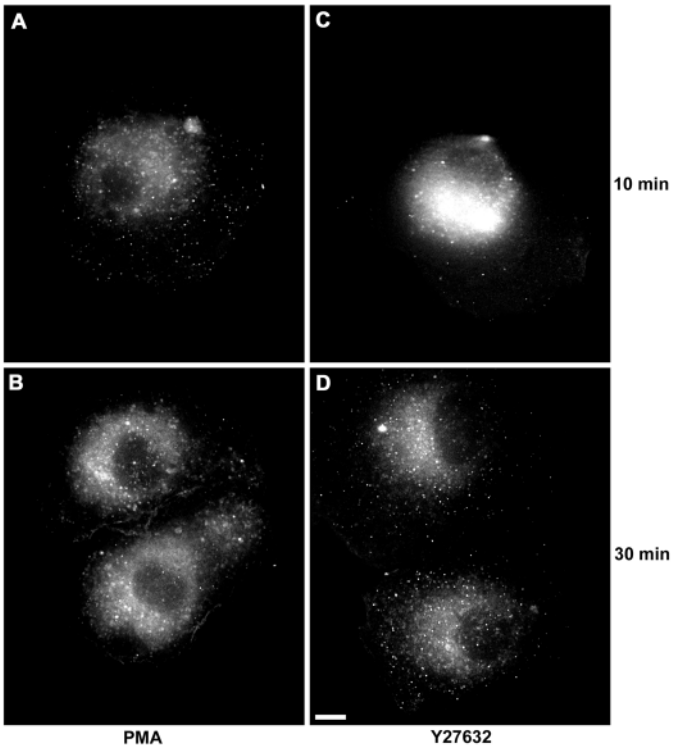

Fig. 4. β_3 Y747 PSSA localization in murine BMMs. Wild-type murine BMMs were adhered to vitronectin-coated coverslips in the presence of PMA (10 ng/ml) (A,B) or Y27632 (10 μM) (C,D). Cells were stained with β_3 Y747 PSSA antibody and FITC-conjugated secondary antibody as described in Materials and Methods at 10- (A,C) and 30-minute (B,D) adhesion time points. Bar, 10 μm.

thought to lie between Rho activation and downstream actin stress fiber formation. We observed equivalent Arp3 organization in K$\alpha_V\beta_3$ Y747,759F cells as compared to K$\alpha_V\beta_3$ WT cells when cells were adhered to fibronectin in the presence of PMA (Fig. 5D), reinforcing our previous observation that the requirement for β_3 Y747 phosphorylation is ligand selective.

To further explore the requirement of β_3 Y747 phosphorylation for Arp3 organization into podosomes, we utilized TAT-fusion peptides to competitively inhibit β_3 phosphorylation-dependent events. TAT-Yp and TAT-Y/F peptides used in Fig. 1 were pre-incubated with K$\alpha_V\beta_3$ WT cells, followed by adhesion to vitronectin. TAT-Yp reduced Arp3 localization to podosomes and limited actin stress fiber formation (Fig. 5I,K). Minimal reduction in Arp3 organization was seen in cells pre-incubated with TAT-Y/F peptide, perhaps attributable to inhibitory effects of other β_3 cytoplasmic motifs in the TAT-peptide.

Phosphorylated Pyk2 colocalizes with total β_3 over course of adhesion to vitronectin

β_3 Y747 PSSA staining to vitronectin decreased over adhesion time in the presence of PMA (Fig. 2). We proposed that this decrease was due to the masking of the Y747 phosphorylation site by adaptor or signaling proteins that form a receptor complex on β_3 Y747 phosphorylation. We double-labeled K$\alpha_V\beta_3$ WT cells with anti-β_3 antibody, 1A2, paired with numerous antibodies to proteins downstream of integrin activation, such as talin, α-actinin, vinculin, Vav and phosphoinositide 3-kinase. We detected organization of each protein into adhesion contacts but did not observe strong colocalization of any of these proteins with β_3. By contrast, active Pyk2 did colocalize with β_3. Pyk2 is a nonreceptor protein tyrosine kinase closely related to focal adhesion kinase that has been shown to couple integrins to their downstream effectors, including $\alpha_V\beta_3$ in osteoclasts (Duong et al., 2000). Pyk2 Y402 phosphorylation is required for integrin-mediated Rac activation (Gismondi et al., 2003). We examined the relationship between β_3 and active Pyk2 in K$\alpha_V\beta_3$ WT and K$\alpha_V\beta_3$ Y747,759F cells adherent to vitronectin in the presence of PMA. We double-labeled cells with anti-β_3 monoclonal antibody, 1A2 (Fig. 6A-C,J-L), and Pyk2 Y402 PSSA (Fig. 6D-F,M-O) and observed

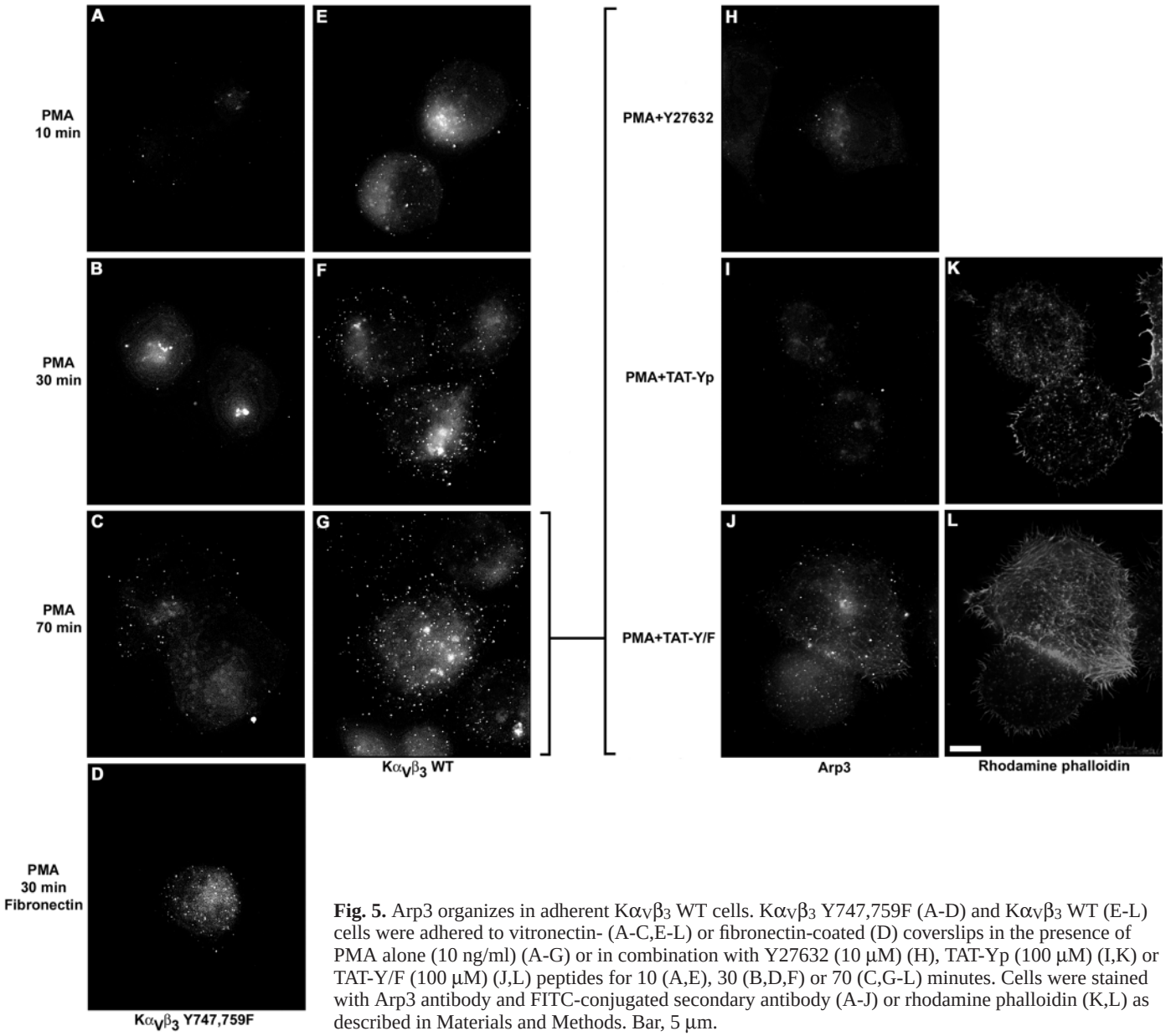

Fig. 5. Arp3 organizes in adherent $K\alpha_V\beta_3$ WT cells. $K\alpha_V\beta_3$ Y747,759F (A-D) and $K\alpha_V\beta_3$ WT (E-L) cells were adhered to vitronectin- (A-C,E-L) or fibronectin-coated (D) coverslips in the presence of PMA alone (10 ng/ml) (A-G) or in combination with Y27632 (10 μM) (H), TAT-Yp (100 μM) (I,K) or TAT-Y/F (100 μM) (J,L) peptides for 10 (A,E), 30 (B,D,F) or 70 (C,G-L) minutes. Cells were stained with Arp3 antibody and FITC-conjugated secondary antibody (A-J) or rhodamine phalloidin (K,L) as described in Materials and Methods. Bar, 5 μm.

aggregation of Y402 phosphorylated Pyk2 to adhesion contacts comparable to those containing Y747-phosphorylated β_3 after 10 minutes of adhesion (Fig. 2E). Over the adhesion time course, an increase in Pyk2 Y402 PSSA staining (Fig. 6F) was seen along with colocalization of total β_3 (Fig. 6I). These data suggest that active Pyk2 is recruited to adhesion contacts containing phosphorylated β_3 and may be in closest proximity to β_3. Over an identical adhesion time course with $K\alpha_V\beta_3$ Y747,759F cells, Y402-phosphorylated Pyk2 neither organized into podosomes (Fig. 6O) nor colocalized with β_3 (Fig. 6R). These data provide evidence for β_3 Y747 phosphorylation-dependent organization of Y402 phosphorylated Pyk2 into podosomes and colocalization with β_3.

Pyk2, Arp3 and vinculin present in adhesion contacts

Unlike fibroblast cell lines that form focal adhesions, leukocytes form podosomes – smaller adhesive structures that we have shown to contain Y747 phosphorylated β_3. After 70 minutes of adhesion to vitronectin in the presence of PMA, we observed actin stress fiber formation (Fig. 2C), Pyk2 Y402 PSSA organization and colocalization with total β_3 (Fig. 6F,I) and Arp3 organization (Fig. 5G). Here, we double-labeled $K\alpha_V\beta_3$ WT cells to determine the proximity of proteins within the signaling complex on β_3. Vinculin was present in adhesion contacts throughout the course of adhesion, but was especially evident in mature contacts in the cell periphery (Fig. 7A,G).

In Fig. 5, Arp3 exhibited progressive organization into podosomes over the course of adhesion. After 70 minutes of adhesion, we detected colocalization of Arp3 with both vinculin (Fig. 7C) and Pyk2 (Fig. 7F) in $K\alpha_V\beta_3$ WT cells. Total Pyk2 staining was largely diffuse (Fig. 7D) compared with vinculin and Arp3. In addition, we observed strong colocalization between Pyk2 and Arp3 (Fig. 7F). However, we

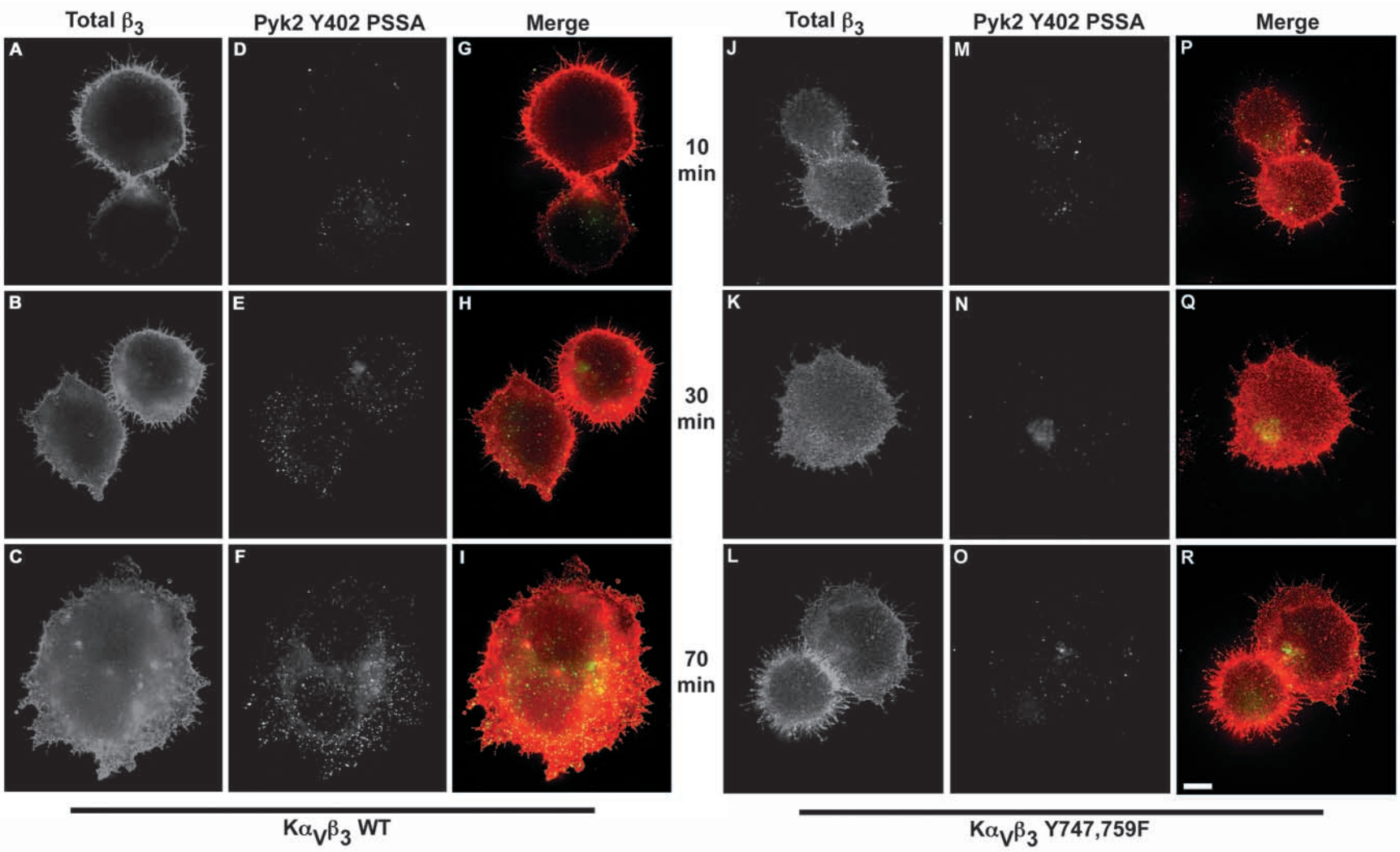

Fig. 6. Phosphorylated Pyk2 colocalizes with β_3 in mature adhesion contacts. K$\alpha_V\beta_3$ WT (A-I) and K$\alpha_V\beta_3$ Y747,759F (J-R) cells were adhered to vitronectin-coated coverslips in the presence of PMA (10 ng/ml) for 10, 30 or 70 minutes. Cells were double-labeled as described in Materials and Methods with anti-β_3 monoclonal antibody (A-C,J-L) and Pyk2 Y402 PSSA (D-F,M-O) with TRITC-conjugated anti-mouse (red, β_3) and FITC-conjugated anti-rabbit (green, Pyk2 Y402 PSSA) secondary antibodies. Merging of images (G-I,P-R) reveals the organization of Pyk2 Y402 PSSA into podosomes (F) and colocalization of total β_3 and Pyk2 Y402 PSSA (I) after 70 minutes of adhesion in K$\alpha_V\beta_3$ WT cells, but not in K$\alpha_V\beta_3$ Y747,759F cells (O,R). Bar, 5 μm.

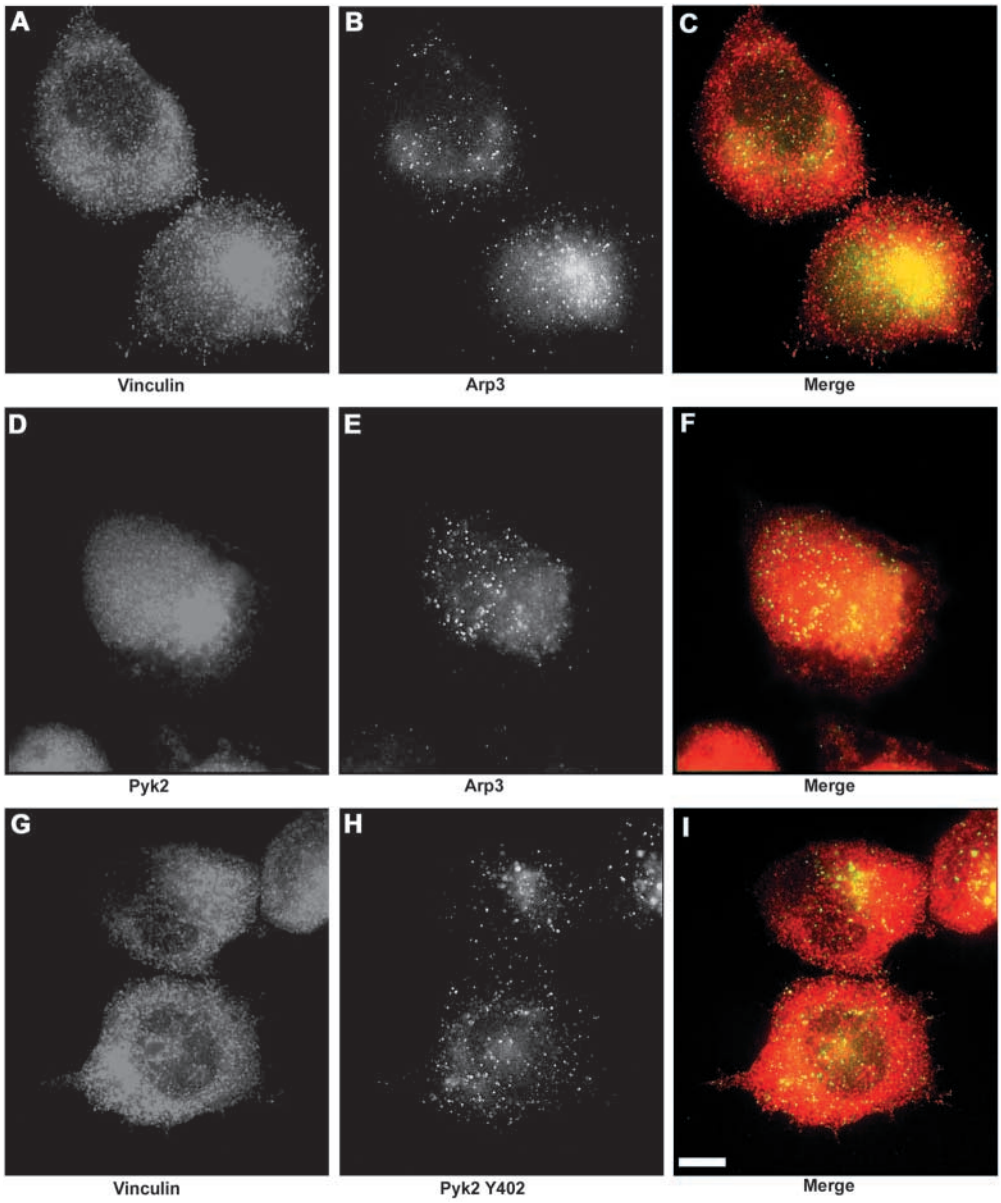

Fig. 7. Pyk2, Arp3 and vinculin are present in $\alpha_V\beta_3$ adhesion contacts. K$\alpha_V\beta_3$ WT cells were adhered to vitronectin-coated coverslips for 70 minutes in the presence of PMA (10 ng/ml). Cells were double-labeled as described in Materials and Methods for vinculin and Arp3 (A-C), Pyk2 and Arp3 (D-F), or vinculin and Pyk2 Y402 PSSA (G-I) antibodies with TRITC-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies. Merging of images (C,F,I) reveals colocalization of the respective proteins in adhesion contacts. Bar, 5 μm.

believe that the subset of Pyk2 that colocalized with Arp3 (Fig. 7F) was the Y402-phosphorylated form. Owing to antibody incompatibility, we could not double-label cells for Pyk2 Y402 PSSA and Arp3. Instead, we double-labeled cells with Pyk2 Y402 PSSA and vinculin, revealing colocalization (Fig. 7I). Combining these data with total β_3/Pyk2 Y402 PSSA co-localization (Fig. 6I), we propose that vinculin, Arp3 and Y402-phosphorylated Pyk2 are present in β_3-containing adhesion contacts. Importantly, β_3 Y747 phosphorylation appears to play a crucial role in the assembly of this adhesion complex into podosomes during adhesion to vitronectin.

Discussion

Cell adhesion and migration require the coordination of integrin adhesion sites with the actin cytoskeleton. This coordination is especially dynamic during leukocyte immunosurveillance to effect a rapid response to inflammation and also during platelet thrombus formation following vascular injury. Current models of cell adhesion and migration separate the formation of integrin adhesion sites and their signaling from the assembly of actin stress fibers. These models predict that integrin signaling culminates in the aggregation of cytoplasmic integrin binding partners that serve as docking sites for actin filaments preformed in the cytosol. However, recent evidence suggests that the final steps in integrin signaling may result in recruitment of actin assembly machinery to the adhesion site, a teleologically advantageous event. De Mali et al. (De Mali et al., 2002) showed sequestration of Arp2, a known component of the Arp2/3 actin nucleation complex, to adhesion sites via integrin-tethered vinculin. A requirement for this association was shown for membrane protrusion. A recent review predicted the presence of membrane anchors at which the ratcheting of actin fiber formation exerted a membrane protrusive force (Pollard and Borisy, 2003). A convergence between preformed actin and actin formed at the membrane to accomplish filopodial extension has also been shown (Vignjevic et al., 2003). As activation of both Arp subunits and formins, established actin nucleators, requires the activity of the Rho GTPases, and as this activity can be both generated and localized to integrin adhesion sites, it seems likely that the mature integrin adhesion site functions as an actin nucleation foci. Anchoring of the growing end of actin filaments to the adhesion site would provide a rapid and directed assembly of actin into stress fibers at the appropriate subcellular locale.

Hematopoietic cell types exhibit a regulated adhesion, requiring multiple input signals to prevent inappropriate thromboses or unwarranted inflammation. Unique signaling

pathways and hematopoietic-specific isomers of various signaling molecules contribute to the regulation of integrin-mediated adhesion in these cell types. We have characterized one event in these regulated adhesion pathways. Phosphorylation of Y747 of the β_3 cytoplasmic tail occurs as a result of integrin-ligand interaction. In leukocytes, this phosphorylation is required for PKC-dependent adhesion to vitronectin. Mutation of this tyrosine prevents hematopoietic cell adhesion both in vitro and in vivo, but has no effect on the adhesion of nonhematopoietic cell types and does not affect normal development. The defective adhesion is due to a failure of the ligand-occupied mutant integrin to activate Rho. Accordingly, these cells do not form actin stress fibers. Interestingly, the Y747F mutation of β_3 does not affect the ability of the receptor to support cell adhesion to fibronectin, with concordant activation of Rho and stress fiber formation. This suggests a very selective regulatory event in the process of leukocyte adhesion. Furthermore, the Y747F mutant β_3 provides an excellent tool to evaluate integrin regulation of the actin cytoskeleton as the affinity regulation of the receptor does not differ from wild type and the defective assembly of an actin cytoskeleton is restricted to the ligand vitronectin. In this study, we have used phosphorylation-specific β_3 antibodies and phosphorylation-deficient β_3 integrins to show a selective pathway for the recruitment of actin assembly machinery to the $\alpha_V\beta_3$ adhesion site.

We have previously shown that K$\alpha_V\beta_3$ Y747,759F cells exhibit neither actin stress fibers nor achieve firm adhesion on vitronectin (Blystone et al., 1997). However, these cells do possess the appropriate signaling molecules and actin assembly machinery for adhesion as can be observed on their adhesion to fibronectin (Butler et al., 2003). To better understand the role of β_3 Y747 phosphorylation for adhesion to vitronectin, we began by localizing this phosphorylation event in adhering cells. Although K$\alpha_V\beta_3$ WT cells displayed a firmly adherent phenotype after 1 hour of adhesion to vitronectin in the presence of PMA, β_3 Y747 phosphorylation was an early event, occurring within minutes of exposure to ligand. Phosphorylated β_3 appeared in punctate structures typical of leukocyte integrin adhesion contacts and were distributed widely throughout the cell. At early time points there was a concentration of phosphorylated β_3 at the cell periphery, forming an incomplete ring at the base of filopodial extensions. These punctate structures, termed podosomes, are hematopoietic integrin adhesion structures and analogous to focal contacts. Podosomes contain many of the same molecules as focal contacts, including α-actinin and vinculin, but also contain hematopoietic-specific signaling proteins, such as Vav1 and Pyk2. As adhesion progressed, β_3 Y747 phosphorylation diminished in direct opposition to the formation of actin stress fibers. In primary cells, phosphorylated β_3 appeared in analogous contacts. These cells, however, have a shorter time course to achieve firm adhesion and exhibit β_3 phosphorylation at earlier time points accordingly. As in the K$\alpha_V\beta_3$ WT cell line, β_3 phosphorylation diminished over time of adhesion in murine BMMs. No significant reactivity of the β_3 Y747 PSSA was detected in K$\alpha_V\beta_3$ Y747,759F cells under any conditions.

For adhesion to vitronectin, β_3 Y747 phosphorylation is required for Rho activation (Butler et al., 2003). Rho GTPase activity initiates the signaling events that result in actin stress fiber formation. To determine if the β_3 Y747 phosphorylation-dependent activation of Rho was responsible for the failure of K$\alpha_V\beta_3$ Y747,759F cells to form stress fibers on vitronectin, we monitored the organization of actin assembly machinery with respect to β_3 Y747 phosphorylation. Surprisingly, we found that Arp3 organized into podosomes over time of adhesion to vitronectin in cells expressing wild-type $\alpha_V\beta_3$ but failed to organize in K$\alpha_V\beta_3$ Y747,759F cells. This defective Arp3 organization was solely due to the β_3 Y747F mutation and the failure of this receptor to activate Rho on vitronectin, as K$\alpha_V\beta_3$ Y747,759F adhesion to fibronectin, which results in active Rho, resulted in Arp3 organization. In addition, Arp3 organization, as well as actin stress fiber formation, was prevented by the presence of peptide mimetics of the phosphorylated β_3 cytoplasmic tail. These data suggest that the presence of phosphorylated β_3 in podosomes is a prerequisite for Arp3 localization.

Rho kinase (ROCK) is a Rho GTPase effector that is required for the initiation of actin assembly and presumably lies between Rho and Arp2/3 in this signaling pathway. Inhibition of ROCK resulted in the complete failure of Arp3 to organize into adhesion contacts. Interestingly, ROCK inhibition also induced a sustained phosphorylation of β_3 for the duration of the time course. This suggests that ROCK activity contributes to feedback mechanisms to dephosphorylate β_3. Alternatively, β_3 Y747 phosphorylation may recruit molecules to the β_3 cytoplasmic tail, progressively blocking epitope availability. If this is the case, recruitment of molecules downstream of ROCK are likely to be responsible for this steric hindrance. In mature podosomal structures, we observed colocalization of Arp3 with Pyk2 in K$\alpha_V\beta_3$ WT cells. Pyk2 plays regulatory and adaptor roles between integrins and their downstream signaling partners in osteoclast-like cells (Pfaff and Jurdic, 2001). Interestingly, over time of K$\alpha_V\beta_3$ WT cell adhesion to vitronectin, we observed increased Pyk2 Y402 PSSA organization into podosomes and colocalization with β_3 that did not occur in K$\alpha_V\beta_3$ Y747,759F cells. Vinculin, an integrin-associated and actin-binding protein (Jockusch and Isenberg, 1981), colocalized with both Arp3 and Pyk2 Y402 in mature adhesive contacts. In our biochemical studies, vinculin coprecipitates with β_3 from adherent K$\alpha_V\beta_3$ WT cells at equivalent levels throughout an adhesion time course to vitronectin (data not shown). Similarly, by immunofluorescence we were unable to visualize any significant changes in vinculin organization over the adhesion time course. Although this does not preclude a vinculin dependence for Arp2/3 organization as reported by DeMali et al. (DeMali et al., 2002), it suggests that Arp2/3 translocation to the podosome requires additional events.

With this combined evidence of colocalization, we propose that vinculin, Arp3 and Y402-phosphorylated Pyk2 are present in adhesive contacts containing Y747 phosphorylated β_3 integrin. As Arp3 presents a β_3 Y747 phosphorylation dependency for organization into podosomes and is part of a large protein complex, it is a strong candidate for the steric masking of Y747-phosphorylated β_3 epitopes on completion of adhesion site signaling. The requirement of β_3 Y747 phosphorylation for $\alpha_V\beta_3$-mediated adhesion to vitronectin appears to lie in the activation and recruitment of actin nucleators for cytoskeletal assembly. Examination of the β_3 Y747-phosphorylation-independent recruitment of Arp2/3 on $\alpha_V\beta_3$ attachment to fibronectin will be of future interest.

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