PKD1/PKC μ promotes α v β 3 integrin recycling and delivery to nascent focal adhesions

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To identify kinases that regulate integrin recycling, we have immunoprecipitated α v β 3 integrin from NIH 3T3 fibroblasts in the presence and absence of primaquine (a drug that inhibits receptor recycling and leads to accumulation of integrins in endosomes) and screened for co-precipitating kinases. Primaquine strongly promoted association of α v β 3 integrin with PKD1, and fluorescence microscopy indicated that integrin and PKD1 associate at a vesicular compartment that is downstream of a Rab4-dependent transport step. PKD1 association was mediated by the C-terminal region of the β 3 integrin cytodomain, and mutants of β 3 that were unable to recruit PKD1 did not recycle in a PDGF-dependent fashion. Furthermore, suppression of endogenous PKD1 levels by RNAi, or over-expression of catalytically inactive PKD1 inhibited PDGF-dependent recycling of α v β 3 from early endosomes to the plasma membrane and blocked recruitment of α v β 3 to newly formed focal adhesions during cell spreading. These data indicate that PKD1 influences cell migration by directing vesicular transport of the α v β 3 integrin heterodimer.

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Introduction

Integrins are heterodimeric transmembrane receptors that mediate interactions between cells and the extracellular matrix (ECM). Integrins transmit signals across the plasma membrane, and engagement of integrins with the ECM activates numerous signalling pathways that influence a range of biological processes, including cell proliferation, migration and apoptosis (Schwartz and Baron, 1999). Correspondingly, intracellular signalling pathways can influence integrin function by increasing the affinity for ligand (affinity modulation) (Shattil, 1999) and initiating clustering of active integrins into macromolecular structures such as focal adhesions/complexes and fibrillar adhesions (Zamir and Geiger, 2001). The rho subfamily of GTPases have a

well-established role in regulating integrin clustering, and phosphatidylinositol 3-kinases (PI(3)Ks), H-ras (Kinashi *et al*, 2000), PKCs (Kolanus and Seed, 1997) and more recently ERK (Roberts *et al*, 2003) (possibly via the activation of calpain) are also implicated in the activation of integrins. Clearly any kinase found to associate with an integrin would be of interest in determining the pathways that regulate integrin function. Indeed PKC- α , src family kinases, pp125^{FAK} (Eliceiri *et al*, 2002), ERK (Ahmed *et al*, 2002; Roberts *et al*, 2003) and ILK (Delcommenne *et al*, 1998) have all been shown to associate physically with integrins and to regulate their function.

Kinases are now known to influence the distribution and function of integrins by controlling their transport through the endocytic and receptor recycling pathways. Internalisation of β 1 integrins is regulated by PKC- α (Ng *et al*, 1999), and PKC- ϵ (Ivaska *et al*, 2002) has a role in controlling an endosomal transport step that permits recycling of integrin to the plasma membrane. In our laboratory we have found that integrins recycle in two time domains, and that the signalling pathways that regulate these processes are divergent (Roberts *et al*, 2001). Under basal conditions (30 min of serum starvation), internalised α 5 β 1 and α v β 3 heterodimers are transported through early endosomes to the perinuclear recycling compartment, and return to the plasma membrane by a pathway that is regulated by Rab11 and PKB/Akt via the inhibition of GSK-3 β (Roberts *et al*, 2004). This is termed 'long-loop' recycling. Following stimulation with PDGF, α v β 3 (but not α 5 β 1) is rapidly recycled directly to the plasma membrane without the involvement of Rab11, thus engaging in a 'short-loop' cycle of endocytosis and recycling. As the PKB/GSK-3 β axis has only minimal effect on 'short-loop' recycling of α v β 3 (Roberts *et al*, 2004), we are interested in elucidating the kinase cascades responsible for this event.

The association of kinases with integrins can be either constitutive (i.e. bound to the heterodimer at all times and at numerous locations including endosomes and focal adhesions), as is the case for Src family kinases (Arias-Salgado *et al*, 2003) and Syk (Woodside *et al*, 2001), or regulated. Kinases mediating growth factor-stimulated recycling might be predicted to associate with the integrin at the cytoplasmic face of an intracellular vesicle, and then dissociate following its delivery to the plasma membrane. To identify signalling moieties that form such transient associations with integrins, we have treated cells with primaquine, a drug that inhibits receptor recycling and leads to accumulation of integrin in endosomes (Roberts *et al*, 2001), and then screened for kinases that co-precipitate with α v β 3 integrin. We have found that addition of primaquine strongly promotes association of α v β 3 with PKD1, a kinase related to PKC that is proposed to play a role in the regulation of fission of transport vesicles from the *trans*-Golgi network (TGN). In this paper, we present data that support a critical role for PKD1 in regulating recycling of α v β 3 integrin via the 'short loop',

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and on recruitment of the integrin to newly forming focal adhesions.

Results

Association of kinases with integrins following addition of primaquine

We have previously shown that the function of $\alpha\text{v}\beta 3$ integrin is influenced by a growth factor-regulated recycling pathway directing the integrin to the plasma membrane (Roberts *et al*, 2001). We hypothesised that kinases responsible for this would be likely to associate with the heterodimer at an endosomal compartment, and then dissociate following delivery to the plasma membrane. As the receptor recycling inhibitor primaquine causes cycling integrins to accumulate rapidly within endosomes, we have sought to identify kinases that associate with $\alpha\text{v}\beta 3$ integrin on the cytoplasmic face of these endosomal vesicles following the treatment of cells

with this drug. NIH 3T3 fibroblasts were serum-starved for 30 min and then challenged with PDGF in the presence and absence of primaquine. Cells were lysed in a buffer containing 0.5% (v/v) Triton X-100 and 0.25% (v/v) Igepal CA-630, and $\alpha\text{v}\beta 3$ heterodimers were isolated by immunoprecipitation. These immunoprecipitates were analysed using the Kinexus 1.2 protein kinase screen that tests for numerous signalling kinases (Pelech and Zhang, 2002). A selection of kinases co-immunoprecipitated with $\alpha\text{v}\beta 3$, and prominent among these were PKC- ζ , PKC- β and ERK1 (Figure 1A). A brief treatment (5 min) with primaquine altered the profile of $\alpha\text{v}\beta 3$ -associated kinases such that there was a substantial enrichment of PKD1/PKC μ and, to a lesser extent, PKC ϵ . We verified these results by Western blotting and confirmed that PKD1 associated with $\alpha\text{v}\beta 3$ only in the combined presence of PDGF and primaquine, and that this kinase did not co-immunoprecipitate with $\alpha 5\beta 1$ (Figure 1B). Moreover, we found that PKC- ζ associated with both $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ hetero-

Figure 1 Association of kinases with integrins following addition of primaquine. NIH 3T3 fibroblasts were transfected with $\alpha\text{v}\beta 3$ or $\alpha 5\beta 1$ integrin alone (**A**, **B**), or $\alpha\text{v}\beta 3$ in combination with GFP-PKD1 (**C**, **D**). Cells were serum-starved, challenged with 10 ng/ml PDGF-BB for 10 min in the absence and presence of 0.6 mM primaquine (PQ), and lysed in a buffer containing 0.5% (v/v) Triton X-100 and 0.25% (v/v) Igepal CA-630. Primaquine was added 5 min following PDGF addition and 5 min prior to lysis. Lysates were immunoprecipitated (IP) using magnetic beads coupled to monoclonal antibodies against human $\beta 3$ or $\alpha 5$ integrin (**A**–**C**), PKD or GFP (**D**), or control antibody (RG-16; **B**–**D**). Immobilised material was analysed using the Kinexus 1.2 protein kinase screen (**A**) or Western blotting (**B**–**D**).

dimers and that this was not augmented by primaquine (Figure 1B), indicating that this aPKC was a less promising candidate for a specific regulator of growth factor-regulated $\alpha\beta$ 3 recycling than PKD1 and PKC ϵ . These analyses also revealed that GFP-PKD1 associated with $\alpha\beta$ 3 immunoprecipitates in a fashion that was dependent on the prior incubation of cells with PDGF and primaquine (Figure 1C), indicating that this GFP-tagged kinase behaves similarly to the endogenous kinase.

To further confirm this association, we expressed GFP-PKD1 and immunoprecipitated the kinase from cells that had been treated with PDGF and primaquine using antibodies recognising either PKD1 or GFP. Western blotting of these immunoprecipitates indicated that β 3 (but not β 1) integrin co-precipitated with GFP-PKD1 but not with a control antibody and that addition of primaquine promoted this association (Figure 1D).

Inhibition of receptor recycling promotes the accumulation of PKD1 and $\alpha\beta$ 3 integrin at intracellular vesicles

We studied the intracellular distribution of $\alpha\beta$ 3 and GFP-PKD1 using fluorescence microscopy. Following treatment with PDGF, GFP-PKD1 was observed in a diffuse cytoplasmic distribution and did not appreciably colocalise with $\alpha\beta$ 3 (Figure 2A). However, 5 min following the addition of primaquine, the integrin and PKD1 became closely colocalised in a set of subplasmalemmal vesicles (Figure 2B). It is notable that this brief exposure to primaquine was sufficient to disassemble focal adhesions completely, highlighting the dynamic nature of these structures and the speed with which integrins are trafficking through the endosomal pathway (the internalisation of $\alpha\beta$ 3 integrin has a $t_{1/2}$ of between 5 and 10 min; Roberts *et al*, 2001). A 10 min exposure to primaquine induced more extensive colocalisation of $\alpha\beta$ 3 with PKD1 in vesicles that were distributed throughout the cell (Figure 2C). Longer exposure to primaquine caused cell-rounding and detachment at a rate that correlated with the internalisation of matrix receptors required for cell adhesion (not shown).

Role of the C-terminal region of β 3 cytodomain in recruitment of PKD1

A number of studies have shown that the cytodomains of integrin β subunits are responsible for recruiting signalling kinases and cytoskeletal proteins to the heterodimer (LaFlamme *et al*, 1997). As initial pull-down experiments indicated that GST- β 3^{737–762} (Figure 3A) was able to recruit PKD1 (Figure 3B), we proceeded to further define regions of the cytodomain responsible for this association. We employed an assay in which GST-cytodomain fusion proteins were immobilised on the surface of microtitre wells. These were then incubated with cell lysates, and bound PKD1 was detected by ELISA. Using this assay, we found that GST- β 3^{727–748} did not bind to PKD1, whereas GST- β 3^{749–762} bound to the kinase to the same extent as GST- β 3^{747–762} (Figure 3C). This indicated that the PKD1-binding site resided exclusively within the C-terminal 14 amino acids of the integrin.

To highlight individual residues necessary for recruiting PKD1, we constructed a series of β 3 cytodomain mutants in which amino acids at or near the C-terminus were either deleted or replaced with alanines (Figure 3A). Although removal of Gly⁷⁶¹ and Thr⁷⁶² from the C-terminus ablated

Figure 2 Colocalisation of PKD1 and $\alpha\beta$ 3 integrin at intracellular vesicles following inhibition of receptor recycling. NIH 3T3 fibroblasts were transfected with $\alpha\beta$ 3 integrin in combination with GFP-PKD1. Cells were serum-starved and challenged with 10 ng/ml PDGF-BB in the absence (A) and presence (B, C) of 60 μ M primaquine (PQ) for 5 min (B) or 10 min (C). Following fixation, cells were permeabilised in 0.2% (v/v) Triton X-100 and stained for β 3 integrin by indirect immunofluorescence using a Texas red-conjugated secondary antibody (red) and GFP-PKD1 was visualised directly (green). Colocalisation of the two fluorophores is shown in yellow in the merged panels. The areas encompassed by the white boxes in (B) and (C) are enlarged four-fold in (B') and (C'), respectively. Images are presented as a single confocal optical slice centred approximately 0.5 μ m above the plane of the substratum. Bar, 10 μ m.

PKD1 binding (Figure 3D), substitution of these residues for alanine did not significantly reduce kinase recruitment (Figure 3E). Replacement of Tyr⁷⁵⁹ with alanine, however, ablated association with PKD1 (Figure 3E). Tyr⁷⁵⁹ is known to be phosphorylated *in vivo* and this reduces binding of β 3 integrins to certain cytoplasmic ligands, such as Shc (Cowan *et al*, 2000). We, therefore, investigated the effect of a phosphomimetic substitution at this position. Indeed, replacement of Tyr⁷⁵⁹ with glutamic acid completely ablated kinase binding (Figure 3E), indicating the requirement for an

Figure 3 Association of PKD1 with the $\beta 3$ integrin cytodomain. (A) Description and nomenclature of the GST- $\beta 3$ integrin cytodomain fusion proteins employed for the present study. GST fusion proteins were expressed in *E. coli* strain BL-21 and purified as described previously (Woods *et al*, 2002). (B) Lysates from NIH 3T3 fibroblasts were incubated with magnetic beads conjugated to GST, or GST- $\beta 3^{727-762}$. Immobilised material was analysed by Western blotting with anti-PKD1 (upper panel), and the loading of the GST fusion proteins was confirmed by Western blotting for GST (lower panel). (C–F) GST and GST-cytodomain fusion proteins were immobilised on the surface of microtitre wells. These were then incubated with either cell lysate (C–E) or biotinylated talin-FERM domain (residues 86–410; mouse sequence) (F). Bound PKD1 was detected by ELISA using antibodies recognising PKD1 (C–E) and binding of the biotinylated talin-FERM domain was detected with HRP-conjugated streptavidin (F).

unphosphorylated tyrosine at this position for efficient recruitment of PKD1. Taken together, these data indicate that (a) the PKD1-binding site resides exclusively within the C-terminal 14 amino acids of the $\beta 3$ cytodomain, (b) an unphosphorylated Tyr at position 759 is essential for PKD1 recruitment and (c) Tyr⁷⁵⁹ must be flanked C-terminally by three amino acids. However, the presence of Gly⁷⁶¹ and Thr⁷⁶² is not mandatory, as alanines at these positions will suffice.

Talin is now established to be recruited to focal adhesions via an interaction between its FERM domain and the membrane proximal NXXY motif in the $\beta 3$ integrin cytodomain (Ulmer *et al*, 2003). We found that $\beta 3$ mutants that did not

recruit PKD1 ($\beta 3^{727-760}$, $\beta 3^{Y759A}$ and $\beta 3^{Y759E}$), bound strongly to the talin FERM domain (Figure 3F), indicating that these mutations disrupted neither the overall structure and/or folding of the cytodomain nor the function of the membrane proximal NXXY motif.

Catalytically inactive PKD1 associates with $\alpha\beta 3$ in the absence of primaquine and traps the integrin in intracellular vesicles

Rey *et al* (2001) have reported that following agonist stimulation kinase-dead PKDs associate with cellular membranes and become trapped, presumably due to a requirement for

Figure 4 Catalytically inactive PKD1 associates with $\alpha\beta$ 3 in the absence of primaquine. (**A**, **B**) NIH 3T3 fibroblasts were transfected with $\alpha\beta$ 3 integrin in combination with GFP-PKD1 or GFP-PKD^{D733A}. Cells were serum-starved, challenged with 10 ng/ml PDGF-BB for 10 min in the absence and presence of 0.6 mM primaquine (PQ), lysed and immunoprecipitated (IP) using magnetic beads coupled to monoclonal antibodies against human β 3 integrin, PKD1, GFP or control antibody (RG-16). Immobilised material was analysed by Western blotting. (**C**) Cells were transfected with PKD1 or PKD^{D733A} and fractionated on 10–40% (v/v) iodixanol gradients. The linearity of the gradient was confirmed by refractometry (●). The sedimentation profile of PKD1 (■) and β 3 integrin (▲; red line) was determined by Western blotting followed by densitometric scanning. These profiles represent the mean values from a pool of four experiments. The sedimentation positions of intracellular compartments were determined by Western blotting with antibodies against the following markers: calreticulin (ER), Rab11 and Rab11 (endosomes) and biotinylated cell surface proteins (plasma membrane).

substrate or autophosphorylation in membrane dissociation of the kinase. We employed a mutant of PKD1, PKD^{D733A}, that is catalytically inactive, and determined the ability of this to associate with immunoprecipitated $\alpha\beta$ 3 integrin. We found that PKD^{D733A} had a greatly increased ability to co-immunoprecipitate with $\alpha\beta$ 3 and, interestingly, did so in the absence of primaquine (Figure 4A and B). We studied the localisation of GFP-PKD^{D733A} with respect to $\alpha\beta$ 3 integrin by fluorescence microscopy. Consistent with the immunoprecipitation data, we found that GFP-PKD^{D733A} co-localised closely with $\alpha\beta$ 3 in a set of subplasmalemmal vesicles. These often appeared to have a tubular morphology and were observable in both the absence (Figure 5A) and presence (Figure 5B) of primaquine. In some cells, PKD^{D733A} and $\alpha\beta$ 3 integrin colocalised in vesicles that were more evenly distributed throughout the

cell (Figure 5C), similar to that observed following longer (10 min) exposures to primaquine (Figure 2C). To confirm that association of PKD and $\alpha\beta$ 3 was not restricted to a single immortalised cell line, we investigated the distribution of PKD1 and $\alpha\beta$ 3 in primary cultured mouse embryo fibroblasts (MEFs). Indeed, GFP-PKD^{D733A} and $\alpha\beta$ 3 integrin colocalised closely in MEFs, and this was seen to occur in subplasmalemmal vesicles (Figure 5D), indicating that these proteins are associated with each other in these primary cultured cells. We have also performed these experiments with another mutant of PKD1, PKD^{S744/748A}, that lacks kinase activity due to the substitution of critical phosphorylation sites in the kinase activation loop. We found this to behave similarly to PKD^{D733A} in both $\alpha\beta$ 3 immunoprecipitation and colocalisation experiments (not shown).

Figure 5 Colocalisation of $\alpha\beta 3$ integrin with catalytically inactive GFP-PKD^{D733A}. NIH 3T3 fibroblasts (A–C) or primary cultured MEFs (D) were transfected with $\alpha\beta 3$ integrin in combination with GFP-PKD^{D733A}. Cells were serum-starved and challenged with 10 ng/ml PDGF-BB in the absence (A, C, D) and presence (B) of 60 μ M primaquine (PQ). Cells were stained for $\beta 3$ integrin (red) and GFP-PKD1 was visualised directly (green). Colocalisation of the two fluorophores is shown in yellow in the merged panels. The areas encompassed by the white boxes are enlarged four-fold in (A') and (B') and two-fold in (D'). Bar, 10 μ m.

To obtain biochemical data supporting the colocalisation of PKD1 and $\alpha\beta 3$ at an endomembrane compartment, we employed iodixanol density gradient centrifugation. On these gradients, $\alpha\beta 3$ integrin was seen to reside primarily in three membrane fractions: the plasma membrane (PM), Rab4- and Rab11-positive endosomes and the endoplasmic reticulum (ER) and these did not co-sediment appreciably with overexpressed wild-type PKD1 (PKD^{WT}) (Figure 4C, left panels). In contrast, following expression of PKD^{D733A}, a proportion (≈ 20 –30%) of cellular $\alpha\beta 3$ co-sedimented with the inactive kinase in a membrane fraction that was buoyant near the mid-point of the gradient (Figure 4C, right panels, red bar). Taken together, these data indicate that PKD1 is recruited to $\alpha\beta 3$ at a subplasmalemmal compartment that is distinct from the plasma membrane, the Rab4- or Rab11-positive endosomes and the ER, and that dissociation of the integrin–kinase complex requires the catalytic activity of PKD1.

PDGF-dependent $\alpha\beta 3$ recycling is dependent on PKD1

We used RNA interference (RNAi) to suppress cellular levels of PKD1. Expression of the PKD1-RNAi vector (mu6pro-PKD1) reduced levels of PKD1 by approximately six-fold, whereas empty vector (mu6pro) and a control hairpin RNA (mu6pro-CON) were ineffective in this regard (Figure 6A). We therefore determined the effect of mu6pro-PKD1 on recycling of $\alpha\beta 3$ from early endosomes to the plasma membrane using the ELISA-based method that we have

employed previously (Roberts *et al*, 2001). Indeed, RNAi of PKD1 strongly opposed the ability of PDGF to stimulate integrin delivery to the plasma membrane (Figure 6B).

Catalytically inactive PKD1s display an enhanced ability to bind to $\alpha\beta 3$ at a vesicular compartment, indicating the possibility that these mutant kinases might oppose recycling of the integrin. Expression of PKD^{WT} had little effect on the basal and PDGF-stimulated components of $\alpha\beta 3$ recycling (Figure 6B). In contrast, expression of catalytically inactive PKD1s, PKD^{D733A} and PKD^{S744/748A}, tended to raise the basal levels of recycling, but inhibited PDGF-driven integrin recycling (Figure 6B). To evaluate the PDGF-dependent component of recycling, we have presented the data from Figure 6B following subtraction of the appropriate basal values. This analysis revealed that either RNAi of PKD1, or expression of PKD^{D733A} and PKD^{S744/748A} suppressed this value by approximately 70% (Figure 6C).

The data in Figure 3 indicate that truncation of the $\beta 3$ cytodomain or substitution of Tyr⁷⁵⁹ opposed the ability of the integrin to recruit PKD1. We tested the behaviour of $\alpha\beta 3$ heterodimers with mutated β subunits in the recycling assay. From this analysis, it was clear that the ability of an integrin to recycle in response to PDGF correlated closely with its capacity to bind PKD1. In this regard, $\alpha\beta 3$ ^{T762A} and $\alpha\beta 3$ ^{C761A} (which bind to PKD1) recycled efficiently following addition of PDGF (Figure 6C). In contrast, $\alpha\beta 3$ ^{1–760}, $\alpha\beta 3$ ^{Y759A} and $\alpha\beta 3$ ^{Y759E} (which do not bind to PKD1) had reduced PDGF-dependent recycling (Figure 6C).

Figure 6 PDGF-stimulated recycling of $\alpha\text{v}\beta 3$ integrin requires association with active PKD1. **(A)** NIH 3T3 fibroblasts were transfected with mu6pro, mu6pro-CON or mu6pro-PKD1 using the Amaxa 'Nucleofector'. Cells were lysed 24 and 48 h following transfection and the lysates were probed for the presence of PKD1, PKC- ζ and vinculin (as a loading control) by Western blotting. **(B, C)** Cells were transfected with $\alpha\text{v}\beta 3$ integrin or $\alpha\text{v}\beta 3$ with mutated $\beta 3$ subunits, in combination with the mu6pro RNAi vectors or PKD1 constructs as indicated. 'Short-loop' recycling of $\alpha\text{v}\beta 3$ in the presence and absence of 10 ng/ml PDGF-BB was determined as described previously (Roberts *et al*, 2001). Values are mean $\pm$ s.e.m., $n > 5$. To evaluate the PDGF-dependent component of recycling, the data from (B) are presented following subtraction of the appropriate basal values (C).

In addition to 'short-loop' recycling, $\alpha\text{v}\beta 3$ also recycles in a 'long loop', which proceeds via the perinuclear (Rab11-positive) recycling compartment. We have found that 'long-loop' recycling of $\alpha\text{v}\beta 3$ is not reduced by truncation of the $\beta 3$

integrin subunit to Asn⁷⁵⁶ (a construct that does not associate with PKD1), nor by expression of PKD^{D733A} (not shown). These data indicate that recruitment of active PKD1 is necessary for 'short-loop' PDGF-driven recycling from early

endosomes, but that this kinase is unlikely to contribute to 'long-loop' recycling of integrins.

PKD and $\alpha\text{v}\beta 3$ associate downstream of a Rab4-regulated step

As PDGF-stimulated recycling of $\alpha\text{v}\beta 3$ requires Rab4 (Roberts *et al*, 2001), we tested the effect of expression of dominant-negative mutants of Rab4 on the association of $\alpha\text{v}\beta 3$ with PKD1. Rab4^{S22N} and Rab4^{N121I} both abolished the ability of PKD1 to co-immunoprecipitate with $\alpha\text{v}\beta 3$ (Figure 7A). Moreover, we were unable to detect convincing colocalisation of $\alpha\text{v}\beta 3$ and PKD^{D733A} following expression of Rab4^{S22N} (Figure 7B). By contrast, expression of dominant-negative Rab11 and treatment of cells with wortmannin (both manipulations that target 'long-loop' recycling) do not inhibit co-immunoprecipitation of integrin and PKD1 (Figure 7A).

To determine whether this effect of dominant-negative Rab4s was due to a requirement for Rab4 in PKD activation, we probed Western blots with an antibody that detects PKD only when phosphorylated at Ser⁹¹⁶. As this residue is an autophosphorylation site, its phosphorylation can be taken as measure of activation of cellular PKD (Matthews *et al*, 1999a). PDGF clearly induced PKD autophosphorylation, as reported previously, and Rab4^{S22N} did not oppose this (Figure 7C). These data suggest that Rab4 does not have a role in the activation of the PKD *per se*, but acts to mediate the transport of $\alpha\text{v}\beta 3$ to a compartment where association with PKD1 is initiated. It should also be noted that primaquine did not alter cellular levels of phospho-Ser⁹¹⁶PKD (Figure 7C) and is therefore unlikely to block integrin recycling by inhibiting PKD1, or any other signalling pathway leading to its activation.

A role for PKD1 in the delivery of $\alpha\text{v}\beta 3$ to newly forming focal adhesions

Expression of a kinase inactive form of PKD1 has been recently shown to compromise fibroblast motility (Prigozhina and Waterman-Storer, 2004). As overexpression of inactive forms of kinases can result in 'sideways' inhibition of other pathways under control of the same upstream kinases (Lawlor and Alessi, 2001), we wished to confirm the role of PKD1 in cell migration using RNAi. Cells expressing the control RNAi vector closed the wound by 24 h (Figure 8A and B). In contrast, cells expressing mu6pro-PKD1 had reduced migration, and were only able to achieve $\approx 50\%$ wound closure after 24 h (Figure 8A and B). We therefore examined the distribution of $\alpha\text{v}\beta 3$ on the surface of migrating cells following suppression of PKD1. In cells transfected with control vector, migrating cells displayed a typical fan-like morphology with $\alpha\text{v}\beta 3$ localised to focal adhesions at the leading edge (Figure 8C). Following suppression of PKD1, migrating cells had altered morphology with $\alpha\text{v}\beta 3$ being no longer concentrated at the leading edge, but present in focal adhesions that were distributed more uniformly on the lower surface of the cell (Figure 8C). Taken together, these data indicated that following RNAi of PKD1 cells migrated with reduced efficiency, and that this may be due to their inability to transport $\alpha\text{v}\beta 3$ to newly forming focal adhesions at the leading edge.

Work by Zaidel-Bar *et al* (2003) has shown that proteins are recruited to focal adhesions during cell migration in a defined sequence: with $\alpha\text{v}\beta 3$ integrin and paxillin arriving

Figure 7 Association of $\alpha\text{v}\beta 3$ with PKD1 requires the activity of Rab4. (A) NIH 3T3 fibroblasts were transfected with $\alpha\text{v}\beta 3$ integrin in combination with Rab4, Rab4^{S22N}, Rab4^{N121I} or Rab11^{N124I}. Cells were serum-starved in the absence and presence of 100 nM wortmannin (WMN), challenged with 10 ng/ml PDGF-BB in the presence of 0.6 mM primaquine (PQ), lysed and immunoprecipitated (IP) using magnetic beads coupled to monoclonal antibodies against human $\beta 3$ integrin. Immobilised material was analysed by Western blotting. (B) Cells were transfected with $\alpha\text{v}\beta 3$ integrin in combination with GFP-PKD^{D733A} and Rab4^{S22N}, serum-starved and challenged with 10 ng/ml PDGF-BB. Cells were stained for $\beta 3$ integrin (red) and GFP-PKD1 was visualised directly (green). The areas encompassed by the white box in (B) is enlarged four-fold in (B'). Bar, 10 μm . (C) Cells were transfected with GFP-PKD1 in combination with Rab4 or Rab4^{S22N}. Following serum starvation, the cells were challenged with 10 ng/ml PDGF in the absence or presence of 0.6 mM primaquine. The cellular content of GFP-PKD1 and phospho-Ser⁹¹⁶GFP-PKD1 was determined by Western blotting.

early while zyxin and vinculin are recruited as relative latecomers. As the data in Figure 8C indicate that PKD1 might be required for transport of $\alpha\text{v}\beta 3$ to nascent focal adhesions during migration, we examined recruitment of $\alpha\text{v}\beta 3$ to focal adhesions formed shortly after attachment of cells to fibronectin. In cells expressing the control RNAi vector or wild-type PKD1, $\alpha\text{v}\beta 3$ was recruited to focal adhesions within 45 min of plating (Figure 8D). In contrast, if PKD1 were suppressed by RNAi or if cells were expressing PKD^{D733A}, little $\alpha\text{v}\beta 3$ was recruited to newly formed focal

Figure 8 Incorporation of $\alpha v\beta 3$ integrin into newly forming focal adhesions requires association with active PKD1. (**A**, **B**) NIH 3T3 fibroblasts were transfected with mu6pro-CON or mu6pro-PKD1 using the Amaxa Nucleofector and allowed to grow to confluence over 48 h. Following wounding, the cells were allowed to migrate in DMEM supplemented with 1% (v/v) FCS and 10 ng/ml PDGF-BB. Photographs of the wounds were taken at various times post-wounding and the degree of wound closure was determined by delineation of the wound envelope using the 'NIH image' software (values are mean $\pm$ s.e.m., $n = 10$ fields). (**C**) Confluent monolayers of NIH 3T3 fibroblasts expressing $\alpha v\beta 3$ integrin in combination with mu6pro-CON or mu6pro-PKD1 were wounded as for (**A**) and the cells were allowed to migrate for 4 h. Surface $\alpha v\beta 3$ was visualised by indirect immunofluorescence (red) and cells were counterstained with phalloidin (green). Surface-only $\alpha v\beta 3$ staining was obtained by addition of the primary antibody prior to detergent permeabilisation. Bar, 10 μm . (**D**) NIH 3T3 fibroblasts were transfected with $\alpha v\beta 3$ integrin or $\alpha v\beta 3$ with mutated $\beta 3$ subunits, in combination with the PKD1-RNAi vector (mu6pro-PKD1) or appropriate control vectors (mu6pro, mu6pro-CON), PKD1 or catalytically inactive PKD as indicated. Following trypsinisation, cells were allowed to adhere to fibronectin-coated coverslips for 45 min or 24 h. $\beta 3$ integrin was visualised by indirect immunofluorescence (red). Bar, 10 μm .

adhesions, and most of the integrin was visible in the body of the cell (Figure 8D).

We tested the ability of $\alpha\text{v}\beta 3$ heterodimers with mutated β subunits to be incorporated into newly forming focal adhesions. This revealed that the ability of an integrin to be recruited to newly formed focal adhesions correlated closely with its capacity to bind PKD1. For instance, $\alpha\text{v}\beta 3^{\text{T762A}}$ and $\alpha\text{v}\beta 3^{\text{G761A}}$ (which bind to PKD1) were rapidly incorporated into newly formed focal adhesions (Figure 8D). In contrast, $\alpha\text{v}\beta 3^{\text{Y759A}}$ and $\alpha\text{v}\beta 3^{\text{Y759E}}$ (that do not associate with PKD1) remained in the perinuclear region 45 min following adhesion of cells to fibronectin (Figure 8D). It should be noted that this reduction in recruitment of $\alpha\text{v}\beta 3$ to nascent focal adhesions is not due to blockade of focal adhesion assembly *per se* as (a) under all these conditions $\alpha\text{v}\beta 3$ was seen in focal adhesions if the cells were allowed to spread for 24 h (Figure 8D), and (b) the level of paxillin in newly formed focal adhesions is unaffected by expression of PKD^{D733A} or RNAi of PKD1 (not shown).

These data support the proposal that association of active PKD1 with $\alpha\text{v}\beta 3$, and its consequent recycling via a PKD1-dependent pathway is necessary to recruit rapidly this integrin into newly forming focal adhesions during cell spreading and migration.

Discussion

Previous studies from our laboratory indicate that $\alpha\text{v}\beta 3$ is rapidly transported from early endosomes to the plasma membrane in response to PDGF (Roberts *et al*, 2001). To identify signalling moieties that mediate this, we screened for kinases immunoprecipitating specifically with $\alpha\text{v}\beta 3$ following blockade of integrin recycling. We report that PKD1 was particularly abundant in these $\alpha\text{v}\beta 3$ immunoprecipitates, and fluorescence microscopy confirmed that $\alpha\text{v}\beta 3$ and PKD1 associate at a vesicular compartment that is likely to be downstream of a Rab4-dependent transport step. The interaction between PKD1 and $\alpha\text{v}\beta 3$ involved the C-terminal region of the $\beta 3$ cytodomain and mutants of $\beta 3$ that were unable to recruit PKD1 were not recycled to the plasma membrane in response to PDGF. Suppression of endogenous PKD1 by RNAi or expression of catalytically inactive PKDs also blocked PDGF-driven $\alpha\text{v}\beta 3$ recycling, and opposed the delivery of $\alpha\text{v}\beta 3$ to newly forming focal adhesions/complexes.

PKD1- $\alpha\text{v}\beta 3$ association

Kinases that associate with integrin β subunit cytoplasmic tails play a role in regulating integrin function. Indeed, a number of studies have reported PKC α to be localised at sites of integrin contact with the ECM, and also to vesicles involved in integrin trafficking (Ng *et al*, 1999). More recently, association of $\beta 1$ integrins with PKC α has been shown to involve a direct interaction between the kinase and regions of the $\beta 1$ cytodomain that correspond to the membrane proximal and distal NPXY motifs (Parsons *et al*, 2002). A number of factors led us to propose that PKD1 associates with $\beta 3$ integrin in a different way from that reported for $\beta 1$ and PKC α . Firstly, we do not find PKD1 to co-immunoprecipitate with $\alpha 5\beta 1$ integrin. Secondly, our data show that mutation of the extreme membrane distal NXXY motif of $\beta 3$ is sufficient to abrogate PKD binding (PKC α requires the disruption of both

membrane proximal and distal NPXY motifs), and thirdly it is necessary to use *n*-octyl- β -D-glucopyranoside as a detergent to demonstrate $\beta 1$ integrin-PKC α binding (not shown), whereas the $\beta 3$ integrin-PKD1 complex survives cell lysis in the somewhat harsher combination of Triton X-100 and Igepal CA-630.

Other kinases bind directly to integrin β cytodomains and these include ILK (Delcommenne *et al*, 1998), pp125^{FAK} (Eliceiri *et al*, 2002), and those of the Src (Arias-Salgado *et al*, 2003) and Syk (Woodside *et al*, 2001) families. ILK and pp125^{FAK} bind to $\beta 1$ and $\beta 3$ (and possibly other) integrins, whereas p60^{src} and Syk require the extreme C-terminal 14 amino acids of $\beta 3$ (and not $\beta 1$), and this association survives lysis in Igepal CA-630-containing buffers (Arias-Salgado *et al*, 2003). Thus the region of $\beta 3$ that associates with p60^{src} and Syk appears to be similar to that required for recruiting PKD1. However, the substitution of Tyr⁷⁵⁹ for Phe in the $\beta 3$ cytodomain has no effect on the interaction of $\beta 3$ integrin with c-Src (Arias-Salgado *et al*, 2003), indicating that the precise determinants for binding this tyrosine kinase differ from those that permit recruitment of PKD1. Additionally, it must be noted that studies on the p60^{src}- $\beta 3$ integrin complex have been performed in platelets that express high levels of Src family kinases (Obergfell *et al*, 2002), or in CHO cells following overexpression of these kinases (Arias-Salgado *et al*, 2003). The fibroblasts employed for the present study do not express high levels of src family kinases, and accordingly we do not find p60^{src} to be detectable in $\alpha\text{v}\beta 3$ immunoprecipitates unless the kinase is overexpressed (not shown). Preliminary data suggest that bindings of PKD1 and p60^{src} to $\beta 3$ are mutually exclusive, and it will be interesting to determine the respective contributions of these two kinases to $\beta 3$ integrin function in a cell type that expresses significant quantities of both PKD1 and p60^{src}.

The data presented in this paper do not resolve whether the association of PKD1 and $\alpha\text{v}\beta 3$ is direct. Proteins that are capable of binding directly to the $\beta 3$ cytodomain may serve to link PKD to the integrin. In this regard, the PKC-adaptor protein, RACK1, certainly binds to PKC ϵ to mediate its association with $\beta 1$ integrin (Besson *et al*, 2002), and it is possible that RACK may bind to PKD1. However, due to the fact that RACK binds to a membrane proximal K000 motif that is absent in our GST- $\beta 3$ integrin cytodomain fusion proteins (Liliental and Chang, 1998), this is unlikely to be the mechanism by which $\alpha\text{v}\beta 3$ recruits PKD1.

PKD1, integrin traffic and cell migration

Previous studies from our laboratory show that the PKB/GSK3 β axis has a strong influence on 'long-loop' recycling of integrins, but only minimal effect on 'short-loop' recycling of $\alpha\text{v}\beta 3$ in response to PDGF (Roberts *et al*, 2004). Conversely, PKD1 appears to be responsible for the majority of the 'short-loop' recycling, but does not regulate the 'long loop', indicating that there are key differences in the cellular machinery that controls these two recycling pathways. So how might PDGF act via PKD1 to regulate $\alpha\text{v}\beta 3$ recycling? It is clear that PDGF invokes the activity of both phospholipase C and PKC to activate PKD1 in fibroblasts (Rey *et al*, 2001). Indeed, we find that treatment with either U73122 (phospholipase C inhibitor) or GF109203X (PKC inhibitor) completely inhibits recruitment of PKD1 to $\alpha\text{v}\beta 3$, and these agents correspondingly block the ability of PDGF to drive integrin recycling (not

shown). This is not surprising, as it has been known for some time that the PLC/DAG/PKC pathway plays a major role in PKD activation, and that PKC is required for this. More recently, it has been shown that PKC ϵ activates PKD (Waldron and Rozengurt, 2003), and the fact that we find PKC ϵ to co-precipitate with α v β 3 is consistent with a role for this isoform of PKC in activating PKD1 and recruiting it to an integrin-recycling vesicle. So what might this compartment be? Although the PKD1· α v β 3 integrin compartment has a subplasmalemmal tubulo-vesicular morphology, we have not been able to demonstrate convincing colocalisation of PKD with a number of endosomal markers (Rab GTPases, EEA1 and the transferrin receptor), even following primaquine treatment. PKD has been shown to have a role in regulating transport from the TGN to the plasma membrane (Liljedahl *et al*, 2001). Prigozhina and Waterman-Storer (2004) have recently proposed that PKD1-mediated transport from the TGN to the leading edge of migrating cells delivers a regulatory molecule key to cell migration, and these authors suggest that this may be an integrin. As we provide clear evidence of a role for PKD1 in the return of endocytosed integrin to the plasma membrane, it is possible that α v β 3 recycles via a Rab4-dependent step that delivers it to the TGN or another post-Golgi compartment that is involved with anterograde vesicular transport.

PKD1 has been localised to sites of active matrix proteolysis during the invasion of breast cancer cells into gelatin (Bowden *et al*, 1999). The soluble matrix protease, MMP-2, and its cognate activator, MT1-MMP, have been shown to bind to α v β 3 integrin (Deryugina *et al*, 2001) and, moreover, MT1-MMP is known to recycle to the plasma membrane via the TGN (Wang *et al*, 2004). This raises the exciting possibility that PKD1 may act to target not only α v β 3 integrin but also matrix metalloproteases to invadopodia and other sites of matrix invasion during angiogenesis and metastasis.

In conclusion, our studies have identified an association between an integrin (α v β 3) and a kinase (PKD1) that occurs in a compartment likely to represent a vesicular recycling intermediate. We propose that PKD1 regulates the vesicular transport of the integrin, which, in turn, influences its incorporation into focal adhesions. These data highlight the need to characterise the signalling machinery involved in integrin recycling, in order to fully understand complex cellular processes such as invasion and metastasis.

Materials and methods

Materials

Monoclonal mouse anti-human β 3 integrin (clone VI-PL2) and α 5 integrin (clone VC5) were purchased from Pharmingen (San Diego, CA). Polyclonal rabbit anti-PKC μ (sc-639) was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-Ser⁹¹⁶PKD1 was as described by Matthews *et al* (1999a). The sample preparation and parameters of the Kinexus protein kinase screen were as described by Pelech and Zhang (2002). Texas red-conjugated anti-mouse immunoglobulins were from Southern Biotechnology (Birmingham, AL). Magnetic sheep anti-mouse IgG Dynabeads (DynaL, Oslo, Norway) and bovine serum albumin (BSA) were from First Link (UK) Ltd. NHS-SS-biotin (21331) and enhanced chemiluminescence reagents (ECL) were from Pierce and Warriner Ltd (Chester, Cheshire, UK). Cell culture medium and Maxisorb 96-well plates were from Life Technologies (Rockville) and fetal calf serum (FCS) was from Sera-Q (Tunbridge Wells, Kent, UK). Fugene 6 transfection reagent was from Roche Diagnostics GmbH (Mannheim, Germany). PDGF-BB (100-14B) was from PreproTech

Inc. (Rocky Hill, NJ). Streptavidin-conjugated horseradish peroxidase (HRP) was from Amersham Biosciences (Little Chalfont, Bucks). Primaquine diphosphate (16039-3) was from Aldrich Chem. Co. (Milw., WI). Iodixanol was purchased as Optiprep from Axis-Shield PoC AS (Oslo, Norway). All other reagents were purchased from Sigma Chemical Co (Poole, Dorset, UK).

Expression plasmids

α v, β 3, α 5 and β 1 integrins, and Rab4, S22NRab4, N121IRab4 and N124IRab11 were in pcDNA3 as described by Roberts *et al* (2001). GFP-PKD1, GFP-PKD^{D733A} and GFP-PKD^{S744/748A} were in the pEF-plink2-GFP_{C3} expression vector and are as described in Matthews *et al* (1999b). The mU6pro vector was used for the expression of RNA duplexes (Vojtek *et al*, 2003). A gene-specific sequence exclusive to murine PKD1 was targeted (available on request). All plasmids were purified by CsCl banding prior to transfection.

Cell culture and transfection

NIH 3T3 mouse fibroblasts and primary cultures of MEFs were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FCS and 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B at 37°C with 10% CO₂. Cells were grown to 50% confluence, fed with fresh DMEM containing 10% (v/v) FCS and transfected using Fugene 6 according to the manufacturer's instructions. The ratio of Fugene 6 to DNA was maintained at 3 μ l Fugene 6:1 μ g DNA. Immunoprecipitations, integrin recycling and cell-spreading assays were carried out 24 h post-transfection.

For the RNAi studies, transfections were carried out using the Amaxa 'Nucleofector' system. Briefly, cells were grown to 80% confluence, removed by trypsinisation, washed in PBS and resuspended in Amaxa solution 'R' together with 10 μ g DNA of mu6pro, mu6pro-CON or mu6pro-PKD1. Following electroporation (in the 'Nucleofector'; program T-20), the cells were re-plated in eight-well dishes. All RNAi experiments were carried out 48 h post-transfection.

Expression and purification of GST-integrin cytodomain fusion proteins

PCR-amplified DNA fragments corresponding to the indicated regions of the human sequence of β 3 integrin were subcloned into the EcoRI-XhoI site of the pGEX-4T-1 vector. GST fusion proteins were expressed in *Escherichia coli* strain BL-21 and purified as described previously (Woods *et al*, 2002).

Immunoprecipitations and pull-downs

Cells were grown to 90% confluence, serum-starved for 30 min and treated with PDGF (10 ng/ml) for 10 min with or without the inclusion of primaquine (0.6 mM) where appropriate. Primaquine was added 5 min following PDGF addition and 5 min prior to lysis. Following this, cells were washed twice in ice-cold PBS, lysed and subjected to immunoprecipitation or pull-downs as described previously (Roberts *et al*, 2003).

Microtitre ELISA

GST-integrin cytodomain fusion proteins were bound at saturating concentrations to wells of microtitre plates (Immunol. 2; Dynatech Labs, Chantilly, VI) in 0.05 M Na₂CO₃ pH 9.6 at 4°C and the wells were blocked with PBS containing 0.1% (v/v) Tween 20 (PBS-T). For detection of binding to PKD1, lysates of NIH 3T3 fibroblasts (50 μ l) were prepared as described above for immunoprecipitation, and were added to the wells and incubated for 1 h. After three washes with PBS-T, PKD1 was detected by serial incubations with rabbit anti-PKD and HRP-conjugated anti-rabbit IgG, followed by chromogenic reaction with ortho-phenylenediamine. For measurement of binding to talin, 50 μ l of PBS-T containing biotinylated talin-FERM domain (residues 86–410) at 50 μ M (a generous gift from Prof. DR Critchley and Mr BC Patel, University of Leicester) was added to the blocked microtitre wells and incubated at 4°C overnight. Bound talin-FERM domain was detected by incubation with streptavidin-conjugated HRP in PBS-T containing 1% (w/v) BSA for 1 h at 4°C.

Iodixanol gradient centrifugation

Cells were grown to 60% confluence, lysed and fractionated on a linear gradient of 10–40% iodixanol as described previously (Woods *et al*, 2002). Proteins were precipitated from the gradient fractions with 20% TCA and the migration positions of PKD1, β 3 integrin and

markers of intracellular compartments were determined by Western blotting with antibodies against the following markers: calreticulin (ER), Rab11 and Rab11 (endosomes) and biotinylated cell surface proteins (plasma membrane).

Integrin recycling assay and immunofluorescence

These were performed as described previously by Roberts *et al* (2001, 2003).

Cell migration assay

NIH 3T3 fibroblasts were transfected with $\mu 6\text{pro-CON}$ or $\mu 6\text{pro-PKD1}$ using the Amaxa Nucleofector, plated on 3 cm tissue culture plates and allowed to grow to confluence over 48 h. Using a 1 mm thick pipette tip, crosses were scraped into the confluent monolayer. The cells were washed with PBS and allowed to migrate in DMEM supplemented with 1% (v/v) FCS and 10 ng/ml PDGF-BB at 37°C. Photographs of the wounds were taken at 0, 2, 4, 6, 8, 10, 18 and 24 h post-wounding. Wound closure was determined by quantification of these images using the 'NIH image' software.

Cell spreading assays

Glass coverslips were coated overnight at 4°C with fibronectin (Sigma, F-1141) at a concentration of 10 $\mu\text{g/ml}$ and then blocked

with 2% (w/v) BSA. Cells were transfected with $\alpha\text{v}\beta 3$ or $\alpha\text{v}\beta 3$ with mutated $\beta 3$ subunits in conjunction with the $\mu 6\text{pro}$ RNAi vectors or GFP-PKD constructs. At 24 h following transfection, the cells were harvested by trypsinisation and collected by centrifugation in the presence of 20 $\mu\text{g/ml}$ soyabean trypsin inhibitor. Cell suspensions were added to ligand-coated coverslips in serum-free DMEM containing 10 ng/ml PDGF-BB for 45 min or in DMEM containing 10% (v/v) FCS for 24 h. Attached cells were fixed and examined by immunofluorescence as described above.

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