PKC α regulates β 1 integrin-dependent cell motility through association and control of integrin traffic

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Protein kinase C (PKC) has been implicated in integrin-mediated spreading and migration. In mammary epithelial cells there is a partial co-localization between β 1 integrin and PKC α . This reflects complexes between these proteins as demonstrated by fluorescence resonance energy transfer (FRET) monitored by fluorescence lifetime imaging microscopy and also by coprecipitation. Constitutive complexes are observed for the intact PKC α and also form with the regulatory domain in an activation-dependent manner. Expression of PKC α causes upregulation of β 1 integrin on the cell surface, whereas stimulation of PKC induces internalization of β 1 integrin. The integrin initially traffics to an endosomal compartment in a Ca²⁺/PI 3-kinase/dynamin I-dependent manner and subsequently enters an endocytic recycling pathway. This induction of endocytosis by PKC α is a function of activity and is not observed for the regulatory domain. PKC α , but not PKC α regulatory domain expression stimulates migration on β 1 integrin substrates. This PKC α -enhanced migratory response is inhibited by blockade of endocytosis.

Keywords: endocytosis/FRET/GFP/integrin/migration/
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Introduction

Various integrin receptors for extracellular matrix (ECM) have been implicated in providing proliferative and cell survival signals (Meredith *et al.*, 1993; Boudreau *et al.*, 1995; Crouch *et al.*, 1996; Frisch *et al.*, 1996a,b; Malik and Parsons, 1996; Udagawa and McIntyre, 1996; Wary *et al.*, 1996; Ng *et al.*, 1997; Bourdoulous *et al.*, 1998) as well as mediating cell migration towards specific ECM proteins and soluble stimulants/growth factors (Klemke *et al.*, 1994; Yun *et al.*, 1996; Hauenberger *et al.*, 1997; Rabinovitz and Mercurio, 1997; Schneller *et al.*, 1997; Meng and Lowell, 1998; Rigot *et al.*, 1998; Saka *et al.*, 1998; Werr *et al.*, 1998). Largely through pharmacological studies, protein kinase C (PKC) has been implicated as a key molecule involved in integrin-mediated cell spreading

and migration (Woods and Couchman, 1992; Klemke *et al.*, 1994; Yebra *et al.*, 1995; Timar *et al.*, 1996). For example, the enhanced motility of T-cells due to β 1 integrin crosslinking can be blocked by a prior incubation with the PKC effector site inhibitor calphostin C (Hauenberger *et al.*, 1997). Similarly, incubation of colon carcinoma cells with the phorbol ester tetradecanoyl phorbol acetate (TPA) for 5 h at a concentration that activates but does not cause PKC down-regulation increases their haptotactic response to fibronectin, collagen, laminin and vitronectin (Rigot *et al.*, 1998).

The extent of ligand–receptor interaction and the outside-in signal that ensues are generally dependent upon the ectodomain conformation (affinity) and clustering/oligomerization (avidity) of the receptor as well as the subsequent processing of the ligand-bound receptor by the cell (Bazzoni and Hemler, 1998). β 1 integrin seems to exist in a dynamic conformational equilibrium between high and low affinity states for ligand binding; both states display bivalent cation dependence and can be modulated by various inhibitory and stimulatory antibodies (reviewed by Humphries, 1996). Inhibitory antibodies appear to function as allosteric inhibitors of ligand engagement (Fogerty *et al.*, 1990; Mould *et al.*, 1996). An example of a stimulatory antibody directed against the integrin β 1 subunit is the mAb 12G10 which recognizes a conformational epitope within the putative β A domain expressed only in the ligand-competent and ligand-occupied receptor conformers (Mould *et al.*, 1995, 1998). Stimulatory antibodies like 12G10 therefore appear to function by stabilizing signalling conformations of the integrin. To some extent, inhibitory antibodies, which recognize ligand attenuated epitopes, and stimulatory antibodies, which recognize ligand-stabilized epitopes, are the converse of each other. The notion that the ectodomain conformation of β 1 integrins can be modified by changes to the intracellular portions of the receptors (so-called ‘inside-out’ signalling) (Crommie and Hemler, 1998; Mastrangelo *et al.*, 1999) is supported by the finding that transfectants expressing a splice variant of β 1 integrin known as β 1B, which lacks both NPXY motifs in two distal cytoplasmic sites (cyto-2 and cyto-3) (Retta *et al.*, 1998), or a dominant negative β 1 cytoplasmic domain–IL2R chimera (Mastrangelo *et al.*, 1999), have substantially reduced activated integrin on the cell surface, as recognized by the mAb 12G10, while the total surface β 1 integrin expression remains the same. Truncation of specific residues in the cytoplasmic domain of the α v subunit also has an impact on the conformation and ligand binding of α v β 3 integrin (Filardo and Cheresh, 1994).

Little is known about the life cycle of β 1 integrin beyond activation and ligand binding. By immunofluorescence, the bulk of ECM-interacting integrins (α 6 β 4, β 1, α IIb β 3 and α V β 3) at steady state appear to reside in an intracellular

vesicular compartment which is at present uncharacterized (Lawson and Maxfield, 1995; Rabinovitz and Mercurio, 1997; Miranti *et al.*, 1998). The distribution and redistribution of integrins through various processes such as surface diffusion, internalization and recycling to the leading front, as well as ripping release from the cell rear have been thought to be involved in the propagation of directional cell movement (Lawson and Maxfield, 1995; reviewed by Lauffenburger and Horwitz, 1996; Sanchez-Madrid and del Pozo, 1999). Nevertheless, the control of the various stages of the integrin receptor life cycle with respect to its activation, internalization and subsequent intracellular trafficking is poorly understood.

The activities of various protein serine/threonine kinases such as protein kinase A, β -adrenergic receptor kinases and PKC have previously been shown to influence the endocytosis, trafficking and hence desensitization/resensitization of receptors (Hausdorff *et al.*, 1990; Inglese *et al.*, 1993; Lefkowitz, 1993; Shih and Malbon, 1996). In the present study we have found PKC α to be an important cytoplasmic component that interacts physically with activated β 1 integrin and regulates its exocytosis to the plasma membrane. It is also established that PKC α controls internalization through a Ca²⁺- and phosphatidylinositol 3-kinase (PI3K)-dependent, dynamin I-controlled endocytic pathway. Furthermore, PKC α -induced upregulation of integrin-dependent cell migration was found to be blocked under conditions that prevented internalization of the receptor complex, identifying a critical role for PKC α in dynamic control of integrin function.

Results

PKC α associates with activated β 1 integrin which traffics through an endosomal recycling compartment

The antibodies employed in this study, 12G10 and mAb13, distinguish at least two major sub-populations of integrins: the 12G10+ population represents activated and ligand-occupied integrins and the mAb13+ population represents unoccupied integrins (Mould *et al.*, 1995, 1998). Consistent with their ability to detect these different conformers, immunocytochemical analysis of 12G10 and mAb13 epitope expression in MCF7 cells revealed overlapping but distinct compartments (Figure 1A). For the purposes of discussion, the 12G10-positive population is referred to as activated, while the mAb13-positive population is referred to as ligand unoccupied. The 8E3 monoclonal is a pan- β 1 integrin antibody.

To investigate the effect of PKC α expression on β 1 integrin we first determined the localization of β 1 integrin in relation to that of green fluorescent protein (GFP)-PKC α . In untreated MCF-7 cells activated β 1 integrin was found predominantly in a perinuclear/pericentriolar compartment with tubulovesicular structures distributed through the cytoplasm (Figure 1B). There was also some plasma membrane staining with some cells displaying a concentration at extended filopodia (see arrows, Figure 1B). This localization compared with a punctate distribution of GFP-PKC α that only partly overlapped with the β 1 integrin compartments. Treatment of cells with the PKC activator TPA caused the redistribution of PKC α to the plasma membrane and to a perinuclear region (two examples are

Fig. 1. Co-localization of PKC α with activated (12G10-positive) β 1 integrin. (A) Two populations of β 1 integrin are observed with the monoclonal antibodies 13 and 12G10. (B) Partial co-localization of intracellular activated (12G10-positive) β 1 integrin with GFP-PKC α . Arrows in the right hand panel show the localization of activated integrin receptors in the filopodia of MCF7 cells. mAb13 is an anti- β 1 integrin antibody that detects the ligand-unoccupied conformer. (C) After TPA (400 nM) stimulation for 20 min, both GFP-PKC α and 12G10-positive integrin accumulate at the plasma membrane and then internalize into distinct perinuclear compartments.

shown in Figure 1C). Coincident with this, the β 1 integrin also redistributed to the plasma membrane and to a distinct perinuclear compartment. Consequently, the main site of co-localization between the two proteins following TPA stimulation of PKC α is at the plasma membrane.

The location of the β 1 integrin was investigated using markers to various defined compartments (Figure 2A and B). The lack of any apparent co-localization with giantin, p63, Rab-7 and β -COP indicates that the perinuclear/pericentriolar structure is not Golgi, endoplasmic reticulum or the intermediate compartment. Treatment with brefeldin A (5 μ g/ml) at 37°C for 20 min did not cause redistribution or dispersal of the integrin-containing structures, consistent with a non-Golgi localization (data not shown). In breast carcinoma cells such as MCF-7 and MDA-MB-231, the steady-state distribution of activated β 1 integrin was, however, found to co-localize with that of both the endogenous transferrin receptor and fibronectin (Figure 2A). Incubation of cells at 20°C for 30 min led

A

B

Fig. 2. Identification of the steady-state compartment of activated $\beta 1$ integrin in MCF-7 cells as the endocytic recycling compartment. **(A)** The lack of co-localization between 12G10–Cy3.5-positive $\beta 1$ integrin (red) and fluorescent staining (green) with polyclonal antibodies against giantin (Golgi complex), p63 (endoplasmic reticulum), Rab-7 (late endosomes) or β -COP (Golgi complex), indicates that the perinuclear/pericentriolar structure is not Golgi, endoplasmic reticulum or the intermediate compartment. The steady-state distribution of activated $\beta 1$ integrin is, however, found to co-localize extensively with that of both endogenous transferrin receptor and fibronectin. The bottom panels show the effects of low temperature treatment or nocodazole on the steady-state distribution of 12G10-positive integrin. **(B)** The uptake and intracellular trafficking of both $\beta 1$ integrin and the transferrin receptor were followed using Cy3.5-labelled 12G10 and FITC–Tf. MCF-7 cells were pulsed with 12G10 and Tf and then the labelling chased for the times indicated. Refer to Materials and methods for a more detailed description.

to a dramatic redistribution of activated $\beta 1$ integrin to the plasma membrane (Figure 2A). However, treatment with nocodazole (5 μ g/ml) at 37°C for 2 h resulted in the

dispersal of these structures to form numerous, punctate vesicles, which were smaller and less tubular than the integrin-containing vesicles in untreated cells. Plasma

membrane staining by 12G10 was also reduced in nocodazole-treated cells.

This behaviour of $\beta 1$ integrin is reminiscent of that described for recycling receptors (Gruenberg and Maxfield, 1995). To assess this directly, we compared the localization of $\beta 1$ integrin with that of the transferrin receptor, which is known to reside within the pericentriolar, endocytic recycling compartment. Localization of these proteins was detected in parallel through the uptake and intracellular trafficking of Cy3.5-labelled 12G10 and FITC-transferrin (Tf), following pulse-chase labelling of MCF-7 cells. During the initial 10 min of chase at 37°C, both 12G10–Cy3.5 and FITC–Tf were found predominantly at the plasma membrane and juxta-membrane vesicular structures (Figure 2B). After 20 min, these labelled structures were found to concentrate in a perinuclear location. The near-complete co-localization of both 12G10–Cy3.5 and FITC–Tf throughout the entire time course of pulse-chase suggests that both activated integrin and transferrin receptors traffic through a similar endocytic, recycling compartment.

The precise localization of $\beta 1$ integrin was examined by immunoelectron microscopy after staining with anti- $\beta 1$ integrin mAbs followed by rabbit anti-mouse

IgG1/protein A–gold. Of the two breast carcinoma cell lines that were investigated, MCF-7 and MDA-MB-231, the latter gave a more distinct staining with the pan- $\beta 1$ integrin antibody 8E3 probably due to a difference in the levels of $\beta 1$ integrin expression between the two cell lines (Figure 3A). Staining was found mainly at the filopodial extensions of the plasma membrane as well as in large intracellular vesicular structures, which can be morphologically classified as multivesicular bodies (MVBs). In contrast, there was no detectable labelling of other membranous structures including the mitochondria, Golgi, *trans*-Golgi, endoplasmic reticulum and the nucleus. Figure 3A (upper panel) shows the corresponding immunofluorescence image of MDA-MB-231 stained with 8E3 (total integrin pool). As for MCF-7 cells, MDA-MB-231 cells showed co-localization of 12G10-positive integrin with the transferrin receptor (Figure 3B). As shown, this recycling compartment was also positive for the marker Rab11. By immunogold-electron microscope (EM) staining, the activated (12G10-immunoreactive) integrin pool was also localized to the filopodia and endosomes within the MVBs. The staining of 12G10 immunogold labelling was specific but weak compared with that of 8E3.

Because of the partial overlap in localization of

GFP-PKC α and activated β 1 integrin we determined whether these proteins in fact form a complex. To assess this possibility *in situ*, we employed fluorescence lifetime imaging microscopy (FLIM) to determine the extent of fluorescence resonance energy transfer (FRET) between the GFP-PKC α (donor) and β 1 integrin (12G10-Cy3.5 acceptor). The spatial separation (R_0) between GFP and Cy3.5 at which the resonance energy transfer efficiency is 50% is 5.7 nm. Detectable energy transfer can, however, occur up to 9 nm (Ng *et al.*, 1999). FRET results in a shortening of the GFP (donor) fluorescence lifetime which

is measured by two independent parameters, the phase shift (τ_p) and relative modulation depth (τ_m) (reviewed by Bastiaens and Squire, 1999; Ng *et al.*, 1999). A detailed description of these parameters and the biophysical principles of FLIM is, however, beyond the scope of this article and can be found in the aforementioned review (Bastiaens and Squire, 1999). For the interpretation of the results, it is taken that the β 1 integrin is present in excess of the GFP-PKC α , i.e. it is not limiting for FRET. This is shown here by the finding that there is no correlation between the intensity of integrin staining and the

Fig. 3. Concentration of β 1 integrin in endosomal vesicles within multivesicular bodies (mvb) and on filopodial extensions at the plasma membrane. (A) Immunofluorescence staining of β 1 integrin in MDA-MB-231 cells by the pan- β 1 integrin mAb 8E3 is shown in the upper panel, alongside a light microscope view. In the lower panels, immunoelectron micrographs are shown, revealing the localization of β 1 integrin to endosomes within multivesicular bodies and at filopodia (8E3 was used at 100 μ g/ml). (B) MDA-MB-231 cells were stained as in (A), but with 12G10 (used at 500 μ g/ml). The immunofluorescence images confirm that in MDA-MB-231 cells there is co-localization between 12G10-positive β 1 integrin and endogenous transferrin receptor (green) as well as Rab11 (a marker of endocytic recycling compartment) in perinuclear vesicular structures.

Fig. 4. PKC α - β 1 integrin association in MCF7 cells detected by FLIM. (A) MCF7 cells were transiently transfected with a GFP-tagged PKC α construct, stimulated with TPA (400 nM) and fixed after 20 min of incubation at 37°C. Cells were then either left as controls (no 12G10-Cy3.5) or stained with mAb 12G10 (12G10-Cy3.5) or (B) mAb13 (mAb13-Cy3.5). The fluorescence images from the donor (GFP-PKC α WT) and acceptor (β 1 integrin) are shown. Eff represents the FRET efficiency pseudocolour scale which only applies to the +mAb lifetime maps ($\text{Eff} = 1 - \tau_{da}/\tau_d$, where τ_{da} is the lifetime map of the donor in the presence of acceptor and τ_d is the average lifetime of the donor in the absence of acceptor). τ is the average of τ_p and τ_m and its pseudocolour scale applies to all four lifetime maps. The cumulative lifetimes of GFP-PKC α alone and that measured in the presence of the acceptor fluorophore are plotted on the 2D histograms in the lowest panels of both (A) and (B). The two distinct lifetime populations on the 2D histograms in (B) illustrate the cell-to-cell variability of the relatively inefficient PKC α -ligand-unoccupied (mAb13-positive) integrin interaction as measured by FLIM as described in the Results.

Fig. 5. PKC α regulatory domain- β 1 integrin association in MCF7 cells detected by FLIM. MCF7 cells were transiently transfected with a GFP-tagged PKC α regulatory (reg.) domain construct (this includes the variable domain V3), stimulated with TPA (400 nM) and fixed after 20 min of incubation at 37°C, then either left as controls or stained with mAb 12G10 (A, mAb 12G10-Cy3.5) or mAb13 (B, mAb13-Cy3.5). The fluorescence images from the donor (GFP-PKC α WT) and acceptor (β 1 integrin) are shown. Eff represents the FRET efficiency. For derivation of Eff, see Figure 4 legend. The Eff pseudocolour scale only applies to the +mAb lifetime maps whereas τ is the average of τ_p and τ_m and its pseudocolour scale applies to all four lifetime maps. The cumulative lifetimes of GFP-PKC α reg. domain alone and that measured in the presence of the acceptor fluorophore are plotted on the 2D histograms in the lowest panel. The distinct lifetime populations in the post-TPA (12G10-Cy3.5) (A) and pre-TPA (mAb13-Cy3.5) (B) 2D histograms illustrate the cell-to-cell variability of the FRET efficiency. The majority of cells (five of seven) in the pre-TPA (mAb13-Cy3.5) panel in (B) do, however, show a significant degree of protein-protein interaction manifested by a reduction in lifetime compared with the no antibody control.

occurrence of FRET (see for example the 12G10 direct images and the lifetime images in Figure 4). GFP-PKC α lifetime measurements for unstained, transfected MCF-7 cells showed a fairly uniform average lifetime [$(\tau_m + \tau_p)/2 = \langle \tau \rangle$] (Figure 4A). When these cells were stained additionally with Cy3.5-labelled 12G10 (12G10-Cy3.5) post-fixation, it was evident that τ for GFP was decreased in the interior of the cell with a punctate pattern of unaltered GFP-PKC α lifetime at the cell periphery (Figure 4A). Thus PKC α is in part complexed to this population of β 1 integrin. Upon TPA treatment for 20 min, the extent of complex formation is increased with a further reduction in the GFP-PKC α lifetime within the cell interior, where up to 44% FRET efficiency was detected. Owing to the non-confocal nature of the images, a direct correlation between co-localization and FRET between PKC α and the 12G10-reactive β 1 integrin in the same juxta-membrane vesicular structures after TPA stimulation is more readily observed near the cell margins. In the centre of the cells, the lifetime presented represents the average of donor lifetimes at both the plasma membrane and intracellular vesicles throughout the whole depth of the cell, making it harder to correlate the co-association and FRET data visually. The specific changes in τ_m and τ_p are shown graphically in the bottom panels, illustrating the decreases in both modulation (m) and phase (p) lifetimes.

The demonstration of FRET between GFP-PKC α and activated β 1 integrin provides direct evidence of complex formation *in vivo*. Next, we addressed the question of whether the formation of the PKC α - β 1 integrin complex is conformation dependent by examining the interaction between GFP-PKC α (donor) and the subset of β 1 integrin which is immunoreactive with the Cy3.5-labelled mAb13 (acceptor), i.e. the ligand-unoccupied population (Mould *et al.*, 1996). FRET was observable in some cells as illustrated in Figure 4B. However, while FRET from GFP-PKC α to the 12G10-recognized integrin was uniformly observed among all the fields of cells studied, only a minority of cells (two of seven and four of 12 before and after TPA treatment, respectively) demonstrated a significant interaction between PKC α and the mAb13-positive integrin, hence the heterogeneity of lifetime distribution in the 2D histogram. It is evident that PKC activation with TPA does not alter the extent of complex formation with this integrin population.

In order to assess the requirements for PKC α - β 1 integrin interaction, a GFP-PKC α regulatory domain construct (GFP-PKC α^{reg}) was employed. In the absence of any PKC α stimulus, the lifetime of the GFP-PKC α^{reg} is not influenced by staining fixed cells with 12G10-Cy3.5 (Figure 5A). Thus the regulatory domain itself has little constitutive capacity to interact with activated β 1 integrin. However, on TPA treatment, the GFP-PKC α^{reg} becomes complexed to the activated β 1 integrin. In contrast, Figure 5B shows interaction between GFP-PKC α^{reg} and the population of ligand-unoccupied β 1 integrin. The extent of this interaction is reduced upon TPA treatment, as the regulatory domain associates increasingly with the 12G10-positive conformer (see Figure 5A).

To corroborate these findings, we determined whether a β 1 integrin-PKC α complex could be demonstrated by co-immunoprecipitation. Immunoprecipitation of the full-

Fig. 6. *In vivo* and *in vitro* association of full-length PKC α or its regulatory domain with β 1 integrin. (A) Coprecipitation of full-length PKC α or its regulatory domain (RD with the variable domain V3) with endogenous MCF-7 cell β 1 integrin detected by mAb13. MC5 is a murine mAb that recognizes an epitope within the V3 region of PKC α . The upper and lower panels show the presence of integrin and PKC α in the immunocomplexes derived from control cells (lanes 1 and 3) or cells pretreated with TPA (lanes 2 and 4). (B) *In vitro* integrin pull-down by immunoprecipitates containing the PKC α regulatory domain (refer to Materials and methods). The M4 antibody is specific for the PKC α regulatory domain. The upper and lower panels show the presence of integrin and GFP-PKC α regulatory domain (RD) in the complexes derived from GFP-PKC α RD incubation in the absence of extract (lane 5) or extracts from control cells (lane 6) or cells transfected with β 1 integrin (lane 7).

length PKC α or its regulatory domain, with the antibody MC5 and Western analysis with Mab13 reveals that β 1 integrin can be co-immunoprecipitated with PKC (Figure 6). The coprecipitation of β 1 integrin with PKC α prior to activation is consistent with the complex formation (PKC α -12G10 and to a lesser extent, PKC α -mAb13) evinced by FLIM. Based upon the FLIM data, the β 1 integrin that is coprecipitated with GFP-PKC α^{reg} is presumably in the ligand-unoccupied conformation. Interestingly, coprecipitation may occur in part through post-extraction interaction of these proteins, since the regulatory domain can interact with β 1 integrin in cell extracts. Thus a pull-down using immobilized PKC α^{reg} can coprecipitate β 1 integrin (Figure 6). This observation indicates that PKC α^{reg} contains a region sufficient for β 1 integrin binding.

PKC α regulates the cellular distribution of β 1 integrin

To determine the influence of PKC α on cell surface expression of β 1 integrin, fluorescence-activated cell sorting (FACS) analysis was performed (Figure 7). A comparison between GFP-PKC α and empty vector-transfected cells showed that there was an increase in the cell surface expression of β 1 integrin associated with PKC α overexpression. This effect was most evident in comparing the mean fluorescent intensities for vector- (61 arbitrary

Fig. 7. Regulation of the cell surface expression and cycling of $\beta 1$ integrin (and its activated subset) by PKC α . (**A** and **B**) Overexpression of either the full-length PKC α (GFP-PKC α) or its regulatory domain with the variable region V3 (RD) causes an upregulation of the total (8E3-positive) cell surface $\beta 1$ integrin expression (with a greater increase in the 12G10-positive, activated, subset) compared with transfection with control vector alone. (**C**) The effect of TPA (400 nM) treatment (20 min at 37°C) on surface integrin expression is shown. The upregulation of 12G10-positive integrin on the surface of GFP-PKC α -transfected cells was initially detected in cells FACSsorted according to the GFP fluorescence (Figure 7A) and subsequently confirmed by two-colour FACS (B and C). In contrast, transfection with the CD2-PKM domain (catalytic domain of PKC α fused to the ectodomain of CD2) does not upregulate $\beta 1$ integrin expression. The negative cut-off for fluorescence was determined by staining cells with an IgG1 isotype control followed by an anti-mouse Ig-phycoerythrin (PE) conjugate.

fluorescence units) and PKC α - (135 arbitrary fluorescence units) transfected cells. The upregulation of 12G10-positive integrin on the surface of GFP-PKC α -transfected cells was initially detected in cells FACSsorted according to the GFP fluorescence (Figure 7A) and subsequently confirmed by two-colour FACS analysis (GFP versus surface labelling by an anti- $\beta 1$ integrin antibody) on whole cell populations in more than three independent experiments. The increase in cell surface expression of $\beta 1$ integrin was evident on detection with either 12G10 (activated subset) or 8E3 (total pool). The percentage of $\beta 1$ integrin molecules that were 12G10 positive was increased in GFP-PKC α -expressing cells compared with vector controls from 65% (34/52) to 81% (67/83), indicating that there is also an increase in activated $\beta 1$ integrin compared with

the bulk population (Figure 7C). Transient transfection with GFP-PKC α^{reg} increased the proportion of $\beta 1$ integrin that was 12G10 positive to 74% (40/54) from 57%, in the vector-transfected control (21/37), along with a moderate increase of total cell surface integrin expression (Figure 7B). The notion that activated $\beta 1$ integrin expression is influenced by the regulatory domain of PKC α , is further supported by the lack of any apparent change in 12G10 expression when a PKC α construct without the regulatory domain (CD2-PKM) is expressed (compare with vector-only profiles).

Under all conditions, TPA treatment of MCF-7 cells caused a redistribution of $\beta 1$ integrin from the cell surface. Thus treatment led to a modest decrease in 8E3 staining (52 to 41% and 83 to 52% in GFP and GFP-PKC α -

A

B

expressing cells, respectively) and a greater concomitant effect on 12G10-positive $\beta 1$ integrin (2- and 2.4-fold decreases in GFP and GFP-PKC α -positive cells, respectively). The greater change in the 12G10-positive $\beta 1$ integrin subset relative to the total pool (8E3-positive) indicates that the active conformer is removed more readily from the cell surface.

To determine whether 12G10-positive $\beta 1$ integrin was internalized following PKC activation, the uptake of Cy5-labelled fibronectin (FN-Cy5) into GFP-PKC α -expressing cells was monitored by immunofluorescence after a 20-min pulse labelling at 37°C in the presence of 400 nM TPA, followed by cell fixation and antibody (12G10-Cy3.5) staining. TPA treatment of GFP-PKC α -expressing cells led to a time-dependent relocation of both GFP-PKC α and 12G10-positive $\beta 1$ integrin. Initial movement was to the cell surface, a process taking 5–10 min (Figure 8A). On further exposure (20 min) internalization occurred and the distinct perinuclear locations of the $\beta 1$ integrin and PKC α became evident. Throughout the time course of the experiment, FN-Cy5 taken up by the cell was co-localized with the activated integrin (an example is shown in Figure 8B, left upper panel). The temporal behaviour varied within populations of unsynchronized cells; however, the sequence of events and patterns of responses are typical. Following TPA treatment, all cells become rounded up, as evident from the Z-series projection (Figure 8A). Evidence that the $\beta 1$ integrin was internalized in response to TPA was provided by the finding that an extensive and time-dependent uptake of fibronectin was observed (Figure 8A). This occurred in GFP-PKC α -expressing cells and was not seen at this level of detection in untransfected cells (an example is shown in Figure 8B, left upper panel).

The PKC α -dependent uptake of fibronectin was inhibited by the PI3K inhibitor LY294002, by chelation of cytosolic Ca²⁺ with BAPTA or by a dominant negative (K44A) mutant of dynamin I (Figure 8B). Furthermore, it was not supported by GFP-PKC α^{reg} despite this protein's ability to form a stable association with $\beta 1$ integrin upon TPA treatment *in vivo* (see above). Similarly, a PKC α kinase-dead mutant (PKC $\alpha^{\text{kin-}}$) was unable to promote fibronectin uptake. Hence the PKC α -driven internalization of activated integrin receptors required its full catalytic potential. As observed for the 12G10-Cy3.5-positive $\beta 1$ integrin staining (Figure 2B), in GFP-PKC α -expressing cells, FN-Cy5 trafficked through the same endocytic compartment as FITC-Tf following its internalization. Figure 8B shows the endocytic vesicles which contained both ligands after a 20-min pulse-chase at 37°C in the presence of 400 nM TPA, followed by cell fixation.

GFP-PKC α expression promotes $\beta 1$ integrin-dependent migration

The evidence presented above indicates that PKC α is recruited to $\beta 1$ integrin receptor complexes. This association is in part dependent upon the regulatory domain. Conversely the cell surface 12G10-positive integrin can be internalized in a PKC α kinase activity-dependent manner. To assess how this PKC-dependent behaviour may influence integrin-dependent function, we analysed the migratory behaviour of MCF-7 cells in a modified Transwell chamber assay. GFP-PKC α was transfected into cells and the proportion of transfected cells migrating or not migrating on different matrix substrates was scored (Figure 9). Among GFP-PKC α -transfected cells, there was an enrichment of GFP-PKC α -positive cells in the migrating population when cells were plated on the $\beta 1$ integrin substrates: fibronectin (FN), laminin (Lam) and collagen (Coll). In Figure 9, the percentage of cells in the upper chamber of each transfected well was given the arbitrary value of unity while that of the migrated cells was expressed as a proportion of the non-migrating transfected percentage, for comparison of results from independent experiments. In a typical experiment, 6% (SD 2%, No. of fields = 6) of cells in the upper chamber expressed GFP-PKC α while the percentage of expressors among the migrated cells in wells coated with fibronectin was enriched to 17% (SD 6%). Vitronectin is not a ligand for $\beta 1$ integrin and no enrichment of GFP-PKC α -positive cells was observed among the migrating population. One of the vitronectin receptors $\alpha v \beta 5$ is, however, present in MCF-7 cells (Meyer *et al.*, 1998). Cells transfected with GFP vector alone showed neither positive nor negative enrichment among the migrating and non-migrating populations. When the dominant negative dynamin I (K44A) construct was co-transfected with GFP-PKC α (3:1 ratio), the effect of PKC α expression on migration was blocked. This indicates that the active cycling of $\beta 1$ integrin contributes to the migratory response. Consistent with this, neither the kinase-defective PKC α nor the PKC α regulatory domain increased the ability of transfected cells to migrate.

Discussion

The results here identify an interaction between PKC α and $\beta 1$ integrin. This is established by FLIM *in vivo* and confirmed by co-immunoprecipitation of the proteins from cell extracts. This association occurs preferentially with the active conformer of $\beta 1$ integrin (12G10 positive) and is enhanced by recruitment of PKC α to membranes by TPA. The PKC α association correlates with altered

Fig. 8. PKC α -driven internalization of the fibronectin-activated integrin complex and its control. (A) Left and central panels, 12G10-Cy3.5/GFP-PKC α (in the *x-y* and *x-z* planes) show the time-course of TPA (400 nM)-induced redistribution of both activated integrin and GFP-PKC α in MCF-7 cells that have been fixed, permeabilized and stained with Cy3.5-labelled 12G10 at different time points as indicated. The *x-z* images show the change in morphology during the course of PKC activation. Right panels, FN-Cy5/GFP-PKC α (in the *x-y* plane only) shows a parallel time course of TPA-induced fibronectin (Cy5-labelled) uptake from media (refer to 'Fibronectin uptake and integrin/transferrin pulse-chase experiments' in Materials and methods). These cells are stained with 12G10-Cy3.5 after fixation and permeabilization. There is an almost complete co-localization between 12G10-Cy3.5 and FN-Cy5 staining at each of the time points (see B, left upper panel which depicts the co-localization of 12G10-Cy3.5 and FN-Cy5 staining after 20 min of TPA treatment). (B) PKC α -driven internalization of the fibronectin-activated integrin complex requires its functional catalytic domain (neither the regulatory domain nor the kinase-defective form are sufficient) and can be abrogated by PI3K inhibition (pretreatment with 10 μ M LY294002 for 20 min), chelation of cytosolic Ca²⁺ (pretreatment with 10 μ M BAPTA-AM ester for 30 min) or by a dominant negative (DN) K44A mutant of dynamin I (ratio of PKC:DN Dyn plasmids that are coinjected is 1:2).

Fig. 9. PKC α overexpression enhances haptotactic responses towards $\beta 1$ integrin substrates in MCF-7 cells. Upper panel shows an example of GFP-PKC α -transfected cells that migrated through the insert (coated with fibronectin on the underside) into the lower chamber of a Transwell (the GFP fluorescence image is on the left, while the right shows the corresponding phase image). Lower panel, the percentage of cells in the upper chamber of each transfected well was given the arbitrary value of unity while that of the migrated cells was expressed as a proportion of the non-migrating transfected percentage. Untransformed raw data were analysed by two way analysis of variance which takes into account the variability between experiments (significant differences and the corresponding p values are shown). Refer to Materials and methods for a more detailed description. Coll, collagen; FN, fibronectin; Kin-PKC α , K368M kinase-defective PKC α (Pears and Parker, 1991); Lam, laminin; PKC α /DN Dyn, cells cotransfected with GFP-PKC α and a dominant negative (K44A) mutant of dynamin I (in a 1:3 ratio); Vitro, vitronectin.

behaviour of the $\beta 1$ integrin, which is partly redistributed from its recycling vesicular compartment to the plasma membrane. Additionally, on stimulation by TPA the cell surface $\beta 1$ integrin is internalized and traffics through an endosomal compartment. The expression of PKC α leads to the enhanced $\beta 1$ integrin-dependent migration of MCF-7 cells. This response requires fully functional PKC α and is blocked under conditions that cause inhibition of the PKC α -driven $\beta 1$ integrin internalization. Thus we conclude that PKC α interacts physically with $\beta 1$ integrin to promote the movement of the receptor to the cell surface and that PKC α catalytic activity is then responsible for integrin internalization. It is this dynamic behaviour of the integrin that is required for its promotion of cell migration.

The activated integrin-ligand complex is endocytosed via a PKC α -catalysed, Ca^{2+} - and PI3K-dependent endocytic pathway. Intracellular Ca^{2+} concentration was also recently shown to affect the movement of intracellular vesicular structures containing $\alpha v\beta 3$ integrins via calcineurin (Lawson and Maxfield, 1995). More recently, Ca^{2+} was reported also to promote endosomal fusion *in vitro*

via its effect on the calmodulin-dependent kinase (Colombo *et al.*, 1997). The reason for the PI3K dependency of integrin cycling is not completely understood but phosphatidylinositol 3,4,5- P_3 has been shown to bind to the PH domain of dynamin I and consequently activate its GTPase activity *in vitro* (Barylko *et al.*, 1998). The phosphoinositide binding potential of dynamin I has been shown to be essential for the internalization of recycling proteins such as transferrin (Achiriloaie *et al.*, 1999; Vallis *et al.*, 1999). In addition, the rate of recycling of activated integrin receptors, like the transferrin receptor, may be slowed down by PI3K inhibition, or more specifically, the inhibition of phosphatidylinositol 3-kinase (Siddhanta *et al.*, 1998).

The dramatic redistribution of activated $\beta 1$ integrin to the plasma membrane after a short incubation at 20°C implies that there is continuous traffic of integrin to the cell surface which is at least partially resistant to low temperature, as well as a temperature-sensitive internalization process. In the light of its sensitivity to the dominant negative K44A dynamin I mutant (Ringerike *et al.*, 1998), we conclude that the internalization of activated $\beta 1$ integrin probably operates through clathrin-mediated endocytosis or possibly via the fission of caveolae from the plasma membrane (Oh *et al.*, 1998). The endocytosis of another recycling protein, transferrin, has also been shown to be abolished by a dominant negative mutant of dynamin I (N272) which lacks most of the GTP binding domain (Achiriloaie *et al.*, 1999). The inhibition of integrin endocytosis at 20°C suggests that this process may be mediated by the same subpopulation of clathrin-coated pits which is responsible for the internalization of other receptors, e.g. the $\beta 2$ -adrenergic receptor (Cao *et al.*, 1998). The low temperature-resistant exocytosis of activated $\beta 1$ integrin to the plasma membrane and the lack of effect of brefeldin A treatment would indicate that the main steady-state, perinuclear/pericentriolar compartment is not the *trans*-Golgi apparatus. This conclusion is confirmed by the lack of co-localization with Golgi markers.

No appreciable degree of integrin degradation was seen during the course of the cell labelling experiments with Cy5-fibronectin, in which MCF-7 cells were preincubated at 37°C with 10 $\mu\text{g}/\text{ml}$ of cycloheximide for 2–4 h (Figure 8A and B). This indicates that the internalized, activated integrin receptors might traffic through a recycling endosomal pathway rather than a late endosomal/lysosomal system (Ghosh *et al.*, 1998). Indeed there is a lack of co-localization between 12G10-positive integrin and late endosomes. The transferrin/integrin pulse-chase experiments provide definitive evidence that the ligand competent/occupied integrin traffics through the recycling endosomal compartment. By electron microscopy, the main steady-state localization of both the total (8E3-positive) and the activated (12G10-positive) integrin pools is at filopodia and in endosomes associated with MVBs. Internalized transferrin has been shown to pass through similar endosomal structures within the MVBs, which function as sorting endosomes during the early stages of their maturation (Futter *et al.*, 1996).

From the FLIM studies, it can be concluded that GFP-PKC α is recruited to an active $\beta 1$ integrin complex and that this complex formation is promoted by the stable

membrane association of GFP-PKC α , driven by its activator TPA. In the absence of TPA, GFP-PKC α can still complex activated β 1 integrin, unlike the GFP-PKC α^{reg} protein, which preferentially associates with the ligand-unoccupied integrin conformer. This difference between intact GFP-PKC α and GFP-PKC α^{reg} indicates that either the kinase domain contributes to the PKC α -activated integrin complex formation or the regulatory domain conformation is distinct in the intact protein. However, in the presence of TPA *in vivo*, the regulatory domain is sufficient for complex formation with the activated integrin to occur.

The enhanced surface expression of the 12G10-immunoreactive β 1 integrin in PKC α -overexpressing cells is at least in part due to an overall upregulation of integrin exocytosis, as shown by the pan- β 1 integrin antibody 8E3. The ligand-competent/occupied integrin conformer is, however, shown to turn over more quickly, with respect to both the exocytosis and internalization processes. This is akin to the finding that the high affinity epidermal growth factor (EGF) receptor is internalized more quickly compared with the bulk population and that the elimination of a major PKC phosphorylation site (T654) of this receptor significantly reduces its rate of internalization (Felder *et al.*, 1992). Previous studies with phorbol esters have demonstrated an upregulation of cell surface TS2/16-immunoreactive β 1 integrin, which can be abrogated by a prior incubation with nocodazole (Chun *et al.*, 1997). Nocodazole is shown here to disperse the integrin-containing vesicular structures as well as prevent the steady-state accumulation of activated integrin at the plasma membrane. The effect of microtubule depolymerization may be to destabilize/disperse the Rab11-containing recycling endosomal structures as shown previously (Casanova *et al.*, 1999). Intracellular vesicles containing β 2 integrins can also be stimulated to translocate to the cell surface by phorbol ester treatment in U937 cells (Kiley and Parker, 1997; Kiley *et al.*, 1997). The mechanism by which PKC brings about an upregulation of integrin exocytosis may be related to its ability to activate ARF-dependent PLD (Geny and Cockcroft, 1992; Whatmore *et al.*, 1996). Consistent with the effects seen here, it has been established that the regulatory domain of PKC α is sufficient for allosteric activation of PLD (Singer *et al.*, 1996).

Stimulation of the conventional and novel PKC isotypes in colon carcinoma cells increases the cell migratory response to fibronectin, collagen, laminin and vitronectin indiscriminately (Rigot *et al.*, 1998). In contrast, transient transfection experiments in this study demonstrate an enhanced haptotactic response specifically towards β 1 integrin substrates in PKC α -overexpressing MCF-7 cells. In fact, PKC α overexpression in MCF-7 cells confers a dramatic increase in random motility. Untransfected MCF-7 cells fail to migrate across the filter in a control chamber [where the undersurface of the chamber is coated with bovine serum albumin (BSA) alone] after a 36 h incubation. However, PKC α -transfected cells migrate across the BSA-coated filters and the migrating cells are found to be consistently (100%) GFP-PKC α positive (T.Ng and P.J.Parker, unpublished results). Morphologically, the loss of activated integrin from the site of attachment to cell substratum in TPA-stimulated, PKC α -

transfected MCF-7 cells is seen to be followed by distinct cell shape changes (such as cell rounding). This, coupled with the observation that activated integrin-containing structures are found to extend into the filopodia (Figure 2; Rabinovitz and Mercurio, 1997; Miranti *et al.*, 1998), provide further evidence that the active cycling of β 1 integrin may contribute to the migratory response. The structural determinants in β 1 integrin that mediate the PKC α -enhanced haptotactic responses have yet to be defined but may reside in the NPXY-containing cytoplasmic domains (Filardo *et al.*, 1995; Saka *et al.*, 1998; Sakai *et al.*, 1998).

A further physiological implication of these studies relates to the general observation that the signalling function of a growing number of surface receptors such as the β 2-AR (Daaka *et al.*, 1998) and insulin-like growth factor-1 (IGF-1) receptor (Lin *et al.*, 1998) are affected by the ability of these receptors to internalize and subsequently traffic intracellularly. For instance, the endocytosis of both β 2-AR and IGF1 receptor is clathrin mediated and inhibitable by the K44A mutant of dynamin I. A consequence of the blockade of receptor internalization is the impairment of either receptor to induce mitogen-activated protein kinase (MAPK) activation. A similar effect was obtained using a dominant negative mutant of β -arrestin 1 (S412D) to inhibit IGF-1 receptor endocytosis (Lin *et al.*, 1998). Given the very same sensitivity of the integrin internalization machinery to the dominant negative effect of K44A dynamin I, the 'outside-in' signalling responses elicited by integrin heterodimers may similarly be affected by conditions which can disrupt the normal cycling of these receptors.

Materials and methods

Cell culture and transfection

Human breast carcinoma cells (MCF-7 and MDA-MB-231 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C, in a 10% CO₂ atmosphere. In addition, the MCF-7 cultures were grown in media supplemented with insulin (10 μ g/ml). Cells were transfected using Lipofectant (Life Technologies). Transfected cells were stimulated as indicated in the text and figure legends, 48 h after transfection.

Plasmid constructs

Myc- and GFP-tagged PKC α constructs were obtained by subcloning the PKC α coding sequence into the *Eco*RI sites of a modified pCDNA3 vector (Clontech) and a pZeoSV-derived vector (Invitrogen), permitting fusion of a myc coding sequence (ATGGAGCAGAAGTCATATCGGAGGAGGACCTAGGGCCCGAATTC) and the coding sequence of GFP, respectively, at the N-terminus of PKC α . In order to fuse PKC α in-frame with the myc and GFP tags, the p-Babe vector (Alvaro *et al.*, 1997) containing human PKC α was used as a template in a PCR using 5' primer 5'-GGGAATTCCAGCAAGCTTGGTTGGGGGGGGGACC-3' (for insertion in pCDNA3) or 5'-GGGAATTCCAGCAAGCTTGGTTGGGGGGGGGACC-3' (for insertion in pZeoSV) and a 3' primer 5'-CATATCGCAGGTGTCACATTTCATCCCTTGATGGAT-3' corresponding to the 391–420 bp of the PKC α coding sequence. The 455/4 bp PKC α N-terminal amplified sequences were digested at their unique *Ban*I restriction site. The resulting 265/4 bp sequences corresponding to the N-terminal sequence of PKC α were then ligated to the C-terminal part of PKC α obtained from a combined *Ban*I and *Eco*RI digestion of the original p-Babe vector. The resulting ligation product containing the full-length sequence of PKC α was digested with *Eco*RI and ligated to the *Eco*RI site of the corresponding vectors. The integrity of the PCR amplified N-terminal sequence of PKC α was verified by sequencing. The construction of the CD2-PKM plasmid has been described previously (Garcia-Paramio *et al.*, 1998). The GFP-tagged PKC α regulatory domain (with variable domain V3) construct was kindly provided by D.Joubert (INSERM U469, Montpellier, France).

The dominant negative (K44A) mutant of dynamin I and the full-length chicken β 1 integrin plasmids were generous gifts of S.Schmidt (Scripps, La Jolla, CA) and Dr A.Horwitz (University of Illinois, Urbana, IL), respectively.

Antibodies and direct conjugation to fluorophores or protein G-Sepharose

MC5 is a murine mAb that recognizes the V3 region of PKC α (Young *et al.*, 1988). The monoclonal antibody M4 (a gift of S.Jaken, Lake Placid, NY), is directed against the PKC α regulatory domain and was used as ascites fluid. The characterization of the mouse anti-human integrin mAb 12G10, which recognizes an activation epitope within the putative A domain region has been described previously (Fogerty *et al.*, 1990; Mould *et al.*, 1995, 1998). The rat anti-human β 1 antibody mAb13 recognizes the ligand-unoccupied forms of the integrin and blocks ligand binding (Humphries, 1996; Mould *et al.*, 1996). 8E3 is a pan- β 1 integrin murine mAb. Direct conjugation of proteins/antibodies to the fluorophores Cy3 and Cy3.5/Cy5 (Amersham Life Science) was performed at pH 9 as described previously (Bastiaens and Jovin, 1998). Anti-Rab 7 polyclonal antiserum was a gift from M.Zerial (EMBL, Heidelberg). Rabbit anti-Rab11 was kindly provided by Dr S.Tooze (ICRF, London). Goat anti-transferrin receptor and anti-fibronectin polyclonal antibodies were from Santa Cruz Biotechnology. mAb MC5 was coupled to protein G-Sepharose (Pharmacia) at pH 9 (in 0.2 M sodium borate buffer) using dimethyl pimelimidate according to the manufacturer's protocol.

Immunoprecipitation, Western blotting and in vitro integrin pull-down

Myc-tagged PKC α or GFP-PKC α^{reg} was immunoprecipitated from the whole cell lysates of MCF-7 cells using 10–20 μ g of protein G-coupled MC5 as described previously (Kanner *et al.*, 1989), except that 1% (w/v) *n*-octyl β -D-glucopyranoside was used instead of NP-40. The immunoprecipitates were denatured in sample buffer (Laemmli, 1970), separated on an 8% polyacrylamide gel under reducing conditions and transferred electrophoretically to PVDF membrane. Blots were then probed with the rat anti-integrin mAb13 (2–4 μ g/ml) followed by a donkey anti-rat-HRP conjugate (Amersham) in the presence of 0.5 μ g/ml mouse IgG to block immunoreactivity with the precipitating antibody. The lower part of the same membrane was probed with MC5 or M3 (used at 2 μ g/ml and 1/1000 dilution of ascites, respectively) to detect PKC α or its regulatory domain. Incubation with primary antibodies was performed overnight at 4°C. Detection was with ECL (Amersham) according to recommended procedures.

For *in vitro* pull-down assays, immunoprecipitates containing GFP-PKC α^{reg} protein were washed thoroughly with modified RIPA buffer (Kanner *et al.*, 1989), then left to tumble overnight at 4°C in the same buffer (negative control) or in freshly lysed detergent extracts of MCF-7 cells alone or cells which had been transiently transfected with a full-length chicken β 1 integrin construct (Reszka *et al.*, 1992). The GFP-PKC α^{reg} protein complexes were then washed three times with RIPA buffer, once with Tris-saline buffer (pH 7.4) and subsequently resuspended in Laemmli sample buffer.

Fibronectin uptake and integrin/transferrin pulse-chase experiments

For fibronectin uptake studies, MCF-7 cells transiently transfected with various PKC α constructs were pretreated with cycloheximide (10 μ g/ml) for 2–4 h to inhibit the synthesis of new proteins, including integrin receptors. Cells were then incubated at 4°C with media containing 50 μ g/ml Cy5-labelled fibronectin, with or without 50 μ g/ml FITC-labelled transferrin (Sigma) for 30 min, washed with phosphate-buffered saline (PBS), then stimulated with TPA (400 nM) at 37°C for various lengths of time before paraformaldehyde fixation, permeabilization and staining with 12G10–Cy3.5. The pulse-chase experiments were performed as previously described (Mayor *et al.*, 1993). Briefly, untransfected MCF-7 cells on coverslips were serum starved for 30 min, pulsed for 5 min with Cy3.5-labelled 12G10 and FITC-Tf at 37°C, then the excess ligand/antibody was washed off with medium at 4°C. The pulsed cells were chased for various periods of time (up to 80 min) at 37°C, fixed in 2% (w/v) paraformaldehyde for 15 min at room temperature, then examined by confocal microscopy.

Immunocytochemical staining and confocal microscopy

Immunocytochemical staining was performed as described elsewhere (Kiley *et al.*, 1997) except for the following modifications. Cells were permeabilized with 0.2% (v/v) Triton X-100/PBS following fixation in 4% (w/v) paraformaldehyde. Primary antibodies were diluted 1:200 in

PBS containing 1% BSA, except for the fluorophore-conjugated antibodies which were used at 1:20–1:50. The secondary conjugates used were Cy5-conjugated donkey anti-goat IgG (1:200) and Texas Red/Cy3-conjugated donkey anti-rabbit IgG (1:400) (Jackson ImmunoResearch Laboratories, West Grove, PA). Confocal images were acquired on a confocal laser scanning microscope (model LSM 510, Carl Zeiss Inc.) equipped with a 63 $\times$ /1.4Plan-APOCHROMAT oil immersion objective. Each image represents a 2-dimensional projection of sections in the *z*-series, taken across the depth of the cell at 0.2 μ m intervals.

Immunogold electron microscopy

Cells were scraped from culture plates and fixed in 4% (w/v) paraformaldehyde for 1 h, followed by 2% (w/v) paraformaldehyde overnight at 4°C. Cells were processed and ultrathin sections were collected as described (Gorlich *et al.*, 1995). Sections were incubated in the following order: anti- β 1 integrin mAb (12G10/8E3, at 500 or 100 μ g/ml respectively), rabbit-anti mouse IgG1 (1/100), protein A-gold 10 nM particles (1/100). Antibody reagents were diluted in PBS containing 1% (w/v) BSA. After antibody labelling, sections were examined using a Jeol 1010 microscope.

FLIM measurements

A detailed description of the FLIM apparatus used for FRET determination in this work can be found elsewhere (Squire and Bastiaens, 1999). The lifetime instrument performs phase and modulation based imaging fluorimetry by microscopy. All images were taken using a Zeiss Plan-APOCHROMAT 100 $\times$ /1.4NA phase 3 oil objective and the homodyne phase sensitive images recorded at a modulation frequency of 80.218 MHz. For experiments involving the anti-integrin IgG–Cy3.5 donor/acceptor FRET pair, the donor (GFP-PKC α or GFP-PKC α^{reg}) was excited using the 488 nm line of a Argon/Krypton laser and the resultant fluorescence separated using a combination of dichroic beamsplitter (Q 505 LP; Chroma Technology Corp.) and narrow band emitter filter (BP514/10; Lys & Optik). Acceptor images (anti-integrin mAb–Cy3.5) were recorded using a 100 W mercury arc lamp (Zeiss Attoarc) as a source of sample illumination combined with a high Q Cy3 filter set (exciter: HQ 535/50, dichroic: Q 565 LP, emitter: HQ 610/75 LP; Chroma Technology Corp.).

Flow cytometric analysis and FACS sorting

Transiently transfected MCF-7 cells were detached from plates using EDTA then, without fixation, stained with either an anti- β 1 integrin mAb or mouse IgG isotype control, followed by an anti-mouse IgG–RPE conjugate. Cells were analysed on the same day by a FACScan, as previously described (Ng *et al.*, 1995). All antibody incubation and washing steps were carried out at 4°C. Cell purification by FACS sorting according to GFP fluorescence was performed using Becton Dickinson FACS Vantage.

Transwell chamber haptotactic migration assays with immunofluorescence microscopy

MCF-7 cells were transiently transfected with a vector control or various PKC α constructs. After 20 h, cells were detached from culture plates with trypsin, washed three times with serum-free medium supplemented with glutamine and 0.5% (w/v) BSA (migration buffer), then replated at 10⁶ cells/ml on to the inserts of 8 μ m pore size Transwell chambers (Costar, Cambridge, MA) according to the manufacturer's instructions. The underside of the inserts was precoated by filling the lower chamber with either 0.1% (w/v) BSA or different integrin substrates (vitronectin 5 μ g/ml; fibronectin, laminin and collagen, 10 μ g/ml) and incubation overnight at 4°C. After 20 h, cells were trypsinized from both the top and underside of the inserts and then washed three times in migration buffer. The cell suspensions obtained, along with the fluid from either the upper or lower chambers, were centrifuged and cytospins recovered on coverslips before mounting on a glass slide. The percentage of GFP-, GFP-PKC α - or GFP-PKC α^{reg} -transfected cells that had migrated through the insert was compared with that of the non-migrating population that remained in the upper chamber. For the untagged kinase-defective PKC α , immunofluorescence staining with a PKC α C-terminus-specific polyclonal antibody was performed to ascertain the percentage of transfected cells in each chamber. The cells in six or more low power fields per slide were counted with the aid of a fluorescence microscope. Data from three independent experiments were pooled and analysed by two way analysis of variance using the statistical software MINITAB.

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