The p85 subunit of phosphoinositide 3-kinase is associated with β -catenin in the cadherin-based adhesion complex

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Cell adhesion is fundamental to establishing and maintaining the discrete tissues in multicellular organisms. Adhesion must be sufficiently strong to preserve tissue architecture, whilst having the capacity to readily dissociate to permit fundamental processes, such as wound repair, to occur. However, very little is known about the signalling mechanisms involved in temporary down-regulation of cell adhesion to facilitate such processes. Cadherins are the principal mediators of cell–cell adhesion in a wide variety of tissues and species and form multi-protein complexes with cytosolic and cytoskeletal proteins to express their full adhesive capacity. In the present study we report that

the p85 subunit of phosphoinositide 3-kinase (PI 3-kinase) is associated with the cadherin-based adhesion complex in human epithelial cells. The interaction of p85 with the complex is via β -catenin. We also show that the interaction of p85 and β -catenin is direct, involves the N-terminal Src homology domain 2 of p85 and is regulated by tyrosine phosphorylation. These data suggest that PI 3-kinase may play a role in the functional regulation of the cadherin-based adhesion complex.

Key words: cell–cell adhesion, epithelia, PI 3-kinase, protein interactions.

INTRODUCTION

In epithelial tissues the cells are tightly bound together into sheets called epithelia. Epithelia form a boundary between discrete compartments within an organism and the interface between the organism and the environment. The development and maintenance of epithelia require selective cell–cell and cell–extracellular matrix adhesion. Adhesion must be strong enough to maintain tissue architecture yet be dynamic enough to permit essential processes, such as morphogenesis or cell division, to occur. Cell adhesion is achieved by a superfamily of transmembrane glycoproteins collectively known as cell adhesion molecules (CAMs), which are responsible for adhesion at a diverse range of cellular junctions (reviewed in [1]). A subfamily known as the cadherins mediate homotypic Ca^{2+} -dependent intercellular adhesion in the majority of cell types [2]. The cadherin family can be divided into four subfamilies: classical cadherins, which are responsible for cell–cell adhesion at the zonula adherens; desmosomal cadherins, which are responsible for cell–cell adhesion at the desmosomal junctions; and protocadherins and cadherin-related proteins whose functions are not fully elucidated [2]. The superfamily is structurally characterized by conserved 110-amino-acid repeat sequences (cadherin repeats) present within the extracellular domain [3,4]. Individual cadherins are differentially expressed during embryonic development and mediate intercellular interactions in distinct cell types.

The classical cadherins, which include epithelial, placental and neuronal cadherin (E-, P- and N- cadherin respectively), share a conserved structure consisting of a large ecto-domain, a single membrane-spanning region and a short cyto-domain. The adhesive capacity of cadherins depends on homotypic interaction with cadherins expressed on neighbouring cells [5,6], together

with the association of the cyto-domain with components of the actin cytoskeleton [7,8]. A family of cytoplasmic proteins called the catenins facilitates interaction of the cadherin cyto-domain with the actin cytoskeleton. β - or γ -catenin binds directly to the cadherin cyto-domain whilst α -catenin links this complex to the actin cytoskeleton [9]. The entire complex is termed a cadherin-based adherens junction and represents a focal point at which the cytoskeletons of adjacent cells are linked. The cyto-domains of the classical cadherins share a high degree of sequence similarity suggesting they perform a common function in cells.

The activation of certain cell surface receptors, such as the epidermal growth factor (EGF) receptor (EGFR) [10,11] and the hepatocyte growth factor receptor ('c-MetR') [12,13], as well as phosphorylation of components of the adherens junction [14–16], have been suggested to modulate intercellular adhesion.

Class 1A phosphatidylinositol 3-kinases (PI 3-kinases) are comprised of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110) [17,18], and generate $\text{PtdIns}(3,4,5)\text{P}_3$ in intact cells [19,20]. PI 3-kinase is activated in a phosphotyrosine-dependent manner via Src homology domain 2 (SH2 domain)-mediated recruitment to tyrosine kinase receptors or docking proteins [21,22]. SH2 domains mapped within the p85 subunit specifically recognize the phosphotyrosine binding motif pYXXM [23]. The production of $\text{PtdIns}(3,4,5)\text{P}_3$ has been suggested to be critical in regulating a wide variety of cellular processes, including mitogenesis, cell survival and secretion. Many of these processes are dependent on negative modulation of cadherin-based adhesion, therefore suggesting a possible role for PI 3-kinase in the regulation of the cadherin-based adhesion complex.

PI 3-kinase has previously been reported to associate with a component of the cadherin-based adhesion complex upon form-

Abbreviations used: 3-AT, 3-aminotriazole; DMEM, Dulbecco's modified Eagle's medium; E-cadherin, epithelial cadherin; EGF, epidermal growth factor; EGFR, EGF receptor; FCS, foetal calf serum; GSK-3 β , glycogen synthase kinase-3 β ; GST, glutathione S-transferase; MDCK, Madin–Darby canine kidney; PI 3-kinase, phosphoinositide 3-kinase; SH2 domain, Src homology domain 2; SHP1, SH2-containing inositol phosphatase-1; SPR, surface plasmon resonance; SVKs, simian virus 40-transformed human keratinocytes.

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ation of intercellular junctions in Madin–Darby canine kidney (MDCK) cells [24]. It is well established that EGF treatment of epithelial cells causes the destabilization of cadherin-based cell adhesion complexes and a consequent reduction in intercellular adhesion, leading to increased cell motility [10,11]. Furthermore, the EGFR has been reported to co-associate with the cadherin-based adhesion complex in epithelial cell lineages via a direct interaction with β -catenin [25]. Additionally, the EGFR has been demonstrated to associate with PI 3-kinase activity following activation with EGF [26,27], and p85 has been demonstrated to interact with EGFR in a phosphorylation-dependent manner [28]. In the present study we demonstrate specific, tyrosine phosphorylation-dependent binding of PI 3-kinase to β -catenin.

MATERIALS AND METHODS

Cells

Scc-12F and Ha-ras transfected Scc-12F human skin keratinocytes were kindly provided by L. S. Young (CRC Institute for Cancer Studies, University of Birmingham, U.K.). Ha-ras transfected Scc-12F cells have been previously characterized [29]. MDCK epithelial cells were kindly provided by A. J. Ridley (Ludwig Institute, London, U.K.). Simian virus 40-transformed human keratinocytes (SVKs) were kindly provided by A. B. Rickinson (CRC Institute for Cancer Studies, University of Birmingham, U.K.). HN5 human squamous cell carcinoma cells were kindly provided by GlaxoSmithKline (Stevenage, U.K.). EJ bladder carcinoma cells were obtained from the European Collection of Cell Cultures (Porton Down, U.K.). LA29 fibroblasts, containing a temperature-sensitive Src kinase, were kindly donated by M. J. Welham (Department of Pharmacology, University of Bath, U.K.). LA29 cells were cultured at the non-permissive temperature of 39.5 °C and transferred to 35 °C (permissive temperature) for 30 min for Src activation. LA29 cells have been previously characterized [30,31]. Scc-12F keratinocytes and SVKs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing glutamax, 5% (v/v) foetal calf serum (FCS) and 400 ng/ml hydrocortisone. All other lines were maintained in DMEM containing glutamax and 10% (v/v) FCS. All cell lines were cultured in a humidified incubator at 37 °C, with a fixed 5% CO₂ atmosphere.

Antibodies

Monoclonal mouse anti-(E-cadherin) was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal rabbit anti-p85 and mouse anti-phosphotyrosine (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Mouse monoclonal anti-p85 (U13) was purchased from Serotec (Kidlington, Oxford, U.K.). Rabbit anti-(β -catenin) was purchased from Sigma (Poole, Dorset U.K.) and goat polyclonal anti-EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Immunoprecipitation from cell lysates

Cells were grown in the appropriate medium until they reached 80% confluence. They were then quiesced for 48 h in DMEM containing 0.5% FCS. Cells were washed and stimulated with 100 ng/ml EGF for 10 min [in DMEM containing 20 mM Hepes (pH 7.4), 0.1% BSA and 0.0375% (w/v) sodium bicarbonate]. Whole-cell lysates were obtained by scraping cells into hypotonic lysis buffer [50 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 2 mM sodium pervanadate, 50 mM sodium fluoride, 40 μ g/ml aprotinin, 40 μ g/ml leupeptin, 25 mM benzamide, 0.1 mM PMSF and 40 mM tetrasodium

pyrophosphate] and centrifuging at 13000 g for 15 min at 4 °C. Cytosolic cell fractions were obtained by scraping confluent cell monolayers into ice-cold PBS and probe sonicating for two periods of 15 s at an intermediate setting, placing the lysates on ice between rounds of sonication. Lysates were then transferred to ultracentrifuge tubes and centrifuged at 300000 g for 15 min at 4 °C. The cytosolic fraction (supernatant) was then harvested. Protein concentration was determined using the Bio-Rad assay.

Immunoprecipitations were performed by mixing the primary antibody with 400 μ g of pre-cleared lysate protein for 1 h at 4 °C. Immunocomplexes were captured by the addition of 20 μ l of Protein G–Sepharose, which was tumbled with the lysate for 2 h at 4 °C. The antibody complexes were isolated by centrifugation, the supernatants removed and the beads washed as follows: 2 $\times$ 0.5 ml of hypotonic lysis buffer; 1 $\times$ 0.5 ml of hypotonic lysis buffer + 0.5 M NaCl; 1 $\times$ 0.5 ml of hypotonic lysis buffer + 0.5 M LiCl; and 2 $\times$ 0.5 ml of hypotonic lysis buffer. The beads were resuspended in 2 $\times$ Laemmli buffer and boiled for 5 min. Proteins were separated by SDS/PAGE and transferred on to PVDF membranes in a semi-dry transfer tank. PVDF membranes were blocked in buffer [10 mM Tris/HCl (pH 7.5), 0.1 M NaCl, and 0.05% (v/v) Tween 20] containing 5% (w/v) non-fat milk powder. Blots were probed with primary antibody and washed five times in buffer prior to incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at 25 °C. Immunoreactive bands were visualized by ECL[®].

Immunocomplex PI 3-kinase assays

Immunoprecipitations were prepared as described above and PI 3-kinase activity was measured in buffer containing sodium cholate [0.5% (w/v)] and phosphatidylinositol (2 mM). Reactions were initiated by addition of 5 μ Ci of [γ -³²P]ATP, incubated for 15 min at 37 °C prior to addition of chloroform and methanol, and ³²P-labelled phosphoinositides were extracted into the chloroform phase. The organic phase was dried *in vacuo* and separated by TLC. The radioactivity was visualized by PhosphorImager analysis (Molecular Dynamics).

Affinity purifications from cell lysates

Lysates were prepared as previously described and 400 μ g of protein was mixed with 3 μ g of purified glutathione S-transferase (GST) fusion protein or free GST for 1 h at 4 °C. Complexes were captured by incubation with GSH–Sepharose beads by mixing for 1 h at 4 °C. The supernatant was removed and the beads were washed with lysis buffer as described above. Interacting proteins were released in Laemmli buffer, separated by SDS/PAGE and subjected to Western-blot analysis.

Surface plasmon resonance (SPR)

Protein–protein interactions measured by SPR employed a BIAcore 3000 instrument and carboxymethyl dextran surfaces (CM5). Proteins were immobilized using covalent coupling, and non-specific binding was assessed using BSA. The binding of GST–p85 (1–5 μ M) to GST– β -catenin (approx. 10 ng of catenin was immobilized) was performed at flow rates of between 5 and 20 μ l of PBS/min. Surfaces could be effectively regenerated using glycine (pH 3). Data obtained were analysed using BIAevaluation 3 software.

Yeast two/three-hybrid analysis

Saccharomyces cerevisiae strain p56Ylck4.1 was generated for yeast two/three-hybrid analysis as described previously [32]. Expression of the third component, p56Lck, is regulated from

the yeast *MET3* promoter, which is repressed in the presence of exogenous methionine. DNA encoding the cytoplasmic and transmembrane portions of E-cadherin was cloned into the yeast shuttle vector pYTH9. pYTH9/E-cadherin was then introduced into strain p56Ylck4.1 by homologous recombination at the Trp locus using a standard lithium acetate/poly(ethylene glycol) yeast protocol to create yeast strain yLck4.1/E-cadherin. Successful integration was verified by PCR analysis. Full length human β -catenin [donated by M. West, GlaxoSmithKline (U.K.)] was subcloned into episomal activation (pACT) and binding (pAS) domain yeast vectors and sequenced. Full-length p85 α in pVL1393 [donated by S. Young, GlaxoSmithKline (U.K.)] was subcloned into pACT and sequenced. A pACT/p85 β construct (lacking 189 N-terminal bases) was isolated from an unrelated yeast three-hybrid screen. Yeast strain yLck4.1/Shp.SH2 [expressing the first 221 amino acids, including the tandem SH2 domains, of murine SH2-containing inositol phosphatase-1 (SHP1)] is as described previously [33]. Competent yeast cells were prepared and transformed using standard yeast protocols [34]. Selection for two/three-hybrid positive yeast was undertaken by growing cells on minimal medium supplemented with yeast nitrogen base, glucose and appropriate amino acids. Interactions were assessed using the standard *Escherichia coli* *LacZ* and yeast *HIS3* reporter genes.

Yeast growth reporter assay

Transformed yeast were plated on to minimal growth medium supplemented with 10 mM 3-aminotriazole (3-AT) in the presence or absence of methionine and left to grow for 3–4 days at 30 °C.

Quantitative β -galactosidase activity assays

Yeast cultures were grown to mid-exponential phase and harvested by centrifugation, and the cells were subsequently lysed in 200 μ l of lysis buffer [0.1 M Tris/HCl (pH 7.5)/0.05% (v/v) Triton X-100] by repeated freeze–thawing in liquid nitrogen. β -Galactosidase activity was assayed as described previously [35].

RESULTS

Two epithelial cell lines, 12F human keratinocytes and MDCK cells, were used to assess the association of various components of the cadherin-based adhesion complex in response to EGF treatment. Figure 1(A) shows that EGF stimulates a rapid increase in tyrosine phosphorylation of the EGFR in both cell lines. Figure 1(B) illustrates that the regulatory subunit of a type 1A PI 3-kinase (p85) is associated with the EGFR in MDCK cells. However, the association of p85 with the EGFR was not enhanced by tyrosine phosphorylation (similar results were obtained in human keratinocytes). The EGFR does not contain a tyrosine within a consensus binding motif for p85, and our results showing the unresponsiveness of the interaction to EGF support the view that the association of p85 and the EGFR may not be direct in epithelial cells.

Using this system, we analysed the effect of EGF on the association of various components of the cadherin-based adhesion complex with p85 and the EGFR in both 12F human keratinocytes and MDCK cells. Immunoprecipitations for components of the adherens complex were prepared from epithelial cell lysates. Figures 2(A) and 2(E) illustrate that the p85 subunit of PI 3-kinase and E-cadherin are associated in both the 12F keratinocyte and MDCK epithelial cells. Figures 2(B) and 2(F) show that E-cadherin is also associated with β -catenin in both

Figure 1 EGF stimulates tyrosine phosphorylation of the EGFR in epithelial cells but does not enhance association of p85

(A) MDCK cells and the human keratinocyte cell line 12F were stimulated with EGF (100 ng/ml for 10 min). The EGFR was immunoprecipitated (IP) from the whole-cell lysates (WCL) and EGFR tyrosine phosphorylation status was assessed by Western-blot analysis using an anti-phosphotyrosine (anti-pY) antibody. (B) EGFR immunoprecipitates (IP) were prepared from MDCK cells as described in the materials and methods section and Western blotted for the p85 subunit of PI 3-kinase.

epithelial cell types. Figures 2(C) and 2(G) show that E-cadherin can also be found associated with the EGFR in epithelial cells. Figures 2(D) and 2(H) illustrate that although endogenous p85 can be immunoprecipitated with endogenous E-cadherin, purified recombinant E-cadherin cannot be used to affinity-purify p85. Figure 2 also shows that the associations between these proteins did not require activation of the EGFR.

The data in Figures 1 and 2 illustrate that a complex protein interaction network exists in epithelial cells involving EGFR, E-cadherin, β -catenin and the p85 subunit of PI 3-kinase, and support the possibility that these proteins may exist in a multi-component complex. To illustrate that such a complex may exist, Figure 3 demonstrates that the EGFR, β -catenin and E-cadherin are simultaneously associated in A431 epithelial cells.

The interaction between endogenous E-cadherin and p85 (Figure 2) may be mediated by an undetected protein present in the immunoprecipitation, such as the EGFR or β -catenin. However, the direct association of EGFR with p85 remains controversial. A previous report suggested that p85 associates with cytosolic β -catenin in ras-transformed mouse keratinocytes, suggesting a key role for ras in a p85– β -catenin interaction [36]. To investigate whether ras transformation was necessary for the interaction of β -catenin and p85 in human cells, immuno-

Figure 2 Multiple interactions between the EGFR, p85, β -catenin and E-cadherin in epithelial cells

Anti-p85 immunoprecipitates (IP) were prepared from EGF-treated (100 ng/ml; 10 min) Scc-12F (A–D) and MDCK (E–H) epithelial whole-cell lysates (WCL) and Western blotted with an anti-[E-cadherin (Ecad)] antibody. Anti-(E-cadherin) immunoprecipitates were similarly prepared and probed with anti-(β -catenin) (B and F). Anti-EGFR immunoprecipitates were probed with anti-(E-cadherin) (C and G). Affinity precipitates were prepared using GST–E-cadherin and were probed with anti-p85 (D and H). Results shown are from one experiment that is representative of two experiments.

precipitates were performed from cytosolic fractions of 12F keratinocytes, Ha-ras-transformed keratinocytes and EJ bladder carcinoma cells, and interactions were assessed by Western-blot

analysis. EJ bladder carcinoma cells are transformed with mutant Ha-ras. Figure 4 shows that p85 was able to immunoprecipitate β -catenin from all of these cell lines and that this interaction was

Figure 3 Simultaneous association of E-cadherin, EGFR and β -catenin

Whole-cell lysates (WCL) were prepared from EGF-treated A431 cells (100 ng/ml; 10 min) and E-cadherin (Ecad) was immunoprecipitated (IP). The immunoprecipitates were analysed by Western blotting, probed simultaneously with goat anti- β -catenin and goat anti-EGFR, and visualized using horseradish peroxidase-conjugated anti-goat and ECL[®].

reciprocal. The interaction of p85 and β -catenin in human keratinocytes supports the previous report in mouse keratinocytes [36]. However, the interaction between p85 and β -catenin in the human cells occurred in the absence of ras and therefore it is unlikely that the GTPase plays a direct role in the interaction in human cells.

The association of the regulatory subunit of PI 3-kinase with the β -catenin of the adherens complex suggests that the adherens complex may be a site for $\text{PtdIns}(3,4,5)\text{P}_3$ synthesis following association of the catalytic p110 subunit of PI 3-kinase with p85. We therefore assessed PI 3-kinase activity in E-cadherin and p85 immunoprecipitates from keratinocyte lysates treated with EGF. Figure 5 illustrates that E-cadherin immunoprecipitates contained very little PI kinase activity and that EGF did not stimulate this activity. Conversely, ligation of the EGFR stimulated PI 3-kinase activity in p85 immunoprecipitates by 5-fold. These results illustrate that the interaction of p85 of PI 3-kinase with proteins from the adherens complex occurs in the absence of an active catalytic subunit.

It was possible that an interaction between β -catenin and p85 was peculiar to 12-F keratinocytes and MDCK cells. Therefore lysates were prepared from additional human epithelial cell lines including HN5, two clones of SVKs (SVK1 and SVK2) and MDCK cells. Figure 6 shows that a GST fusion protein of p85

(GST-p85) affinity-purified endogenous β -catenin from cell lysates (previously cleared with GST) and, similarly, a GST fusion protein of β -catenin (GST- β -cat) affinity-purified endogenous p85. The observed heavy band was due to non-specific antibody cross-reaction.

The co-precipitation (Figures 1–3) and affinity purification (Figure 6) of p85 and β -catenin illustrated the potential for interaction within the cadherin-based adhesion complex. However, the possibility that an intermediary protein, other than ras, was involved in the association could not be completely excluded. Therefore the direct association of p85 and β -catenin was tested using two independent methods. Firstly, SPR studies were used to assess whether purified recombinant β -catenin interacted with recombinant p85 directly. GST fusion proteins were expressed in bacteria and purified by means of the affinity tag and, in some cases, gel-filtration chromatography. Figure 7(A) shows that covalent immobilization of β -catenin to a carboxymethyl dextran did not generate a surface able to bind proteins non-specifically, since recombinant GST did not interact with the surface. Similarly neither GST-c-Jun N-terminal kinase nor BSA bound to these surfaces (results not shown). Figure 7(B) illustrates that GST-p85 (1 μM) bound specifically to the β -catenin and did not interact with immobilized GST, BSA or the surface chemistry. The specificity of the interaction between β -catenin and p85 was also observed at concentrations of p85 up to 5 μM and at flow rates of 5–20 $\mu\text{l}/\text{min}$.

The second method used to evaluate direct interaction of β -catenin with p85 utilized directed yeast two- and three-hybrid analysis. In directed yeast two-hybrid analysis an interaction between two proteins results in expression of *E. coli LacZ* and yeast *HIS3* reporter genes under control of the *GAL1* promoter. When activated, the *HIS3* reporter permits yeast growth on histidine-negative media in the presence of the inhibitor 3-AT (10 mM). The *LacZ* gene encodes β -galactosidase and allows quantitative measurement of protein–protein interactions. In yeast three-hybrid analysis, phosphorylation-dependent interactions can be investigated by simultaneous expression of a third component, a tyrosine kinase. By regulating expression of a kinase using the yeast *MET3* promoter, which is repressed in the presence of exogenous methionine, phosphorylation-dependent interactions can be investigated. Phosphorylation-dependent interactions are therefore observed only in media lacking methionine. Yeast strain p56Ylck4.1 expresses full-length murine p56Lck and has been previously characterized

Figure 4 Interaction of endogenous p85 and β -catenin is independent of Ha-ras

Anti-p85 (A) and anti- β -catenin (B) immunoprecipitates (I. P.) were prepared from cytosolic fractions of Scc-12F, Ha-ras transformed Scc-12F (12F-Ras) and EJ bladder carcinoma cells (Ha-ras transformed). Anti-p85 immunoprecipitates were Western blotted with anti- β -catenin, and anti- β -catenin immunoprecipitates were probed with anti-p85. Results shown are from one experiment that is representative of two experiments.

Figure 5 E-Cadherin immunoprecipitates do not contain PI 3-kinase activity

Lysates were prepared from human keratinocytes stimulated with EGF (100 ng/ml; 10 min). E-cadherin (E-cad) and p85 were immunoprecipitated (IP) and phosphoinositide kinase activity was determined *in vitro* by quantifying production of 32 P-labelled phosphatidylinositol phosphate using PhosphorImaging. Control (Con) immunoprecipitations contained no primary antibody. The results shown are representative of three experiments.

using tyrosine phosphorylation-dependent interactions involving the T-cell receptor zeta chain [32]. To assess the protein interactions involved in the adhesion complex, the cytoplasmic domain of E-cadherin was cloned into the integrating yeast shuttle vector pYTH9 and introduced into the p56Ylck4.1 yeast genome by site-directed homologous recombination at the *trp1-901* locus to produce yeast strain p56Ylck4.1/E-cadherin. p85 and β -catenin were introduced into yeast strains p56Ylck4.1 or p56Ylck4.1/E-cadherin using episomal yeast vectors.

Figure 8(A) shows that growth of the p56Ylck4.1/E-cadherin yeast is only supported by simultaneous expression of β -catenin p56Ylck4.1/E-cadherin yeast transformed with p85 α or p85 β cannot overcome the growth inhibition provided by 3-AT (10 mM). Conversely, transformation of p56Ylck4.1 yeast with β -catenin and either p85 α or p85 β supported growth of the yeast on 3-AT indicating a positive interaction by two-hybrid analysis.

Figure 7 Direct association of p85 with β -catenin

Recombinant GST- β -catenin, recombinant GST and BSA were covalently immobilized to a CM5 (BIAcore) chip to approximately equal molarities. The binding of 1 μ M recombinant GST (A) or 1 μ M recombinant GST-p85 (B) was assessed using SPR in a BIAcore 3000 instrument. Direct interactions were observed at concentrations between 1 and 5 μ M and at flow rates of between 5 and 20 μ l/min.

Expression of p56Lck had no effect on the interaction. Quantitative analysis of the protein interactions by a liquid β -galactosidase activity assay (product of the *LacZ* reporter gene)

Figure 6 Interactions between recombinant and endogenous p85 and β -catenin can be observed in other epithelial cells

Recombinant GST- β -catenin (β -cat) or GST-p85 were incubated with lysates prepared from SVKs (SVK1 and SVK2), squamous cell carcinoma (HN5), Scc-12F and MDCK cells. GST- β -catenin affinity precipitations were Western blotted with anti-p85 antibody. GST-p85 affinity precipitations were probed with anti- β -catenin antibody. Results shown are from one experiment that is representative of two experiments.

Figure 8 Interaction between p85 and β -catenin is negatively regulated by tyrosine phosphorylation

(A) The interactions between E-cadherin, β -catenin (β -cat) and p85 were assessed in the presence (—Met) or absence (+Met) of p56Lck using the standard yeast two-hybrid growth reporter assay. (B) Shows quantification of the interactions between E-cadherin (Ecad), β -catenin and p85 using β -galactosidase activity expressed from the product of the *LacZ* reporter gene in the presence or absence of p56Lck. β -Galactosidase activity (arbitrary units) was quantified spectrophotometrically (absorbance at 420 nm) from yeast cell lysates using the chromogenic substrate *o*-nitrophenyl β -D-galactopyranoside in triplicate ($\pm$ S.D.). Results presented are means of triplicate experiments. (C) LA29 fibroblasts, expressing a temperature-sensitive src, were grown to confluence at 39.5 °C then switched to the permissive temperature of 35 °C for 30 min, and cytosolic and whole-cell lysates (WCL) were prepared. Anti-p85 immunoprecipitates were probed with anti-(β -catenin), and anti-(β -catenin) immunoprecipitates were probed with anti-p85. Results shown are from one experiment that is representative of two experiments.

is shown in Figure 8(B). Co-expression of E-cadherin and β -catenin leads to strong expression of β -galactosidase activity consistent with their well characterized association in the cadherin-based adhesion complex. This interaction was unaffected by the activity of p56Lck tyrosine kinase. E-cadherin failed to interact with either p85 α or p85 β , in support of our previous observations. Both p85 α and p85 β interacted with β -catenin leading to similar levels of expression of β -galactosidase activity. The interaction of p85 with β -catenin was decreased in the presence of p56Lck activity, suggesting that tyrosine phosphorylation negatively regulates this interaction. SHP1 has been reported to interact with p85 β in response to p56Lck activity [36]. As a control, p85 β interaction with the SH2 domains of SHP1 was entirely phosphorylation-dependent indicating the integrity of the p56Lck system. Supporting evidence for tyrosine-phosphorylation negatively regulating the p85– β -catenin interaction was obtained in LA29 fibroblasts that express a Src kinase which is activated upon temperature shift to 35 °C

(permissive temperature). Figure 8(C) indicates that the p85– β -catenin interaction is reduced, but not ablated, upon incubation at the permissive temperature, directly mirroring data obtained in the yeast system.

To further define the interaction of p85 with β -catenin, GST fusion proteins of the domains of p85 were used to affinity-purify endogenous E-cadherin or β -catenin from keratinocyte lysates. Figure 9(A) shows that interaction between p85 and β -catenin predominantly involves the N-terminal SH2 domain of p85 with a small contribution from the SH3 domain. The C-terminal SH2 domain appears to play no part in the interaction. Figure 9(B) shows that this pattern of interaction is repeated when these constructs are used to immunopurify E-cadherin from cell lysates. Since we have shown that p85 and E-cadherin do not interact directly the presence of E-cadherin in the GST–p85 domain affinity precipitates reflects the interaction of p85 with β -catenin and β -catenin association with E-cadherin. The possibility that the recombinant C-terminal SH2 domain of p85 was inactive was

Figure 9 β -catenin predominantly interacts with the N-terminal SH2 domain of p85

Recombinant GST fusion proteins expressing the SH3 domain, C-terminal SH2 (C-SH2) domain or the N-terminal SH2 (N-SH2) domain of p85 were incubated with SVK lysates. The fusion proteins were affinity purified using immobilized GSH and the interaction of (A) β -catenin and (B) E-cadherin was assessed by Western-blot analysis (C) Lysates were prepared from A431 cells treated with EGF, and the N-SH2 and C-SH2 fusion proteins were used to affinity purify the EGFR.

excluded because, as Figure 9(C) shows, both p85 SH2 domains demonstrated EGF-dependent association with the EGFR.

DISCUSSION

Our initial experiments illustrate that the p85 subunit of PI 3-kinase associates with proteins found in the cadherin-based adhesion complex. Co-association of p85 with β -catenin has been reported in mouse keratinocytes expressing activated Ha-ras [37]. The expression of Ha-ras in the mouse cells caused re-localization of β -catenin from the adhesion complex to the cytoplasm and potentiated the β -catenin–p85 interaction. However, in human keratinocytes the interaction between β -catenin and p85 displays clear differences between the two species. Firstly, we found that the interaction of p85 and β -catenin was not restricted to a cytosolic pool of these proteins and importantly ras was not necessary for this interaction to occur.

The possibility that p85 interacted with E-cadherin was excluded, since these proteins could not be co-precipitated and they didn't interact by yeast two-hybrid analysis. This suggested that p85 associated with proteins from the adherens complex via interaction with β -catenin. Three independent methods were used to investigate the specificity and regulation of such an interaction. The results confirmed that p85 and β -catenin could be co-precipitated, and that recombinant p85 and β -catenin interacted directly when analysed by SPR and yeast two-hybrid analysis.

Yeast three-hybrid analysis revealed that the interaction of β -catenin with p85 was reduced by co-expression of the p56Lck src-family tyrosine kinase. We observed a similar, partial reduction in the interaction between p85 and β -catenin when src expression was induced in LA29 fibroblasts (Figure 8C). Previous reports have suggested that src family tyrosine kinases phosphorylate components of the adherens junction, including β -catenin [14,15]. These data show that interaction between β -catenin and the p85 subunit of PI 3-kinase does not require tyrosine phosphorylation, but can be modified by it [38].

Analysis of the primary structure of β -catenin reveals no consensus tyrosine residues within binding motifs for either of the SH2 domains from p85 or a binding motif for the SH3 domain of p85. The core region of β -catenin contains 12 copies of a 42-amino-acid sequence termed the armadillo repeat, which undertakes protein interactions involving β -catenin. Deletion mutagenesis has mapped the binding of cadherins, adenomatous-polyposis coli protein, T-cell factor transcription factors, axin

and the EGFR tyrosine kinase domain to this region. In all cases these different binding partners recognize overlapping portions of a minimum of 6–7 armadillo repeats within the first 10 repeats. Although these binding partners of β -catenin show no significant similarity overall, they all share a short acidic sequence (Ser-Ser-Leu) that may represent part of a β -catenin binding site. However, both p85 α and p85 β were able to interact with β -catenin, yet only p85 β contains a Ser-Ser-Leu motif, suggesting this motif is not involved in interactions between p85 and β -catenin. In addition Ser-Ser-Leu is found in the C-terminal SH2 domain, which our analysis showed was not involved in the interaction with β -catenin.

The observation that p85 is associated with β -catenin in the cadherin-based adhesion complex suggests that PI 3-kinase may play a role in regulating or mediating the functions of the complex. Since PI 3-kinase has been implicated in processes requiring modulation of intercellular adhesion, common regulatory pathways may be implicated. Small molecular mass GTPases and actin cytoskeleton remodelling appear to constitute targets for both PI 3-kinase and cadherin-based adhesion complex function. Although our experiments showed that there was no apparent PI 3-kinase catalytic activity in the adherens complex, it is possible that PI 3-kinase activity is only associated with newly assembled adhesion complexes [24]. Therefore the p85 associated with proteins of the adherens complex may serve as a potential docking site for p110, that is regulated by future events requiring production of 3-phosphorylated inositides.

We demonstrated that the cytoplasmic β -catenin–p85 interaction in human keratinocytes was unaffected by activated Ha-ras transformation. Cytoplasmic levels of β -catenin are tightly regulated within the cell by a multi-protein 'destruction complex' that binds to β -catenin, targeting it for proteolysis by the ubiquitination system in a process dependent on glycogen synthase kinase-3 β (GSK-3 β) activity (reviewed in [39]). A physiological role for the p85 subunit may be to stabilize β -catenin when released by the adhesion complex into the cytoplasm by preventing binding of the 'destruction complex' as originally proposed [37]. Additionally or alternatively, PI 3-kinase activation may serve to inhibit the catalytic activity of GSK-3 β . A known downstream target of PI 3-kinase is the serine/threonine protein kinase B (PKB/Akt) [40,41], which in turn has been demonstrated to phosphorylate and inactivate GSK-3 β [42]. PKB activation would therefore prevent the GSK-3 β -mediated targeting of β -catenin for destruction, leading to the accumulation of cytosolic β -catenin.

Despite lacking a recognized nuclear localization signal, several studies have reported nuclear translocation of PI 3-kinase in response to a number of stimuli (reviewed in [43]). Thus a possible physiological function of a β -catenin–PI 3-kinase interaction could be to translocate PI 3-kinase from the cytoplasm in to the nucleus. β -Catenin is known to translocate to the nucleus and interact with members of the T-cell factor/lymphoid enhancing factor family of transcription factors to *trans*-activate the expression of certain target genes [39,44]. PI 3-kinase could therefore translocate to the nucleus complexed with β -catenin and take part in nuclear signalling. It is possible that nuclear PI 3-kinase activity may potentiate or modify β -catenin-mediated transcription.

The present study reveals that the 85 kDa regulatory subunit of PI 3-kinase is able to directly interact with cytosolic and adherens junction-complexed β -catenin and is independent of activated Ha-ras expression. Furthermore, this interaction involves the N-terminal SH2 domain of p85 and is negatively regulated by tyrosine phosphorylation.

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