Tyrosine Phosphorylation of Human Keratinocyte β-Catenin and Plakoglobin Reversibly Regulates their Binding to E-Cadherin and α-Catenin

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We show that tyrosine phosphorylation, produced by incubation of normal human keratinocytes with the tyrosine phosphatase inhibitor peroxovanadate, directly and reversibly regulates the association of β-catenin and plakoglobin with E-cadherin and α-catenin. Prior studies have demonstrated a correlative, but not causal, association between increased tyrosine phosphorylation and decreased adherens junction mediated cell-cell adhesion. We observed that (i) binding of tyrosine phosphorylated β-catenin and plakoglobin to E-cadherin and to α-catenin was substantially reduced, but could be restored *in vitro* by removal of phosphate from β-catenin and plakoglobin with added tyrosine phosphatase, and (ii) tyrosine phosphorylation of β-catenin and plakoglobin was associated with decreased cell–cell adhesion. These findings support a direct and causal role for tyrosine phosphorylation of β-catenin and plakoglobin in regulating adherens junction mediated cell–cell adhesion. We propose that tyrosine phosphorylation of specific and probably different residues is responsible for regulating the binding of β-catenin or plakoglobin to (i) E-cadherin and (ii) α-catenin. Additionally, because β-catenin and plakoglobin have both structural and regulatory functions, the data raise the possibility that β-catenin or plakoglobin released from the adherens junctions by tyrosine phosphorylation may transduce a signal to the nucleus regarding the adhesive state of the cell. ***Key words: adherens junction/adhesion/keratinocyte. J Invest Dermatol 117:1059–1067, 2001***

In the skin, keratinocytes migrate from the basal layer upward to the topmost layer, the stratum corneum, undergoing a morphologically well-described pattern of differentiation. This migration can take from 4 wk in normal tissues to as little as 4 d in inflammatory hyperplastic conditions such as psoriasis (Van Scott and Ekel, 1963; Weinstein and Van Scott, 1965). Despite this constant state of movement, the tissue maintains its structural integrity, presumably due to the ability of adjacent keratinocytes to break and reform their adhesive contacts with each other in a controlled fashion.

In keratinocytes, adherens junctions are composed of cadherin (Takeichi, 1988; 1991), β-catenin or plakoglobin, and α-catenin (Ozawa *et al*, 1989; Nagafuchi *et al*, 1991; Butz and Kemler, 1994). β-Catenin or plakoglobin bind to the cytoplasmic tail of E-cadherin and to α-catenin (Aberle *et al*, 1994; Hulsken *et al*, 1994). α-Catenin in turn binds to the actin cytoskeleton (Ozawa *et al*, 1990; Rimm *et al*, 1995). This assemblage, by forming a continuous macromolecular complex between the external cell–cell contacts and the internal actin cytoskeleton, functions to maintain both cell–cell adhesion and tissue integrity. β-Catenin and plakoglobin represent potential candidates for the dynamic regulation of keratinocyte cell–cell adhesion. They have a central role in forming two key structures that link adjacent keratinocytes, the adherens junction and the desmosome. Additionally, they have been shown to participate in Wnt-mediated signal transduction (Funayama *et al*, 1995; Karnovsky and Klymkowsky, 1995; Miller and Moon, 1996). Thus, they may provide a link between the sensing of cell–cell adhesion or the disruption of cell–cell adhesion, and subsequent cell fate/epithelial differentiation events that occur as a consequence of, or are directed by, the presence or absence of cell–cell adhesion. For example, the principle of contact inhibition of cell growth presumably requires signaling from the cell surface to the nucleus to alter the pattern of gene transcription from active growth/cell division to quiescence.

Human β-catenin and plakoglobin are homologs of the *Drosophila melanogaster* protein Armadillo, to which they are structurally and functionally similar (Peifer and Wieschaus, 1990; Peifer *et al*, 1992). During their function in the two core processes of cell–cell adhesion and signal transduction, β-catenin, plakoglobin, and Armadillo interact with a large number of proteins including: (i) the adherens junction proteins E-cadherin and α-catenin (Aberle *et al*, 1994), (ii) the Cdc42 and Rac1 effector protein IQGAP1 (Fukata *et al*, 1999), (iii) the actin bundling protein fascin (Tao *et al*, 1996), (iv) the transcription factors T cell factor (TCF) (Molenaar *et al*, 1996) and lymphoid enhancing factor (LEF) (Behrens *et al*, 1996; Huber *et al*, 1996), (v) the nuclear protein Pontin 52 (Bauer *et al*, 1998a), (vi) the tumor suppressor protein adenomatous polyposis coli (APC) (Rubinfeld *et al*, 1993; 1995), (vii) presenilins (Zhou *et al*, 1997; Yu *et al*, 1998; Zhang *et*

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Abbreviations: APC, adenomatous polyposis coli protein; IP, immunoprecipitation; LEF, lymphoid enhancing factor; PTP, protein tyrosine phosphatase; TCF, T cell transcription factor.

al, 1998), (viii) serine/threonine specific protein kinases (glycogen synthase kinase-3), (ix) the epidermal growth factor (EGF) (Hoschuetzky *et al*, 1994) and c-erbB-2 (Kanai *et al*, 1995) receptor tyrosine kinases, and (x) axin (Kodama *et al*, 1999) and conductin (Behrens *et al*, 1998).

Post-translational modification of β-catenin and plakoglobin represents a potential mechanism by which these various protein–protein interactions are regulated. β-Catenin, plakoglobin, and *Drosophila* Armadillo are phosphoproteins that contain potential serine, threonine, and tyrosine phosphorylation sites. Increased tyrosine kinase activity is associated with decreased cell–cell adhesion (Matsuyoshi *et al*, 1992; Behrens *et al*, 1993; Hamaguchi *et al*, 1993; Soler *et al*, 1998). This decreased cell–cell adhesion may be due in part to alterations in the adherens junction (Volberg *et al*, 1991; 1992). Increased tyrosine phosphorylation of E-cadherin, β-catenin, and plakoglobin was observed when leukemic cells transfected with an E-cadherin construct were treated with the tyrosine phosphatase inhibitor peroxovanadate. This was associated with decreased cell–cell adhesion and dissociation of α-catenin from the E-cadherin–β-catenin and E-cadherin–plakoglobin complexes (Ozawa and Kemler, 1998). Decreased binding to E-cadherin of *in vitro* pp60 c-src tyrosine phosphorylated β-catenin has recently been reported (Roura *et al*, 1999). In contrast, recent work has suggested that tyrosine phosphorylation is associated with increased stability of adherens junction protein association/binding and increased cell–cell adhesion in murine keratinocytes (Calautti *et al*, 1998).

Because of our interest in understanding how keratinocyte cell–cell adhesion is regulated and because of the apparent conflicting reports regarding the effect of tyrosine phosphorylation on cell–cell adhesion and the adherens junction, we sought to investigate the regulation of cell–cell adhesion in a normal human keratinocyte tissue culture system. These nonimmortalized primary cells may more closely approximate the *in vivo* biology than the immortalized cell lines used in most prior studies. Here we report that (i) tyrosine phosphorylation of the adherens junction components β-catenin and plakoglobin causes them to dissociate from E-cadherin and α-catenin, (ii) this dissociation correlates with decreased keratinocyte cell–cell adhesion, and (iii) *in vitro* tyrosine dephosphorylation of β-catenin and plakoglobin results in the reassociation of β-catenin and plakoglobin with E-cadherin and α-catenin. Prior studies have demonstrated associative, but not causal, links between tyrosine phosphorylation and adherens junction protein association. In this report, we demonstrate that the effect of tyrosine phosphorylation on the adherens junction is reversible, providing support for such a causal link. These *in vitro* reconstitution experiments are consistent with a mechanism by which tyrosine phosphorylation of β-catenin and plakoglobin reversibly regulates the association of adherens junction protein components. Thus, tyrosine phosphorylation of β-catenin and plakoglobin probably represents one aspect of the dynamic regulation of keratinocyte cell–cell adhesion.

MATERIALS AND METHODS

Materials Monoclonal antibodies to human β-catenin and plakoglobin (N-terminal), a horseradish peroxidase conjugated monoclonal antiphosphotyrosine antibody (RC20-HRPO conjugate), a polyclonal rabbit antihuman α-catenin antibody, and protein G sepharose beads were from Zymed (San Francisco, CA). Monoclonal antibodies to human E-cadherin and a C-terminal monoclonal antibody to human plakoglobin were from Transduction Laboratories (Lexington, KY). For Western blot detection, secondary goat antimouse and goat antirabbit horseradish peroxidase conjugated IgG and enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). For immunofluorescent staining, secondary fluorescein isothiocyanate (FITC) and rhodamine conjugated goat antimouse/rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant *Yersinia enterocoltica* protein tyrosine phosphatase (PTP) was from Roche-Boehringer Mannheim (Indianapolis, IN). Electrophoresis reagents were from Bio-Rad (Hercules, CA). All other reagents were of standard grade from Sigma (St. Louis, MO).

Tissue culture Primary human keratinocytes established from human neonatal foreskins were obtained from Cascade Biologics (Portland, OR) and cultured according to the recommended procedure using NHK 154 medium (0.2 mM $CaCl_2$) supplemented with human keratinocyte growth supplement (Cascade Biologics) and penicillin, streptomycin, and amphotericin B (Cascade Biologics) according to the manufacturer's recommendations. Cells were expanded for two passages and third passage keratinocytes were used for all experiments. For immunoprecipitation (IP) experiments, cultures were grown on Falcon 100 × 20 mm polystyrene Petri dishes to 70%–80% confluence. Cells were treated with or without peroxovanadate (0.5 mM Na_3VO_4, 3 mM H_2O_2) for 0–60 min prior to harvesting.

Confocal immunofluorescent microscopy For immunofluorescent staining, normal human keratinocytes were grown on ethanol sterilized glass coverslips. Cells were treated with or without peroxovanadate (0.5 mM Na_3VO_4, 3 mM H_2O_2) for 0–60 min prior to fixation in 3.7% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS: 137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 0.9 mM $CaCl_2$) for 5 min at room temperature, permeabilized with 0.25% Trition X-100 in DPBS for 10 min at room temperature, and blocked with 2% bovine serum albumin (BSA) in DPBS for 15 min at room temperature. Fixed cells were incubated with primary antibody overnight at 4°C in a humidity chamber. Each primary antibody (E-cadherin, α-catenin, β-catenin, and plakoglobin) was diluted 1:100 in antibody dilution buffer (2% BSA, 0.01% Tween 20, 0.1% NaN_3, in DPBS). After overnight incubations with primary antibody, the coverslip fixed cells were washed three times for 10 min each in DPBS at 4°C, blocked with 2% BSA in DPBS at 4°C for 20 min, incubated with FITC or rhodamine conjugated goat antimouse or goat antirabbit IgG secondary antibodies diluted 1:100 in antibody dilution buffer for 90 min at 4°C in the dark, washed three times for 10 min each in DPBS at 4°C, and then mounted with polyvinyl alcohol mounting medium [10% (wt/vol) polyvinyl alcohol, 25% (vol/vol) glycerol, 50 mM Tris-Cl, pH 8.5]. Images were obtained using a Zeiss laser scanning confocal microscope. For double-labeled images, data from the two channels were superimposed using LSM 3.8 software, with the FITC channel in green and the rhodamine channel in red as described by Loureiro and Peifer (1998).

IP Cell extracts were prepared from normal human keratinocytes in tissue culture at 70%–80% confluence. Each plate (100 × 20 mm) of cells was harvested on ice in 1 ml lysis buffer [1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 mM NaF, 10 μM E-64, 100 μM leupeptin, 10 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride], with or without peroxovanadate (1 mM Na_3VO_4, 3 mM H_2O_2). All subsequent steps were done at 4°C. Insoluble material was removed by high-speed centrifugation. Protein concentration in extracts was determined by Bradford assay (Bio-Rad, Laboratories, Hercules, CA). Detergent soluble extracts were subjected to IP with the following primary antibodies at the indicated concentrations: E-cadherin (3 μg per ml), α-catenin (3 μg per ml), β-catenin (3 μg per ml), C-terminal plakoglobin (3 μg per ml). A 50% slurry of protein G beads was then added to the extract (1:10 vol/vol) and incubated overnight at 4°C with gentle rotation. The beads were then pelleted in a microfuge for 15 s and each washed three times with a 65-fold volume excess of lysis buffer. Samples were eluted from the beads by boiling 15 μl beads in 15 μl sodim dodecyl sulfate (SDS) Laemmli sample buffer containing β-mercaptoethanol for 3 min. Typically, 5 μl of sample was loaded per lane and separated by 10% SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) followed by electrotransfer to nitrocellulose according to established procedures. Nitrocellulose membranes were then blocked with 3% BSA in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and probed for 1 h at room temperature with primary antibody diluted to the manufacturer's recommendations in TBST (β-catenin, 1:1000; N-terminal plakoglobin, 1:1000; antiphosphotyrosine, 1:2500; α-catenin, 1:1000; E-cadherin, 1:2500; C-terminal plakoglobin, 1:2000). Blots were then washed three times in TBST and incubated with horseradish peroxidase conjugated secondary antibody diluted 1:10,000 in TBST, washed three times in TBST, and developed with enhanced chemiluminescence reagents. Horseradish peroxidase conjugated antiphosphotyrosine antibodies were developed directly.

Tyrosine phosphatase activity Measurement of tyrosine phosphatase activity in keratinocyte cell extracts was based on the molybdate–malac green–phosphate complex formed with released free phosphate utilizing the tyrosine phosphatase assay system from Promega (Madison, WI) used

according to the manufacturer's recommendations. Endogenous free phosphate in the extracts was removed prior to assay by gel filtration chromatography on Sephadex G-25 spin columns. Levels of released free phosphate were determined after 30 min incubations at 37°C.

Reconstitution experiments Immunoprecipitates from peroxovanadate-treated cells were split into equal samples. One half was washed three times with lysis buffer (peroxovanadate free) to remove peroxovanadate, and then incubated with *Yersinia enterocoltica* PTP [25 mU per ml in 20 mM Tris-Cl, pH 7.2, 150 mM NaCl, 0.1 mg per ml BSA, and 0.1% (vol/vol) 2-mercaptoethanol], in a total volume of 50 μl for 15 μl beads, for 30 min at 37°C; the other half underwent mock incubation with PTP buffer, but no added PTP. The beads were then washed three times in lysis buffer. PTP-treated and mock-incubated proteins were incubated for 2 h at 4°C with gentle rotation with fresh keratinocyte cell lysates from either control or peroxovanadate-treated cells, respectively. Protein G beads were then collected and washed three times with lysis buffer, and the samples were eluted from the beads by boiling in reducing SDS Laemmli sample buffer. Samples were separated by 10% SDS-PAGE, followed by electrotransfer to nitrocellulose, and probed with antibodies to E-cadherin, α-catenin, β-catenin, plakoglobin, and phosphotyrosine as described above.

To demonstrate that the conditions used for IP resulted in saturation of protein G bound antibody by antigen (E-cadherin, α-catenin, β-catenin, or plakoglobin), immune complexes generated from IP of keratinocyte extracts using monoclonal antibodies to each adherens junction component (E-cadherin, α-catenin, β-catenin, or plakoglobin) were split into equal fractions. One half of each IP reaction was then re-incubated with fresh keratinocyte extracts under conditions used for the reconstitution experiments, separated by SDS-PAGE, and analyzed by Western blot detection using the respective antibody. No additional E-cadherin, α-catenin, β-catenin, or plakoglobin could be detected after re-incubation of IPs with fresh extract, indicating that the conditions used for the initial IP were saturating. Additionally, to demonstrate that antigen in the added back extracts was not displacing bound antigen in the initial IP reactions, β-catenin and plakoglobin immune complexes from nonperoxovanadate treated-keratinocytes were split into equal fractions. One half of each IP reaction was then re-incubated with fresh extracts from peroxovanadate-treated keratinocytes under conditions used for the reconstitution experiments. Reactions were separated by SDS-PAGE and analyzed by Western blot detection using β-catenin or plakoglobin antibody. Bands of retarded migration corresponding to tyrosine phosphorylated β-catenin and plakoglobin were not observed when immune complexes containing nontyrosine phosphorylated β-catenin and plakoglobin were re-incubated with extracts from peroxovanadate-treated cells, indicating that the tyrosine phosphorylated β-catenin and plakoglobin in the peroxovanadate-treated extracts was not displacing bound nontyrosine phosphorylated β-catenin and plakoglobin from the protein G localized antibody.

RESULTS

Cell–cell adhesion is disrupted and adherens junction protein distribution is altered in peroxovanadate-treated normal human keratinocytes Exposure of cells in culture to peroxovanadate, a potent inhibitor of tyrosine phosphatases, results in a rapid increase in tyrosine phosphorylation of cellular proteins (Kadota *et al*, 1987; Ruff *et al*, 1997). We used peroxovanadate to investigate the effect of tyrosine phosphorylation on keratinocyte cell–cell junctions. Keratinocytes, 70%–80% confluent, were exposed to peroxovanadate. Addition of this reagent to the culture medium disrupted cell–cell adhesion; cell membranes on adjacent cells retracted and individual keratinocytes rounded up (**Fig 1*a***). Immunofluorescent confocal microscopy was used to examine the distribution of the adherens junction proteins E-cadherin, α-catenin, β-catenin, and plakoglobin. In nontreated control keratinocytes, staining for E-cadherin, α-catenin, β-catenin, and plakoglobin was primarily at the cell membrane in regions of cell–cell contact. For the most part, the staining for these proteins colocalized (**Fig 1*a***). In contrast, in peroxovanadate-treated cells, the distribution of adherens junction proteins was significantly altered: (i) E-cadherin staining at the cell membrane decreased, whereas punctate cytoplasmic staining increased, similar to the redistribution of E-cadherin that has been described during mitosis in Madine–Darby canine kidney cells (Bauer *et al*, 1998b) and suggesting partial internalization of E-cadherin; (ii) α-catenin staining was markedly reduced at the cell membrane without a compensatory increase in either cytoplasmic or nuclear staining; (iii) there was decreased β-catenin staining at the cell membrane and increased cytoplasmic staining of β-catenin; nuclear staining of β-catenin appeared essentially the same in treated and control keratinocytes; (iv) there was decreased staining at the cell membrane and increased cytoplasmic and nuclear staining of plakoglobin.

The adherens junction complex is disrupted and β-catenin and plakoglobin are tyrosine phosphorylated in peroxovanadate-treated keratinocytes To further explore the effects of tyrosine phosphorylation on the redistribution of adherens junction proteins, we used monoclonal antibodies to investigate the ability of E-cadherin to coimmunoprecipitate α-catenin, β-catenin, and plakoglobin in extracts from control and peroxovanadate-treated keratinocytes. The amount of α-catenin, β-catenin, and plakoglobin coimmunoprecipitating with E-cadherin was significantly reduced in E-cadherin immunoprecipitates from peroxovanadate-treated cells relative to controls (**Fig 1*b***). Thus, peroxovanadate treatment was associated with a decreased ability of the adherens junction proteins α-catenin, β-catenin, and plakoglobin to form a functional adherens junction complex with E-cadherin.

Peroxovanadate treatment resulted in tyrosine phosphorylation of β-catenin and plakoglobin as detected by retarded migration on SDS-PAGE and by Western blot detection with antiphosphotyrosine antibodies; E-cadherin and α-catenin were not detected with antiphosphotyrosine antibodies from either control or peroxovanadate-treated cells (**Fig 2**). Additionally, in peroxovanadate-treated cells, the time courses of (i) keratinocyte dyshesion, (ii) redistribution of β-catenin and plakoglobin, and (iii) tyrosine phosphorylation of β-catenin and plakoglobin were similar (**Fig 2*a–c***). These results suggested that tyrosine phosphorylation of β-catenin and plakoglobin was associated with decreased keratinocyte cell–cell adhesion and that this dyshesion was associated with altered subcellular distribution of the adherens junction proteins.

Tyrosine phosphorylation of β-catenin and plakoglobin reversibly decreases their binding to E-cadherin and α-catenin We hypothesized that if tyrosine phosphorylation of β-catenin or plakoglobin functioned as a biochemical switch to regulate the association of adherens junction proteins, and if tyrosine phosphorylation disrupted this association, then removal of phosphate from tyrosine residues of β-catenin and plakoglobin should promote reassociation of the adherens junction complexes (E-cadherin–β-catenin–α-catenin and E-cadherin–plakoglobin–α-catenin complexes). To investigate this causal relationship, *in vitro* reconstitution experiments were performed. Normal human keratinocytes were grown to 70%–80% confluence and incubated in the presence or absence of peroxovanadate for 60 min prior to harvesting. Detergent soluble cell extracts were prepared from control and peroxovanadate-treated cells and subjected to IP with antibodies to E-cadherin, α-catenin, β-catenin, and plakoglobin. The immunoprecipitates from peroxovanadate-treated cells were split into equal fractions; half the sample was treated with PTP and the other half underwent mock incubation (buffer, but no added phosphatase). The beads were then washed and exposed to fresh keratinocyte extracts. Because human keratinocytes have endogenous tyrosine phosphatase activity (data not shown), reconstituting mock-incubated samples with control extracts would result in the addition of phosphatase and thus not differ significantly from those samples treated with exogenous PTP. To avoid addition of endogenous phosphatase, peroxovanadate-treated extracts (in which endogenous tyrosine phosphatase activity is inhibited) were used to reconstitute the mock-incubated samples; control extracts were used to reconstitute PTP-treated samples. After extensive washing, the samples were analyzed by SDS-PAGE

and Western blot. The results of this experiment are shown in **Fig 3**.

From control keratinocytes (i) monoclonal antibodies to E-cadherin coimmunoprecipitated α-catenin, β-catenin, and plakoglobin (**Fig 3*a***, *lane C*), (ii) monoclonal antibodies to α-catenin coimmunoprecipitated E-cadherin, β-catenin, and plakoglobin (**Fig 3*b***, *lane C*), (iii) monoclonal antibodies to β-catenin coimmunoprecipitated E-cadherin and α-catenin, but not plakoglobin (**Fig 3*c***, *lane C*), and (iv) monoclonal antibodies to plakoglobin coimmunoprecipitated E-cadherin and α-catenin, but not β-catenin (**Fig 3*d***, *lane C*). These data suggest that in keratinocytes in tissue culture (i) β-catenin binds to E-cadherin and α-catenin and (ii) plakoglobin similarly binds to E-cadherin and α-catenin; this reflects the core structure of the adherens junction in these cells.

When E-cadherin, α-catenin, β-catenin, or plakoglobin were immunoprecipitated from peroxovanadate-treated cells, β-catenin and plakoglobin were tyrosine phosphorylated; this correlated with a decrease in the association of (i) β-catenin with α-catenin and E-cadherin and (ii) plakoglobin with α-catenin and E-cadherin (**Fig 3*a–d***, *lane V*). When immunoprecipitated tyrosine phosphorylated β-catenin and plakoglobin were treated with the tyrosine-specific phosphatase PTP, and then re-exposed to α-catenin and E-cadherin present in detergent soluble extracts from keratinocytes that had not been treated with peroxovanadate, adherens junction complexes were reconstituted; we observed that (i) phosphate was removed from both β-catenin and plakoglobin as evidenced by their increased mobility on SDS-PAGE and by the loss of reactivity with antiphosphotyrosine antibodies (**Fig 3*a–d***, *lanes V/PTP*), and (ii) the E-cadherin–β-catenin–α-catenin (**Fig 3*a***, ***b***, ***c***, *lanes V/PTP*) and the E-cadherin–plakoglobin–α-catenin (**Fig 3*a***, ***b***, ***d***, *lanes V/PTP*) complexes reformed.

No reassociation of the adherens junction complexes was observed with tyrosine phosphorylated β-catenin and plakoglobin. β-Catenin and plakoglobin immunoprecipitates from peroxovanadate-treated cells that underwent mock incubations without added PTP remained tyrosine phosphorylated and failed to significantly reassociate with E-cadherin and α-catenin (**Fig 3*c–d***, *lane V*). Furthermore, when re-exposed to peroxovanadate-treated extracts, neither immunoprecipitated E-cadherin (**Fig 3*a***, *lane V*) nor α-catenin (**Fig 3*b***, *lane V*) substantially reformed adherens junction complexes because both β-catenin and plakoglobin, in the added back extracts from peroxovanadate-treated cells, are tyrosine

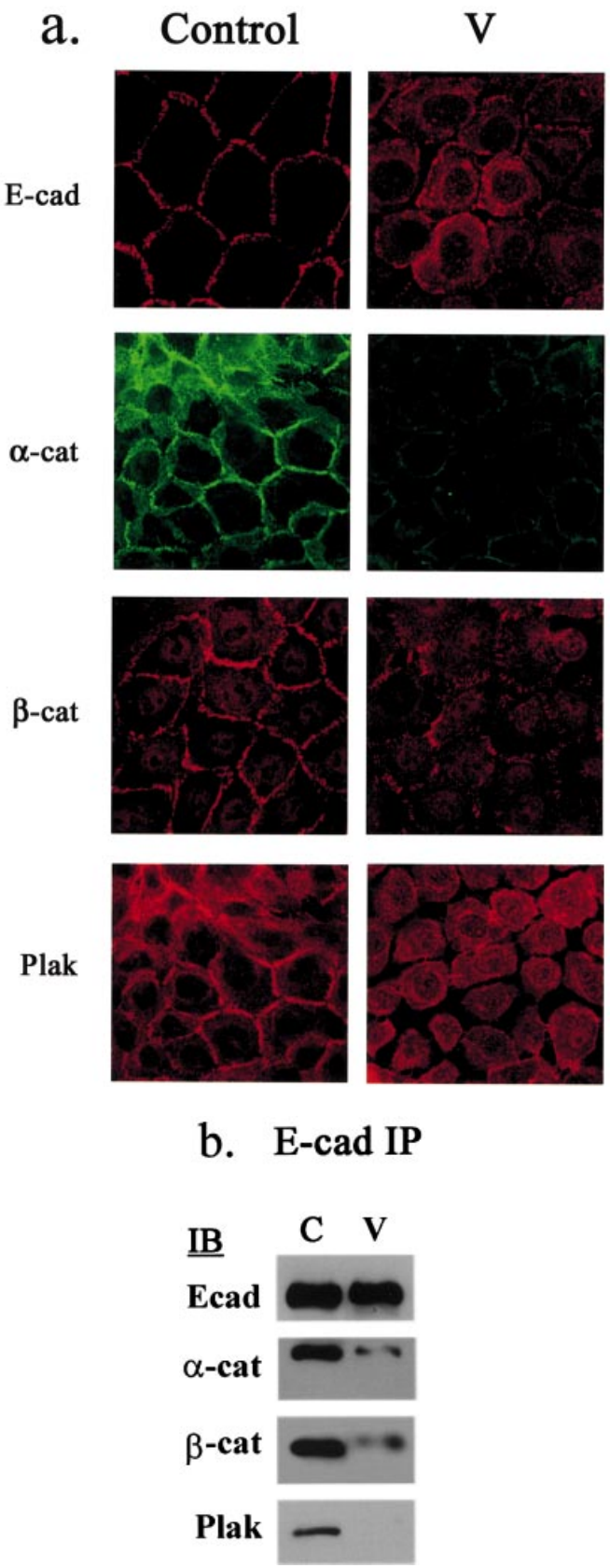

Figure 1. Peroxovanadate-induced changes in keratinocyte cell–cell adhesion and adherens junctions. (*a*) Exposure of keratinocytes to peroxovanadate results in decreased cell–cell adhesion and a redistribution of adherens junction proteins. Keratinocytes incubated in the presence (*column V*) or absence (*column Control*) of the tyrosine phosphatase inhibitor peroxovanadate were fixed and stained with monoclonal antibodies to E-cadherin (*row E-cad*), α-catenin (*row α-cat*), β-catenin (*row β-cat*), and plakoglobin (*row Plak*), followed by rhodamine (E-cad, β-cat, and Plak) or FITC (α-cat) conjugated secondary antibodies, and then examined by confocal laser microscopy. The cell membranes of control keratinocytes are adjacent to one another and the predominant staining pattern for each adherens junction protein is cell membrane. In contrast, the cell membranes of peroxovanadate-treated keratinocytes are retracted from one another consistent with decreased cell–cell adhesion. In peroxovanadate-treated cells, there is a reduction in membrane staining for E-cadherin, α-catenin, β-catenin, and plakoglobin, with a compensatory increase in cytoplasmic staining for E-cadherin, β-catenin, and plakoglobin. In treated cells, the overall signal for α-catenin is markedly reduced. Interestingly, after peroxovanadate treatment, there is an increase in the nuclear signal from plakoglobin suggesting that plakoglobin released from the membrane by tyrosine phosphorylation may translocate to the nucleus. (*b*) Decreased association of E-cadherin with α-catenin, β-catenin, and plakoglobin is observed when E-cadherin is immunoprecipitated from peroxovanadate-treated keratinocytes. E-cadherin immunoprecipitates from control (C) or peroxovanadate (V) treated keratinocytes were separated by SDS-PAGE and analyzed by Western blot by sequential probing of the membrane using monoclonal antibodies to E-cadherin (E-cad), α-catenin (α-cat), β-catenin (β-cat), and plakoglobin (Plak). After peroxovanadate treatment, there is a marked reduction in the ability of E-cadherin to coimmunoprecipitate α-catenin, β-catenin, and plakoglobin.

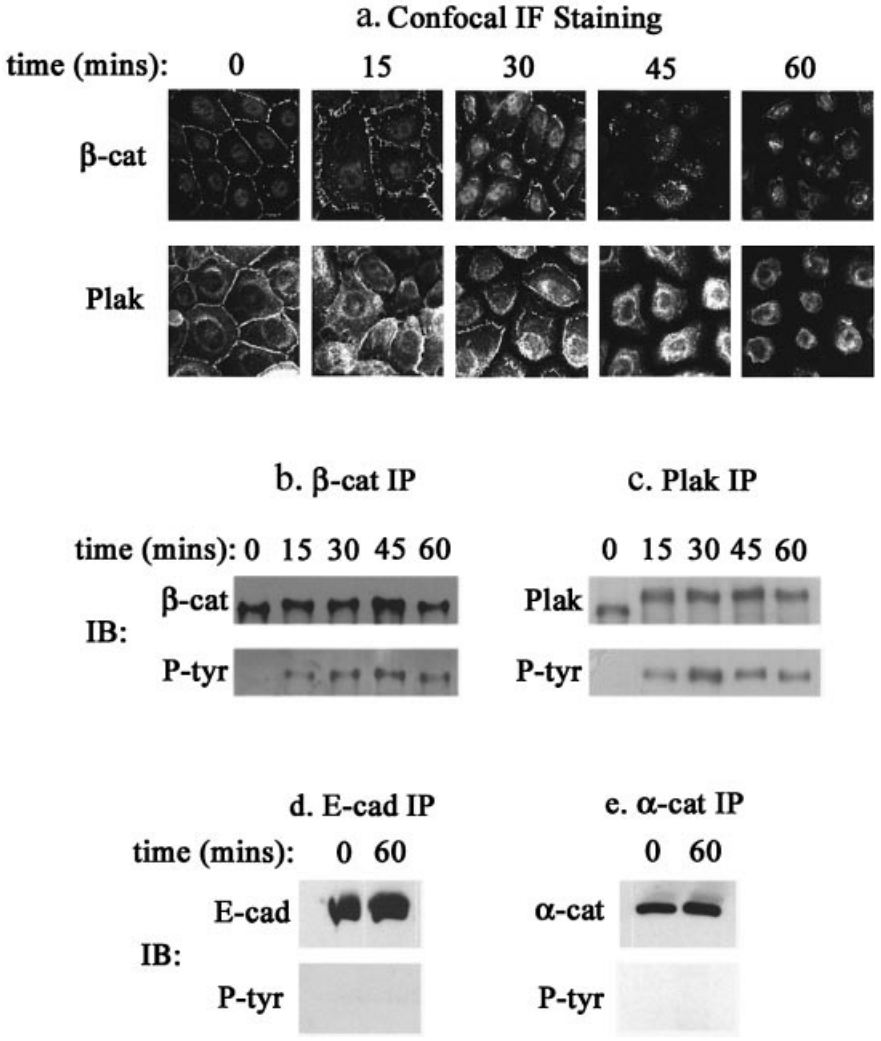

Figure 2. Loss of cell–cell adhesion and tyrosine phosphorylation of β-catenin and plakoglobin are rapid changes, occurring within 15 min of exposure of keratinocytes to peroxovanadate. (*a*) Confocal immunofluorescent micrographs of keratinocytes treated with peroxovanadate for 0, 15, 30, 45, and 60 min and stained with monoclonal antibodies to either β-catenin (β-cat) or plakoglobin (Plak). Peroxovanadate treatment causes (i) a progressive disruption in cell–cell adhesion, with cell membranes on adjacent cells retracted and keratinocytes round up, and (ii) a redistribution of β-catenin and plakoglobin from the cell membrane to the cytoplasm and nucleus. (*b*), (*c*) The time course of tyrosine phosphorylation of β-catenin and plakoglobin immunoprecipitated from peroxovanadate-treated keratinocytes parallels the observed decreases in keratinocyte cell–cell adhesion. β-catenin (*b*, β-cat IP) and plakoglobin (*c*, Plak IP) were immunoprecipitated from keratinocytes treated with peroxovanadate for 0, 15, 30, 45, and 60 min. IPs were separated by SDS-PAGE and analyzed by Western blot detection with monoclonal antibodies to β-catenin (β-cat), plakoglobin (Plak), and phosphotyrosine (p-tyr). Tyrosine phosphorylation of β-catenin and plakoglobin, as detected by retarded migration in SDS-PAGE and by reaction with antiphosphotyrosine antibodies, can be seen within 15 min of exposure of confluent keratinocytes to peroxovanadate. (*d*), (*e*) E-cadherin and α-catenin are not tyrosine phosphorylated in control and peroxovanadate-treated keratinocytes. E-cadherin (*d*, E-cad IP) and α-catenin (*e*, α-cat IP) were immunoprecipitated from keratinocytes treated with peroxovanadate for 0 and 60 min. IPs were separated by SDS-PAGE and analyzed by Western blot detection with monoclonal antibodies to E-cadherin (E-cad), α-catenin (α-cat), and phosphotyrosine (p-tyr).

phosphorylated and thus have markedly diminished E-cadherin and α-catenin binding.

The observed reassociation of adherens junction proteins is not due to the binding of adherens junction complexes in the added back extracts to unsaturated antibody If free E-cadherin, α-catenin, β-catenin, or plakoglobin antibody binding sites were present after the initial IP, i.e., the antibody was not saturated, then antigen present in the fresh extracts would bind to unoccupied antibody binding sites. In this assay, binding of E-cadherin–β-catenin–α-catenin or E-cadherin–plakoglobin–α-catenin complexes in the added back extract to unoccupied antibody would result in increased detection of E-cadherin and α-catenin and would yield similar results to true reconstitution of the adherens junction complex. To demonstrate that the conditions used for IP resulted in saturation of protein G bound antibody by antigen (E-cadherin, α-catenin, β-catenin, or plakoglobin), E-cadherin, α-catenin, β-catenin, or plakoglobin immunoprecipitates were split into equal fractions. One half of each respective IP reaction was then re-incubated with fresh keratinocyte extracts under conditions mimicking the reconstitution experiments, separated by SDS-PAGE, and analyzed by Western blot detection using the respective antibody. If after the initial IP the antibody was not saturated, then antigen present in the fresh extracts would bind to unoccupied antibody resulting in more E-cadherin, α-catenin, β-catenin, or plakoglobin being detected in the IP reactions that had undergone re-incubation with fresh extracts. The same amount of E-cadherin, α-catenin, β-catenin, and plakoglobin was detected whether or not the initial IP had undergone re-incubation with fresh extracts (**Fig 4*a***), demonstrating that antibody was saturated under the conditions used for the initial IP. Thus, the appearance of E-cadherin–β-catenin–α-catenin and E-cadherin–plakoglobin–α-catenin complexes in the reconstituted samples was not due to the binding of adherens junction complexes to unsaturated binding sites on the protein G bound antibodies.

The observed reassociation of adherens junction complexes is not due to antigen exchange The displacement of antibody bound non-E-cadherin–α-catenin associated β-catenin and plakoglobin by E-cadherin–α-catenin associated β-catenin and plakoglobin from the added back extract would also mimic reconstitution. Therefore, it was necessary to demonstrate that antigen in the added back extracts was not displacing bound antigen in the initial IP. Tyrosine phosphorylated β-catenin and plakoglobin from extracts of peroxovanadate-treated extracts exhibit retarded migration on SDS-PAGE, react with antiphosphotyrosine antibodies, and can be differentiated from nontyrosine phosphorylated β-catenin and plakoglobin from control extracts. This property was used to determine the source of β-catenin or plakoglobin detected in the immune complex (extract from initial IP *versus* added back fresh extract).

β-Catenin and plakoglobin immunoprecipitates from nonperoxovanadate-treated keratinocytes were split into equal fractions; one half was then re-incubated with fresh extracts from peroxovanadate-treated keratinocytes under conditions mimicking the reconstitution experiments. Reactions were separated by SDS-PAGE and analyzed by Western blot detection using β-catenin or

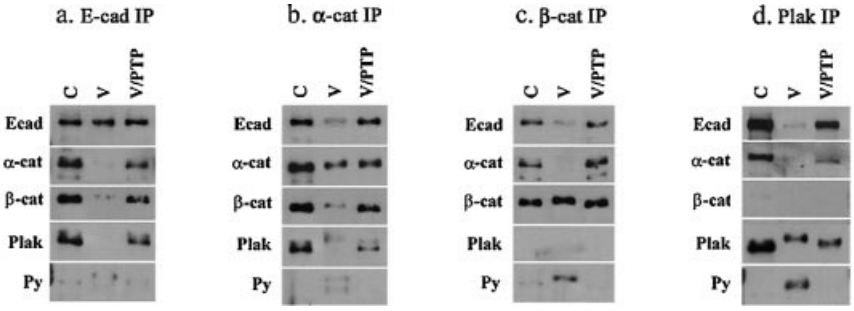

Figure 3. Tyrosine phosphorylation of β-catenin and plakoglobin reversibly regulates their association with E-cadherin and α-catenin. Detergent soluble extracts from keratinocytes treated with (columns V and V/PTP) or without (columns C) peroxovanadate, were immunoprecipitated with antibodies to E-cadherin (*a*, E-cad IP), α-catenin (*b*, α-cat IP), β-catenin (*c*, β-cat IP), and plakoglobin (*d*, Plak IP). Peroxovanadate induced tyrosine phosphorylation of β-catenin and plakoglobin, but not E-cadherin or α-catenin; only the region of the gel corresponding to the migration of β-catenin and plakoglobin is shown in the phosphotyrosine blots. Tyrosine phosphorylation of β-catenin and plakoglobin decreased their ability to co-IP with E-cadherin and α-catenin. Peroxovanadate-treated immunoprecipitates were then either treated with protein tyrosine phosphatase and re-exposed to fresh keratinocyte extracts from control cells (columns V/PTP) or mock incubated with buffer, but no added phosphatase, and re-exposed to fresh keratinocyte extracts from peroxovanadate-treated cells (columns V). Adherens junction complexes reformed after PTP-treatment (*lanes V/PTP*); whereas no significant reassociation wass seen if phosphate was not removed from tyrosine residues of antibody bound β-catenin and plakoglobin (*c*, *d*, *lanes V*) or if tyrosine-phosphorylated β-catenin and plakoglobin from peroxovanadate-treated extracts were added back to antibody-bound E-cadherin or α-catenin (*a*, *b*, *lanes V*). Therefore, reconstitution of the adherens junction required tyrosine dephosphorylation of β-catenin and plakoglobin. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and sequentially probed with antibodies to E-cadherin (rows E-cad), α-catenin (rows α-cat), β-catenin (rows β-cat), plakoglobin (rows Plak), and phosphotyrosine (rows Py). Blots were stripped and reblocked between each successive probe.

plakoglobin antibody. If antigen exchange was occurring, then the IP reactions that were re-incubated with extracts from peroxovanadate-treated keratinocytes should contain an additional band of retarded mobility corresponding to tyrosine phosphorylated β-catenin or plakoglobin originating from the peroxovanadate extracts. No such band was observed demonstrating that, under the conditions used for the reconstitution experiments, antigen in the added back extracts was not displacing antibody bound antigen from the initial IP (**Fig 4*b***). These results indicate that tyrosine phosphorylation of β-catenin and plakoglobin can reversibly regulate the formation of E-cadherin–β-catenin–α-catenin and E-cadherin–plakoglobin–α-catenin complexes.

DISCUSSION

Peroxovanadate caused (i) opposing cell membranes on adjacent adherent keratinocytes to retract from one another and the cells to assume a more rounded shape, morphologic changes consistent with decreased keratinocyte cell–cell adhesion (Volberg *et al*, 1992), (ii) a decrease in membrane associated E-cadherin, α-catenin, β-catenin, and plakoglobin, and a concomitant increase in the cytoplasmic staining of β-catenin and plakoglobin and in the nuclear staining of plakoglobin, (iii) tyrosine phosphorylation of β-catenin and plakoglobin, and (iv) markedly decreased binding of β-catenin and plakoglobin to E-cadherin and α-catenin. The *in vitro* removal of phosphate by PTP treatment of β-catenin and plakoglobin resulted in the reassociation of β-catenin and plakoglobin with E-cadherin and α-catenin.

No tyrosine phosphorylation of E-cadherin and α-catenin occurs when keratinocytes are treated with peroxovanadate; therefore, both control and peroxovanadate-treated keratinocyte extracts were used as a source for nontyrosine phosphorylated E-cadherin and α-catenin. In contrast, extracts prepared from normal human keratinocytes have endogenous tyrosine phosphatase activity; this endogenous tyrosine phosphatase activity is inhibited in extracts prepared from peroxovanadate-treated keratinocytes. The presence in keratinocyte extracts of endogenous PTP activity necessitated the reconstitution of PTP and mock-incubated samples with different extracts. PTP-treated samples were reconstituted with extracts from control keratinocytes; however, incubating immunoprecipitated phosphorylated β-catenin and plakoglobin with

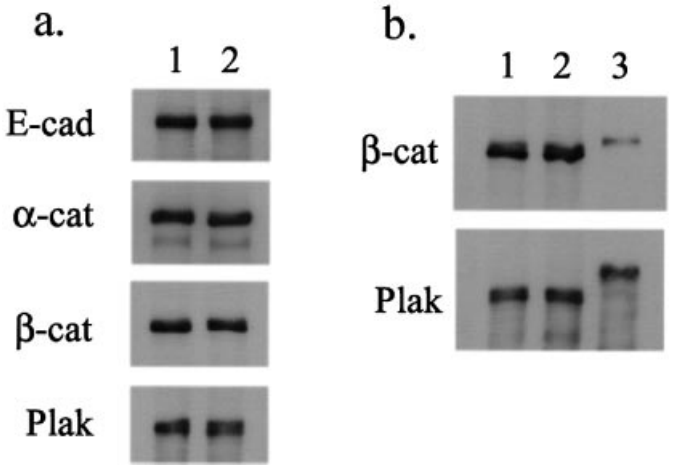

Figure 4. Antibody saturation and antigen exchange controls for reconstitution experiments. (*a*) Antibody saturation control. Immune complexes from keratinocyte extracts (*lane 1*) are compared with immune complexes from keratinocyte extracts that had been additionally incubated with fresh extracts under conditions used for reconstitution experiments (*lane 2*). No additional protein is immunoprecipitated when immune complexes are re-incubated with fresh extracts, indicating that the conditions used for immunoprecipitation result in antigen saturation of antibody binding sites. E-cad, E-cadherin IP followed by E-cadherin IB; α-cat, α-catenin IP followed by α-catenin IP; β-cat, β-catenin IP followed by β-catenin IB; Plak, plakoglobin IP followed by plakoglobin IB. (*b*) Antigen exchange control. SDS-PAGE followed by western blot detection of immune complexes from non-peroxovanadate-treated keratinocyte extracts (*lane 1*) compared with immune complexes from non-peroxovanadate-treated keratinocyte extracts that had been additionally incubated with fresh extracts from peroxovanadate-treated cells (*lane 2*). β-catenin and plakoglobin immunoprecipitated from peroxovanadate-treated keratinocytes (*lane 3*) are shown for comparison. No additional band of retarded migration is seen in immune complexes re-incubated with peroxovanadate-treated extracts, indicating that antigen exchange is not occurring. β-cat, β-catenin IP followed by β-catenin IB; Plak, plakoglobin IP followed by plakoglobin IB.

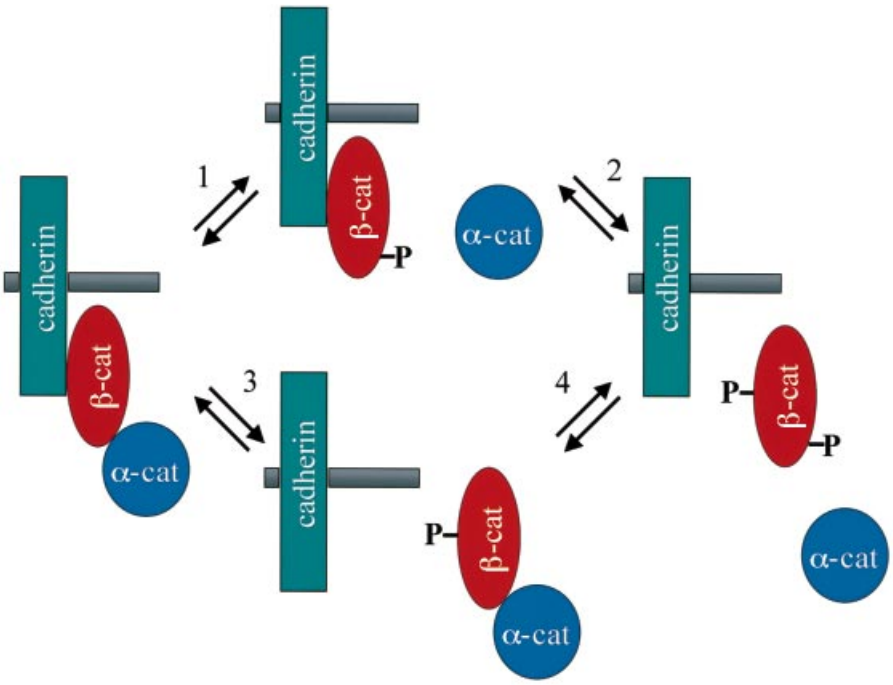

Figure 5. A model for the reversible regulation by tyrosine phosphorylation of adherens junction protein binding. Two separate tyrosine phosphorylation events are proposed to regulate the binding of β-catenin (or plakoglobin) to E-cadherin and α-catenin. In reactions 1 and 4, phosphorylation of a tyrosine residue in the amino terminus of β-catenin, perhaps mediated by EGF receptor or related tyrosine kinases, disrupts β-catenin–α-catenin binding, whereas in reactions 2 and 3, phosphorylation of a different tyrosine residue in the Armadillo repeat region of β-catenin, perhaps mediated by src family kinases, disrupts β-catenin–E-cadherin binding. Because each reaction is reversible, tyrosine phosphorylation of the adherens junction proteins β-catenin and plakoglobin represents one possible mechanism by which cells can dynamically regulate their ability to adhere to one another.

control extracts containing endogenous phosphatase would be analogous to adding exogenous PTP. To control for the presence or absence of phosphatase activity in the reconstitution experiments required reconstituting mock-incubated samples with peroxovanadate-treated extracts (a phosphatase-free source of E-cadherin and α-catenin). Because keratinocyte extracts, not isolated components, were used to reconstitute adherens junction complexes and because of the necessity to reconstitute PTP- and mock-incubated samples with different extracts, we investigated if other events might explain the observed reassociation of β-catenin/plakoglobin with E-cadherin and α-catenin. Controls demonstrated that neither binding of adherens junction complexes in the added back extracts to unsaturated antibody nor antigen exchange were occurring. Although not definitive, the results are strongly suggestive of a mechanism by which tyrosine phosphorylation of β-catenin and plakoglobin disrupts cell–cell adhesion by causing the dissociation of the E-cadherin–β-catenin–α-catenin and E-cadherin–plakoglobin–α-catenin complexes. Taken together, these observations support a causal role for tyrosine phosphorylation of β-catenin and plakoglobin in regulating keratinocyte cell–cell adhesion and adherens junction function.

The ability to detect a small amount of tyrosine phosphorylated β-catenin and plakoglobin that could still associate with either E-cadherin or α-catenin after peroxovanadate treatment may be due to phosphorylation of additional tyrosine residues in β-catenin and plakoglobin that are not involved in regulating the association of the adherens junction proteins. Alternatively, it may be that the association of β-catenin/plakoglobin with E-cadherin and α-catenin is regulated by the phosphorylation of not one, but at least two, tyrosine residues. Phosphorylation of one tyrosine residue might regulate the interaction of β-catenin/plakoglobin with α-catenin and phosphorylation of a second tyrosine residue might regulate the interaction of β-catenin/plakoglobin with E-cadherin. This would explain our ability to capture tyrosine phosphorylated (i) β-catenin–E-cadherin, (ii) β-catenin–α-catenin, (iii) plakoglobin–E-cadherin, and (iv) plakoglobin–α-catenin intermediates.

A mechanism by which two separate tyrosine phosphorylation events regulate the association of (i) β-catenin/plakoglobin with E-cadherin and (ii) β-catenin/plakoglobin with α-catenin is supported by the observations of other investigators. Under varying experimental conditions, some have observed a tyrosine phosphorylation associated disruption of the E-cadherin–β-catenin complex from either α-catenin (Takahashi *et al*, 1997; Ozawa and Kemler, 1998) or the α-catenin-related protein vinculin (Hazan and Norton, 1998), whereas others have observed disruption of β-catenin (Kinch *et al*, 1995; Balsamo *et al*, 1996) or plakoglobin from cadherin subsequent to tyrosine phosphorylation. Roura and coworkers demonstrated decreased *in vitro* binding of $pp60^{c-src}$ tyrosine-phosphorylated recombinant β-catenin to the cytoplasmic domain of E-cadherin (Roura *et al*, 1999). The $pp60^{c-src}$ modified residues in β-catenin were tyr-86 and tyr-654. Interestingly, the major site of phosphorylation, tyr-86, had no effect on E-cadherin binding, whereas their data suggest that phosphorylation of the minor phosphorylation site at tyr-654 decreased the binding of β-catenin to E-cadherin (Roura *et al*, 1999). α-Catenin binds to the amino terminus of β-catenin (Aberle *et al*, 1994) and it may be that phosphorylation of tyr-86 of β-catenin, in proximity to the α-catenin binding site, decreases β-catenin's binding to α-catenin.

A model in which two separate residues regulate the association of either E-cadherin or α-catenin with β-catenin/plakoglobin would be consistent with our data and the above-cited studies (**Fig 5**). In this model, phosphorylation of an N-terminal tyrosine residue of β-catenin or plakoglobin decreases the binding to α-catenin but not E-cadherin, whereas phosphorylation of a tyrosine residue within the Arm repeat region of β-catenin or plakoglobin decreases the binding to E-cadherin but not α-catenin. The same or different tyrosine kinases may be responsible for each phosphorylation event.

Increased kinase activity and decreased phosphatase activity are two *in vivo* mechanisms that may regulate the adherens junction function of β-catenin and plakoglobin. Several tyrosine kinases are candidates for regulating the association of β-catenin and plakoglobin with E-cadherin and α-catenin. Tyrosine phosphorylation by src family kinases may regulate the binding of β-catenin/plakoglobin to E-cadherin (Behrens *et al*, 1993; Roura *et al*, 1999), whereas EGF receptor tyrosine phosphorylation of β-catenin and plakoglobin may mediate the dissociation of α-catenin from the E-cadherin–β-catenin–α-catenin and the E-cadherin–plakoglobin–α-cateinin adherens junction complexes (Hoschuetzky *et al*, 1994; Takahashi *et al*, 1997; Hazan and Norton, 1998). Other kinases, such as the cytoplasmic tyrosine kinase Tyk2, may also regulate β-

catenin (Ruff *et al*, 1997). Additionally, several phosphatases have been reported to associate with adherens junctions and may also have a role in regulating adherens junction structure by a mechanism in which they maintain β-catenin and plakoglobin in a dephosphorylated state; these include (i) a member of the leukocyte antigen related protein tyrosine phosphatase family (Kypta *et al*, 1996; Muller *et al*, 1999), (ii) the receptor PTP μ (Brady-Kalnay *et al*, 1995; Brady-Kalnay *et al*, 1998), (iii) the receptor PTP κ (Fuchs *et al*, 1996), and (iv) the receptor PTP λ (Cheng *et al*, 1997).

β-Catenin, plakoglobin, and the *Drosophila* homolog Armadillo are capable of transducing signaling events from the cell surface to the nucleus (Karnovsky and Klymkowsky, 1995; Behrens *et al*, 1996; Huber *et al*, 1996; Molenaar *et al*, 1996; van de Wetering *et al*, 1997). Current models (for reviews, see Cavallo *et al*, 1997; Ben-Ze'ev and Geiger, 1998; Willert and Nusse, 1998; Novak and Dedhar, 1999) suggest that, in the case of human β-catenin, binding of the secreted morphogen Wnt to the frizzled transmembrane receptor suppresses the proteosome-mediated degradation of cytoplasmic β-catenin, perhaps by disrupting the ability of the serine threonine kinase GSK-3, the tumor suppressor protein APC, and conductin to target β-catenin for ubiquitination and proteosome-mediated degradation. Consequently, cytoplasmic levels of β-catenin are increased, driving the formation of a bipartite transcription factor between β-catenin and the architectural transcription factors TCF or LEF, which then activate transcription of downstream target genes.

We observed increased nuclear localization of plakoglobin as a consequence of its dissociation from E-cadherin at the cell membrane. Because β-catenin and plakoglobin are known to mediate signaling events, increased nuclear localization of plakoglobin in peroxovanadate-treated keratinocytes suggests the possibility that the release of plakoglobin from the adherens junction is associated with altered transcriptional activity in the nucleus. As suggested by our confocal data, events at the cell surface that disrupt cell–cell adhesion or, more specifically, the adherens junction might result in translocation of plakoglobin to the nucleus. Here, plakoglobin may directly transduce the signal that ''informs'' the cell that adhesion has been disrupted. If this is indeed the case, then this family of proteins (β-catenin, plakoglobin, Armadillo), by virtue of their ability to function in cell–cell adhesion as well as in DNA transcription, may serve as a direct link between events at the cell surface, namely the sensing of cell–cell adhesion *versus* dyshesion, and altered cell growth/differentiation. An interesting question raised by this suggestion is whether all β-catenin/plakoglobin signaling is equivalent or whether adherens junction *versus* Wnt-mediated signaling regulates different transcriptional events.

Our results suggest a direct and reversible role for tyrosine phosphorylation of β-catenin and plakoglobin on the association of the adherens junction complexes formed by E-cadherin, β-catenin or plakoglobin, and α-catenin. Our data, in the context of the cited studies, support a model in which at least two tyrosine phosphorylation events regulate the association of these complexes. One disrupts the binding of α-catenin to either β-catenin or plakoglobin; the second disrupts the binding of E-cadherin to either β-catenin or plakoglobin. Mapping the residues on β-catenin and plakoglobin that are modified by phosphorylation will be important to furthering our understanding of how this post-translational modification regulates adherens junction mediated cell–cell adhesion.

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