

Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell–cell adhesion

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The Rho GTPase and Fyn tyrosine kinase have been implicated previously in positive control of keratinocyte cell–cell adhesion. Here, we show that Rho and Fyn operate along the same signaling pathway. Endogenous Rho activity increases in differentiating keratinocytes and is required for both Fyn kinase activation and increased tyrosine phosphorylation of β - and γ -catenin, which is associated with the establishment of keratinocyte cell–cell adhesion. Conversely, expression of constitutive active Rho is sufficient to promote cell–cell adhesion through a tyrosine kinase- and Fyn-dependent mechanism, trigger Fyn kinase activation, and induce tyrosine phosphorylation of β - and γ -catenin

and p120^{cas}. The positive effects of activated Rho on cell–cell adhesion are not induced by an activated Rho mutant with defective binding to the serine/threonine PRK2/PKN kinases. Endogenous PRK2 kinase activity increases with keratinocyte differentiation, and, like activated Rho, increased PRK2 activity promotes keratinocyte cell–cell adhesion and induces tyrosine phosphorylation of β - and γ -catenin and Fyn kinase activation. Thus, these findings reveal a novel role of Fyn as a downstream mediator of Rho in control of keratinocyte cell–cell adhesion and implicate the PRK2 kinase, a direct Rho effector, as a link between Rho and Fyn activation.

Introduction

Cadherin-mediated cell–cell adhesion plays a crucial function in establishment and maintenance of organized tissues. Although the extracellular domain of cadherins is essential for connecting neighboring cells through calcium-dependent homophilic interactions, the cadherin intracellular domain is crucial for regulating the strength of cell–cell adhesion. This latter aspect of cadherin function is regulated by a complex cross-talk of intracellular signaling pathways (Provost and Rimm, 1999; Gumbiner, 2000). The epidermis provides an excellent experimental system to investigate cell–cell adhesion control in stratified epithelia. The continuous vertical differentiation program of this tissue involves highly dynamic changes in cell–cell adhesion (O’Keefe et al., 1987; Lewis et

al., 1994; Tao et al., 1996). Two major families of signaling proteins have been implicated in positive regulation of E-cadherin-dependent cell–cell adhesion in keratinocytes: Rho-like GTPases (Braga et al., 1997) and Fyn/Src tyrosine kinases (Calautti et al., 1998).

Rho-like GTPases play an important role as regulators of cell–cell adhesion in a manner which varies substantially depending on cell type and cellular context (Hall, 1998). In keratinocytes, both Rac and Rho activities are required for cell–cell junction formation (Braga et al., 1997). In these cells, Rac has been implicated upstream of Rho in remodeling of cortical actin, whereas Rho appears required for cadherin clustering at the cell membrane via an actin-independent mechanism (Braga et al., 1997). The downstream mechanisms responsible for Rho-mediated cell–cell adhesion and the direct Rho effectors involved have not been defined.

Tyrosine phosphorylation of adherens junction and desmosome components have also been implicated in cell–cell adhesion control (Provost and Rimm, 1999; Gumbiner, 2000). Mitogenic growth factor stimulation of receptor tyrosine kinases frequently causes disassembly of adherens junctions together with tyrosine phosphorylation of adherens junction components such as β - and γ -catenin and p120^{cas} (Kanner et al., 1991; Reynolds et al., 1994; Shibamoto et al., 1994, 1995). Similar effects are induced by expression of oncogenic Src kinase (Behrens et al., 1993; Hamaguchi et

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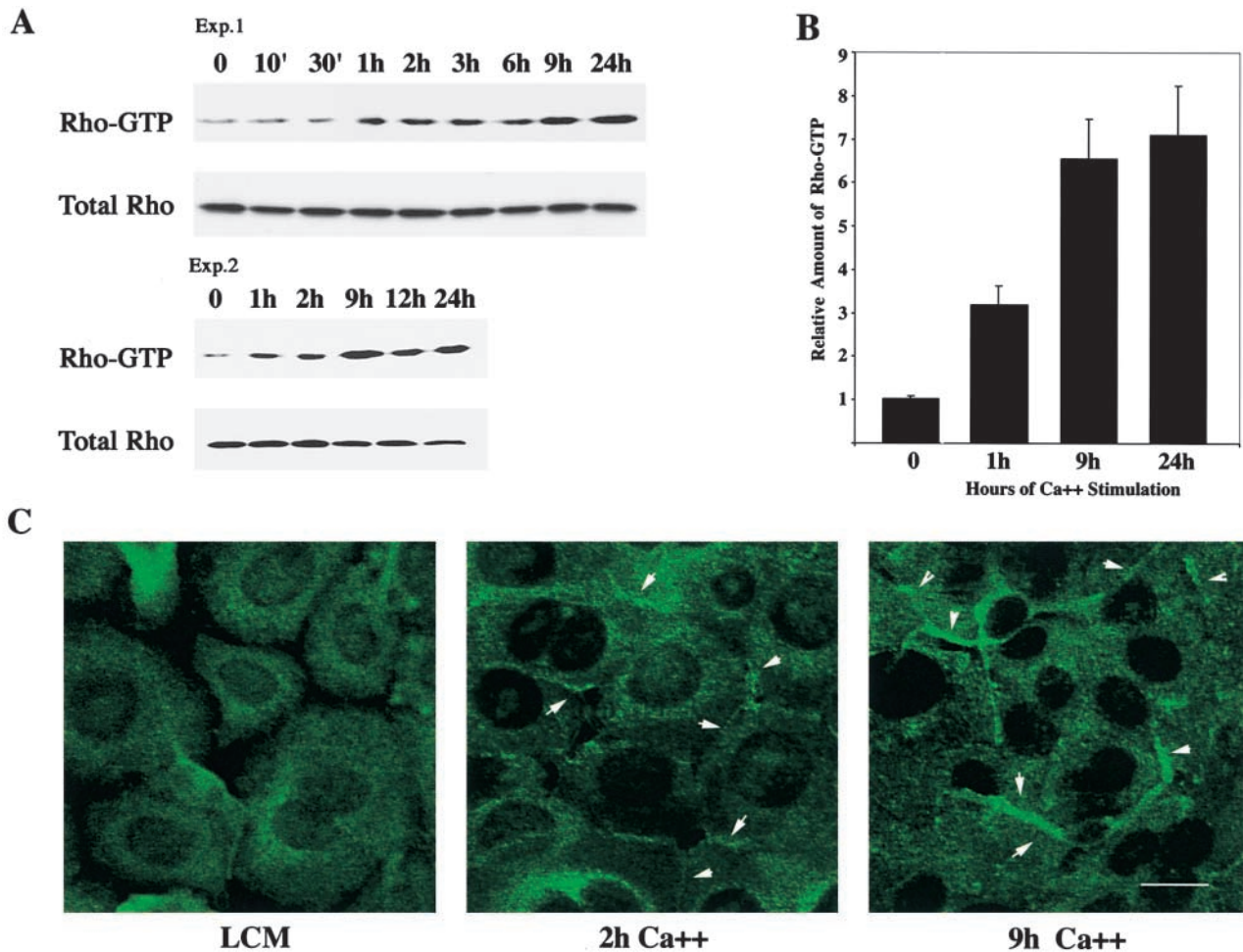


Figure 1. Endogenous Rho activity increases in calcium-induced keratinocyte differentiation. (A) Total cell extracts from mouse primary keratinocytes under low calcium conditions and at various times of calcium exposure were incubated with beads coupled to the Rho-binding domain of rhotekin, which binds specifically to activated Rho. Pull-down assays were analyzed by 12.5% SDS-PAGE and immunoblotting with antibodies against RhoA (top), alongside corresponding total cell extracts, for normalization of total RhoA protein levels (bottom). The results of two independent experiments are shown. (B) Densitometric scanning of the autoradiographs was used to determine relative levels of RhoA activation versus protein amounts. Average Rho activation from three independent experiments, including the ones shown in A, was calculated. Bars refer to value variation among experiments. (C) Keratinocytes under low calcium conditions and at 2 and 9 h of high calcium exposure were fixed in 4% paraformaldehyde and processed for immunofluorescence with anti-Rho A polyclonal antibodies and FITC-conjugated secondaries. Samples were analyzed by confocal microscopy using the same light exposure and image capture conditions. Arrows point to areas of cell-cell contacts in calcium-treated keratinocytes with detectable Rho-specific signal. Bar, 20 μ m.

al., 1993; Calautti et al., 1998) even if in this case disassembly of adherens junctions may be independent of β -catenin phosphorylation and involve tyrosine phosphorylation of other molecules (Takeda et al., 1995). In contrast to the effects of growth factor receptors or oncogenic Src, endogenous tyrosine kinases of the Src family, and Fyn in particular, play a positive role in cell-cell junction formation in keratinocytes both in vitro and in vivo (Calautti et al., 1998). In fact, the establishment of keratinocyte cell-cell adhesions is associated with increased tyrosine phosphorylation of β - and γ -catenin and p120^{cas}, and adherens junction formation is impaired in cells treated with tyrosine kinase inhibitors or lacking Fyn, either alone or in combination with Src (Calautti et al., 1998).

Thus, in keratinocytes both small GTPases of the Rho family and Fyn/Src tyrosine kinases have been implicated independently in positive regulation of cell-cell adhesion.

Here, we show that these two signaling pathways are functionally and biochemically connected and point to the PRK2 kinase, a direct Rho effector (Bishop and Hall, 2000), as a link between Rho and Fyn activation.

Results

Endogenous Rho activity increases with keratinocyte differentiation and is necessary for the establishment of cell-cell adhesion and underlying tyrosine phosphorylation events

Exposure of mouse primary keratinocytes to increased extracellular calcium concentrations provides a well-defined model for the switch between keratinocyte growth and differentiation (Dotto, 1999). To determine whether endogenous Rho activity is regulated during this process, primary keratinocytes under basal conditions and at various times of

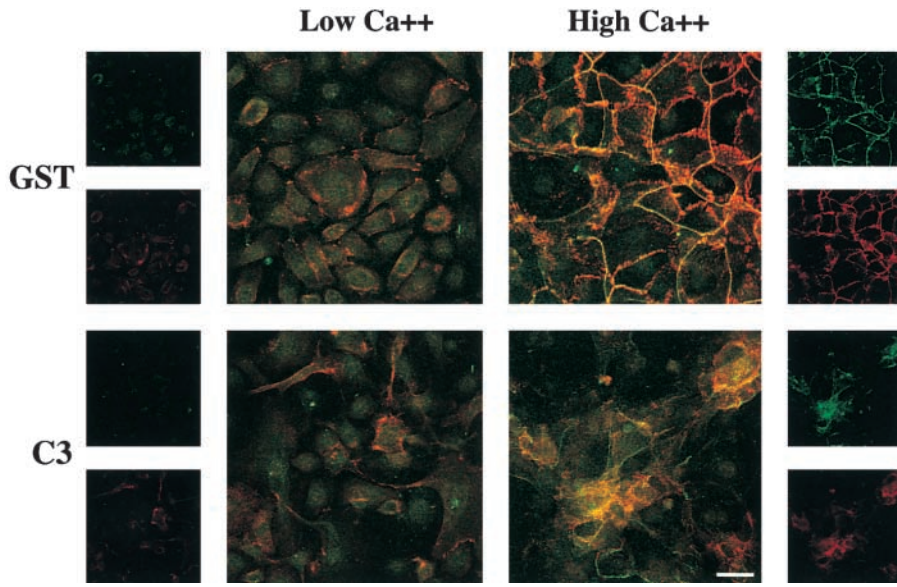


Figure 2. Endogenous Rho activity is required for recruitment of E-cadherin and tyrosine-phosphorylated proteins at cell–cell borders. Keratinocytes were pretreated with a GST-C3 fusion protein (bottom) or GST control (top) for 1.5 h and either exposed to 2 mM CaCl₂ (High Ca²⁺) for 9 h or kept in medium at low calcium concentrations (0.05 mM) (Low Ca²⁺). Cells were briefly preextracted in 0.2% Triton CSK buffer before paraformaldehyde fixation and double stained with E-cadherin-specific monoclonal antibodies followed by Texas red-conjugated secondary antibodies (red) and with FITC-conjugated antiphosphotyrosine antibodies (green). A similar pattern of staining was obtained without preextraction procedure. Samples were analyzed by confocal microscopy using same light exposure and image capture conditions, and green and red images (small panels) were superimposed; sites

of overlapping staining are visualized as yellow (large panels). Each image is the projection of eight focal planes spanning the whole depth of the culture (0.8 μm between individual plans). Note that some cell–cell borders stain only weakly with antiphosphotyrosine antibodies. These correspond to immature borders at the zipper stage as can be visualized at higher magnification. Bar: (large panels) 15 μm; (small panels) 3.3 μm. Similar results were obtained in two other experiments.

calcium exposure (2 mM) were analyzed by pull-down assay with the Rho-binding domain of rhotekin, which specifically binds the GTP-bound activated form of Rho (Ren et al., 1999). Rho activity was found to be induced by 1 h of calcium treatment with a further progressive increase up to 24 h (Fig. 1, A and B). In parallel, immunofluorescence analysis revealed that a fraction of Rho becomes localized to cell–cell contacts in calcium-treated keratinocytes (Fig. 1 C).

To determine whether endogenous Rho activity is required for calcium-induced cell–cell adhesion and underlying tyrosine phosphorylation events (Calautti et al., 1998), primary keratinocytes were pretreated with C3 exotoxin, a specific Rho inhibitor (Boquet, 1999), and subsequently treated with

calcium for 9 h or maintained under low calcium conditions. Cells were stained with antibodies against phosphotyrosine and E-cadherin. Although calcium treatment of control keratinocytes resulted in the expected colocalization of E-cadherin and tyrosine phosphorylated proteins at cell–cell borders (Fig. 2, top), this was impaired significantly in the C3-treated keratinocytes (Fig. 2, bottom).

For direct biochemical determinations, C3-treated and control keratinocytes were processed for immunoprecipitation with E-cadherin antibodies followed by sequential immunoblotting with antibodies against phosphotyrosine and β- and γ-catenin. High calcium exposure induced the expected tyrosine-phosphorylation of β- and γ-catenin in con-

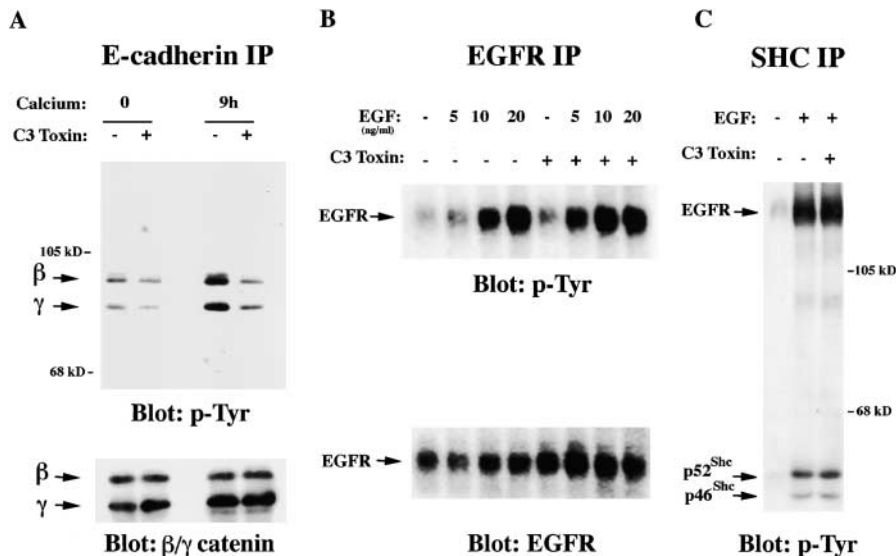


Figure 3. Endogenous Rho activity is required for tyrosine phosphorylation of β- and γ-catenin in response to increased extracellular calcium. (A) Keratinocytes were pretreated for 1.5 h with either GST-C3 (+) or GST control proteins (–) and exposed to 2 mM CaCl₂ for 9 h or maintained under low calcium conditions (0). Cell lysates were immunoprecipitated with anti-E-cadherin antibodies followed by immunoblotting with antibodies against phosphotyrosine or β- and γ-catenin as indicated. (B) Keratinocytes in low calcium medium were serum-starved for 18 h and then pretreated for 1.5 h with either GST-C3 (+) or GST proteins (–). Cells were stimulated for 10 min with EGF at the indicated concentrations followed by immunoprecipitation with antibodies against the EGF receptor (EGFR) and immunoblotting with the same antibodies or antibodies against phosphotyrosine as indicated. (C) Keratinocytes were treated as in B followed by immunoprecipitation with antibodies against SHC and immunoblotting with antiphosphotyrosine antibodies. The position of the p46 and p52 isoforms of SHC and of the EGF receptor are indicated.

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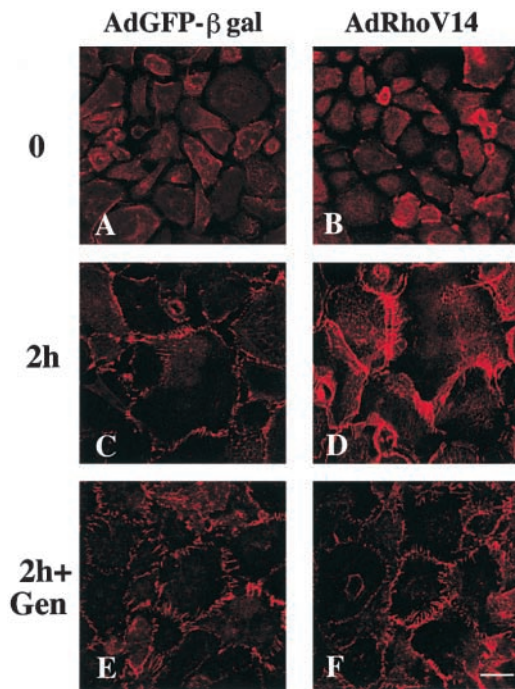


Figure 4. Constitutively active Rho promotes keratinocyte cell–cell adhesion by a tyrosine kinase-dependent mechanism. Keratinocytes were infected with a control adenovirus expressing the GFP and β -gal proteins (AdGFP- β -gal) or an adenovirus expressing a constitutively active RhoA mutant together with GFP (AdRhoV14). Cells were kept in low calcium medium (0) or exposed to high calcium for the last 2 h (2 h) before termination of the experiment (48 h after infection). Parallel experiments were performed with cells pretreated with the tyrosine kinase inhibitor Genistein (100 μ M) for 1 h before calcium exposure (2 h + Gen). 48 h after infection, samples were briefly preextracted with 0.2% Triton CSK buffer, fixed in para-formaldehyde, and stained with E-cadherin-specific antibodies followed by Texas red-conjugated secondary antibodies. The higher E-cadherin signal in RhoV14-expressing cells is due to increased association of E-cadherin with Triton-insoluble cell–cell adhesion structures, since RhoV14 expression causes no increase in E-cadherin expression levels (unpublished data). Samples were analyzed by confocal microscopy using the same light exposure and image capture conditions. Images result from the projection of eight focal plans. Similar results were obtained in three other experiments. Bar, 15 μ m.

control cells, whereas C3 treatment almost completely blocked this response (Fig. 3 A). The C3 inhibition was specific for the calcium-induced tyrosine phosphorylation events, since EGF-induced autophosphorylation of the EGF receptor and phosphorylation of its substrate SHC was not impaired by C3-treatment (Fig. 3, B and C).

Activated Rho promotes cell–cell adhesion in a tyrosine kinase-dependent manner

The above experiments indicated that endogenous Rho function is required for the establishment of cell–cell adhesion and associated tyrosine phosphorylation events. To test whether increased Rho activity promotes cell–cell adhesion in a tyrosine phosphorylation-dependent manner, cells were infected with an adenovirus expressing a constitutively active RhoV14 mutant. Consistent with calcium providing the initial trigger for adherens junction formation (O’Keefe et al., 1987), RhoV14 expression in keratinocytes under low calcium conditions failed to induce cell–cell junctions as assessed by immunofluorescence with anti-E-cadherin antibodies (Fig. 4, A and B). However, upon high calcium exposure formation of mature E-cadherin junctions was significantly anticipated by RhoV14 expression. In fact, RhoV14-expressing keratinocytes showed strong continuous E-cadherin staining at cell–cell borders by 2 h of calcium exposure, whereas control cells showed only the typical zipper-like structures characteristic of immature adherens junctions (Calautti et al., 1998; Vasioukhin et al., 2000) (Fig. 4, C and D). Levels of E-cadherin, β - and γ -catenin, and p120^{cas} were not increased by RhoV14 expression (unpublished data). In a time course of cell–cell junction formation, we showed previously that in calcium-treated keratinocytes the transition from the immature zipper-like structures to fully formed cell–cell junctions can be suppressed by tyrosine kinase inhibition (Calautti et al., 1998). Similarly, the “maturation” effect of RhoV14 expression on cell–cell junction formation was blocked by treatment with the tyrosine kinase inhibitor Genistein. Both control and Rho-expressing cells were arrested by this compound at the zipper-like stage at 2 h of calcium treatment and at later times (Fig. 4, E and F; unpublished data).

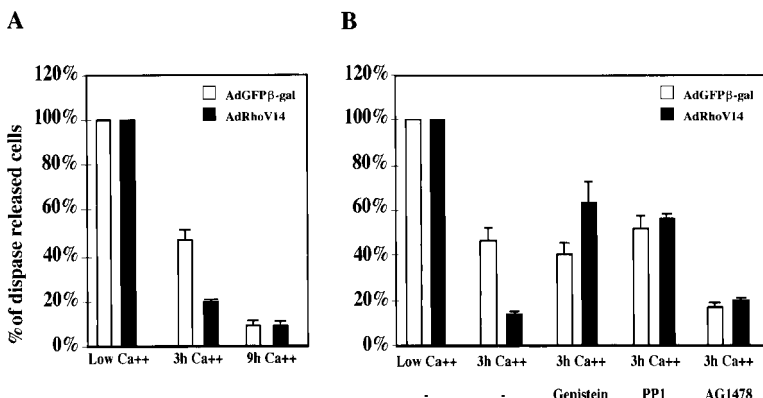
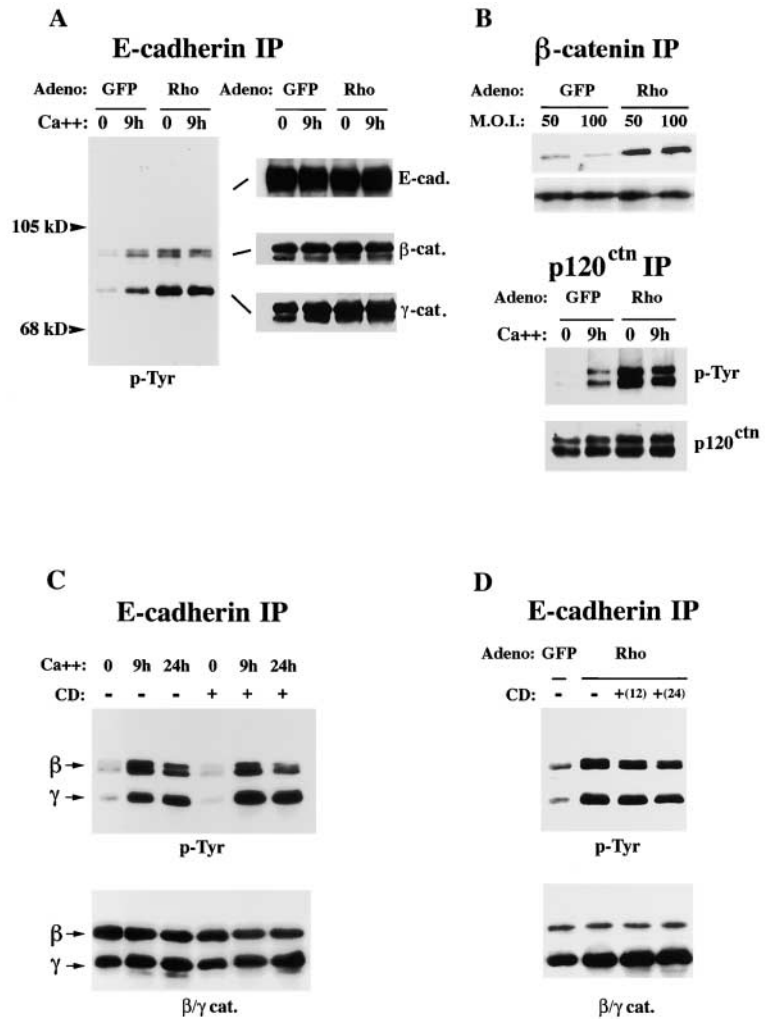


Figure 5. Quantitative measurement of keratinocyte cell–cell adhesion as a function of RhoV14 expression and tyrosine phosphorylation. (A) Triplicate samples of primary keratinocytes infected with control (AdGFP- β -gal; white bars) or RhoV14-expressing (AdRhoV14; black bars) adenoviruses were kept under low calcium conditions (Low Ca²⁺) or treated with calcium for 3 or 9 h. Data are expressed as the percentage of single cells released by mechanical disruption after disperse treatment versus total number of cells recovered after subsequent treatment of the same samples with trypsin. The difference in single cell release from the RhoV14-expressing keratinocytes versus same cells treated with Genistein was found to be statistically significant ($P < 0.004$) as assessed by Kruskal-Wallis test. (B) A similar assay was performed

with adenovirus-infected keratinocytes under low calcium conditions or incubated with calcium for 3 h in the absence or the presence of Genistein (100 μ M), PP1 (5 μ M), or AG1478 (5 μ M). Inhibitors were added to the medium 12 h after adenovirus infection, and cells were kept in low calcium medium or exposed to 2 mM CaCl₂ for the last 3 h before termination of the experiment (48 h after infection).

Figure 6. Activated Rho induces tyrosine phosphorylation of β - and γ -catenin and p120^{ctn}. (A)

Keratinocytes were infected with the control AdGFP- β -gal or AdRhoV14 adenoviruses and kept in low calcium medium (0) or exposed to 2 mM CaCl₂ for the last 9 h (9 h) before termination of the experiment (48 h after infection). Cell extracts were immunoprecipitated with antibodies against E-cadherin, and the immune complexes were analyzed by sequential immunoblotting with antibodies against phosphotyrosine, E-cadherin, β -catenin, and γ -catenin as indicated. The slight increase in total levels of γ -catenin in the RhoV14-expressing keratinocytes was not reproducibly observed in other experiments (for instance in C). (B) Extracts from keratinocytes infected with the AdGFP- β -gal and AdRhoV14 adenoviruses were immunoprecipitated with antibodies against β -catenin (top) or p120^{ctn} (bottom) followed by sequential immunoblotting with antibodies against phosphotyrosine and the corresponding proteins as indicated. The β -catenin immunoprecipitates were derived from keratinocytes under low calcium conditions infected with adenoviruses at two different multiplicity of infection (moi). The p120^{ctn} immunoprecipitates were from adenovirally infected keratinocytes with or without calcium treatment for 9 h. The slightly lower levels of p120^{ctn} tyrosine phosphorylation in the RhoV14-expressing keratinocytes after calcium treatment were not seen in other experiments. (C) Keratinocytes were pretreated for 1.5 h with either solvent alone (-) or 1 μ M cytochalasin D (CD; +) and either kept in low calcium medium (0) or exposed to 2 mM CaCl₂ for 9 or 24 h. Cell lysates were immunoprecipitated with E-cadherin-specific antibodies followed by immunoblotting with antibodies against phosphotyrosine and β - and γ -catenin as indicated. (D) Keratinocytes were infected with either the AdGFP- β -gal or AdRhoV14 adenoviruses and either untreated (-) or treated with 1 μ M cytochalasin D (CD) 12 or 24 h before termination of the experiment (48 h after infection). Cell lysates were analyzed as in C. Preliminary experiments showed that the indicated concentration and time of cytochalasin D treatment was sufficient to totally disrupt actin cables (as visualized by FITC-conjugated phalloidin) without affecting cell viability or AdRhoV14 expression.



We previously developed a quantitative cell–cell adhesion assay based on dispase, a protease that degrades preferentially extracellular matrix proteins, which function as anchors of keratinocytes to the substratum (Calautti et al., 1998). Dispase treatment of keratinocytes in low calcium medium causes detachment of all cells as single cell suspension. Conversely, keratinocytes switched to high calcium medium for 9 h or longer have formed strong cell–cell adhesions and are much more resistant to dispase, eventually detaching from the dish as a confluent sheet of cells. Under low calcium conditions, dispase treatment of both control and RhoV14-expressing keratinocytes caused the expected release of all cells singly into suspension, whereas by 9 h of calcium treatment they were mostly resistant (Fig. 5 A). Instead, after 3 h of calcium treatment only 20% of Rho-expressing keratinocytes were released into suspension as single cells as opposed to 47% of the control. The greater resistance of Rho-expressing keratinocytes to dispase at 3 h of calcium exposure was reduced to control levels by treatment with the broad specificity tyrosine kinase inhibitor Genistein and the inhibitor of Src family kinases PP1 (Hanke et al., 1996; Fig. 5 B), providing a first indication that

these kinases are involved. In contrast to Genistein or PP1, treatment with the EGF receptor inhibitor AG1478 did not affect the dispase resistance of Rho-expressing keratinocytes but rather increased that of control cells consistent with an enhancement of cell–cell adhesion by suppression of mitogenic signals (Gumbiner, 2000). None of the indicated inhibitors affected the expression of the virally transduced Rho V14 gene as assessed by immunoblotting (unpublished data).

Activated Rho induces tyrosine phosphorylation of β - and γ -catenins and p120^{ctn} independently of the integrity of the actin cytoskeleton

Underlying the above effects, activated Rho may induce the specific tyrosine phosphorylation events connected with the establishment of cell–cell adhesion (Calautti et al., 1998). In fact, immunoblot analysis of E-cadherin immunoprecipitates showed that tyrosine phosphorylation of β - and γ -catenin was substantially increased in RhoV14-expressing keratinocytes under low calcium conditions to the same levels as after high calcium exposure (Fig. 6 A).

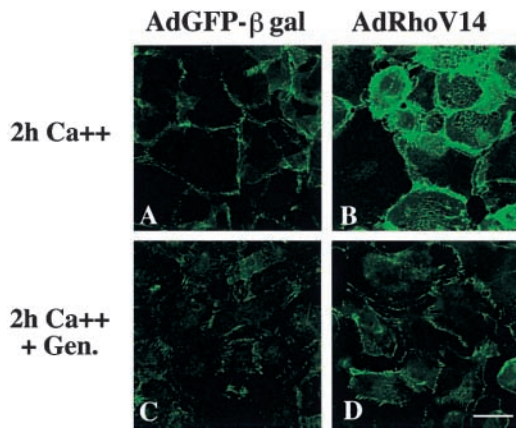


Figure 7. Constitutively active Rho promotes recruitment of p120^{ctn} to cell-cell adhesions in a tyrosine kinase-dependent manner. Keratinocytes were infected with AdGFP- β -gal or AdRhoV14 adenoviruses and exposed to high calcium concentrations for the last 2 h (2 h) before termination of the experiment (48 h after infection) (top). Parallel experiments were performed with cells pretreated with the tyrosine kinase inhibitor Genistein (100 μ M) for 1 h before calcium exposure (2 h Ca⁺⁺ + Gen) (bottom). Samples were processed for immunofluorescence analysis with anti-p120^{ctn} antibodies and FITC-conjugated secondaries as in the legend to Figs. 2 and 4. Samples were analyzed by confocal microscopy using same light exposure and image capture conditions. Images result from the projection of eight focal plans. Parallel analysis of keratinocytes under low calcium conditions and at 9 h of calcium treatment yielded a pattern of staining for p120^{ctn} similar to that observed for E cadherin (as shown in Fig. 4). Bar, 20 μ m.

p120^{ctn} associates with E-cadherin less strongly than β - and γ -catenin so that it is recovered from E-cadherin complexes only after immunoprecipitation under low stringency conditions and can best be analyzed by direct immunoprecipitation with the corresponding specific antibodies (Calautti et al., 1998). We found that tyrosine phosphorylation levels of p120^{ctn}, like directly immunoprecipitated β -catenin, were also substantially elevated in RhoV14-expressing keratinocytes (Fig. 6 B). The localization of p120^{ctn} to the cell membrane is dependent on its association with cadherins (Thoreson et al., 2000), which is in turn positively regulated by p120^{ctn} tyrosine phosphorylation (Calautti et al., 1998; Anastasiadis and Reynolds, 2000). Immunofluorescence analysis revealed that at early times of calcium treatment RhoV14 expression enhanced significantly recruitment of p120^{ctn} to cell-cell junctions in a tyrosine kinase-dependent manner (Fig. 7).

Expression of RhoV14 induced the expected increase in actin cables, which could be disrupted by cytochalasin D treatment (unpublished data). By contrast, cytochalasin treatment did not block the establishment of calcium-induced cell-cell contacts and had no effects on the induction of β - and γ -catenin tyrosine phosphorylation by either increased extracellular calcium or active Rho (Fig. 6, C and D).

Activated Rho induces tyrosine phosphorylation of β - and γ -catenin and p120^{ctn} through a Fyn/Src-dependent mechanism

We showed previously that keratinocyte differentiation is associated with induction of Fyn kinase activity (Calautti et al., 1995), and Fyn either alone or in combination with Src

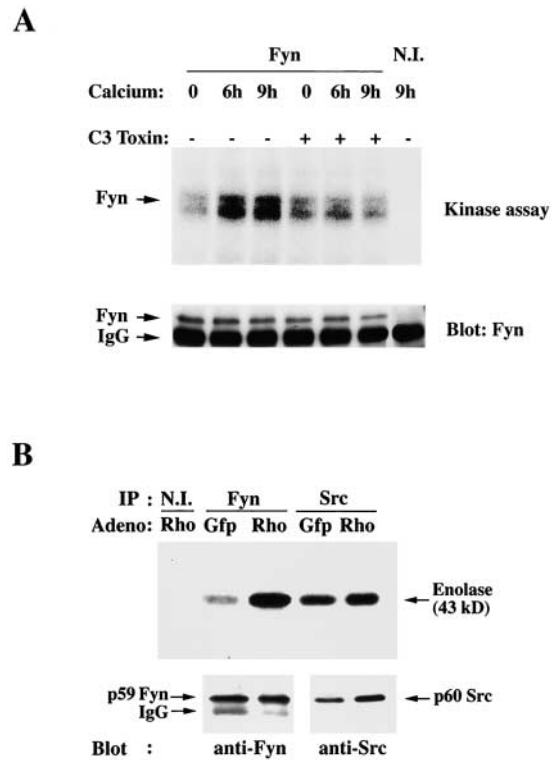
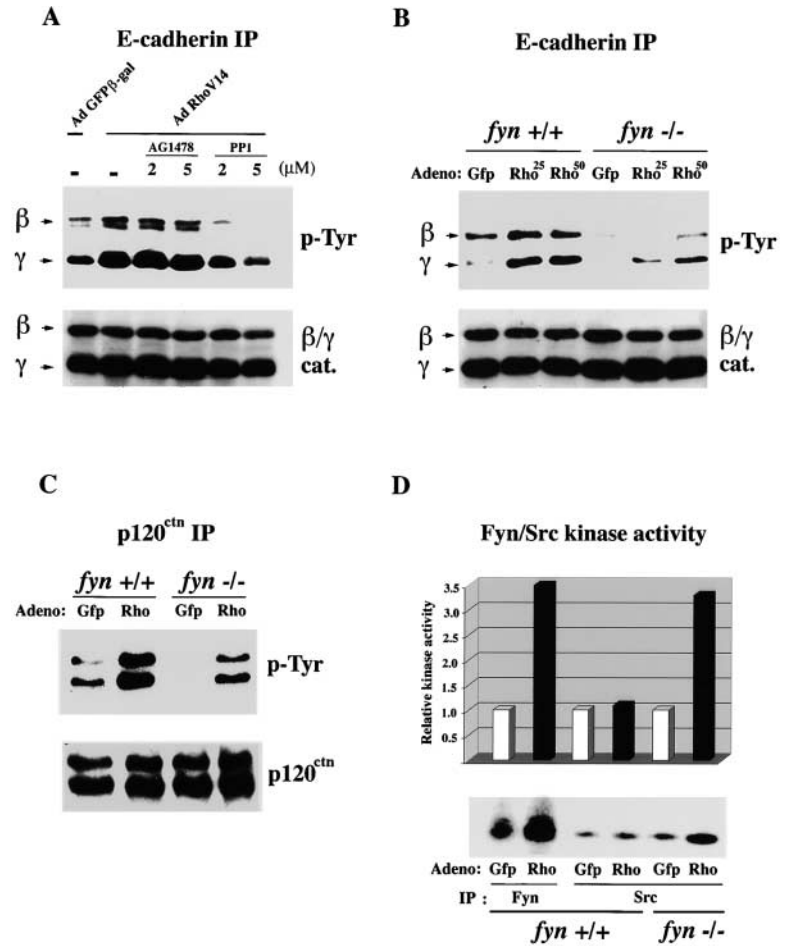


Figure 8. Rho activity is both required and sufficient for induction of Fyn tyrosine kinase activity. (A) Keratinocytes were pretreated for 1.5 h with either the GST-C3 (+) or GST proteins (-). Cells were maintained under low calcium conditions or exposed to 2 mM CaCl₂ for the indicated times and immunoprecipitated with Fyn-specific antibodies or nonimmune IgG (N.I.) control. Half of the immune complexes were processed for in vitro kinase assay with [γ -³²P]ATP without exogenous substrate (top) and half analyzed by immunoblotting with anti-Fyn antibodies (bottom). Positions of Fyn and IgG heavy chains are indicated. Densitometric scanning of the autoradiographs after normalization for Fyn protein amounts indicated that Fyn activity as measured by the autophosphorylation signal was induced 3.6-fold in control cells after 6 and 9 h of calcium treatment, whereas in the C3-pretreated cells Fyn activity increased only 1.2-fold after calcium exposure. (B) Keratinocytes infected with control AdGFP- β -gal and AdRhoV14 adenoviruses were immunoprecipitated with anti-Fyn or anti-Src antibodies or with nonimmune rabbit IgGs (N.I.) as indicated. Half of the immune complexes were subjected to in vitro kinase assays with [γ -³²P]ATP and enolase as exogenous substrate (top); half of the immune complexes were processed for immunoblotting with Src- or Fyn-specific antibodies (bottom). The enolase bands were excised from the gel, and their ³²P content was determined by direct scintillation counting and normalized for kinase protein amounts as determined by densitometric scanning of the immunoblots. The ratio of Fyn kinase activity in Rho-expressing versus control keratinocytes was 3.2, whereas the ratio of Src kinase activity was 1.2. Similar results were obtained in three other experiments.

is required for β - and γ -catenin and p120^{ctn} tyrosine phosphorylation (Calautti et al., 1998). Calcium treatment induced Fyn kinase activation in control keratinocytes, whereas no such induction was observed in cells concomitantly treated with the C3 toxin (Fig. 8 A). Conversely, Fyn kinase activity was induced by RhoV14 expression in keratinocytes under low calcium conditions, whereas little or no increase of Src activity was detected (Fig. 8 B). In parallel with these results, treatment with the Src family kinase in-

Figure 9. Increased catenin tyrosine phosphorylation by activated Rho is mediated by Fyn/Src kinases. (A) Keratinocytes were infected for 48 h with the AdGFP-β-gal or AdRhoV14 adenoviruses. 12 h after infection, cells were treated with solvent alone (–) or with the AG1478 or PP1 inhibitors at the indicated concentrations. Cell extracts were immunoprecipitated with antibodies against E-cadherin followed by immunoblotting with antibodies against phosphotyrosine and β- and γ-catenin as indicated. (B) Primary keratinocytes derived from *fyn*^{-/-} and *fyn*^{+/+} mice were infected with control AdGFP-β-gal or AdRhoV14 adenoviruses at an moi of 25 and 50 (Rho²⁵ and Rho⁵⁰, respectively). Cell lysates were analyzed as in A. (C) Keratinocytes from *fyn*^{-/-} and *fyn*^{+/+} mice were infected with AdGFP-β-gal and AdRhoV14 adenoviruses and immunoprecipitated with antibodies against p120^{ctn} followed by immunoblotting with the same antibodies or antibodies against phosphotyrosine as indicated. (D) Keratinocytes from *fyn*^{-/-} and *fyn*^{+/+} mice were infected with AdGFP-β-gal and AdRhoV14 adenoviruses as above and immuno-precipitated with antibodies against Fyn or Src followed by in vitro kinase assays without exogenous substrates. The graph shows quantification of Fyn and Src activity as determined by densitometric scanning of the autophosphorylation signal after normalization for protein amounts. Similar results were observed by in vitro kinase assays in the presence of enolase as exogenous substrate (unpublished data).



hibitor PP1 reduced tyrosine phosphorylation of β- and γ-catenin in RhoV14-expressing keratinocytes to levels similar to the control, whereas treatment with the EGF receptor inhibitor AG1478 had no effect (Fig. 9 A).

Primary keratinocytes derived from Fyn-deficient mice exhibit a flatter morphology, lack of stratification (Calautti et al., 1995), and suppression of β- and γ-catenin tyrosine phosphorylation relative to wild-type controls (Calautti et al., 1998). RhoV14 expression in these cells induced tyrosine phosphorylation of β- and γ-catenin and p120^{ctn} to a substantially lesser extent than in wild-type controls (Fig. 9, B and C), indicating that Fyn plays an important even if not exclusive role in these tyrosine phosphorylation events. Our previous work indicates that functional compensation between the Fyn and Src kinases in keratinocytes can occur (Calautti et al., 1998), and this could also apply to mediation of the Rho-dependent effects. In fact, RhoV14 expression, which induced Fyn but not Src activity in wild-type cells, induced Src activity in Fyn-negative cells (Fig. 9 D).

Endogenous PRK2 activity increases with keratinocyte differentiation and is required for establishment of cell–cell adhesion and associated tyrosine phosphorylation

We showed previously that the Fyn tyrosine kinase is activated in keratinocytes by increased PKC activity (Cabodi et al., 2000). Among the direct Rho effectors, the closely related PKN and PRK2 serine/threonine kinases share signifi-

cant homology with PKCs in their catalytic region (Bishop and Hall, 2000). To test whether PRK2/PKN mediates the effects of activated Rho on cell–cell adhesion, keratinocytes were transfected with plasmid expression vectors for either RhoV14 or the RhoV14-Y42C mutant, which is selectively defective in PRK2/PKN binding (Sahai et al., 1998). Both vectors also carried a green fluorescent protein (GFP)* reporter gene for identification of transfected cells. Transfected cultures were exposed to calcium for 2 h to trigger the initial calcium-dependent events. Immunofluorescence confirmed that at this early time of calcium exposure, RhoV14 expression promotes recruitment of E-cadherin to cell–cell junctions (Fig. 10 A). Importantly, RhoV14 expression in single isolated cells was sufficient for this effect. Unlike RhoV14, expression of the RhoV14-Y42C mutant even in immediately adjacent cells failed to promote recruitment of E-cadherin to cell–cell junctions (Fig. 10 A). Transfection of the RhoV14 and RhoV14-Y42C vectors resulted in identical protein expression and enhanced focal adhesion formation similarly (Fig. 10, B and C).

To test whether endogenous PRK2 and/or PKN activities increase with differentiation, keratinocyte extracts were immunoprecipitated with the corresponding antibodies followed by in vitro kinase assays. PRK2 kinase activity was induced at 1 h of

*Abbreviations used in this paper: GFP, green fluorescent protein; GST, glutathione S-transferase; moi, multiplicity of infection.

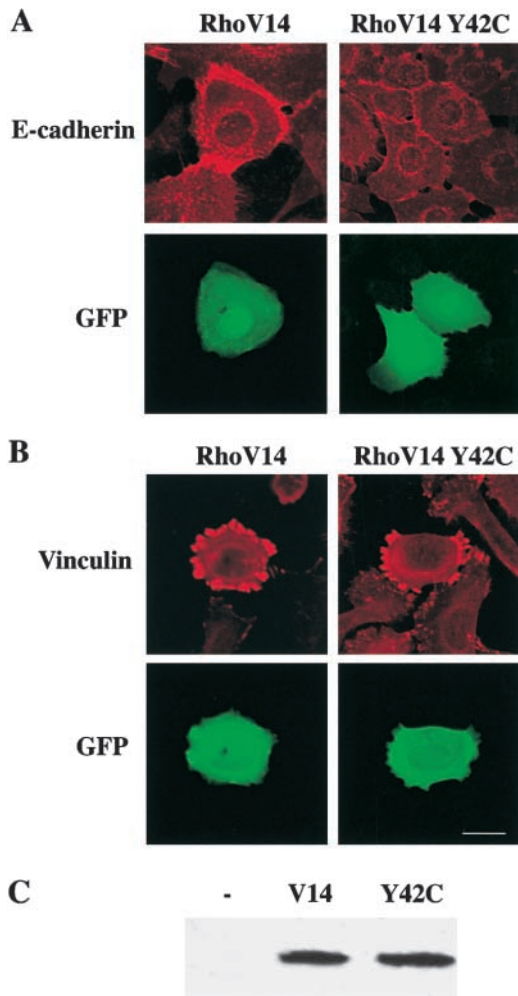


Figure 10. The enhanced recruitment of E-cadherin to cell–cell adhesions by activated Rho is dependent on PRK2/PKN binding. (A) Primary keratinocytes were transfected with plasmid vectors concomitantly expressing GFP for identification of transfected cells and activated RhoV14 (left) or the RhoV14-Y42C mutant (right). 2 d after transfection, cultures were treated with calcium for 2 h before fixation and processed for immunofluorescence with anti-E-cadherin antibodies and rhodamine red-coupled secondaries. Samples were analyzed by confocal microscopy using same image capture conditions. Images are representative of two independent experiments in which a minimum of 20–30 transfected cells were analyzed for each condition. (B) Keratinocytes transfected with the same vectors as in A and maintained under low calcium conditions were processed for immunofluorescence with antivinculin antibodies for focal adhesion visualization. (C) HeLa cells were transfected with the same vectors as in A and B and analyzed by immunoblotting with antibodies against the myc-tagged Rho proteins. Bar, 20 μ m.

calcium treatment, remaining at elevated levels until at least 24 h of high calcium exposure. The extent of PRK2 induction by calcium was similar to that caused by RhoV14 expression (Fig. 11, A and B). PKN kinase activity was only transiently induced at 1 h of calcium treatment and to a limited extent (Fig. 11 C).

Increased PRK2 expression induces catenin tyrosine phosphorylation and Fyn activation

To test whether increased PRK2 activity induces some of the Rho effects on cell–cell adhesion, we constructed a PRK2-

expressing adenovirus. The minimal amount of the PRK2 adenovirus required to infect all keratinocytes and induce the effects described below resulted in an approximately threefold increase of PRK2 expression over endogenous levels (Fig. 12 A). As with cells expressing RhoV14, at early times of calcium exposure keratinocytes infected with the PRK2 adenovirus showed enhanced cell–cell adhesion (Fig. 12, B and C).

Tyrosine phosphorylation of β - and γ -catenin and p120^{cas} was also induced by PRK2 overexpression (Fig. 13 A, top), this effect being suppressed by treatment with the Fyn/Src inhibitor PP1 (Fig. 13 B). Immunoprecipitation of Fyn and Src followed by *in vitro* kinase assay showed that Fyn but not Src kinase activity was induced by PRK2 overexpression (Fig. 14, A and B). Increased tyrosine kinase activity was also observed *in vitro* after incubation of Fyn immunoprecipitates with a recombinant protein corresponding to the constitutively active domain of PKN (highly homologous to PRK2) compared with incubation of the same immunoprecipitates with a kinase-dead mutant form of PKN (Yoshinaga et al., 1999; Fig. 14 C). A greater induction was observed with Fyn immunoprecipitates derived from serum-starved keratinocytes versus cells in fully supplemented medium due to higher basal levels of Fyn activity in the latter condition.

Discussion

We have demonstrated previously that establishment of keratinocyte cell–cell adhesion is linked to increased tyrosine phosphorylation of E-cadherin-associated catenins and that the Fyn tyrosine kinase plays a key role in this process (Calautti et al., 1998). Studies from another group indicate that the activities of the Rac and Rho GTPases are also essential for keratinocyte adherens junction formation (Braga et al., 1997) similar to what has been reported for the MDCK epithelial kidney cell line (Takaishi et al., 1997). Here, we show that the positive function of Rho in keratinocyte cell–cell adhesion is closely linked to downstream tyrosine phosphorylation events. Increased tyrosine phosphorylation of β - and γ -catenin and p120^{cas} is dependent on endogenous Rho function and can be triggered by increased Rho activity. Rho activity is also required and sufficient for Fyn kinase activation, and Fyn in turn is an essential downstream mediator of Rho function in cell–cell adhesion. A link between Rho and Fyn activation in control of cell–cell adhesion is provided by PRK2, a fatty acid/Rho-dependent serine/threonine kinase with a so far elusive function (Bishop and Hall, 2000).

A cross-talk between Rho GTPases and tyrosine kinases has been implicated in several processes, including adhesion to the substrate (Flinn and Ridley, 1996), response to mitogenic growth factors (Belsches et al., 1997), and gene expression (Hill et al., 1995; Mao et al., 1998; Tominaga et al., 2000). The actin cytoskeleton is a crucial mediator of the cross-talk between Rho and Src kinases at least at the level of adhesion to the substrate (Fincham et al., 1996; Murakami et al., 1999). We have shown here a functional interconnection between Rho and Fyn/Src kinases also at the level of cell–cell adhesion, which is independent of the integrity of the actin cytoskeleton. A complex cross-regulation between Rho and Fyn activities at the level of adherens junctions is

PRK2/PKN Kinase Activity

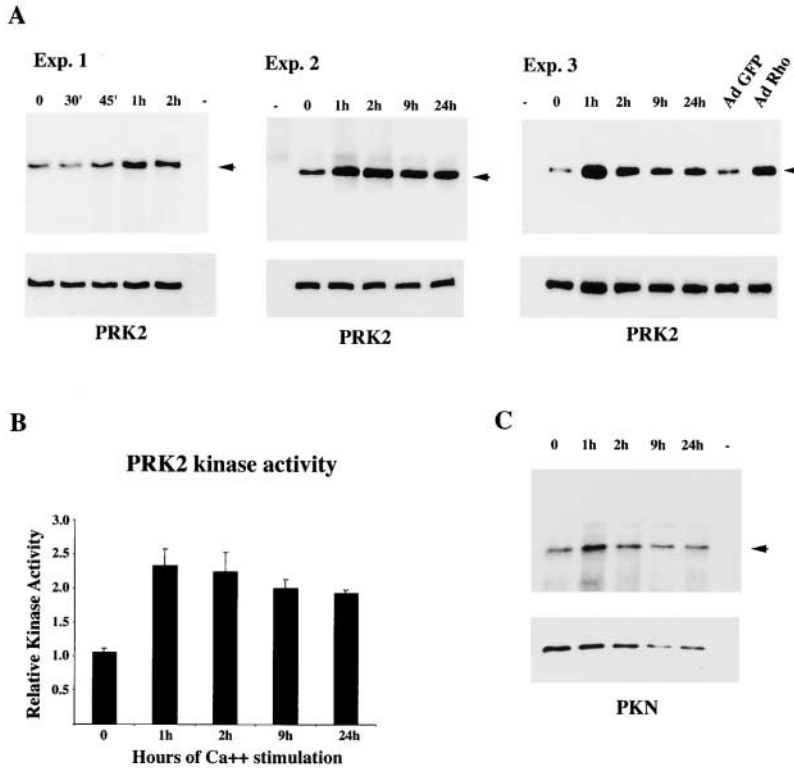


Figure 11. Endogenous PRK2 activity increases with keratinocyte differentiation. (A) Total cell extracts from keratinocytes under low calcium conditions (0) and at various times of high calcium exposure were immunoprecipitated with antibodies against PRK2 or nonimmune IgG control (–) and processed for *in vitro* autophosphorylation kinase assay with [γ -³²P]ATP. Half of the immune complexes were analyzed by immunoblotting with anti-PRK2 antibodies for normalization of kinase amounts (bottom). The results of three independent experiments are shown. Experiment 3 includes a parallel analysis of keratinocytes infected for 48 h with the AdGFP- β -gal and AdRhoV14 adenoviruses. (B) Results were quantified by densitometric scanning of the autoradiographs and normalized for PRK2 protein amounts. Values are expressed as arbitrary units of kinase activity relative to basal levels in keratinocytes under low calcium conditions. Average values of PRK2 kinase activity from the three experiments shown in A were calculated. Bars refer to variation in relative kinase activity at the same time points among different experiments. (C) Total cell extracts from mouse primary keratinocytes under low calcium conditions (0) and at various times of high calcium exposure were immunoprecipitated with antibodies against PKN or nonimmune IgG control (–) and processed for *in vitro* autophosphorylation kinase assay with [γ -³²P]ATP. Half of the immune complexes were analyzed by immunoblotting with anti-PRK2 antibodies for normalization of protein amounts (bottom). By densitometric scanning of the autoradiographs and normalization for PKN protein amounts, PKN kinase activity was found to increase 1.8- and 1.5-fold at 1 and 2 h of calcium treatment, respectively, returning to basal levels thereafter.

likely to exist. In particular, p120^{cas} is a well-established substrate for Src family kinases, and tyrosine phosphorylation of this catenin can promote its association with E-cadherin (Anastasiadis and Reynolds, 2000). p120^{cas} has been implicated as a negative regulator of Rho activity, and these inhibitory effects may be relieved by the increased association of this catenin (through increased tyrosine phosphorylation) with cadherin complexes (Anastasiadis et al., 2000). Our finding that Rho activation also causes Fyn-dependent tyrosine phosphorylation of p120^{cas} points to a possible positive regulatory loop between Rho and Fyn activities mediated by p120^{cas}/cadherin complex formation.

Biochemical activation of a Src family kinase as a consequence of increased Rho activity was not reported previously. We showed recently that PKC- η , a specific PKC isoform whose expression is induced in differentiating keratinocytes, can directly cause Fyn activation (Cabodi et al., 2000). However, the ability of activated Rho to induce tyrosine phosphorylation of β - and γ -catenin is not suppressed by treatment with a PKC- η -specific inhibitor, and tyrosine phosphorylation of these catenins is not induced by PKC- η overexpression (unpublished data). Among direct Rho effectors, the related PKN and PRK2 kinases share significant homology with PKC family members in their catalytic domain (Bishop and Hall, 2000). We have shown that increased PRK2 activity exerts positive effects on cell–cell adhesion similar to those of activated Rho. However, it

causes no suppression of keratinocyte growth nor terminal differentiation marker expression (unpublished data), which are instead induced by PKC- η activation (Cabodi et al., 2000). Thus, although both PRK2 and PKC- η induce Fyn kinase activity their differential biological effects suggest the existence of different pools of Fyn, which may be separately activated by the two kinases and target different substrates.

The activities of endogenous Rho, PRK2, and PKN are induced in keratinocytes at 1 h of calcium treatment. However, although Rho activity further increases at later times of calcium exposure PRK2 activity remains elevated but does not further increase, and PKN activity returns back to basal levels consistent with a complex control of these kinases besides Rho (Yoshinaga et al., 1999; Flynn et al., 2000). The progressive increase of Rho activity starting at 1 h of calcium-induced differentiation parallels that of Fyn (Calautti et al., 1995). Thus, in addition to persistently elevated PRK2 activity other factors likely contribute to Fyn activation, especially at the later times. Recent studies have shown that another class of direct Rho effectors, mDia/Formin proteins (mDia-1 and -2) can associate directly with Src kinases and change their three-dimensional conformation without being sufficient to induce their activation (Tominaga et al., 2000). Thus, by analogy with cooperation of multiple Rho effectors in stress fiber formation or SRF factor activation (Van Aelst and D'Souza-Schorey, 1997; Sahai et al., 1998) it is possible that PKN/PRK2 and mDia-1 and -2 are concom-

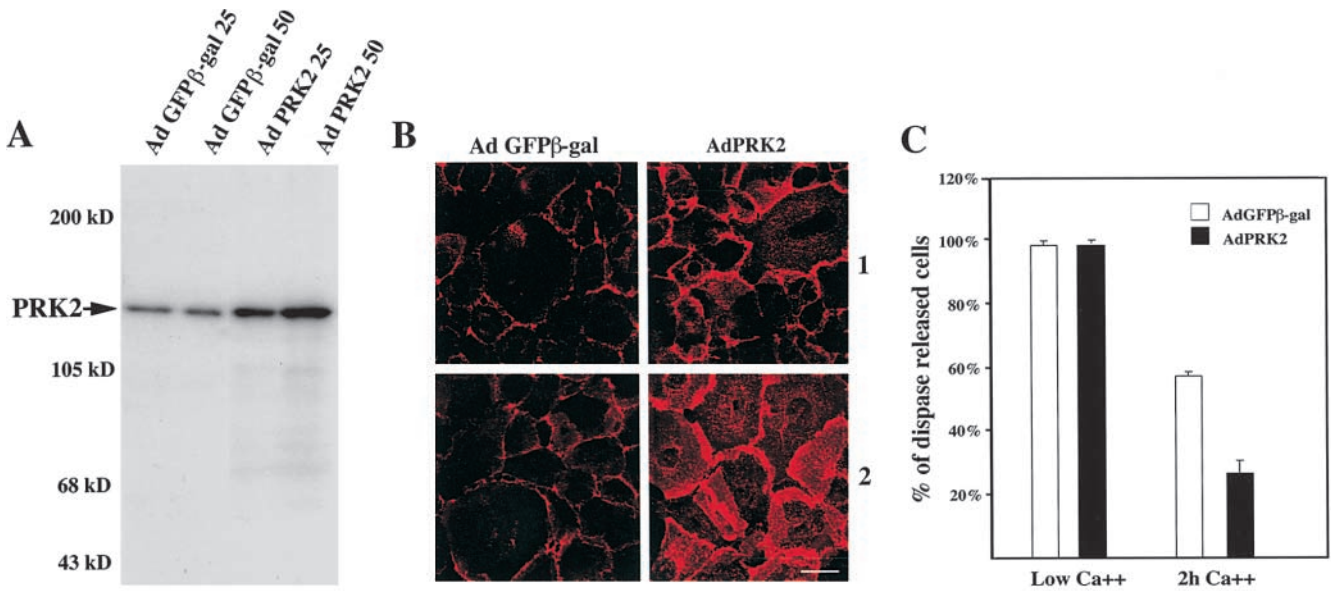


Figure 12. Increased PRK2 activity promotes establishment of keratinocyte cell-cell adhesion. (A) Keratinocytes were infected with either AdGFP-β-gal- or a PRK2-expressing adenovirus at 25 (AdPRK2 25) or 50 moi (AdPRK2 50). 48 h after infection cell extracts were analyzed by 7.5% SDS-PAGE and immunoblotting with anti-PRK2 antibodies. The position of the PRK2 protein is indicated. Densitometric analysis of the autoradiograph revealed an increase of 3.7- and 6-fold in PRK2 expression in cells infected with AdPRK2 at a 25 and 50 moi, respectively, relative to cells infected with the AdGFP-β-gal virus. (B) Keratinocytes infected with the AdGFP-β-gal and AdPRK2 adenoviruses were exposed to high calcium concentrations for 2 h before termination of the experiment (48 h after infection). Samples were processed for immunofluorescence analysis with anti-E-cadherin antibodies and rhodamine red-conjugated secondaries and analyzed by confocal microscopy using the same light exposure and image capture conditions. Each image is the projection of eight focal plans. 1 and 2 refer to images derived from two independent experiments. (C) Triplicate dishes of keratinocytes infected with AdGFP-β-gal (white bars) or AdPRK2 (black bars) adenoviruses for 48 h were kept under low calcium conditions or treated with calcium for 2 h before disperse-based cell-cell adhesion assay (Calautti et al., 1998). Data are expressed as the percentage of single cells released by mechanical disruption after disperse treatment versus total number of cells recovered after subsequent treatment of the same samples with trypsin. Bar, 15 μm.

itantly involved in the Rho-dependent effects on catenin tyrosine phosphorylation and underlying Fyn activation.

Materials and methods

Cells

Primary keratinocytes were isolated from newborn Sencar mice or mice with a disruption of the *fyn* gene (Stein et al., 1992) and genetically matched

wild-type controls and cultured in medium with low calcium concentration (0.05 mM CaCl₂; low calcium medium) as described previously (Calautti et al., 1998). The disperse-based cell-cell adhesion assay was as described previously (Calautti et al., 1998). Bacterially expressed C3 transferase toxin or glutathione *S*-transferase (GST) control proteins were purified according to Dillon and Feig (1995) and delivered into the cells by osmotic shock (Okada and Rechsteiner, 1982). Briefly, cells were incubated for 30 min in 10% PEG 8000, 0.5 M sucrose in low calcium medium without serum, and EGF in the presence of 30 μg/ml of either GST control or GST-C3 proteins followed by incubation in hypotonic medium (60% serum/EGF-free low calcium me-

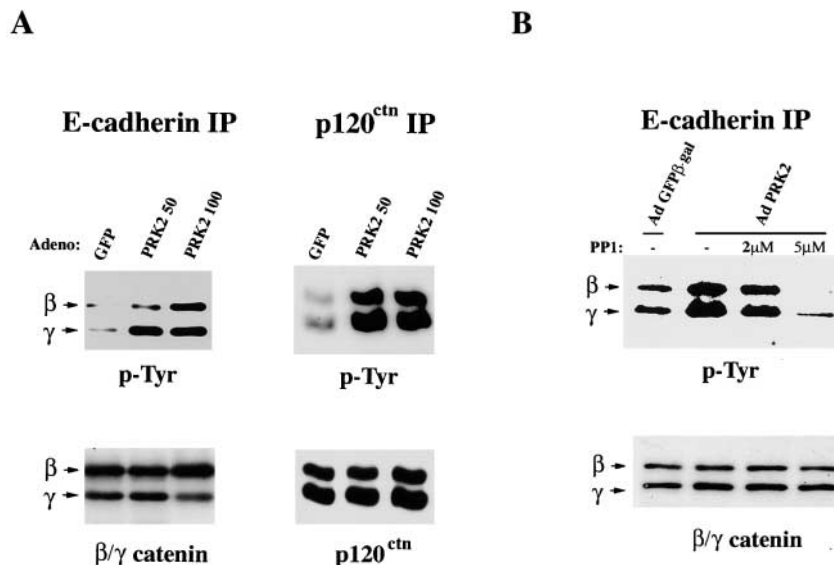


Figure 13. Expression of PRK2 induces catenin tyrosine phosphorylation in a Src family-dependent fashion. (A) Keratinocytes were infected with the AdGFP-β-gal or AdPRK2 adenovirus at 50 (PRK2 50) or 100 moi (PRK2 100). 48 h after infection, cell extracts were immunoprecipitated with antibodies against E-cadherin (left) or p120^{ctn} (right) followed by immunoblotting with antibodies against phosphotyrosine (p-Tyr; top) or β- and γ-catenin (β/γ catenin; bottom left) and p120^{ctn} (bottom right). Similar results were obtained in three independent experiments. (B) Keratinocytes were infected at 100 moi with control (AdGFP-β-gal) or PRK2-expressing (PRK2) adenoviruses. 12 h after infection, cells were treated with solvent alone (-) or the indicated concentrations of the PP1 inhibitor. 48 h after infection, cell extracts were immunoprecipitated with antibodies against E-cadherin followed by immunoblotting with antibodies against phosphotyrosine (p-Tyr; top) or β- and γ-catenin (β/γ catenin; bottom).

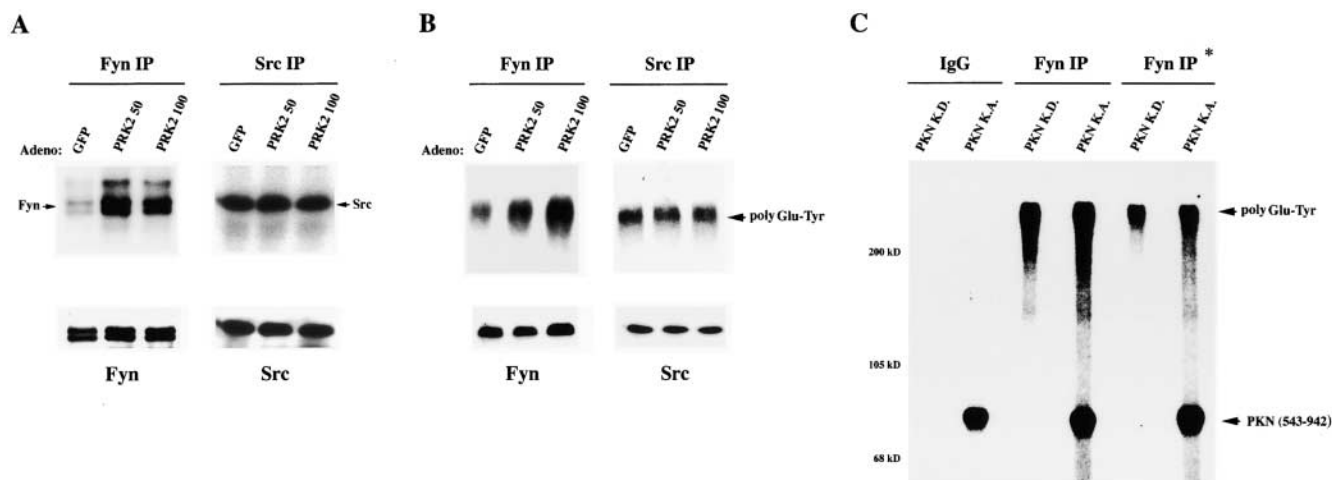


Figure 14. Expression of PRK2 triggers Fyn kinase activation. (A) Keratinocytes were infected with control (GFP) or PRK2-expressing adenovirus at 50 (PRK2 50) or 100 moi (PRK2 100), and 48 h after infection Fyn and Src activities were measured by immunoprecipitation followed by *in vitro* autophosphorylation assay (top). Quantification of the results after normalization for Fyn/Src proteins amounts, as detected by immunoblotting (bottom), revealed a 3.2-fold increase of the Fyn kinase activity in cells infected with the PRK2 adenovirus with no significant increase of Src activity. (B) Keratinocytes were infected and processed as in C except that *in vitro* kinase reactions were performed in the presence of poly(Glu-Tyr) as a tyrosine kinase-specific substrate (top). Quantification of the results after normalization for Fyn and Src protein amounts (bottom) revealed an ~ 2.5 -fold increase in Fyn kinase activity in cells infected with the PRK2 adenovirus with no significant changes in Src kinase activity. Similar results were obtained in three other independent experiments. (C) Keratinocytes kept in fully supplemented low calcium medium or serum-starved for 24 h (asterisk) were immunoprecipitated with either Fyn polyclonal antibodies (Fyn IP) or preimmune rabbit immunoglobulins (IgG). Each immunoprecipitate was divided into two aliquots, and these were incubated with a purified recombinant protein, encompassing the constitutively active GST-PKN kinase domain (543–942 amino acids) (PKN K.A.) or the corresponding kinase-dead mutant GST-PKN(543–942)-K644E (PKN K.D.) (Yoshinaga et al., 1999). After a first incubation in cold ATP, samples were extensively washed and incubated in presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Poly-Glu-Tyr as a tyrosine-kinase-specific substrate. Densitometric analysis of the phosphorylated Poly-Glu-Tyr signal coupled to normalization for Fyn protein amounts revealed a 2.8-fold increase of Fyn activity in samples derived from serum-starved keratinocytes incubated with the kinase-active form of PKN relative to the same samples incubated with the kinase-dead mutant. The increase of Fyn kinase activity in immunoprecipitates from keratinocytes in fully supplemented medium was 1.6-fold. The positions of Poly-Glu-Tyr, the autophosphorylated PKN (543–942), and the molecular weight markers are indicated.

dium; 40% H_2O) for 2 h at 34°C . This medium was replaced with the original medium supplemented with 20 mM Hepes (pH 7.4). After 1.5 h to allow recovery, some cultures were exposed to calcium (2 mM) for an additional 9 h. More than 85% of cells assumed GST fusion proteins as assessed by immunofluorescence with anti-GST antibodies (unpublished data).

Plasmid expression vectors and recombinant adenoviruses

Human cDNAs for the myc-tagged RhoV14 and RhoV14 Y42C mutants (Sahai et al., 1998) and for Flag-tagged wild-type PRK2 (Vincent and Settleman, 1997) were subcloned into the pAdTrack-CMV expression vector, which already carries a cDNA for the GFP for replication-defective adenoviruses generation (He et al., 1998). Conditions for infection of keratinocytes were as reported previously (Cabodi et al., 2000).

Antibodies

Monoclonal antibodies against E-cadherin (clone 36), β -catenin, γ -catenin, pp120^{cas}, Fyn, PRK2, PKN, polyclonal antibodies against SHC, and HRP-conjugated antiphosphotyrosine antibodies (RC20H) were from Transduction Laboratories. Polyclonal antibodies against Fyn (Fyn3), Src (SRC-2) and RhoA, anti-RhoA and anti-c-Myc (9E10) monoclonal antibodies, and affinity purified rabbit and mouse IgGs were from Santa Cruz Biotechnology, Inc. Antivinculin monoclonal antibodies were from Sigma-Aldrich, and anti-Src M327 monoclonal antibodies were from Oncogene Research Products. FITC-conjugated antiphosphotyrosine monoclonals 4G10 were from Upstate Biotechnology.

Immunoprecipitations, kinase assays, and pull-down assays

Conditions for immunofluorescence, immunoprecipitation, immunoblotting, and *in vitro* kinase assays were as described previously (Calautti et al., 1995, 1998). GST fusion proteins encompassing the constitutively active PKN kinase domain (543–942 amino acids) and the corresponding kinase-dead version (PKN 543–942 K644E) were produced and purified as described previously (Yoshinaga et al., 1999). Fyn immunoprecipitates in kinase buffer (20 mM Tris-HCl, pH 7.5, 4 mM MgCl_2) were divided into two aliquots and incubated with either glutathione-Sepharose beads

bound to 20 ng kinase-active GST-PKN or its kinase-dead version (K644E) in 50 μl of kinase buffer supplemented with 100 μM cold ATP. After 30 min at 30°C , samples were diluted into 1 ml of NP-40 lysis buffer, and after washes to remove unincorporated ATP they were incubated for 5 min at 30°C in 50 μl of Fyn kinase buffer (20 mM Hepes, pH 7.4, 5 mM MgCl_2 , 3 mM MnCl_2) supplemented with 10 μg of poly(Glu-Tyr), 1 μM ATP, and 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (6,000 Ci/mM). Pull-down assays with the GST-Rhotekin Rho binding domain coupled to glutathione-agarose beads (Upstate Biotechnology) were performed as described (Ren and Schwartz, 2000).

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