Tyrosine Phosphorylation and Src Family Kinases Control Keratinocyte Cell–Cell Adhesion

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Abstract. In their progression from the basal to upper differentiated layers of the epidermis, keratinocytes undergo significant structural changes, including establishment of close intercellular contacts. An important but so far unexplored question is how these early structural events are related to the biochemical pathways that trigger differentiation. We show here that β-catenin, γ-catenin/plakoglobin, and p120-Cas are all significantly tyrosine phosphorylated in primary mouse keratinocytes induced to differentiate by calcium, with a time course similar to that of cell junction formation. Together with these changes, there is an increased association of α-catenin and p120-Cas with E-cadherin, which is prevented by tyrosine kinase inhibition. Treatment of E-cadherin complexes with tyrosine-specific phosphatase reveals that the strength of α-catenin association is directly dependent on tyrosine phosphorylation. In parallel with the biochemical effects, tyrosine kinase inhibition suppresses formation of cell adhesive structures, and causes a significant reduction in adhesive strength of differentiating keratinocytes. The Fyn tyrosine kinase colocalizes with E-cadherin at the cell membrane in calcium-treated keratinocytes. Consistent with an involvement of this kinase, *fyn*-deficient keratinocytes have strongly decreased tyrosine phosphorylation levels of β- and γ-catenins and p120-Cas, and structural and functional abnormalities in cell adhesion similar to those caused by tyrosine kinase inhibitors. Whereas skin of *fyn*−/− mice appears normal, skin of mice with a disruption in both the *fyn* and *src* genes shows intrinsically reduced tyrosine phosphorylation of β-catenin, strongly decreased p120-Cas levels, and important structural changes consistent with impaired keratinocyte cell adhesion. Thus, unlike what has been proposed for oncogene-transformed or mitogenically stimulated cells, in differentiating keratinocytes tyrosine phosphorylation plays a positive role in control of cell adhesion, and this regulatory function appears to be important both in vitro and in vivo.

As keratinocytes progress from the basal proliferating layer of the epidermis to the immediately adjacent differentiating layer (spinous layer) they lose contact with the extracellular matrix and undergo significant structural changes, such as establishment of close intercellular contacts, desmosome formation and rearrangements of the actin/cytokeratin network. An important but so far unexplored question is how these early structural events are related to the biochemical pathways which trigger differentiation.

Cell–cell contacts among neighboring keratinocytes are mediated mainly by adherens junctions and desmosomes. Adherens junctions contain "classical" cadherins, whereas desmosomes are composed of specialized cadherins, such as desmoglein(s) and desmocollin(s). Each type of junction is linked via several cytoplasmic proteins to different elements of the cytoskeleton. Adherens junctions are associated with the actin cytoskeleton and have been shown to be important for the establishment of cell adhesion and polarization, while desmosomes interact with keratin filaments and impart mechanical strength to the epithelium (Cowin and Burke, 1996).

Adherens junctions in epithelial cells depend on the homophilic, calcium-dependent binding of the extracellular domain of transmembrane cadherins (Takeichi, 1988, 1991). The cytoplasmic domain of E-cadherin forms a complex with β-, γ-, and α-catenins, and this association is essential for the establishment of proper cell adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Nagafuchi et al.,

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1991). β- and γ-catenins share a similar structure (65% identity) (Fouquet et al., 1992), including a 42–amino acid motif repeated 12 or 13 times, originally described in the *Drosophila* segment polarity gene product *Armadillo* (Riggleman et al., 1989). β- and γ-catenins bind directly to E-cadherin in a mutually exclusive fashion (Mathur et al., 1994; Stappert and Kemler, 1994). They also form a complex with α-catenin, a cytoplasmic protein similar to vinculin, which in turn is connected, either directly or indirectly, to the actin network (Knudsen et al., 1995; Rimm et al., 1995). Besides adherens junctions, γ-catenin/plakoglobin is also found in desmosomes, in association with desmosomal cadherins and is thought to modulate their function (Cowin et al., 1986; Troyanovsky et al., 1993). Another catenin, p120-Cas, originally described as a putative substrate of the activated Src tyrosine kinase (Kanner et al., 1990, 1991; Reynolds et al., 1992), has been shown to complex directly with E-cadherin (Reynolds et al., 1994, 1996; Shibamoto et al., 1995). In normal cells only a small proportion of p120-Cas is associated with cadherins (Shibamoto et al., 1995), but in specific circumstances, such as upon *ras* transformation, this catenin displays increased affinity for E-cadherin (Kinch et al., 1995). Interestingly, p120-Cas lacks the ability to bind α-catenin (Daniel and Reynolds, 1995), suggesting that p120-Cas/cadherin complexes are disconnected from the actin cytoskeleton and thus may account, at least partially, for the poor adhesive phenotype of *Ras*-transformed cells.

Elevation of Ca^{2+} concentrations in epithelial cell cultures induces the rapid translocation of cadherins to cell–cell borders and consequent adherens junction and desmosome formation (O'Keefe et al., 1987; Lewis et al., 1994). These changes are accompanied by a reorganization of the cytoskeleton, polarization and, in keratinocyte cultures, stratification. Establishment of adherens junctions has been shown to play a primary role in these processes. In particular, inhibition of adherens junction formation by anti–E-cadherin antibodies (Hodivala and Watt, 1994; Lewis et al., 1994) or by expression of dominant negative cadherin mutants (Amagai et al., 1995), suppresses all later steps, such as desmosome formation, polarization, and stratification. Whereas calcium can trigger initial formation of cadherin-mediated cell adhesion, a second, temperature-dependent step is required for the strengthening of these interactions (Angres et al., 1996). In addition, for stratification to occur, cell contacts need to remain fluid, as movement of cells within a tri-dimensional structure requires continual disruption and reorganization of intercellular junctions, without loss of adhesive strength (Lewis et al., 1994; Tao et al., 1996). The mechanisms that control the strength of cell adhesion and modulate its dynamic state are still poorly understood.

Increased tyrosine phosphorylation of β- and γ-catenin (plakoglobin) and p120-Cas has been previously correlated with the decrease of cell adhesion which occurs upon neoplastic transformation (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Kinch et al., 1995; Papkoff, 1997) or mitogenic growth factor stimulation (Kanner et al., 1991; Hoschuetzky et al., 1994; Reynolds et al., 1994; Shibamoto et al., 1994; for review see Miller and Moon, 1996). However, in no cases was catenin tyrosine phosphorylation shown to be causally linked to decreased cell adhesion. In fact, additional evidence indicates that loosening of cell contacts in *src*-transformed cells cannot be explained by tyrosine phosphorylation of β-catenin, but is likely resulting from tyrosine phosphorylation of other junctional proteins such as ZO-1, ezrin/radixin/moesin, or some other unidentified proteins (Takeda et al., 1995). The small Rho/Rac GTPases have also been implicated in control of cell adhesion. Interestingly, these molecules appear to play a negative suppressive function in MDCK cells (Ridley et al., 1995), but a positive one in keratinocytes (Braga et al., 1997), suggesting that control of cell adhesion may differ significantly among epithelial cells of different types.

Unlike what may occur in oncogene-transformed or mitogenically stimulated cells, we report here that in differentiating keratinocytes, tyrosine phosphorylation plays a positive role in the strengthening of cell adhesion. At least two distinct tyrosine kinase activities are induced in the keratinocyte differentiation process (Calautti et al., 1995). One of these activities is specifically increased by calcium and a number of other divalent cations from the outside of the cell, suggesting that an extracellular cation-sensor mechanism is involved. Induction of this kinase activity occurs within minutes of calcium exposure (Calautti et al., 1995), and correlates with the rapid tyrosine phosphorylation of a ras-GAP associated p62 protein (Filvaroff et al., 1992, 1994), which may be similar, but probably not identical (Medema et al., 1995), to the p62-Dok adaptor protein (Carpino et al., 1997; Yamanashi and Baltimore, 1997). The second tyrosine kinase activity, identified as Fyn, increases only at relatively late times of exposure of keratinocytes to calcium (within 1–6 h) (Calautti et al., 1995). In close parallel with Fyn kinase activation, calcium treatment triggers tyrosine phosphorylation of cortactin, a protein which colocalizes with subcortical actin at sites of cell movement. A significant role of Fyn in keratinocyte differentiation is indicated by the fact that this process is altered in keratinocytes with a disruption of the *fyn* gene (Calautti et al., 1995).

Immunoblotting of keratinocyte cell extracts with anti-phosphotyrosine antibodies indicates that p62 and cortactin are only two of a wider group of proteins which are tyrosine phosphorylated in calcium-induced keratinocyte differentiation (Filvaroff et al., 1990; Calautti et al., 1995; our unpublished observations). We show here that β-, γ-catenin (plakoglobin), and p120-Cas become all strongly tyrosine phosphorylated at early times of calcium-induced keratinocyte differentiation, and that these modifications are linked to an increased association of α-catenin and p120-Cas with E-cadherin. Both biochemical and genetic evidence indicates that tyrosine phosphorylation plays a fundamental role in the changes in cell adhesion associated with keratinocyte differentiation, and that the activity of Fyn and related kinases is involved, both in vitro and in vivo.

Materials and Methods

Cells and Animals

Primary keratinocytes were isolated from newborn Sencar mice or mice with a disruption of the *fyn* (Stein et al., 1992), *src* (Soriano et al., 1991), and *yes* (Stein et al., 1994) genes, and genetically matched wild-type controls. The *yes* mutation results in the production of a small amount of a catalytically inactive fragment of the Yes kinase (Calautti et al., 1995). Cells were cultivated in MEM with 4% Chelex-treated fetal calf serum,

EGF (10 ng/ml; Collaborative Research, Inc., Cambridge, MA), and 0.05 mM $CaCl_2$ (low calcium medium), as described (Hennings et al., 1980; Calautti et al., 1995). For all experiments, cells were used one week after plating. Culture medium was changed for the last time 24 h before the experiment. Keratinocyte differentiation was induced by addition of $CaCl_2$ (to 2 mM). Genistein and Tyrphostin 23 were purchased from BIOMOL (Plymouth Meeting, PA), and then stored in 100-mM aliquots in DMSO at −20°C. PP1 was a gift of Dr. S. Fuji (Tokyo University, Tokyo, Japan) and was stored in DMSO (10 mg/ml) at −20°C.

C57B/129 mice carrying single *fyn*, *src*, or *yes* knockout mutations (Soriano et al., 1991; Stein et al., 1992, 1994) were bred to obtain double heterozygotes for the *fyn*/*src* or *fyn*/*yes* mutations. Double heterozygotes were then bred, and among the offspring, the mice carrying a *fyn*−/− *src*+/− or *yes*−/−*fyn*+/− genotype were intercrossed to obtain *src*−/− *fyn*−/− or *fyn*−/−*yes*−/− double knockout mice, respectively.

Recombinant Adenovirus Infection of Mouse Primary Keratinocytes

Recombinant adenoviruses expressing either a constitutively active c-Src mutant (Tyrosine 527 to phenylalanine substitution) (Ad-src), or a green fluorescent protein (Ad-GFP) were used at a multiplicity of infection of 100. Detailed description of the generation of Ad-Src will be presented elsewhere. In all cases, infection of primary mouse keratinocytes was performed for 1 h in serum- and EGF-free low calcium medium. Keratinocytes were further incubated for 24 h in low calcium medium, and then either kept in this medium or switched to high calcium medium (2 mM $CaCl_2$) for the indicated times.

Antibodies

Monoclonal antibodies against E-cadherin, α-catenin, β-catenin, and pp120-Cas were purchased from Transduction Laboratories (Lexington, KY). Anti–γ-catenin/plakoglobin mAbs (ascites fluid) were a gift of Dr. P. Cowin (New York University, New York, NY). Anti–desmoglein 3 antibodies were a gift of Dr. J. Stanley (University of Pennsylvania, Philadelphia, PA). Anti–plakophilin 1 rabbit antisera were raised against either the head or the *Arm* repeats domain of plakophilin, expressed in *Escherichia coli* as His-tag fusion proteins. Anti-Fyn and anti-Src polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-phosphotyrosine antibodies (RC20H) were purchased from Transduction Laboratories. Anti-phosphotyrosine mAbs 4G10 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Either affinity-purified anti-E1A mAbs (Oncogene Science Inc., Cambridge MA) or affinity-purified rabbit IgG were used as unrelated negative controls.

Immunoprecipitations

Mouse primary keratinocytes were washed twice in PBS containing 1 mM Na_3VO_4 and lysed for 20 min on ice, either in NP-40 lysis buffer for high stringency immunoprecipitations (0.5% NP-40, 50 mM TRIS-HCl, pH 8.0, 120 mM NaCl) or in CSK lysis buffer for low stringency immunoprecipitations (0.2% Triton X-100, 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA), supplemented with 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO), 10 μg/ml leupeptin (Sigma Chemical Co.). Cell lysates were spun for 5 min at 4°C. Samples were normalized for equal amounts of proteins by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Same amounts (0.5–1 mg) were incubated either for 2 h or overnight at 4°C with antibodies (2–4 μg of affinity-purified mAbs or 5 μl of crude ascites fluid). Protein G–agarose beads (60 μl of a 50% suspension; Boehringer Mannheim Corp., Indianapolis, IN) were added and samples were incubated for 45 min at 4°C. Immune complexes were washed five times for high stringency immunoprecipitations and three times for low stringency immunoprecipitations. After the last wash, immune complexes were eluted in boiling Laemmli sample buffer for 3 min, and separated by 7.5% acrylamide/0.173% bis-acrylamide SDS-PAGE.

Immunoprecipitations from skin samples were performed as follows: whole skins from newborn mice were snap-frozen in liquid nitrogen and pulverized with a tissue grinder. The pulverized tissues were solubilized with a Polytron homogenizer in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. Tissue debris was removed by centrifugation for 5 min at 3,000 *g*. The supernatants were further spun for 45 min at 25,000 *g* and incubated sequentially with protein G–agarose and protein A–Sepharose beads for 45 min as a preclearing step. Tissue extracts were normalized for total protein content and for E-cadherin protein by preliminary SDS-PAGE and immunoblotting with anti–E-cadherin antibodies. Normalized extracts (2–5 mg of protein) were immunoprecipitated with relevant antibodies and immune complexes were processed as described above for high stringency immunoprecipitations.

Protein–Tyrosine–Phosphatase Treatment of Cadherin–Catenin Complexes

Keratinocytes were lysed in NP-40 lysis buffer supplemented with inhibitors of proteases and phosphatases as described above. E-cadherin and associated proteins were immunoprecipitated with E-cadherin–specific antibodies. The immune complexes were washed twice in NP-40 lysis buffer without phosphatase inhibitors, and twice in phosphatase buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl). The immune complexes were than resuspended in 50 μl of phosphatase buffer supplemented with 1 mM DTT and 1 mg/ml BSA, and incubated for 30 min at 37°C with 25 mU of *Yersinia enterocolitica* protein tyrosine phosphatase (Boehringer Mannheim Corp.), either in the presence or the absence of tyrosine phosphatase inhibitors (2 mM Na_3VO_4, 2 mM $ZnCl_2$). The reaction was stopped by the addition of 1 ml NP-40 lysis buffer supplemented with 2 mM Na_3VO_4, 2 mM $ZnCl_2$. After one wash in this buffer, the complexes were washed three times with 4 M LiCl, 1 mM Na_3VO_4, and then twice in PBS plus 1 mM Na_3VO_4. Samples were analyzed by SDS-PAGE and immunoblotting.

Immunoblotting

Immunoblotting of protein gels onto PVDF membranes was performed as previously described (Calautti et al., 1995). All blots were blocked for 1 h at room temperature in 5% milk in PBS 0.2% Tween 20, except for anti-phosphotyrosine immunoblots, which were blocked for 1 h in 1% BSA, 10 mM TRIS, pH 7.5, 100 mM NaCl, 0.1% Tween 20. RC20-horseradish–conjugated anti-phosphotyrosine antibodies were used for immunoblotting at a 1:2,500 dilution. HRP-conjugated anti–mouse or anti–rabbit immunoglobulins (Amersham Corp., Arlington Heights, IL) were used as secondary antibodies for all other immunoblots at a 1:2,000–1:5,000 dilution, and blots were developed with the ECL system (Amersham Corp.).

Immunofluorescent Staining of Primary Keratinocytes

Mouse primary keratinocytes were plated on collagen-coated glass coverslips, and cells in low calcium medium or at different times after calcium treatment were fixed for 10 min at room temperature with 2% paraformaldehyde in PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS, blocked in 5% goat serum in PBS. The coverslips were stained with primary antibodies for 1 h at room temperature, followed by isotype-specific FITC- or Texas red–conjugated secondary antibodies (Southern Biotechnology Associates Inc., Birmingham, AL) For the analysis of the detergent-insoluble subcellular localization, keratinocytes were pre-extracted for 5 min on ice in 0.2% Triton X-100–CSK buffer supplemented with inhibitors of proteases and phosphatases before fixation in 2% paraformaldehyde in PBS. Conventional fluorescent microscopy was performed with a Nikon FXA microscope (Melville, NY). Confocal microscopy was performed using a TCS 4D scanner (Leica, Heerbrugg, Switzerland) connected to an inverted LEITZ DM IRB microscope (Oberkonen, Germany). Images were processed using a TCS-NT software package.

Dispase Treatment of Mouse Primary Keratinocytes

Duplicate 60-mm dishes of confluent keratinocyte cultures in either low or high calcium medium were washed twice in PBS and incubated in 2 ml dispase solution in PBS (from *Bacillus polymyxa*; >2.4 units/ml; Boehringer Mannheim Corp.) at 34°C. Cells were analyzed with an inverted transmission microscope at 5-min intervals, for >35 min. Quantification of this assay was performed as follows: after 35 min of dispase treatment, cells from each sample, including single cells released into suspension and detached sheets of cells, were collected by scraping, and then washed twice in PBS. After centrifugation, samples were resuspended in 500 μl of PBS and subjected to mechanical disruption by pipetting 15 times with a 1 ml Pipetman. The remaining aggregates were left to sediment at 1 *g* for 1 min, and the number of single cells into suspension was determined by counting 10 μl of supernatant with an hemocytometer. The whole samples were then centrifuged, and incubated for 7 min at 37°C in 0.25% trypsin, and 2.5 mM EDTA, to release all cells into suspension. Cells were counted as before and used for assay normalization.

Electron Microscopy

Keratinocytes grown on collagen-coated plastic coverslips were fixed in 1% glutaraldehyde; skin samples were fixed in 1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% picric acid in 50 mM cacodylic acid buffer, pH 7.4. All specimens were postfixed in 1% osmium tetroxide/1.5% potassium ferrocyanide, dehydrated, infiltrated, and then embedded in Epon/Araldite. Thin sections were cut, stained with uranyl acetate and lead citrate, and then examined with a transmission electron microscope (1200 EX; JEOL USA Inc., Peabody, MA).

Results

Exposure of Keratinocytes to High Extracellular Calcium Induces Tyrosine Phosphorylation of β- and γ-Catenin, and Increased Association of α-Catenin with E-Cadherin Complexes, with a Time Course That Parallels Cell Junction Formation

As mentioned in the introduction, very little is known about control of cell adhesion in differentiating epithelia. We tested whether there is a specific interconnection between induction of tyrosine phosphorylation and the changes in cell adhesion that occur during calcium-induced keratinocyte differentiation. In an initial set of studies, we examined the cellular localization pattern of total tyrosine phosphorylated proteins in growing versus differentiating keratinocytes. In parallel with our previous biochemical data, immunofluorescence analysis with anti-phosphotyrosine antibodies revealed a substantial increase of tyrosine phosphorylation signal in the differentiating cells, as early as 2 h after calcium exposure. More significantly, calcium treatment resulted in a striking localization of the tyrosine phosphorylation signal at sites of cell–cell contact (not shown). To test whether β-catenin, an essential adherens junction component, shows an increased localization at tyrosine phosphorylation sites, we focused on the detergent-insoluble submembranous cytoskeleton. Keratinocytes under growing versus differentiating conditions were pre-extracted with a 0.2% Triton X-100 buffer before fixation in 2% paraformaldehyde. Cells were then double stained with anti-phosphotyrosine and anti–β-catenin antibodies, and analyzed by confocal microscopy. As shown in Fig. 1 (*top panels*), calcium treatment (9 h) resulted in a significant increase of tyrosine-phosphorylated proteins as well as β-catenin associated with the detergent-resistant, membrane–cytoskeleton compartment. The pattern of staining of anti-phosphotyrosine and anti–β-catenin antibodies was almost totally overlapping in the calcium-treated cells.

To determine whether tyrosine phosphorylation levels of specific adherens junction components are directly increased with differentiation, extracts from keratinocytes under basal growing conditions and at various times after calcium exposure were immunoprecipitated with anti–E-cadherin antibodies, followed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 2 *A* (*left panel*), two tyrosine phosphorylated bands of ~85 and 92 kD were detected in the anti–E-cadherin immunoprecipitates of keratinocytes under basal low calcium conditions. Tyrosine phosphorylation of these bands was strongly increased by 9 h of calcium treatment and persisted at elevated levels for 24 h. These bands comigrate and are likely to correspond to β- and γ-catenin, as judged by reprobing of the same immunoblot with antibodies specific against these proteins (Fig. 2 *A*, *right panels*). Immunoprecipitation of keratinocyte cell extracts with antibodies against β- and γ-catenins followed by anti-phosphotyrosine immunoblotting, confirmed that tyrosine phosphorylation of β- and γ-catenins is directly increased in the differentiating keratinocytes (Fig. 2 *B*).

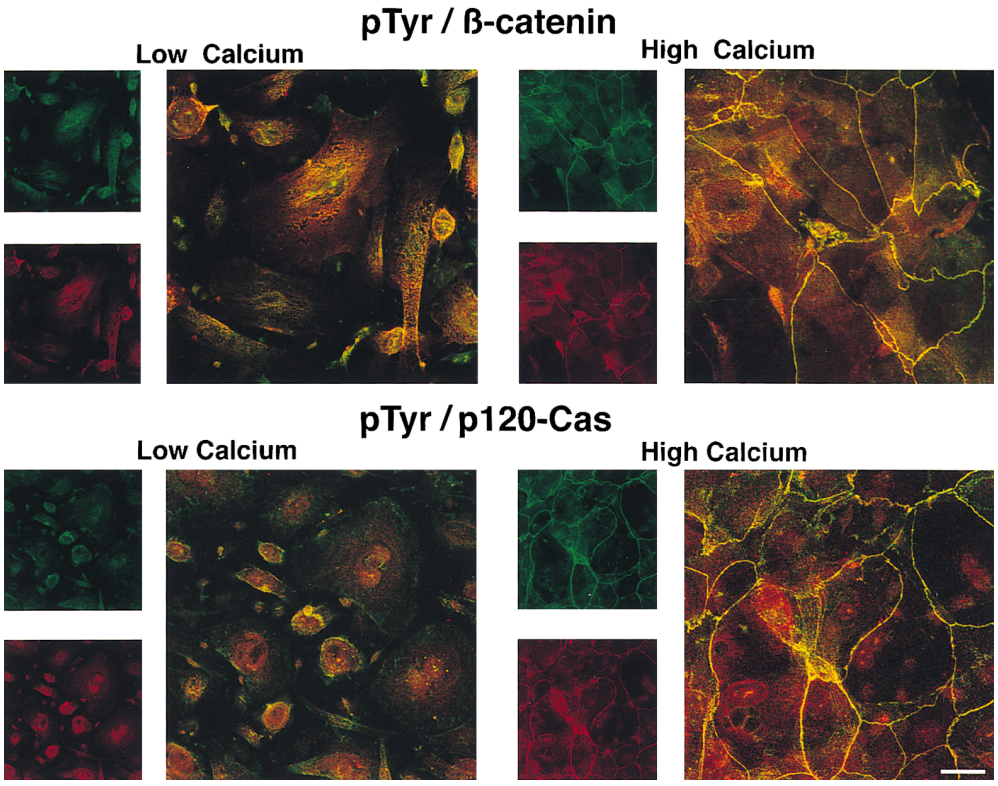

Figure 1. Colocalization of phosphotyrosine with β-catenin and p120-Cas in the cortical cytoskeleton of differentiating keratinocytes. Mouse primary keratinocytes in low calcium medium or at 9 h of calcium exposure were pre-extracted in 0.2% Triton X-100 buffer before paraformaldehyde fixation as described in Materials and Methods. Cells were double stained with anti-phosphotyrosine antibodies and FITC-conjugated secondaries (*green*) and antibodies against either β-catenin (*top panels*) or p120-Cas (*bottom panels*), and Texas red–conjugated secondaries (*red*). Samples were analyzed by confocal microscopy and green and red images (*small panels*) were superimposed (*large panels*), so that sites of staining overlap are visualized as yellow. Bars: (*large panels*) 15 μm; (*small panels*) 33 μm.

No direct tyrosine phosphorylation of E-cadherin itself nor of α-catenin could be detected in the anti–E-cadherin immunoprecipitates. However, even if not directly tyrosine phosphorylated, the amount of α-catenin found in association with E-cadherin was significantly increased by 9 h of calcium treatment, whereas the amounts of β- and γ-catenins associated with E-cadherin remained constant (Fig. 2 *A*, *right panels*). Immunoblotting of total keratinocyte cell extracts indicated that the total amounts of α-catenin, as well as β- and γ-catenins and E-cadherin, remained constant up to 48 h of calcium treatment (data not shown).

Besides adherens junctions, γ-catenin/plakoglobin is also found in desmosomes, and is thought to modulate their function (Cowin et al., 1986; Troyanovsky et al., 1993). γ-Catenin associated with desmosomal cadherins may also be increasingly tyrosine phosphorylated in response to calcium. To test this possibility, keratinocyte cell extracts were immunoprecipitated with anti–desmoglein 3 antibodies, followed by immunoblotting with anti-phosphotyrosine antibodies. A single specific band of ~85 kD was detected, which became strongly tyrosine phosphorylated by 9 h of calcium treatment (Fig. 2 *C*). This band is likely to correspond to γ-catenin, as judged by reprobing of the same immunoblot with antibodies against this protein. As in E-cadherin complexes, the amount of desmoglein-associated γ-catenin did not appear to vary significantly over time (Fig. 2 *C*). Tyrosine phosphorylation of plakophilin 1, another key desmosomal protein that shares structural homology with β- and γ-catenins (Heid et al., 1994), was analyzed by direct immunoprecipitation with anti–plakophilin 1 antibodies followed by anti-phosphotyrosine immunoblotting. Plakophilin 1 was found to be weakly tyrosine phosphorylated in keratinocytes under low calcium conditions, and tyrosine phosphorylation of this protein (at least that recoverable from the detergent-soluble fraction) did not increase with differentiation (data not shown).

Previous reports showed that elevation of Ca^{2+} concentrations in epithelial cell cultures induces the rapid translocation of cadherins to cell–cell borders and consequent cell junction formation (O'Keefe et al., 1987; Lewis et al., 1994). The precise time course of these events is likely to vary as a function of cells and culture conditions. It was important to determine whether the timing of adherens junction and desmosome formation in our primary mouse keratinocyte cultures parallels that of increased tyrosine phosphorylation. Accordingly, cells were analyzed at various times after calcium treatment by immunofluorescence with antibodies against either E-cadherin or plakophilin. Extraction with 0.2% Triton X-100 buffer before fixation ensured detection of these proteins only when assembled into insoluble cell membrane structures. As shown in Fig. 3, by 2 h of calcium treatment E-cadherin and plakophilin showed only an incomplete recruitment into membrane–cytoskeleton structures, whereas by 9 h a very strong signal for these proteins was detected at sites of close intercellular junctions.

Thus, in mouse primary keratinocytes induced to differentiate by calcium, there is an increased tyrosine phosphorylation of proteins at sites of cell–cell adhesion, and this is associated with a specific increase in tyrosine phosphorylation levels of β- and γ-catenins. In contrast, α-catenin is not directly tyrosine phosphorylated, but its association with E-cadherin increases, with a time course that parallels that of β- and γ-catenin tyrosine phosphorylation, as well as that of cell junction formation.

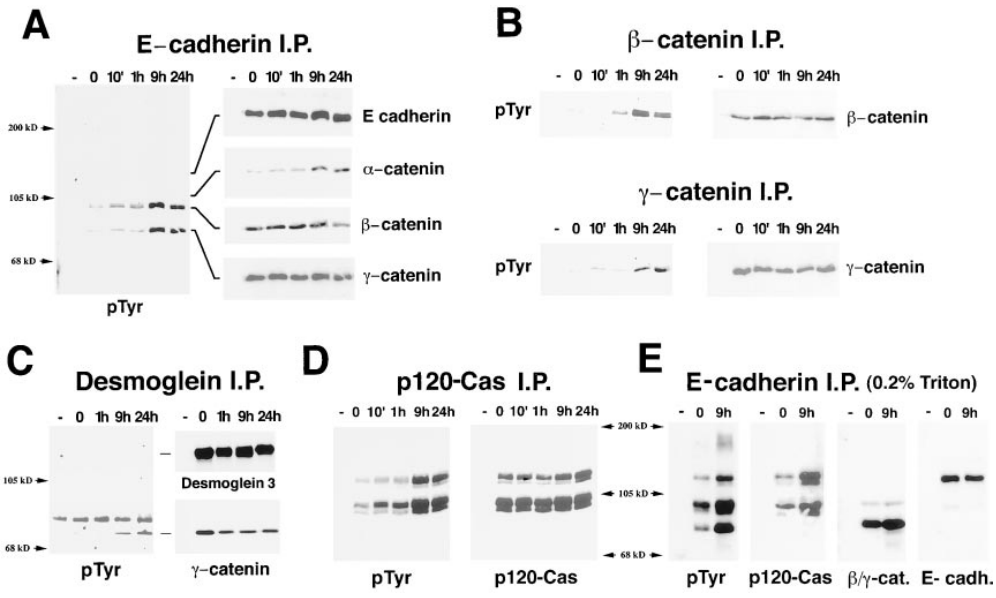

Figure 2. Tyrosine phosphorylation and cadherin association of α-, β-, and γ-catenins and p120-Cas in growing versus differentiating keratinocytes. (*A–D*) Keratinocytes in low calcium medium (*0*), and at various times after calcium addition were lysed in 0.5% NP-40 lysis buffer and immunoprecipitated with antibodies against E-cadherin (*A*), β-catenin, or γ-catenin/plakoglobin (*B*), desmoglein 3 (*C*), or p120-Cas (*D*). In all cases, control immunoprecipitations with unrelated antibodies were included (−). Immune complexes were analyzed by SDS-PAGE and anti-phosphotyrosine immunoblotting (*left panels*). The same blots were subsequently reprobed with antibodies against specific proteins as indicated (*right panels*). Positions of these molecules in the anti-phosphotyrosine immunoblots are indicated. In *C*, the band recognized by anti-phosphotyrosine antibodies that migrates above γ-catenin is nonspecific, since it is also detected in the nonimmune control. (*E*) Association of p120-Cas with E-cadherin, as detected by immunoprecipitation under milder stringency conditions than in the previous experiments. Keratinocytes in low calcium medium (*0*), and at 9 h after calcium addition (2 mM) were lysed in a 0.2% Triton X-100 lysis buffer and immunoprecipitated with E-cadherin–specific monoclonals or with unrelated control monoclonals (−). Immune complexes were analyzed by SDS-PAGE and sequential immunoblotting with antibodies against phosphotyrosine (*p-Tyr*), p120-Cas (*p120-Cas*), a mixture of antibodies against β- and γ-catenins (*β/γ-cat.*) and E-cadherin (*E-cadh.*).

p120-cas Becomes Heavily Tyrosine Phosphorylated in Keratinocytes in Response to Calcium, and Displays Increased Association with E-Cadherin

p120-Cas, another catenin originally described as a substrate of activated Src (Kanner et al., 1990, 1991; Reynolds et al., 1992), can also form a direct complex with E-cadherin at adherens junctions (Reynolds et al., 1994; Shibamoto et al., 1995). However, in normal cells, only a small fraction of p120-Cas is associated with cadherins, and this fraction varies with various cell types (Kinch et al., 1995; Shibamoto et al., 1995). To determine whether in differentiating keratinocytes p120-Cas shows an increased localization at tyrosine phosphorylation sites in the submembranous cy-

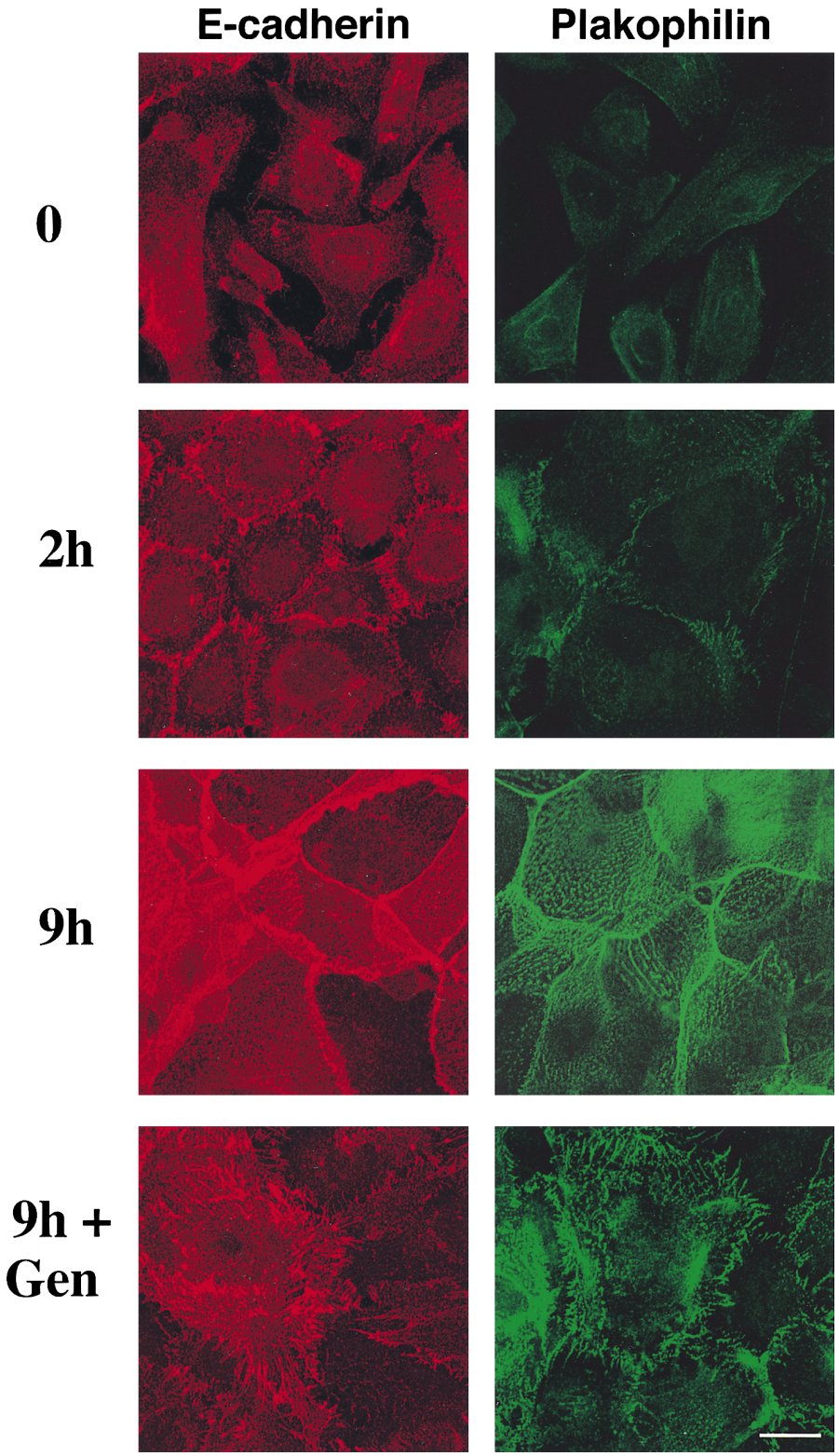

Figure 3. Time course of cell adhesion formation in mouse primary keratinocytes after calcium treatment, and as a function of tyrosine phosphorylation. Mouse primary keratinocytes in low calcium medium or at 2 and 9 h of calcium exposure were pre-extracted in 0.2% Triton X-100 buffer before paraformaldehyde fixation as described in Materials and Methods. Parallel experiments were performed with cells pretreated for 2 h with 100 μM Genistein (*Gen*) and incubated for additional 9 h under high calcium conditions. Cells were stained with antibodies against E-cadherin (*left panels*, *red*) or plakophilin (*right panels*, *green*). Samples were analyzed by confocal microscopy. Each image results from the projection of eight different focal plans of the same field, and is representative of what observed in the individual focal plans. Bar, 15 μm.

toskeleton, we performed the same type of immunofluorescence analysis described above for β-catenin: cells either kept in low calcium medium or switched to high calcium for 9 h were pre-extracted with 0.2% Triton X-100 buffer before fixation, and were double stained with antibodies against phosphotyrosine and p120-Cas. Cells were then analyzed by confocal microscopy. Like β-catenin, in calcium-treated keratinocytes p120-Cas was also recruited into the detergent-insoluble cortical cytoskeleton, and it colocalized with phosphotyrosine at cell–cell borders (Fig. 1, *bottom panels*).

To assess whether tyrosine phosphorylation of p120-Cas is intrinsically increased with differentiation, extracts from keratinocytes under low versus high calcium conditions were immunoprecipitated with an mAb that recognizes all known isoforms of the p120-Cas proteins (Mo and Reynolds, 1996), and the immunocomplexes were analyzed by anti-phosphotyrosine immunoblotting. As shown in Fig. 2 *D*, there was a progressive increase of tyrosine phosphorylation of all four p120-Cas isoforms, which was detectable already by 10 min of calcium exposure, and then became very pronounced by 9 h. Tyrosine phosphorylation of p120-Cas remained elevated at least until 24 h. Reprobing of the same blot with anti-p120 antibodies showed that expression of the two larger isoforms of p120-Cas was only slightly increased by 24 h of calcium treatment, whereas levels of the smaller forms did not vary significantly over time.

As reported for other cells (Kinch et al., 1995), increased tyrosine phosphorylation of p120-Cas may correlate with a higher affinity of these proteins for cadherin–catenin complexes. No p120-Cas/E-cadherin association could be detected in E-cadherin immunoprecipitates performed under standard stringency conditions (not shown). However, p120/E-cadherin complexes may have been disrupted by the detergent concentrations used for those experiments. We reexamined this question by performing anti–E-cadherin immunoprecipitations under milder stringency conditions (lysis in 0.2% Triton X-100 buffer). Immunoprecipitates from keratinocytes in low calcium medium versus cells treated with calcium for 9 h, were separated by SDS-PAGE, blotted, and then sequentially probed with antibodies against phosphotyrosine, p120-Cas, β- and γ-catenins, and E-cadherin (Fig. 2 *E*). Under these conditions, association of all p120-Cas isoforms with E-cadherin was easily detected, and this association was strongly increased in the calcium-treated cells, whereas that of β- and γ-catenins remained mostly unaffected. At least four major tyrosine-phosphorylated bands were detectable in the E-cadherin immunoprecipitate from keratinocytes under low calcium conditions, and tyrosine phosphorylation of these bands was strongly increased upon calcium treatment (Fig. 2 *E*). These bands comigrate and are likely to correspond to the p120-Cas and β- and γ-catenins discussed above. In addition, a fifth tyrosine phosphorylated protein of heavier molecular weight was detectable in the anti-phosphotyrosine immunoblot, which appeared only in the calcium-treated sample and whose identity remains to be assessed.

Thus, calcium treatment of mouse primary keratinocytes induces strong tyrosine phosphorylation of p120-Cas proteins, and this increased phosphorylation correlates with an increased association of all four p120-Cas isoforms with E-cadherin.

Tyrosine Phosphorylation Is Required for the Increased Association of α-Catenin and p120-Cas with E-Cadherin in Differentiating Keratinocytes

The results of the previous sections showed that exposure of keratinocytes to high extracellular calcium concentrations has two important consequences: it induces tyrosine phosphorylation of β- and γ-catenins and p120-Cas proteins, and at the same time, triggers an increased association of α-catenin and p120-Cas with E-cadherin. An important question is whether the two events are connected.

The isoflavone Genistein inhibits specific tyrosine phosphorylation events associated with calcium-induced keratinocyte differentiation (Filvaroff et al., 1990). Tyrphostins represent a class of structurally unrelated tyrosine kinase inhibitors which block EGF-dependent mitogenicity and revert the transformed phenotype of *v-src*–transformed cells (Levitzki, 1992). We initially tested whether treatment of keratinocytes with either Genistein or Tyrphostin 23 could block induction of tyrosine phosphorylation of β- and γ-catenins and p120-Cas in response to calcium, and whether, in turn, association of α-catenin and p120-Cas with E-cadherin was affected. Primary keratinocytes were pretreated for 2 h with Genistein or Tyrphostin 23, or DMSO solvent alone, and then cells were incubated for additional 9 h under either low or high calcium conditions. Cell lysates in 0.5% NP-40 lysis buffer were immunoprecipitated with anti–E-cadherin antibodies and immunoblotted, sequentially, with antibodies against phosphotyrosine, α-, β-, and γ-catenins, and E-cadherin. As shown in Fig. 4 *A*, Genistein treatment had only limited effects on basal tyrosine phosphorylation levels of E-cadherin–associated β- and γ-catenins, while it blocked almost completely tyrosine phosphorylation of these molecules in response to calcium. Tyrphostin treatment had more pronounced inhibitory effects on tyrosine phosphorylation of β- and γ-catenins, both under basal conditions and after calcium exposure. In parallel with these effects, both tyrosine kinase inhibitors blocked the increased association of α-catenin with E-cadherin, which normally occurs in calcium-treated cells (see Fig. 4 *A*, *top right panel*). This effect was specific for the calcium-induced association, as neither inhibitor affected the basal level of α-catenin associated with E-cadherin in cells under low calcium conditions.

To test whether tyrosine phosphorylation is also required for the increased association of p120-Cas with E-cadherin, keratinocytes in low versus high calcium conditions ± tyrosine kinase inhibitors, were lysed under mild detergent conditions (0.2% Triton X-100). Extracts were immunoprecipitated with anti–E-cadherin antibodies and immunoblotted, sequentially, with antibodies against phosphotyrosine, p120-Cas, β- and γ-catenins, and E-cadherin. As shown in Fig. 4 *B*, both inhibitors reduced the basal tyrosine phosphorylation levels of p120-Cas, and blocked any further increase in response to calcium. Genistein treatment had little or no effect on the amount of p120-Cas associated with E-cadherin in cells under basal conditions, while it totally prevented the increased association induced by calcium (Fig. 4 *B*, *top right panel*). Tyrphostin treatment caused a reduction of p120-Cas associated with E-cadherin already in keratinocytes in low calcium me-

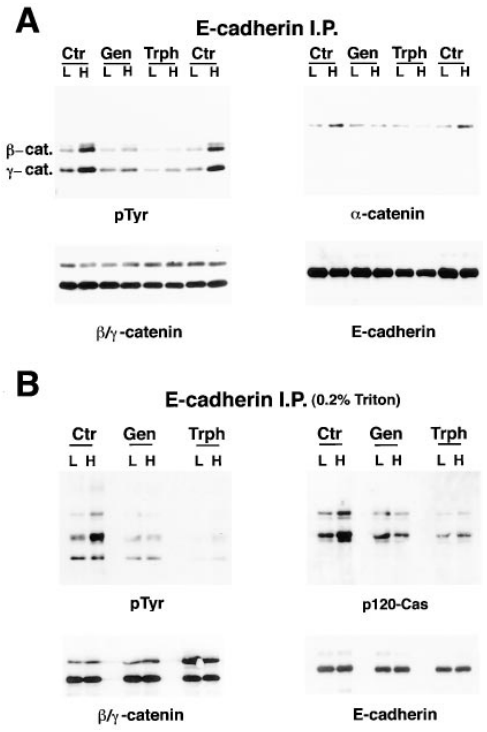

Figure 4. E-cadherin association of α-, β-, and γ-catenins and p120-Cas in growing versus differentiating keratinocytes as affected by tyrosine kinase inhibition. Growing keratinocytes were pretreated for 2 h with either 100 μM Genistein (*Gen*), Tyrphostin 23 (*Trph*), or with DMSO solvent alone (*Ctr*). Incubation was then continued for additional 9 h under either low (*L*) or high (*H*) calcium conditions. Cells were lysed in either 0.5% NP-40 lysis buffer (*A*) or 0.2% Triton X-100 lysis buffer (*B*), and immunoprecipitated with anti–E-cadherin antibodies. Immune complexes were separated by SDS-PAGE and immunoblotted sequentially, as indicated, with antibodies against phosphotyrosine, α-catenin, a mixture of anti–β- and anti–γ-catenin antibodies, antibodies against p120-Cas, and against E-cadherin. In the experiment of *A*, two independent sets of control samples were included, to illustrate the reproducibility of the calcium-induced increase in α-catenin association with E-cadherin under normal conditions.

dium, and prevented any further increase in cells exposed to calcium.

We have previously shown that tyrosine kinase inhibitors disrupt several aspects of keratinocyte differentiation (Filvaroff et al., 1990), and it is still possible that their suppressive effects on α-catenin and p120-Cas association with E-cadherin complexes are not directly resulting from inhibition of tyrosine phosphorylation. To test whether tyrosine phosphorylation of E-cadherin complexes can directly affect α-catenin association, keratinocyte extracts were immunoprecipitated with anti–E-cadherin antibodies, and the immunoprecipitates were treated with a tyrosine-specific phosphatase ± tyrosine phosphatase inhibitors. Preliminary experiments, which included untreated controls, showed that dephosphorylation of β- and γ-catenins by the Yersinia tyrosine phosphatase was totally blocked by the inhibitors at the chosen concentrations (not shown). Immunocomplexes were washed under higher stringency conditions than in the previous experiments using LiCl, which has been shown to affect charge-based interactions of stable protein–protein complexes (El-Baradi et al., 1984). Samples were then analyzed by SDS-PAGE and sequential immunoblotting with antibodies against phosphotyrosine, α-catenin, β- and γ-catenins, and E-cadherin. Tyrosine phosphorylation of β- and γ-catenins was totally abrogated by phosphatase treatment in the absence of the inhibitor, whereas it remained unaffected in its presence (Fig. 5). α-Catenin was readily detected in the complexes treated with phosphatase plus inhibitor, and higher levels of this catenin were found in the complexes derived from calcium-treated versus untreated keratinocytes. By contrast, under the same washing conditions, no α-catenin was left in the complexes treated with phosphatase without inhibitor (Fig. 5 *A*). Levels of β- and γ-catenins were also slightly reduced in these samples, suggesting that tyrosine phosphorylation may also stabilize association of these proteins with E-cadherin, even if to a lesser extent than α-catenin (Fig. 5 *A*). To eliminate this variable, a second independent experiment was performed, where the E-cadherin immunoprecipitated samples were normalized for amounts of β- and γ-catenin levels before immunoblot analysis. Even under these conditions, the amount of α-catenin associated with the E-cadherin complexes was found to be markedly reduced after tyrosine phosphatase treatment (Fig. 5 *B*).

Thus, the increased association of α-catenin and p120-Cas with E-cadherin, which occurs in calcium-treated keratinocytes, is blocked by tyrosine kinase inhibition and the strength of α-catenin association is directly dependent on tyrosine phosphorylation of E-cadherin–associated proteins.

Inhibition of Tyrosine Phosphorylation Interferes with the Normal Cell Adhesive Function of Differentiating Keratinocytes

To assess whether the biochemical alterations induced by tyrosine kinase inhibition were associated with altered cell junction formation, keratinocytes were examined by transmission electron microscopy at 9 h after calcium treatment, at a time when tyrosine phosphorylation of β- and γ-catenins and p120-Cas is highly induced, and close cell–cell contacts are formed. Efficient cell–cell junction and formation was observed in control cells, with cell borders being brought into close juxtaposition (Fig. 6 *A*, *left*; and data not shown). By contrast, in Genistein-treated cells, only very few and incompletely formed cell junctions were detected. Cell borders remained quite distant from each other and were connected by protrusions of neighboring cells in the form of pseudopod-like structures which met without forming any kind of organized adhesive structure (Fig. 6 *A*, *center*). As previously reported (Filvaroff et al., 1990), calcium-induced reorganization of the actin/keratin cytoskeleton was also prevented. Tyrphostin treatment also resulted in suppression of cell junctions, but in this case no pseudopod formation of neighboring cells was detected (Fig. 6 *A*, *right*). Widespread suppression of membrane detergent-insoluble structures containing E-cadherin and plakophilin was confirmed by immunofluorescence analysis of control and tyrosine kinase inhibitor-treated cells with the corresponding antibodies (Fig. 3). Interestingly, incomplete cell adhesion and membrane protrusions

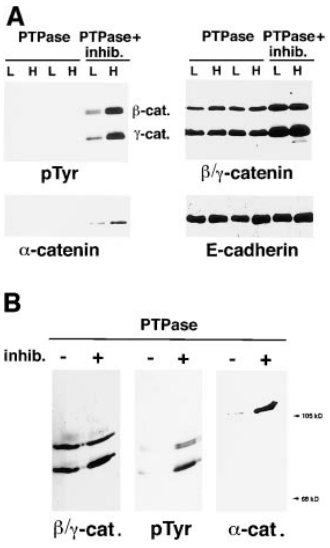

Figure 5. Strength of α-catenin association with E-cadherin complexes as a direct function of tyrosine phosphorylation. (*A*) Keratinocytes in low calcium medium (*L*) and at 9 h after high calcium addition (*H*) were lysed in 0.5% NP-40 lysis buffer, and immunoprecipitated with mAbs against E-cadherin. Immunocomplexes were treated with tyrosine-specific phosphatase (*PTPase*) ± phosphatase-specific inhibitors, followed by washing under high stringency conditions, as described in Materials and Methods. Samples were analyzed by SDS-PAGE and anti-phosphotyrosine immunoblotting (*top panel*). The same blots were subsequently reprobed with antibodies against α-, β-, and γ-catenins, and E-cadherin, as indicated. (*B*) A second independent experiment was performed, similar to the previous one, except that E-cadherin immunoprecipitated samples from keratinocytes at 9 h of calcium treatment were normalized for amounts of β- and γ-catenin levels (by a preliminary immunoblotting experiment), before the immunoblot analysis shown here. Densitometric quantitation of the film indicated that the relative ratio of α-catenin in the immunoprecipitates treated with phosphatase plus versus minus inhibitor was 6.2, while that of β- and γ-catenin was 1.2 and 0.8, respectively.

similar to those of control keratinocytes at 2 h of calcium treatment were observed.

The biochemical and structural alterations of cell junction formation that result from tyrosine kinase inhibition may be associated with some significant functional defects. To explore this possibility, we developed a new functional assay for cohesiveness of cell adhesion. Dispase is a protease that degrades preferentially molecules such as type IV collagen and fibronectin, which function as important attachment sites for keratinocytes to the underlying substratum (Stenn et al., 1989). Dispase treatment is routinely used for skin grafting assays, to detach confluent cultures of well-differentiated keratinocytes as intact sheets of cells. We reasoned that a lack of cohesive strength may be revealed under these conditions, when keratinocytes lose attachment to their support and are connected to each other only through direct intercellular contacts. Treatment of primary keratinocyte cultures in low calcium medium with dispase caused detachment of cells as single cell suspension, consistent with the fact that keratinocytes under these conditions are only very loosely connected with each other. Conversely, keratinocyte cultures switched to high calcium medium for either 9 or 24 h were much more resistant to dispase treatment and eventually (after 25–30 min) detached from the dish as confluent sheet of cells (Fig. 7 *A*). Genistein-treated cultures switched to high calcium conditions behaved differently from the controls. Already by 5 min of dispase treatment when control cells were still unaffected, the Genistein-treated keratinocytes started to detach, not as a sheet of cells, but in a localized fashion, generating the appearance of "holes" in the confluent monolayers (Fig. 7 *A*). Curiously, one or two cells were selectively left at the center of these holes, the nature of which remains to be assessed. A similar pattern of cell detachment was also observed with Tyrphostin-treated keratinocytes (data not shown).

Quantification was achieved by counting the number of single cells released by mechanical disruption after a 35-min dispase treatment, normalized for the total number of cells recovered after subsequent treatment of the same samples with trypsin (Fig. 8). This assay confirmed at the functional level our immunofluorescence findings (Fig. 3) that, in primary mouse keratinocytes cultured under our conditions, cell–cell adhesion is only marginally increased by 2 h of calcium treatment, whereas it is firmly established by 9 h and beyond (Fig. 8 *A*). Genistein and Typhostin treatment caused a >50% reduction in strength of cell adhesion (Fig. 8 *B*). PP1 is a recently developed inhibitor of tyrosine kinases of the Src family (Hanke et al., 1996). Concomitant treatment of keratinocytes with calcium and PP1 caused a drastic inhibition of β- and γ-catenin tyrosine phosphorylation (not shown) and, at the same time, a reduction in strength of cell adhesion comparable to that induced by the other tyrosine kinase inhibitors (Fig. 8 *B*), together with the same pattern of cell detachment (not shown).

Thus, block of tyrosine phosphorylation has important structural and functional consequences on calcium-induced cell adhesion. Formation of normal cell junctions is prevented, and cohesive strength of confluent keratinocyte cultures, as revealed by a novel dispase-based assay, is significantly reduced.

Expression of an Activated Src Oncoprotein Suppresses Keratinocyte Cell–Cell Adhesion through Mechanisms Other Than Disruption of α-Catenin/E-Cadherin Complexes

Previous work with mitogenically stimulated or oncogene-transformed cells established a correlation between increased tyrosine phosphorylation of β- and γ-catenins and p120-Cas and decreased rather than increased strength of cell adhesion (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). However, more recent evidence has suggested that tyrosine phosphorylation of β-catenin in *src*-transformed cells cannot be directly responsible for loosening of cell adhesion, and tyrosine phosphorylation of some other proteins must be involved (Takeda et al., 1995). It was of interest to determine whether this conclusion applies also to keratinocytes. Accordingly, primary mouse keratinocytes were infected with a recombinant adenovirus expressing an activated Src kinase, or a green fluorescent protein vector control. At 24 h after infection, part of the cultures were switched to high calcium medium, and incubation was continued for additional 24 h. The dispase assay described above was used to evaluate the strength of cell adhesion of control versus *src*-transformed keratinocytes, at 24 h after calcium treatment. As reported for other cells, expression of the Src kinase resulted in a marked disruption of cell adhesion (Fig. 8 *C*). Parallel cultures were analyzed for E-cadherin complex formation by immunoprecipitation with anti–E-cadherin

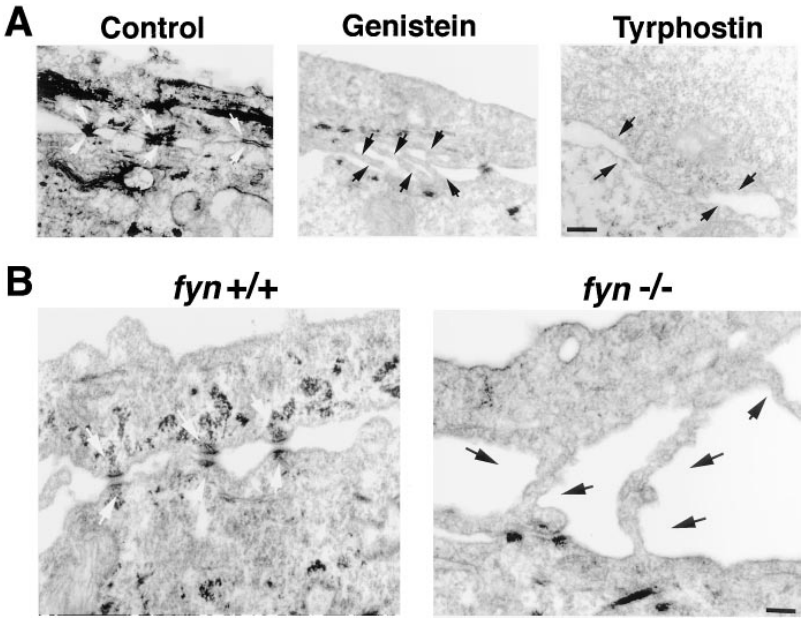

Figure 6. Inefficient cell–cell junction formation in calcium-treated keratinocytes as a consequence of tyrosine kinase inhibition or lack of the Fyn kinase. (*A*) Confluent keratinocyte cultures under growing conditions were either kept as untreated controls (*Control*) or pretreated for 2 h with 100 μM Genistein (*Gen*) or Tyrphostin 23 (*Trph*). Incubation was then continued for additional 9 h under high calcium conditions. Cells were fixed and processed for electron microscopy as described in Materials and Methods. The protrusions from neighboring cells in the Genistein-treated cultures were consistently observed. (*B*) Primary keratinocytes derived from *fyn*−/− mice and wild-type littermates were exposed to high calcium concentrations (2 mM) for 9 h. Cells were fixed and processed for electron microscopy. Note that in the *fyn*−/− cultures, cell borders were far apart and connected by protrusions of the cell membrane similar to those found with genistein-treated keratinocytes (as shown in *A*). Note the presence of well formed cell adhesive junctions in control cells and *fyn*+/+ cells (*A* and *B*, *white arrows*) and the lack of well-formed junctions and of cytoskeleton organization in the tyrosine kinase inhibitor treated and *fyn*−/− cells (*A* and *B*, *black arrows*). Adherens junctions and desmosomes can be defined as electron dense intercellular adhesive structures connected with the actin and keratin cytoskeleton, respectively. Unlike in vivo, in most of our EM photographs of cultured keratinocytes it is hard to see electron-dense junctional structures in connection with either the actin or keratin cytoskeleton, and therefore to conclusively distinguish between adherens junctions and desmosomes. In all cases, the observed alterations (>70% reduction in mature cell junction formation) were observed throughout the dishes (in each case examining at least 10 different fields), and were confirmed in a minimal of two independent experiments. Bars: (*A*) 200 nm; (*B*) 100 nm.

antibodies and sequential immunoblotting with antibodies against phosphotyrosine, α-, β-, and γ-catenins and E-cadherin. β- and γ-catenins were very highly tyrosine phosphorylated in the Src-expressing keratinocytes, whereas their relative amounts remained unchanged (Fig. 9). Importantly, similar levels of α-catenin were found in the E-cadherin complexes from Src expressing versus control keratinocytes, and calcium treatment caused a similar increase of α-catenin association in the two types of cells (Fig. 9). At least two other prominent tyrosine phosphorylated bands were evident in the *src*-transformed keratinocytes, which were not detected in the control (more bands were evident after prolonged exposure of the autoradiograph). One of these bands comigrates and is likely to correspond to E-cadherin. The other, with a size of ~60 kD, was found in Src-expressing keratinocytes after calcium treatment (Fig. 9). Reprobing of the same immunoblot with anti-Src antibodies revealed that this latter protein comigrates and is likely to correspond to constitutively active Src itself, which increasingly associates with E-cadherin complexes in a calcium-dependent manner.

Thus, suppression of cell adhesion in *src*-transformed keratinocytes is not because of disruption of E-cadherin/α-catenin complexes, but correlates with tyrosine phosphorylation of additional proteins not phosphorylated in response to calcium, including E-cadherin itself.

The Fyn Tyrosine Kinase Is Involved in Catenin Phosphorylation and Modulation of Adhesive Function in Differentiating Keratinocytes in Culture

An important question raised by the above results is which kinase(s) are responsible for tyrosine phosphorylation of specific catenins in calcium-induced keratinocyte differentiation. In our previous work, we demonstrated that at least two distinct tyrosine kinases are activated in calcium-induced keratinocyte differentiation, one of which corresponds to Fyn, a specific Src family member (Calautti et al., 1995). An intriguing possibility was that Fyn may be at least partially responsible for tyrosine phosphorylation of β- and γ-catenins and p120-Cas in differentiating keratinocytes. Consistent with this possibility, immunofluorescence analysis of detergent-extracted keratinocytes showed that the after calcium treatment the Fyn kinase colocalizes with E-cadherin at sites of cell adhesion (Fig. 10).

The tyrosine phosphorylation state of E-cadherin–associated molecules was analyzed in primary keratinocytes derived from mice with a disruption of the *fyn* gene versus genetically matched wild-type controls. Cells were either kept in low calcium medium or exposed to high calcium concentrations for various amounts of time. Cell extracts were immunoprecipitated with antibodies against E-cadherin or p120-Cas, followed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 11 *A*, *top panels*). The same immunoprecipitates were then blotted with antibodies against β- and γ-catenins and p120-Cas, to verify amounts of the respective proteins (Fig. 11 *A*, *bottom panels*). Tyrosine phosphorylation of all three catenins was found to be reduced in *fyn*-deficient keratinocytes already under basal conditions. In response to calcium, tyrosine phosphorylation of β- and γ-catenin increased in *fyn*-deficient cells, but to a much lesser extent than in the wild-type controls. Tyrosine phosphorylation of p120-Cas was also strongly reduced in the *fyn*-deficient cells, with phosphory-

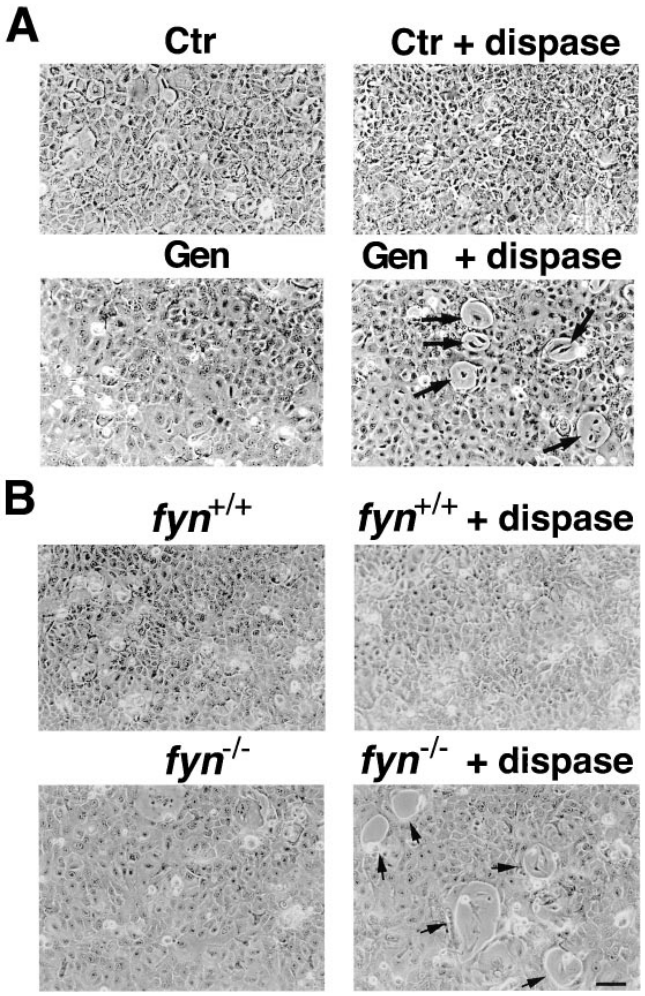

Figure 7. Decreased strength of calcium-induced cell adhesion as a consequence of tyrosine kinase inhibition or lack of the Fyn kinase. (*A*) Primary keratinocytes under growing conditions were either tested as untreated controls (*Ctr*) or pretreated for 2 h with Genistein. Incubation was then continued for additional 24 h under high calcium conditions. Cultures were examined as such (*left panels*) or after dispase treatment for 5 min (*right panels*). *Arrows*, the focal areas of cell detachment that occurred in the tyrosine kinase inhibitor-treated cultures. No such areas were evident in control cultures even after prolonged dispase exposure (>30 min). Instead, control cells eventually detached from the dish as a confluent sheet. Similar results were observed with keratinocyte cultures switched to high calcium conditions for only 9 h (not shown). (*B*) Primary keratinocytes derived from *fyn*−/− mice and wild-type littermates were exposed to high calcium concentrations (2 mM) for 9 h. Cultures were examined as such (*left panels*) or after treatment with dispase for 5 min (*right panels*). *Arrows*, the focal areas of cell detachment that occurred in the *fyn*−/− cultures already at this time. There were no cells missing in the monolayer of *fyn* knockout keratinocytes before dispase treatment. As we previously reported (Calautti et al., 1995), the *fyn* knockout keratinocytes fail to stratify and are larger than normal, which explains the different morphological appearance of these cultures relative to the wild-type controls even before dispase treatment. Similar results were observed in two other independent experiments. Bar, 60 μm.

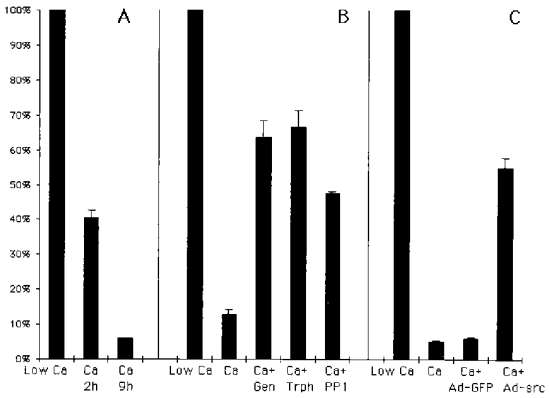

Figure 8. Quantitative measurements of keratinocyte cell adhesion as determined by a novel dispase-based assay. (*A*) Primary keratinocytes under low calcium conditions (*Low Ca*) or treated with calcium for 2 or 9 h (*Ca*) were incubated with dispase for 35 min as described in Materials and Methods. Data are expressed as percentage of single cells released by mechanical disruption after dispase treatment versus total number of cells recovered after subsequent treatment of the same samples with trypsin. (*B*) A similar assay was performed with keratinocytes under low calcium conditions or incubated with calcium for 9 h (*Ca*) in the absence or presence of Genistein (100 μM) (*Gen*), Thyrphostin (100 μM) (*Trph*), or PP1 (1 μg/ml). Cells were preincubated with the inhibitors for 2 h before calcium treatment. (*C*) Keratinocytes under low calcium conditions were infected with a control adenovirus expressing the green fluorescent protein (*Ad-GFP*) or a virus expressing a constitutively active form of c-src (*Ad-src*). 24 h after infection, cells were switched to high calcium medium and incubation was continued for additional 24 h (*Ca*). Cells were analyzed by the dispase assay as before, together with control uninfected cells kept in either low or high calcium medium for 24 h.

lation of the high molecular weight isoforms remaining close to undetectable even in cells under high calcium conditions. The decreased levels of catenin tyrosine phosphorylation appear to be specific for cells lacking the Fyn kinase, as they were not observed in cells with a disruption of the related *yes* or *src* kinase genes (Fig. 11 *A*; data not shown).

This reduction of tyrosine phosphorylation in *fyn*-deficient keratinocytes may have structural and/or functional consequences similar to those caused by tyrosine kinase inhibition. In fact, relative to wild-type controls, electron microscopy of *fyn*-deficient keratinocytes treated with calcium showed a drastic reduction in cell junction formation (Fig. 6 *B*). Cell–cell borders remained widely separated and were connected by pseudopod-like protrusions remarkably similar to those observed with Genistein-treated cells. A functional impairment in cell adhesion was also revealed by the dispase assay with wild type and *fyn* knockout cultures tested at 9 h of calcium treatment (Fig. 7 *B*). Under these conditions, the *fyn*-deficient keratinocytes detached in an uneven fashion, similar to that was observed with tyrosine kinase inhibitor–treated cells.

Thus, in cultured keratinocytes, basal and calcium-induced tyrosine phosphorylation of β- and γ-catenin and p120-Cas is partially dependent on the Fyn kinase. Lack of this kinase has important structural and functional consequences that share substantial similarities with those induced by tyrosine kinase inhibitors.

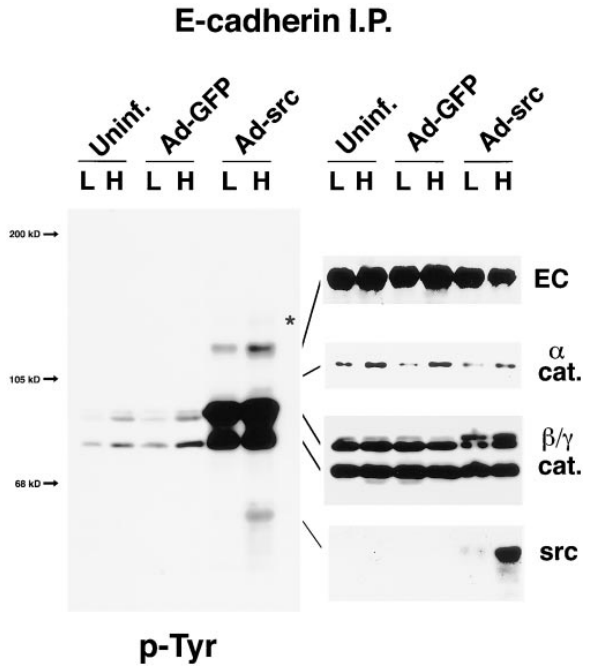

Figure 9. Tyrosine phosphorylation and protein composition of E-cadherin complexes in control versus src-transformed keratinocytes. Keratinocytes under low calcium conditions were infected with either a green fluorescent protein control adenovirus (*Ad-GFP*) or a virus expressing constitutively active c-src (*Ad-src*). 24 h after infection cells were incubated in either low (*L*) or high (*H*) calcium medium for additional 9 h, in parallel with control uninfected cells (*Uninf.*). Keratinocytes were lysed in 0.5% NP-40 lysis buffer, and immunoprecipitated with mAbs against E-cadherin. Samples were analyzed by SDS-PAGE and anti-phosphotyrosine immunoblotting (*p-Tyr*, *left*). The same blot was subsequently reprobed with antibodies against α-catenin (*α-cat.*), β-catenin, and γ-catenin (*β/γ-cat.*), E-cadherin (*EC*), and Src as indicated (*right panels*). The asterisk on the anti-phosphotyrosine immunoblot indicates one of the additional tyrosine phosphorylated proteins that are present in the E-cadherin immunoprecipitates from Src-expressing cells, and which become more evident after prolonged exposures.

Fyn and Src Kinases Are Important Determinants of β-Catenin Tyrosine Phosphorylation and Cell Adhesion in Keratinocytes In Vivo

Unlike the significant alterations found in *fyn*-deficient keratinocytes in culture, the epidermis of *fyn* knockout mice shows a partial reduction of differentiation marker expression (Calautti et al., 1995) but looks otherwise normal. One possible explanation for this discrepancy between the in vitro and in vivo situations could be the capability of other Src family members to compensate for Fyn function in the intact epidermis. We investigated this possibility as it relates to E-cadherin function, by analyzing the skin of mice with a concomitant disruption of the *fyn*/*yes* and *fyn*/*src* genes in comparison with that of wild-type and single knockout littermates. More than 90% of mice with double *fyn*/*src,* and ~70% of mice with *fyn*/*yes* homozygous mutations die soon after birth (Stein et al., 1994), thus limiting our analysis to newborn animals.

Direct biochemical analysis of keratinocytes in the skin is complicated by the presence of other cell types. However, we took advantage of the fact that E-cadherin is preferentially expressed in keratinocytes, to directly immunoprecipitate this protein from total skin extracts and evaluate the in vivo tyrosine phosphorylation pattern of E-cadherin associated proteins by anti-phosphotyrosine immunoblotting. In an initial set of experiments, we compared E-cadherin complexes derived from wild-type newborn skin versus cultured keratinocytes at 9 h of calcium treatment. Immunoprecipitates were normalized for the same amounts of E-cadherin protein and immunoblotted sequentially with antibodies against phosphotyrosine and β- and γ-catenin. Interestingly, the skin-derived E-cadherin complexes contained a single prominent tyrosine phosphorylated band as opposed to the two bands present in the complexes from cultured keratinocytes (Fig. 11 *B*, *left panel*). This different tyrosine phosphorylation pattern reflected the fact that similar amounts of β-catenin were found to be associated with E-cadherin both in vitro and in vivo. By contrast, the association of γ-catenin/plakoglobin with E-cadherin is markedly reduced in vivo, possibly because of a preferential association of this catenin with desmosomal cadherins in the skin (Fig. 11 *B*, *right panel*).

When the skin of single and double knockout mutant mice was analyzed, similar amounts of E-cadherin–associated β-catenin were found in all cases (Fig. 11 *C*, *bottom panel*). Tyrosine phosphorylation levels of this catenin were essentially the same in wild-type mice and mice with single *fyn*, *yes*, or *src* mutations. By contrast, a very drastic reduction of β-catenin tyrosine phosphorylation was observed in mice with a concomitant disruption of the *fyn* and *src* genes, and, to a lesser extent, in *fyn*/*yes* mutants (Fig. 11 *C*, *top panel*).

As discussed above, association of p120-Cas with E-cadherin cannot be detected by coimmunoprecipitation under standard stringency conditions. For a direct analysis of this catenin, total skin extracts were immunoprecipitated with anti–p120-Cas antibodies. Immunoblotting with anti-phosphotyrosine antibodies revealed that levels of tyrosine phosphorylated p120-Cas were essentially normal in the skin of single *yes* knockout mice and were slightly reduced in the skins of mice with a single *fyn* or *src* mutations. By contrast, tyrosine phosphorylation of all forms of p120-Cas was strikingly reduced in the skin of double *fyn*/*src* mutants. A significant but lesser reduction was also observed in the skin of *fyn*/*yes* knockouts (Fig. 11 *D*, *top panel*). Surprisingly, however, in contrast to the intrinsically decreased tyrosine phosphorylation of β-catenin in the presence of constant protein, p120-Cas protein levels, and not only their tyrosine phosphorylation state, were drastically reduced in the double knockouts. This was revealed by immunoblotting with anti–p120-Cas antibodies of the p120-Cas immunoprecipitates (Fig. 11 *D*, *bottom panel*), as well as by immunoblotting of total skin extracts (data not shown).

We tested whether the striking biochemical alterations found in the skins of double *fyn*/*src*-deficient mice were associated with defects in keratinocyte cell adhesion detectable at the histological and/or ultra structural level. Newborn mouse epidermis is composed of multiple stratified layers. In contrast to normal skin, a separation of overlying layers was observed occasionally in histological sections of double *fyn*/*src* mutant skins (data not shown). Much more widespread and significant alterations were

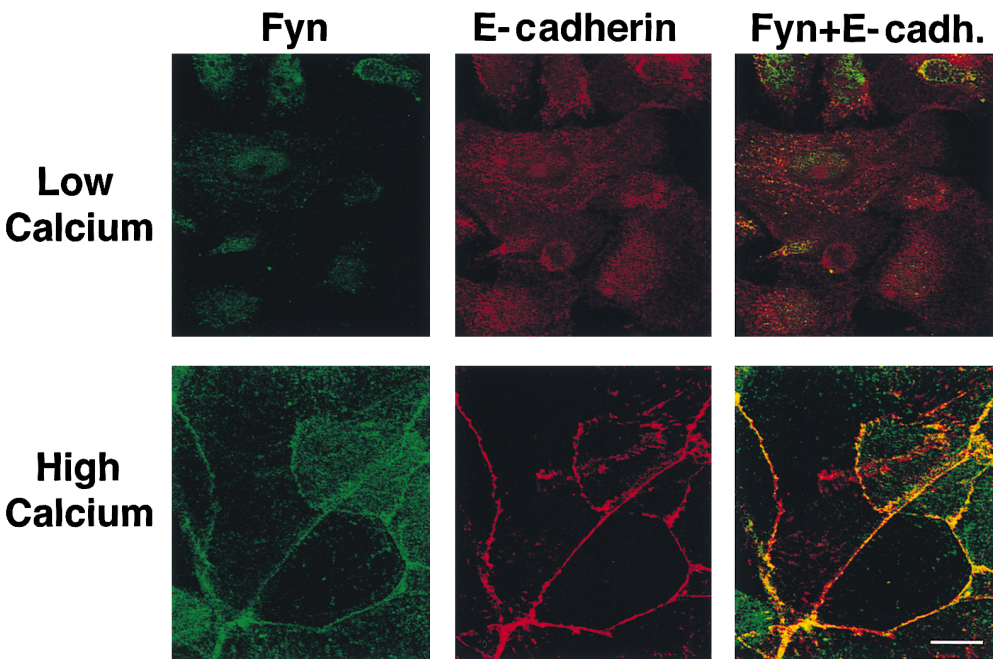

Figure 10. Colocalization of Fyn and E-cadherin at sites of cell–cell adhesion in calcium-treated keratinocytes. Mouse primary keratinocytes in low calcium medium (*top row*) or at 9 h of calcium exposure (*bottom row*) were pre-extracted in 0.2% Triton X-100 buffer before paraformaldehyde fixation as described in Materials and Methods. Cells were double stained with affinity-purified anti-Fyn polyclonal antibodies and FITC-conjugated secondaries (*green*) and mAbs against E-cadherin and Texas red–conjugated secondaries (*red*). Samples were analyzed by confocal microscopy and green and red images were superimposed (*right panels*), so that sites of staining overlap are visualized as yellow. Images correspond to single focal plans. Bar, 15 μm.

revealed by electron microscopy (Fig. 12), with cells at the border between spinosum and granular layers appearing as the most affected. Similar numbers of well-formed desmosomes were present in the epidermis of wild-type and *fyn*/*src* mutant epidermis. However, the tracts of cell membranes between desmosomal junctions, corresponding to sites of adherens junction formation, were usually closely juxtaposed in wild-type skin (Fig. 12 *A*). By contrast, the interdesmosomal spaces were often widely separated in the double knockouts (Fig. 12 *B*), generating the appearance of a series of arches or fenestrations between neighboring cells (Fig. 12 *C*).

In parallel with their lesser biochemical alterations, skins of double *fyn*/*yes* mutant mice showed none of the ultra structural abnormalities in keratinocyte cell adhesion found in the *fyn*/*src* knockouts. However, subtler alterations may also exist in the epidermis of these mice as indicated by their reduced number of layers (data not shown).

Thus, unlike the significant alterations found in culture, keratinocyte cell adhesion in the epidermis of *fyn* deficient mice appears essentially normal. This is likely due to a functional compensation of Fyn with related kinases. In fact, in the skin of mice with a double *fyn*/*src* knockout mutation, tyrosine phosphorylation of β-catenin is intrinsically reduced, p120-Cas is strongly downmodulated, and interdesmosomal spaces are largely increased.

Discussion

We have previously demonstrated that induction of tyrosine phosphorylation is required for keratinocyte differentiation to occur, and that at least two distinct tyrosine kinases are involved, one of which was identified as Fyn. However, only two proteins of ill-defined function were found to be tyrosine phosphorylated in this process, *ras*-GAP–associated p62 and cortactin, and the specific physiological function(s) of tyrosine kinase activation in keratinocyte differentiation remained to be established. Also, the significance of tyrosine phosphorylation for keratinocyte differentiation control in vivo was not assessed. We show here that induction of tyrosine phosphorylation is involved in at least one key aspect of keratinocyte differentiation, i.e., control of cell adhesion, both in cultured cells and in the intact skin.

E-cadherin–mediated adherens junctions provide a primary determinant of increased cell–cell adhesion in keratinocytes (Hodivala and Watt, 1994; Lewis et al., 1994; Amagai et al., 1995). We have found here that three E-cadherin–associated proteins, β- and γ-catenins and p120-Cas, are directly tyrosine phosphorylated at the onset of calcium-induced keratinocyte differentiation, in parallel with establishment of close intercellular contacts and the beginning of stratification. Consistent with the coordinate control of adherens junction and desmosome formation (Hodivala and Watt, 1994; Lewis et al., 1994), Desmoglein-associated γ-catenin also appears to be increasingly tyrosine phosphorylated in response to calcium. We have found that tyrosine phosphorylation of β- and γ-catenins does not affect their association with E-cadherin. In contrast, α-catenin, which is not itself tyrosine phosphorylated, becomes increasingly associated with E-cadherin/catenin complexes, with a time course that parallels that of β- and γ-catenin tyrosine phosphorylation. The increased association of α-catenin with E-cadherin complexes is blocked by tyrosine kinase inhibition, and the strength of α-catenin association depends directly on tyrosine phosphorylation of E-cadherin complexes. α-Catenin provides a direct bridge between cadherin–catenin complexes and the actin cytoskeleton (Rimm et al., 1995). Thus, our findings provide the first evidence that tyrosine phosphorylation can determine the extent of α-catenin association with E-cadherin complexes, and provide an attractive explanation for the fact

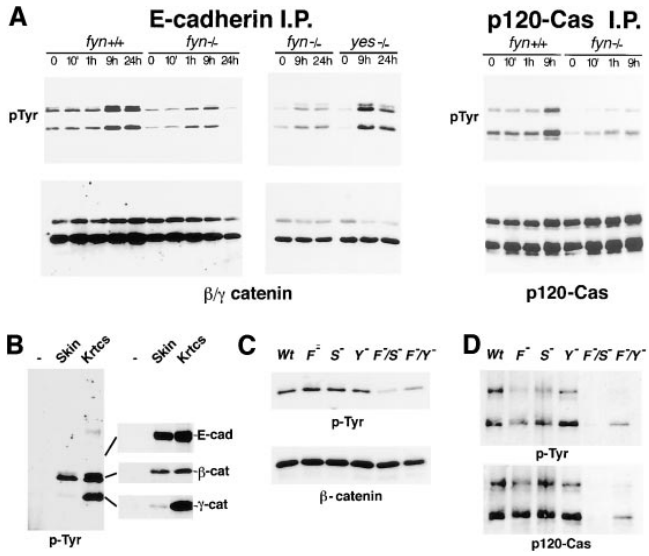

Figure 11. Tyrosine phosphorylation of β- and γ-catenins and p120-Cas in wild-type versus *fyn*−/− keratinocytes, and in the skin of mice with single versus double knockout mutations of *fyn*-related genes. (*A*) Primary keratinocytes derived from *fyn*−/− mice and genetically matched wild-type controls were either kept in low calcium medium (*0*) or exposed to high calcium concentrations (mm) for the indicated amounts of time. Cells were lysed in 0.5% NP-40 lysis buffer, and immunoprecipitated with mAbs against either E-cadherin (*A*) or p120-Cas (*B*). In *A*, a second independent experiment is also shown, with E-cadherin immunoprecipitates from keratinocytes with a disruption of the *fyn* versus *yes* kinase gene. Immune complexes were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies (*top panels*) and with a mixture of antibodies against β- and γ-catenins or anti–p120-Cas (*bottom panels*). (*B*) Pattern of tyrosine phosphorylation and of β- and γ-catenin association in E-cadherin complexes derived from newborn wild-type mouse skin (*Skin*) versus cultured keratinocytes (*Krcts*). Total extracts of newborn mouse skin along with extracts from cultured keratinocytes at 9 h after calcium exposure were immunoprecipitated with anti–E-cadherin antibodies or with unrelated control antibodies (−). Immunoprecipitates were analyzed by SDS-PAGE and sequential immunoblotting with antibodies against phosphotyrosine (*p-Tyr*), β- and γ-catenins and E-cadherin, as indicated. (*C*) tyrosine phosphorylation of E-cadherin associated proteins (β-catenin) in the skin of newborn mice with single versus double knockout mutations of *fyn*-related genes. Newborn mice with single knockout mutations of the *fyn* (F^-), *src* (S^-), and *yes* (Y^-) genes, and with double *fyn*/*src* (F^-/S^-) and *fyn*/*yes* (F^-/Y^-) mutations and wild-type littermates, were killed immediately after birth. Total skin extracts were normalized for E-cadherin amounts by preliminary SDS-PAGE and immunoblotting with E-cadherin antibodies. Normalized extracts were immunoprecipitated with anti–E-cadherin antibodies followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies (*top panel*). The same blot was reprobed with antibodies against β-catenin (*bottom panel*). (*D*) Tyrosine phosphorylation and total protein levels of p120-Cas in the skin of newborn mice with single versus double knockout mutations of *fyn*-related genes. Same amounts of the extracts used above were immunoprecipitated with anti–p120-Cas antibodies followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies (*top panel*). The same blot was reprobed with antibodies against p120-Cas (*bottom panel*).

that in differentiating keratinocytes increased β- and γ-catenin tyrosine phosphorylation is associated with an increased strength of cell adhesion. Control of adherens junction and desmosome formation is intimately connected (Hodivala and Watt, 1994; Lewis et al., 1994; Amagai et al., 1995). Our data indicate that desmosomes may also be controlled by tyrosine phosphorylation, either directly (for instance, by γ-catenin tyrosine phosphorylation) or indirectly (through modulation of adherens junction formation).

In mitogenically stimulated or oncogene-transformed cells, increased tyrosine phosphorylation of β- and γ-catenins has been correlated with a decrease of cell adhesion (Kanner et al., 1991; Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Hoschuetzky et al., 1994; Shibamoto et al., 1994; Kinch et al., 1995). However, additional evidence indicates that loosening of cell contacts in Src-transformed cells cannot be explained by tyrosine phosphorylation of β-catenin, but is likely resulting from tyrosine phosphorylation of some other proteins (Taketa et al., 1995). A similar conclusion is likely to apply to our cells. In fact, in keratinocytes expressing a constitutively active Src kinase, the highly increased tyrosine phosphorylation of β- and γ-catenins does not alter their stoichiometry in E-cadherin complexes, nor the basal or calcium-induced α-catenin association. Given the relatively low tyrosine phosphorylation levels of β-catenin in normal calcium-treated keratinocytes, only a tentative tyrosine phosphopeptide map could be obtained. These results are consistent with the possibility raised by our other findings that (*a*) the residues required for basal α-catenin association are already tyrosine phosphorylated in keratinocytes under low calcium conditions and (*b*) the new residues required for calcium-induced association are not tyrosine phosphorylated by the constitutively active Src kinase. However, the additional possibility should be considered that any positive effects of tyrosine phosphorylation of E-cadherin–associated proteins by Src may be counteracted by tyrosine phosphorylation of other proteins. In fact, we have found that *src*-transformed keratinocytes still respond to calcium but phosphorylate additional proteins, including E-cadherin itself, in an aberrant manner. In addition, constitutively active Src itself was also found to associate with E-cadherin complexes in a calcium-dependent manner. Thus, by analogy with the results of Takeda et al. (1995), tyrosine phosphorylation of proteins other than β- and γ-catenins would seem likely to contribute to suppression of keratinocyte cell adhesion.

Besides its role in cadherin-mediated cell adhesion, β-catenin can play a second independent function in signaling pathways connected with development and cell fate determination (for review see Gumbiner, 1995; Miller and Moon, 1996). A fraction of β-catenin has been reported to localize to the nucleus and to influence, through association with transcription factors such as LEF-1 or Tcf4, gene expression (Behrens et al., 1996; Korinek et al., 1997; Morin et al., 1997). Mice with a disruption (van Genderen et al., 1994) or inappropriate expression (Zhou et al., 1995) of the LEF gene exhibit a significantly altered skin phenotype. Future studies will have to address whether even in keratinocytes β-catenin is involved in complex functions other than cell adhesion, and whether the extent of this involvement can be controlled by tyrosine phosphorylation.

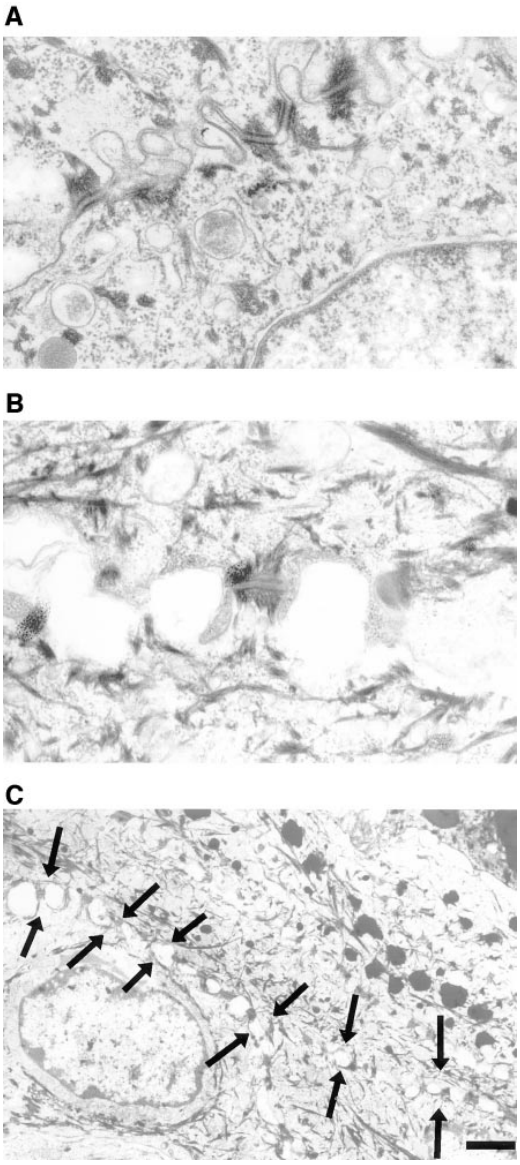

Figure 12. Altered cell–cell adhesion in the skin of double *fyn*/*src* knockouts as directly assessed by ultra structural analysis. Newborn mice with single versus double knockout mutations of the *fyn* (F^-), *src* (S^-), and *yes* (Y^-) genes, and wild-type littermates, were killed immediately after birth and skin samples were processed for electron microscopy. (A) Ultrastructural analysis of skin from a wild-type newborn animal. (*B* and *C*) Ultrastructural analysis of the skin from a double *fyn*/*src* knockout animal at high (*B*) and low (*C*) magnification. Similar alterations were found in the skin of three double *fyn*/*src* knockouts, derived from two independent litters. Arrows point to the interdesmosomal areas of cell detachment found in the *fyn*/*src* mutant skin. The skin of all other mutant animals was found to have desmosomal structures and interdesmosomal spaces similar to the wild-type controls. Bars: (*A* and *B*) 300 nm; (*C*) 1.4 μm.

The other catenin that is found to be directly tyrosine phosphorylated in differentiating keratinocytes is p120-Cas. Four isoforms of p120-Cas have been described, which differ for the presence or the absence of two alternatively spliced sequences (Mo and Reynolds, 1996). All four isoforms are expressed in cultured keratinocytes and become increasingly tyrosine phosphorylated in response to calcium, with kinetics similar to those of β- and γ-catenins. However, whereas the stoichiometry of β- or γ-catenin in E-cadherin complexes is unaffected by calcium treatment, p120-Cas displays an increased association with E-cadherin. The association of p120-Cas is likely to be more unstable than that of β- or γ-catenin, as it could only be detected by immunoprecipitation under mild stringency. As with α-catenin association, inhibition of tyrosine phosphorylation suppressed the increased association of p120-Cas with E-cadherin. This situation in differentiating keratinocytes may be similar to that reported for *ras*-transformed breast epithelial cells, where tyrosine phosphorylation of p120 was correlated with increased association with E-cadherin (Kinch et al., 1995).

p120-Cas binds to E-cadherin in close proximity to the binding site for β- and γ-catenins (Daniel and Reynolds, 1995; Shibamoto et al., 1995). However, unlike these latter molecules, p120-Cas does not bind to α-catenin and thus may function by blocking the interactions of E-cadherin with this latter protein and, indirectly, with the cytoskeleton (Daniel and Reynolds, 1995). An attractive working hypothesis is that tyrosine phosphorylation could play two complementary functions in the changes in cell adhesion connected with keratinocyte differentiation. Tyrosine phosphorylation of β- and γ-catenins, by promoting α-catenin association, would enable cadherin–catenin complexes to become more firmly connected to the actin cytoskeleton, thus enhancing the strength of cell adhesion. By concomitantly increasing E-cadherin/p120-Cas complex formation, tyrosine phosphorylation of these latter molecules would ensure fluidity of cell adhesion, thus allowing stratification. Besides the biochemical data, this hypothesis would be consistent with the functional and structural analysis discussed below.

Previously used assays of cell adhesion depend on dissociation of cells followed by measurement of their reassociation in suspension and/or after centrifugation (Takeda et al., 1995; Angres et al., 1996). The dispase assay that we have developed provides a useful alternative, in that it allows to evaluate epithelial cells as confluent layers, without any previous disruption of already formed cell contacts, and taking into account the cohesive strength of both adherens junction and desmosome formation. Using this approach, we have found that the cohesiveness of keratinocyte cell adhesion induced by calcium is significantly lessened by tyrosine kinase inhibition, and this is associated with a strongly reduced number of well-formed cell junctions. Similar alterations were observed with confluent cultures of *fyn*-deficient keratinocytes, in which calcium-induced tyrosine phosphorylation was strongly decreased, even if not totally suppressed (as discussed further below). In both Genistein-treated and *fyn*-deficient keratinocytes, a distinguishing feature that accompanies the scarcity of intercellular junctions and separation of cell–cell borders, is the formation of pseudopod-like extensions, which may correspond to abortive attempts of cells to establish stable junctions with each other. Interestingly, adherens junction formation has been recently reported to depend on a second signaling pathway involving the *rho* and *rac* GTPases, which control actin/cytoskeleton organization and cell motility (Braga et al., 1997). Thus, it is tempting to suggest

that efficient adherens junctions formation involves at least two complementary, and possibly sequential, steps. Active movement at the cell membrane, leading to bridging protrusions from neighboring cells, would occur in a *rho*- and *rac*-dependent fashion, whereas establishment of fully assembled adherens junctions would be under tyrosine phosphorylation control.

Besides increased strength, fluidity of cell contacts is an important element in keratinocyte differentiation, allowing these cells to stratify (Lewis et al., 1994; Tao et al., 1996). Evidence for tyrosine phosphorylation being involved in dynamic control of cell adhesion is provided by the strongly reduced stratification of keratinocytes treated with tyrosine kinase inhibitors (Filvaroff et al., 1990) or deficient for the Fyn kinase after calcium exposure (Calautti et al., 1995). Whether or not *rho*- and *rac*-dependent pathways are also involved in the stratification process is obviously a relevant question that remains to be assessed.

A final issue raised by our results is which kinase(s) are responsible for tyrosine phosphorylation of β- and γ-catenins and p120-Cas in differentiating keratinocytes. A specific involvement of the Fyn kinase is indicated by the fact that tyrosine phosphorylation of these catenins is significantly reduced in cultured keratinocytes with a disruption of the *fyn* but not *yes* or *src* kinase genes. This fact is consistent with our previous findings that the Fyn kinase is specifically activated in differentiating keratinocytes in culture, and that a number of differentiation parameters are selectively altered in the *fyn*-deficient cells (Calautti et al., 1995). However, Fyn is not the only kinase to be involved, as a lower level of basal and calcium-induced tyrosine phosphorylation of these catenins is still observed in the *fyn*-deficient keratinocytes. We previously showed that a second independent tyrosine kinase is activated in calcium-induced keratinocyte differentiation (Calautti et al., 1995), and it is possible that Fyn and the other calcium-induced kinase cooperate in control of catenin function by phosphorylation. In addition, other Src family members are likely to contribute to catenin tyrosine phosphorylation in keratinocytes. In fact, we have found that functional compensation of Src family members clearly occurs in vivo, as tyrosine phosphorylation levels of β-catenin are essentially normal in the skin of newborn mice with a single *fyn* knockout mutation, whereas they are significantly decreased in the skin of mice with a concomitant disruption of *fyn* and *src* genes. Interestingly, in these same mice, protein levels of p120-Cas, and not simply its tyrosine phosphorylation, are drastically reduced. This points to a so far unexplored control of catenin function by *src*-related kinases, which may be consistent with the already reported upregulation of p120-Cas expression in *src*-transformed cells (Mo and Reynolds, 1996).

In parallel with the biochemical findings, the skin of the double *fyn*/*src* mutants showed striking ultra structural abnormalities, with widely disrupted interdesmosomal spaces at sites where adherens junctions are usually found. By contrast, density and structure of desmosomes were essentially normal. In vitro, our data are consistent with the previous demonstration that impaired adherens junction formation affects desmosome assembly as well (Hodivala and Watt, 1994; Lewis et al., 1994; Amagai et al., 1995). In vivo, the results with the *fyn*/*src* mutant skin suggest that control of adherens junctions and desmosome formation are at least partially independent. This possibility would also be consistent with the strikingly different composition of E-cadherin complexes in the in vitro versus in vivo situation, where the ratio of β- to γ-catenin appears to be markedly increased.

The skin of mice with a concomitant disruption of the *fyn* and *yes* genes showed none of the alterations in cell adhesion found in the skin of *fyn*/*src* mutants, indicating that the Src and Yes kinases can compensate for lack of Fyn to a different extent. In fact, in the *fyn*/*yes* knockout skin tyrosine phosphorylation of β-catenin and expression of p120-Cas were found to be decreased, but to a significantly lesser extent than in the *fyn*/*src* mutants. In addition, other more selective biochemical defects may occur in the *fyn*/*src* knockout epidermis, which could contribute to its striking reduction in cell adhesion.

Taken together, our results indicate that tyrosine phosphorylation, and Fyn-related kinases in particular, play an essential function in one critical aspect of keratinocyte differentiation, i.e., control of cell adhesion. This regulatory function is important not only in vitro but also in vivo. More generally, our findings support the notion that in partially redundant regulatory systems, such as that of Src family kinases, the biochemical response of cells in tissues is much more plastic than in culture, so that a combined in vitro/in vivo approach is necessary to dissect the function of any individual component.

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