fyn tyrosine kinase is involved in keratinocyte differentiation control

Enzo Calautti,^{1,3} Caterina Missero,¹ Paul L. Stein,⁴ Robert M. Ezzell,² and G. Paolo Dotto^{1,5}

¹Cutaneous Biology Research Center, ²Surgery Research Unit, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129 USA, ³Department of Genetics, Biology and Medical Chemistry, University of Torino, Italy and ⁴Wistar Institute, Philadelphia, Pennsylvania 19104 USA

Induction of tyrosine phosphorylation is an early and specific event which is required for mouse keratinocyte differentiation to occur, in response to both calcium and TPA (12-*O*-tetradecanoylphorbol-13-acetate). We report here that there is an increase of tyrosine kinase activity immunoprecipitable with anti-phosphotyrosine antibodies specifically in response to calcium—and a number of other divalent cations—within 2 min of exposure. Such an activity does not correspond to any of the known tyrosine kinases that were tested. A second tyrosine kinase activity is induced in response to both calcium and TPA, and has been identified as *fyn*, a nonreceptor tyrosine kinase of the *src* family. *fyn* activation is induced in keratinocytes within 6 hr of calcium exposure, but already within 2 min of TPA treatment. Cortactin, a p80–85 substrate of *src*- and *fyn*-related kinases that localizes with actin at cell adhesion sites, is increasingly tyrosine phosphorylated in calcium- and TPA-induced differentiation, with a time course which parallels that of *fyn* activation. Keratinocytes with a specific disruption of the *fyn*, but not *yes* kinase gene show no induction of phosphorylation of p80–85 proteins, and are significantly altered in their differentiation response both *in vitro* and *in vivo*. Thus, at least two tyrosine kinase activities are induced in keratinocyte differentiation, one of which has been identified as *fyn* and shown to be specifically involved in this process.

[Key Words: *fyn* tyrosine kinase; calcium; TPA; keratinocyte differentiation; cortactin]

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Control of terminal differentiation in normal epithelial cells is still poorly understood. Mouse primary keratinocytes provide an ideal system to elucidate this process. Addition of calcium to cultured keratinocytes triggers a terminal differentiation program which closely resembles that in the skin (Hennings et al. 1980; Hennings and Holbrook 1983). A gradient of increasing calcium concentrations in the upper epidermal layers (Menon et al. 1985) suggests that this ion could be involved in control of keratinocyte differentiation even *in vivo*. Calcium can exert its effects in two distinct fashions. It can act at the cell surface, favoring processes such as cell-cell and cell-substrate interactions (Luna and Hitt 1992), and possibly triggering a transmembrane calcium-receptor mechanism (Filvaroff et al. 1994). It can also induce intracellular signaling pathways, through direct modifications of intracellular ion concentrations (Hennings et al. 1983; Reiss et al. 1991; and references therein) and phospholipase C (PLC) and protein kinase C (PKC) activation (Moscat et al. 1989; Lee and Yuspa 1991; and references therein).

Like calcium, the skin tumor promoter TPA (12-*O*-tetradecanoylphorbol-13-acetate) can elicit some important aspects of keratinocyte differentiation, such as

growth arrest, loricrin and filaggrin production, epidermal transglutaminase activation, and cornification (Dlugosz and Yuspa 1993; and references therein). However, certain other aspects of keratinocyte differentiation triggered by calcium, such as desmosome formation, keratin bundle reorganization and keratin 1 and 10 expression fail to be induced by TPA (Dlugosz and Yuspa 1993). These biological effects suggest that TPA activates signaling pathways partially overlapping with those triggered by calcium. This possibility is further supported by the fact that keratinocyte variants selected for resistance to calcium-induced differentiation are often also resistant to the effects of TPA (Yuspa et al. 1983; Dotto et al. 1986).

As has been shown in other differentiation and developmentally regulated systems (Pazin and Williams 1992), induction of tyrosine phosphorylation is an early and specific event which is required for keratinocyte differentiation, in response to both calcium and TPA (Filvaroff et al. 1990). In particular, tyrosine phosphorylation of a single protein of ~60 kD is induced in keratinocytes specifically in response to calcium, as early as 5' after exposure, while tyrosine phosphorylation of a second protein of ~80–85 kD is induced by both calcium and TPA. The first protein has been identified as "p62" (Filvaroff et al. 1992), a tyrosine phosphorylated protein

⁵Corresponding author.

which associates with *ras*-GAP (Ellis et al. 1990) and which belongs to a novel family of RNA binding proteins (G. Wong et al. 1992).

Nothing is known about the tyrosine kinases activated in mouse keratinocyte differentiation. We report here that at least two distinct tyrosine kinase activities are involved. One activity can be immunoprecipitated with anti-phosphotyrosine antibodies, and is induced specifically in response to calcium—and a number of other divalent cations—within 2 min of exposure. The identity of this activity remains to be established. The other activity is induced in response to both calcium and TPA, and has been identified as *fyn*, a nonreceptor tyrosine kinase of the *src* family. *fyn* activation is observed a few hours after calcium-induced differentiation, but already within 2 min of TPA treatment.

To date, nine tyrosine kinases of the *src* family have been identified, which share similar structural and biochemical properties, but differ in some of the upstream and downstream signaling molecules with which they interact (Brickell 1992; Bolen 1993). Six of these kinases, *fgr*, *lck*, *blk*, *hck*, *lyn*, *yrk*, are expressed with a high degree of tissue specificity, consistent with their involvement in specialized cellular functions. Three kinases, *src*, *yes* and *fyn*, are widely expressed, and appear to have a general role in cell growth and differentiation, as well as more specific cell-type related functions. Functional compensation between these three kinases has been revealed by gene knockout experiments in mice, with homozygous deletions of a single kinase gene causing limited effects, at sites which do not necessarily correlate with kinase expression levels (Soriano et al. 1991; Appleby et al. 1992; Stein et al. 1992; 1994). However, a finer analysis of the mutant mice can still provide important insights into the role of a given kinase in specific cellular functions.

Disruption of the *fyn* gene was previously found to cause altered T-cell receptor signaling in thymocytes, with mature T cells being only minimally affected (Appleby et al. 1992; Stein et al. 1992). Hippocampal function in *fyn*-negative mice is also abnormal (Grant et al. 1992), while other parts of the nervous system, where *fyn* is also usually expressed, are essentially normal. Here, we show that, in parallel with our biochemical findings, *fyn*-deficient keratinocytes are significantly altered in their differentiation response both in vitro and in vivo. In contrast to these cells, keratinocytes of *yes*-mutant mice exhibit an essentially normal differentiation response.

Thus, two distinct tyrosine kinases are induced in keratinocyte differentiation, one of which has been identified as *fyn* and shown to be specifically involved in this process.

Results

A tyrosine kinase activity immunoprecipitable with anti-phosphotyrosine antibodies is induced at very early times of calcium-induced differentiation

Activation of most tyrosine kinases is associated with

their autophosphorylation. Furthermore these enzymes are often found in association with other tyrosine phosphorylated proteins, including their substrates. We tested whether the tyrosine kinases activated at early times of keratinocyte differentiation could be recognized and immunoprecipitated by anti-phosphotyrosine antibodies. Extracts were prepared from mouse primary keratinocytes under growing conditions and at various times of calcium- or TPA-induced differentiation. Extracts were normalized for protein content and immunoprecipitated with anti-phosphotyrosine monoclonal antibodies PY20 (Glenney et al. 1988). The immunoprecipitates were tested by in vitro kinase assays with enolase as exogenous substrate. As shown in Figure 1, there was a significant increase in tyrosine kinase activity in anti-phosphotyrosine immunoprecipitates from keratinocytes exposed to high calcium medium, relative to those from keratinocytes kept under growing conditions. Increased activity was detectable as early as 2 min after calcium addition, and remained at even higher levels for up to 24 hr (Fig. 1A,B). Tyrosine phosphorylation of the enolase substrate was confirmed by direct phosphoamino acid analysis (Fig. 1C). These results were confirmed by in vitro kinase assays with poly(Glu/Tyr) as an exogenous substrate (data not shown).

In contrast to calcium, there was no induction of tyrosine kinase activity in anti-phosphotyrosine immunoprecipitates from keratinocytes at early times of TPA exposure (Fig. 1D). In parallel with our previous findings on p62 tyrosine phosphorylation (Filvaroff et al. 1994), induction of anti-phosphotyrosine-immunoprecipitable kinase activity was also induced by exposure to magnesium and, to an even greater extent, cobalt and nickel ions, whereas no induction was observed after treatment with the calcium ionophore A23187 (Fig. 1D).

Thus, a tyrosine kinase activity which could be immunoprecipitated with anti-phosphotyrosine antibodies was specifically induced at very early times of calcium—but not TPA—induced differentiation. A similar induction was also observed after exposure of keratinocytes to a number of divalent cations, but not to a calcium ionophore.

The fyn tyrosine kinase is induced at later times of calcium-induced differentiation and at very early times of TPA treatment

The calcium-induced kinase activity could correspond to a known tyrosine kinase. To test this possibility, in vitro kinase assays similar to the ones described above were performed, after immunoprecipitation with antibodies specific for a number of known tyrosine kinases (Table 1). None of the tested kinases was found to be activated within a few minutes of calcium-induced differentiation (data not shown). However, a significant induction of tyrosine kinase activity was found in immunoprecipitates with *cst-1* antiserum starting after 1 hr of calcium exposure and remaining elevated up to 24 hr (Fig. 2). The *cst-1* antiserum recognizes an epitope common to three kinases of the *src* family, *src*, *yes*, and *fyn* (Courtneidge

Figure 1. Anti-phosphotyrosine immunoprecipitable tyrosine kinase activity in ion-treated mouse primary keratinocytes. (A,B) Primary mouse keratinocytes were exposed to high calcium concentrations (2 mM) for the indicated amounts of time. Extracts were immunoprecipitated with anti-phosphotyrosine mAbs Py 20 (+) and control antibodies (-) and tested by in vitro kinase assays in the presence of enolase as exogenous substrate. Phosphorylation products were separated on 10% SDS-PAGE and detected by autoradiography. Positions of molecular size markers and of the enolase substrate (arrow) are indicated. Similar results were obtained in ten independent experiments. (C) Direct phosphoamino acid analysis of the eluted enolase substrate shown in panel A. Internal standard positions for phospho-serine (s), phospho-threonine (t) and phospho-tyrosine (y) are indicated. (D) Keratinocytes were exposed to calcium (2 mM), A23187 (2 μ M), TPA (100 ng/ml), magnesium (5 mM), cobalt (5 mM) and nickel (5 mM) for the indicated amounts of time and immunoprecipitated with anti-phosphotyrosine antibodies. In vitro kinase assays were performed as in the previous experiments, except that they were carried out in the presence of 1 rather than 10 μ M cold ATP.

and Smith 1984). In vitro kinase assays after immunoprecipitation with antibodies specific for each of the nine *src* family members were performed. Increased kinase activity was found only in immunoprecipitates with antibodies specific for *fyn*, starting after 1 hr of calcium-induced differentiation (Fig. 2; Table 1). These results were confirmed by in vitro kinase assays with poly(Glu/Tyr) as an exogenous substrate (data not shown).

Immunoblotting with anti-*fyn* antibodies was used to measure amounts of this protein in total keratinocyte extracts as well as in anti-*fyn* immunoprecipitates at various times of calcium-induced differentiation. No induction of *fyn* protein expression was observed, which could explain the observed increase in *fyn* kinase activity (Fig. 3A,B; data not shown). A detailed time course experiment indicated that intrinsic *fyn* kinase activity was induced by 4 hr of calcium treatment and increased up to four- to fivefold by 24 hr (Fig. 3C). Similar results were obtained in a second independent experiment. *fyn* kinase activity was also induced by exposure of keratinocytes to intermediate calcium concentrations, such as 0.1 or 0.5 mM, and was further induced at higher calcium doses (Fig. 3D). This response was specific for calcium, as other divalent cations such as cobalt or nickel failed to induce *fyn* kinase activity to any detectable extent (data not shown).

Given the partially overlapping effects of calcium and TPA on keratinocytes, it was of interest to determine whether *fyn* kinase activation is specific for calcium-

induced differentiation or also occurs after TPA treatment. We found that the tyrosine kinase activity recovered after immunoprecipitation with the *cst-1* antiserum was significantly induced as early as 2 min after TPA treatment (Fig. 4). Such an activity persisted at elevated levels up to 6 hr. A similar increase in tyrosine kinase activity was observed after immunoprecipitation of TPA-treated keratinocytes with *fyn* specific antibodies (Fig. 4). In contrast to *fyn*, tyrosine kinase activity in *yes* immunoprecipitates was slightly decreased by 10 minutes of TPA exposure (Fig. 4). There was little or no effect on *src* kinase activity at these very early times of TPA exposure, and this activity was only slightly increased at later times (Fig. 4; data not shown). As with calcium, *fyn* protein amounts were not significantly affected by the TPA treatment (data not shown).

Thus, *fyn* kinase activity is progressively induced by exposure of keratinocytes to calcium, in a time- and dose-dependent fashion. In TPA-treated keratinocytes, the *fyn* kinase is also specifically activated, but with a much more rapid time of induction than in the calcium-treated cells.

Cortactin, a p80–85 substrate of src and fyn kinases, is tyrosine phosphorylated in keratinocytes in response to both calcium and TPA

It was previously reported that a protein of ~80–85 kD is increasingly tyrosine phosphorylated in primary kerati-

Table 1. In vitro activity of specific tyrosine kinases in mouse keratinocyte differentiation

Kinase	Calcium	
	low	high
<i>src</i>	+	+
<i>fyn</i>	+	+++
<i>yes</i>	+	+
<i>lck</i>	–	–
<i>blk</i>	–	–
<i>fgr</i>	+	+
<i>hck</i>	+	+
<i>lyn</i>	+	+
<i>yrk</i>	+	+
<i>fak</i>	+	+
<i>eck</i>	+	–
<i>met</i>	+	+
<i>abl</i>	+	+

The listed tyrosine kinases were tested for their activity by in vitro assays with enolase as an exogenous substrate, after immunoprecipitation with kinase-specific antibodies (as described in Materials and methods). Kinase activity was compared in keratinocytes under growing conditions and at various times of calcium-induced differentiation (10 min, 1, 6, 12, and 24 hr). (+ and –) The presence or absence, respectively, of basal activity levels detectable over background (in nonimmune control). (+++) Increased activity relative to basal levels.

nocytes within 5–10' of TPA treatment and a few hours after calcium exposure (Filvaroff et al. 1990). A protein of similar molecular weight, cortactin, was previously identified as being highly tyrosine phosphorylated in cells expressing activated forms of *src*, *fgr* and *fyn* kinases (Wu et al. 1991; Wu and Parsons 1993). Keratinocyte cell extracts were immunoprecipitated with anti-cortactin antibodies followed by immunoblotting with either the same antibodies or antibodies against phosphotyrosine. While cortactin protein levels did not vary to any significant extent, tyrosine phosphorylation of this protein was strongly induced in response to both calcium and TPA (Fig. 5A). Enhanced tyrosine phosphorylation of cortactin was already evident by 10 minutes of TPA exposure (Fig. 5A). In response to calcium, cortactin became increasingly tyrosine phosphorylated after 1 hr of exposure and, by 24 hr, phosphorylation levels were comparable to those found in *src*-transformed fibroblasts (Fig. 5B).

As we previously reported (Filvaroff et al. 1990), immunoblotting of total keratinocyte cell extracts with anti-phosphotyrosine antibodies showed induced phosphorylation of a ~p80–85 protein in the calcium-treated cells. Levels of the tyrosine phosphorylated p80–85 proteins were only marginally reduced by preclearing of the keratinocyte cell extracts with anti-cortactin antibodies, under conditions where cortactin itself was totally removed (Fig. 5C).

Thus, tyrosine phosphorylation of cortactin, a substrate of *src*- and *fyn*-related kinases, is induced in keratinocytes in response to both calcium and TPA, with a

time course which is similar to that of *fyn* activation. Cortactin appears to be only one of a group of ~80–85 kD proteins which are tyrosine phosphorylated upon induction of keratinocyte differentiation.

Keratinocytes of fyn-negative mice exhibit an altered differentiation response under both in vitro and in vivo conditions

fyn activation may play a direct role in control of keratinocyte differentiation or, alternatively, may be a secondary event. To address this question, we compared the differentiation behavior of primary keratinocytes derived from *fyn*-negative mice with that of cells from wild-type

Figure 2. Tyrosine kinase activity associated with *src*-related kinases in calcium-treated keratinocytes. Extracts from keratinocytes at various times of calcium exposure were immunoprecipitated with wide specificity *cst-1* or *fyn*-, *src*-, and *yes*-specific antibodies and tested by in vitro kinase assay with enolase as an exogenous substrate. Reaction products were analyzed by gel electrophoresis and autoradiography (1 day of exposure). Densitometric analysis revealed a 1.2, 1.6, 2.9, 2.8 and 4.3-fold increase of enolase phosphorylation over basal levels in the *fyn* immunoprecipitates from the 1, 6, 8, 12 and 24 hr calcium-treated samples, respectively. Similar results were obtained in at least four independent experiments, except that the slight decrease in *c-src* activity shown here after 1 hr of calcium exposure was not consistently observed.

Figure 3. Induction of intrinsic *fyn* kinase activity by calcium treatment in a time- and dose-dependent fashion. Extracts from keratinocytes at various times of calcium exposure were immunoprecipitated with *fyn*-specific antibodies in the absence (-) or presence (+) of neutralizing peptide. The same immunoprecipitates were tested for in vitro kinase activity with enolase (A) and for *fyn* protein levels by immunoblotting with *fyn*-specific antibodies (B). The positions of *fyn*, of a p72 *fyn*-related protein (da Silva et al. 1993) and of immunoglobulins heavy chains (Abs.) are indicated. (C) detailed time course of *fyn* kinase activation after calcium (2 mM) exposure. Extracts were immunoprecipitated with *fyn*-specific antibodies and processed for enolase kinase assay as in panel A and for *fyn* immunoblot as shown in panel B. Intrinsic *fyn* kinase activity was determined as described in Materials and methods and expressed as

fold of induction relative to basal *fyn* activity in untreated control cells. The graph shows one typical experiment. Similar results were obtained in another independent experiment. (D) *fyn* kinase activation as a function of extracellular calcium concentrations. Primary keratinocytes were treated for 24 hr with the indicated amounts of calcium, and *fyn* kinase activity was determined as described above. Values are expressed as fold of induction relative to basal *fyn* activity in untreated control cells. The graph shows one typical experiment. Similar results were obtained in three other independent experiments.

syngeneic controls. To address the issue of specificity, keratinocytes derived from *yes*-deficient mice were also examined.

Primary keratinocytes from the *fyn* or *yes* mutant mice grew into confluence to approximately the same time as the wild-type controls, indicating that the absence of either kinase has no severe effects on keratinocyte growth under our culture conditions. Morphologically, *fyn*-negative keratinocytes appeared less refractile than wild-type cells already under basal growing conditions (Fig. 6A,B). However, after TPA treatment, the differences between these two types of cells were more apparent; the mutant keratinocytes failed to assume the typical spindle morphology induced by TPA, and were much less refractile than the controls (Fig. 6C,D). Accentuated morphological differences were also found after calcium exposure, with the *fyn*-negative cells failing to stratify to any significant extent, and assuming a much larger size than their wild-type counterparts (Fig. 6E,F). Fluorescent staining for filamentous (F-) actin cables confirmed these differences in size, but failed to show any significant alterations in the actin cytoskeleton organization (Fig. 6G,H). These morphological differences were consistently observed with two independent preparations of *fyn*-negative and positive keratinocytes. Control experiments with wild-type cells under more sparse conditions were also performed to ensure that the observed morphological alterations of the *fyn*-negative keratinocytes were not simply due to cell density variations. The morphological features of the *fyn*-negative keratinocytes were also not observed with the *yes*-mutant cells (data not shown).

Figure 4. Tyrosine kinase activity associated with *src*-related kinases in TPA-treated keratinocytes. Extracts from keratinocytes at various times of TPA exposure (100 ng/ml) were immunoprecipitated with wide specificity *cst-1* or *fyn*-, *src*- and *yes*-specific and control (-) antibodies and tested by in vitro kinase assay with enolase as an exogenous substrate. To verify that the assays were performed in antibody excess, three times more extracts of the 1 hr TPA-treated cells [1h (3×)] were also used for immunoprecipitation with the same amount of *cst-1* antibodies used for the other samples. Reaction products were analyzed by SDS-gel electrophoresis and autoradiography (1-day exposure). Densitometric analysis revealed a 3.3 and 3.1-fold increase in enolase phosphorylation in the anti-*fyn* immunoprecipitates at 5' and 10' of TPA exposure, respectively. Similar results were obtained in two other independent experiments.

Figure 5. Tyrosine phosphorylation of cortactin in growing versus differentiating keratinocytes. (A) Extracts from keratinocytes at various times of TPA (100 ng/ml) or calcium treatment (2 mM) were immunoprecipitated with anti-cortactin antibodies or control antibodies (-) and the immune complexes analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-phosphotyrosine antibodies (α-ptyr). The same blot was stripped and blotted with anti-cortactin antibodies (α-crtc). (B) Detailed time course of cortactin tyrosine phosphorylation after calcium exposure. Samples were immunoprecipitated as described in A and analyzed by anti-phosphotyrosine immunoblotting. Similar analysis was performed with untransformed (3T3) and v-src-transformed (3T3/src) NIH-3T3 fibroblasts. (C) Extracts from keratinocytes under growing conditions and 24 hr after calcium-induced differentiation were analyzed by immunoblotting with anti-phosphotyrosine or anti-cortactin antibodies. Total cell extracts were used directly (total) or after preclearing by immunoprecipitation with anti-cortactin antibodies (precleared). The blots were developed with the ECL system (Amersham).

Biochemically, treatment with calcium-induced tyrosine phosphorylation of a number of proteins, including the p80/85 group, in both wild-type and *yes* mutant keratinocytes, while no such induction was observed in the *fyn*-negative cells (Fig. 7, upper panel). Similarly, TPA-induced phosphorylation of the p80/85 proteins was specifically blocked in the *fyn*-negative keratinocytes (Fig. 7, lower panel).

Epidermal transglutaminase is a well characterized enzymatic marker of keratinocyte differentiation (Thacher and Rice 1985), which is induced in cultured keratinocytes in response to both calcium and TPA, and which is blocked by concomitant treatment with tyrosine ki-

nases inhibitors (Filvaroff et al. 1990). In agreement with these previous findings, enzymatic measurements revealed very little or no induction of transglutaminase activity in calcium- and TPA-treated *fyn*-negative keratinocytes, while this enzyme was readily induced in the wild-type controls (Fig. 8).

Differentiation markers of the intermediate and upper epidermal layers such as keratin 1, involucrin and filaggrin were previously reported to be induced in primary mouse keratinocytes by calcium treatment (Yuspa et al. 1989). Exposure of control cells to 2 mM calcium caused a strong induction of keratin 1, involucrin and filaggrin expression by 12 hr (Fig. 9). These markers were also induced in the *yes*-mutant keratinocytes. In contrast, in

Figure 6. Morphological and actin cytoskeletal characteristics of control and *fyn*-negative keratinocytes under growing and differentiating conditions. Primary mouse keratinocytes from *fyn*-negative mice and genetically-matched controls were grown to confluence in low calcium medium and analyzed under basal conditions and at 24 hr after calcium- or TPA-induced differentiation. (A-F) Contrast phase microscopy of primary keratinocytes derived from control and *fyn*-null mice. (A,B) Control and *fyn*-negative keratinocytes in low calcium medium. (C,D) Control and *fyn*-negative keratinocytes after 24 hr of TPA exposure. (E,F) Control and *fyn*-negative keratinocytes after 24 hr of calcium exposure (area out of focus in panel E is due to the presence of focal stratification which was observed throughout the cultures of control but not of *fyn*-negative keratinocytes). (G,H) Control and *fyn*-negative keratinocytes 24 hr after calcium exposure, after F-actin staining and visualization by confocal microscopy. Bar, 15 μm for A-F and 5 μm for G and H.

Figure 7. Induction of tyrosine phosphorylation by calcium- and TPA-treatment of wild-type, *fyn*- and *yes*-deficient keratinocytes. Primary keratinocytes derived from *fyn*-deficient (*fyn*-), *yes*-deficient (*yes*-) and syngenic control mice (Ctrl.) were exposed to calcium (2 mM) or TPA (100 ng/ml) for the indicated amounts of time. Extracts were normalized for protein concentration and analyzed by gel electrophoresis (7.5% SDS-PAGE) and immunoblotting with anti-phosphotyrosine antibodies. The immunoblot was developed with the ECL system (Amersham). The position of the molecular weight markers and of the p80–85 proteins are indicated.

Figure 8. Transglutaminase induction in control and *fyn*-null keratinocytes. Transglutaminase activity was measured in duplicate cultures of primary keratinocytes from *fyn*-negative mice (black bars) and wild-type controls (white bars), under basal conditions and 24 hr after calcium or TPA exposure. Transglutaminase activity is expressed as folds of induction in treated versus untreated cells. Similar results were obtained in two other independent experiments.

Figure 9. Induction of differentiation markers by calcium treatment of control and *fyn*- and *yes*-deficient keratinocytes. Primary keratinocytes from wild-type controls (w.t.), *fyn*-null, (*fyn*-) and *yes*-deficient (*yes*-) mice were kept under growing conditions in low calcium medium (0), or induced to differentiate by calcium (2 mM) for 12 or 24 hr. The cells were lysed in boiling sample buffer and the extracts were normalized for protein content. Equal amount of proteins were separated on a 7.5% SDS-PAGE, and the expression of keratinocyte differentiation markers was analyzed by immunoblotting with specific antibodies. The same blot was probed with anti-filaggrin antibodies, anti-keratin 1 and anti-keratin 5 as a loading control. The same protein extracts were run on a second identical gel for the involucrin immunoblot, alongside immunoprecipitates with involucrin-specific antiserum (+) or rabbit normal serum (-). Control experiments with cell extract preparations under different reducing conditions, as well as immunofluorescence analysis suggested that the lesser induction of filaggrin and involucrin at 24 hr of calcium treatment may be due to trapping of these proteins in insoluble cross-linked products formed during the differentiation process.

the *fyn*-negative cells, induction of filaggrin expression was almost totally suppressed, and that of keratin 1 was strongly reduced (Fig. 9). Only involucrin induction occurred to a similar extent in *fyn*- and *yes*-deficient cells, and was only slightly reduced relative to the control (Fig. 9). Expression of keratin 5 in cultured mouse keratinocytes is not affected by calcium-induced differentiation. Immunoblotting of the same extracts tested above with anti-K5 antibodies verified that equal amounts of proteins were tested in each case (Fig. 9). In agreement with these changes in cultured cells, keratin 1 and filaggrin were found to be expressed in a significantly reduced number of epidermal layers in newborn *fyn*-negative mice relative to either *yes*-mutant or wild-type animals (Fig. 10). Expression of keratin 5 and involucrin appeared similar in all cases.

Thus, *fyn* null keratinocytes are altered in their differentiation response to both calcium and TPA. These effects appear to be specific as they were not observed in keratinocytes carrying a disruption of the related *yes* kinase gene. In parallel with these findings, the skin of *fyn*-negative mice exhibit reduced expression of specific keratinocyte differentiation markers.

Several findings suggest that the calcium-induced kinase activity which was recovered after anti-phosphotyrosine immunoprecipitation is the one responsible for

A second tyrosine kinase activity activated after 1 hr of calcium-induced differentiation was recovered after immunoprecipitation with *fyn*-specific antibodies. This increased activity was not due to increased *fyn* protein expression. More difficult to rule out is the possibility that the increased activity is due to a kinase which closely associates with *fyn*, rather than to *fyn* itself. In fact, *fyn* has been shown to associate with at least two membrane-bound tyrosine kinases, the PDGF [Kypta et al. 1990] and CSF-1 [Courtneidge et al. 1993] receptors, as well as to a cytoplasmic kinase, *fak* [Cobb et al. 1994]. This latter kinase is intimately connected with cell adhesion/membrane structures which could be among the primary targets of calcium- and TPA-induced differenti-

ation. However, anti-phosphotyrosine immunoblotting of anti-*fak* immunoprecipitates as well as direct in vitro kinase assays indicated that *fak* is not activated upon induction of keratinocyte differentiation (Table 1). Also, immunoblotting of anti-phosphotyrosine immunoprecipitates from growing and differentiating keratinocytes with anti-*fyn* antibodies failed to reveal any close association of *fyn* with other tyrosine phosphorylated proteins, under conditions where the tyrosine kinase activated at very early times of calcium treatment is readily recovered. Intrinsic activation of *fyn* kinase activity in vivo is strongly suggested by the intense tyrosine phosphorylation of its cortactin substrate, which occurs in both calcium- and TPA-treated keratinocytes, as well as by our results with the *fyn*-negative cells (see below).

It has been recently reported that the *c-src* kinase is activated and *c-yes* inhibited in human keratinocytes induced to differentiate by the concomitant treatment with calcium and calcium ionophore A23187 (Zhao et al. 1992, 1993). It is difficult to relate these results with ours, given the different sensitivity of human and mouse keratinocytes to calcium. The specific molecules which become tyrosine phosphorylated in vivo after induction of mouse keratinocyte differentiation, such as p62 and p80/85, were not found in the human keratinocytes (Zhao et al. 1992; E. Calautti and G.P. Dotto, unpubl.). The A23187 calcium ionophore used to induce differentiation of the human cells has a number of calcium-unrelated effects and inhibits, rather than stimulating, tyrosine phosphorylation in differentiating mouse keratinocytes (Filvaroff et al. 1994). Furthermore, the effects on *src* and *yes* activity elicited by A23187 are not specific for keratinocytes, as they were also observed in similarly treated fibroblasts (Zhao et al. 1993).

Treatment of mouse keratinocytes with TPA did not cause an increase in tyrosine kinase activity which is immunoprecipitated by anti-phosphotyrosine antibodies. Instead, treatment with this compound induced activation of the *fyn* kinase, at much earlier times than those observed with calcium. The rapid induction of *fyn* activity (within 2 min of TPA exposure) suggest that this kinase could be directly activated by PKC action. This may represent a novel mechanism of PKC-mediated activation of *src*-related kinases. In fact, treatment of Jurkat T cells with TPA was also reported to cause rapid phosphorylation of the *lck* kinase, on a number of serine and threonine residues, but in that case *lck* kinase was not activated (Luo and Sefton 1990). Conversely, treatment of neuronal (Bjelfman et al. 1990) or myeloid (Gee et al. 1986) cells with TPA was shown to cause *c-src* activation but only at late times, through some indirect mechanisms which result, respectively, in increased *c-src* phosphorylation (mostly on Ser 12) or synthesis.

The molecular mechanisms responsible for *fyn* activation in keratinocyte differentiation remain to be elucidated. Preliminary results indicate that in calcium- but not TPA-treated keratinocytes a *fyn* peptide containing tyrosine 531 is specifically dephosphorylated. *fyn* activity is negatively regulated by phosphorylation of this residue, which is analogous to *src* Tyr 527 (Cheng et al.

1991; Cooper and Howell 1993). A possible mechanism by which *fyn* activation could be selectively achieved could be activation of a specific protein tyrosine phosphatase located in close proximity of the *fyn* kinase. This situation would be analogous to that described in T cells, where a transmembrane tyrosine phosphatase, CD45, has been shown to regulate activity of the *fyn* kinase associated with the T cell receptor (Hurley et al. 1993).

Cortactin was originally identified in *src*- (and *fgr*- and *fyn*-) transformed cells, as a heavily tyrosine phosphorylated p80–85 protein which colocalizes with F-actin at specific sites of cell contact (podosomes or rosettes) (Wu et al. 1991). The multidomain structure of this protein, together with its cellular localization suggest that it may play a role in cytoskeleton/membrane organization. In normal cells, cortactin is phosphorylated mostly on serine and threonine, and is also found in association with F-actin at peripheral cell extensions. Tyrosine phosphorylation of cortactin, also found in growth factor-stimulated cells (Downing and Reynolds 1992; Maa et al. 1992; S. Wong et al. 1992), does not affect its actin-binding properties (Wu and Parsons 1993), but may influence its association with other structural or signal transducing proteins. In keratinocytes, cortactin becomes increasingly tyrosine phosphorylated in response to both calcium and TPA, at times which closely coincide with those of *fyn* activation. The specific connection between phosphorylation of this protein and structural changes associated with keratinocyte differentiation is currently under investigation.

fyn-negative keratinocytes appeared significantly different from control cells after induction of differentiation by calcium and TPA. These morphological/structural changes were associated with—and could be explained by—decreased or absent induction of phosphorylation of a number of proteins, including the p80–85 group, in the *fyn*-negative cells. A strong indication that the *fyn* kinase plays an important and specific role in control of keratinocyte differentiation is provided by the decreased induction of a number of differentiation markers in *fyn*-, but not *yes*-mutant keratinocytes. The latter cells were derived from mice carrying a disruption of the *yes* gene, such that *yes* kinase activity is totally abrogated. These mice express a truncated form of the *yes* protein at low levels and only in certain tissues. This truncated *yes* protein lacks an essential region of the kinase domain and could only act, if at all, as a dominant-negative mutant interfering with the function of other SH2- and SH3-containing proteins. In keratinocytes, this clearly does not happen, as the *yes*-mutant cells behave like the wild-type controls.

The biological relevance of our findings with cultured cells is further substantiated by the fact that in vivo, expression of these markers is significantly reduced in several overlying epidermal layers of newborn mice with a disruption of the *fyn*-, but not *yes*-kinase gene. Similar alterations would be much harder if not impossible to assess in adult mice, because their epidermis is much thinner, consisting only of two or three keratinocyte layers. Functionally, the skin of adult *fyn*-negative mice

produced a normal hyperplastic response to acute TPA treatment [E. Calautti and G.P. Dotto, unpubl.]. In view of the subtle changes that have been previously detected in the immune and nervous systems of these mice [Appleby et al. 1992; Stein et al. 1992; Grant et al. 1992], it is likely that a finer analysis will be required to find functional abnormalities in the skin of these animals. Additionally, the previous studies suggest that compensatory activation of other *src*-related kinases can also take place. Future work will have to address these possibilities, by extending the present analysis to the skin of mice where multiple *src*-related genes have been eliminated.

In conclusion, we have demonstrated that two distinct tyrosine kinase activities are induced at very early times of keratinocyte differentiation, and at least one of them, *fyn*, is directly involved in control of this process. These results can be integrated with previous findings on control of keratinocyte differentiation by calcium and TPA, in the following model (Fig. 11). Treatment of keratinocytes with calcium triggers the very rapid activation of a tyrosine kinase which is in turn responsible for the phosphorylation of the *ras*-GAP associated p62 protein. Activation of this kinase and phosphorylation of p62, are specifically induced by calcium and a number of other divalent cations, and are not linked to increased levels of intracellular calcium, suggesting that a cation-specific receptor, similar to that of other calcium-responsive cells, is involved [Filvaroff et al. 1994].

Besides its direct and specific effects on this pathway, calcium causes phospholipase C activation, a rapid increase in phosphatidylinositol metabolism, subsequent intracellular calcium mobilization and protein kinase C activation [Yuspa 1994]. This sequence of events may ultimately lead to activation of the *fyn* kinase, possibly through decreased phosphorylation of its negative regulatory tyrosine. *fyn* activation is in turn responsible for the phosphorylation of a group of p80/85 proteins, among which cortactin may be involved in specific changes at the cytoskeleton/membrane junction. Treatment of keratinocytes with TPA does not activate the calcium-specific pathway which leads to p62 phosphorylation. Instead, it activates *fyn*, possibly through a direct PKC mechanism which bypasses many of the steps induced by calcium. Even in this case, *fyn* activation leads to cortactin phosphorylation on tyrosine. A clearly outstanding question is how these changes are connected with specific gene expression events involved in the keratinocyte terminal differentiation process.

Materials and methods

Cells

Primary keratinocytes were isolated from newborn Sencar mice and cultivated in minimal essential medium with 4% Chelex-treated fetal calf serum (Hyclone), epidermal growth factor (EGF; 10 ng/ml, Collaborative Research, Inc., Cambridge, MA), and 0.05 mM CaCl_2 (low calcium medium), as described [Hennings et al. 1980; Filvaroff et al. 1990]. For all experiments, cells were used one week after plating. Culture medium was changed

for the last time 24 hr before the experiment. Keratinocyte differentiation was induced by addition of CaCl_2 (to 2 mM) or TPA (100 ng/ml; stored in aliquots of 100 $\mu\text{g/ml}$ in DMSO at -70°C ; purchased from Sigma). A23187 was purchased from Molecular Probes and stored in aliquots of 1 mM in DMSO at -70°C .

Antibodies

Anti-phosphotyrosine mAbs Py20 [Glenney et al. 1988] used for immunoprecipitations were purchased from ICN. Anti-phosphotyrosine mAbs 4G10 [Druker et al. 1989] used for immunoblots were from Upstate Biotechnology Inc. Cst-1 antibodies [Courtneidge and Smith 1984] were a gift of Dr. Sara Courtneidge (EMBL, Heidelberg, Germany); anti-*src* M327 monoclonals [Lipsich et al. 1983] were provided by Dr. Joan Brugge (Ariad Pharmaceuticals, Cambridge, MA.). Anti-*fyn* affinity purified rabbit immunoglobulins (epitope: amino acids 28–48 of human p59^{fyn}) and *fyn* neutralizing peptide were purchased from Santa Cruz Biotechnology, Inc. Anti-*yes* and anti-*yrk* sera [Zhao et al. 1991; Sudol et al. 1993] were a gift of Dr. Marius Sudol (The Rockefeller University, New York). Polyclonal antisera against unique sequences of *lyn*, *fgr* and *hck* [Huang et al. 1991] were a gift of Dr. Joseph Bolen (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). Affinity-purified antiserum against residues 22–51 of *lck* was purchased from UBI. Anti-*blk* serum was provided by Dr. Steve Desiderio (Johns Hopkins University, Baltimore, MD). Anti-cortactin (p80–85) monoclonal antibodies 4F11 [Kanner et al. 1990] were a gift of Dr. Thomas Parsons (University of Virginia Health Sciences Center, Charlottesville, VA). Anti-*fak* monoclonal antibodies [Kanner et al. 1990] were purchased from UBI. Anti-*met* antibodies were purchased from UBI; anti-*abl* antibodies were purchased from Oncogene Science. Anti-involucrin antiserum [Djian et al. 1993] was purchased from Berkeley Antibodies Company (Richmond, CA). Affinity-purified polyclonal antibodies anti-filaggrin, anti-keratin 1 and anti-keratin 5 were a gift of Dr. Stuart Yuspa (National Institutes of Health, Bethesda, MD).

Immunoprecipitations

Mouse primary keratinocytes were washed twice in PBS containing 1 mM Na_3VO_4 and then lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) supplemented with 1 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4 , 10 $\mu\text{g/ml}$ aprotinin (Sigma), 10 $\mu\text{g/ml}$ leupeptin (Sigma) for 20' on ice. Cell lysates were spun for 5' at 4°C . Samples were normalized for equal amounts of proteins (100–300 μg) by a Bradford assay (Bio-Rad), and incubated for 2 hr at 4°C with antibodies. Monoclonal antibodies (1–2 μg) or affinity-purified antisera, or 2–5 μl of total immune serum were used for single immunoprecipitation reaction. Affinity-purified rabbit anti-goat IgG (1–2 μg) [Cappel, West Chester, PA] or 2–5 μl of rabbit normal serum were used for the nonimmune controls. Protein A-Sepharose (60 μl of a 50% suspension; Pharmacia) in RIPA buffer was added and samples were incubated for other 30' at 4°C . Protein A-Sepharose precoupled with affinity-purified rabbit anti-mouse antiserum (Pierce) was used for immunoprecipitation with M327 and 4F11 mAbs.

Kinase assays

Immune complexes for kinase assay were washed twice in RIPA buffer, twice in kinase buffer (20 mM HEPES at pH 7.4; 3 mM MnCl_2 , 5 mM MgCl_2), resuspended in 50 μl of kinase buffer supplemented with 5 μCi of $\gamma^{32}\text{P}$ ATP (6000 Ci/mM; NEN), 1 μM ATP, and 5 μg of acid-denatured enolase and incubated at

30°C for 10'. In some experiments the concentration of cold ATP was 10 μ M. All reactions were stopped with 25 mM cold ATP, 20 mM EDTA. Samples incubated with enolase were supplemented with 2 $\times$ sample buffer, boiled and separated on 10% SDS-PAGE. The gels were fixed in 10% acetic acid:10% methanol, and washed several times with 10% acetic acid to remove unincorporated label. The position of the substrate was detected by coomassie blue staining. Quantitative measurements of *fyn* kinase activity were performed as follows: Enolase bands were excised from gels and γ -³²P incorporation was determined by scintillation counting. *fyn* protein levels were determined by immunoblotting of the same immunoprecipitates tested for kinase assay with anti-*fyn* antibodies, using iodinated protein A as a detection agent. The autoradiograms of the immunoblots were subjected to densitometric analysis using a Molecular Dynamics computing densitometer and the data were processed with a Fast Scan ImageQuant software version 3.0 (Molecular Dynamics). Specific kinase activity was calculated as amounts of γ -³²P incorporation into the enolase substrate per amount of *fyn* protein present in each immunoprecipitate. Values were expressed as fold of induction relative to basal *fyn* activity in control cells.

Immunoblotting

Immune complexes processed for immunoblotting were washed four times in RIPA buffer with inhibitors, then eluted in boiling sample buffer for 3 min and separated electrophoretically on 7.5% or 10% SDS-PAGE alongside 12- to 200-kD molecular size markers (GIBCO). Immunoblotting of protein gels onto nitrocellulose was performed as previously described (Filvaroff et al. 1990). The transfer buffer and the procedures for blocking and washing the filter were described (Kamps and Sefton 1988). Blots were developed with 1 μ Ci/ml of ¹²⁵I-labeled protein A (35 μ Ci/ μ g; ICN) in blocking buffer and exposed for 24–48 hr.

For anti-phosphotyrosine immunoblots, horseradish peroxidase-conjugated anti-mouse immunoglobulins (Amersham) were used as secondary antibodies in a 1:5000 dilution, and blots were developed with the ECL system (Amersham).

Phosphoamino acid analysis

³²P-labeled proteins were transferred to Immobilon-P membrane (Millipore). Filters were exposed for 2–4 hr, and the bands of interest were excised and subjected to hydrolysis by boiling in 5.7 N HCl for 1 hr. Before electrophoresis samples were supplemented with a mixture of cold phosphoserine, phosphothreonine and phosphotyrosine as standards. The resulting phosphoamino acids were resolved by two-dimensional electrophoresis as described by Kamps and Sefton (1989). The position of the standards was determined by Ninhydrin staining.

Actin staining of primary keratinocytes

Primary mouse keratinocytes were fixed for 5' at 37°C with 2% paraformaldehyde in PBS, permeabilized with 0.4% Triton X-100, washed with PBS and incubated for 60 min with Bodipy 581-591 phalloidin (Molecular Probes, Inc., Eugene, OR), 5 U/ml in PBS. The cells were washed four times for 5' in PBS and visualized by laser confocal microscopy (BioRad MRC 600 Confocal Imaging System, attached to a Zeiss Axiovert microscope).

Transglutaminase assay

Primary keratinocytes were prepared from three days old *fyn*-negative mice (Stein et al. 1992) and from control mice of the

same age and same genetic background (C57Bl/129). Cells were plated onto 35-mm dishes, grown to confluence and tested at one week of plating, 24 hr after addition of fresh low calcium medium, or medium plus calcium (2 mM) or TPA (100 ng/ml). Transglutaminase activity was assayed in duplicate samples, by measuring the incorporation of [2,3-³H] putrescine (31.1 Ci/mmole; Dupont, NEN) into casein at pH 9.5, as described by Filvaroff et al. (1990). Background radioactivity was determined from control reactions containing no cell lysate, and was subtracted in each case. Experimental values were normalized for protein amounts (Bradford assay; Bio-Rad Laboratories, Richmond, CA) and expressed as folds of induction in treated versus untreated cells.

Immunofluorescence of newborn mouse skins

Unfixed frozen sections (6 μ m) of three day old newborn mice were air dried before staining. After three PBS washes, the sections were blocked with goat normal serum diluted 1:5 in PBS for 30 min at room temperature. Sections were treated with the avidin/biotin blocking solution (Vector) according to the manufacturer's instruction, and then incubated with the appropriate antibody diluted in 0.3% Triton X-100, 1% BSA in PBS (buffer A) for 2 hr at room temperature. The slides were then washed in PBS and incubated for 1 hr with biotinylated goat anti-rabbit IgG (Vector) diluted 1:1000 in buffer A. After several PBS washes the sections were incubated for 30 min with fluorescein-conjugated streptavidin (Amersham) diluted 1:100 in buffer A, washed extensively in PBS and mounted with 0.1 M *n*-propylgallate in glycerol. The slides were examined with a Zeiss Axiovert microscope.

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Note added in proof

Like *yes*-deficient keratinocytes, primary keratinocytes derived from *src*-negative mice (kindly provided by Dr. Pat Preisig) were found to be indistinguishable from wild-type controls in their differentiation response, as evaluated by morphological characteristics, tyrosine phosphorylation pattern, and differentiation marker expression.

References

- Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{fyn}. *Cell* 70: 751–763.

- Bjelfman, C., G. Meyerson, C.A. Cartwright, K. Mellström, U. Hammerling, and S. Pahlman. 1990. Early activation of endogenous pp60^{src} kinase activity during neuronal differentiation of cultured human neuroblastoma cells. *Mol. Cell. Biol.* **10**: 361–370.
- Bolen, J.B. 1993. Nonreceptor tyrosine protein kinases. *Oncogene* **8**: 2025–2031.
- Brickell, P.M. 1992. The p60^{c-src} family of protein-tyrosine kinases: Structure, regulation, and function. *Crit. Rev. Oncogenesis* **3**: 401–446.
- Cheng, S.H., P.C. Espino, J. Marshall, R. Harvey, J. Merrill, and A.E. Smith. 1991. Structural elements that regulate pp59^{c-fyn} catalytic activity, transforming potential, and ability to associate with Polyomavirus middle-T antigen. *J. Virol.* **65**: 170–179.
- Cobb, B.S., M.D. Schaller, T.-H. Leu, and J.T. Parsons. 1994. Stable association of pp60^{src} and pp59^{fyn} with the focal adhesion-associated protein tyrosine kinase, pp125^{FAK}. *Mol. Cell. Biol.* **14**: 147–155.
- Cooper, J.A. and B. Howell. 1993. The when and how of *src* regulation. *Cell* **73**: 1051–1054.
- Courtneidge, S.A., R. Dhand, D. Pilat, G.M. Twamley, M.D. Waterfield, and M.F. Roussel. 1993. Activation of *src* family kinases by colony stimulating factor-1, and their association with its receptor. *EMBO J.* **12**: 943–950.
- Courtneidge, S.A. and A.E. Smith. 1984. The complex of polyoma virus middle-T antigen and pp60^{c-src}. *EMBO J.* **3**: 585–591.
- da Silva, A.J. and C.E. Rudd. 1993. A 72-Kilodalton *fyn*-related polypeptide (p72^{fyn-R}) binds to the antigen-receptor/CD3 (TcR/CD3) complex. *J. Biol. Chem.* **268**: 16537–16543.
- Djian, P., M. Phillips, K. Easley, E. Huang, M. Simon, R.H. Rice, and H. Green. 1993. The involucrin gene of the mouse and rat: Study of their shared repeats. *Mol. Biol. Evol.* **10**: 1136–1149.
- Dlugosz, A.A. and S.H. Yuspa. 1993. Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. *J. Cell Biol.* **120**: 217–225.
- Dotto, G.P., M.Z. Gilman, M. Maruyama, and R.A. Weinberg. 1986. c-myc and c-fos expression in differentiating mouse primary keratinocytes. *EMBO J.* **5**: 2853–2857.
- Downing, J.R. and A.B. Reynolds. 1992. PDGF, CSF-1, and EGF induce tyrosine phosphorylation of p120, a pp60^{src} transformation-associated substrate. *Oncogene* **6**: 607–613.
- Druker, B.J., H.J. Mamon, and T.M. Roberts. 1989. Oncogenes, growth factors and signal transduction. *New Engl. J. Med.* **321**: 1383–1391.
- Ellis, C., M. Moran, F. McCormick, and T. Pawson. 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature* **343**: 377–381.
- Filvaroff, E., D.F. Stern, and G.P. Dotto. 1990. Tyrosine phosphorylation is an early and specific event involved in primary keratinocyte differentiation. *Mol. Cell. Biol.* **10**: 1164–1173.
- Filvaroff, E., E. Calautti, F. McCormick, and G.P. Dotto. 1992. Specific changes of Ras GTPase Activating Protein (GAP) and a GAP-associated p62 protein during calcium-induced keratinocyte differentiation. *Mol. Cell. Biol.* **12**: 5319–5328.
- Filvaroff, E., E. Calautti, M. Reiss, and G.P. Dotto. 1994. Functional evidences for an extracellular calcium receptor mechanism triggering tyrosine kinase activation associated with mouse keratinocyte differentiation. *J. Biol. Chem.* **269**: 21735–21740.
- Gee, C.E., J. Griffin, L. Sastre, L.J. Miller, T.A. Springer, H. Piwnica-Worms, and T.M. Roberts. 1986. Differentiation of myeloid cells is accompanied by increased levels of pp60^{c-src} protein and kinase activity. *Proc. Natl. Acad. Sci.* **83**: 5131–5135.
- Glenney, J.R. Jr., L. Zokas, and M.P. Kamps. 1988. Monoclonal antibodies to phosphotyrosine. *J. Immunol. Methods* **109**: 277–285.
- Grant, S.G.N., T.J. O'Dell, K.A. Karl, P.K. Stein, P. Soriano, and E.R. Kandel. 1992. Impaired long-term potentiation, spatial learning, and hippocampal development in *fyn* mutant mice. *Science* **258**: 1903–1910.
- Hennings, H. and K.A. Holbrook. 1983. Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture: An ultrastructural study. *Exp. Cell Res.* **143**: 127–142.
- Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S.H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**: 245–254.
- Hennings, H., K.A. Holbrook, and S.H. Yuspa. 1983. Factors influencing calcium-induced terminal differentiation in cultured mouse epidermal cells. *J. Cell. Physiol.* **116**: 265–281.
- Huang, M.-M., J.B. Bolen, J.W. Barnwell, S.J. Shattil, and J.S. Brugge. 1991. Membrane glycoprotein IV (CD36) is physiologically associated with the *fyn*, *lyn*, and *yes* protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci.* **88**: 7844–7848.
- Hurley, T.R., R. Hyman, and B.M. Sefton. 1993. Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the *lck*, *fyn*, and *c-src* tyrosine protein kinases. *Mol. Cell. Biol.* **13**: 1651–1656.
- Kamps, M.P. and B.M. Sefton. 1988. Identification of multiple novel polypeptide substrates of the *v-src*, *v-yes*, *v-fps*, *v-ros*, and *v-erb-B* oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. *Oncogene* **2**: 305–316.
- . 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates the analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* **176**: 22–27.
- Kanner, S.B., A.B. Reynolds, R.R. Vines, and J.T. Parsons. 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci.* **87**: 3328–3332.
- Kypta, R.M., Y. Goldberg, E.T. Ulug, and S.A. Courtneidge. 1990. Association between the PDGF receptor and members of the *src* family of tyrosine kinases. *Cell* **62**: 481–492.
- Lee, E. and S.H. Yuspa. 1991. Changes in inositol phosphate metabolism are associated with terminal differentiation and neoplasia in mouse keratinocytes. *Carcinogenesis* **12**: 1651–1658.
- Lipsich, L., A.J. Lewis, and J.S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. *J. Virol.* **48**: 352–360.
- Luna, E.J. and A.L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. *Science* **258**: 955–963.
- Luo, K. and B.M. Sefton. 1990. Analysis of the sites in p56^{lck} whose phosphorylation is induced by tetradecanoyl phorbol acetate. *Oncogene* **5**: 803–808.
- Maa, M.-M., L.K. Wilson, J.S. Moyers, R.R. Vines, J.T. Parsons, and S.J. Parsons. 1992. Identification and characterization of a cytoskeleton-associated, epidermal growth factor sensitive pp60^{src} substrate. *Oncogene* **7**: 2429–2438.
- Menon, G.K., S. Grayson, and P.M. Elias. 1985. Ionic calcium reservoirs in mammalian epidermis: Ultrastructural localization by ion-capture cytochemistry. *J. Invest. Dermatol.* **84**: 508–512.

- Moscat, J., T.P. Fleming, C.J. Molloy, M. Lopez-Barahona, and S.A. Aaronson. 1989. The calcium signal for Balb/MK keratinocyte terminal differentiation induces sustained alterations in phosphoinositide metabolism without detectable protein kinase C activation. *J. Biol. Chem.* **264**: 1122–11235.
- Pazin, M.J. and L.T. Williams. 1992. Triggering signaling cascades by receptor tyrosine kinases. *Trends in Biochem. Sci.* **17**: 374–378.
- Reiss, M., L.R. Lipsey, and Z.L. Zhou. 1991. Extracellular calcium-dependent regulation of transmembrane calcium fluxes in murine keratinocytes. *J. Cell. Physiol.* **147**: 281–291.
- Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the *c-src* proto-oncogene leads to osteopetrosis in mice. *Cell* **64**: 693–702.
- Stein, P., H.M. Lee, S. Rich, and P. Soriano. 1992. pp59^{fyn} mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell* **70**: 741–750.
- Stein, P.L., H. Vogel, and P. Soriano. 1994. Combined deficiencies of *src*, *fyn*, and *yes* tyrosine kinases in mutant mice. *Genes & Dev.* **8**: 1999–2007.
- Sudol, M., H. Greulich, L. Newman, A. Sarkar, J. Sukegawa, and T. Yamamoto. 1993. A novel *yes*-related kinase, *yrk*, is expressed at elevated levels in neural and hematopoietic tissues. *Oncogene* **8**: 823–831.
- Thacher, S.M. and R.H. Rice. 1985. Keratinocyte-specific transglutaminase of cultured human epidermal cells: Relation to cross-linked envelope formation and terminal differentiation. *Cell* **40**: 685–695.
- Wong, G., O. Muller, R. Clark, L. Conroy, M.F. Moran, P. Polakis, and F. McCormick. 1992. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. *Cell* **69**: 551–558.
- Wong, S., A.B. Reynolds, and J. Papkoff. 1992. Platelet activation leads to increased *c-src* kinase activity and association of *c-src* with an 85-kDa tyrosine phosphoprotein. *Oncogene* **7**: 2407–2415.
- Wu, H., A.B. Reynolds, S.B. Kanner, R.R. Vines, and J.T. Parsons. 1991. Identification and characterization of a novel cytoskeleton-associated pp60^{src} substrate. *Mol. Cell. Biol.* **11**: 5113–5124.
- Wu, H. and J.T. Parsons. 1993. Cortactin, an 80/85 Kilodalton pp60^{src} substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell Biol.* **120**: 1417–1426.
- Yuspa, S.H. 1994. The pathogenesis of squamous cell cancer: Lessons learned from studies of skin carcinogenesis- Thirty-third G. H. A. Clowes Memorial Award Lecture. *Cancer Res.* **54**: 1178–1189.
- Yuspa, S.H., H.M. Kulesz-Martin, T. Ben, and H. Hennings. 1983. Transformation of epidermal cells in culture. *J. Invest. Dermatol.* **81**: 162s–168s.
- Yuspa, S.H., A.E. Kilkenney, P.M. Steinert, and D.R. Roop. 1989. Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J. Cell. Biol.* **109**: 1207–1217.
- Zhao, Y., H. Baker, S.I. Walaas, and M. Sudol. 1991. Localization of p62^{c-yes} protein in mammalian neural tissues. *Oncogene* **6**: 1725–1733.
- Zhao, Y., M. Sudol, H. Hanafusa, and J. Krueger. 1992. Increased tyrosine kinase activity of *c-src* during calcium-induced keratinocyte differentiation. *Proc. Natl. Acad. Sci.* **89**: 8298–8302.
- Zhao, Y., H. Uyttendaele, J.G. Krueger, M. Sudol, and H. Hanafusa. 1993. Inactivation of *c-yes* tyrosine kinase by elevation of intracellular calcium levels. *Mol. Cell. Biol.* **13**: 7507–7514.

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References

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