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Increased tyrosine kinase activity of c-Src during calcium-induced keratinocyte differentiation

(non-receptor-type tyrosine kinase/epidermis/protein tyrosine phosphatase/protooncogene)

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ABSTRACT **In cultured human epidermal keratinocytes, induction of differentiation by Ca^{2+} and ionophore treatment was found to result in rapid elevation of c-Src tyrosine kinase activity and inactivation of the c-Yes tyrosine kinase. Activation of c-Src kinase was accompanied by tyrosine dephosphorylation, which might be explained by a rapid increase in intracellular protein-tyrosine phosphatase activity. Ca^{2+}-induced differentiation was also associated with altered tyrosine phosphorylation of several cellular proteins and correlated with a marked redistribution of intracellular phosphotyrosine from membrane and adhesion sites to the nucleus. Some of the c-Src protein was also found in the nucleus after Ca^{2+} treatment, and Ca^{2+}-activated c-Src bound to three cellular proteins (120 kDa, 65 kDa, and 34 kDa). In agreement with these results, immunohistochemistry on human epidermis revealed an increase in c-Src expression and tyrosine phosphorylation in cells undergoing differentiation, which strongly suggests a possible role of non-receptor-type tyrosine kinases in epithelial cell maturation.**

Protein-tyrosine kinases are thought to regulate critical events of cell growth and tissue structure. Analysis of genetic mutants suggests a critical role for receptor-type tyrosine kinases in embryonic development and tissue morphology (1, 2). Non-receptor-type tyrosine kinases, typified by $p60^{c\text{-}src}$(c-Src), $p62^{c\text{-}yes}$(c-Yes), and related kinases, are believed to participate in cellular growth signaling pathways, since specific mutations that activate their intrinsic kinase activity lead to altered cell growth or neoplasia (3–5). Although c-Src and c-Yes are closely related tyrosine kinases, their expression differs greatly during embryonic development and in specific cell types (6–9). The expression of Src family tyrosine kinases in cell types with sustained growth potential (e.g., lymphoid cells and epithelial cells) suggests a potential role for these kinases in regulation of cell growth or tissue development in postembryonic life (7, 10).

Observations that overexpression of c-Src in kidney epithelial cells alters their epithelial structure (11) and that Ca^{2+}-induced differentiation of murine epidermal keratinocytes (KCs) is accompanied by increased tyrosine phosphorylation of cellular proteins early in the differentiation process (12) suggest that tyrosine kinases may have important functions in epithelial cells. c-Yes is highly expressed in a variety of adult epithelial cell types (8, 13). To examine whether Ca^{2+}-induced differentiation can alter the activity of tyrosine kinases in cultured KCs, the kinase activities of c-Src and c-Yes were investigated. We found that the activity of these tyrosine kinases was rapidly altered at an early stage of KC differentiation. In addition, the level and location of tyrosine-phosphorylated proteins were significantly modified, implicating their important roles in epithelial differentiation.

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MATERIALS AND METHODS

Cells and Antibodies. Human KCs were cultured from foreskin epidermis in growth factor-supplemented MCDB 153 medium (KGM; Clonetics; ref. 13). A431 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (GIBCO) (8). The medium was replaced with basic MCDB 153 medium the night before incubation with 1.0 mM $CaCl_2$ and 10 μM 4-bromo-Ca^{2+}-ionophore A23187 (Sigma). Anti-Src antibody was monoclonal antibody (mAb) 327 (14); anti-Yes antibody was a polyclonal antibody described previously (8). Anti-phosphotyrosine (*P*-Tyr) polyclonal antibody was generated against v-Abl *P*-Tyr-containing peptides (15) by D. Sternberg (Rockefeller University, NY). The anti-*P*-Tyr 4G10 mAb was purchased from UBI (Lake Placid, NY).

Immunoassays. Cells were lysed in 150 mM RIPA buffer with protease inhibitors (13). c-Src or c-Yes was immunoprecipitated from 50 μg of cell lysate by mAB 327 or anti-Yes antibody. The autophosphorylation kinase assay was performed as described (16). For the substrate kinase assay, 2 μg of enolase (Sigma) was used as the exogenous substrate (9). Western blot analysis followed the procedure described previously (8). Anti-*P*-Tyr Western blotting was performed on either anti-Src immunoprecipitates or total lysates with a polyclonal anti-*P*-Tyr antibody.

$^{32}P_i$ Labeling. Cultures were labeled in phosphate-free KGM with 1 mCi of $^{32}P_i$ per ml (1 Ci = 37 GBq) for 3 hr and then treated with Ca^{2+}/ionophore. Cells were lysed in cold 150 mM RIPA, and equivalent amounts of lysate were used for immunoprecipitation with mAb 327.

Protein-Tyrosine Phosphatase (PTPase) Assay. Cells were lysed in Nonidet P-40 buffer (1% Nonidet P-40/150 mM NaCl/1% Trasylol, 2.5 mM EDTA/20 mM Tris·HCl, pH 8.0/1 mM leupeptin/1 mM antipain). ^{32}P-labeled Raytide was prepared as described (17), using baculovirus-expressed v-Src (18). PTPase activity was assayed by measuring the release of $^{32}P_i$ from ^{32}P-labeled Raytide by Cerenkov counting.

Glycerol Gradient Centrifugation. A431 cells were lysed in RIPA buffer. A 10–30% continuous glycerol gradient was prepared in a final volume of 4.6 ml in RIPA buffer. Protein separation was achieved by ultracentrifugation at 44,000 rpm (100,000 × *g*) for 16 hr in a SW 50.1 rotor (Beckman). Gradient fractions were 200 μl in size, and *in vitro* kinase assays were performed on mAb 327 immunoprecipitates from every other fraction. The molecular mass of proteins in each fraction was estimated by sedimentation of monomeric/multimeric phosphorylase b in parallel gradients.

Abbreviations: *P*-Tyr, phosphotyrosine; KC, keratinocyte; PTPase, protein-tyrosine phosphatase; mAb, monoclonal antibody.

Immunohistochemistry. For immunofluorescent staining, KCs were subcultured onto glass coverslips and then treated with Ca^{2+}/ionophore. The coverslips were fixed in 10% formalin in phosphate-buffered saline for 30 min and permeablized with 0.5% Triton X-100 in phosphate-buffered saline. Secondary antibodies were fluorescein isothiocyanate-conjugated goat $F(ab')_2$ fragments to mouse immunoglobulins (Tago). Immunohistochemistry on cryostat sections of adult human skin was performed as described (13).

RESULTS

Induction of KC Differentiation by Ca^{2+}/Ionophore. Rapidly proliferating cultured human KCs, which resemble undifferentiated basal epidermal KCs, can be induced to differentiate by the addition of Ca^{2+} and ionophore (19, 20). As shown in Table 1, Ca^{2+}/ionophore-induced differentiation of human KCs was assessed at different times by quantification of cornified envelopes, a structure formed only in differentiated KCs (20–22). Cell viability was assessed in parallel by trypan blue dye exclusion. Within 3–6 hr of the addition of Ca^{2+}/ionophore, significant accumulation of cornified envelopes occurred (>50% of cells), whereas cells remained viable (>95%) within this time frame. Unlike murine KCs, which are induced to differentiate by an elevation of the extracellular Ca^{2+} concentration (23), human KCs in culture do not differentiate with an elevated concentration of extracellular Ca^{2+} unless ionophore is added (20, 24).

Kinase Activity of c-Src and c-Yes After Ca^{2+}/Ionophore Treatment. The kinase activities of c-Src and c-Yes in human KCs treated with Ca^{2+}/ionophore were examined by an *in vitro* kinase assay. The kinase activity of c-Src, as detected by autophosphorylation, increased 5- to 6-fold after 30 min of treatment (Fig. 1*A*, lane 2). Its kinase activity toward an exogenous substrate, enolase, was also elevated to the same degree (Fig. 1*B*, lanes 2 and 3). After 6 hr of treatment, the kinase activity of c-Src decreased to the initial level (Fig. 1 *A* and *B*, lane 5). Western blot analysis on the same cell lysates showed that the protein levels of c-Src remained unaffected by the treatment (Fig. 1*C*).

In contrast to the c-Src kinase, the activity of c-Yes kinase declined at least 10-fold within 10 min (Fig. 1*D*, lane 2). The inactivation of c-Yes will be discussed in a separate report.

Activation of c-Src by Tyrosine Dephosphorylation. Since it is known that c-Src kinase activity is up-regulated by dephosphorylation at a tyrosine residue near the carboxyl terminus (3, 4, 25), we examined the level of *in vivo* phosphorylation of c-Src before and after Ca^{2+}/ionophore treatment. c-Src was immunoprecipitated from ^{32}P-labeled KCs (Fig. 2*A*). As shown in Fig. 2*A*, 30 min after the addition of Ca^{2+}/ionophore, the phosphorylation of c-Src was greatly reduced.

Table 1. Cell death and cornified envelope formation induced by Ca^{2+}/ionophore in cultured KCs

Time, hr	Dead cells, %	Cornified envelopes, %
0	0.4	0
0.5	0.5	0
1	1.0	0
3	3.0	17
6	4.0	53
24	38.0	Clumps

Cultured KCs were treated with Ca^{2+}/ionophore as described in *Materials and Methods* for the indicated times. For cell death measurement, cells were stained with trypan blue for 2 min and washed with phosphate-buffered saline three times before trypsinization. Dead cells were counted with a hemocytometer. For measurement of cornified cells, cells were boiled in 1% SDS for 10 min, and cornified cells were counted by a hemocytometer.

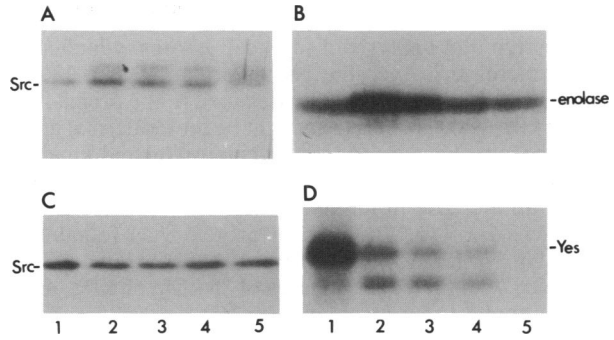

FIG. 1. Kinase activity of c-Src and c-Yes proteins in human KCs after Ca^{2+}/ionophore treatment. (*A–D*) Lane 1, untreated cells; lane 2, cells treated for 30 min; lane 3, cells treated for 1 hr; lane 4, cells treated for 3 hr; lane 5, cells treated for 6 hr. (*A*) c-Src immunoprecipitation with mAb 327 followed by *in vitro* kinase assay (autophosphorylation). (*B*) Use of enolase as an exogenous substrate in an *in vitro* kinase assay. (*C*) Western blot with mAb 327. (*D*) Immunoprecipitation with c-Yes antibody followed by *in vitro* kinase assay (autophosphorylation).

The level of *P*-Tyr of c-Src *in vivo* was also examined by immunoprecipitation with mAb 327 from KCs, followed by Western blot analysis with a polyclonal anti-*P*-Tyr antibody (Fig. 2*B*). Compared with untreated cells (Fig. 2*B*, lane 1), c-Src in cells treated with Ca^{2+}/ionophore showed a sharp decrease in tyrosine phosphorylation (Fig. 2*B*, lane 2). This suggests that elevation of intracellular Ca^{2+} reduces tyrosine phosphorylation of c-Src *in vivo* and thus elevates its kinase activity. Tyrosine dephosphorylation of c-Src was also observed in A431 cells after Ca^{2+}/ionophore treatment (see Fig. 4*B*).

Tyrosine Phosphorylation of Cellular Proteins and the Level of Cellular PTPase Activity. It has been reported that Ca^{2+}-induced differentiation of murine KCs is accompanied by increased tyrosine phosphorylation of cellular proteins (12). Since our data showed that in human KCs c-Src is activated and c-Yes is inactivated after Ca^{2+} treatment, we examined

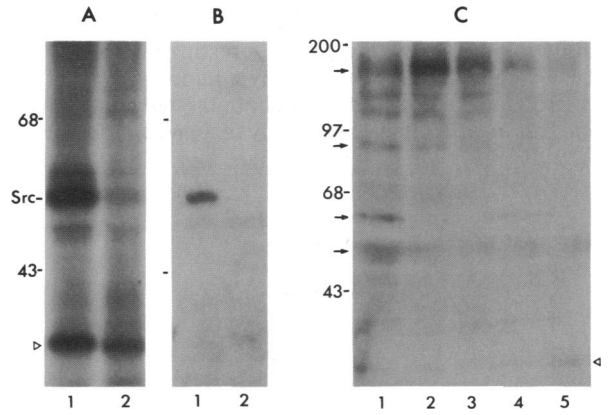

FIG. 2. Tyrosine dephosphorylation of c-Src after Ca^{2+}/ionophore treatment. (*A*) KCs were labeled with $^{32}P_i$, and c-Src protein was immunoprecipitated by mAb 327. Lane 1, untreated cells; lane 2, cells treated for 30 min. A 34-kDa protein coprecipitated with c-Src is indicated by an arrowhead. (*B*) c-Src was immunoprecipitated from unlabeled KCs and immunoblotted with polyclonal anti-*P*-Tyr antibody. Lane 1, untreated cells; lane 2, cells treated for 30 min. (*C*) Anti-*P*-Tyr Western blot on total cell lysates. Lane 1, untreated KCs; lane 2, cells treated for 10 min; lane 3, cells treated for 30 min; lane 4, cells treated for 1 hr; lane 5, cells treated for 3 hr. Bands corresponding to 180 kDa, 85 kDa, 65 kDa, 60 kDa, and 34 kDa are indicated by arrows.

the *P*-Tyr content of cellular proteins. Fig. 2*C* is the anti-*P*-Tyr Western blot analysis of total cell lysates, which shows a rapid increase in tyrosine phosphorylation of a 180-kDa protein in 10 min (lane 2), which declined to the original level after 1 hr (lane 4). This protein might be the epidermal growth factor receptor because immunoprecipitation and kinase assay with an anti-epidermal growth factor receptor antibody showed an increase in its kinase activity (data not shown). The level of *P*-Tyr of a 34-kDa protein was found to be elevated in 3 hr (lane 5). On the other hand, the level of *P*-Tyr of proteins with molecular masses of 85 kDa, 65 kDa, and 60 kDa was found to be reduced (Fig. 2*C*).

The dephosphorylation of c-Src may be explained by the activation of intracellular PTPases or by the inhibition of a carboxyl-terminal Src kinase, which phosphorylates tyrosine-527 of c-Src (26). We found a rapid increase in overall intracellular PTPase activity, which peaked at 30 min (Fig. 3), and this increase (Fig. 3, lane 4) could be inhibited by the PTPase inhibitor sodium vanadate (Fig. 3, lane 8).

Ca^{2+}-Induced c-Src Activation in A431 Cells. The A431 cell line is derived from a human epidermoid carcinoma (27). Ca^{2+}/ionophore treatment of A431 cells also resulted in the activation of c-Src and inactivation of c-Yes. As shown in Fig. 4*A*, lane 2, a 30-min treatment with Ca^{2+}/ionophore resulted in a 3- to 5-fold increase in c-Src kinase activity. Three proteins (120 kDa, 65 kDa, and 34 kDa) were coprecipitated with activated c-Src and phosphorylated *in vitro* (Fig. 4*A*, lanes 2–4). These three proteins were also detectable in the gel shown in Fig. 1*A* after a longer exposure (data not shown).

Similar to KCs, activation of the c-Src kinase in A431 cells was also accompanied by tyrosine dephosphorylation of c-Src itself (Fig. 4*B*). After treatment with Ca^{2+}/ionophore for 2 hr, *in vivo* tyrosine phosphorylation of c-Src was almost undetectable (lane 2). However, the 34-kDa protein that was coprecipitated by mAb 327 showed an increase in its *P*-Tyr content (Fig. 4*B*, lane 2).

The nature of c-Src's interaction with cellular proteins was further investigated by glycerol gradient sedimentation. In Ca^{2+}/ionophore-treated A431 cells, two different c-Src complexes peaked in fractions 8–10 and 10–13 (Fig. 5*B*). In one complex, c-Src was found in association with cellular proteins of 34 kDa and 65 kDa (in fractions 8–10) and, in the other, with a 120-kDa protein (in fractions 10–13). Therefore, the three proteins detected in Fig. 4*A* are likely to be in two separate complexes with c-Src. Interestingly, c-Src present in these complexes constitutes only a minor portion of total c-Src protein (fractions 4 and 5 in both treated and untreated cells). This *in vitro* kinase assay only recognized proteins that

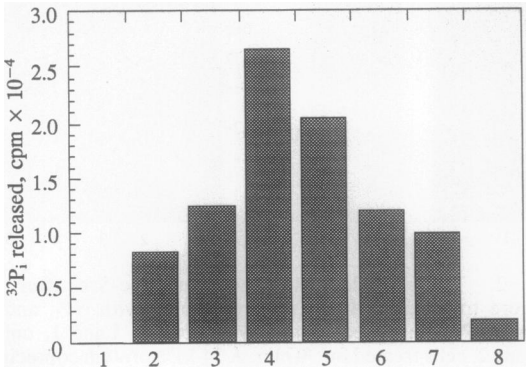

FIG. 3. Alteration of total PTPase activity in KCs after treatment with Ca^{2+}/ionophore. Seventy-eight nanograms of total protein lysate was used in each assay. Lane 1, reaction mix without lysate; lane 2, untreated cells; lane 3, cells treated for 10 min; lane 4, cells treated for 30 min; lane 5, cells treated for 1 hr; lane 6, cells treated for 3 hr; lane 7, cells treated for 6 hr; lane 8, sodium vanadate was added into lysate of lane 4 prior to the PTPase assay.

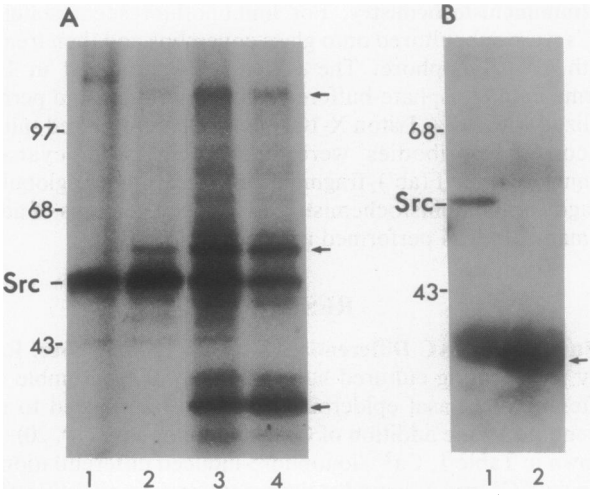

FIG. 4. Ca^{2+} activation of the c-Src kinase in A431 cells. (*A*) Lane 1, untreated cells; lane 2, cells treated with Ca^{2+}/ionophore for 1 hr; lane 3, cells treated for 3 hr; lane 4, cells treated for 6 hr. Proteins coprecipitated with c-Src are indicated by arrows. (*B*) Anti-*P*-Tyr Western blot on mAb 327 immunoprecipitates from cells untreated (lane 1) or treated with Ca^{2+}/ionophore for 2 hr (lane 2). An increase in *P*-Tyr of a 34-kDa protein that coprecipitated with c-Src is indicated by an arrow. Molecular size markers (in kDa) are shown.

can be phosphorylated by c-Src, which does not rule out the possible existence of other proteins in the c-Src complexes.

Localization of *P*-Tyr and c-Src in Differentiating KCs. Given the changes in the activity of c-Yes and c-Src kinases, as well as tyrosine phosphatase activity upon Ca^{2+} treatment, we explored the cellular localization of *P*-Tyr and c-Src in differentiating KCs by immunofluorescence or histochemistry with specific antibodies. In cultured KCs, *P*-Tyr is conspicuous in focal adhesions, at opposing plasma membranes, and in the perinuclear cytoplasm (Fig. 6 *A* and *B*). c-Src is visualized in the perinuclear cytoplasm and at

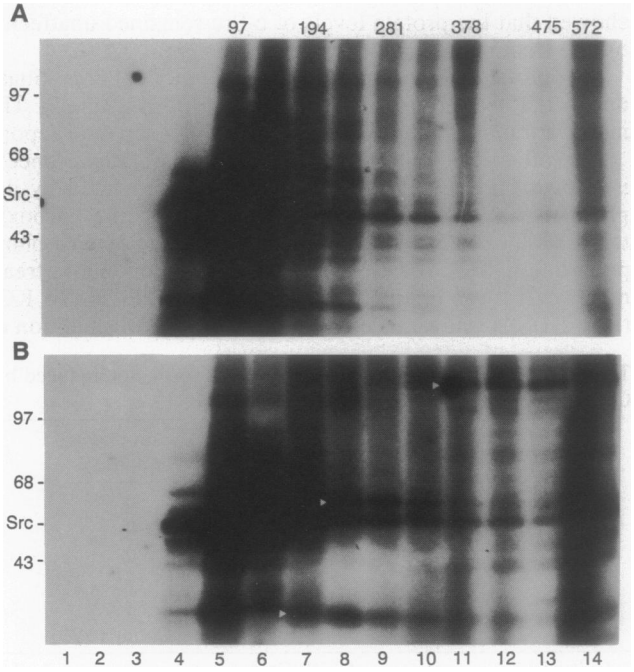

FIG. 5. Glycerol gradient sedimentation analysis of c-Src/cellular protein complexes in A431 cells. Lysates of untreated cells (*A*) or treated cells (*B*) were fractionated on glycerol gradients. The molecular mass is marked on top of *A*. c-Src complexes were visualized after *in vitro* kinase reaction. Proteins of 120 kDa, 65 kDa, and 34 kDa are indicated by white arrowheads in *B*.

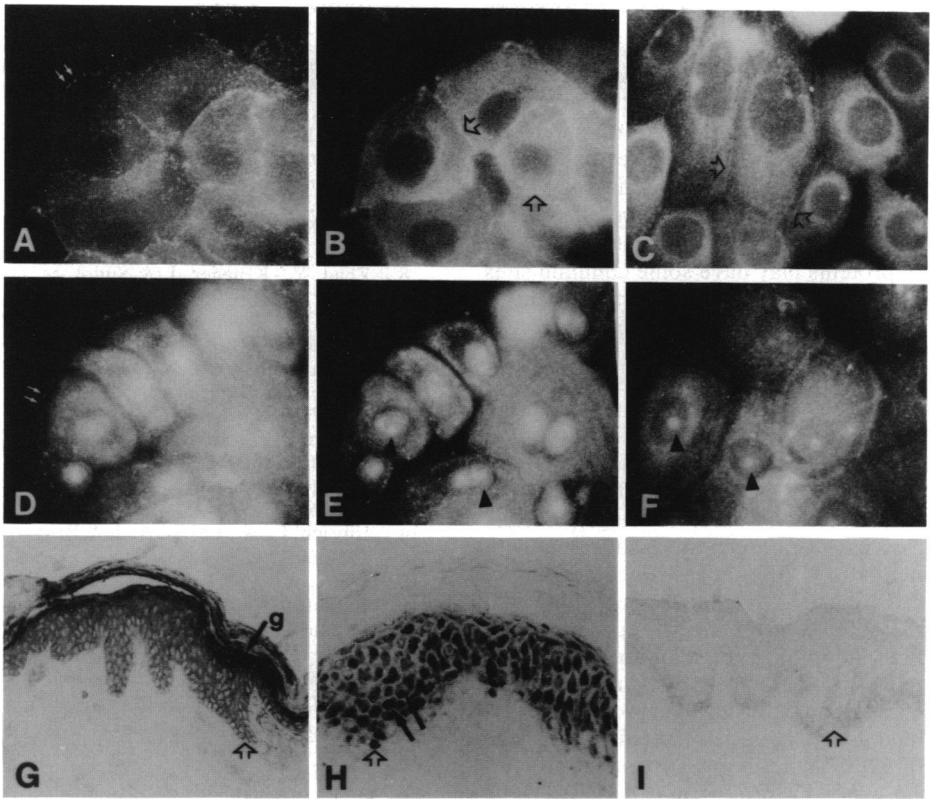

FIG. 6. Intracellular localization of c-Src and *P*-Tyr in KCs after Ca^{2+}/ionophore treatment and in human skin. (*A–C*) Control cells. (*D–F*) Cells treated for 2 hr. *A*, *B*, *D*, and *E* were stained with 4G10 anti-*P*-Tyr antibody; *C* and *F* were stained with mAb 327. The focal plane is at the cell substratum (*A* and *D*) or at the midcellular level (*B*, *C*, *E*, and *F*). Arrows in *A* and *D* indicate focal adhesions. Open arrows in *B* and *C* indicate plasma membrane. Arrowheads in *E* and *F* indicate nuclei with nucleolar staining. Cryostat sections of adult human skin were incubated with anti-*P*-Tyr (*G*), mAb 327 (*H*), or control monoclonal anti-cytokeratin 8.12 (*I*; Sigma). Open arrows in *G–I* indicate the basal layer; the solid arrow in G indicates the granular layer (g); the solid arrow in H indicates nuclear staining. (*A–F*, ×1260; *G* and *I*, ×180; *H*, ×360.)

opposing plasma membranes (Fig. 6*C*). Upon induction of differentiation by Ca^{2+}/ionophore, there are time-related alterations in the intracellular location of *P*-Tyr (Fig. 6 *D* and *E*) and c-Src (Fig. 6*F*). *P*-Tyr in focal adhesions and plasma membranes largely disappeared by 10 min of treatment (data not shown) and was followed by an accumulation of *P*-Tyr within the nucleus by 1–2 hr of treatment (Fig. 6*E*). The c-Src protein also accumulates in the nucleus within 1–2 hr, but some fraction of intracellular c-Src remains at the plasma membrane and in the perinuclear cytoplasm (Fig. 6*F*). Because the nuclear localization of c-Src was surprising, we performed subcellular fractionation and found that c-Src was detectable in Triton X-100-treated A431 nuclei after Ca^{2+}/ionophore incubation. In addition, glucuronyl transferase, another membrane-associated enzyme, was never found in nuclei after immunofluorescence staining. Moreover, nuclei of differentiated KCs in psoriatic scale also reacted with mAb 327 (data not shown).

To further explore the level of c-Src protein and *P*-Tyr in keratinocyte differentiation, cryostat sections of adult human skin were incubated with specific mAbs. *P*-Tyr labeling was evident in all viable epidermal layers, but appeared to increase in intensity near the granular epidermal layer, in which the final stages of KC differentiation occur (Fig. 6*G*). The c-Src protein was also abundant in differentiating keratinocytes in the spinous epidermal layers (Fig. 6*H*), where it was detected within nuclear and perinuclear cytoplasmic areas. Another c-Src-specific antibody GD11 also showed abundant reactivity within the upper spinous and granular epidermal layers (data not shown), whereas a control monoclonal antibody showed no specific epidermal staining (Fig. 6*I*).

DISCUSSION

An increase in the intracellular concentration of Ca^{2+} induces a variety of effects that are cell-type specific. Effects include activation of mast cell degranulation, augmented lymphocyte mitogenesis, and induction of terminal differentiation in KCs (28–30). While many of these effects are thought to be mediated by Ca^{2+} binding proteins such as protein kinase C and calmodulin (29), Ca^{2+}-induced negative regulation of PTPases has also been observed (30). Data presented in this paper indicate that Ca^{2+}/ionophore treatment of human KCs leads to alteration of two cellular protein-tyrosine kinases: rapid activation of c-Src and inactivation of c-Yes. The kinase activity of c-Src has been reported to be regulated in a negative fashion, primarily by phosphorylation of a carboxyl-terminal tyrosine residue (tyrosine-527) (3–5). A marked reduction in tyrosine phosphorylation of Ca^{2+}-activated c-Src kinase, along with an increase in intracellular PTPase activity, is consistent with this concept. The Ca^{2+}-induced activation of c-Src could be responsible for the increased tyrosine phosphorylation of p85 and p62 in murine KCs induced to differentiate with Ca^{2+} (12), as well as the proteins observed in this report that are coprecipitated with activated c-Src.

To confirm that the activation of c-Src kinase was due to an increase in the intracellular Ca^{2+} concentration rather than an increase in the concentration of other cations, we treated cells with ionomycin, a Ca^{2+}-specific ionophore and obtained essentially the same result. We were also able to detect c-Src activation with various reagents that release Ca^{2+} from intracellular stores, such as thapsigargin and angiotensin (data not shown). Thus, the activation of c-Src is a conse-

quence of an elevation of the intracellular Ca^{2+} concentration. Three cellular proteins (120 kDa, 65 kDa, and 34 kDa) coprecipitated with Ca^{2+}-activated c-Src in A431 cells, as well as in KCs. It is tempting to speculate that an activated c-Src kinase might relate to some aspects of terminal differentiation in KCs through association with specific cellular proteins. v-Src, which has a constitutively activated kinase activity, associates with numerous cellular proteins of approximately the same sizes (5). Thus enzymatically active cellular and viral Src proteins may have some common sites for interaction with cellular proteins, although the biochemical identity of the proteins associated with c-Src remains to be determined.

In cultured, proliferating KCs, c-Src appears to be localized in juxtanuclear cytoplasmic areas and at the plasma membrane, like in a variety of other cell types (31–36). It is remarkable that *P*-Tyr accumulates within the nucleus of Ca^{2+}/ionophore-treated KCs and, likewise, that c-Src is localized in or near the nucleus of differentiating epidermal KCs. Although c-Src has been seen in association with nuclear envelope membranes (32) and with centrisomes of mitotic cells (35), c-Src has not previously been observed within the nucleus of cells. Several recent reports suggest that tyrosine phosphorylation might regulate the function of some nuclear proteins. cdc2, lactose dehydrogenase, or androgen receptor are examples of nuclear proteins whose function or subcellular localization is affected by tyrosine phosphorylation (37–39). Furthermore, two other protein-tyrosine kinases, the c-Abl IV protein and the FER gene product, have also been localized to the cell nucleus (40, 41). Thus protein-tyrosine kinases such as c-Src might affect gene expression or other cellular functions upon conditional nuclear localization. The reduced abundance of *P*-Tyr at cell adhesion points and at the plasma membrane might also be important in conveying signals that are associated with growth cessation or with changes in cell shape and cytoskeletal rearrangements that are part of progressive cell differentiation in the epidermis. It should be acknowledged that other tyrosine kinases such as the epidermal growth factor receptor, insulin-like growth factor receptor, or c-Yes, which are highly expressed in basal KCs (13, 42, 43), might play important roles in transducing proliferation-related signals. Thus, protein-tyrosine phosphorylation might have dual roles in regulating both KC proliferation and differentiation, depending upon the epidermal layer in which a specific kinase or phosphatase is activated and depending upon the intracellular sites at which tyrosine phosphorylation is altered.

Our data indicate dynamic changes in protein-tyrosine phosphorylation in differentiating KC. The activation of c-Src is accompanied with changes in the subcellular distribution of *P*-Tyr-containing proteins and c-Src itself, both of which accumulate in the nucleus. Our data thus link Ca^{2+} signaling with altered tyrosine phosphorylation. Given the roles of Ca^{2+} in differentiation-related cell signaling, this suggests a potentially important role of tyrosine phosphorylation in epidermal differentiation.

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