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Specific Changes of Ras GTPase-Activating Protein (GAP) and a GAP-Associated p62 Protein during Calcium-Induced Keratinocyte Differentiation

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Induction of tyrosine phosphorylation occurs as an early and specific event in keratinocyte differentiation. A set of tyrosine-phosphorylated substrates which transduce mitogenic signals by tyrosine kinases has previously been identified. We show here that of these substrates, the Ras GTPase-activating protein, GAP, is specifically affected during calcium-induced keratinocyte differentiation. As early as 10 min after calcium addition to cultured primary mouse keratinocytes, GAP associates with tyrosine-phosphorylated proteins and translocates to the membrane. In addition, a GAP-associated protein of ~62 kDa (p62) becomes rapidly and heavily tyrosine phosphorylated in both membrane and cytosolic fractions. This protein corresponds to the major tyrosine-phosphorylated protein that is induced in differentiating keratinocytes as early as 5 min after calcium addition. p62 phosphorylation was not observed after exposure of these cells to epidermal growth factor, phorbol ester, or transforming growth factor β. In contrast, PLCγ and PI3K were tyrosine phosphorylated after epidermal growth factor, but not calcium, stimulation. Thus, changes of Ras GAP and an associated p62 protein occur as early and specific events in keratinocyte differentiation and appear to involve a calcium-induced tyrosine kinase.

Very little is known about early regulatory mechanisms underlying epithelial cell differentiation. Understanding this process is of great importance, especially since its disruption is closely associated with epithelial tumor development.

Mouse primary keratinocytes are an ideal system for studying epithelial cell differentiation. Addition of calcium to these cells induces a program of terminal differentiation similar to that which occurs in vivo, including growth arrest, induction of antigenic and enzymatic markers, cornification, and stratification (25, 29).

Although the later events involved in keratinocyte differentiation have been well described, the only known early biochemical changes are ion influx (26–28) and increased phosphatidylinositol metabolism (31, 46, 67). In addition, we have recently shown that induction of tyrosine phosphorylation is an early and specific event which appears to be required for keratinocyte differentiation to occur (18).

Tyrosine phosphorylation is one way by which growth factor receptors and several oncogenes transmit their mitogenic signals (12, 62). Tyrosine kinase oncogenes are able to substantially alter the ability of specialized cells to differentiate (1, 59), and changes in tyrosine phosphorylation occur during differentiation (3, 6, 18, 20, 21). Recently, a tyrosine kinase previously identified as a transforming oncoprotein has been shown to mediate nerve growth factor-induced differentiation of neuronal cells (33, 37). Thus, tyrosine phosphorylation is involved in the transduction of signals for both growth and differentiation.

A set of specific signal-transducing molecules, including Ras GTPase activating protein (GAP), phospholipase C gamma (PLCγ), and phosphatidylinositol 3-kinase (PI-3K), which are associated with and become tyrosine phosphorylated by a number of tyrosine kinases, have been identified (for a review, see reference 12). The biochemical activities of these substrate molecules suggest that they act as important signal transducers.

We report here that of these substrates, Ras GAP associates with tyrosine-phosphorylated proteins and translocates to the membrane during calcium-induced keratinocyte differentiation. In addition, a GAP-associated protein of 62 kDa (p62) becomes rapidly and heavily tyrosine phosphorylated. This protein corresponds to the major tyrosine-phosphorylated protein that is induced in differentiating keratinocytes as early as 5 min after calcium addition. p62 phosphorylation was not observed after exposure of these cells to epidermal growth factor (EGF), phorbol ester, or transforming growth factor β (TGF-β). In contrast, PLCγ and PI3K were tyrosine phosphorylated after EGF, but not calcium, stimulation. Thus, changes of Ras GAP and a GAP-associated p62 protein occur as early and specific events in keratinocyte differentiation and appear to involve a calcium-induced tyrosine kinase.

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), EGF (10 ng/ml; Collaborative Research, Inc., Cambridge, Mass.), and 0.05 mM $CaCl_2$ as previously described (18, 29). Harvey sarcoma virus infection was carried out as previously described (15).

SDS-polyacrylamide gel electrophoresis and immunoblotting. Samples were suspended in electrophoresis sample

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buffer, boiled, run on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to nitrocellulose as previously described (18). The transfer buffer and procedures for blocking and washing the filter were as previously described (32). Blots were developed by incubation in blocking buffer containing 1 μCi of ^{125}I-protein A (35 μCi/μg; ICN) per ml and exposed for 3 to 7 days. Longer exposures did not improve detection. For detection of PI-3K, the ECL (chemoluminescence) kit from Amersham was used. The antiphosphotyrosine antibodies used for immunoblotting, kindly provided by David F. Stern, are polyclonal antibodies prepared against the synthetic copolymer phosphotyrosine-Gly-Ala as previously described (32). The antiphosphotyrosine antibodies used for immunoprecipitation (PY20, purchased from ICN) are mouse monoclonal antibodies which are specific for phosphobenzene derivatives such as phosphotyrosine and phenyl phosphate (22). The anti-PI-3K antibodies, raised in rabbits against the 85-kDa subunit of PI-3K, were purchased from Upstate Biotechnology, Inc., Lake Placid, N.Y. The anti-PLCγ antibodies were kindly provided by John Knopf (Genetics Institute, Cambridge, Mass.) (56). The polyclonal, anti-human recombinant GAP antibodies have been previously described (24).

Antiphosphotyrosine immunoprecipitation. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in EBC buffer (50 mM Tris Cl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM Na_3VO_4). Lysates were incubated on ice for 10 min and spun for 5 min, and protein assays were performed on the supernatant. A total of 1 μl of PY20 antibody (1 mg/ml) or (as a negative control) 1 μl of affinity-purified rabbit anti-goat immunoglobulin G (Cappel, West Chester, Pa.) per ml of extract was used. After 2 h of incubation at 4°C, 75 μl of a 50% solution of protein A-Sepharose (Pharmacia) in EBC buffer was added. After 30 min at 4°C, samples were washed 5 times with NET-N buffer (20 mM Tris HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM PMSF). Samples were resuspended in 50 μl of 2× sample buffer and boiled for 3 min.

Anti-GAP, anti-PLCγ, or anti-PI3K immunoprecipitations. Proteins were immunoprecipitated essentially as previously described (16), with minor modifications. Briefly, cells were washed twice with PBS and lysed in 1 ml of lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM PMSF, 200 μM sodium orthovanadate, 100 mM sodium fluoride). Lysates were incubated on ice for 5 min and spun for 5 min, and equal amounts of protein (determined by a Bradford protein assay [Bio-Rad]) were incubated for 1 h at 4°C with antiserum followed by a 30-min incubation with 100 μl of 20% protein A-Sepharose. The resulting immune complexes were washed three times with 20 mM HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, and 1 mM sodium orthovanadate, resuspended in 50 μl of 2× sample buffer, and boiled briefly.

Cell fractionation. Subcellular localization of GAP was determined essentially as previously described (43) with minor modifications. Briefly, cells were rinsed twice with cold PBS containing 1 mM Na_3VO_4 and lysed in 0.5 ml of hypotonic lysis buffer (20 mM HEPES buffer [pH 7.4], 5 mM sodium PP_i, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg [each] of aprotinin and leupeptin per ml). Cells were collected by scraping, and samples were Dounce homogenized by using a tight-fitting pestle with 40 strokes. After centrifugation at 1,000 × *g* for 5 min, the supernatant fraction was ultracentrifuged at 100,000 × *g* for 45 min. The cytosolic (S-100) supernatant fraction was removed, and the pellet was resuspended in hypotonic lysis buffer and recentrifuged as described above. The pellet (P-100) was then resuspended in 0.5 ml of lysis buffer (as for anti-GAP immunoprecipitation). Samples were normalized for protein content (Bio-Rad). A total of 10 μg of protein of each fraction was used for Western immunoblot analyses, and 100 μg was used for immunoprecipitations.

RESULTS

GAP and p62 tyrosine phosphorylation in differentiating keratinocytes. GAP is a known regulator (and perhaps effector) of *ras* function (10, 39), which becomes tyrosine phosphorylated in response to growth factor stimulation or transformation by tyrosine kinase oncogenes (16, 34, 43, 45). Growing mouse keratinocytes kept in medium at low calcium concentrations (0.05 mM, low-calcium medium) can be induced to differentiate by the addition of calcium (2 mM, high-calcium medium) (29). To test whether GAP becomes tyrosine phosphorylated during this process, extracts from keratinocytes in low- or high-calcium medium were immunoprecipitated with either anti-GAP or anti-phosphotyrosine antibodies and immunoblotted with the reciprocal antibodies (Fig. 1). A significant and progressive increase in anti-phosphotyrosine-precipitable GAP was detected on anti-GAP immunoblots starting within 10 min of calcium addition (Fig. 1A). GAP could not be detected as a distinct band on anti-phosphotyrosine immunoblots of anti-GAP immunoprecipitations from keratinocytes (Fig. 1B), while it could be found under similar conditions in *src*-transformed fibroblasts (Fig. 1C). Thus, the calcium-induced increase in anti-phosphotyrosine-precipitable GAP may be due to association of GAP with other tyrosine-phosphorylated proteins. Direct tyrosine phosphorylation of GAP may occur but remains below detectable levels.

Immunoprecipitation of keratinocyte extracts with anti-GAP antibodies followed by anti-phosphotyrosine blotting detected a broad band (Fig. 1B) which migrated above the GAP protein seen on GAP Western blots. Detection of this band on anti-phosphotyrosine blots did not increase after calcium treatment. Similarly, a GAP-associated protein with a molecular mass of ~190 kDa was detected by anti-phosphotyrosine antibodies under basal (low-calcium) conditions but did not seem to be significantly modified during calcium-induced differentiation. In contrast, a GAP-associated protein of ~62 kDa became heavily tyrosine phosphorylated within 10 min of calcium treatment, and its phosphorylation seemed to further increase with time (Fig. 1B). Both the 62- and the 190-kDa proteins comigrated with previously described GAP-associated proteins (p62 and p190) found in fibroblasts transformed with v-*src* (Fig. 1C) (16).

One of the earliest effects associated with induction of keratinocyte differentiation by calcium is tyrosine phosphorylation of a single detectable protein of ~60 kDa (18). If this protein corresponds to the GAP-associated p62 protein, it should be specifically removed from total keratinocyte extracts by immunoprecipitation with anti-GAP antibodies. Extracts from keratinocytes in low- or high-calcium medium were incubated with either anti-GAP or nonimmune antibodies, and immunoprecipitates and supernatants (obtained after incubation with protein A-Sepharose beads) were analyzed by gel electrophoresis and immunoblotting with anti-

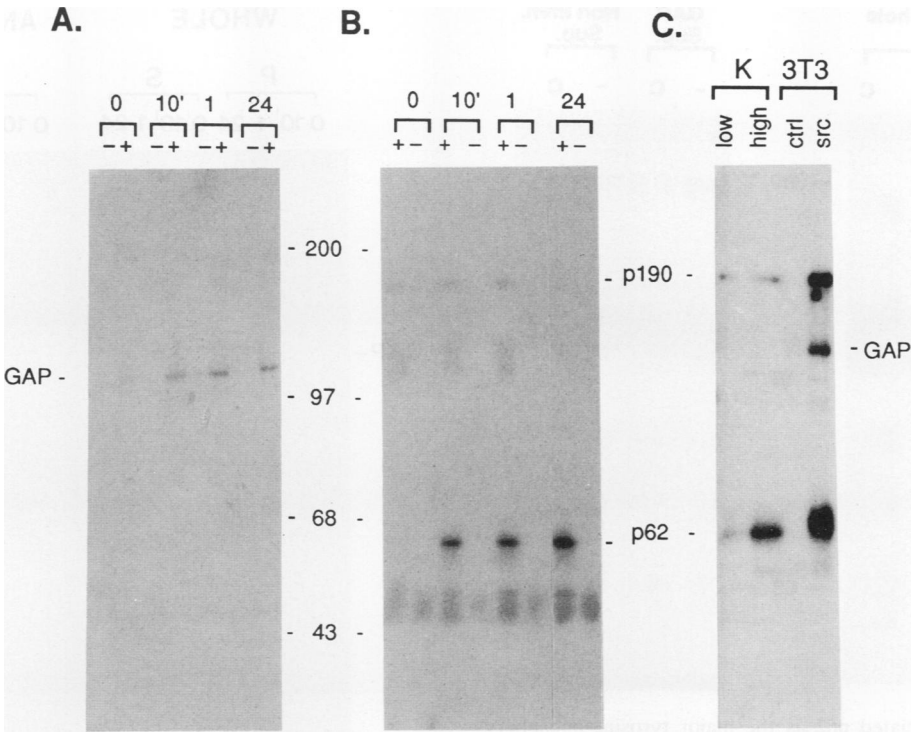

FIG. 1. Tyrosine phosphorylation of GAP and GAP-associated proteins in differentiating keratinocytes or in *src*-transformed fibroblasts. Cell extracts from keratinocytes which were either untreated or exposed to calcium for 10 min, 1 h, or 24 h were immunoprecipitated with Py 20 anti-phosphotyrosine (+) or control, affinity-purified rabbit antibodies (−) and immunoblotted with anti-GAP antibodies (A) or immunoprecipitated with anti-GAP (+) or control, rabbit whole serum (−) and immunoblotted with polyclonal anti-phosphotyrosine antibodies (B). (The 24-h time comes from the same experiment and the same blot as the other times.) (C) Cell extracts from normal (ctrl) or *src*-transformed (src) NIH 3T3 cells were compared with cell extracts from keratinocytes in low (low)- or high (high)-calcium medium for 1 h by immunoprecipitation and blotting as in panel B. The positions for GAP, p62, and p190 are indicated. Numbers refer to molecular size markers in kilodaltons.

phosphotyrosine antibodies. The calcium-inducible 62-kDa protein detectable in the anti-GAP immunoprecipitate was found to comigrate with a similar tyrosine-phosphorylated protein present in whole keratinocyte extracts (Fig. 2, left panel). This protein was specifically removed from the supernatant of the anti-GAP immunoprecipitate, while it remained detectable in the supernatant of the nonimmune control (Fig. 2, right panel).

Thus, calcium-induced differentiation of keratinocytes led to a progressive increase in anti-phosphotyrosine-precipitable GAP and strong tyrosine phosphorylation of a GAP-associated p62 protein. This latter protein appeared to be the major tyrosine-phosphorylated protein induced at very early times of keratinocyte differentiation.

GAP membrane translocation. During growth factor stimulation of cells, GAP has been shown to translocate to the cell membrane (43, 45) where it may interact with critical regulatory molecules. To determine whether GAP moves to the membrane during keratinocyte differentiation, cell extracts from keratinocytes in low- or high-calcium medium were separated into membrane and cytoplasmic fractions and immunoblotted with anti-GAP antibodies. The amount of GAP associated with the particulate fraction increased progressively, starting 10 min after calcium addition, with a concomitant decrease in the cytosolic fraction (Fig. 3, WHOLE). Anti-GAP immunoblots of anti-phosphotyrosine immunoprecipitations of these fractions confirmed this result and showed that anti-phosphotyrosine-precipitable GAP was found predominantly in the particulate fraction (Fig. 3, ANTI-PTYR).

To test whether the strong tyrosine phosphorylation of the GAP-associated p62 protein was also associated with membrane translocation, cell fractions were immunoprecipitated with anti-GAP antibodies and blotted with anti-phosphotyrosine antibodies. Similar amounts of p62 could be immunoprecipitated with anti-GAP antibodies from both fractions (Fig. 4, ANTI-GAP), suggesting that phosphorylated p62 associates with GAP both at the membrane and in the cytosol. Unlike p62, other tyrosine-phosphorylated proteins of ~80, 125, and 130 kDa associate with GAP specifically at the membrane (Fig. 4, ANTI-GAP), even though these proteins seemed to be present in both fractions (Fig. 4, WHOLE). Thus, during keratinocyte differentiation, GAP appeared to move to the membrane where it associated specifically with other tyrosine-phosphorylated proteins, while cellular distribution of a GAP-associated p62 protein did not change.

State of phosphorylation of GAP and p62 after treatment of keratinocytes with other agents. In keratinocytes, modification of GAP and p62 may be specific for calcium-induced differentiation or may occur after treatment with other agents which induce mitogenesis, growth arrest, and/or some aspects of differentiation. EGF has been shown to induce tyrosine phosphorylation of GAP and p62 in fibroblasts (16, 45) and is mitogenic for these cells as well as for keratinocytes. The primary effect of TGF-β on keratinocytes

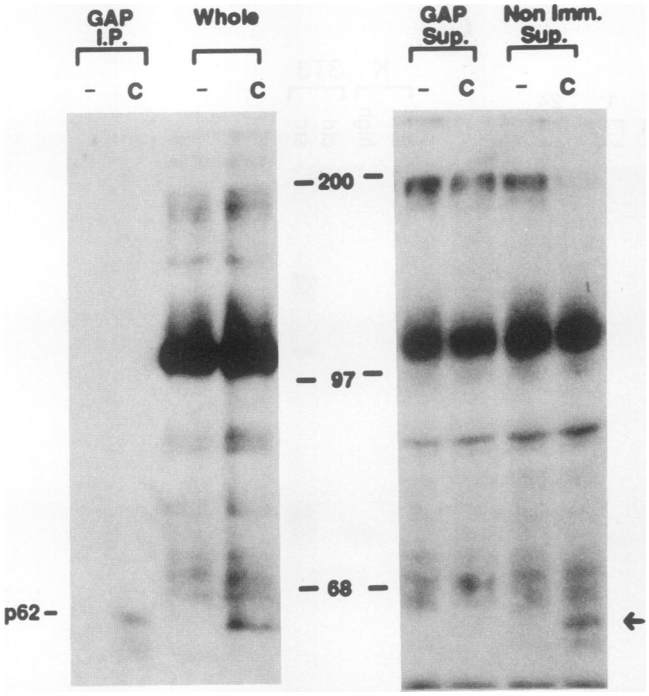

FIG. 2. GAP-associated p62 as the major tyrosine-phosphorylated protein induced at early times of keratinocyte differentiation. Keratinocytes were either untreated (−) or exposed to high-calcium medium for 1 h (C). Cell extracts were immunoprecipitated with anti-GAP antibodies or affinity purified rabbit anti-goat immunoglobulin G. Immunoprecipitates and supernatants were analyzed by gel electrophoresis and immunoblotting with anti-phosphotyrosine antibodies as in Fig. 1. Whole cell extracts (Whole) were also used for comparison. Samples in the left and right panels are from the same experiment but were run on two different gels. Positions for GAP- and differentiation-associated p62 proteins are indicated.

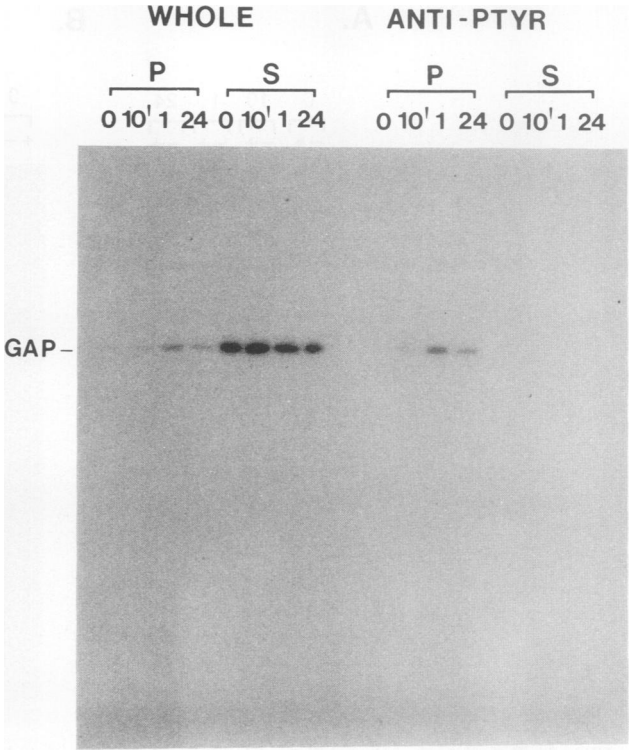

FIG. 3. Subcellular localization of GAP in differentiating keratinocytes. Cell extracts from keratinocytes in low-calcium medium (0) treated with calcium for various times (10 min and 1 and 24 h) were fractionated into particulate (P) or soluble (S) fractions, either loaded directly onto the gel (WHOLE) or immunoprecipitated with anti-phosphotyrosine antibodies (ANTI-PTYR) prior to electrophoresis, and immunoblotted with anti-GAP antibodies.

is growth arrest (13), but this protein can also induce some markers of differentiation (41, 42, 53). The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces a program of keratinocyte differentiation which partially overlaps that induced by calcium (64).

Keratinocytes were treated for 10 min or 1 h with calcium, EGF, TGF-β, or TPA and proteins were immunoprecipitated with either anti-GAP or anti-phosphotyrosine antibodies. After electrophoresis and transfer to nitrocellulose, samples were probed with reciprocal antibodies. Compared with the calcium effect, EGF treatment resulted in only a very slight increase in anti-phosphotyrosine-immunoprecipitable GAP. No such effects were detected after TGF-β or TPA treatment (Fig. 5A). Similarly, phosphorylation of p62 was observed only after calcium, but not EGF, TGF-β, or TPA treatment of keratinocytes (Fig. 5B). Thus, these changes of GAP and p62 appear to be specific for calcium-induced differentiation, since no similar effects were observed in keratinocytes treated with other agents which affect growth or differentiation.

GAP and p62 modifications in *ras*-transformed keratinocytes. In fibroblasts, a transforming *ras* protein has been shown to block signal transduction by the platelet-derived growth factor (PDGF) receptor (8, 51, 52, 68), including inhibition of PDGF-induced GAP phosphorylation (34). In primary mouse keratinocytes, oncogenic *ras* blocks some aspects of keratinocyte differentiation (59, 60, 65) but not others (41, 66) and may interfere with the calcium-induced modifications of GAP and p62.

Primary keratinocytes were infected with Harvey sarcoma virus 1 week prior to the experiment under conditions which result in a high level of expression of the v-*ras* p21 gene product (11a). Extracts from these cells were immunoprecipitated and immunoblotted as before. After calcium addition, *ras*-transformed keratinocytes showed the same pattern of GAP and p62 modifications as the untransformed controls. That is, calcium treatment of *ras*-transformed cells caused an increase in the amount of anti-phosphotyrosine-precipitable GAP (Fig. 6A, left), tyrosine phosphorylation of p62 (Fig. 6A, right), and translocation of GAP to the membrane (Fig. 6B, left) where it associated with the specific tyrosine-phosphorylated proteins described above (Fig. 6B, ANTI-GAP). Therefore, unlike its effects on mitogenically stimulated fibroblasts, the transforming *ras* oncoprotein does not seem to interfere with the GAP and p62 modifications associated with calcium-induced keratinocyte differentiation.

State of PLCγ and PI-3K tyrosine phosphorylation. Besides GAP, other previously identified signal-transducing molecules (for a review, see reference 12) might also be modified during keratinocyte differentiation. The apparent induction of PLC activity associated with keratinocyte differentiation (31, 46, 57) may be due to tyrosine phosphorylation of PLCγ, the only PLC isoform known to be tyrosine phosphorylated (38, 40, 58) and hence activated (48, 56). To test this

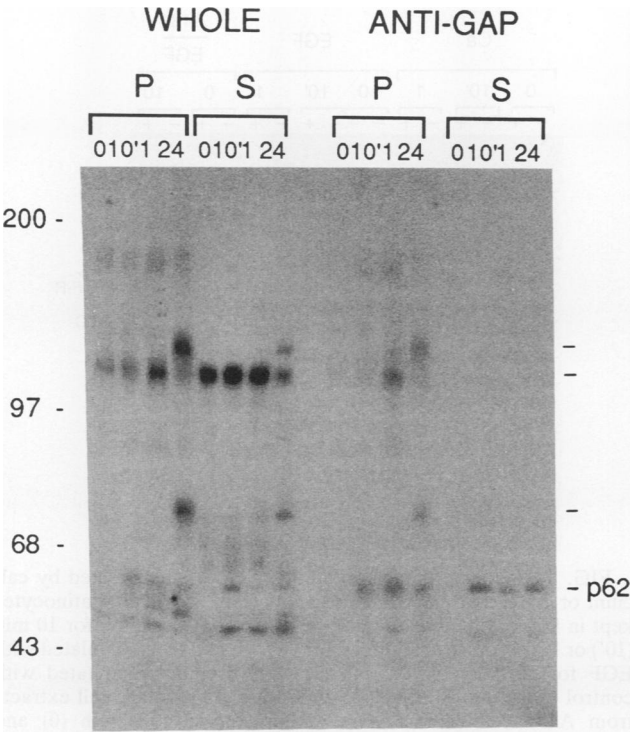

FIG. 4. Subcellular localization of tyrosine-phosphorylated and/or GAP-associated proteins in differentiating keratinocytes. Cell extracts from keratinocytes treated with calcium for various amounts of time (10 min and 1 or 24 h) were fractionated into particulate (P) and soluble (S) fractions and were analyzed directly (WHOLE) or immunoprecipitated with anti-GAP antibodies (ANTI-GAP) prior to electrophoresis. Anti-phosphotyrosine antibodies were used for immunoblotting. Molecular size markers are indicated on the left (in kilodaltons), while on the right are the positions for the p62 protein and the other bands of ~80, 120, and 130 kDa discussed in text.

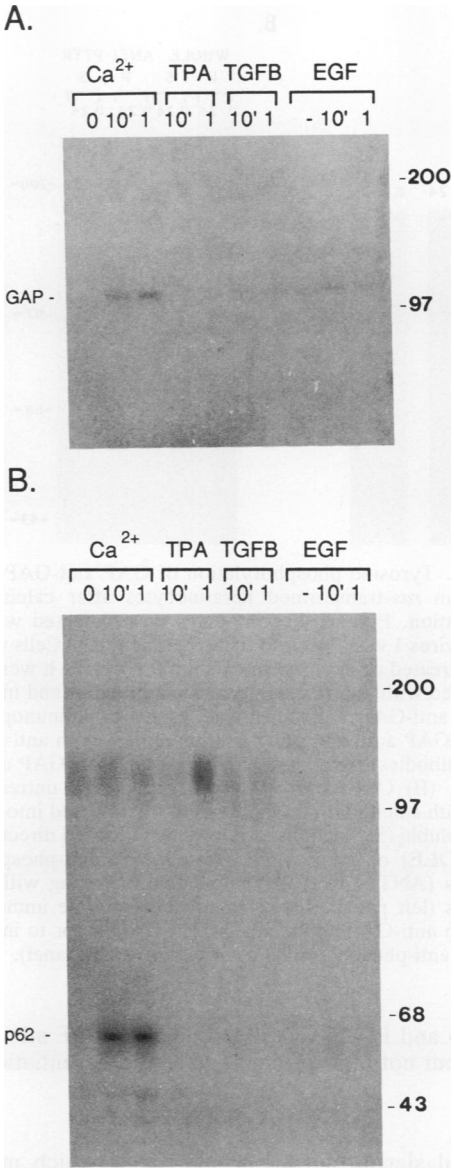

FIG. 5. Specific modification of GAP and p62 by calcium but not by other agents which induce differentiation, growth arrest, or mitogenesis. Cell extracts from keratinocytes untreated (0) or treated with calcium (Ca^{2+}), TPA (100 ng/ml), TGF-β (TGFB) (10 ng/ml), or EGF (80 nM) for 10 min (10') or 1 h (1) were immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with anti-GAP antibodies (A) or immunoprecipitated with anti-GAP antibodies and immunoblotted with anti-phosphotyrosine antibodies (B). The positions of GAP and p62 are as indicated.

possibility, extracts from keratinocytes treated with calcium or EGF were immunoprecipitated with anti-PLCγ antibodies and immunoblotted with anti-phosphotyrosine antibodies (Fig. 7). The epidermoid carcinoma cell line A431 was used as a positive control. When stimulated with EGF, these cells showed increased phosphorylation of two proteins of the expected molecular masses for the EGF receptor (180 kDa) and PLCγ (145 kDa). (Overexpression of the EGF receptor in A431 cells [23] may explain its significant level of basal phosphorylation as well as why it was also found in normal serum controls.) EGF treatment of keratinocytes for 10 min resulted in tyrosine phosphorylation of similar proteins. These proteins were no longer detectable by 1 h after exposure to EGF. In contrast, neither of these proteins became phosphorylated during calcium-induced differentiation (Fig. 7).

Calcium treatment of keratinocytes leads to increased PI metabolism (31, 46, 57, 67), suggesting that a PI kinase might be activated. By analogy to mitogenically stimulated cells (12, 17, 50), the 85-kDa subunit of the PI-3K might become tyrosine phosphorylated after calcium treatment of keratinocytes. To test this possibility, extracts from keratinocytes treated with calcium or EGF were immunoprecipitated with anti-p85 antibodies and immunoblotted with anti-phosphotyrosine or anti-p85 antibodies (Fig. 8). Calcium treatment did not cause tyrosine phosphorylation of p85, while this protein became phosphorylated by EGF treatment of keratinocytes as well as A431 cells. As with PLCγ, phosphorylation of p85 was detected 10 min after EGF treatment but was no longer present by 1 h.

In mitogenically stimulated cells, phosphorylation of p85 correlates with an increase in PI kinase activity in anti-phosphotyrosine immunoprecipitates (14, 35). Calcium addition to keratinocytes did not result in a significant increase in PI kinase activity, while such an increase was observed after EGF treatment of A431 cells (data not shown).

In conclusion, in keratinocytes, tyrosine phosphorylation

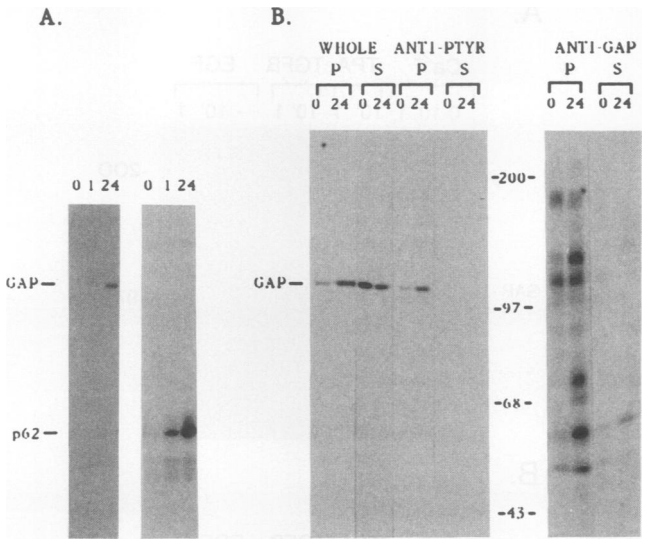

FIG. 6. Tyrosine phosphorylation of GAP and GAP-associated proteins in *ras*-transformed keratinocytes after calcium-induced differentiation. Primary keratinocytes were infected with Harvey sarcoma virus 1 week prior to the experiment. (A) Cells which were either untreated or exposed to calcium for 1 or 24 h were immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with anti-GAP antibodies (left panel) or immunoprecipitated with anti-GAP antibodies and immunoblotted with anti-phosphotyrosine antibodies (right panel). The positions for GAP and p62 are indicated. (B) Cell extracts from keratinocytes untreated (0) or treated with calcium for 24 h (24) were fractionated into particulate (P) and soluble (S) fractions. Samples were loaded directly onto the gel (WHOLE) or immunoprecipitated with anti-phosphotyrosine antibodies (ANTI-PTYR) prior to immunoblotting with anti-GAP antibodies (left panel). These same samples were immunoprecipitated with anti-GAP antibodies (ANTI-GAP) prior to immunoblotting with anti-phosphotyrosine antibodies (right panel).

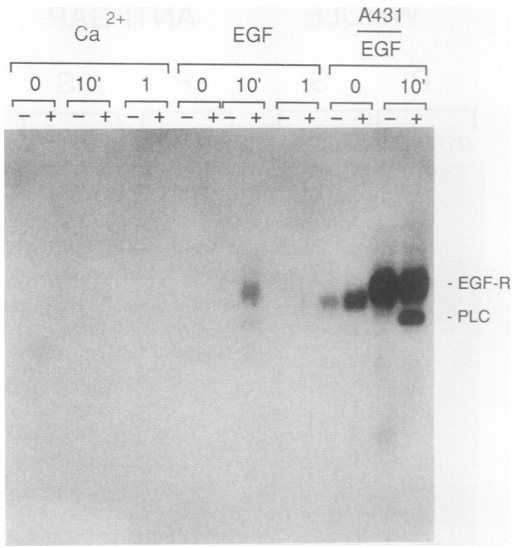

FIG. 7. Tyrosine phosphorylation of PLCγ1 as affected by calcium or EGF treatment. Cell extracts from primary keratinocytes kept in low-calcium medium (0) or treated with calcium for 10 min (10′) or 1 h (1) or serum starved for 24 h (0) and then stimulated with EGF for 10 min (10′) or 1 h (1) were immunoprecipitated with control (−) or anti-PLCγ1 (+) antibodies. In parallel, cell extracts from A431 cells which were serum starved for 24 h (0) and stimulated with EGF for 10 min (10′) were similarly immunoprecipitated. Anti-phosphotyrosine antibodies were used for immunoblotting.

of PLCγ and PI-3K was detected after EGF stimulation of growth but not after calcium-induced differentiation.

DISCUSSION

Several signal-transducing molecules which may be altered in their activity by tyrosine phosphorylation have been identified (for a review, see reference 12). We have shown here that of these molecules, GAP undergoes specific changes during calcium-induced keratinocyte differentiation. The amount of GAP which is immunoprecipitable by anti-phosphotyrosine antibodies progressively increases and is associated with its translocation to the membrane. In addition, one GAP-associated protein of ~62 kDa, p62, becomes heavily tyrosine phosphorylated. These effects of calcium treatment are not observed after exposure of keratinocytes to other agents such as EGF, TGF-β, or TPA. Thus, modification of GAP and p62 is a specific event in keratinocyte differentiation and involves a tyrosine kinase which is activated, directly or indirectly, by calcium.

The amount of GAP that can be immunoprecipitated with anti-phosphotyrosine antibodies increased significantly during calcium-induced keratinocyte differentiation. However, no induction of tyrosine phosphorylation of GAP could be detected on anti-phosphotyrosine immunoblots. Thus, in differentiating keratinocytes, GAP may be immunoprecipitated by anti-phosphotyrosine antibodies because of its association with other tyrosine-phosphorylated proteins rather than as a result of its direct tyrosine phosphorylation. Even in *src*-transformed or mitogenically stimulated cells, only a small fraction (3 to 4%) of GAP is tyrosine phosphorylated (45). Tyrosine phosphorylation of GAP may not be necessary for it to form complexes with other proteins (11). Rather, tyrosine phosphorylation of other proteins may increase their affinity for the SH2 domains of GAP (2, 44).

Since most anti-phosphotyrosine-immunoprecipitable GAP was found at the membrane, association with other tyrosine-phosphorylated proteins may be involved in the translocation of GAP to the membrane. Changes in cellular localization and/or association with other proteins or phospholipids could modify the function of GAP (for a review, see references 10 and 40). Given that GAP is able to increase the GTPase activity of *ras* (40), variations in GAP activity might be associated with alterations in *ras* activation. In three independent experiments, the amount of GTP-bound *ras* was found to increase approximately twofold during calcium-induced keratinocyte differentiation (our unpublished observation). This slight activation may have biological significance, as it is comparable to that of 3T3 cells stimulated with fetal bovine serum or PDGF (54, 55). The *ras* protein may control keratinocyte differentiation in a manner analogous to that observed for differentiation of other cells (5, 7, 19, 47, 49). Furthermore, *ras* may act synergistically with GAP, as has been shown in atrial membranes where these proteins uncouple the muscarinic receptor from the potassium channel (63).

Besides GAP, two GAP-associated proteins of 62 and 190 kDa (p62 and p190) become tyrosine phosphorylated after EGF stimulation of cells overexpressing the EGF receptor or after transformation with tyrosine kinase oncogenes (11, 16, 45). Tyrosine phosphorylation of p62 and p190 in normal

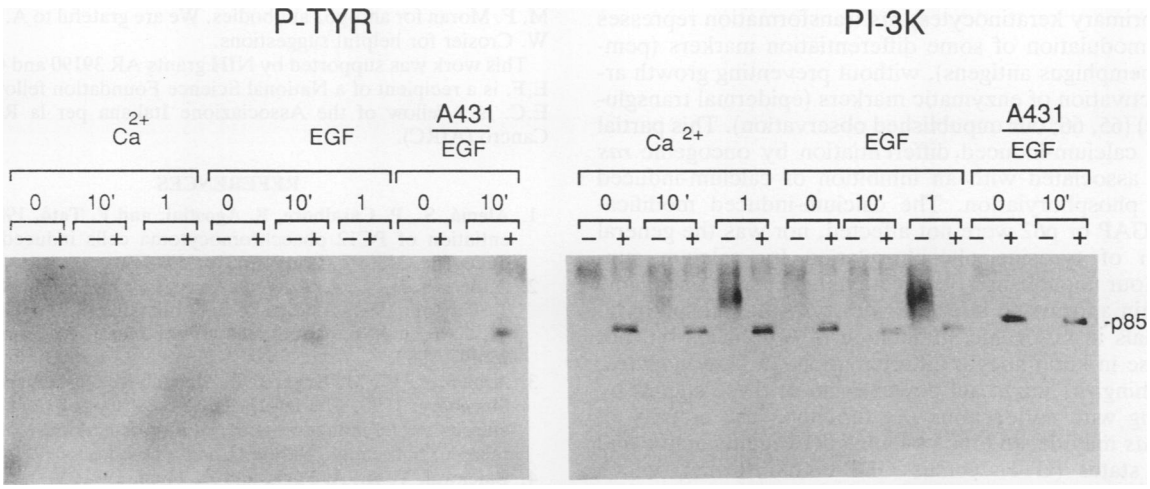

FIG. 8. Tyrosine phosphorylation of the p85 subunit of PI-3K as affected by calcium or EGF treatment. Extracts from cells treated as in Fig. 6 were immunoprecipitated with control (−) or anti-p85 (+) antibodies and immunoblotted with anti-phosphotyrosine antibodies (P-TYR) or the same anti-p85 antibodies used for immunoprecipitation (PI-3K). The position of p85 is indicated.

cells in response to mitogenic stimuli is hard to detect (our unpublished observation). Similarly, growth stimulation of keratinocytes with EGF did not lead to detectable tyrosine phosphorylation of p190 or p62. In contrast, induction of differentiation by calcium caused rapid and heavy tyrosine phosphorylation of p62. This protein appears to be the major tyrosine-phosphorylated protein that is induced at very early times of keratinocyte differentiation. This, together with the fact that tyrosine kinase-specific inhibitors significantly interfere with keratinocyte differentiation (18), suggests that phosphorylation of p62 is more than coincidental and may play a direct regulatory role in keratinocyte differentiation.

In contrast to p62, the weak level of phosphorylation of p190 detected on anti-phosphotyrosine immunoblots was not significantly increased by calcium treatment. Although it is possible that p190 becomes phosphorylated on serine or threonine residues after calcium treatment, no significant increase in phosphorylation of p190 was detected in GAP immunoprecipitates of in vivo-labelled keratinocytes (our unpublished observations).

The recent isolation of cDNA clones for p62 (61) and p190 (55) should be of great help in elucidating their function. GAP seems to form separate complexes with these proteins (45), and this differential association could have specific functional effects. In particular, association with p190 in the cytoplasm may significantly decrease GAP activity (45). The purpose of binding to p62 is not known. The fact that only a small fraction of p62 (10% in transformed fibroblasts [45]) is associated with GAP suggests that p62 might have a role of its own, other than as a regulator and/or effector of GAP. Consistent with this possibility is our finding that in keratinocytes, tyrosine phosphorylation of p62 is accompanied neither by its translocation to the membrane nor by its specific association with GAP at the membrane.

The predicted amino acid sequence of the p62 protein present in *src*-transformed fibroblasts has several similarities with those of nuclear proteins involved in RNA metabolism (61). An important question is whether the p62 protein found in *src*-transformed fibroblasts is identical or closely related to the one we have described here. Available anti-p62 antibodies are much less efficient than anti-GAP antibodies in immunoprecipitating tyrosine-phosphorylated p62 from *src*-transformed fibroblasts (our unpublished observations). No tyrosine-phosphorylated p62 was detected with these antibodies in differentiating keratinocytes. However, given the lower level of p62 phosphorylation present in these cells, it is hard to reach a definitive conclusion, and anti-p62 antibodies with a substantially higher affinity will have to be obtained.

In keratinocytes, changes of GAP and its associated protein, p62, seem to be specific to calcium treatment. Similar effects were not observed after mitogenic stimulation with EGF or after exposure to other agents (TGF-β or TPA) which cause growth arrest and/or some partial aspects of keratinocyte differentiation. Growth arrest is an important aspect of the keratinocyte terminal differentiation program. TGF-β is a potent growth inhibitor of keratinocytes which has been found to induce differentiation of these cells in some cases (41, 42, 53), even if not in others (13). TGF-β treatment of keratinocytes did not stimulate tyrosine phosphorylation of p62, GAP, or of any other proteins (our unpublished observation), suggesting that TGF-β triggers signaling mechanisms distinct from those caused by calcium.

TPA elicits a program of keratinocyte differentiation which partially resembles that triggered by calcium (64). Exposure to TPA induces some of the same enzymatic markers as calcium treatment, as well as similar changes in DNA and protein synthesis (61, 64). However, some important aspects of keratinocyte differentiation which are caused by calcium, such as desmosome formation and stratification, are not reproduced by TPA (18, 64). In keratinocytes, the overall pattern of protein phosphorylation induced by TPA overlaps only partially with that stimulated by calcium (60a). We have previously shown that in keratinocytes TPA causes rapid tyrosine phosphorylation of a protein of ~80 kDa that also seems to be phosphorylated after calcium treatment but with different kinetics (18). Another protein of ~65 kDa is phosphorylated after calcium but not TPA treatment (18). Similarly, TPA did not affect GAP or p62, suggesting that TPA and calcium either activate separate tyrosine kinases or affect the substrate specificity of the same kinase differently.

In fibroblasts, expression of a transforming *ras* protein leads to an attenuated response to PDGF (8, 51, 52, 68), including a loss of PDGF-induced GAP phosphorylation

(34). In primary keratinocytes, *ras* transformation represses calcium modulation of some differentiation markers (pemphigoid/pemphigus antigens), without preventing growth arrest or activation of enzymatic markers (epidermal transglutaminase) (65, 66; our unpublished observation). This partial block of calcium-induced differentiation by oncogenic *ras* was not associated with an inhibition of calcium-induced tyrosine phosphorylation. The calcium-induced modifications of GAP or p62 were not affected, nor was the general induction of tyrosine phosphorylation by calcium suppressed (our unpublished observations). Thus, an oncogenic *ras* protein appears to interfere with tyrosine phosphorylation signals in mitogenic stimulation of fibroblasts but not with those in keratinocyte differentiation. In keratinocytes, transforming *ras* might act downstream of these signals by interfering with endogenous *ras* function. The activity of normal *ras* may depend on a balance between its active and inactive states (GTP- versus GDP-bound forms), which would be disrupted by a constitutively active *ras*. Alternatively, *ras* transformation may interfere with keratinocyte differentiation by a more indirect mechanism, involving, for example, critical alterations in gene expression (4, 9).

Growth stimulation of keratinocytes with EGF caused transient phosphorylation of PLCγ and the p85 subunit of PI-3K, while neither of these proteins was phosphorylated after induction of keratinocyte differentiation. While tyrosine phosphorylation of PLCγ is well established (38, 40, 58), that of PI-3K is variably found. This enzyme is tightly associated with the PDGF receptor and, in response to PDGF stimulation, appears to be directly tyrosine phosphorylated in some cells (36), even if not in others (30). In several cell types, PI-3K associates with the EGF receptor to a much lesser extent than with the PDGF receptor (12). The observed tyrosine phosphorylation of PI-3K in EGF-treated keratinocytes raises the interesting possibility of a tight interaction of this enzyme with the EGF receptor in this particular cell type.

Our observations suggest that the increase in PLC activity observed in calcium-induced keratinocyte differentiation (31, 46, 57) involves mechanisms other than tyrosine phosphorylation. For instance, calcium (or other ion) influx in response to increased extracellular calcium might stimulate PLC directly, as calcium ionophores are capable of mimicking the effect of calcium on PI metabolism (31). Similarly, the apparent increase in PI kinase activity in differentiating keratinocytes (31, 46, 67) cannot be explained by tyrosine phosphorylation of the 85-kDa subunit of PI-3K.

No tyrosine kinase tested to date modifies GAP and p62 without affecting other signal-transducing molecules such as PLCγ and PI-3K (12). The selectivity of substrates observed during calcium-induced keratinocyte differentiation suggests that a new type of tyrosine kinase may be involved. Alternatively, keratinocytes may activate a known kinase by a novel calcium-specific mechanism. The maintenance of tyrosine phosphorylation of p62 (up to at least 24 h) also seems unusual. This effect could be due to constant stimulation of a kinase or the result of inactivation of a specific phosphatase. Identification of the enzyme(s) involved in this process will help elucidate the signal transduction mechanisms underlying keratinocyte differentiation.

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REFERENCES

1. **Alemá, S., P. Casalbore, E. Agostini, and F. Tatô.** 1985. Differentiation of PC12 phaechromocytoma cells induced by v-src oncogene. Nature (London) **316**:557–559.
2. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase C γ1, GAP, and Src to activated growth factor receptors. Science **250**:979–982.
3. **Aroian, R. V., M. Koga, J. E. Mendel, Y. Ohshima, and P. W. Sternberg.** 1990. The let-23 gene necessary for Caenorhabditis elegans vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature (London) **348**:693–699.
4. **Baichwal, V. R., A. Park, and R. Tijan.** 1991. V-src and EJ ras alleviate repression of c-jun by a cell-specific inhibitor. Nature (London) **352**:165–168.
5. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. Cell **42**:841–848.
6. **Basler, K., and E. Hafen.** 1988. Control of photoreceptor cell fate by the sevenless protein requires a functional tyrosine kinase domain. Cell **54**:299–311.
7. **Benito, M., A. Porras, A. R. Nebreda, and E. Santos.** 1991. Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes. Science **253**:565–568.
8. **Benjamin, C. W., W. G. Tarpley, and R. R. Gorman.** 1987. Loss of platelet-derived growth factor-stimulated phospholipase activity in NIH-3T3 cells expressing the EJ-ras oncogene. Proc. Natl. Acad. Sci. USA **84**:546–550.
9. **Binetruy, B., T. Smeal, and M. Karin.** 1991. Ha-ras augments c-jun activity and stimulates phosphorylation of its activation domain. Nature (London) **351**:122–127.
10. **Bollag, G., and F. McCormick.** 1991. Regulators and effectors of ras proteins. Annu. Rev. Cell Biol. **7**:601–632.
11. **Bouton, A. H., S. B. Kanner, R. R. Vines, H.-C. R. Wang, J. B. Gibbs, and J. T. Parsons.** 1991. Transformation by $pp60^{src}$ or stimulation of cells with epidermal growth factor induces the stable association of tyrosine-phosphorylated cellular proteins with GTPase-activating protein. Mol. Cell. Biol. **11**:945–953.

11a. **Brissette, J., C. Missero, S. H. Yuspa, and G. P. Dotto.** Mol. Carcinog., in press.

12. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64**:281–302.
13. **Coffey, R. J., Jr., N. J. Sipes, C. C. Bascom, R. Graves-Deal, Y. Pennington, B. E. Weissman, and H. L. Moses.** 1988. Growth modulation of mouse keratinocytes by transforming growth factors. Cancer Res. **48**:1596–1602.
14. **Courtneidge, S. A., and A. Heber.** 1987. An 81 kd protein complexed with middle T antigen and pp60c-src: a possible phosphatidylinositol kinase. Cell **50**:1031–1037.
15. **Dotto, G. P., R. A. Weinberg, and A. Ariza.** 1988. Malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus and its modulation by surrounding normal cells. Proc. Natl. Acad. Sci. USA **85**:6389–6393.
16. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343**: 377–381.
17. **Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** 1991. cDNA cloning of a novel 85kD protein that has SH2 domains and regulated binding of PI3-kinase to the PDGF β-receptor. Cell **65**:75–82.
18. **Filvaroff, E., D. 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.
19. **Fortini, M. E., M. A. Simon, and G. M. Rubin.** 1992. Signalling

by the sevenless protein tyrosine kinase is mimicked by ras1 activation. Nature (London) **355**:559–561.
20. **Frank, D. A., and A. C. Sartorelli.** 1988. Biochemical characterization of tyrosine kinase and phosphotyrosine phosphatase activities of HL-60 leukemia cells. Cancer Res. **48**:4299–4306.
21. **Gee, C. E., J. Griffin, L. Sastre, L. F. Miller, T. A. Springer, H. Piwnica-Worms, and T. M. Roberts.** 1986. Differentiation of myeloid cells is accompanied by increased levels of pp60c-src protein and kinase activity. Proc. Natl. Acad. Sci. USA **83**: 5131–5135.
22. **Glenney, J. R., L. Zokas, and M. P. Kamps.** 1988. Monoclonal antibodies to phosphotyrosine. J. Immunol. Methods **109**:277–286.
23. **Haigler, H., J. F. Ash, S. J. Singer, and S. Cohen.** 1978. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. Proc. Natl. Acad. Sci. USA **75**:3317–3321.
24. **Halenbeck, R., W. J. Crosier, R. Clark, F. McCormick, and K. Koths.** 1990. Purification, characterization, and Western blot analysis of human GTPase activating protein (GAP) from native and recombinant sources. J. Biol. Chem. **265**:21922–21928.
25. **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.
26. **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.
27. **Hennings, H., K. A. Holbrook, and S. H. Yuspa.** 1983. Potassium mediation of calcium-induced terminal differentiation of epidermal cells in culture. J. Invest. Dermatol. **81**:50s–55s.
28. **Hennings, H., F. J. Kruszewski, S. H. Yuspa, and R. W. Tucker.** 1989. Intracellular calcium alterations in response to increased external calcium in normal and neoplastic keratinocytes. Carcinogenesis **10**:777–780.
29. **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.
30. **Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger.** 1992. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. Mol. Cell. Biol. **12**:981–990.
31. **Jaken, S., and S. H. Yuspa.** 1988. Early signals for keratinocyte differentiation: role of Ca^{2+}-mediated inositol lipid metabolism in normal and neoplastic epidermal cells. Carcinogenesis **9**:1033–1038.
32. **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.
33. **Kaplan, D. R., B. L. Hempstead, D. Martin-Zanca, M. V. Chao, and L. F. Parada.** 1991. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science **252**:554–558.
34. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell **61**:125–133.
35. **Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. Cantley, and T. M. Roberts.** 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell **50**:1021–1029.
36. **Kavanaugh, W. M., A. Klippel, J. A. Escobedo, and L. T. Williams.** 1992. Modification of the 85-kilodalton subunit of phosphatidylinositol-3 kinase in platelet-derived growth factor-stimulated cells. Mol. Cell. Biol. **12**:3415–3424.
37. **Klein, R., S. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid.** 1991. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell **65**:189–197.
38. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. Cell **57**:1101–1107.
39. **McCormick, F.** 1990. The world according to GAP. Oncogene **5**:1281–1283.
40. **Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase c-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell **57**:1109–1122.
41. **Missero, C., E. Filvaroff, and G. P. Dotto.** 1991. Induction of transforming growth factor B1 resistance by the EIA oncogene requires binding to a specific set of cellular proteins. Proc. Natl. Acad. Sci. USA **88**:3489–3493.
42. **Missero, C., S. Ramon y Cajal, and G. P. Dotto.** 1991. Escape from TGF-β control and oncogene cooperation in skin tumor development. Proc. Natl. Acad. Sci. USA **88**:9613–9617.
43. **Molloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, J. B. Gibbs, and S. A. Aaronson.** 1989. PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London) **342**:711–714.
44. **Moran, M. F., C. A. Koch, D. A. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson.** 1990. Src homology domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. USA **87**:8622–8626.
45. **Moran, M. F., P. Polakis, F. McCormick, T. Pawson, and C. Ellis.** 1991. Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21-ras GTPase-activating protein. Mol. Cell. Biol. **11**:1804–1812.
46. **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**:11228–11235.
47. **Nakagawa, T., M. Mabry, A. de Bustros, J. N. Ihle, B. D. Nelkin, and S. B. Baylin.** 1987. Introduction of v-Ha-ras oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. Proc. Natl. Acad. Sci. USA **84**:5923–5927.
48. **Nishibe, S., M. I. Wahl, S. M. T. Hernandez-Sotomayor, N. K. Tonks, S. G. Rhee, and G. Carpenter.** 1990. Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science **250**:1253–1256.
49. **Noda, M., M. Ko, A. Ogura, D.-G. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. Nature (London) **318**:73–75.
50. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85kD proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI-3kinase. Cell **65**:91–104.
51. **Parries, G., R. Hoebel, and E. Racker.** 1987. Opposing effects of a ras oncogene on growth factor-stimulated phosphoinositide hydrolysis: desensitization to platelet-derived growth factor and enhanced sensitivity to bradykinin. Proc. Natl. Acad. Sci. USA **84**:2648–2652.
52. **Rake, J. B., M. A. Quiñones, and D. V. Faller.** 1991. Inhibition of platelet-derived growth factor-mediated signal transduction by transforming ras. J. Biol. Chem. **266**:5348–5352.
53. **Reiss, M., and A. C. Sartorelli.** 1987. Regulation of growth and differentiation of human keratinocytes by type β transforming growth factor and epidermal growth factor. Cancer Res. **47**: 6705–6709.
54. **Satoh, T., M. Endo, M. Nakafuku, T. Akiyama, T. Yamamoto, and Y. Kaziro.** 1990. Accumulation of p21rasGTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. Proc. Natl. Acad. Sci. USA **87**:7926–7929.
55. **Settleman, J., V. Narasimhan, L. C. Foster, and R. A. Weinberg.** 1992. Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. Cell **69**:539–549.
56. **Sultzman, L., C. Ellis, L.-L. Lin, T. Pawson, and J. Knopf.** 1991.

Platelet-derived growth factor increases the in vivo activity of phospholipase C-γ1 and phospholipase C-γ2. Mol. Cell. Biol. **11**:2018–2025.

57. **Tang, W., V. A. Ziboh, R. Isseroff, and D. Martinez.** 1988. Turnover of inositol phospholipids in cultured murine keratinocytes: possible involvement of inositol triphosphate in cellular differentiation. J. Invest. Dermatol. **90**:37–43.
58. **Wahl, M. I., N. E. Olashaw, S. Nishibe, S. G. Rhee, W. J. Pledger, and G. Carpenter.** 1989. Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase c-γ in quiescent BALB/3T3 cells. Mol. Cell. Biol. **9**:2934–2943.
59. **Weissman, B., and S. A. Aaronson.** 1985. Members of the *src* and *ras* oncogene families supplant the epidermal growth factor requirement of BALB/MK-2 keratinocytes and induce distinct alterations in their terminal differentiation program. Mol. Cell. Biol. **5**:3386–3396.
60. **Weissman, B. E., and S. A. Aaronson.** 1983. BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. Cell **32**:599–606.

60a. **Wirth, P. J., S. H. Yuspa, S. S. Thorgeirsson, and H. Hennings.** 1987. Induction of common patterns of polypeptide synthesis and phosphorylation by calcium and 12-*O*-tetradecanoylphorbol-13-acetate in mouse epidermal cell culture. Cancer Res. **47**:2831–2838.

61. **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.
62. **Yarden, U., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinases. Annu. Rev. Biochem. **57**:443–478.
63. **Yatani, A., K. Okabe, P. Polakis, R. Halenbeck, F. McCormick, and A. M. Brown.** 1990. ras p21 and GAP inhibit coupling of muscarinic receptors to atrial K+ channels. Cell **61**:769–776.
64. **Yuspa, S. H., T. Ben, H. Hennings, and U. Lichti.** 1982. Divergent responses in epidermal basal cells exposed to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. Cancer Res. **42**:2344–2349.
65. **Yuspa, S. H., A. E. Kilkenny, J. Stanley, and U. Lichti.** 1985. Keratinocytes blocked in phorbol ester-responsive early stage of terminal differentiation by sarcoma viruses. Nature (London) **314**:459–462.
66. **Yuspa, S. H., W. Vass, and E. Scolnick.** 1983. Altered growth and differentiation of cultured mouse epidermal cells infected with oncogenic retrovirus: contrasting effects of viruses and chemicals. Cancer Res. **43**:6021–6030.
67. **Ziboh, V. A., R. R. Isseroff, and R. Pandey.** 1984. Phospholipid metabolism in calcium-regulated differentiation in cultured murine keratinocytes. Biochem. Biophys. Res. Commun. **122**: 1234–1240.
68. **Zullo, J. N., and D. V. Faller.** 1988. p21 v-*ras* inhibits induction of c-*myc* and c-*fos* expression by platelet-derived growth factor. Mol. Cell. Biol. **8**:5080–5085.