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Tyrosine Phosphorylation Is an Early and Specific Event Involved in Primary Keratinocyte Differentiation

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Very little is known about early molecular events triggering epithelial cell differentiation. We have examined the possible role of tyrosine phosphorylation in this process, as observed in cultures of primary mouse keratinocytes after exposure to calcium or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Immunoblotting with phosphotyrosine-specific antibodies as well as direct phosphoamino acid analysis revealed that induction of tyrosine phosphorylation occurs as a very early and specific event in keratinocyte differentiation. Very little or no induction of tyrosine phosphorylation was observed in a keratinocyte cell line resistant to the differentiating effects of calcium. Treatment of cells with tyrosine kinase inhibitors prevented induction of tyrosine phosphorylation by calcium and TPA and interfered with the differentiative effects of these agents. These results suggest that specific activation of tyrosine kinase(s) may play an important regulatory role in keratinocyte differentiation.

Tyrosine phosphorylation provides a basic mechanism by which a variety of cellular receptors transmit their responses to mitogens or growth factors (36). Several oncogene products possess an intrinsic tyrosine kinase activity which is required for their transforming effects (6). Besides transformation, these same oncogenes are able to cause substantial alterations in the differentiation program of specialized cell types (2, 44, 45). Taken together, these findings suggest that tyrosine phosphorylation plays a major role in control of cellular proliferation and might play an equally important role in cellular differentiation. Direct biochemical evidence in support of the latter possibility, however, is only recently emerging (4).

Mouse primary keratinocytes provide an ideal system with which to study regulation of epithelial cell differentiation. Addition of calcium to these cells in culture induces a rapid and homogeneous response, triggering a terminal differentiation program which closely resembles that of differentiating keratinocytes in vivo (17, 20). Use of a primary culture system eliminates the possibility of alterations in critical regulatory events that might occur during establishment of cells in culture. In addition, keratinocyte variants are available that are resistant to the differentiating effects of calcium and that can be directly compared with their normal primary counterparts (see, for instance, references 12, 29, and 47).

A link between this differentiation program and in vivo skin carcinogenesis is provided by the fact that a differentiation response similar to that induced by calcium is also elicited in primary keratinocytes by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (48), and calcium-resistant variants are often also resistant to the effects of TPA (12, 48, 49, 51). Furthermore, a differentiation resistance phenotype in keratinocytes is often associated with acquisition of tumorigenic properties (34, 47).

This report represents an attempt to assess the possible involvement of tyrosine phosphorylation in control of keratinocyte differentiation. From our results, it appears that protein phosphorylation, as detected by phosphotyrosine-specific antibodies and phosphoamino acid analysis, occurs as a very early and specific event in calcium- and TPA-induced keratinocyte differentiation. Furthermore, results of experiments using a calcium-resistant cell line or tyrosine kinase inhibitors further support the hypothesis that tyrosine phosphorylation plays a crucial role in keratinocyte differentiation.

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MATERIALS AND METHODS

Cells. Primary keratinocytes were isolated from newborn BALB/c or 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, Mass.), and 0.05 mM $CaCl_2$ as described by Hennings et al. (20). TPA was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used in dimethyl sulfoxide solution. Genistein, purchased from ICN Biochemicals (Cleveland, Ohio), was solubilized in 100% ethanol and stored at −20°C. Herbimycin A (in ethanol) was kindly provided by H. Hanafusa. Calcium, TPA, and genistein were added directly to cells (confluent, 7 to 10 days after plating) without concomitant change of medium. In all experiments in which genistein or TPA was used, control samples were treated with solvent alone (ethanol or dimethyl sulfoxide). Spectrophotometric measurements revealed that the concentration of calcium in the medium was not affected by addition of genistein.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Samples were suspended in electrophoresis sample buffer (5 mM sodium phosphate [pH 7.0], 2% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue), incubated at 100°C for 3 min, and analyzed by electrophoresis on discontinuous 10% acrylamide–0.13% bisacrylamide gels. The gels were washed for 5 min in water and once in transfer buffer containing SDS and sodium orthovanadate (0.5 mM), and proteins were transferred to nitrocellulose by electrophoresis for 2 h at 50 V in the cold. The transfer buffer and procedures for blocking and washing the filter were as described by Kamps and Sefton (24). Blots were developed by incubation in blocking buffer containing 1 μCi of ^{125}I-protein A (35 μCi/μg; ICN) per ml. The antiphosphotyrosine antibodies used in these studies, kindly provided by M. Kamps and B. M. Sefton, are polyclonal antibodies raised

against a synthetic copolymer of phosphotyrosine, alanine, and glycine and have been shown to recognize a number of substrates for viral tyrosine kinases in immunoblots (24).

Fractionation. Cells were fractionated by the method of Burr et al. (7). Briefly, they were extracted in 100 μl of extraction buffer (1% Triton X-100, 10 mM 1,4-piperazinediethanesulfonic acid (PIPES) [pH 6.8], 100 mM KCl, 300 mM sucrose, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethanesulfonyl fluoride, aprotinin [Sigma] at 100 kallikrein inhibitor units per ml). The soluble fraction was removed from the dish and centrifuged at 12,000 × *g* for 1 min. Proteins were precipitated by addition of 1 ml of cold acetone. The pellet was dissolved in 200 μl of boiling sample buffer. Proteins left on the dish were washed once with extraction buffer and then dissolved in 400 μl of boiling sample buffer. Samples were boiled for 3 min and then analyzed by immunoblotting.

Whole-cell phosphoamino acid analysis. Cells were switched to phosphate-free medium with or without calcium for 3 h before the addition of carrier-free ^{32}P (1 mCi/ml). Genistein treatment was initiated 2 h before this and was continued throughout the experiment. After 21 h of labeling, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in phosphate-buffered RIPA (10 mM Tris hydrochloride [pH 7.4], 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 1% aprotinin, 1 mM ATP, 1 mM sodium orthovanadate). Phosphoamino acid analysis was carried out by acid hydrolysis and two-dimensional thin-layer electrophoresis as described previously (10, 40).

Elution of phosphoamino acids from thin-layer cellulose plates. Blue tips with Omnifit sinters were used to vacuum up the cellulose in the areas corresponding to the phosphoamino acids. 1.9 buffer (2.2% formic acid, 7.8% glacial acetic acid) was injected into tips with a syringe, and phosphoamino acids were eluted by spinning in a tabletop centrifuge at 1,500 rpm for 2 min. After three such washes with 1.9 buffer, 200 μl of water was added to eluate. Samples were transferred to scintillation vials and counted in 10 ml of Ecoscint.

Isolation and phosphoamino acid analysis of specific bands. Primary keratinocytes were incubated in low (0.05 mM)- or high (2 mM)-calcium medium for 20 h, switched to phosphate-free medium (at the same calcium concentration) for 30 min, and then incubated with carrier-free ^{32}P (1 mCi/ml) for 4 h. Cells were washed twice with ice-cold PBS (calcium free), lysed in phosphate-buffered TG-VO_4 (1% Triton X-100, 10% glycerol, 1% aprotinin, 100 μM sodium orthovanadate), and immunoprecipitated with normal whole serum or an antiphosphotyrosine antibody (24). ^{32}P-labeled samples were run on a polyacrylamide gel in parallel with similarly treated unlabeled samples and transferred to Immobilon filters (Millipore Corp., Bedford, Mass.). The unlabeled side of the blot was probed with antiphosphotyrosine antibodies and ^{125}I-protein A (as above) and aligned (after autoradiographic exposure) with the hot side of the gel in order to locate the ^{32}P-labeled areas corresponding to bands 1 and 2. The appropriate areas were cut from the radiolabeled samples and extracted from the membrane as described by Kamps and Sefton (25). Briefly, the excised membrane fragments were wet in methanol, washed in distilled water twice for 5 min each, hydrolyzed in HCl at 110°C for 2 h, and lyophilized overnight. Samples were run in two-dimensional thin-layer electrophoresis as described previously (10, 40).

Labeling of cells to measure ^{32}P incorporation. Genistein- and/or calcium-treated primary keratinocytes were cultured for 30 min in phosphate-free medium (with genistein, calcium, or both) and then incubated with 10 μCi of carrier-free $^{32}P_i$ (Dupont, NEN Research Products, Boston, Mass.) per ml for 5 h. Cells were washed three times with PBS, solubilized in electrophoresis sample buffer, incubated at 100°C for 3 min, and analyzed by electrophoresis on discontinuous 10% acrylamide–0.13% bisacrylamide gels.

DNA synthesis assay. Synthesis of DNA was measured by [^3H]thymidine (1 μCi/ml; Dupont, NEN) incorporation for 1 h. Cells were lysed by treatment with NaOH (0.2 M) for 10 min at 4°C, and trichoacetic acid (TCA)-precipitable counts were determined.

Electron microscopy. Cell cultures were washed in PBS and fixed in a solution of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min to 1 h at room temperature. They were washed, incubated at 0.1 M phosphate buffer for 15 to 30 min, and postfixed in a 1:1 solution of 2% osmium tetroxide and 0.2 M phosphate buffer (pH 7.4) for 30 to 40 min. Dehydration was carried out by passage through a succession of alcohols (30-50-70-95%) for 2 to 5 min each, followed by 100% ethanol for 15 to 30 min. They were then placed in a 1:1 solution of 100% alcohol-Lx resin for 1.5 h at room temperature, followed by pure Lx treatment for 1.5 h. Once embedded in Beem capsules, they were put into the oven at 65°C overnight. Thin sections were cut with an LKB Huxley Ultramicrotome, stained with saturated uranyl acetate and lead citrate, and examined with a Zeiss 10C transmission electron microscope.

Transglutaminase assay. Transglutaminase activity was assayed by measuring the incorporation of [2,3-^3H]putrescine into casein at pH 9.5 by a slight adaptation of the method of Yuspa et al. (50). Briefly, cells were lysed by freeze-thawing in PBS containing 1% Triton X-100 and 10 mM dithiothreitol and incubating at 37°C for 5 min. Transglutaminase was assayed on 50-μl samples of the lysate by adding the following: 100 μl of 100 mM sodium borate (pH 9.5), 10 μl of 100 mM $CaCl_2$, 10 μl of 10 mM EDTA, 20 μl of casein (20 mg/ml; made fresh and sonicated briefly; Calbiochem-Behring, La Jolla, Calif.), and 10 μl of [2,3-^3H]putrescine (31.1 Ci/mmol; Dupont, NEN). Sample and blanks (containing the PBS–Triton X-100–dithiothreitol solution only) were incubated for 20 min at 37°C, and 80-μl samples were spotted on Whatman 3MM disks and immediately immersed in ice-cold 10% TCA. Filter papers were gently agitated through three TCA washes for 10 min each, rinsed with ice-cold absolute ethanol, and dried briefly. Radioactivity bound to casein that precipitated on filter paper was counted for 10 min. Background radioactivity from controls (containing no cell lysate) was subtracted from all samples. Protein concentration was determined by a colorimetric protein assay (BioRad Laboratories, Richmond, Calif.).

RESULTS

Pattern of tyrosine phosphorylation in differentiating keratinocytes as detected by immunoblotting. If the mechanism whereby extracellular calcium initiates keratinocyte differentiation involves tyrosine phosphorylation, the patterns and/or degrees of protein phosphorylation in calcium-stimulated versus unstimulated cells should be different. Moreover, these modifications should occur early in the sequence of events induced by calcium. To examine this possibility, we compared the pattern of tyrosine phosphorylation in total extracts from growing and differentiating primary keratinocytes by immunoblotting with specific antibodies to phosphotyrosine. Use of these antibodies provides a very sensitive method to detect intracellular changes in tyrosine

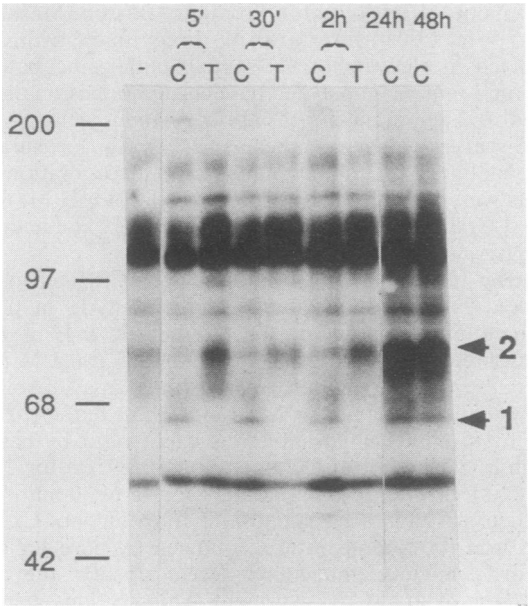

FIG. 1. Effects of calcium and TPA on tyrosine phosphorylation in primary keratinocytes. Calcium (2 mM) or TPA (100 ng/ml) was added to primary keratinocyte cultures for various amounts of time. Whole-cell extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antiphosphotyrosine antibodies (24, 39) as described in Materials and Methods. Extracts were from untreated cells (-) or from cells treated with calcium (C) or TPA (T) for the indicated amounts of time. Preincubation of the antiphosphotyrosine antibodies with phosphotyrosine, but not with tyrosine, significantly inhibited immunorecognition of bands 1 and 2 (data not shown). These bands were also recognized by antiphosphotyrosine antibodies raised against a different immunogen (9; data not shown).

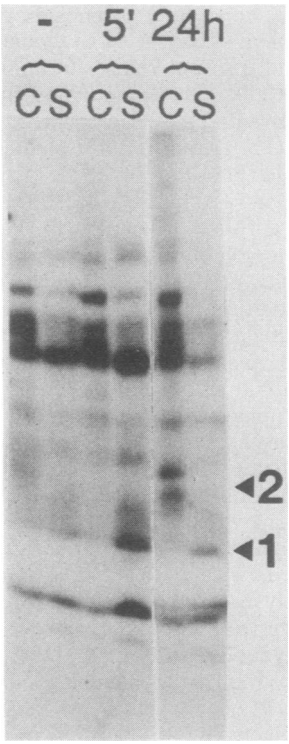

FIG. 2. Cellular localization of calcium-specific tyrosine-phosphorylated proteins. Cells were fractionated by the method of Burr et al. (7) as described in Materials and Methods. Shown is an immunoblot of extracts from untreated cells (-) or cells exposed to calcium for 5 min (5') or 24 h (24h). Lanes: S, supernatant (soluble fraction); C, cytoskeleton (detergent-insoluble fraction).

phosphorylation in response to a variety of agents (see, for instance, references 9, 24, 27, and 39).

Cell extracts were prepared from cells growing in medium at low calcium concentrations (0.05 mM; low-calcium medium) or cells induced to differentiate by addition of calcium (2 mM; high-calcium medium) for various amounts of time. In low-calcium medium, several discrete bands appeared after prolonged autoradiographic exposure of the immunoblots (1 week) (Fig. 1). A similar number of bands could be seen on blots of unstimulated fibroblasts exposed for the same lengths of time (data not shown).

In addition to these basal bands, one band with an approximate molecular mass of 65 kilodaltons (kDa) was specifically found in calcium-treated cells (Fig. 1, lanes C) but was completely absent in cells maintained in low-calcium medium (Fig. 1, lane -). This band (labeled 1 in Fig. 1) will be referred to as band 1. Unlike many of the changes induced by calcium, such as decreased DNA synthesis, cornified envelope formation, and sloughing of cells, which occur after many hours (20), phosphorylation of band 1 occurred within 5 min of calcium addition (Fig. 1) and thus appeared to be one of the very early effects of calcium. In addition, even after >90% inhibition of protein synthesis by cycloheximide treatment, band 1 quickly appeared in response to calcium (data not shown).

After 24 h in high-calcium medium, it was possible to detect a strong increase in intensity of a second, broad band of 80 to 85 kDa that in some experiments ran as a doublet. This band (labeled 2 in Fig. 1) will be referred to as band 2. Band 2, unlike band 1, was detectable with lower intensity in cells cultured in low-calcium medium (Fig. 1). The two bands differed in their approximate subcellular location, band 2 being found in a cytoskeletal, Triton X-100-insoluble fraction (5, 7), whereas band 1 was located primarily in the soluble fraction (Fig. 2). Appearance of band 1 could be reversed after cultivation of cells in high-calcium medium for 24 h, followed by a switch to lower-calcium conditions for another 24 h, whereas induction of band 2 could not be reversed under similar conditions (data not shown).

Calcium and TPA might induce keratinocyte differentiation through related pathways. If so, the patterns of such protein modifications after exposure to these two agents might be similar. At 5 to 30 min after addition of TPA, there was induction of a band migrating at the same position as band 2; band 1, on the other hand, was not detected at any time point after TPA treatment (Fig. 1).

If these protein modifications are directly involved in keratinocyte differentiation, then a calcium-resistant cell line might be expected not to show such changes. One such cell line, Pam 212, was derived from keratinocytes that underwent spontaneous malignant transformation in culture (47). Unlike primary keratinocytes, which undergo a complete arrest of DNA synthesis after switch from low (0.05 mM)- to high (2 mM)-calcium medium, Pam 212 cells show a 10-fold increase in DNA synthesis after this switch (data not shown). Under these conditions, there was little or no induction of band 1 phosphorylation and no induction of band 2 (Fig. 3). Band 2 phosphorylation was, however, readily increased after treatment with TPA (Fig. 3), in

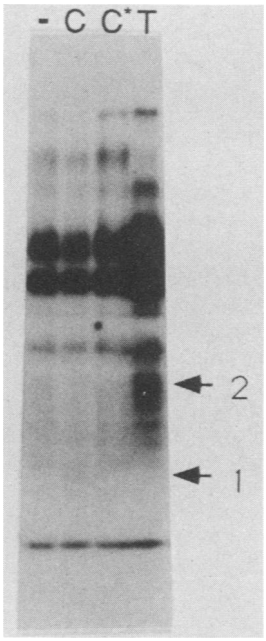

FIG. 3. Tyrosine phosphorylation in Pam 212 cells, a calcium-resistant cell line, in response to calcium or TPA. Total-protein extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antiphosphotyrosine antibodies (24, 39) as described in Materials and Methods. The weak induction of band 1 seen here at 1 h and not at 24 h was not consistently found in other experiments. Extracts were from untreated cells (-), and from cells exposed to calcium (2 mM) for 1 (C) and 24 (C*) h or treated with TPA (100 ng/ml) for 1 h (T).

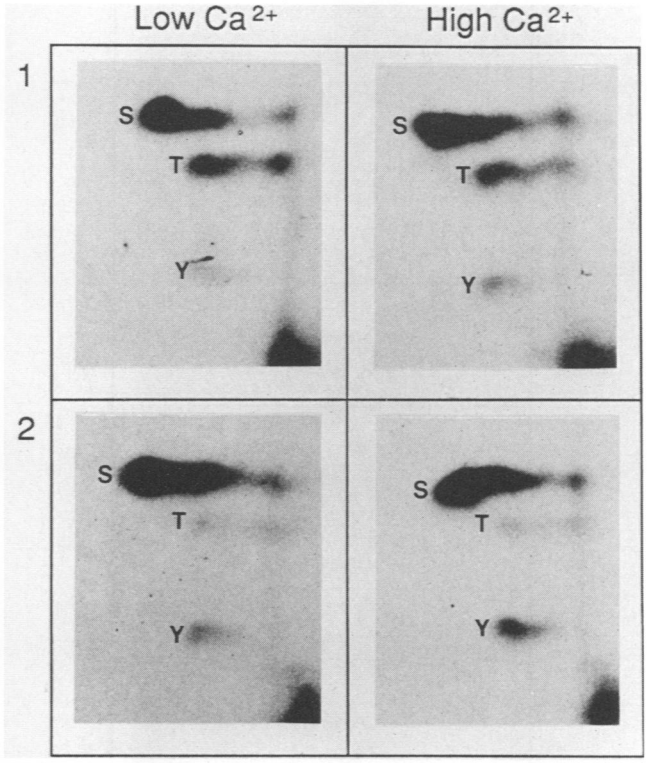

FIG. 4. Effects of calcium on phosphoamino acid composition of proteins comigrating with bands 1 and 2. Cells were labeled with $^{32}P_i$ for 4 h before immunoprecipitation with antiphosphotyrosine antibodies as described in Materials and Methods. After gel electrophoresis and blotting onto an Immobilon membrane, the areas of the filter corresponding to bands 1 and 2 (located as described in Materials and Methods) were cut, and the proteins were eluted, hydrolyzed, and resolved by two-dimensional electrophoresis on thin-layer cellulose plates. Electrophoresis in the first dimension was at pH 1.9 (anode at the left), and that in the second dimension was at pH 3.5 (anode at the top). Nonlabeled phosphoamino acid standards mixed in with the samples were located by ninhydrin staining. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Thin-layer plates were exposed to preflashed film (Kodak X-Omat R) for 14 days at −70°C, using an intensifying screen. Shown are phosphoamino acid analyses of the areas of the filter corresponding to band 1 (row 1) or band 2 (row 2) from cells kept in low (0.05 mM)- or high (2 mM)-calcium medium for 24 h.

agreement with the fact that Pam 212 cells are still pharmacologically responsive to this agent (47).

We conclude that calcium-induced differentiation of keratinocytes is associated with induction of two specific bands detected by immunoblotting with antiphosphotyrosine antibodies. The biochemical and positional properties of the corresponding proteins may be very different. Phosphorylation of a protein comigrating with band 2 is triggered in response to TPA, though with different kinetics. Finally, calcium-induced phosphorylation of band 1 and 2 is minimal or absent in the calcium-resistant Pam 212 cells.

Pattern of tyrosine phosphorylation in differentiating keratinocytes as detected by phosphoamino acid analysis. Since antiphosphotyrosine antibodies can cross-react with other epitopes, we attempted to measure directly the level of tyrosine phosphorylation in protein(s) comigrating with bands 1 and 2. Cultures of cells in low- or high-calcium medium were labeled with $^{32}P_i$ for the last 4 h of the 24-h incubation. After immunoprecipitation with antiphosphotyrosine antibodies, gel electrophoresis, and blotting onto an Immobilon membrane, the areas of the filter corresponding to bands 1 and 2 (located as described in Materials and Methods) were cut and eluted for phosphoamino acid analysis (25).

Very little phosphotyrosine could be found in the region of the filter corresponding to band 1 in extracts of cells incubated in low-calcium medium, whereas some phosphotyrosine was present in the area corresponding to band 2 (Fig. 4). This finding is consistent with the fact that band 2, but not band 1, could already be detected by antiphosphotyrosine antibodies under low-calcium conditions. With extracts in high-calcium medium, there was a significant increase in phosphotyrosine content in both band 1 and band 2 areas (Fig. 4), confirming the results obtained by Western blotting (immunoblotting).

Although Western blotting showed only two bands that were phosphorylated specifically in response to calcium, other proteins might become phosphorylated on tyrosines as well. The extent of total tyrosine phosphorylation in growing versus differentiating keratinocytes was therefore determined directly by phosphoamino acid analysis. As expected, cells maintained in low-calcium medium contained very little phosphotyrosine (0.07% of total phosphoamino acids in one experiment and 0.02% in a second one; Fig. 5A). After addition of calcium for 24 h, the relative proportion of phosphotyrosines was significantly increased (0.16% of total phosphoamino acids in experiment 1 and 0.37% in experiment 2; Fig. 5B). If bands 1 and 2 were the only proteins to become tyrosine phosphorylated in response to calcium, the percentage of total phosphotyrosine would not be likely to change significantly. Thus, our results suggest that proteins

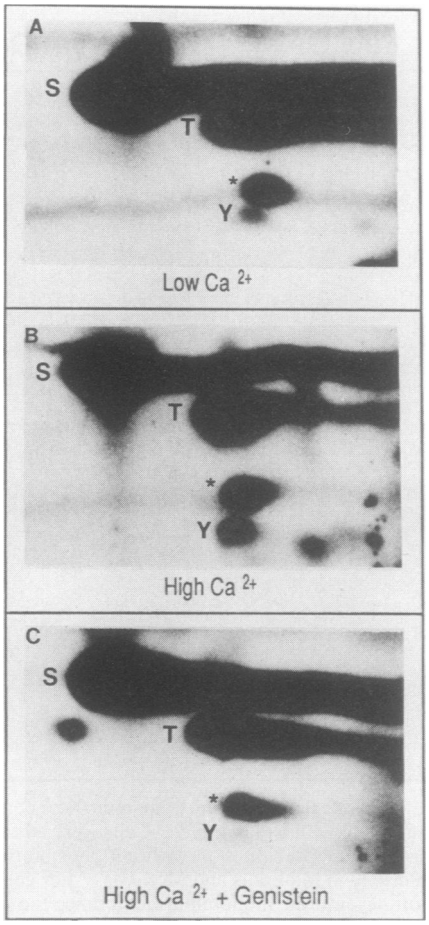

FIG. 5. Effects of calcium on whole-cell phosphoamino acid composition. Cells in low-calcium medium or exposed to calcium or calcium and genistein for 24 h were labeled and processed as described in Materials and Methods. Phosphoamino acid analysis was carried out by acid hydrolysis and two-dimensional thin-layer electrophoresis as described for Fig. 4. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine; *, an unidentified compound usually detected in whole-cell phosphoamino acid analyses (10).

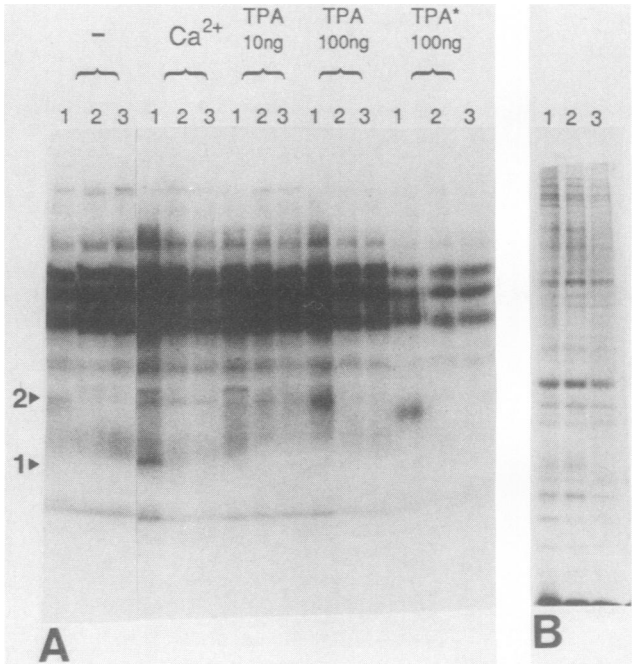

FIG. 6. Effects of genistein on calcium- and TPA-induced phosphorylation. (A) Tyrosine phosphorylation. Primary keratinocytes were incubated with genistein (30 or 60 μg/ml) for 2 h before addition of calcium (2 mM for 24 h) or TPA (10 ng/ml for 24 h, 100 ng/ml for 1 h, or 100 ng/ml for 24 h [TPA*]). Shown are immunoblots of extracts from untreated cells (lanes 1) or cells pretreated with genistein at 30 (lanes 2) or 60 (lanes 3) μg/ml. (B) Total protein phosphorylation. Keratinocytes switched to high-calcium medium for 24 h in the absence (lane 1) or presence of genistein at 30 (lane 2) or 60 (lane 3) μg/ml were labeled in vivo with 10 μCi of $^{32}P_i$ per ml for 5 h. As in all other experiments, genistein was added to cells 2 h before calcium addition. Total-cell extracts were normalized for protein content and analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. In a separate experiment, TCA precipitation of equal amounts of proteins showed that genistein had no effect on total ^{32}P incorporation even at 60 μg/ml (data not shown).

corresponding to bands 1 and 2 belong to a larger group of proteins phosphorylated on tyrosine in keratinocytes in response to calcium.

Concomitant inhibition of tyrosine kinase activation and keratinocyte differentiation. One question raised by our results is whether specific activation of tyrosine kinase(s) is merely coincidental with the keratinocyte response to calcium and TPA or is an important event that is required for this process to occur. One way to address this problem would be to use specific tyrosine kinase inhibitors and evaluate their biological effects. The isoflavone genistein was chosen for most of these studies because it was shown to specifically block a number of tyrosine kinases (1, 31).

Exposure of keratinocytes to genistein 2 h before (and during) calcium or TPA exposure was sufficient to drastically or totally inhibit induction of band 1 and 2 phosphorylation (Fig. 6A). The calcium- and TPA-induced tyrosine phosphorylations were particularly sensitive to the inhibitor, as effects on the background bands were significantly less.

To verify that genistein was a specific inhibitor of tyrosine phosphorylation under our experimental conditions, cells in high-calcium medium, with or without genistein, were metabolically labeled with $^{32}P_i$, and the total pattern of protein phosphorylation was examined by gel electrophoresis. Under these conditions, almost all ^{32}P would be esterified to serine or threonine residues, and only a negligible fraction would be esterified to tyrosines (10). Genistein treatment had no effect on the total pattern of protein phosphorylation of differentiating keratinocytes (Fig. 6B). In addition, whole-cell phosphoamino acid analysis showed that cells treated with this compound and exposed to high-calcium medium for 24 h (Fig. 5C) had phosphotyrosine levels comparable to those of cells kept in low-calcium medium (Fig. 5A.). Thus, genistein was able to block the calcium-induced increase in phosphotyrosines of many proteins, including bands 1 and 2.

In parallel with its specific effects on tyrosine phosphorylation, genistein interfered substantially with several parameters of differentiation as induced by calcium or TPA. Calcium addition to genistein-treated keratinocytes resulted in morphological changes rather different from those induced in untreated controls (Fig. 7) but remarkably similar to those observed with calcium-resistant cell lines, such as Pam 212 (not shown). In fact, cells exposed to genistein and calcium (Fig. 7d) did not lose their cellular borders, nor did they stratify as did cells exposed only to calcium (Fig. 7c). Electron microscopic analysis of the latter cells revealed a

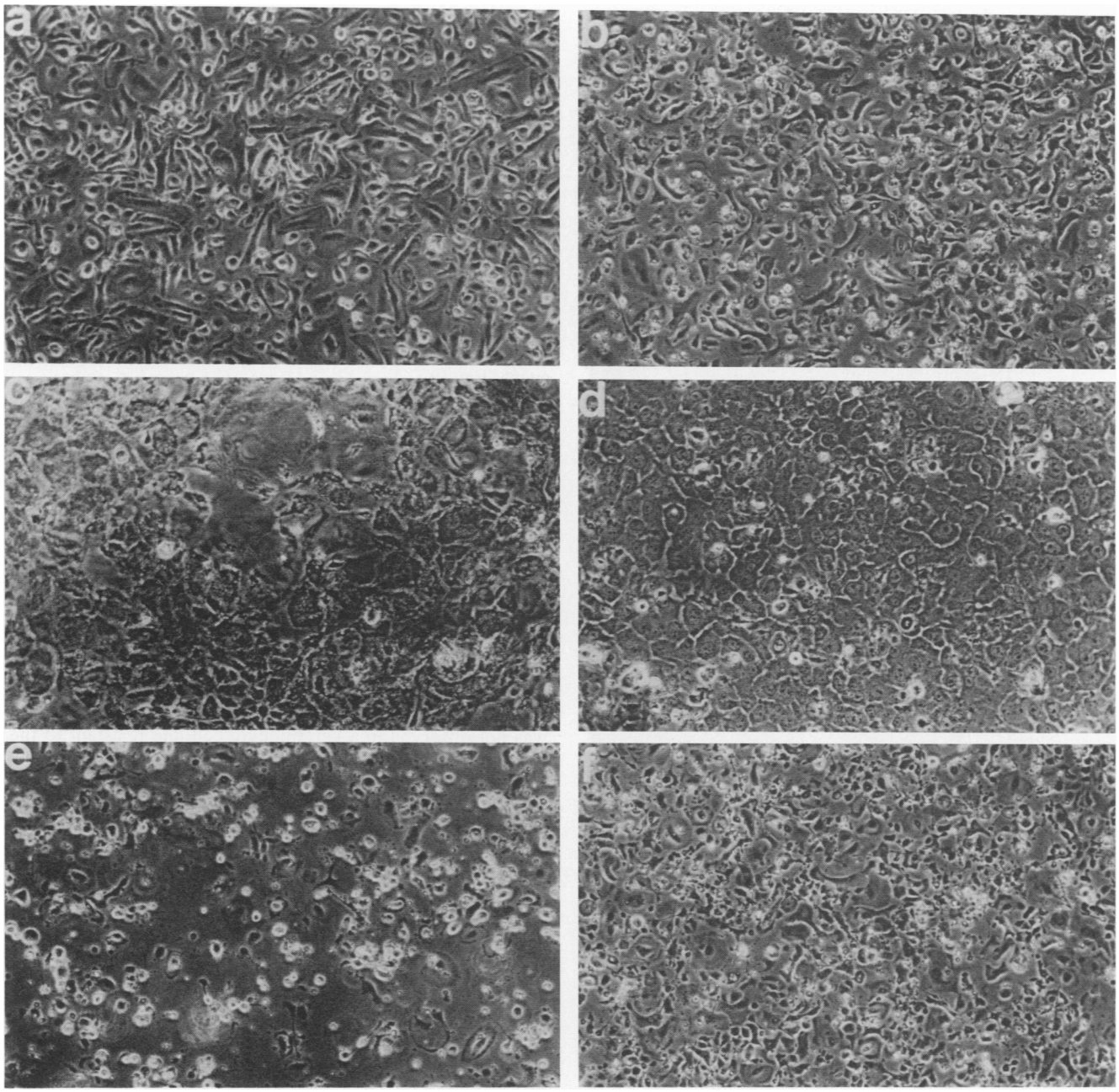

FIG. 7. Effects of genistein on keratinocyte morphological differentiation. Shown are phase-contrast photographs of keratinocytes in low-calcium (0.05 mM) medium (a), cells in low-calcium medium with genistein (30 μg/ml) for 24 h (b), cells in high-calcium (2 mM) medium for 24 h (c), cells preincubated with genistein (30 μg/ml) for 2 h before addition of calcium for 24 h (d), cells in low-calcium medium with TPA (100 ng/ml) for 24 h (e), and cells preincubated with genistein (30 μg/ml) for 2 h before addition of TPA (100 ng/ml) for 24 h (f). Magnification, ×95.

large number of well-formed desmosomes and keratin bundles directed toward the cell membrane (Fig. 8A). In contrast, cells pretreated with genistein before calcium addition showed large intercellular spaces with mostly abortive desmosome formation and no organization of keratin filaments (Fig. 8B). Similar results were obtained by immunofluorescence of cells with pemphigus antiserum which recognizes a calcium-inducible desmosomal component (37, 38). Intense membrane staining was detected in calcium-treated keratinocytes, whereas only diffuse staining, comparable to that of the low-calcium control, was detectable in calcium- and genistein-treated cells (data not shown).

TPA induced similar morphological changes in both genistein-treated (Fig. 7f) and control (Fig. 7e) keratinocytes. In the latter case, however, a large fraction of keratinocytes detached from the dish after 24 h of exposure to TPA (100 ng/ml) (Fig. 7e), whereas the TPA- and genistein-treated cells still remained attached as an intact monolayer (Fig. 7f). Direct counting of cells or of radioactivity, after labeling with [^{3}H]thymidine for 24 h before the TPA and genistein

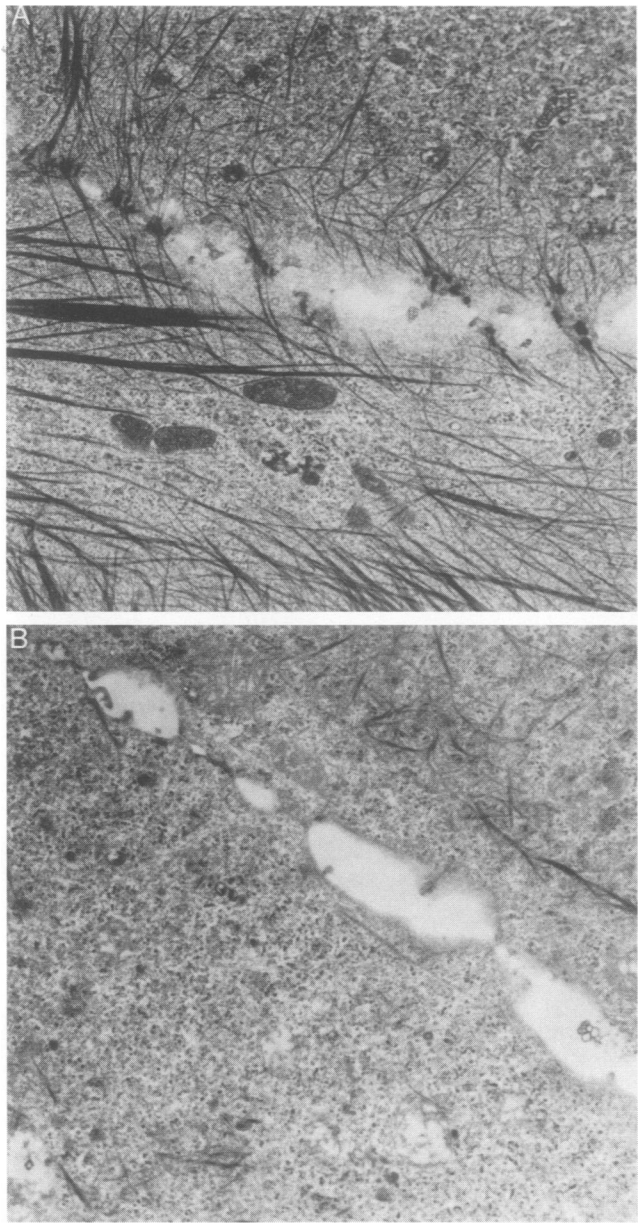

FIG. 8. Effects of genistein on calcium-induced ultrastructural changes. Shown are electron micrographs of keratinocytes in high-calcium medium for 24 h (a) or pretreated with genistein (30 μg/ml) for 2 h before addition of calcium for 24 h (b). Cells were fixed and processed for electron microscopic analysis as described in Materials and Methods. Note the close association of adjacent cells within the cell layer, abundant keratin filaments, and complete desmosome formation in panel a and the wide intercellular spaces, lack of keratin filament organization, and incomplete desmosome formation in panel b. Magnification, ×6,765.

treatment, confirmed that TPA caused a >50% detachment of cells from the dish and that this effect could be totally blocked by pretreatment with genistein.

Epidermal transglutaminase is an important biochemical marker of keratinocyte differentiation (21, 41), which is normally induced by calcium and TPA under both complete and minimal medium conditions (Fig. 9). Genistein substantially reduced calcium- or TPA-mediated transglutaminase induction under conditions in which protein synthesis remained totally unaffected (Fig. 9).

The compound herbimycin A was used as a second, unrelated tyrosine kinase inhibitor (32, 42, 43) to confirm the results obtained with genistein. Herbimycin A was able to block induction of band 1 and band 2 phosphorylation by calcium or TPA in a manner analogous to that of genistein at a concentration (0.5 μg/ml) that did not affect total protein phosphorylation (data not shown). In parallel with these effects, treatment of keratinocytes with this inhibitor was sufficient to protect these cells from the effects of TPA. Herbimycin A, like genistein, was able to block the TPA-induced detachment of cells (>50% after 24 h of exposure to TPA [100 ng/ml]). Transglutaminase induction by TPA was also blocked by the herbimycin A pretreatment (Fig. 9). In contrast, herbimycin A pretreatment had little effect on the morphological changes induced by calcium (data not shown) and was not able to block transglutaminase induction by this agent (Fig. 9).

Thus, there is a strong correlation between the ability of genistein to interfere with some important aspects of calcium- and TPA-induced differentiation of keratinocytes in culture and its inhibition of tyrosine phosphorylation. Herbimycin A was also able to block some specific tyrosine phosphorylation events (band 1 and 2 induction) and protected keratinocytes from some of the differentiating effects of TPA but not calcium.

DISCUSSION

Induction of tyrosine phosphorylation in differentiating keratinocytes was detected by immunoblotting with antiphosphotyrosine antibodies as well as by direct phosphoamino acid analysis. Increased tyrosine phosphorylation was not observed in a keratinocyte cell line resistant to the differentiating effects of calcium, whereas the differentiative response of normal keratinocytes was substantially altered by a tyrosine kinase-specific inhibitor. These results demonstrate that induction of tyrosine phosphorylation occurs as an early and specific event in keratinocyte differentiation as induced by calcium and TPA and might be required for this process to occur.

Modulation of tyrosine phosphorylation, which is clearly involved in control of cell growth (36), may also help regulate cell differentiation. This conclusion would be consistent with the finding that morphological differentiation of the neuronal PC12 cell line by nerve growth factor is accompanied by rapid and transient tyrosine phosphorylation (27), and similar differentiation can be induced by introduction of the v-*src* oncogene into these cells (2). In HL-60 leukemia cells, induction of differentiation is associated with modulation of total tyrosine phosphorylation (13) and increased c-*src* tyrosine kinase activity (3). Inhibition of tyrosine kinases can also result in induction of differentiation in mouse embryonal carcinoma (F9) and erythroleukemia (MEL) cells (26) and in K562 human leukemic cells (22).

Only two specific bands (bands 1 and 2) were detected by immunoblotting of keratinocyte extracts after induction of differentiation by calcium or TPA. Wirth et al. have characterized total protein phosphorylations after in vitro or in vivo treatment of keratinocytes with calcium or TPA (46). In their hands, phosphorylation of an 85-kDa protein was increased five- to sixfold by both calcium and TPA. Although its phosphorylation sites were not determined, this protein might correspond to our band 2. Similarly, other tyrosine kinase substrates have been described which might correspond to our proteins based on their similar molecular weights. In particular, ezrin, an 80-kDa component of mi-

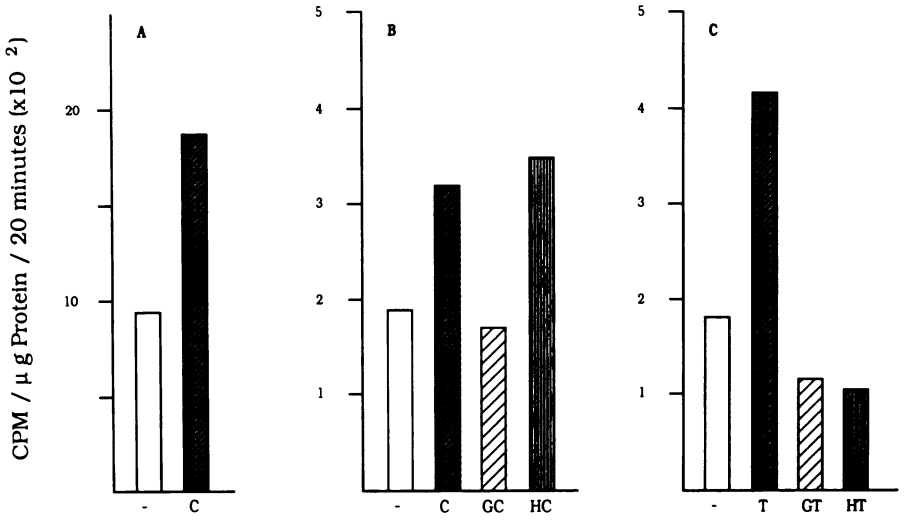

FIG. 9. Induction of transglutaminase activity as a marker of keratinocyte differentiation. Transglutaminase activity was assayed by measuring the incorporation of [2,3-^{3}H]putrescine into casein at pH 9.5 by a slight adaptation of the method of Yuspa et al. (50) as described in Materials and Methods. All samples were done in duplicate, and background radioactivity from controls (containing no cell lysate) was subtracted from all samples. Values shown are the averages of duplicate samples with a standard deviation of less than 10%. Similar results were obtained in at least two other independent experiments. In experiments in which inhibitors were used, controls (low or high calcium) were also treated with solvent (ethanol, 3 μl/ml). (A) Cells in minimal medium (without EGF or serum) at a low calcium concentration (0.05 mM) (-) for 48 h or after addition of calcium (2 mM) (C) for the last 24 h. (B) Cells in low (-)- or high (C)-calcium medium for 24 h compared with cells pretreated with genistein (GC) or herbimycin A (HC) for 2 h before addition of calcium for 24 h. (C) Cells in low-calcium medium (-) or exposed to TPA (100 ng/ml) for 24 h (T) compared with cells pretreated with genistein (GT) or herbimycin A (HT) for 2 h before addition of TPA for 24 h. Pretreatment with genistein or herbimycin A did not affect protein synthesis (as measured by [^{35}S]methionine incorporation) but did decrease DNA synthesis (as measured by [^{3}H]thymidine uptake) to ~10%. A similar decrease occurred in cells cultured in EGF–serum-free medium for 24 h, in which the differentiative response to calcium seemed to be intact (panel A).

crovillar core proteins phosphorylated on tyrosine (15), could be related to band 2 protein(s). This possibility would be also consistent with the apparent association of the band 2 protein with the cytoskeleton.

Bands 1 and 2 likely represent only a subset of several proteins phosphorylated on tyrosines after induction of keratinocyte differentiation. This view is suggested by the substantial increase in total tyrosine phosphorylation in differentiating keratinocytes versus their growing counterparts. The fact that only two specific bands were detected by blotting with antiphosphotyrosine antibodies could be explained by low-level phosphorylation of other proteins, not detectable by immunologic techniques, or by phosphorylation of proteins comigrating with those already detectable in cells under growing conditions.

Relative to other potential substrates, the significance of band 1 and 2 phosphorylation in response to calcium and TPA is not clear. In fact, two independent tyrosine kinase inhibitors (genistein and herbimycin A) could block induction of both bands, and this effect correlated with a block of the keratinocyte response to TPA (Table 1). Calcium-induced differentiation, however, seemed to be affected by only one of the two inhibitors (genistein). These different biological effects could be due to different enzyme and substrate specificities of the two inhibitors. It has been shown, for instance, that the sensitivity of several known tyrosine kinases to one tyrosine-specific inhibitor, a synthetic hydroxycinnamamide derivative, varies greatly (35). In addition, the biochemical mechanism by which herbimycin A is able to block tyrosine kinases in vivo, but not in vitro (43), is not understood. Genistein has been shown to specifically block the in vitro tyrosine kinase activity of the EGF receptor, pp60$^{v\text{-}src}$, and pp110*gag-fes* with no effects on serine- and threonine-specific protein kinases, such as cyclic AMP-dependent protein kinase, phosphorylase kinase, and protein kinase C (1). We have shown here that even in vivo genistein did not change the total pattern of protein phosphorylation (mostly serine and threonine) but did specifically inhibit the tyrosine phosphorylation events observed after induction of keratinocyte differentiation. It is possible, however, that even in this case some of the biological effects of the inhibitor are due to interference with biochemical pathways other than tyrosine phosphorylation (C. Linassier, M. Pierre, J.-B. Le Peco, and J. Pierre, Biochem. Pharmacol., in press). Genistein and herbimycin A could also interfere with keratinocyte differentiation indirectly, through inhibition of DNA synthesis. We note, however, that cultivation of keratinocytes in minimal medium resulted in a similar block of DNA synthesis, without interference with other parameters of differentiation (Fig. 9A). Keratinocyte proliferation, but not differentiation, has also been reported to be blocked after exposure to transforming growth factor β (8, 33).

It would be important to establish the nature of the

TABLE 1. Biochemical and biological effects of tyrosine kinase inhibitors

Induction of:	Induction[a] in cells exposed to:					
	Ca^{2+}			TPA		
	Con	G	H	Con	G	H
Tyrosine phosphorylation						
Band 1	+	−	−	−	−	−
Band 2	+	−	−	+	−	−
Differentiation	+	−	+	+	−	−

[a] Measured in untreated control cells (Con) and cells treated with genistein (G) or herbimycin A (H).

tyrosine kinase(s) that is activated during keratinocyte differentiation by calcium or TPA. Specific phosphorylation of bands 1 and 2, detected on immunoblots after exposure to calcium or TPA, occurred in the apparent absence of growth factors (data not shown). In addition, EGF treatment of keratinocytes did not stimulate phosphorylation of band 1 or band 2 (data not shown). This finding raises the interesting possibility that a direct increase in intracellular calcium concentrations, or some changes in other ion concentrations concomitant with the calcium influx (18, 19), might activate these kinases. Calcium-calmodulin-dependent kinases, including one of the tyrosine kinase category (28), have been described (16). Alternatively, calcium influx might stimulate these tyrosine kinases through primary interaction with some other cellular enzyme. It has been reported, for instance, that calcium-induced differentiation is accompanied by induction of phospholipase C activity (23) and increased phosphoinositide metabolism (30), which could then trigger a cascade of events leading to activation of a tyrosine kinase. Protein kinase C might also be involved in this process, as suggested by the ability of TPA to stimulate tyrosine phosphorylation (11, 14). As shown here, however, TPA induces a pattern of tyrosine phosphorylation only partially similar to that elicited by calcium (phosphorylation of a protein[s] comigrating with band 2 but not band 1) and with substantially different kinetics. In addition, use of a calcium-resistant keratinocyte line revealed a specific loss of band 1 and 2 phosphorylation in response to calcium but not TPA, suggesting that the responses to the two agents can be dissociated genetically as well as biochemically.

Very little is known about early molecular events triggering epithelial cell differentiation. The results presented here indicate that tyrosine phosphorylation might have an important role in regulation of keratinocyte differentiation as induced by calcium and TPA. The potential relevance of these findings for differentiation of these cells in vivo could be assessed by direct inhibition of tyrosine phosphorylation in intact skin.

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