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The Tumor Promoter 12-*O*-Tetradecanoylphorbol-13-Acetate and the *ras* Oncogene Modulate Expression and Phosphorylation of Gap Junction Proteins

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Gap junctional intercellular communication is inhibited in response to tumor promoters and oncogene transformation, suggesting that loss of this function is an important step in tumor formation. To elucidate the molecular mechanisms responsible for this inhibition, we examined the expression of gap junction proteins and mRNA in mouse primary keratinocytes after treatment with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and/or *ras* transformation. During normal cell growth, keratinocytes express the α_1 (connexin 43) and β_2 (connexin 26) proteins. Within 5 min of TPA treatment, the α_1 protein became rapidly phosphorylated on serine residues and its expression was dramatically reduced by 24 h. The β_2 protein, after an initial increase in expression, was also significantly reduced 24 h after treatment with TPA. *ras* transformation caused changes similar to those induced by TPA. The α_1 protein underwent an increase in serine phosphorylation, although its expression declined only slightly, while β_2 expression was greatly reduced. The effects of TPA and *ras* on α_1 expression were additive; treatment of *ras*-transformed cells with TPA resulted in increased α_1 phosphorylation, with greatly decreased protein levels, much lower than those generated by either agent alone. These data provide a likely explanation for the similar and synergistic inhibition of gap junctional intercellular communication by phorbol esters and *ras*.

Gap junctions are transmembrane channels that directly link the cytoplasms of adjacent cells (3, 21, 35). While gap junctions from different tissues are not identical, they share a basic structural organization. Six protein subunits, termed connexins, form a hemichannel or connexon in the plasma membrane of one cell, and connexons from adjacent cells then pair to generate a complete channel. Gap junctional intercellular communication (GJIC) mediates transfer of ions and low-molecular-weight metabolites (<1,000 daltons; 35) which are thought to regulate growth and differentiation, as well as maintain normal tissue homeostasis (34, 42). Antibodies to gap junction proteins were found to interfere with development when injected into amphibian embryos (51).

Many lines of evidence suggest that GJIC plays a role in tumor formation. Tumor promoters have been shown to inhibit GJIC both in vitro (37, 54) and in vivo (26, 27), and the ability to communicate is lost in many types of tumor cells (53). Transformation of cells by the *src*, *ras*, *myc*, and *neu* oncogenes (2, 5, 7, 12, 16) also leads to suppression of GJIC, showing that a decrease in GJIC is a common result of oncogene activation.

Several connexins from different tissues have been cloned in recent years (4, 20, 24, 31, 41, 58), yielding much information concerning the structure, membrane topology, and tissue distribution of the gap junction proteins. However, relatively little is understood about the molecular mechanisms that control GJIC in both normal and transformed cells. Modulation of junctional communication by the *src* oncogene and retinoic acid was shown to be associated with posttranslational modifications of the gap junction proteins (11, 44, 49). Retinoic acid treatment results in increases in both GJIC and phosphorylation of α_1 (connexin 43; 44), while inhibition of GJIC by *src* correlates with tyrosine phosphorylation of α_1 (11, 18, 49).

Mouse primary keratinocytes provide an ideal system for studying the role of GJIC in carcinogenesis. Cells of primary origin have not yet undergone the many changes which occur during establishment in culture and are amenable to the study of transformation in vitro, in vivo, and after transplantation onto an animal (see, for instance, references 6, 15, 23, 36a, 45, and 56). Gap junctional communication in this system is dramatically reduced after treatment with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or transformation with the *ras* oncogene (12). Furthermore, treatment of *ras*-transformed primary keratinocytes with TPA completely abolishes GJIC (12). The similar and synergistic effects of *ras* and TPA on GJIC suggest that inhibition of junctional communication represents an initial step in keratinocyte transformation.

In this study, the molecular mechanisms by which TPA and *ras* regulate GJIC in mouse primary keratinocytes were investigated. These agents affected both synthesis and modification of the gap junction proteins. Inhibition of GJIC by TPA or the *ras* oncogene correlated with both phosphorylation of α_1 (connexin 43) and a decline in α_1 and β_2 (connexin 26) expression. The effects of TPA and *ras* on α_1 expression were additive; TPA treatment of *ras*-transformed cells resulted in enhanced α_1 phosphorylation and marked reduction of its protein, to a level much lower than that generated by either agent alone. Inhibition of GJIC by *ras* or TPA in other cell types may occur through similar effects on the production and processing of gap junction proteins.

MATERIALS AND METHODS

Cells and viruses. Primary keratinocytes isolated from newborn Sencar mice were cultivated in low-calcium (0.05

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mM) minimal essential medium supplemented with 4% Chelex-treated fetal calf serum and epidermal growth factor (10 ng/ml; Collaborative Research, Inc., Cambridge, Mass.) as previously described (23). Cells were infected with either Moloney leukemia virus (MoLV) or Harvey sarcoma virus (HaSV) 2 days after plating, as previously described (15), and used 1 week after infection. TPA (Chemsyn Science Laboratories, Lenexa, Kans.) was dissolved and stored in dimethyl sulfoxide (100 μg/ml).

RNase protection assays. Total RNA was isolated as previously described (9). Plasmids pBSmα_1 and pBSmβ_2 were used to produce antisense probes to the α_1 and β_2 connexin mRNAs. pBSmα_1 consists of the 2.2-kb *Eco*RI fragment of the mouse α_1 cDNA (40) subcloned into the *Eco*RI site of pBS(KS$^+$) (Stratagene, LaJolla, Calif.). This construct was digested with *Cla*I to generate an 855-base antisense probe. pBSmβ_2 contains the 0.8-kb *Eco*RI fragment of the mouse β_2 cDNA (40) ligated into the pBS(KS$^+$) *Eco*RI site. This plasmid was linearized with *Bgl*II to create the 559-base β_2 antisense probe. Single-stranded antisense riboprobes were labelled with [α-^{32}P]UTP (400 Ci/mmol; Amersham) by using T7 RNA polymerase. Samples containing equal amounts of RNA (10 μg, as determined by spectrophotometry and ethidium-stained agarose gels) were hybridized to the ^{32}P-labelled riboprobes and added in excess to the gap junction transcripts, and RNase protection assays were performed as previously described (1). Protected RNA-RNA hybrids were resolved on 6% polyacrylamide gels containing 7 M urea. This type of analysis yields multiple, specific, RNase-resistant fragments. These fragments are likely to result from limited degradation at the 3′ ends or intramolecular RNase digestion at AT-rich regions of the RNA-RNA hybrids (1).

Immunoblotting. Crude gap junction preparations were obtained by NaOH fractionation. Briefly, 10^7 cells were washed with phosphate-buffered saline (PBS), scraped in 0.02% EDTA, centrifuged (5 min; Eppendorf) to pellet the cells, and suspended in 0.375 ml of 0.1 M $NaHCO_3$ (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium molybdate, 10 mM sodium fluoride, and 10 mM sodium PP_i. An equal volume of 40 mM NaOH (freshly prepared) was added; the sample was sheared by passage through a 25-gauge needle, incubated on ice for 30 min, and centrifuged (30 min; Eppendorf). The NaOH-insoluble pellets were washed with the $NaHCO_3$ buffer described above, recentrifuged for 30 min, and suspended in 30 μl of Laemmli sample buffer (32). Samples were not boiled before electrophoresis to prevent aggregation of β_2 protein. Protein samples (normalized by Coomassie blue staining following sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis) were electrophoresed on SDS–12.5% polyacrylamide gels (32) and transferred to nitrocellulose membranes (50 V, 3 h) as described by Kamps and Sefton (28). The immunoblots were stained with Ponceau S to ensure that the amount of transferred protein was the same for each sample. Immunoblots were processed by the method of Milks et al. (36). Immune complexes were detected with ^{125}I-labelled protein A and visualized by autoradiography. α_1 antibody α_1S and β_2 antibody β_2J were previously described (43). The specificity of the antisera was demonstrated in preliminary experiments by incubating separate blots with either preimmune, anti-α_1, or anti-β_2 serum. Only α_1 and β_2 gap junction antibodies gave reproducible signals on blots.

Immunoprecipitation and phosphoamino acid analysis. Primary keratinocytes were washed twice with PBS, preincubated with phosphate-free medium for 30 min, and labelled for 4 h with carrier-free $H_3{}^{32}PO_4$ (1 mCi/ml; Amersham). Immunoprecipitation of α_1 protein was carried out as follows. Cells were washed with PBS, incubated in 1 ml of 0.02% EDTA (37°C, 5 min), scraped, and centrifuged (5 min at room temperature [RT]; Eppendorf). Pellets were suspended in 0.4 ml of buffer B (10 mM Na_2HPO_4 [pH 7.0], 0.15 M NaCl, 10 mM EDTA, 10 mM NaF, 10 mM sodium PP_i, 10 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 0.5% Nonidet P-40, 0.1% SDS), incubated for 5 min (RT), precleared with prewashed Pansorbin cells (10% [wt/vol]; incubated for 10 min at RT after vortexing), and recentrifuged (5 min at RT). Antibody (10 μl) was added, and the mixture was rocked overnight at 4°C. Preswollen protein A–Sepharose CL-4B (50 μl at 50% [vol/vol]; Pharmacia) was added, and the mixture was incubated for an additional hour at 4°C. Samples were centrifuged (1 min at RT), washed twice with buffer B containing 0.35 M NaCl, recentrifuged, and washed twice with buffer C (buffer B containing 0.2% Nonidet P-40 and lacking SDS) and then twice with buffer A (buffer B without SDS and Nonidet P-40). Immunoprecipitated proteins were resuspended in sample buffer (30 μl) (32).

^{32}P-labelled samples and similarly treated unlabelled samples were electrophoresed on an SDS-polyacrylamide gel and transferred to an Immobilon membrane (Millipore Corp., Bedford, Mass.). The unlabelled portion of the blot was probed with α_1 antibodies and ^{125}I-labelled protein A as described above and aligned with the portion of the blot containing the ^{32}P-labelled samples. Regions of the blot corresponding to the 44- and 46-kDa α_1 species were excised, rehydrated in methanol, washed twice in distilled water, hydrolyzed with HCl (110°C, 2 h), and lyophilized (29). Individual ^{32}P-labelled phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis as previously described (10).

Immunofluorescence. Keratinocytes were fixed in methanol (5 min at −20°C), washed with PBS, and incubated in 100% acetone for 7 min at −20°C. Cells were then washed with PBS, incubated with preimmune serum or the α_1S antibody diluted 1:75 or 1:100 in PBS for 1 h at 37°C in a humidified box, and extensively washed with PBS. Fluorescein isothiocyanate-conjugated F(ab′)$_2$ goat anti-rabbit immunoglobulin G (Cappel Laboratories, West Chester, Pa.) diluted 1:100 in PBS was added and incubated for 30 min at 37°C in a light-tight, humidified box. Following incubation with the secondary antibody, cells were washed with PBS and mounted in PBS containing 0.1% *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine and 90% glycerol. All PBS washes were repeated 15 times.

Phosphatase digestion of α_1. α_1 protein was immunoprecipitated from untreated and TPA-treated (100 ng/ml, 2 h) keratinocytes as described above, except that samples were washed twice with phosphatase reaction buffer (50 mM Tris-Cl [pH 8.0], 10 mM $MgCl_2$, 150 mM NaCl) supplemented with 0.1% Triton X-100–0.05% SDS–2 mM phenylmethylsulfonyl fluoride; suspended in 20 μl of phosphatase reaction buffer containing 1% SDS, 1% 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride; and heated (60°C, 3 min). Samples were then divided in half (10 μl), diluted with 40 μl of phosphatase reaction buffer, and incubated for 3 h at 37°C with or without 3 U of molecular biology grade calf intestine alkaline phosphatase (Boehringer Mannheim; 38). Sodium orthovanadate (0.1 M; Fisher Scientific, Fair Lawn, N.J.) was dissolved in water. The reaction was terminated by addition of sample buffer. Samples were

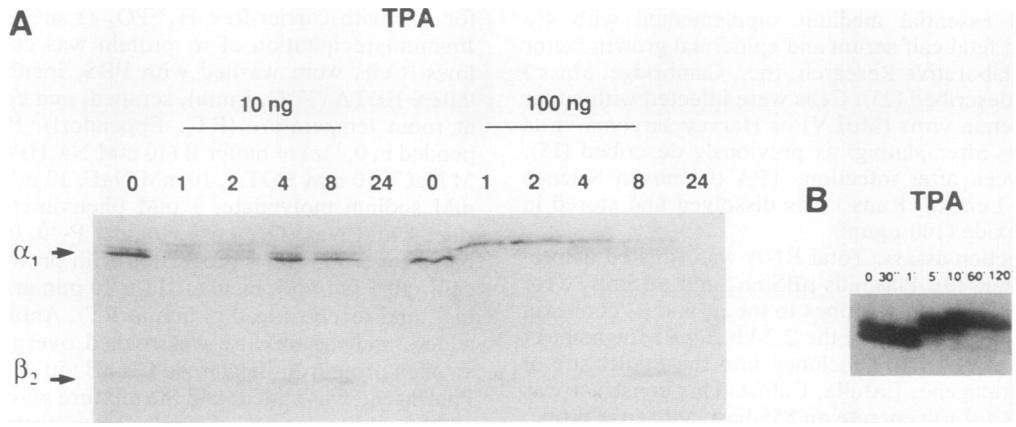

FIG. 1. Profile of α_1 and β_2 gap junction proteins in TPA-treated keratinocytes. (A) Cells were incubated in the presence of TPA (10 or 100 ng/ml) for the indicated times (hours). Proteins were isolated by NaOH fractionation, electrophoresed, and immunoblotted with anti-α_1 and anti-β_2 antibodies. The α_1 and β_2 gap junction proteins (arrows) migrated at the expected positions, as verified by molecular weight standards, as well as comigration with purified α_1 and β_2 proteins. (B) Pattern of α_1 phosphorylation in keratinocytes at early times after TPA exposure. Keratinocytes were treated with TPA (100 ng/ml) for the indicated times, and gap junction proteins were isolated by NaOH fractionation. The crude gap junction preparations were analyzed by immunoblotting as described in Materials and Methods.

electrophoresed on 12.5% acrylamide–0.10% bis SDS gels and analyzed by immunoblotting as described above.

RESULTS

Effects of TPA on gap junction protein expression and modification. In our initial experiments, we found that murine primary keratinocytes express both the α_1 (4) and β_2 (58) gap junction genes but not the β_1 gene (31, 41; data not shown). To elucidate the molecular mechanism responsible for inhibition of GJIC by phorbol esters, the gap junction protein profile was examined following exposure to TPA. α_1 protein is posttranslationally phosphorylated in several cell types to two more slowly migrating forms of 44 and 46 kDa (the nonphosphorylated form is 42 kDa; 11, 38, 39). In proliferating keratinocytes, the predominant form of α_1 protein migrated as a 42-kDa species (Fig. 1A). The 44- and 46-kDa forms were present as minor fractions of α_1 protein. Following treatment with 10 ng of TPA per ml for 1 to 2 h, the higher-molecular-weight species (44 and 46 kDa) increased relative to the 42-kDa protein, resulting in equal distribution of α_1 among the three forms (Fig. 1A). By 4 to 8 h after treatment, most of the α_1 protein was converted back to the 42-kDa species. After 24 h, there was a dramatic reduction in the total amount of the protein (Fig. 1A).

Gap junction protein localization was examined before and 2 h after TPA treatment by indirect immunofluorescence (Fig. 2). The characteristic punctate staining of large connexons at the cell membrane which was observed in untreated keratinocytes (Fig. 2a) was lost in TPA-treated cells (Fig. 2b). An increase of cytoplasmic staining with perinuclear localization was observed after TPA treatment. While no attempt was made to quantify the signal strength, it was consistently observed that the intensity of immunofluorescence was greater in TPA-treated samples than in controls. The reason for this difference is not known, but several possibilities can be suggested. These include (i) the lack of detection of small gap junction plaques by immunofluorescence and/or (ii) different accessibility of epitopes in TPA-treated versus untreated samples.

More drastic effects on the α_1 protein profile were observed with TPA at 100 ng/ml, which caused a dramatic shift of most, if not all, of this protein to the more slowly migrating 46-kDa species (Fig. 1A). In contrast to the transient modification observed with 10 ng of TPA per ml, the 46-kDa form predominated even at the later time points (4 to 8 h). Similar to TPA at 10 ng/ml, the total amount of the α_1 protein declined to barely detectable levels by 24 h. A second, more detailed time course of the response to TPA treatment at 100 ng/ml confirmed these results and revealed that the α_1 protein shifted to the 46-kDa form as early as 5 min after TPA exposure (Fig. 1B).

To determine whether the mobility differences of the various α_1 species are due to phosphorylation, immunoprecipitated α_1 protein from control and TPA-treated cells (100 ng of TPA per ml for 2 h) was digested with alkaline phosphatase and electrophoresed on a low-bisacrylamide gel (12.5% acrylamide–0.10% bis). This gel system allows better separation of the various α_1 forms. Alkaline phosphatase converted the 44- and 46-kDa species to the faster-migrating 42-kDa form present in control samples, and the migration of the 42-kDa form was not altered by phosphatase treatment (Fig. 3). Addition of vanadate prevented the phosphatase from altering the mobility of the 46-kDa species (data not shown).

To identify the amino acid phosphorylated in response to TPA, the α_1 protein was immunoprecipitated from ^{32}P-labelled cells and transferred to Immobilon membranes. No ^{32}P-labelled 42-kDa species was observed in either TPA-treated or control cells, confirming that this was the nonphosphorylated form. The 46-kDa species induced by TPA was excised and subjected to phosphoamino acid analysis, which revealed phosphorylation exclusively on serine residues (Fig. 4A). This result was confirmed by direct phosphoamino acid analysis of the α_1 protein in crude gap junction preparations. Gap junctions, extracted by sodium hydroxide fractionation of ^{32}P-labelled and unlabelled cells, were electrophoresed and transferred to an Immobilon membrane, and the unlabelled portion of the blot was incubated with α_1 antibodies. Immune complexes were detected with ^{125}I-labelled protein A and aligned with the portion of the blot containing the ^{32}P-labelled samples. Regions of the blot corresponding to the ^{32}P-labelled α_1 protein from both control and TPA-treated samples were excised and subjected to

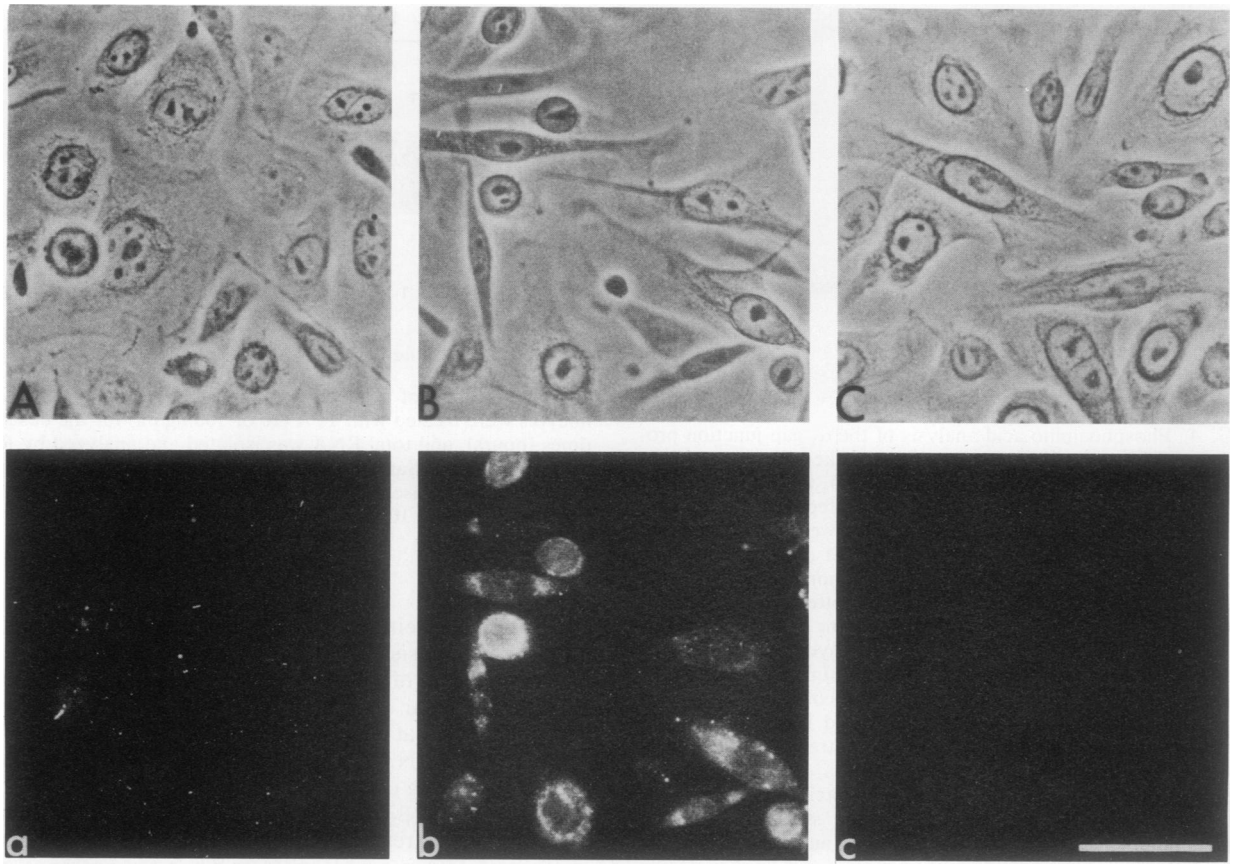

FIG. 2. Immunofluorescence staining of the α_1 protein in TPA-treated keratinocytes. Cells were fixed and reacted with the α_1S antibody as described in Materials and Methods. Panels: A, untreated keratinocyte B, keratinocytes exposed to 10 ng of TPA per ml for 2 h; C, TPA-treated keratinocytes incubated with preimmune serum. Panels A, B and C are phase-contrast images, and a, b, and c are indirect immunofluorescence images. Bar, 40 μm.

phosphoamino acid analysis. The only phosphorylated amino acid detected was serine, and the amount of phosphorylated serine increased significantly upon TPA treatment (Fig. 4B and C).

GJIC was measured by the scrape-loading–dye transfer technique (17) at various times after TPA treatment to determine whether early phosphorylation of α_1 correlates with inhibition of junctional communication (Fig. 5). Addition of 10 ng of TPA per ml resulted in a substantial decrease in GJIC by 1 h, which paralleled the appearance of the 46-kDa α_1 species. Conversion of the phosphorylated α_1 protein pattern to control levels by 8 h correlated with a substantial increase in junctional communication, even though GJIC was not restored to 100% of the control levels. In cells treated with 100 ng of TPA per ml, α_1 phosphorylation was detected as early as 5 min posttreatment (Fig. 1B) and GJIC was already significantly reduced. By 2 h, α_1 was maximally phosphorylated and GJIC reached its lowest level. Thus, phosphorylation of α_1 may be directly responsible for inhibition of GJIC at early times after TPA treatment.

The β_2 protein is expressed at rather low levels in primary keratinocytes. In contrast to that of α_1, the electrophoretic mobility of β_2 did not change after TPA treatment (Fig. 1A). Furthermore, comparison of crude gap junction preparations from ^{32}P-labelled and unlabelled cells revealed no significant ^{32}P signal in the region near β_2 in either untreated or

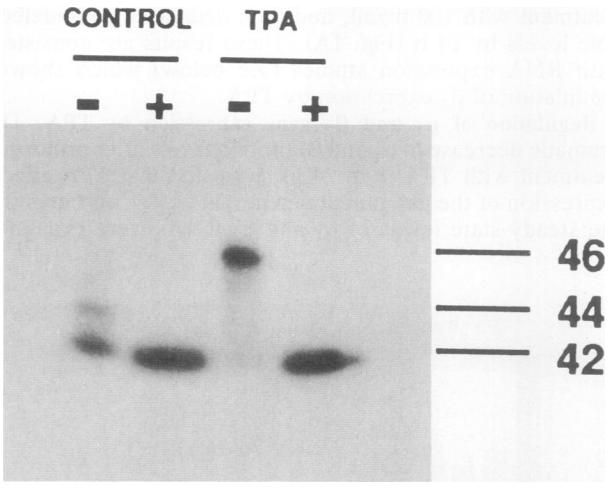

FIG. 3. Identification of α_1 phosphorylated forms by alkaline phosphatase digestion. The α_1 gap junction protein was immunoprecipitated from either control cells or cells treated with 100 ng of TPA per ml for 2 h. Immunoprecipitations were incubated with (+) or without (−) calf intestine alkaline phosphatase as described in Materials and Methods. Samples were analyzed by electrophoresis on 12.5% acrylamide–0.10% bis SDS gels for greater resolution of the α_1 forms and immunoblotted as described in the legend to Fig. 1. The various α_1 species are indicated.

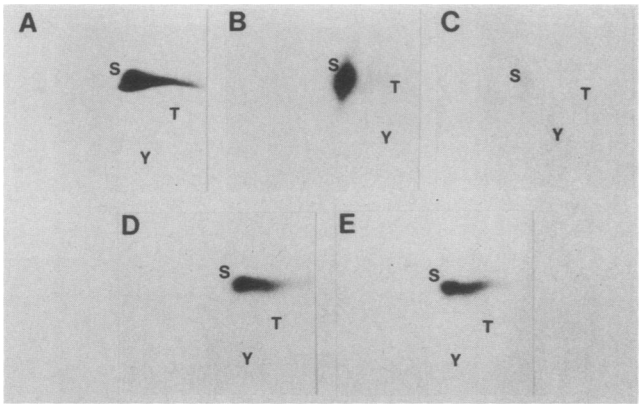

FIG. 4. Phosphoamino acid analysis of the α_1 gap junction protein from TPA-treated or *ras*-transformed keratinocytes. Cells which were metabolically labelled with $H_3{}^{32}PO_4$ for 4 h were either treated with 100 ng of TPA per ml for 2 h or infected with HaSV as described in Materials and Methods. Samples were either immunoprecipitated with α_1 antibodies (A, D, and E) or fractionated by using NaOH treatment (B and C), electrophoresed on a 12.5% acrylamide–0.10% bis SDS gel, and immunoblotted onto Immobilon membranes. Regions of the filters corresponding to the phosphorylated α_1 forms were excised, eluted, and hydrolyzed for two-dimensional phosphoamino acid analysis. (A) Immunoprecipitated 46-kDa α_1 from TPA-treated cells. (B) Region corresponding to the 46-kDa α_1 in TPA-treated, NaOH-fractionated keratinocytes. (C) Region corresponding to the 46-kDa α_1 in untreated, NaOH-fractionated cells. (D) Immunoprecipitated 44-kDa α_1 from *ras*-transformed keratinocytes. (E) The 46-kDa α_1 protein immunoprecipitated from *ras*-transformed cells. Internal standards for the phosphoamino acids serine (S), threonine (T), and tyrosine (Y) are indicated.

TPA-treated samples (data not shown), suggesting that the β_2 protein was not phosphorylated in response to TPA. The β_2 protein levels seemed to increase to a maximum at 4 h after treatment with 10 ng of TPA per ml, or at 8 h after treatment with 100 ng/ml, and then decreased to undetectable levels by 24 h (Fig. 1A). These results are consistent with RNA expression studies (see below) which showed modulation of β_2 expression by TPA.

Regulation of α_1 and β_2 gene expression by TPA. The dramatic decrease in α_1 and β_2 protein levels after prolonged treatment with TPA (8 to 24 h) suggested that TPA affects expression of the gap junction genes as well. Consequently, the steady-state levels of α_1 and β_2 RNAs were examined

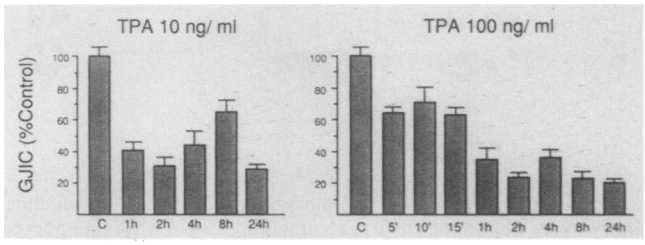

FIG. 5. GJIC in keratinocytes after exposure to TPA. Cells were treated with either 10 or 100 ng of TPA per ml for the indicated times, and GJIC was measured by the scrape-loading–dye transfer technique (17). For each condition, the numbers of secondary dye recipient cells were counted in four microscopic fields, and the values shown represent 16 independent measurements. Standard deviations are indicated by bars. C, control.

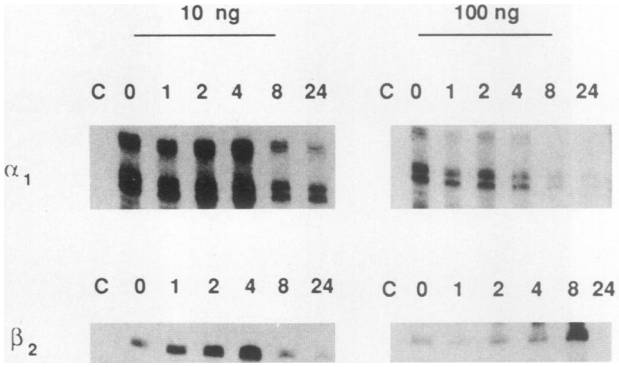

FIG. 6. Effect of TPA on α_1 and β_2 RNA expression. Keratinocytes were treated with TPA (10 or 100 ng/ml) for the designated times (hours), and total RNA was isolated and analyzed by RNase protection assay. Samples treated with 100 ng of TPA per ml and probed for α_1 transcripts contained 5 μg of total RNA. All other samples contained 10 μg of total RNA. C, tRNA control.

after addition of either 10 or 100 ng of TPA per ml by RNase protection analysis. Following treatment with 10 ng of TPA per ml, the level of α_1 RNA was significantly reduced by 8 h (Fig. 6). A similar α_1 RNA profile was seen when cells were exposed to TPA at a higher concentration (100 ng/ml; Fig. 6; 5 μg of total RNA, rather than 10 μg, was used in this analysis, yielding a lower basal α_1 signal).

In the case of β_2, the steady-state level of RNA continuously increased, reaching a peak at either 4 h in the presence of 10 ng of TPA per ml (Fig. 6) or 8 h in the presence of 100 ng of TPA per ml (Fig. 6). Similar to the α_1 mRNA, the β_2 message then declined to barely detectable levels by 24 h. These variations in RNA expression paralleled the protein profile and indicated that, in addition to inducing phosphorylation of α_1, TPA modulated the steady-state levels of both the α_1 and β_2 gap junction transcripts.

Gap junction expression and modification following *ras* transformation. Transformation of mouse primary keratinocytes by the activated *ras* oncogene introduced via infection with HaSV results in partial disruption of the differentiation program of these cells in vitro (8, 56) and a tumorigenic phenotype in vivo (15, 36a, 45). Since GJIC in these transformed keratinocytes is significantly reduced (70 to 80%; 12), the effect of *ras* transformation on the expression of gap junction RNA and protein was examined.

The concentrations of α_1 mRNA and protein were only slightly reduced in HaSV-infected cells compared with those of control cultures infected with helper MoLV alone (Fig. 7A and B). In contrast, the levels of β_2 mRNA and protein were greatly reduced in HaSV-infected keratinocytes, similar to the pattern observed after TPA treatment.

Transformation by *ras* also resulted in an altered pattern of phosphorylation of the α_1 protein (Fig. 7B). Whereas the α_1 protein in MoLV-infected cells was present as the 42- and 44-kDa species, the α_1 found in HaSV-infected cells was equally distributed among its three forms. This pattern closely resembled that observed in cells treated with TPA at 10 ng/ml. To identify the phosphorylated amino acid residue, the α_1 gap junction protein was immunoprecipitated from ^{32}P-labelled, *ras*-transformed keratinocytes. No ^{32}P label was found in the 42-kDa α_1 form, while both the 44- and 46-kDa species were labelled. These bands were excised and subjected to phosphoamino acid analysis. Phosphoserine

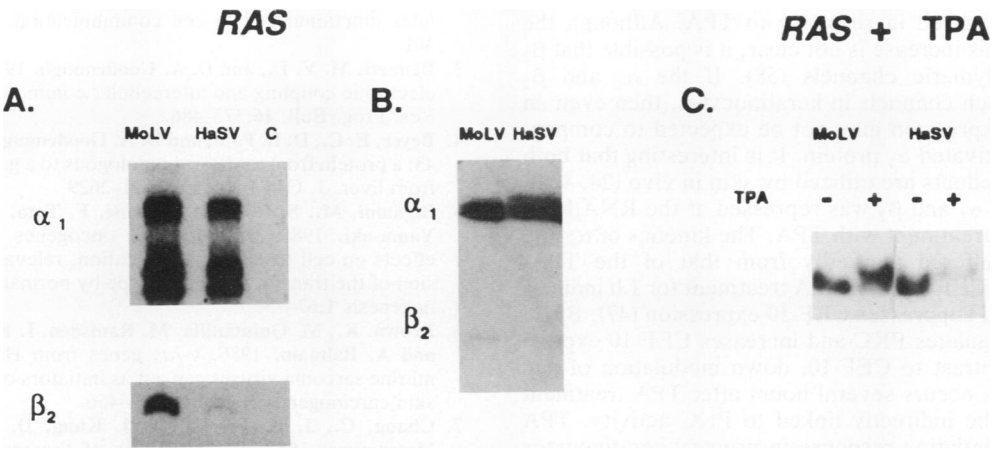

FIG. 7. Expression of α_1 and β_2 RNAs and proteins in *ras*-transformed primary keratinocytes. Total RNA and protein were isolated from primary keratinocytes 1 week after infection with either MoLV or HaSV. (A) RNase protection analysis of α_1 and β_2 RNAs as described in Materials and Methods. C, tRNA control. (B) Protein profile of α_1 and β_2 gap junction proteins as determined by immunoblot analysis as described in the legend to Fig. 1. (C) Additive effects of *ras* and TPA on α_1 protein expression. Keratinocytes were infected with MoLV or HaSV for 1 week. Infected cells were either untreated (−) or exposed to 10 ng of TPA per ml for 1 h (+). Gap junction proteins which were extracted by NaOH fractionation were subjected to immunoblot analysis as described in Materials and Methods.

was the only amino acid detected in both the 44- and 46-kDa α_1 species (Fig. 4D and E).

Treatment of *ras*-transformed keratinocytes with 10 ng of TPA per ml for 1 h caused, in addition to phosphorylation of α_1 protein, a substantial reduction in the α_1 concentration. The total level of α_1 protein in TPA-treated, *ras*-transformed cells was much lower than that found in TPA-treated, MoLV-infected control cells (Fig. 7C). Thus, the effects of *ras* and TPA on α_1 expression were additive and these molecular events are consistent with synergistic inhibition of GJIC by these agents (12).

DISCUSSION

In this report, we show that TPA treatment and *ras* transformation of mouse primary keratinocytes results in both phosphorylation of α_1 protein and decreased expression of the α_1 and β_2 genes. These results provide a molecular explanation for the decrease in GJIC that *ras* and TPA cause in these primary cells and suggest a possible mechanism for the GJIC inhibition produced by these agents in other cell types.

Phosphorylation of α_1 has been implicated in the regulation of gap junctional communication. Treatment of the mouse cell line C3H 10T1/2 with a synthetic retinoid, a potent inhibitor of experimental carcinogenesis, increased both GJIC and phosphorylation of α_1 (at unidentified residues; 44). In a separate study, serine phosphorylation was shown to correlate with increased GJIC, as certain communication-deficient cell lines were unable to phosphorylate α_1 on serine residues to generate the 46-kDa form (39). The kinase(s) responsible for these modifications is not known. The fact that TPA-induced serine phosphorylation of α_1 was associated with inhibition of GJIC, rather than an increase, suggests that the phosphorylated sites in TPA-treated cells are different from the sites associated with increased communication. An alternative explanation is that the cellular compartment where phosphorylation occurs differentially alters the properties of the gap junction protein (e.g., stability, targeting, etc).

Several mechanisms could account for the decreased communication associated with α_1 modification in TPA-treated cells. Phosphorylation could interfere with connexon formation, pairing of connexons in neighboring cells, or gating of assembled channels (the ability of a channel to open and close). Phosphorylation of α_1 does not appear to interfere with membrane insertion, since the 46-kDa form fractionates with membranes. The rapid effects of TPA on GJIC suggest that TPA is likely to influence existing cell surface junctional proteins. The fact that the amount of α_1 protein recognizable by immunofluorescence on the cell surface is decreased 2 h after treatment with TPA is consistent with the notion that phosphorylation induces a conformational change at the cell membrane.

TPA and related tumor promoters are thought to exert most, if not all, of their effects through activation of one or more forms of protein kinase C (PKC), the TPA receptor (30). PKC may be directly responsible for TPA-induced phosphorylation of α_1. Such PKC activity is consistent with the early time course of phosphorylation and the fact that α_1 was entirely phosphorylated on serine residues. Several potential PKC modification sites are present in the carboxy terminus of the α_1 protein (20). Further, addition of PKC to electrically coupled cell pairs resulted in loss of junctional conductance (48).

Treatment of keratinocytes with TPA at 10 ng/ml caused transient phosphorylation of α_1 (1 to 2 h), which then returned to control levels (at 8 h). This correlates with the initial inhibition of GJIC (12), which is followed by an increase in junctional communication by 8 h. The fact that GJIC did not return to 100% of the control levels may be due to the presence of small amounts of the 46-kDa form of α_1. Alternatively, the conformation of α_1 after dephosphorylation may be altered, interfering with its ability to form functional channels.

In addition to α_1, primary keratinocytes expressed the β_2 gap junction protein at rather low levels. Our results suggest that the β_2 protein is not modified by phosphorylation in TPA-treated keratinocytes. It has been previously shown that β_2 is not phosphorylated in hepatocytes or by purified cyclic AMP-dependent protein kinase, PKC, or calcium/calmodulin-dependent protein kinase II (46, 50). β_2 expres-

sion initially increased in response to TPA. Although the significance of this increase is not clear, it is possible that β_2 forms heteropolymeric channels (58). If the α_1 and β_2 proteins form such channels in keratinocytes, then even an increase in β_2 expression may not be expected to compensate for the inactivated α_1 protein. It is interesting that both the α_1 and β_2 products are utilized by skin in vivo (24, 42a).

Expression of α_1 and β_2 was repressed at the RNA level upon prolonged treatment with TPA. The kinetics of α_1 and β_2 repression differed markedly from that of the TPA-repressible gene CEF-10 (47). TPA treatment for 1 h induces PKC activity and suppresses CEF-10 expression (47). By 24 h, TPA down regulates PKC and increases CEF-10 expression (47). In contrast to CEF-10, down modulation of gap junction proteins occurs several hours after TPA treatment and appears to be indirectly linked to PKC activity. TPA induces a differentiation response in primary keratinocytes similar to that of calcium (13, 19, 22, 55, 57), and calcium, like prolonged TPA treatment, suppresses gap junction expression (unpublished data). Thus, the effects of TPA on gap junction expression may result from induction of some aspects of keratinocyte differentiation.

The *ras* oncogene was previously shown to cause a 70 to 80% decrease in GJIC in primary keratinocytes and completely inhibit communication in synergism with TPA (12). In this study, we found that *ras*, like TPA, induced serine phosphorylation of α_1 and that the phosphorylation pattern resulting from *ras* transformation was similar to the pattern induced by 10 ng of TPA per ml. While it is not known which kinase(s) is activated by *ras*, one possible candidate is PKC, as *ras* has been shown to act through PKC-dependent (as well as PKC-independent) pathways (33, 52). Like TPA, *ras* did not appear to induce modification of β_2, but it caused substantial down modulation of its expression.

The effects of *ras* and TPA were additive. In *ras*-transformed cells treated with TPA, phosphorylation of the α_1 protein increased and its expression was reduced to a level lower than that found in cells exposed to either agent alone. Such a rapid effect on α_1 expression may be due to protein destabilization. However, decreased levels of α_1 mRNA 15 to 30 min after treatment of *ras*-transformed cells with TPA were also observed (unpublished data). Cooperation between *ras* and TPA has been shown both in vitro in focus formation of cells (14, 25) and in vivo in induction of skin tumors (6). The additive effects of *ras* and TPA in blocking GJIC (12) via modulation of gap junction proteins may contribute to clonal expansion of initiated cells during neoplastic development.

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