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Switch in gap junction protein expression is associated with selective changes in junctional permeability during keratinocyte differentiation

(mouse primary keratinocytes/calcium/connexins/channels)

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ABSTRACT **Gap junctional communication provides a mechanism for regulating multicellular activities by allowing the exchange of small diffusible molecules between neighboring cells. The diversity of gap junction proteins may exist to form channels that have different permeability properties. We report here that induction of terminal differentiation in mouse primary keratinocytes by calcium results in a specific switch in gap junction protein expression. Expression of α_1 (connexin 43) and β_2 (connexin 26) gap junction proteins is down-modulated, whereas that of β_3 (connexin 31) and β_4 (connexin 31.1) proteins is induced. Although both proliferating and differentiating keratinocytes are electrically coupled, there are significant changes in the permeability properties of the junctions to small molecules. In parallel with the changes in gap junction protein expression during differentiation, the intercellular transfer of the small dyes neurobiotin, carboxyfluorescein, and Lucifer yellow is significantly reduced, whereas that of small metabolites, such as nucleotides and amino acids, proceeds unimpeded. Thus, a switch in gap junction protein expression in differentiating keratinocytes is accompanied by selective changes in junctional permeability that may play an important role in the coordinate control of the differentiation process.**

Differentiation involves a complex set of events that must be coordinated among neighboring cells. Coordination of this process is likely to be mediated, at least in part, by the direct cell-to-cell exchange of small molecules via gap junctions. Gap junction proteins (connexins) form transmembrane channels when a hemichannel, termed a connexon, in one cell pairs with a connexon in an adjacent cell (1). Connexins compose a family of proteins, and the expression of each connexin depends on both cell type and time of development (2, 3). The channels formed by different gap junction proteins have distinct unitary conductances (4), suggesting that these channels have different physiological properties. The present study was undertaken to investigate the role of gap junctional communication (GJC) in keratinocyte differentiation.

Dye-transfer studies with cultured keratinocytes (5) and intact skin (6, 7) implicated gap junctions in the control of epidermal differentiation, although these studies reached conflicting conclusions. In a mixed population of cultured human keratinocytes, undifferentiated (involucrin-negative) cells transferred dye more frequently than did differentiated (involucrin-positive) cells (5). In intact human skin, however, dye coupling was more extensive between differentiated (suprabasal) cells than between undifferentiated (basal) cells (7). These studies did not identify the gap junction proteins responsible for dye transfer.

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Cultured mouse primary keratinocytes provide a useful model for the study of epithelial cell differentiation. Keratinocytes proliferate in medium with low calcium concentrations but upon calcium addition initiate a program of terminal differentiation. This differentiation program is similar to that observed in the upper layers of the epidermis *in vivo* and includes growth arrest, the induction of specific markers, stratification, and cornification (8, 9). We report here that terminally differentiating keratinocytes undergo a switch in gap junction protein expression and a concomitant change in the transfer of specific molecules. This switch in gap junction gene expression and junctional permeability suggests that gap junctions may play a role in the coordinate control of keratinocyte differentiation.

MATERIALS AND METHODS

Primary Cell Cultures. Mouse primary keratinocytes were isolated from 2- to 3-day-old Sencar mice and maintained in low calcium (0.05 mM) minimal essential medium/4% Chelex-treated fetal calf serum/epidermal growth factor at 10 ng/ml (Collaborative Research) as described (8). Differentiation was induced by adding $CaCl_2$ to a final concentration of 2 mM to confluent monolayers.

RNase Protection Assays. Total RNA was isolated from primary keratinocytes (10) and analyzed by RNase protection assays (11). RNase protection analysis yields multiple, specific, RNase-resistant fragments that are likely to result from intramolecular RNase digestion at A+T-rich regions or limited degradation at the 3′ ends of the RNA–RNA hybrids (11). The α_1, β_2, and glyceraldehyde-3-phosphate dehydrogenase probes have been described (12, 13). The β_3 and β_4 probes were prepared from mouse cDNAs obtained by reverse transcriptase PCR (14). The oligonucleotides used to clone β_3 gap junction protein corresponded to nt 284–299 and the reverse complement of 790–810 of the mouse cDNA (15). To clone the β_4 gap junction gene, oligonucleotides were used corresponding to −3 to 23 and the reverse complement of 799–816 (16). Identities of the cloned cDNAs were confirmed by sequencing.

Immunoblotting. Crude gap junction preparations were obtained by sodium hydroxide (NaOH) fractionation as described (12). Samples were normalized for total protein after SDS/PAGE (17) by staining with Coomassie blue. Protein extracts were electrophoresed on SDS/12.5% polyacrylamide gels and transferred to nitrocellulose membranes according to Kamps and Sefton (18). Immunoblots were processed and developed with ^{125}I-labeled protein A as described

Abbreviation: GJC, gap junctional communication.
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(12). The specificity of each antiserum has been demonstrated (19).

Microinjection Experiments. Neurobiotin transfer was determined by coinjecting 5% neurobiotin and 0.4% rhodamine dextran (both from Molecular Probes) into keratinocytes using a Zeiss Axiovert 35 microscope and an Eppendorf microinjector (model 5242). In all microinjection experiments rhodamine dextran was used to identify the injected cell. After microinjection, cells were washed with fresh medium and incubated for 15 min. The cells were then washed with phosphate-buffered saline (PBS) and fixed with 4% (wt/vol) paraformaldehyde. After fixation, the keratinocytes were permeabilized with PBS/2% bovine serum albumin/0.25% Triton X-100 (2 hr, 37°C), washed two times with PBS, incubated with fluorescein isothiocyanate-conjugated streptavidin (Pierce)/PBS/0.25% Triton X-100 (30 min, 37°C), and washed two times with PBS. Cells were monitored for fluorescence with a Nikon Microphot-FXA microscope. Transfer of Lucifer yellow was measured by microinjecting 0.4% Lucifer yellow (Molecular Probes) and 0.4% rhodamine dextran into primary keratinocytes. Cells were then washed with fresh medium, incubated for 15 min, and examined by fluorescence microscopy.

Cytidine 5′-triphosphate or methionine transfer was measured by coinjection of 0.8% rhodamine dextran with either cytidine 5′-[α-[^{35}S]thio]triphosphate [1455 Ci/mmol; New England Nuclear; 12.5 mCi/ml (1 Ci = 37 GBq); 20 fl per injection] or [^{35}S]methionine (1175 Ci/mmol; New England Nuclear; 395 μCi/ml; 20 fl per injection) into primary keratinocytes. Cells were then washed with fresh medium, incubated for 15 min, and fixed with 3% paraformaldehyde. Emulsion autoradiography was performed for 3 days at 4°C. Label-acquiring cells were identified by using a combination of dark-field, bright-field, and phase-contrast microscopy.

Electrophysiology. Conductances of keratinocyte cell pairs were measured by the double whole-cell patch-clamp technique (20) using two Axopatch 1C patch clamps. Just before each experiment the cell culture medium was replaced with mammalian Ringer solution (140 mM NaCl/4.5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$, pH 7.2 with NaOH), and the cells were incubated for 20 min. Patch pipettes were filled with potassium aspartate internal solution (160 mM potassium aspartate/1.1 mM EGTA/2 mM $MgCl_2$/0.1 mM $CaCl_2$/10 mM Hepes, pH to 7.2 with KOH), and for each experiment, one of the pair of pipettes was filled with potassium aspartate Ringer solution/20 μm 5,6-carboxyfluorescein (Molecular Probes). Cells were visualized on a Nikon Diaphot inverted microscope equipped with Hoffman modulation optics, epifluorescent illumination, and a Cohu model 5000 SIT television camera (Cohu, San Diego), enabling the detection of very low levels of fluorescent emission. Thus, both dye transfer and conductance were determined simultaneously in the same pairs of cells. To measure junctional conductances, both cells were first held at −40 mV for 50 ms. Voltage in cell 1 was then increased to −20 mV for 100 ms and returned to −40 mV for 50 ms. The voltage in cell 2 was next increased to −20 mV for 100 ms and then returned to the holding potential of −40 mV. Junctional currents were thus seen as downward deflections from the holding current, and junctional conductance was calculated as the junctional current divided by 20 mV. All experiments were done at room temperature, and dye fill of the cell patched with the dye-containing pipette was always observed within 1–2 min after the whole-cell configuration was obtained.

RESULTS

Gap Junction Expression in Proliferating and Differentiating Keratinocytes. Gap junction protein expression has been seen to vary during skin development in the overlying epidermal layers and the epidermal adnexa (19). We further investigated this differential expression under well-defined culture conditions. Gap junction gene expression was compared in proliferating keratinocytes (0.05 mM calcium, low-calcium medium) and at various times after calcium-induced differentiation (2 mM calcium, high-calcium medium) using ribonuclease protection assays. As we had reported (12), growing keratinocytes express the α_1 (connexin 43) and β_2 (connexin 26) gap junction protein genes but do not express the β_1 (connexin 32) protein gene. The induction of keratinocyte differentiation by calcium significantly decreased both α_1 and β_2 mRNA levels by 6 hr. After 72 hr, expression of these transcripts was virtually undetectable (Fig. 1*A*).

α_1 and β_2 protein levels were analyzed by immunoblot analysis. During keratinocyte proliferation, the α_1 gap junction protein exists predominantly as a nonphosphorylated form of ≈42 kDa and, to a lesser extent, as two phosphorylated species of 44 and 46 kDa (12). In keratinocytes treated with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate or transformed with the *ras* oncogene, the 42-kDa species is converted into the higher-molecular weight forms, and this increase in α_1 phosphorylation is associated with the inhibition of GJC (12). As shown in Fig. 1*B*, the induction of differentiation by calcium also affected the ratio of the different α_1 forms, but the major effect was a progressive decrease in the amount of α_1 protein. The steady-state level of α_1 was substantially reduced by 24 hr after calcium addition and was undetectable by 72 hr. Proliferating keratinocytes also express low levels of the β_2 protein, as has been reported (12). The β_2 connexin was also down-modulated by 24–72 hr of calcium treatment (Fig. 1*B*). Thus, the levels of the α_1 and β_2 proteins during calcium-induced differentiation correlated with the expression patterns observed for their respective transcripts. Consistent with these results, α_1 gap junction plaques were easily detected on the surface of growing keratinocytes by indirect immunofluorescence but were absent 24 hr after calcium addition (data not shown).

The β_3 (connexin 31; refs. 15, 21), β_4 (connexin 31.1; refs. 16, 22), and α_4 (connexin 37 or 37.6; refs. 22, 23) gap junction genes were reported also to be expressed in skin and in certain keratinocyte cell lines. Therefore, the primary keratinocyte cultures were examined for expression of these connexins during growth and differentiation. β_3 mRNA was present at very low levels in proliferating keratinocytes and at early times after calcium addition. However, 8 hr after calcium treatment, β_3 transcript levels markedly increased and reached a maximum by 3 days (Fig. 1*A*). These high levels persisted for up to 7 days (data not shown). Consistent with these results, the β_3 protein was not detectable by immunoblotting until 3 days after calcium addition (Fig. 1*B*). In proliferating keratinocytes, the level of β_4 mRNA was very low. Similar to the β_3 expression pattern, β_4 RNA was significantly induced 24 hr after calcium addition and progressively increased up to 7 days (Fig. 1*A*). Expression of the α_4 gene was not detected in cultured keratinocytes, and it was found primarily in the dermis *in vivo* (data not shown).

Changes in Junctional Permeability During Differentiation. Because there was a switch in gap junction protein expression during keratinocyte differentiation, it was important to determine if there was also a change in GJC. This possibility was examined by microinjection experiments. Neurobiotin is a small compound (M_r 323) that has been used to measure GJC (24). Rhodamine dextran is a large fluorescent compound (M_r 10,000) that cannot pass through gap junction channels and, therefore, serves as a marker for the injected cells. After microinjection into growing keratinocytes, neurobiotin readily moved to several layers of neighboring cells. In contrast, 72 hr after calcium-induced differentiation the intercellular transfer of neurobiotin was reduced to undetectable levels (Fig. 2). Identical results were obtained after

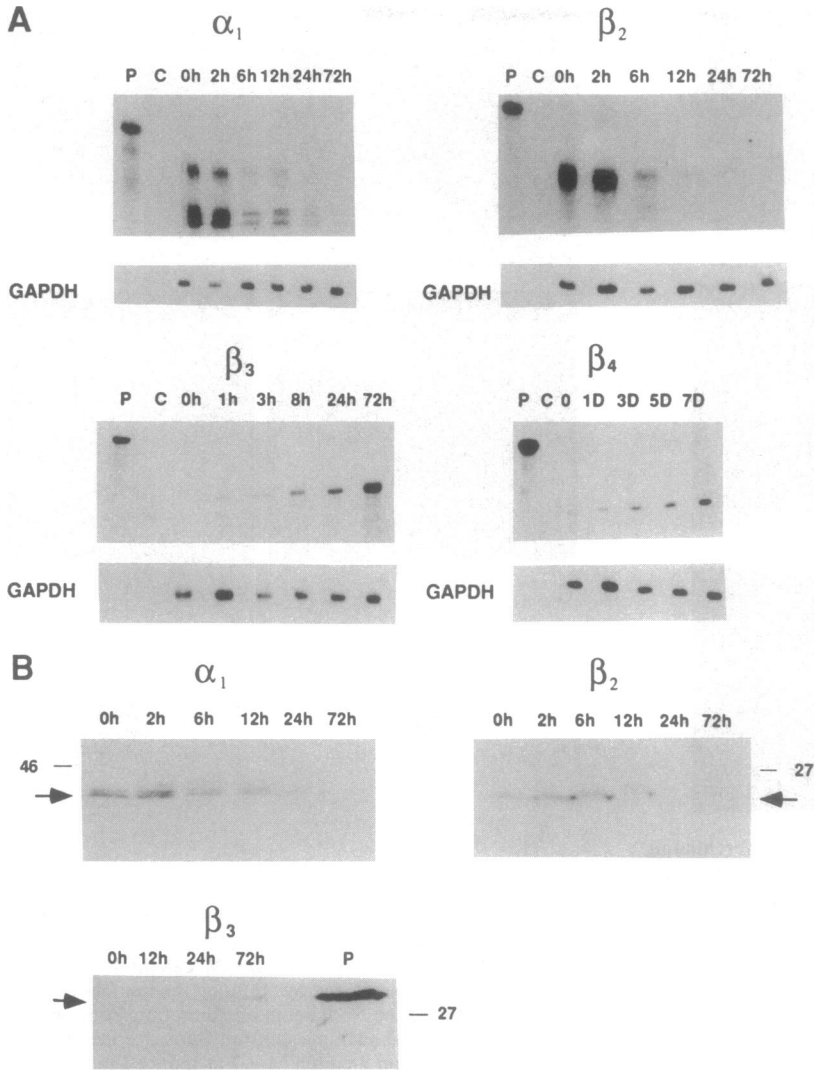

FIG. 1. Switch in connexin gene expression during calcium-induced keratinocyte differentiation. (*A*) Total RNA was isolated from keratinocytes before and at various times after calcium addition (hours for α_1, β_2, and β_3 RNA and days for β_4 RNA). Ribonuclease protection assays were done with ^{32}P-labeled RNA probes specific for α_1 (10 μg of RNA per lane), β_2 (10 μg of RNA per lane), β_3 (10 μg of RNA per lane) or β_4 (30 μg of RNA per lane) connexins. An antisense probe to glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH; 0.1 μg of RNA per lane) was used to confirm that equal amounts of RNA were present in each reaction. P, probe; C, tRNA control. Gels were autoradiographed for 12 hr at −70°C, except the gel for β_4 connexin which was autoradiographed at −70°C for 3 days. (*B*) Crude gap junction preparations were isolated by NaOH fractionation of keratinocytes under proliferating conditions and at various times [hours (h)] after calcium addition. Equal quantities of proteins were electrophoresed on SDS/12.5% polyacrylamide gels and analyzed by immunoblotting with either anti-α_1, anti-β_2, or anti-β_3 antibodies. Samples for the β_3 immunoblot were run in parallel with human placenta extracts (P), which provided a well-characterized source of β_3 protein (21). Positions of α_1, β_2, β_3 proteins and size markers are indicated.

microinjection of Lucifer yellow, a slightly larger compound (M_r 457), that is also routinely used to analyze junctional communication (25). Transfer of Lucifer yellow between cells was readily detectable in proliferating keratinocytes, whereas it was not detectable in differentiating cells (data not shown). A similar decrease in Lucifer yellow dye transfer was previously reported for differentiating (involucrin-positive) versus undifferentiating (involucrin-negative) human keratinocytes (5).

Gap junctions are permeable to nucleotides and amino acids (26–28). Therefore, we determined whether the transfer of these molecules was also blocked in differentiating keratinocytes. Cells in low- or high-calcium medium were coinjected with rhodamine dextran and cytidine 5′-[α-[^{35}S]thio]triphosphate (M_r 484). After a period of 15 min to allow incorporation of the injected ^{35}S-labeled CTP into cellular RNA, cultures were fixed. ^{35}S-labeled CTP transfer was determined by emulsion autoradiography. Like other phosphorylated compounds, ^{35}S-labeled CTP cannot be taken up from the medium by cells. Thus, only ^{35}S-labeled CTP that has been microinjected can be incorporated into RNA. The labeled CTP was transferred between keratinocytes in both proliferating and differentiating cultures, although transfer was somewhat greater between growing cells (Fig. 3). Proliferating and differentiating keratinocytes transferred ^{35}S-labeled CTP with an average radius of 3.7 ± 0.91 and 2.6 ± 0.96 cells, respectively. Control injections were done with an ^{35}S-labeled DNA fragment (447 bp), and, as expected, the DNA was not transferred (data not shown). Similar microinjection experiments were done with [^{35}S]methionine (M_r 149). As with ^{35}S-labeled CTP, [^{35}S]methionine transfer was approximately equal in both growing and differentiating cells (data not shown).

Gap Junctional Conductances of Growing and Differentiating Keratinocytes. Double whole-cell patch-clamp studies were done in combination with dye transfer to directly measure junctional conductances (20). Fig. 4 shows that the junctional conductances of proliferating and differentiating keratinocytes were not significantly different. Simultaneous measurement of 5,6-carboxyfluorescein (M_r 376) transfer and junctional conductance in the same cell pairs showed that in proliferating keratinocytes, dye transfer occurred (9 out of 10 cell pairs), even when the conductance was much lower than the highest conductance seen for differentiating cells. However, none of the cell pairs grown in high-calcium medium showed evidence of dye transfer (5 out of 5 cell pairs), confirming the results obtained with neurobiotin and Lucifer yellow (Fig. 2). Thus, the difference in dye transfer seen in cells grown in low- vs. high-calcium medium is not a result of a difference in junctional conductance. Due to the large calcium buffering capacity of the cells and the fact that the cells are electrically coupled, it is unlikely that the observations are related to changes in calcium concentrations in the medium. Furthermore, no significant changes in the selectivity of GJC were observed at early times after calcium addition when the intracellular calcium concentration is already increased (29).

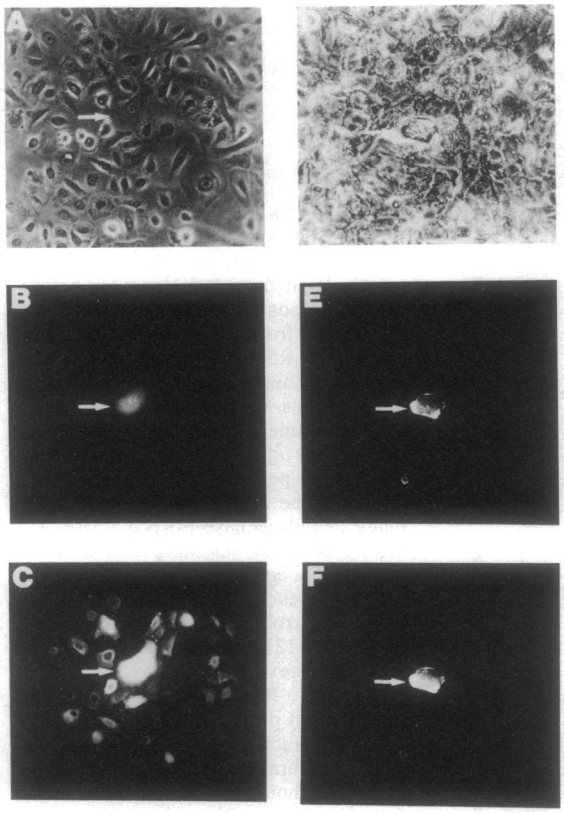

FIG. 2. Transfer of neurobiotin in growing or differentiating keratinocytes. Neurobiotin (5%) and rhodamine dextran (0.4%) were coinjected into primary keratinocytes before and 3 days after calcium addition. GJC was quantitated by counting the number of cells that acquired neurobiotin (in one direction) from single injected cells identified by rhodamine dextran fluorescence. Primary keratinocytes in culture are heterogeneous with respect to size; this size heterogeneity does not appear to affect dye transfer but may be responsible for uneven intensity of fluorescent staining. Keratinocytes do have processes, which may explain the appearance of bright cells not in obvious contact with any other cells. Transfer was measured from at least 25 injected cells for each experimental condition, and similar results were obtained in at least two independent experiments. Neurobiotin was transferred to an average of 3.3 ± 0.80 keratinocytes per injected cell grown in low-calcium medium. In cells incubated for 3 days in the presence of 2 mM calcium, neurobiotin was only occasionally transferred to a single adjacent cell (3 out of 25 injected cells). Proliferating (*A–C*) or differentiating keratinocytes (*D–F*) were examined by phase-contrast (*A, D*), rhodamine dextran (*B, E*), and neurobiotin (*C, F*) fluorescence microscopy. Arrows denote injected cells. (×630.)

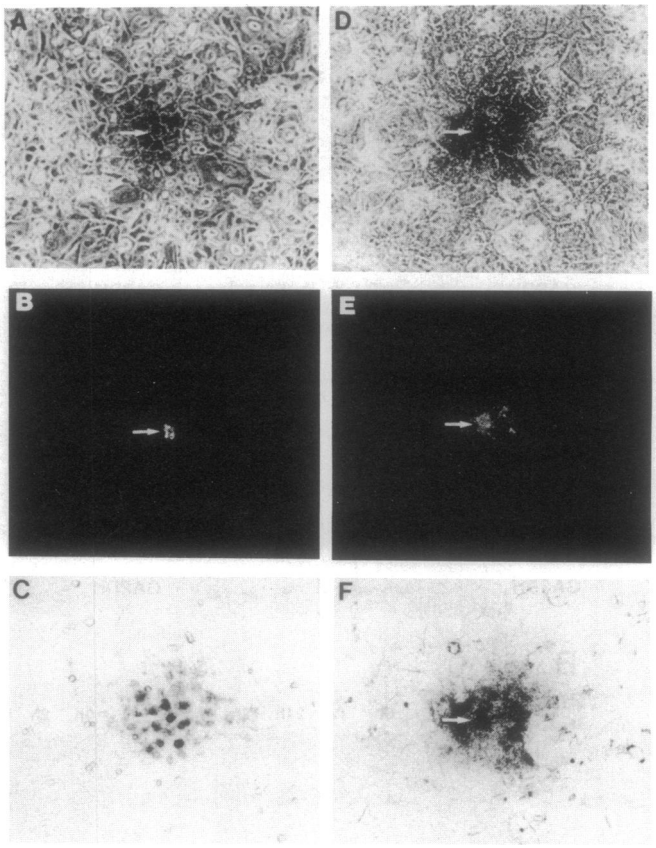

FIG. 3. Transfer of CTP in proliferating and differentiating keratinocytes. Rhodamine dextran and cytidine 5′-[α-[^{35}S]thio]triphosphate were coinjected into either growing or differentiating keratinocytes (72 hr after addition of 2 mM calcium). Transfer of ^{35}S-labeled CTP was quantitated by counting the number of neighboring cells that acquired the ^{35}S label in one direction from single injected cells identified by rhodamine dextran fluorescence. Keratinocytes grown in low-calcium medium (*A–C*) or induced to differentiate for 72 hr with 2 mM calcium (*D–F*) were examined by phase-contrast microscopy (*A, D*), rhodamine dextran fluorescence (*B, E*), and bright-field microscopy (*C, F*). Arrows indicate injected cells. (×590.)

DISCUSSION

In the present study, we show that not only do terminally differentiating keratinocytes undergo a specific switch in gap junction protein expression, but this switch is associated with changes in gap junction permeability. In particular, the transfer of CTP and methionine, but not of neurobiotin, carboxyfluorescein, or Lucifer yellow, indicates that GJC in differentiating keratinocytes is selectively altered. Because the molecular weight of CTP (M_r 484) is similar to that of Lucifer yellow (M_r 457), it seems likely that the specificity of transfer depends on the conformation, charge, and hydration radius of the molecule, in addition to its size (1, 30). However, we cannot rule out the possibility that a mono- or diphosphate is removed from the CTP, reducing its molecular weight. Selectivity in gap junction permeability was initially observed in the mammalian embryo (31), in the early amphibian embryo (32), at the segmental border in insect epidermis (33), and in the unidirectional transfer of dye in neurons (34). However, the molecular mechanisms responsible for channel selectivity have not been previously identified. In keratinocytes, calcium is likely to modulate GJC in at least two ways—indirectly, by inducing E-cadherin expression that brings cells close together (35, 36) and directly, as we report here, by altering connexin gene expression.

The influence of other factors on the observed changes in gap junction permeability should be considered. The fact that very well-coupled (electrically) differentiated keratinocytes did not transfer dye, whereas very poorly coupled undifferentiated cells did suggests that the changes in GJC cannot be accounted for by a reduction in gap junction numbers. Also, no substantial increase in gap junction plaques was observed by electron microscopy (data not shown). Furthermore, we measured cell size by confocal microscopy and found that the keratinocytes undergo a 30% increase in volume during differentiation. Such an increase in cell size cannot account for the differences in dye transfer. If junctional conductance remained constant, the rate of dye transfer should have been reduced by only 30%. In proliferating cells, dye spread was observed in <10 min after injection, but transfer was not observed in the differentiating cells even 1 hr after injection. Thus, a more plausible explanation for our results is that the different channel permeabilities are due to the specific switch in gap junction protein expression. In fact, recent data

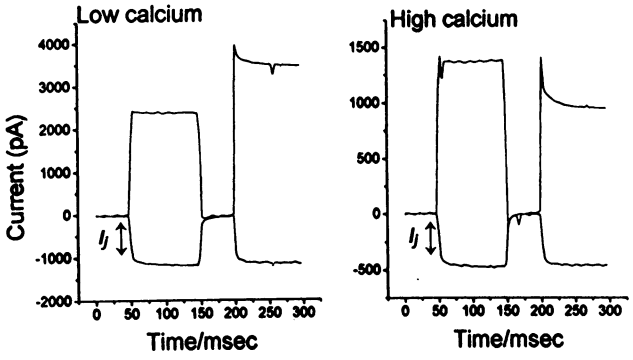

FIG. 4. Junctional conductances of keratinocyte cell pairs. Electrical properties of proliferating and differentiating keratinocytes (3 days after addition of 2 mM calcium) were measured by the double whole-cell patch-clamp method as described. Conductance measurements were obtained for 13 cell pairs grown in low-calcium medium and 5 cell pairs grown in high-calcium medium for 3 days. The average conductance for 13 cell pairs grown in low-calcium medium was 20 nS. In 9 out of 10 of these 13 pairs, dye transfer was seen. For the three remaining pairs no record of dye transfer was made before the experiment was terminated by loss of one or more seals. The lowest conductances observed were 1–2 nS, whereas the highest junctional conductance was 73 nS. In differentiating cell pairs, the average junctional conductance was 17 nS with a population SE of 9.7 nS. Conductances ranged from 5.6 nS to 32 nS. Dye transfer was not seen between any of these cell pairs. Although the average conductance of the low-calcium pairs was greater than that of a high-calcium pair, a given high-calcium cell pair was often found to have a higher conductance than a given low-calcium cell pair. The tracings show the current responses (with holding currents subtracted) of the cells in low calcium (*Left*) and high calcium (*Right*). The junctional current (I_j) is indicated.

suggests that restoration of α_1 expression in differentiating keratinocytes is sufficient for Lucifer yellow dye transfer (J.L.B., unpublished work).

While all previous studies have reported differences solely in single channel conductances, we demonstrate here that expression of different gap junction gene products results in different permeabilities. Consequently, there is now an opportunity to identify the molecular mechanisms responsible for differences in junctional permeability throughout this multigene family. Secondly, this work demonstrates that a change in junctional permeability is associated with a specific biological event, that being differentiation of keratinocytes. Several parameters of keratinocyte differentiation (including growth arrest, cornified envelope formation, keratin 1 and 10 expression, and transglutaminase activity) are induced in primary cultures within 24 hr of calcium exposure, and these parameters progressively increase thereafter (8, 9). Thus, both the biochemical and morphological changes characteristic of keratinocyte differentiation *in vitro* correlate with the progressive down-modulation of α_1 and β_2 gap junction mRNA and induction of β_3 and β_4 gap junction mRNA expression. The profile of α_1 gap junction expression observed *in vitro* is also consistent with the localization of the α_1 gap junction protein in the lower epidermal layers *in vivo* (19, 37). The patterns of β_2 and β_3 expression *in vitro* and *in vivo* are harder to reconcile. In newborn rodent skin, the β_2 gap junction protein was variably localized to the intermediate (19) or upper epidermal layers (37), whereas the β_3 protein was below detectable levels and found only at later developmental times in the sebaceous glands (19). In culture, however, the β_2 protein was found in growing keratinocytes, and β_3 protein was induced in differentiated cells. This difference could be attributed to the greater sensitivity of the biochemical methods used in the present study or alterations of gene expression due to cultivation. Future studies may identify the precise relationship that exists between the modulation of GJC and keratinocyte differentiation, as well as the specific cytoplasmic molecules that regulate this process by moving from cell to cell.

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