Multiple gap junction genes are utilized during rat skin and hair development

BORIS RISEK, F. GEORGE KLIER and NORTON B. GILULA*  
Department of Cell Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, USA  
*Author for correspondence

Summary

The expression of four different gap junction gene products (α1, β1, β2, and β3) has been analysed during rat skin development and the hair growth cycle. Both α1 (Cx43) and β2 (Cx26) connexins were coexpressed in the undifferentiated epidermis. A specific, developmentally regulated elimination of β2 expression was observed in the periderm at E16. Coinciding with the differentiation of the epidermis, differential expression of α1 and β2 connexins was observed in the newly formed epidermal layers. α1 connexin was expressed in the basal and spinous layers, while β2 was confined to the differentiated spinous and granular layers. Large gap junctions were present in the basal layer, while small gap junctions, associated with many desmosomes, were typical for the differentiated layers. Although the distribution pattern for α1 and β2 expression remained the same in the neonatal and postnatal epidermis, the RNA and protein levels decreased markedly following birth. Hair follicle development was marked by expression of α1 connexin in hair germs at E16. Following β2 detection at E20, the expression increased for both α1 and β2 in developing follicles. A cell-type-specific expression was detected in the outer root sheath, in the matrix, in the matrix-derived cells (inner root sheath, cortex and medulla) and in the dermal papilla. In addition, α1 was specifically expressed in the arrector pili muscle, while sebocytes expressed both α1 and β3 (Cx31) connexin. β1 connexin (Cx32) was not detected at any stage analysed. The results indicate that multiple gap junction genes contribute to epidermal and follicular morphogenesis. Moreover, based on the utilization of gap junctions in all living cells of the surface epidermis, it appears that the epidermis may behave as a large communication compartment that may be coupled functionally to epidermal appendages (hair follicles and sebaceous glands) via gap junctional pathways.

Key words: gap junctions, skin, hair, rat, connexin.

Introduction

Gap junctions (GJs) are specialized clusters of plasma membrane channels that provide pathways for bidirectional movements of small molecules (metabolic coupling) and ions (electrical coupling) between adjacent cells (Gilula et al., 1972). Thus, GJs provide the mechanism for a direct cell-to-cell transfer (GJ-mediated intercellular communication, GJIC) of molecular information by forming functional syncytia, also known as ‘communication compartments’ (Lo and Gilula, 1979; Pitts and Kam, 1985). Consequently, GJIC might play a role in the coordination of physiological processes that take place during development, regeneration, proliferation and differentiation (for review, see Guthrie and Gilula, 1989).

Molecular characterization of cDNA clones encoding GJ proteins (connexins) has provided evidence for a multigene family of related proteins forming intercellular channels (for recent review, see Kumar and Gilula, 1992). Based on the amino acid sequences in combination with topological analysis, it has been possible to propose a general topology for connexin structure. Accordingly, each polypeptide subunit contains four transmembrane domains with cytoplasmic amino and carboxy termini. A hemichannel (connexon) is an oligomer of six subunits in a single cell membrane, while a complete GJ channel is formed by association of two connexons, each contributed by a different cell. Although a large body of information has emerged regarding the structure and diversity of GJ gene products, little is known about the functional properties of the individual products and their biological significance. A cell-type-specific coexpression, as well as a correlation between connexin expression and germ-layer origin, was reported for three members of the GJ multigene family (Risek and Gilula, 1991). The effort to describe connexin expression in terms of cell-type specificity and germ-layer origin has been incomplete and inconclusive, since new members of the GJ multigene family are still being identified.

Although the morphological changes during embryonic and fetal skin development are well documented, little is known about biochemical events that accompany the differentiation of the ectodermally derived single-layered epithe-
limum into a highly organized, multilayered squamous epidermis. Thus far, keratin proteins are the best characterized cellular markers whose expression coincides with the course of epidermal development and terminal differentiation (Fuchs and Green, 1980; Banks-Schlegel, 1982). Ultrastructural identification of GJs in different compartments of wool follicles (Orwin et al., 1973), as well as in developing and mature human skin (Breathnach, 1971; Gabbiani et al., 1976; Caputo and Pelucchetti, 1977), indicates that GJs may play a role in the synchronization of processes that take place during the growth, differentiation and remodeling of the epidermis and epidermal appendages. Supportive evidence for a regulatory role of GJs in epidermis comes from a dye-transfer analysis of communicating compartments in the newborn mouse (Kam et al., 1986; Pitts et al., 1987) and adult human skin (Salomon et al., 1988). However, since these studies were performed prior to the discovery of the GJ multigene family, it was not known which GJ genes are utilized during skin development.

Based on a recent report describing a coexpression of α1 and β2 connexins in developing rat skin (Risek and Gilula, 1991), the present study was undertaken to define more clearly the contribution of GJ gene products to cutaneous biology. By using a combination of immunohistochemistry, electron microscopy, immunoblot and northern blot analysis, GJ expression was analysed during the following relevant stages of skin development: (a) differentiation of embryonic stem cells into epidermis and hair follicles, (b) epidermal and follicular development and (c) the hair growth cycle.

Materials and methods

Animals and tissue collection

Timed pregnant Wistar rats (220-250 g body weight), with a gestational period of 22 days, were obtained from Simonsen (Gilroy, CA). The presence of a uterine plug was defined as day 0 of gestation. The animals were maintained individually on a 12 hour light/dark cycle, and killed by decapitation at the following stages of embryonic development: E12, E14, E16, E18, E19, E20 and E21. Three pregnant animals were used at each gestational stage. For postnatal development, rats were killed at 3, 6, 9, 12, 16, 19, 25, 40 and 55 days of age reflecting different stages of the first and second hair growth cycle.

Isolation of fetal skin

Entire fetuses (n=5) were used for immunohistochemistry at early and mid stages of development (E12-E16). At later stages, ventral and dorsal skin (n=5) was removed intact, and used separately for immunohistochemistry. For protein and transcript analysis, skin (dorsal and ventral) was collected from 20 fetuses, divided into two equal parts, frozen in liquid nitrogen and stored at -70°C.

Skin isolation from newborn and postnatal rats

Pieces of mid-dorsal skin were used for immunohistochemical analysis. Skin was isolated from 10 newborn and 3-day-old (d+3) animals, from 6 animals at each stage until 19 days of age, and from four animals for d+25, d+40 and d+55 developmental stages. Rats older than 6 days were shaved before skin isolation.

Immunohistochemistry

Whole embryos (E12, E14 and E16) or pieces of skin (ventral and dorsal for E18 and E20 stages, dorsal for newborn and postnatal stages) were embedded in OCT compound (Tissue-tek, Miles Lab., Inc., Naperville, IL) and frozen in an isopentane/dry-ice bath. Skin samples were appropriately oriented during the freezing procedure to obtain longitudinal sections of hair follicles. Fresh frozen tissues were stored at -70°C until use. Samples were sectioned on a cryostat (3 to 5 μm in thickness), collected on gelatinized slides, and processed for indirect immunohistochemistry by using α1S, β1S, β2J and β3S peptide antibodies. The preparation and characterization of affinity-purified α1S, β1S, and β2J peptide antibodies has been described (Milk et al., 1988; Risek et al., 1990). β3S peptide antibodies were prepared by immunizing rabbits with a synthetic peptide (12 mer) conjugated to keyhole limpet hemocyanin (Pacific Biomarine Labs, Venice, CA) as described by Milk et al. (1988). The peptide sequence (NH2-DLEAVPADDKLQ-COOH) corresponds to the carboxy terminus of the β3 (connexin31) gap junction protein as determined by analysis of a rat cDNA clone (Hoh et al., 1991). This sequence extends from residues 250 to 261. Peptide antibodies were affinity-purified using the β3S peptide as a matrix, and analyzed for reactivity and specificity on frozen sections of rat skin and placenta, the two organs containing β3 RNA as reported by Hoh et al. (1991). These antibodies bound to antigen in placenta (data not shown) and in sebocytes of rat skin (this study). The specificity was demonstrated by the addition of β3S peptide (50 μg/ml) to the incubation reaction to compete with the immunolabel. Following characterization, peptide antibodies, as well as corresponding preimmune IgG molecules, were routinely used for indirect immunohistochemistry (Risek et al., 1990). Mounted specimens were examined on a Zeiss Axioskop microscope equipped with epifluorescence. All photographs were recorded with Kodak T-MAX 400 black-and-white film.

Immunoblot analysis

Following alkali-extraction (Hertzberg, 1984) of frozen skin and protein determination (Lowry et al., 1951), homogenates were solubilized in a loading buffer (by vortexing at room temperature for 60 minutes), and separated by a 12.5% SDS-PAGE using the procedure of Laemmli (1970). Some gels were stained with Coomassie blue, while others were subjected to immunoblot analysis as described by Milk et al. (1988). For quantitative analysis of α1 and β2 GJ proteins at each developmental stage, the nitrocellulose blots were incubated sequentially with α1S and β2J peptide antibodies. Following localization of the 43kDa M1 protein by 125I-protein A to the 43 and 26kDa M1 protein. For determination of bound 125I-protein A to the 43 and 26kDa M1 proteins, respectively, the nitrocellulose blot was cut 0.5 cm below the 43x10^3 M1 band, and the lower portion of the membrane was reincubated with β2J peptide antibodies. The immunoblotted 26x10^3 M1 protein was localized with the same procedure as the 43x10^3 M1 protein. For determination of bound 125I-protein A to the 43 and 26x10^3 M1 proteins, respectively, the nitrocellulose blot was cut 0.5 cm below and above the center of the signal, and measured in a γ-counter (LS 3801, Beckman). The data were corrected by subtracting the ‘background’ value, which corresponded to the non-specifically bound radioactivity of an area of membrane that was equivalent in size to the area used for the bound antibody/antigen complex determination.

Isolation of total RNA and normalization of poly(A)+ RNA

The second portion of the skin samples was used for RNA isolation. Frozen skin was pulverized in liquid nitrogen, homogenized in guanidine isothiocyanate buffer (Fishier), and sedimented through a CsCl gradient by ultracentrifugation (Chirgwin et al., 1979). The RNA concentrations were determined by absorbance at 260 nm, and normalized for poly(A)+ RNA content (Risek et al., 1990) using the procedure of Harley (1987). The concentrations were then adjusted according to the results of oligo(dT)
hybridization in order to apply equal amounts of poly(A)+ RNA for northern blot analysis. Finally, the content of the normalized total RNA samples was verified by a second oligo(dT) hybridization.

**Northern blot analysis**

Total RNA aliquots containing equal amounts of poly(A)+ RNA were separated by electrophoresis using 1% agarose/0.6 M formaldehyde gels, stained with ethidium bromide and transferred to nylon membranes (MSI). Following baking for 2 hours at 80°C under vacuum, the membranes were hybridized with two different GI cDNA probes: (a) a rat αGI cDNA: a clone isolated from a rat granulosa cell cDNA library (Risek et al., 1990) coding for a 4.3×10^6 Mr protein; and (b) a mouse β2 GI cDNA: a clone isolated from a mouse liver cDNA library (Nishi et al., 1991) that codes for a 2.6×10^5 Mr protein. In addition, blots were rehybridized using two human cDNA probes coding for glucose-6-phosphate dehydrogenase (Uirano and Beutler, 1988) and β-actin (Gunning et al., 1983), respectively. Hybridization and washing conditions were essentially the same as described previously (Risek et al., 1990). Blots were exposed at −70°C using Kodak XAR-5 film with an intensifying screen at variable time intervals to determine the linearity in X-ray response. Signal intensities were quantified by densitometry using an LKB laser scanner (Ultrascan IL, Bromma, Sweden).

**Thin-section and freeze-fracture electron microscopy**

Dorsal skin from various developmental stages was cut into small pieces (approx. 1×2 mm) and fixed either in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.4 (samples for thin sections), or in 5% paraformaldehyde/PBS (samples for freeze-fracture) for 3 hours at room temperature. The fixed tissues were rinsed several times in the corresponding buffer and processed for thin-section or freeze-fracture electron microscopy, respectively.

**Thin-sections**

For thin-sections, the samples were subsequently treated with OsO₄, tannic acid, uranyl acetate (en bloc) and embedded in Epon. The sections were cut with a diamond knife on an LKB Ultratome V, mounted on copper grids and post-stained with lead citrate.

**Freeze-fracture**

Tissues were treated for 2-4 hours at room temperature with 25% glycerol in 0.1 M sodium cacodylate buffer (pH 7.4) before rapid freezing in Freon 22 and storage in liquid nitrogen. The samples were freeze-fractured at −120°C and replicated with a Balzers BAF 400 (Balzers AG, Liechtenstein). The platinum-carbon replicas were floated on sodium hypochlorite (Fisher) followed by distilled water, and mounted on 400 mesh grids (Pelco). All electron microscope observations were made with a Hitachi H600.

**Results**

**Development of rat skin**

The development of rat skin, with particular emphasis on the epidermis and hair follicles, was analysed morphologically using hematoxylin- and eosin-stained fresh-frozen sections of dorsal and ventral rat fetal skin at different developmental stages. Morphological characteristics were used to monitor the dynamic changes and to identify the four prenatal stages of epidermal development according to the criteria described for human epidermal development (Dale and Holbrook, 1987). The prenatal development of rat skin was subdivided into four stages by correcting the duration of each prenatal stage for the gestational period of 22 days in the rat: (1) the embryonic period (from 12 to 14 days of gestation; E12-E14); (2) the period of epidermal stratification (E14-E16); (3) the period of follicular keratinization (E16-E18); and (4) the period of interfollicular keratinization (E18-E20).

At E12, the developing ventral and dorsal epidermis was identified as a double-layered, undifferentiated epithelium consisting of an inner (basal) and outer (periderm) layer (Fig. 1A). Stratification occurred around E14 in ventral regions, where the epidermis contained several layers (Fig. 1B). At E16, primary hair germs (the earliest morphologically recognizable precursors of hair follicles) were formed and condensation of dermal fibroblasts was observed around these structures (Fig. 1C). Subsequently, the fibroblasts were incorporated into a pocket, forming the aggregates of dermal papilla anlage (Fig. 1D). The E18 stage was characterized by the differentiation of the s. intermedium into spinous and granular layers, while the epidermal maturation was marked by the appearance of cornified cells below the degenerated periderm at E20 (period of interfollicular keratinization; Fig. 1E). The differentiated spinous and granular layers were very prominent, and the epidermis reached its maximal thickness at this time (except for the s. corneum, see below). The follicular buds progressed through a rapid morphogenesis from E18 to E20. The elongated follicles contained short hair cones, and the dermal papilla was completely engulfed by the hair bulb (Fig. 1E). The epidermis of newborn rats resembled the E20 epidermis with regard to the thickness of cellular layers (Fig. 1F); however, the periderm disappeared completely and the cornified layer increased markedly in thickness. The postnatal stages shown in Fig. 1G-I were selected according to the period of the hair growth cycle in the rat (Butcher, 1934; Chase, 1954). At the mid-anagen (d+6), all hair follicles were developed and many contained fully keratinized hair that penetrated the epidermis (Fig. 1G). The s. corneum reached its maximal thickness, which amounted to more than one-half of the entire epidermis. The cellular layers of the epidermis decreased in thickness with progressing age, as shown in Fig. 1H for the late anagen (d+15) of the first hair growth cycle. Following this stage, morphological changes were no longer recognizable in the epidermis. Consequently, skin analysis during the resting period of the second growth cycle (telogen d+55) revealed a very thick cornified layer, while the cellular strata amounted to about one-third of the thickness of the entire epidermis (Fig. 1I).

**Expression of α₁ and β₂ connexins in pre- and postnatal skin**

(a) **Epidermis**

The results of the immunohistochemical analysis are illustrated in Fig. 2 and summarized in Table 1. The early epidermal development (E12-E14) was marked by coexpression of α₁ and β₂ connexins in the periderm, s. intermedium and s. basale (Fig. 2B-F). A developmentally specific downregulation of β₂ was detected in the periderm around E16, when α₁ appeared in the primordia of hair follicles. The differentiation of the s. intermedium into spinous and gran-
ular layers at E18 was reflected by high levels of $\alpha_1$ and $\beta_2$ antigens in the newly formed layers (Fig. 2K,L). Characteristic for the mature epidermis at E20 was the absence of $\beta_2$ in the basal layer (Fig. 2O). $\beta_2$ antigen was detectable in the hair cone and in the outer root sheath surrounding the hair bulb of some follicles (data not shown). Thus, in contrast to the coexpression of $\alpha_1$ and $\beta_2$ during early epidermal development, $\alpha_1$ and $\beta_2$ connexins were differentially expressed in basal and apical strata of the mature epidermis. The differential expression coincided with the maturation and differentiation process at the end of the interfollicular keratinization period.
α₁ and β₂ connexins were analysed in the interfollicular epidermis at different anagen stages (early, mid and late anagen) and at one resting stage (telogen at d+25) of the first growth cycle to correlate cyclic changes of the epidermal GJ expression during different stages of follicular growth. For the purpose of this analysis, one growing stage (anagen at d+40) will be compared to one resting stage (telogen at d+55) of the second growth cycle. Relative to the late fetal stage (E20), the epidermis of newborn and postnatal rats was marked by a dramatic reduction of α₁ and β₂ antigen. This reduction, which reflected the program of terminal differentiation in the fully developed epidermis, was marked by low levels of α₁ in the proliferating basal and early differentiating spinous layers, while β₂ was confined to the differentiated spinous and granular layers (Fig. 2Q,R). However, at resting stages of the hair cycle, β₂ antigen was also observed in some regions of the basal layer (Table 1). Connexins were not observed in the terminally differentiated s. corneum.

The immunohistochemical results of the E20 epidermis were consistent with an ultrastructural analysis by freeze-fracture and thin-section electron microscopy (Fig. 3). Gap junctions were observed in all cellular layers with the highest frequency observed in the basal layer (Fig. 3A,B). Basal junctions were larger in size than those observed in the spinous and granular layers (Fig. 3C,D), and they were not associated with desmosomes. Desmosomes were present in all cellular strata of the epidermis, but with a higher frequency in the spinous and granular layers.

(b) Hair follicles

The results of the follicular α₁ and β₂ expression during the growing and resting stages of the first and second hair cycle are illustrated in Figs 4 and 5 and summarized in Table 1. Following parturition, the expression of both α₁ and β₂ connexins increased in early and mid anagen hair follicles with appearance of α₁ in dermal papilla at d+10. Fig. 4 illustrates a cell-type-specific use and distribution of α₁ and β₂ connexins in the lower and middle portions of active hair follicles at the late anagen stage (d+15), when follicles were attaining their maximal size. α₁ was coexpressed with β₂ in the outer root sheath, in partially keratinized layers of the inner root sheath, as well as in the cortex and proliferating matrix. A cell-type-specific expression was observed for α₁ in fibroblasts of the dermal papilla, and for β₂ in the medulla of hair.

Following the growth period, quiescent follicles containing club hair were formed by processes that are not well understood. Disintegration of the inner root sheath and a dramatic reduction in size (which amounted to about one-third of its previous length) were characteristic features of the resting follicles. The medulla was no longer present, and hair growth ceased due to termination of mitotic activity of the matrix cells. A brush-like, anchoring club was formed at the base of the hair follicle, surrounded by a capsule of partially keratinized cells. The cells of the dermal papillae were more condensed and formed rounded clusters. The expression of α₁ in resting hair follicles of the first growth cycle (telogen, d+25) is illustrated in Fig. 5B. Note that β₂ was not detectable in resting follicles (Fig. 5C).

The follicles re-entered the growth period of the next cycle by downgrowth of the secondary germ, while an elongated column of epithelial cells developed in the opposite direction. The newly formed matrix redifferentiated, giving rise to the layers of the inner root sheath and hair shaft (cuticle, cortex and medulla). The expression pattern of α₁ and β₂ connexins in growing hair follicles during the second growth cycle (anagen at d+40) was essentially the same as described for the anagen of the first growth cycle at d+15 (Fig. 5E,F). By analogy, the follicular expression of α₁ and β₂ connexins during the resting period of the second hair cycle (telogen at d+55) resembled the resting period of the first cycle at d+25 (Fig. 4H,I).

(c) Sebaceous gland and arrector pili muscle

The sebaceous gland developed as an outgrowth of the follicular outer root sheath during early postnatal development. At about the same time, the arrector pili muscle was recognizable as bundles of smooth muscle fibres attached to the connective tissue sheath surrounding the hair follicle. The formation of the pilosebaceous apparatus, together with the arrector pili muscles at mid anagen, was marked by expression of α₁ and β₂ connexins in sebaceous glands and α₁ in smooth muscle (Table 1). The expression levels for both α₁ and β₂ increased slightly during glandular maturational growth, with no detectable changes at different stages of the hair growth cycle (Fig. 6). α₁ and β₃ connexins were coexpressed in peripheral sebocytes, while the more differentiated, centrally located cells were marked by β₃ expression only. In contrast to the extensive α₁ staining, the β₃ immunostaining was more limited, indicative of small β₃-containing GJ plaques. The α₁ expression in the arrector pili muscle was relatively high with non-detectable changes during postnatal development, and it remained constant at...
Fig. 2. Immunolocalization of $\alpha_1$ and $\beta_2$ connexins in fetal and adult rat skin. Fetuses at different stages of development were fresh frozen, sectioned and treated with either $\alpha_1$S or $\beta_2$J peptide antibodies. (A-C) Embryonic period (E12): $\alpha_1$ and $\beta_2$ antigen in the double-layered, undifferentiated epithelium. (D-F) Period of epidermal stratification (E14): $\alpha_1$ and $\beta_2$ in the multilayered epidermis. (G-I) Period of follicular keratinization (E16): $\alpha_1$ in the periderm, s. intermedium, s. basale, and in the primary hair germ; $\beta_2$ was present in the s. intermedium and basal layer. (J-L) Period of interfollicular keratinization (E18): $\alpha_1$ in basal, spinous and granular layers, and in the developing hair follicle; $\beta_2$ was detectable in the basal (arrow) and differentiated spinous and granular layers. (M-O) Period of epidermal cornification (E20): $\alpha_1$ in the basal, spinous and granular layers, and in the growing hair follicle; $\beta_2$ was present in the spinous and granular layers. Note the absence of $\beta_2$ in the basal layer and in the follicle. (P-R) Mature epidermis (d+40): $\alpha_1$ was present in the basal and spinous layers, and $\beta_2$ in the spinous and granular layers. Scale bar, 50 µm.
Gap junction genes and skin development

Analysis of $\alpha_1$ and $\beta_2$ GJ proteins in developing rat skin

Skin samples at different stages of development were analysed by immunoblotting in order to compare with the results of immunohistochemical analysis. A semiquantitative comparison was made for the different developmental stages by using equal amounts of alkali-insoluble material for immunoblotting.

Coomassie blue staining revealed a large amount of partially insoluble material with an apparent relative molecular mass of about $140 \times 10^3 \ M_r$, which increased in abundance with age (Fig. 7A). The immunoblot analysis using $\alpha_1S$ and $\beta_2J$ peptide antibodies revealed dynamic modulations of $\alpha_1$ and $\beta_2$ expression (Fig. 7B,C). The levels of both $\alpha_1$ and $\beta_2$ proteins were markedly reduced during the transition from fetal to neonatal skin (for relative comparison between different stages, the amount of newborn (NB) sample was defined as ‘1’; Fig. 7D). Following a further decrease during early postnatal development (d+3), the protein levels increased gradually during growing stages of the first hair cycle. After reaching maximal levels at late stages (d+12 through d+16), both proteins decreased in abundance during the period of follicular regression (catagen, d+19), and reached their lowest levels at the resting period (d+25). $\alpha_1$ and $\beta_2$ were high in abundance during the growing period of the second hair cycle (anagen at d+40), and decreased to the level of NB skin during the resting period (telogen at d+55) of the second growth cycle.

Analysis of $\alpha_1$ and $\beta_2$ GJ RNA in developing rat skin

The expression of $\alpha_1$ and $\beta_2$ connexins was extended to the RNA level using northern blot analysis. The earliest stage analysed was E19; the other stages were the same as those
selected for protein analysis. To compare the transcript levels between different developmental stages, the transcript level in the newborn (NB) skin sample was defined as ‘1’ (Fig. 8). The α1 (3.3 kb) and β2 (2.8 kb) RNA profiles resembled very closely the corresponding protein pattern. Both transcripts were elevated in fetal skin, low during early postnatal development and increased gradually during the growing period of the first hair cycle. Following the maximal abundance (6.5-fold for α1, and 6.0-fold for β2) at d+16, the levels declined during the period of follicular regression (d+19), to the lowest values at resting stage (d+25). The α1 and β2 RNAs analysed at two representative stages of the second growth cycle (anagen at d+40 and telogen at d+55), were modulated similarly to that observed during the growing and resting stages of the first growth cycle. Following hybridization with the α1 and β2 GJ cDNA probes, the same blots were rehybridized with human cDNA probes for glucose-6-phosphate dehydrogenase (G6PDH) and β-actin. The expression pattern of the G6PDH RNA (2.8 kb) is shown in Fig. 8D,F. This RNA was modulated in the same manner as the α1 and β2 transcripts, except that the changes were less pronounced. The reprobing of the northern blots with the human β-actin cDNA resulted in detection of muscle (1.4 kb, α-actin) and non-muscle (2.0 kb, β- and γ-actin) RNA isomers (Fig. 8E,F). The changes of muscle (derived from skeletal and smooth muscle) and non-muscle actin isomers at the RNA level reflected the relative contribution of muscle and non-muscle tissue to the total poly(A)+ RNA pool. The increased amount of α-actin at the resting stages of hair growth was most likely not due to a higher expression level of this RNA, but rather due to a greater contribution of muscle tissue mass to the total poly(A)+ RNA at times when the contribution of hair follicles was low. Conversely, the contribution of muscle mass to the total poly(A)+ RNA pool was lower during the follicular growth period. The profile of total poly(A)+ RNA, as averaged from five different experiments, is shown in Fig. 8F. Ideally, since equal amounts of total poly(A)+ RNA were used for this analysis, the resulting profile should have been a straight line. The fluctuations of about 1.5-fold above the NB level at fetal stages, and at d+12 through d+19 reflect the range of experimental error. Changes in RNA levels within this range should not be regarded as experimentally or developmentally relevant.

**Discussion**

The results of the present study are integrated in Table 1 and Fig. 9. Several novel observations have resulted from analysing the sequence and pattern of connexin expression during rat skin development. First, α1 and β2 connexins were differentially expressed during the morphogenesis of epidermis and hair follicles. Both α1 and β2 connexins were coexpressed in the undifferentiated epidermis at early fetal stages (<E16), whereas a differential expression coincided with the epidermal differentiation and maturation process. Second, both α1 and β2 connexins were coexpressed in pluripotential stem cells of the embryonic basal layer before their commitment to differentiate into epidermal and follicular compartments. The differential expression coincided with the epidermal and follicular differentiation pathway. Third, the follicular expression levels of α1 and β2 connexins were modulated in relation to the hair growth cycle.

**Fig. 3.** Freeze-fracture and thin-section electron micrograph of fetal rat epidermis at E20. Large gap junctions (gj) were detected in the basal layer (A,B), while smaller gap junctional structures were present in the spinous and granular layers (C,D). Note the association of a gap junction with desmosomes (d) in the differentiated epidermal layers. Scale bar, 200 nm.
Gap junction genes and skin development

The resting stages were marked by the absence of \( \beta_2 \) connexin in hair follicles.

Expression of connexins during morphogenesis of epidermis and hair follicles

The expression of GJ proteins was intimately related to the morphologic changes during both the ontogenesis and the terminal differentiation of the epidermis and hair follicles. The embryonic and the early fetal stages (<E16) were marked by the coexpression of \( \alpha_1 \) and \( \beta_2 \) connexins in the basal and intermediate cell layer, as well as in the periderm. The initial evidence for a developmentally regulated GJ expression was obtained at E16 (period of follicular keratinization), when \( \beta_2 \) was specifically down-regulated in the periderm. This stage-specific elimination of \( \beta_2 \) expression probably represents a differential regulatory mechanism, since \( \alpha_1 \) expression was not affected in the periderm at this stage. The peridermal \( \alpha_1 \) expression persisted until this layer began to degenerate at about E18. The E18 stage was one of the most decisive stages during the course of epidermal morphogenesis, since at this time the rather undifferentiated s. intermedium differentiated into spinous and granular layers with a concomitant restriction of proliferative activity to the basal layer. These events were clearly reflected at the level of connexin expression. While the differentiation of the intermediate layer was accompanied by high levels of \( \alpha_1 \) and \( \beta_2 \) in the newly formed spinous and granular layers, the restricted proliferative activity of basal cells was reflected by a down-regulation of \( \beta_2 \) expression. The gradual down-regulation of \( \beta_2 \) in the basal layer, which resulted in the absence of \( \beta_2 \) connexin in these cells of the mature epidermis, is in accord with the loss of pluripotential properties of basal cells as suggested by Kopan and Fuchs (1989) based on the keratin expression pattern. Follicular morphogenesis was marked by a specific \( \alpha_1 \) expression in hair germs, which were morphologically recognizable at E16 as downgrowths of basal cells. This developmentally regulated, stage-specific onset of \( \alpha_1 \) expression persisted until about E20, when \( \beta_2 \) connexin appeared in the ORS of some advanced follicles. The expression pattern and the differential regulation of \( \alpha_1 \) and \( \beta_2 \) coexpression indicates that GJIC may play a fundamental role in the morphogenetic processes that occur during the course of epidermal and follicular proliferation, growth and differentiation. The potential role of GJ gene products in morphogenetic events, together with cell- and substrate-adhesion molecules, has been integrated in a unifying ‘morphoregulator’ hypothesis (Edelman, 1984; 1992). Furthermore, the modulation pattern of follicular GJ expression during the hair growth cycle suggests that GJIC may contribute to the mechanism/s controlling follicular morphogenesis. In this context, it is worthwhile to note the delay of \( \alpha_1 \) expression by dermal papilla cells (the expression occurred at about d+10), since these cells have inductive properties during both hair follicle formation and hair cycling (Davidson and Hardy, 1952; Oliver, 1966; Jahoda et al., 1984).

Fig. 4. Immunolocalization of \( \alpha_1 \) and \( \beta_2 \) connexins in hair follicles of a 15-day-old rat (late anagen). Oblique and longitudinal sections of hair follicles illustrating the distribution of \( \alpha_1 \) and \( \beta_2 \) antigen in different follicular compartments. (A,C) \( \alpha_1 \) antigen was present in the outer (ors) and inner root sheath (irs; Huxley’s, hx and Henle’s layer, hn), cortex (ct) and dermal papilla (dp). Note the absence of \( \alpha_1 \) in the cuticle (cu) and medulla (md). (B,D) \( \beta_2 \) was present in the outer and inner root sheath, cortex and medulla. Note the absence of \( \beta_2 \) in the cuticle and dermal papilla. Scale bar, 50 µm.
Fig. 5. Immunolocalization of $\alpha_1$ and $\beta_2$ connexins in hair follicles during different stages of the hair cycle (dorsal skin). (A-C) Hair follicles of a 25-day-old rat (telogen): $\alpha_1$ antigen was in the dermal papilla (dp), in the secondary hair germ (arrow), and in the outer capsule (arrowhead) of the club hair. Note the absence of $\beta_2$ antigen in the resting follicles. (D-F) Hair follicles of a 40-day-old rat (anagen of the 2. hair cycle): $\alpha_1$ was present in the hair bulb and in the differentiated cell layers of the inner root sheath and cortex (ct); $\beta_2$ was localized in the bulbar region and in differentiated cell layers of the inner root sheath, cortex and medulla (md). (G-I) Hair follicles of a 55-day-old rat (telogen of the 2. hair cycle): $\alpha_1$ was present in the dermal papilla, the tertiary hair germ (arrow), and the outer capsule (arrowhead) of the club hair. $\beta_2$ antigen was not detectable in the resting follicle. apm, arrector pili muscle; cu, cuticle; mx, matrix; sbg, sebaceous gland. Scale bar, 50 µm.
Gap junction genes and skin development

Possible physiological and developmental implications
The data from the present study indicate that GJs may mediate the metabolic cooperativity between the more and less differentiated cell types of the interfollicular and follicular epidermis. Accordingly, the epidermis and epidermal appendages, components forming the pilosebaceous unit (see Fig. 9), may be considered as a functional syncytium for the passage of small molecules and ions by utilizing $\alpha_1$ and $\beta_2$ GJ channels. Supportive evidence for a large epidermal communication compartment has been provided by ultrastructural identification of GJs in frog skin epithelium (Shahin and Blankemeyer, 1989) where the distribution, frequency and size of GJs were quite similar to the ultrastructural data as reported here for the E20 epidermis. However, observations have also been made that support the concept of small epidermal communication compartments (Kam et al., 1986; Pitts et al., 1988), as deduced from the pattern of GJIC in prenatal and neonatal mouse epidermis.

Fig. 6. Immunolocalization of $\alpha_1$ and $\beta_3$ connexins in the sebaceous gland and arrector pili muscle (dorsal skin, telogen d+55). Both $\alpha_1$ and $\beta_3$ were present in sebaceous gland (sbg), while only $\alpha_1$ was expressed in the arrector pili muscle (apm). Scale bar, 20 µm.

Fig. 7. Modulation of $\alpha_1$ and $\beta_2$ GJ protein levels in developing rat skin during different stages of the hair cycle. (A) Coomassie blue staining of NaOH-insoluble skin homogenates (50 µg protein/lane) isolated from prenatal, neonatal and postnatal rats. Marker proteins (110, 84, 47, 33, 24, and 16×10^3 Mr) are shown on the left. See Material and methods for immunoblot analysis and quantitation of bound radioactivity. (B) Immunoblot of a gel identical to that in A, incubated with affinity-purified $\alpha_1$S peptide antibodies. (C) Immunoblot of the lower portion of a gel identical to that in A, incubated with affinity-purified $\beta_2$J peptide antibodies. (D) $\alpha_1$ and $\beta_2$ protein abundance at different stages of skin development relative to the signal intensity detected in samples of newborn rats, which was defined as ‘1’.

Fig. 8. Modulation of $\alpha_1$ and $\beta_2$ GJ RNA levels in developing rat skin during different stages of hair cycle. Total RNA was isolated from prenatal, neonatal and postnatal rats and used for northern blot analysis following poly(A)^+ RNA normalization. (A) Ethidium bromide staining of total RNA (18 S rRNA is shown). (B) Detection of $\alpha_1$ RNA (3.3 kb), (C) $\beta_2$ RNA (2.8 kb), (D) glucose-6-phosphate dehydrogenase (G6PDH) RNA (2.8 kb) and (E) $\beta$- and $\alpha$-actin RNA (2.0 or 1.4 kb, respectively). Signal intensities were quantitated by densitometry following several film exposures for each transcript and graphically presented in F. (F) Signal intensities of different RNAs relative to the RNA sample of newborn rats, which was defined as ‘1’.
using the dye-transfer analysis. Electrophysiological studies should be useful in the future to define the actual size of a functional epidermal communication compartment. As originally noted by Orwin et al. (1973) analysing wool follicles, and also in this study, the follicular distribution of GJs indicates that intercellular communication may be compartmentalized within this structure. The compartmentalization appears to be a consequence of the keratinization process, since the loss of GJs occurred in the keratinized regions of the wool and hair follicles. Supportive evidence for a compartmentalized GJIC was recently provided by dye-transfer analysis of follicular communication compartments (Kam and Hodgins, 1992).

It is of interest to note the similarity of the β2 expression pattern in the developing epidermis and mature hair follicles to the glycogen distribution during mouse development (Hardy, 1952). This close similarity might indicate that β2 channels could provide an intercellular pathway for the transport of glucose and its metabolites. Furthermore, the rapid transport of radiolabeled amino acids throughout the bulbar regions of the hair (Bern et al., 1955) and wool follicles (Wilson et al., 1971) could be explained by the presence of direct intercellular communication pathways created by α1 and β2 GJ channels.

α1 connexin was constitutively expressed at high levels, independent of the hair cycle, in arrector pili muscles. In this region, α1 channels could provide low-resistance pathways between muscle cells for synchronous contraction. The contraction of pili muscles ultimately contributes to the vertical orientation of hair follicles. This constitutive expression should be compared with a rapidly inducible α2 expression in smooth muscle of the uterine myometrium at the onset of labor, when the smooth muscle contraction is required for the expulsion of the fetus (Risek et al., 1990; Risek and Gilula, 1991).

The results of the present study indicate that some basic questions related to the biological significance and the diversity of the GJ multigene family may be addressed by studying skin development. Some of these questions should focus on the control mechanisms for developmentally regulated, cell-type-specific coexpression of different GJ genes. The cyclicity and dynamic properties of follicular hair growth may provide an important experimental model for studying some significant aspects of GJ gene expression.

The authors are grateful for the technical assistance of Jessica Van Leeuwen, Robert Safarik, Jason B. Miller and John Leopart. We are also grateful to Dr Raphael Kopan and Dr Karen A. Holbrook for their constructive suggestions during the course of this study. This study was supported by grants from the National Institutes of Health (GM 37904) and Johnson and Johnson Co. (J-188).

References


(Accepted 24 July 1992)
Fig. 9 Schematic representation of a pilosebaceous unit illustrating the spatial expression of multiple GJ gene products at the anagen stage of the hair growth cycle.

Colour code: blue, $\alpha_1$; yellow, $\beta_2$; red, $\beta_3$; green, $\alpha_1 + \beta_2$; purple, $\alpha_1 + \beta_3$. Keratinized regions of the epidermis and hair follicles are shown in black. $\beta_1$ GJ antigen was not detected in the pilosebaceous unit.

Letter code: apm, arrector pili muscle; ct, cortex; cu, cuticle; dp, dermal papilla; hn, Henle’s layer; hs, hair shaft; hx, Huxley’s layer; irs, inner root sheath; md, medulla; mx, matrix; ors, outer root sheath; sb, stratum basale; sbg, sebaceous gland; sc, stratum corneum; sg, stratum granulosum; ss, stratum spinosum.