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Structural Proteins in Perinatal Rat Epidermis: Characterization of a High Molecular Weight Prekeratin

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ABSTRACT. The major water-insoluble proteins of perinatal rat epidermis have been examined by gel electrophoretic techniques. Particular focus has been placed on that family of epidermal structural proteins called keratins which are characterized by mol wt between 40 and 70 kD. Analysis of these proteins by 2-dimensional PAGE revealed the largest member of this family (M_r = 63 kD) to consist of a series of isoelectric variants with isoelectric points ranging between 7.3 and 5.9. Antibodies raised in rabbits against this protein were specific by immunoblot analysis and exhibited no cross-reactivity with keratins isolated from human foreskin epidermis under the same extraction conditions. Ontogenetic examination by Western blot was performed on extracts of whole fetal rat skin from d 17 to d 19 of gestation. Expression of the protein was seen only after the 18th gestational d. Posttranslational modification of neonatal rat keratins by phosphorylation was examined under *in vitro* conditions at two different ambient temperatures (23 and 37°C). Overall phosphorylation was markedly increased at the higher temperature. A similar qualitative pattern of keratin phosphorylation was seen after *in vivo* labeling at nest temperature (35°C). In both the *in vitro* and *in vivo* experiments, the major radiolabeled moiety was the 63 kD epidermal protein. In summary, insoluble proteins between 40 and 70 kD have been examined in perinatal rat epidermis. The tissue localization, solubility, phosphorylation status, ontogenetic appearance, and mol wt of the 63 kD protein are consistent with the identification of an epidermal prekeratin. We hypothesize that this protein is an important molecular precursor of stratum corneum formation in the perinatal rat. (*Pediatr Res* 25: 542–547, 1989)

Abbreviations

IEF, isoelectric focusing
pI, isoelectric point

The epidermis constitutes a resilient yet flexible tissue interface between the organism and the environment. Many of the important functions of this interface (water retention, high electrical resistance, barrier formation to microorganism invasion) are resident not in the deeper, living cell layers, but in the outermost, nonnucleated layer called the stratum corneum. The orderly process by which the stratum corneum is formed is generally called "keratinization" (1, 2).

In the premature infant this process of keratinization is incomplete (3). Consequently, the multiple functions subserved by a well-keratinized epidermis are missing or only partially present. The premature infant, therefore, experiences problems with increased water loss, heat conservation, risk of infection, and local trauma. Rational clinical management of these problems is enhanced by knowledge of the normal ontogeny and the sequence of molecular events underlying the keratinization process. Moreover, this information provides a logical basis for interfacing exogenous sensor devices with the developing skin.

Recently, we have focused on the perinatal rat as a model of integumental maturation and growth factor action (4–6). This animal model offers a major advantage for the investigation of cutaneous development in that the perinatal period is a time of orderly, hyperplastic epidermal growth and differentiation (7). Moreover, the hair follicles, which form an insulatory layer of fur in older animals, erupt after birth in the rat (8). Consequently, the state of the interfollicular epidermis in the late gestational and early neonatal rodent is comparable to that of the developing human infant. Although the neonatal rat is an important developmental animal model, it is, at present, much less studied in terms of keratin biology than the mouse or the human.

In the epidermis, the keratins form a general family of water insoluble proteins which aggregate to form intermediate filaments (9) during the process of keratinization. These intermediate filament subunits range in mol wt between 40 and 70 kD (10). When extracted under nonreducing conditions, they are collectively called "prekeratins" (11), although the generic term "keratin" is often used for routine purposes. In this study, we have focused on the 1D and 2D gel characterization and posttranslational modification (phosphorylation) of keratins in neonatal rat epidermis. In particular, we have examined the high mol wt (M_r = 63 kD) member of the keratin family as studies in other systems (mouse and human) have implicated the higher mol wt, basic keratins as specific markers of the keratinization process (10). The ontogeny of the high mol wt (63 kD) protein has been examined by immunoblot analysis using a specific anti-rat antibody which does not cross-react with human keratins. Finally, we report on the effect of ambient temperature to influence keratin phosphorylation as well as the relative patterns of *in vivo* versus *in vitro* keratin phosphorylation in neonatal rat epidermis.

MATERIALS AND METHODS

Tissue preparation. Timed gestation Sprague-Dawley rats were obtained on the 14th d of pregnancy from Zivic-Miller Laboratories Inc. (Zelienople Park, PA). Animals were housed with a 12-h light/dark cycle and given routine laboratory food *ad libitum*. Mothers routinely delivered on d 22 of gestation. Newborn

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pups were undisturbed until the time of experimentation. For experiments using fetal tissue, mothers were anesthetized with Metofane (Pitman-Moore Inc., Washington Crossing, NJ) on the indicated day of gestation. Pups were delivered by Cesarian section, and their skins were gently blotted dry. Neonatal or fetal rats were killed by decapitation, and sections measuring approximately 2.5 × 2 cm were excised from the dorsal surface. Hypodermal tissue was removed with forceps and the tissues were either prepared for epidermal separation or frozen on dry ice and stored at −20°C. Isolated epidermal samples were obtained by submerging the whole skin sections in normal saline at 60°C for 20 s as described by Baumberger *et al.* (12) or by incubation in 20-mM dithiothreitol for 20 min as described by Epstein *et al.* (13). The sections were blotted dry, and dermis and epidermis were separated with forceps. The epidermal samples were then washed once in saline, frozen on dry ice, and stored at −20°C.

Fresh human foreskins were obtained after routine circumcision, with the approval of the University of Cincinnati Institutional Review Board for Human Research. Epidermal samples were obtained by heat separation, processed, and stored as described above.

In vitro phosphorylation. Dorsal skin sections, prepared as above, were placed epidermal side down on a McIlwain tissue chopper (Brinkman Instruments, Westburg, NY) and cut into strips (20 mm × 1.0 mm). These strips were incubated for 60 min at 37°C on a rocker platform in magnesium-free 20 mM Tris-buffered Hanks' balanced salt solution containing 1-mM $CaCl_2$ and 200 μCi ^{32}P-orthophosphoric acid/mL. The strips were then washed in nonradioactive media, blotted dry, frozen on dry ice, and stored at −20°C. Isolated epidermal and dermal samples were obtained by making the radioactive media 20 mM in dithiothreitol for the final 20 min of incubation as described by Epstein *et al.* (13). The strips were then washed in nonradioactive media, and the epidermis was separated from the dermis with forceps. Tissues were washed, blotted dry, frozen on dry ice, and stored at −20°C.

In vivo phosphorylation. Pups were removed from their mothers and placed in a temperature-controlled isolette (C-86 incubator, Air-Shields) at 35°C for 1 h before injection. The dorsal area of each pup was injected subcutaneously with 300 μCi ^{32}P-orthophosphoric acid. After 4 h, the animals were removed from the isolette and immediately killed by decapitation, and epidermal samples were isolated by the heat-stripping method described above.

Protein extraction. Tissue samples, 100 mg or less, were homogenized on ice for 1 min in motor-driven Duall Tissue Grinders (size 020, Kontes, Vineland, NJ) using 10 vol of primary extraction buffer (50-mM Tris, pH 7.3, 190-mM NaCl, 1-mM phenylmethylsulfonyl fluoride). The homogenates were heated to 100°C for 5 min, then centrifuged for 30 min at 4000 × *g* and 4°C. The primary supernatants (containing Tris-soluble proteins) were removed and the pellets were homogenized (1 min on ice) in 10 vol of secondary extraction buffer (50-mM Tris, pH 7.3, 2% SDS, 1-mM phenylmethylsulfonyl fluoride) and centrifuged as above. Secondary supernatants were transferred to 1.5 mL Eppendorff tubes and stored at 4°C. Protein concentrations were measured by Peterson's modification of the Lowry method (14) using BSA as standard.

Gel electrophoresis. Proteins were separated according to their mol wt on 7.5% or 11% SDS-polyacrylamide slab gels using the discontinuous buffer system of Laemmli (15) as modified by Smith (16). Proteins were visualized by staining with Coomassie brilliant blue or by silver staining (17). Radioactive proteins were localized by autoradiography on Kodak XAR film (Eastman Kodak, Rochester, NY) after drying the stained gels in a Bio-Rad gel dryer (Bio-Rad Laboratories, Richmond, CA). Mol wt standards (Dalton Mark VII L from Sigma Chemical Co., St. Louis, MO, or ^{14}C-methylated proteins from Amersham International, Amersham Corp., Arlington Heights, IL), were used for mol wt estimation as described by Weber and Osborn (18). Two-dimensional electrophoresis (19) of the secondary extract was carried out using the modifications described by Sun and Green (20).

Antibody generation and immunoblotting. Antibody production and immunoblotting procedures were performed essentially as described by Kronenberg and Clark (21). Briefly, individual bands corresponding to the 63 kD protein were excised from 1D polyacrylamide gels and emulsified in Freund's adjuvant. The emulsified fragments were injected intradermally into multiple sites over the dorsal region of a female New Zealand White rabbit (Hazelton, Denver, PA). The animal was bled serially after the 4th wk.

RESULTS

Extraction of neonatal rat skin in 2% SDS under nonreducing conditions yields a series of distinct proteins in the mol wt region between 40 and 70 kD (Fig. 1). These proteins are insoluble in water and are localized exclusively to the epidermis (see Fig. 5). The solubility characteristics and M_r of this family of proteins are consistent with the known properties of the epidermal keratins. Analysis of these proteins by 2-dimensional PAGE (Fig. 2) revealed a series of isoelectric variants, with the higher mol wt species exhibiting numerous more basic forms. Examination of the 63 kD protein revealed a series of at least 5 isoelectric variants ranging in pI from 5.9 to 7.3.

Antibodies raised in rabbits against the 63 kD epidermal protein were immunologically specific as compared with other epidermal proteins and with keratins isolated from human neonatal foreskin epidermis (Fig. 3). No cross-reaction was observed with other tissue extracts or purified preparations of neurofilaments (data not shown). The ontogenetic expression of this protein was examined by Western blot analysis using homoge-

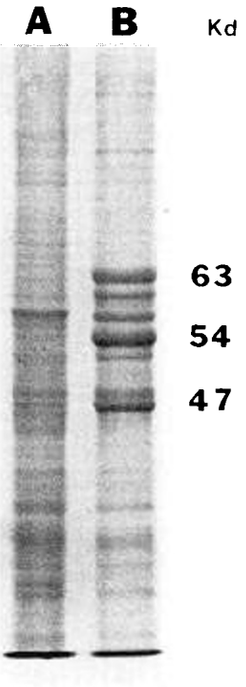

Fig. 1. One-dimensional electrophoresis of Tris-soluble (*lane A*) *vs.* Tris-insoluble (*lane B*) proteins extracted from the dorsal skin of newborn rat pups. Equal amounts of protein (200 μg) were electrophoresed on a 7.5% SDS-containing polyacrylamide gel and stained with Coomassie blue. The region of the presumptive epidermal keratins is shown at the far right with M_r between 63 and 47 kD.

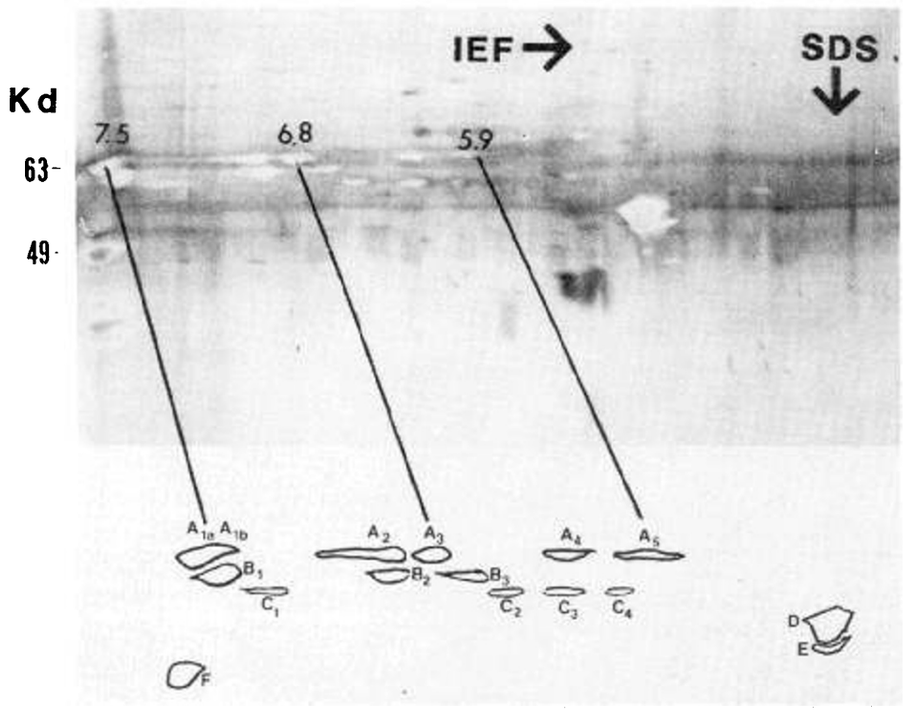

Fig. 2. Isoelectric variants of proteins isolated from the epidermis of a 1-d-old rat. Two-dimensional gel electrophoresis was performed on 100 μg of the sample using an 11% polyacrylamide slab gel as the second dimension. Proteins were separated by IEF in the first dimension as indicated. The identification of the silver-stained prekeratin isoelectric variants is shown schematically in the *inset*.

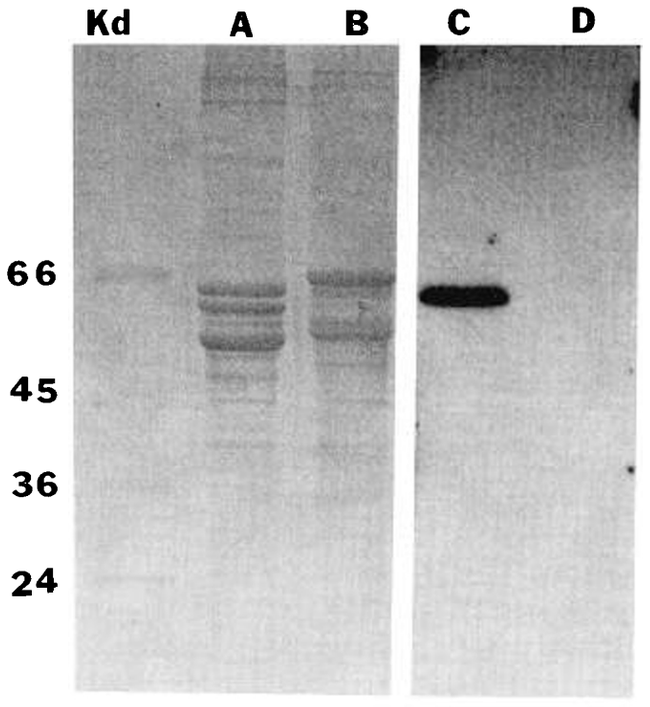

Fig. 3. Comparison of Tris-insoluble proteins isolated from newborn rat and human epidermis using SDS-PAGE and immunoblot analysis. Amounts of 75 μg of newborn rat (*lanes A* and *C*) or newborn human (*lanes B* and *D*) epidermal proteins were electrophoresed on an 11% SDS-polyacrylamide gel. *Lanes A* and *B* were stained with Coomassie blue. *Lanes C* and *D* were electrophoretically transferred to nitrocellulose, immunoblotted with rabbit anti-63 kD subunit antibody and ^{125}I-labeled protein A, then autoradiographed for 72 h.

nates of whole skin obtained from fetal rats of 17, 18, and 19 d of gestation. Whole skin, rather than epidermis, was used for this study because of the difficulty in obtaining pure epidermal isolates in the younger age groups. The results indicate the presence of the 63 kD epidermal protein beyond the 18th d of gestation (Fig. 4).

Differential phosphorylation of proteins was examined in epidermis, dermis, and whole skin sections from 1-d-old rat pups. As shown in Figure 5, incorporation of radiolabel was greatest into proteins of the epidermis with M_r between 69 and 46 kD. The two major phosphorylated epidermal proteins exhibited M_r of 63 and 51 kD, respectively. The overall pattern of phosphorylation was similar in isolated epidermis and in whole skin indicating that the epidermal stripping procedure had no major effect on phosphorylation status. 2D gels and autoradiograms were also performed on the phosphorylated samples (data not shown). The 63 kD phosphoprotein corresponded to the isoelectric variants marked A1-A5 in Figure 2. The most basic form was unlabeled.

The pattern of epidermal protein phosphorylation was also examined under *in vivo* conditions and contrasted with simultaneous experiments performed *in vitro*. Figure 6 shows representative autoradiograms obtained after epidermal exposure to ^{32}P under the two experimental test conditions. The *in vivo* experiment was performed with the animal maintained at 35°C in a temperature-controlled isolette. The effect of *in vitro* temperature alteration on phosphorylation status was examined and the results shown in Figure 7. As demonstrated, diminution of incubation temperature from 37 to 23°C resulted in a marked decrease in protein phosphorylation with no apparent change in the qualitative pattern of radiolabeling.

DISCUSSION

The epidermis of the newborn plays a significant role in perinatal physiology. This thin yet tough membrane forms the

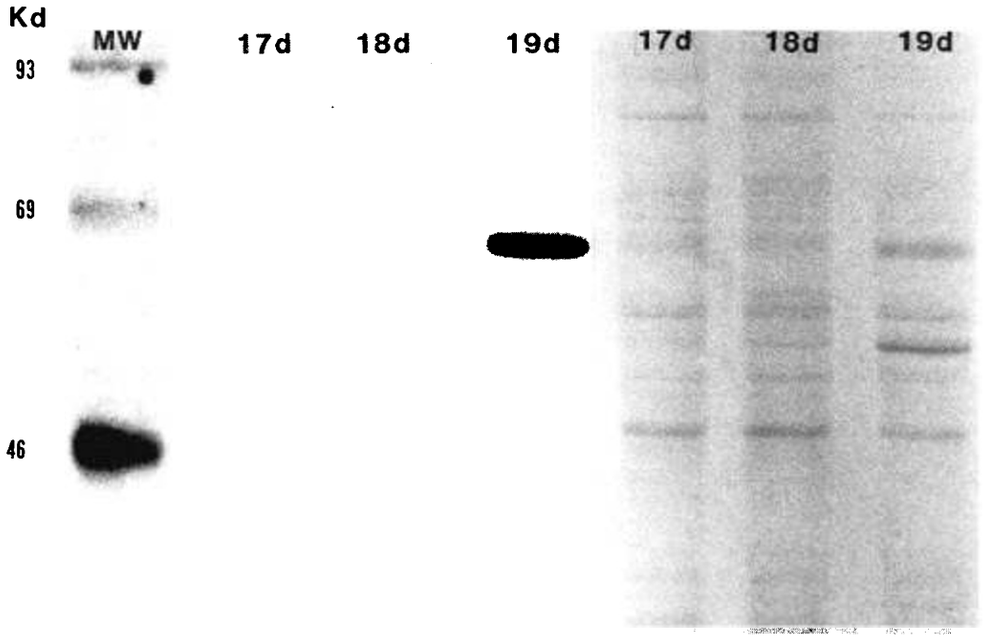

Fig. 4. Ontogenetic analysis by Western blot of the 63 kD epidermal protein in homogenates of whole skin from 17-, 18-, and 19-d gestation fetal rats. Amounts of 25 μg of protein from each homogenate were electrophoresed on 7.5% polyacrylamide gels. One gel was stained with Coomassie blue (*right panel*) and the other was transferred to nitrocellulose for immunoblotting with an antibody raised against the 63 kD keratin subunit (*left panel*).

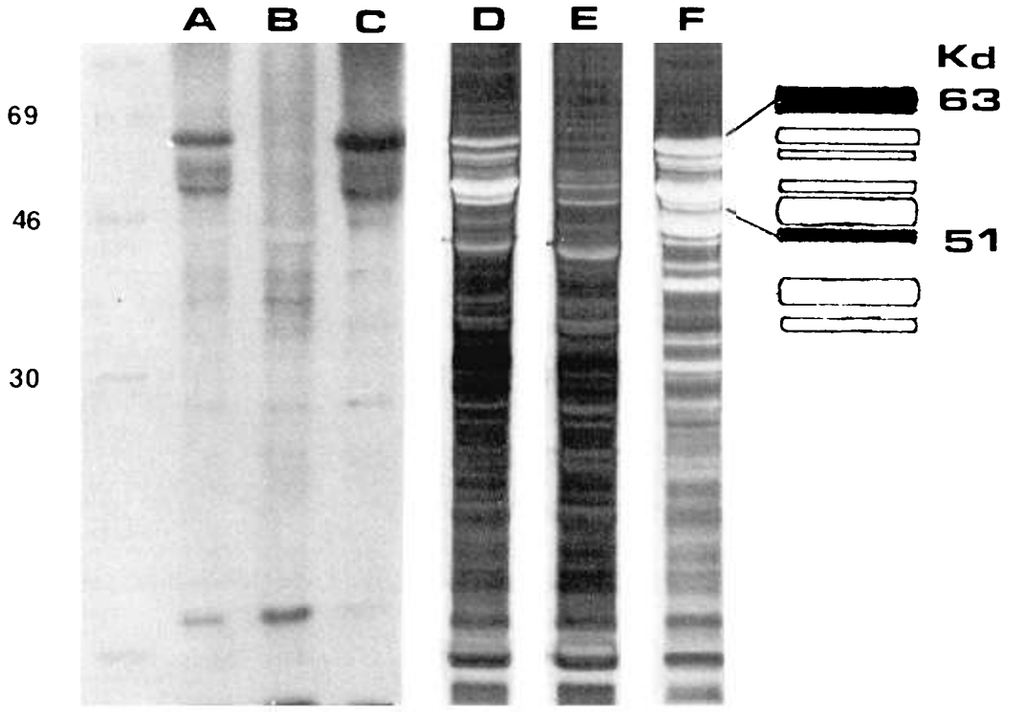

Fig. 5. Differential phosphorylation of epidermal and dermal proteins in skin sections from 1-d-old rats. Freshly-excised dorsal skin strips were phosphorylated at 23°C before harvesting and processing of isolated epidermal and dermal samples as described in "Materials and Methods." 1D silver-stained gels and corresponding autoradiograms are shown. *Lanes C* and *F*: epidermal extracts; *B*, *E*: dermal extracts; *A*, *D*: whole skin extracts (epidermis + dermis). Preferential incorporation of radiolabel into the keratin region is illustrated by the autoradiograms (*lanes A* and *C*). Radioactive mol wt standards are shown on the left. The location of the two major phosphorylated epidermal proteins is shown in the schema to the right. The 63 kD epidermal phosphoprotein corresponds to the isoelectric variants marked A1-A5 in Figure 2.

natural limit between the infant and his environment. Among its many functions, the epidermis acts as a barrier to prevent the postnatal loss of heat and water (22); it contains physical, biochemical, and cellular defenses against invasion by pathogenetic microorganisms (23–25); and it forms major derivative structures such as hair shafts, which are important transducers of sensory information (26). In addition, the epidermis constitutes the physical interface for a multitude of electronic sensing devices including transcutaneous blood gas electrodes, temperature probes, and cardiorespiratory monitors.

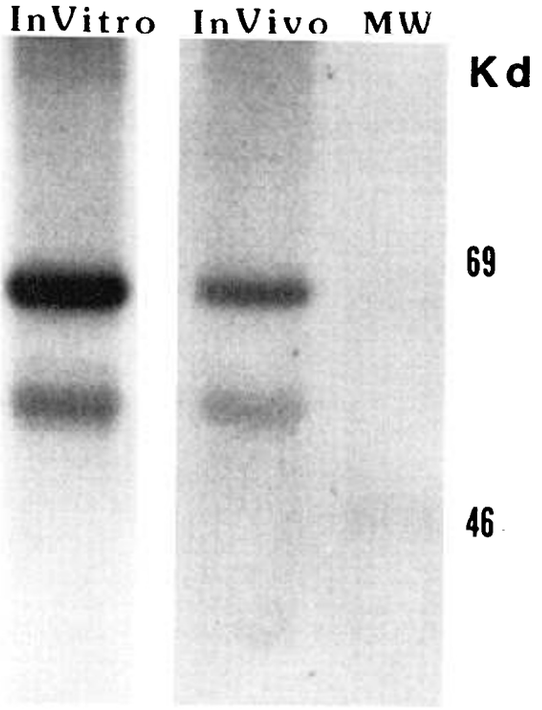

Fig. 6. Comparison of *in vitro* versus *in vivo* phosphorylation patterns of Tris-insoluble proteins in newborn rat epidermis. Proteins were phosphorylated and extracted as described in the Methods section. 150 μg of total protein were electrophoresed on an 11% polyacrylamide gel followed by autoradiography of the dried gel. Autoradiography time was 13 h for the *in vitro* sample and 96 h for the *in vivo* sample.

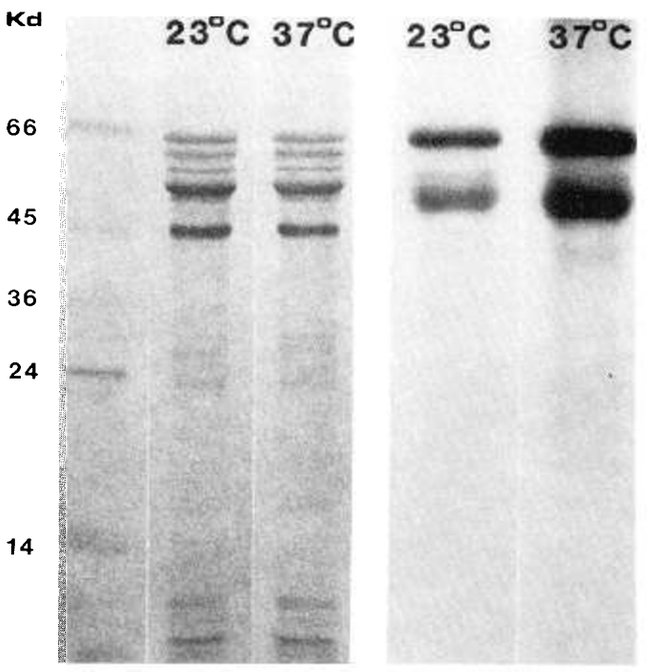

Fig. 7. Effect of temperature on *in vitro* phosphorylation of epidermal proteins in the newborn rat. Homogenates of ^{32}P-labeled epidermis containing 50 μg of protein were electrophoresed on a 11% polyacrylamide gel which was subsequently stained with Coomassie blue, dried and autoradiographed for 18 h. The stained gel is shown on the left and the corresponding autoradiogram on the right. The increased phosphorylation at 37°C is evident.

Molecular-level understanding of perinatal skin functions requires characterization of the key participant proteins. The major gene products and structural proteins of the mammalian epidermis are the keratins. These proteins are importantly involved in the process of terminal differentiation which culminates in the formation of the stratum corneum. In general, the keratins comprise the largest subgroup of a family of structural proteins which share the ability to form intermediate filaments, 8–10 nm in diameter (9). The keratins range in mol wt between 40 and 70 kD and are expressed only in epithelial tissues, where they form the genetic hallmark for this tissue subtype (10). Within any given epithelial cell, the specific group of keratin subunits expressed can be divided into acidic (pI < 5.5) and neutral-basic (pI > 6) groups known as type I and type II subunits, respectively (10, 27). Our 2-dimensional gel analyses (Fig. 2) indicate that neonatal rat epidermal proteins are similarly divisible into two classes based on their pI with the larger species being more basic that the smaller mol wt proteins.

This gel pattern is very similar to that seen when keratins from human epidermal keratinocytes are separated by mol wt and pI (27). The largest human keratin, for example, has a reported mol wt of 67–68 kD and consists of a series of charge variants extending into the basic range (27, 28). To date, most work on epidermal keratins has been performed on human, bovine, and murine sources. In various studies in the rat, the largest epidermal keratin has been reported to vary in mol wt from 65 000 to 68 000 (29–32). Our data are consistent with an M_r = 63 kD for the largest keratin species. As keratins are known to be expressed in "subunit pairs" with the type II (basic) protein usually 7–10 kD larger than its type I (acidic) partner (10, 27), our data would be consistent with a complementary acidic keratin of mol wt equal to 53–56 kD. The importance of the high mol wt subunit keratin pairs derives from the observation that these subunits are markers of epidermal differentiation. Thus, lower epidermal cell layers, as well as epidermal cells in culture, synthesize smaller mol wt keratin pairs whereas the upper, more differentiated, layers synthesize pairs of larger mol wt keratins (10, 33).

In this study, we have focused primarily on characterization of the large basic 63 kD rat epidermal keratin. This protein, as shown by the immunoblot in Figure 3, is immunologically distinct from other rat and human epidermal keratins. Moreover, the ontogenetic expression of the protein (Fig. 4) is consistent with the known sequence of epidermal growth and differentiation in the perinatal rat (7). Thus, in the rat, the epidermis undergoes a remarkable period of hyperplastic growth beginning on approximately the 17th d of gestation. Stratum corneum formation is apparent on the 19th d (29) and by term (d 22) the epidermis of the rat is as thick as it will be for the remainder of the animal's life. As growth and differentiation are precisely linked in this tissue, perinatal rat epidermis provides a useful and accessible model within which to study developmental controls.

One of the known posttranslational modifications of keratin is phosphorylation (33). Figure 5 indicates the general pattern of phosphorylation observed when neonatal rat skin is exposed to ^{32}P *in vitro*. Under these conditions, the major phosphoproteins observed are epidermal in origin and include the 63 kD protein as a prominent component. The general qualitative pattern of phosphorylation is identical under both *in vivo* and *in vitro* radiolabeling conditions (Fig. 6). This finding in the neonatal animal supports the work of Gilmartin *et al.* using the adult (34).

We also examined the effect of ambient temperature to alter the phosphorylation of epidermal proteins in the rat. As shown in Figure 7, a lower incubation temperature diminished phosphorylation under *in vitro* conditions, but the general phosphoprotein pattern was similar at both 23 and 37°C. At present, it is unknown whether temperature dependent phosphorylation of epidermal structural proteins has any physiologic significance. One possibility is that temperature-dependent alterations in keratin phosphorylation accompany changes in epidermal biomechanical function. In this regard, we have demonstrated an *in vitro* effect of temperature (23 to 37°C) to alter tension-related

properties of whole neonatal rat skin (35) and epidermis (36). Further work will need to be directed at understanding the overlap between physical and biomolecular factors regulating keratin phosphorylation at the cellular level both *in vitro* and *in vivo*.

Previous studies of keratin phosphorylation have implicated a wide variety of potential endogenous biomediators; *e.g.* calcium, EGF, cyclic AMP (37–40). One particularly intriguing possibility relates to the regulation of stratum corneum formation and the relationship of keratin to the intermediate filament-associated matrix protein, filaggrin. This protein has been demonstrated to exist as a very high mol wt (>350 000) phosphorylated precursor called profilaggrin (41). This precursor undergoes dephosphorylation and proteolytic clipping into multiple identical smaller forms, which then associate with keratins to form insoluble complexes required for the keratinization process and stratum corneum formation (42, 43). Whether the phosphorylation-dephosphorylation of the high mol wt keratins is an important aspect of this process remains to be determined.

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