Histidine-rich Proteins As Molecular Markers of Epidermal Differentiation*

(Received for publication, October 21, 1977, and in revised form, March 13, 1978)

Richard D. Rail,† Glenn K. Walker,§ and Isadore A. Bernstein†
From the Department of Biological Chemistry and the Cellular Chemistry Laboratory of the Department of Environmental and Industrial Health, The University of Michigan, Ann Arbor, Michigan 48109

Cells of the basal, spinous, and granular layers of the mammalian epidermis represent morphological stages in the differentiation of keratinocytes to form the outer cornified layer of the skin. In the newborn rat, concomitant with the transition of granular into cornified cells, there is a conversion of histidine-rich protein I (HRP), localized in keratinocytes of the epidermal granular layer, into HRP,. This transition from HRP to HRP provides a biochemical marker for this particular differentiative event.

HRP and HRP, have similar amino acid compositions which are unusually high in polar amino acids (greater than 80 mol %) and histidine (approximately 7 mol %). As estimated from gel filtration studies in the presence of sodium dodecyl sulfate, HRP exhibits a broad range of molecular weights from approximately 60,000 to greater than 10⁶ whereas HRP, has an apparent molecular weight between 60,000 and 80,000. Peptide mapping of a tryptic digest of a mixture of HRP labeled with [¹⁴C]histidine and HRP, labeled with [²H]histidine shows that of 18 peptides which are significantly labeled with [²H] and a similar number labeled with [¹⁴C] 11 of the [²H]-peptides chromatograph coincidentally with [¹⁴C]-peptides.

HRP accounts for about 40% of the total nondialyzable [²H] in the epidermis after exposure in vivo for 1 h to [²H]histidine and about 30% after a similar exposure to an equally labeled mixture of the 20 common [²H]-amino acids. HRP constitutes only about 4% of the epidermal protein which is soluble in 8 M urea, 0.2 M Tris, pH 8.5, whereas HRP is a major component and represents 35% of this extract. HRP, is present primarily in the cells of the lower cornified layers as demonstrated by autoradiography of histologically "pure" stratum corneum and isolation of the substance from such tissue preparations. HRP is localized in the cells of the granular layers.

Available data support the hypothesis that the product of translation, HRP, is polymerized rapidly in the granular cells to a large polymer of heterogeneous size, HRP, which is degraded to a smaller molecule, HRP, when the granular cell differentiates into a cornified cell, presumably, under genetic and/or epigenetic control. The conversion of HRP into HRP, can be used to study those regulatory processes which mediate epidermal keratinization, a specific example of eukaryotic differentiation.

Epidermal keratinocytes undergo a series of morphological and biochemical changes during their migration from the germinative basal layer to the outer cornified layer of the mammalian skin which results in the formation of stratified layers of cells (basal, spinous, granular, and cornified layers) representing stages of differentiation of the same cell type (1). Autoradiographic data have demonstrated that certain labeled amino acids, e.g. glycine and histidine, are incorporated initially in the upper, more differentiated layers of the epidermis, while other amino acids, e.g. leucine, phenylalanine, and methionine, localize initially in the lower layers of the epidermis (2-4). These data were interpreted (5, 6) as suggesting that a protein(s) rich in histidine and/or glycine was being preferentially synthesized in the upper layers of the epidermis. In support of this hypothesis, an unusual protein high in histidine (approximately 7%) and soluble in 8 M urea, pH 8.5, as well as in 0.1 N HCl04, was isolated from the upper layers of the cutaneous epidermis of the newborn rat (5). The [²H] fraction accounts for 35 to 50% of the total nondialyzable [²H]-histidine incorporated into the epidermis 1 h after the subcutaneous injection of the tracer, and is localized in the epidermal layer immediately below the outer cornified layer, i.e. the granular layer (Fig. 1A) (7, 8).

The present paper concerns the properties of the above HRP, now designated HRP, and reports the isolation and characterization of another HRP form, HRP, from the stratum corneum. HRP, is apparently derived from HRP. Since HRP is localized in the granular layer and HRP is localized in the cornified layers, the conversion of HRP to HRP is assumed to be a molecular marker of the differentiation of a granular into a cornified keratinocyte. The conversion of HRP species provides a model in which to study the genetic/epigenetic "control processes" that regulate epidermal (eukaryotic) differentiation (9).

EXPERIMENTAL PROCEDURES

Methods

Preparation of Epidermis—Newborn rats (CFN strain, originally obtained from Careforth Farms, Inc., Rockland County, N. Y.) at 1 to 3 days of age, derived from a randomly inbred colony maintained in...
this laboratory, were killed by cervical dislocation and placed on ice. The skins were excised and spread with the stratum corneum down on an ice-chilled Petri dish, and the subcutaneous fat and blood vessels were removed by scraping with a dull scalpel.

The epidermis was obtained by a modification of the method of Baumberger et al. (10). Approximately 10 skins were immersed in 100 ml of 0.24 M NH₄Cl, pH 8.5, at 0°C, for 15 min with gentle stirring. After removal from the solution, the skins were spread out on a cold Petri dish with the stratum corneum down and the dermis was then teased from the epidermis with forceps. Epidermal tissue was always prepared fresh.

Preparation of Nonkeratinized and Keratinized Epidermal Cells—The epidermis remaining after removal of the dermis contained the more superficial spinous, granular, and cornified cell layers, but did not contain the lowermost, relatively undifferentiated keratinocytes (basal and lower spinous cell layers) (11). Thorough scraping of the lower side of the epidermis with the edge of a piece of tinocytos (basal and lower spinous cell layers) (11). Thorough scraping but did not contain the lowermost, relatively undifferentiated keratinized or keratinized cells (separated by scraping) or on the isolated epidermis directly. The precipitation of HRP₁ was carried out for 15 min at room temperature, rather than at 4°C overnight, as was done by earlier workers (5, 7, 12), since significant amounts of HRP₁ were also precipitated at 4°C and would have contaminated the preparation of HRP₁. This did not occur at room temperature (8).

Determination of Protein and Radioactivity—Protein determination was performed with a Technicon autoanalyzer system using the method of Lowry et al. (13) as described by Mandl (14). Samples were prepared for measurement of protein concentration and radioactivity determination by adding 1 volume of 0.2 M NaOH, 1% ARW-7 (wetting agent-detergent from Technicon Corp.) and heating the sample in a boiling water bath for 5 min. Radioactivity determinations were performed by adding samples prepared in the above manner to about 10 ml of PCS (Phase-Combining System, Packard Instruments, Inc.) in minivials and counting in a Packard Tri-Carb scintillation counter. Efficiency of counting was 90% for ¹⁴C and 35% for ³²P.

Gel Filtration Chromatography—Gel filtration chromatography was performed using Sepharose 2B or 6B agarose gels (Pharmacia Fine Chemicals, Inc.). Data are reported in terms of Kᵣ, and Vᵣ (15). All chromatographic columns (2.5 x 40 cm) were eluted at room temperature with a mixture of 0.1% SDS, 0.1 M NaCl, 0.001 M EDTA, and 0.01 M Tris, pH 8.5. When included in the above buffer, the concentration of 2-mercaptoethanol was 0.1%. Fractions collected were equal to approximately 2.5% of the total column volume. Void volumes were determined using dextran blue 2000 (Pharmacia Fine Chemicals, Inc.).

Samples were prepared for SDS-gel filtration chromatography by making the samples 1.0% in SDS, 0.1 M in NaCl, and 0.01 M in Tris, pH 8.5, heating in a boiling water bath for 5 min, stirring at room temperature for 3 h, and dialyzing overnight (2 x 50 volumes) at room temperature against the column-eluting buffer. When used in the above sample buffer, the concentration of 2-mercaptoethanol was 3%.

Peptide Analysis—Samples for peptide analysis were dissolved in 0.2 M ammonium carbonate, pH 8.8, containing 0.02 mg/ml of trypsin which had been treated with diphenylcarbamyl chloride to remove chymotryptic activity (Sigma Chemical Co., Catalogue No. T-1005). Samples (1 to 2 mg of protein/ml) were incubated at room temperature for approximately 16 h with the addition at 2 and 6 h of 0.2 ml of trypsin solution (1 mg/ml of trypsin in 0.01 N HCl) per 10 ml of digest medium. Samples to be co-chromatographed were digested as a mixture. In order to remove the salt, samples were then lyophilized for 12 h, resolubilized in a volume of 0.3 M acetic acid equal to the volume of the original digest, and again lyophilized for 12 h before being dissolved in the initial column buffer and applied to the chromatographic column. Peptide analysis was carried out on a column (0.6 x 20 cm) of Aminex A-5 (Bio-Rad) by a modification of a Technicon peptide analysis procedure (16).

Fig. 2. The isolation procedure was applied to preparations of nonkeratinized or keratinized cells (separated by scraping) or on the isolated epidermis directly. The precipitation of HRP₁ was carried out for 15 min at room temperature, rather than at 4°C overnight, as was done by earlier workers (5, 7, 12), since significant amounts of HRP₁ were also precipitated at 4°C and would have contaminated the preparation of HRP₁. This did not occur at room temperature (8).

FIG. 1. A, histology of the skin of the newborn rat. SC, stratum corneum; G, granular cell layer; S, spinous cell layer; B, basal cell layer; f, fibroblast nucleus in the dermis. (H & E, ×1900). B, stratum corneum obtained by scraping techniques. The dark staining in the lower layers is eosinophilic. (H & E, ×2000). C, autoradiogram of stratum corneum obtained from a newborn rat exposed to [³²P]histidine for 24 h in vivo. Note the band of silver grains in the lower portion of the stratum corneum. (X 2000).

Fig. 2. Flow chart for isolation of HRP₁ and HRP₂ (5, 8).
gradient was supplied by a Buchler Varigrad: chamber one, 150 ml of 0.2 M pyridine, pH 3.1 (16.1 ml of pyridine and 278 ml of glacial acetic acid/liter); chamber two, 150 ml of 0.5 M pyridine, pH 4.1 (40.2 ml of pyridine and 138 ml of glacial acetic acid/liter); chamber three, 150 ml of distilled water; chamber four, 150 ml of 2.0 M pyridine, pH 5.1 (121 ml of pyridine and 144 ml of glacial acetic acid/liter).

**Determination of Amino Acids**—Amino acid composition was determined by the method of Spackman et al. (17) using a Beckman 120C automated amino acid analyzer. Protein fractions were hydrolyzed in 6 N HCl at 110°C for 24 h. Samples were not submitted to oxidation prior to hydrolysis since amino acids containing sulfur were present in negligible amounts, if at all, in the fractions of interest (18, 19). Losses of threonine and serine during hydrolysis were 5 and 10%, respectively, under the conditions used and appropriate corrections were made.

**SDS-Polyacrylamide Gel Electrophoresis—**SDS-polyacrylamide gel electrophoresis was carried out by a modification of the method of Weber and Osborn (20) using 7.5% acrylamide. Gels were stained with 0.1% Coomasie G-250 in a mixture of 50% methanol and 7% acetic acid for 8 h at room temperature, followed by electrophoretic destaining utilizing transverse electrophoresis in a mixture of 30% methanol and 10% acetic acid for 20 min. Gels were further destained by diffusion in 10% acetic acid.

**Radioactive Labeling of Newborn Rat Epidermis in Vivo**—Newborn rats were injected subcutaneously with 20 or 40 μl of the isotonic solutions in 0.01 N HCl. Injection of these small amounts of acid did not appear to exert any deleterious effects and injection of isotonic solutions adjusted to pH 7.5 with 0.05 M sodium phosphate produced results which were not different from those obtained when the acidified solutions were used. [14C]Histidine was always administered at 20 μCi (20 μl) per animal. [3H]Histidine was injected at 1 μCi (20 μl) per animal except when labeled HRP was prepared for peptide analysis. In this case, 2 μCi (40 μl) were given to each animal. In all experiments, where the period of labeling in vivo exceeded 3 h, the animals were handled with clean surgical gloves, the injection sites were covered with 5% Formvar dissolved in 1,2-dichloroethylene, and the animals were returned to the mother.

**Materials**

All general reagents used were reagent grade. Ultrapure urea was purchased from Schwarz/Mann and prepared fresh on the day of use. Sodium dodecyl sulfate was recrystallized once (21). Reagent grade acrylamide was recrystallized once (22). The [14C]histidine (1 Ci/mmol, 1 mCi/ml) and [3H]histidine (300 mCi/mmol, 50 μCi/ml) were obtained from Schwarz/Mann, and a mixture of trinitiated l-amino acids (0.1 mCi/ml) from New England Nuclear. Pyridine was purified by refluxing with ninhydrin followed by distillation (23) within 1 week of use.

**RESULTS**

**Gel Filtration Chromatography of HRP**—Fig. 3 shows a chromatogram on Sepharose 2B of HRP isolated from nonkeratinized cells. HRP was labeled in vivo by [3H]histidine for 24 h and [14C]histidine for 1 h. It had previously been shown that when radioactive histidine is used only histidine residues are labeled in HRP (19). Elution profiles of protein, [3H], and [14C] were determined at 20 μCi (20 μl) per animal. The [3H]histidine was injected at 1 μCi (20 μl) per animal except when labeled HRP was prepared for peptide analysis. In this case, 2 μCi (40 μl) were given to each animal. In all experiments, where the period of labeling in vivo exceeded 3 h, the animals were handled with clean surgical gloves, the injection sites were covered with 5% Formvar dissolved in 1,2-dichloroethylene, and the animals were returned to the mother.

**Distribution of Radioactivity and Protein in the Urea-Gel Electrophoresis**—Distribution of radioactivity and protein of HRP isolated from whole epidermis were essentially the same as those obtained for HRP isolated from the nonkeratinized cells.

**Gel Filtration Chromatography of HRP**—Gel filtration chromatography of HRP isolated from keratinized cells—HRP isolated from stratum corneum was purified by ethanol precipitation and subsequent SDS-gel filtration chromatography. The addition of 0.5 volume of 95% ethanol to a solution of HRP and cooling of the mixture for 2 h at 4°C resulted in the precipitation of essentially all (95%) of the [3H]histidine incorporated by 24 h, but only 65% of the protein. Fig. 4A shows a chromatogram of ethanol-precipitated HRP on Sepharose 6B. The HRP had been obtained from the epidermis of animals which had been exposed to [3H]histidine for 24 h and [14C]histidine for 1 h. The protein and [3H]histidine of HRP were retarded on the gel under the above conditions, being eluted in the region of bovine serum albumin elution. No significant amount of [14C] appeared in this fraction. Treatment of HRP with 2 mercaptoethanol and inclusion of 2-mercaptoethanol in the eluting buffer did not alter the above elution profile. HRP isolated directly from whole epidermis gave essentially the same elution profile of radioactivity and protein under the above conditions. When the leading edge of the elution profile from Sepharose 6B, as indicated in Fig. 4A, was collected, re-chromatographed on Sepharose 6B (Fig. 4B), and submitted to SDS-polyacrylamide gel electrophoresis, all the protein entered the gel and moved as a single sharp band (Fig. 5). On the second passage through Sepharose 6B, the trailing nonradioactive contaminant was absent and the specific activity across the peak varied by no more than the counting error.

**Amino Acid Composition of HRP**—Table I shows that the amino acid compositions of HRP1 and HRP2 are similar and unique but do exhibit minor variations. Arginine, lysine, glutamic acid, serine, threonine, histidine, aspartic acid, and glycine account for approximately 90 mol % of the residues present in both cases. Nonpolar amino acids are present in low amounts. As obtained from the whole epidermis by the same isolation procedure, both HRP species have more lysine and different amounts of proline than is true when the proteins are isolated from the separated strata. HRP1 also has less alanine when isolated from whole epidermis.

**Distribution of Radioactivity and Protein in the Urea-
Histidine-rich Proteins in Epidermal Differentiation

Fig. 4. SDS-gel filtration chromatography of HRPi isolated from keratinized cells. Animals were exposed to [3H]histidine for 24 h and [14C]histidine for 1 h in vivo. The column (2.5 x 40 cm) of Sepharose 6B was eluted with a mixture of 0.1% SDS, 0.1 M NaCl, 0.001 M EDTA, and 0.05 M Tris, pH 8.5, at room temperature. V0 = 75 ml. A, initial fractionation of HRPi fraction. Arrows indicate fractions combined for further purification. B, rechromatography of the combined fractions of the leading edge of the profile shown in (A). - - - - A, [3H]histidine; --- A, [14C]histidine.

TABLE I
Amino acid composition (residues/100 residues) of various HRPi fractions

<table>
<thead>
<tr>
<th>Source of HRP</th>
<th>HRPi (n = 1)</th>
<th>HRPi (n = 2)</th>
<th>Purified HRPi (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.9</td>
<td>0.7 ± 0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>6.9</td>
<td>7.4 ± 0.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>14.0</td>
<td>12.9 ± 0.5</td>
<td>13.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.0</td>
<td>4.5 ± 0.2</td>
<td>5.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.7</td>
<td>6.2 ± 0.1</td>
<td>6.5</td>
</tr>
<tr>
<td>Serine</td>
<td>15.1</td>
<td>16.4 ± 0.2</td>
<td>16.9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>21.9</td>
<td>21.5 ± 0.2</td>
<td>20.8</td>
</tr>
<tr>
<td>Proline</td>
<td>0.4</td>
<td>2.2 ± 0.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4</td>
<td>14.5 ± 0.2</td>
<td>13.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>10.8</td>
<td>11.3 ± 0.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Valine</td>
<td>0.7</td>
<td>0.3 ± 0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.8</td>
<td>1.0 ± 0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.5</td>
<td>0.5 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.0</td>
<td>0.3 ± 0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

- Preparations of HRPi applied to Sepharose 6B.
- HRPi from leading edge of Sepharose 6B elution profile (Fig. 4A).
- Standard deviation.
- Not detectable.

incorporation of [3H] at approximately 1 h after the injection of the tracer (Fig. 6). A gradual decrease in this net incorporation followed which extended to 24 h, the longest time period used in this study. Relatively little [3H]histidine was incorporated into HRPi initially (0 to 8 h), but at 24 h after administration of labeled histidine, HRPi accounted for 35 to 40% of the nondialyzable [3H] in the urea-soluble protein. The net incorporation of [3H] into HRPi at 24 h was roughly three times greater than the maximal net incorporation of [3H] into HRPi at any given time, suggesting the possibility that there is a flux of labeled histidine through HRPi into HRPii. While HRPi accounted for less than 10% of the nondialyzable HClO4-soluble [3H]histidine at 1 h after injection of the tracer, at 24 h it represented approximately 85%.

HRPi appears to represent a major portion of the de novo protein synthesis in the upper layers of the epidermis since when a tritiated amino acid mixture with approximately 5% of the total [3H] present in each of the 20 common amino acids was used as the tracer, HRPi incorporated about 25% of the
TABLE II

Distribution of protein and radioactivity (relative per cent) during the preparation of HRP₁ and HRP₃ from whole epidermis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Relative activity of ³H found in HRP₁ when only ³H histidine was used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactivity</td>
<td>mg/g epidermis</td>
<td>%</td>
</tr>
<tr>
<td>whole epidermis</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Urea-soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nondialyzed</td>
<td>94.3 ± 0.9</td>
<td>96.2 ± 1.0</td>
</tr>
<tr>
<td>Dialyzed</td>
<td>85.9 ± 2.0</td>
<td>84.3 ± 1.5</td>
</tr>
<tr>
<td>HClO₄-soluble</td>
<td>41.2 ± 6.5</td>
<td>44.8 ± 7.2</td>
</tr>
<tr>
<td>HRP₁ fraction</td>
<td>4.1 ± 1.8</td>
<td>38.1 ± 6.8</td>
</tr>
<tr>
<td>HRP₃ fraction</td>
<td>35.0 ± 4.4</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>Urea-insoluble</td>
<td>4.5 ± 1.3</td>
<td>9.55</td>
</tr>
</tbody>
</table>

Table III shows that 70% of the protein, 86% of the [³H]histidine and 96% of the [¹⁴C]histidine in HRP₁ came from the nonkeratinized cells, while 93% of the [³H]histidine and 90% of the [¹⁴C]histidine in HRP₃ were from the keratinized cells. These data support the view that HRP₁ from HRP₁ were labeled with [¹⁴C]. Eleven ³H-containing peptides from HRP₁ co-chromatographed with [¹⁴C]-labeled peptides of HRP₃.

Localization of HRP₁ in the Nonkeratinized Cells and HRP₃ in the Stratum Corneum—Table III illustrates the distribution of protein and labeled histidine among the various fractions when HRP₁ and HRP₃ were isolated from the nonkeratinized cells and the keratinized cells, respectively. These data approximate the values for HRP₁ and HRP₃ obtained when the isolations were carried out on the whole epidermis (Table II). Table III shows that 70% of the protein, 86% of the [³H]histidine and 96% of the [¹⁴C]histidine in HRP₁ came from the nonkeratinized cells, while 93% of the [³H]histidine and 90% of the [¹⁴C]histidine in HRP₃ were from the keratinized cells. These data support the view that HRP₁

Table III illustrates the distribution of protein and labeled histidine among the various fractions when HRP₁ and HRP₃ were isolated from the nonkeratinized cells and the keratinized cells, respectively. These data approximate the values for HRP₁ and HRP₃ obtained when the isolations were carried out on the whole epidermis (Table II). Table III shows that 70% of the protein, 86% of the [³H]histidine and 96% of the [¹⁴C]histidine in HRP₁ came from the nonkeratinized cells, while 93% of the [³H]histidine and 90% of the [¹⁴C]histidine in HRP₃ were from the keratinized cells. These data support the view that HRP₁ from HRP₁ were labeled with [¹⁴C]. Eleven ³H-containing peptides from HRP₁ co-chromatographed with [¹⁴C]-labeled peptides of HRP₃.

Localization of HRP₁ in the Nonkeratinized Cells and HRP₃ in the Stratum Corneum—Table III illustrates the distribution of protein and labeled histidine among the various fractions when HRP₁ and HRP₃ were isolated from the nonkeratinized cells and the keratinized cells, respectively. These data approximate the values for HRP₁ and HRP₃ obtained when the isolations were carried out on the whole epidermis (Table II). Table III shows that 70% of the protein, 86% of the [³H]histidine and 96% of the [¹⁴C]histidine in HRP₁ came from the nonkeratinized cells, while 93% of the [³H]histidine and 90% of the [¹⁴C]histidine in HRP₃ were from the keratinized cells. These data support the view that HRP₁ from HRP₁ were labeled with [¹⁴C]. Eleven ³H-containing peptides from HRP₁ co-chromatographed with [¹⁴C]-labeled peptides of HRP₃.

Localization of HRP₁ in the Nonkeratinized Cells and HRP₃ in the Stratum Corneum—Table III illustrates the distribution of protein and labeled histidine among the various fractions when HRP₁ and HRP₃ were isolated from the nonkeratinized cells and the keratinized cells, respectively. These data approximate the values for HRP₁ and HRP₃ obtained when the isolations were carried out on the whole epidermis (Table II). Table III shows that 70% of the protein, 86% of the [³H]histidine and 96% of the [¹⁴C]histidine in HRP₁ came from the nonkeratinized cells, while 93% of the [³H]histidine and 90% of the [¹⁴C]histidine in HRP₃ were from the keratinized cells. These data support the view that HRP₁ from HRP₁ were labeled with [¹⁴C]. Eleven ³H-containing peptides from HRP₁ co-chromatographed with [¹⁴C]-labeled peptides of HRP₃.
is primarily localized in the nonkeratinized cells while HRP$_{II}$ is essentially localized in the stratum corneum. To support this conclusion, autoradiography of the scraped stratum corneum labeled in vivo for 24 h with $[^{3}H]$histidine was done. As shown in Fig. 1C, there were large numbers of silver grains over the lower cornified layers, but few over the upper layers of this stratum. The stratum corneum contains no radioactivity that is demonstrable by autoradiographic techniques when the labeling period is less than 6 h (2). The appearance of autoradiographically detectable $[^{3}H]$histidine in the keratinized layers coincides temporally with the appearance of significant amounts of $[^{3}H]$histidine in the HRP$_{II}$ fraction (Fig. 6) and supports the localization of HRP$_{II}$ in the stratum corneum.

DISCUSSION

HRP$_{I}$ and HRP$_{II}$ represent major products of epidermal differentiation, since together they account for 40 to 45% of the epidermal protein which is soluble in 8 M urea, 0.9 M Tris, pH 8.5, and represent approximately 12.5% of the wet weight of the tissue. As judged by the relatively high degree of labeling of HRP$_{I}$ by a tritiated amino acid mixture in vivo, synthesis of HRP$_{I}$ represents a major portion of the total protein synthesis in the cells of the upper layers of the epidermis. Based on its elution profile on Sepharose 2B in SDS-containing buffers (Fig. 3), HRP$_{I}$ appears to exist in a range of molecular weights estimated to extend from approximately 60,000 to as high as 10$^{6}$. The high molecular weight of HRP$_{II}$ could not be reduced by common protein denaturants, e.g. 8 M urea, 6 M guanidinium chloride, or by reduction with 2-mercaptoethanol in the presence of SDS. HRP$_{I}$ and HRP$_{II}$ are different entities since when isolated from the same epidermis, they show different specific radioactivities (Table II) and in experiments carried out over a period of 24 h of exposure to labeled histidine, the initially highly labeled HRP$_{I}$ decreased in specific radioactivity while HRP$_{II}$ became increasingly radioactive (Fig. 6). At each time point in these experiments, the same amounts of HRP$_{I}$ and HRP$_{II}$ were isolated per g of epidermis.

Since the molecular weights of single, unaltered polypeptides of ribosomal origin are rarely larger than 125,000 to 150,000, the above results suggest that HRP$_{I}$ represents a polymer containing a form of HRP (HRP$_{I}$) which is encoded in mRNA and is linked by covalent, non-disulfide bonds in the large molecule. No HRP$_{II}$ was observed in the present investigation in vivo. However, in previous studies in vitro (24), in a puromycin-sensitive reaction, labeled histidine was incorporated into a small molecule that was soluble in 0.1 N HClO$_{4}$ and remained soluble at pH 4.5. The small molecule was then converted in the presence of puromycin to a larger molecule which was soluble in HClO$_{4}$ but was precipitated at pH 4.5.

The working hypothesis that HRP$_{I}$ is a polymer of heterogeneous size consisting of various numbers of subunits (HRP$_{I}$) is based upon (a) the constant specific activity in counts per min/mg of protein across the peak when HRP$_{I}$ labeled with radioactive histidine was chromatographed on Sepharose 2B (Fig. 3) and (b) the similarity in the unique amino acid composition of HRP$_{I}$ at various stages during its purification (8) and the presence in the newborn rat epidermis of a protein oligomeric series with molecular weights from $10^{5}$ to $6 \times 10^{4}$ which has essentially the same amino acid composition as HRP$_{I}$.

Previous studies (19) have indicated the absence of carbohydrates. The nature of the putative linkages in HRP$_{I}$ is not known, although it has been reported that HRP$_{I}$ contains lysine residues with the ε-amino group blocked in an undetermined manner (25). Since the granular layer of the epidermis is known to contain large amounts of transglutaminase, the enzyme responsible for forming γ-glutamyl-ε-lysine linkages from polypeptide-bound glutamine and lysine (26-28), interchain γ-glutamyl-ε-lysine cross-links may play a role in forming the HRP$_{I}$ polymer and be broken in the conversion of HRP$_{I}$ to HRP$_{II}$. Work in progress to determine the composition of the tryptic peptides should clarify the nature of the linkages.

The absence of disulfide linkages in HRP is supported by the results of earlier studies (a) in which the amino acid analysis of HRP$_{I}$ included a procedure to oxidize cysteine to cysteic acid but no cysteic acid was observed and (b) in which HRP was isolated from animals which had been exposed to $^{35}$S-cysteine for 1 h in vivo but no $^{35}$S was found in the HClO$_{4}$-soluble, pH 4.5-insoluble tissue fraction (18). The absence of cysteine has been confirmed by other investigators (29-32).

The present results with respect to the molecular weight of HRP, conflict with those of previous workers (5, 7, 12) who observed molecular weights ranging from 30,000 to 390,000. Lower molecular weights for HRP$_{I}$ were obtained in the present investigation only when the protein was exposed to sodium carbonate buffers, e.g. 0.05 M Na$_{2}$CO$_{3}$, for 12 to 24 h at room temperature (8) as was done in the original procedure (5). This step was omitted in the present procedure. The basis for this phenomenon is not understood. It did not occur at room temperature in 0.1 M acetic acid or 0.05 M NH$_{4}$OH with or without 0.1% SDS (8).

Attempts to isolate HRP, from the liver of newborn rats by extraction with urea followed by exposure to 0.1 N HClO$_{4}$ and gel filtration chromatography in SDS have been unsuccessful (8, 32).

The epidermal preparations from which HRP$_{I}$ was isolated in this study included upper spinous, granular, and cornified keratinocytes. However, isolation of HRP$_{I}$ from epidermal preparations which contained only granular and cornified cells did not result in a significant decrease in the yield of HRP$_{I}$ preparations (8) and HRP$_{II}$ could not be isolated in significant quantities from the stratum corneum (Table III). These data imply that HRP$_{I}$ is localized in the granular layer of the epidermis, and that the initial autoradiographic localization of $[^{3}H]$histidine in the granular layers is in large part a result of the synthesis of HRP$_{I}$, since HRP$_{II}$ accounts for approximately 40% of the nondialyzable $[^{3}H]$histidine incorporation in the upper layers of the epidermis (Table III). The initial autoradiographic localization of $[^{3}H]$histidine in the granular layer is absent in psoriasis, a clinical human disease, in which keratolytin, the morphologic entity responsible for the granules of these cells, and HRP are absent (33).

The fate of HRP has been unclear in the past, since no protein fraction with the properties of HRP could be isolated from preparations of stratum corneum (6). It now seems possible that HRP$_{I}$ is converted into the smaller HRP$_{II}$ (M, = 60,000 to 80,000) as granular keratinocytes become cornified and that by the previously utilized techniques, HRP$_{I}$ was extracted along with HRP$_{I}$ (5). HRP$_{II}$ appears to be present exclusively in the cornified layer (Table III and Fig. 1C).

HRP$_{II}$ may be derived from HRP$_{I}$ since (a) the amino acid compositions of HRP$_{I}$ and HRP$_{II}$ are similar (Table I); (b) the histidine-containing tryptic peptides of HRP$_{I}$ and HRP$_{II}$ bear a high degree of similarity (Fig. 7); (c) significant amounts of $[^{3}H]$histidine are not incorporated into HRP$_{II}$ until 6 to 8 h after injection of the tracer—a time at which HRP$_{I}$ is already highly labeled but experiencing a gradual decrease in specific activity (Fig. 6); (d) the appearance of $[^{3}H]$histidine in HRP$_{II}$ at 6 to 8 h after administration of the tracer coincides
temporally with the initial detection of $^1$H in the stratum corneum by autoradiographic techniques (2); and (e) HRP$_{II}$ is localized in older, more differentiated keratinocytes than is HRP$_I$. Proof that HRP$_I$ arises from HRP$_{II}$ must await the results of a future study of the process.

Recently, Dale reported (29) the isolation from the stratum corneum of a basic protein having a molecular weight of 50,000 and an amino acid composition similar to that of HRP$_I$ and HRP$_{II}$. This basic protein has a high level of histidine and no sulfur and might be formed from HRP$_I$. The protein isolated by Dale may be the unlabeled protein which followed HRP$_{II}$ in the elution from Sepharose 6B (Fig. 4A). This nonradioactive contaminant of HRP$_{II}$ probably had a composition similar to HRP$_{II}$ since the material submitted to chromatography on Sepharose 6B had an amino acid composition only slightly different from that of the purified HRP$_{II}$ although the contaminant represented a significant fraction of the total protein put on the column. If this contaminant (HRP$_{II}$?) is indeed derived from HRP$_{II}$, it should become labeled at some time subsequent to 24 h after exposure to radioactive histidine in vivo.

Histidine-rich proteins have also been isolated by Freedberg and colleagues from the epidermis of newborn rat by extraction with deoxycholate followed by molecular sieve chromatography (30,31) and by Balmain (34) from the epidermis of the adult mouse after treatment of the animal with phorbol esters.

A model for HRP biosynthesis, based on the above data, is shown in Fig. 8. HRP$_I$, the putative "ribosomal" form of HRP (24), is synthesized in the granular layer of the epidermis and polymerized to form HRP$_I$. Concomitant with the conversion of granular keratinocytes to cornified keratinocytes, HRP$_I$ is converted to HRP$_{II}$. The conversion of HRP$_I$ to HRP$_{II}$ is possibly the result of a specific type of proteolytic cleavage.

The epidermis offers a system in which it is technically easy to isolate subpopulations of keratinocytes which have, and have not, undergone one major morphological differentiative step. These subpopulations of keratinocytes present the advantages that they can be isolated from the same animal, therefore serving as their own controls, and that most experiments can be run in vitro without physiologic perturbation of the organ. The above features are in contrast to those of other systems currently used to study eukaryotic differentiation, e.g. the erythroid system in which there are difficulties in obtaining large numbers of cells in a given stage of differentiation, e.g. psoriasis, ichthyosis vulgaris, and other ichthyoses (3,36) and appears to be affected when the normal equilibrium of the epidermis is perturbed artificially by treatment with tumor-promoting phorbol esters (37).

The biosynthesis of HRP in the granular layer (7) and the synthesis of a leucine-rich protein (38) in the lower layers represent two metabolic systems associated with different morphological events in the epidermal differentiative process. HRP$_I$ is synthesized in cells containing keratohyalin granules and appears to become a major component of the sulfur-poor type (39, 40) of this structural element (12) while the leucine-rich protein is synthesized in cells making tonofilaments and is a component of this structure (41). Whereas HRP$_I$ is formed in granular cells which have nuclei, HRP$_{II}$ is initially seen primarily in the lower cornified cells which have lost their nuclei. Whether the conversion of HRP$_I$ to HRP$_{II}$ is related to the loss of the nuclei remains to be ascertained. If so, the metabolic event could provide an entree for studying this morphologic transformation. Formation of $\gamma$-glutamyl-lysine cross-links (26-28) also occurs as the granular cell differentiates into a cornified cell. The cross-link is involved in the development of the cornified envelope of the latter cell (28,42,44). The relationship of any one of these metabolic events to the formation of the keratin molecule which is the major component of the stratum corneum, is also yet to be elucidated. It is possible that the keratin structure as described by Steinert et al. (45) actually contains the leucine-rich protein since this protein has been shown to be localized in the stratum corneum by immunocytotechnical techniques (38, 41) and the leucine-rich protein and keratin subunits have similar amino acid compositions (38, 46). HRP, on the other hand, may play a functional role in the organization of the keratin structure, since HRP$_I$ is synthesized in cells at the differentiative stage which precedes cornification and HRP$_{II}$ is converted to HRP$_{II}$ at the time keratin fibers are formed. The $\gamma$-glutamyl-lysine cross-link could be responsible for the increased rigidity which the cellular membrane achieves as the granular cell cornifies (42). The availability of keratinocyte cultures, which differentiate in vitro to form tonofilaments, keratohyalin (47), and cornified envelopes (48) should be useful for clarifying the relationships between the various biochemical and morphological steps in epidermal differentiation.

Acknowledgments—Our special thanks are extended to Doctor William P. Winter and Dorothy Sweet for assistance in performing the amino acid analyses and to Mark Krauss for assistance with the electrophoretic analyses.

REFERENCES
Histidine-rich Proteins in Epidermal Differentiation

Histidine-rich proteins as molecular markers of epidermal differentiation.
R D Ball, G K Walker and I A Bernstein


Access the most updated version of this article at
http://www.jbc.org/content/253/16/5861.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/253/16/5861.citation.full.html#ref-list-1