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Evolution

Primate involucrins: Antigenic relatedness and detection of multiple forms

(keratinocytes/unusual solubility/cell-free translation)

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ABSTRACT **Hominoid apes (gorilla, chimpanzee, orangutan, gibbon), Old World monkeys (rhesus, cynomolgus), New World monkeys (owl, cebus), and a prosimian (lemur) express involucrin-like proteins in cultured keratinocytes. Primate involucrins can be precipitated with trichloroacetic acid, resolubilized at pH 8, and subsequently retain aqueous solubility in 67% ethanol. Polyacrylamide gel electrophoresis of keratinocyte extracts after this rapid partial purification has revealed in each species tested one (chimpanzee, orangutan, gibbon) or two (gorilla, rhesus, owl, cebus) antigenically crossreactive proteins that migrate in the vicinity of human involucrin. In the species examined further (gorilla, chimpanzee, rhesus), poly(A)$^+$ mRNA isolated from the cultures directed the cell-free translation of polypeptides with mobilities similar to those extracted from the cells. From five cynomolgus monkeys, three different electrophoretic profiles were obtained, suggesting the existence of different alleles. Quantitative comparisons by a sensitive enzyme-linked immunosorbent assay indicated that certain primate involucrins have a higher density of antigenic determinants than the human protein, whereas others lack some determinant(s). In contrast to those from other species, all of which showed substantial crossreactivity, the lemur protein was minimally immunoreactive by immunoblotting and not clearly detected by solid-phase assay. The electrophoretic and antigenic differences displayed throughout the primate order suggest that this protein has been subject to relatively rapid evolution.**

A characteristic feature of keratinocyte terminal differentiation is the formation of a layer of enzymatically crosslinked protein immediately beneath the plasma membrane (1). Such crosslinked envelope structures are present in all human stratified squamous epithelia and are observed in the cornified layer of mammalian, avian, reptilian, and amphibian epidermis (2). Studies of envelope formation in cultured human epidermal cells have identified the protein involucrin as a prominent envelope constituent (3). Reflecting its high glutamine content (4), this protein is an excellent substrate for a particulate transglutaminase, which stabilizes envelope structures with ε-(γ-glutamyl)lysine crosslinks (5, 6).

Rodents (6–8) and lagomorphs (9) express a particulate transglutaminase that is biochemically, functionally, and immunochemically related to that in humans. To date, however, a protein antigenically or biochemically related to involucrin has not been observed in species lower than primates (10, 11). As a starting point to discern whether other species express transglutaminase substrates related to human involucrin, we have investigated whether similar proteins are identifiable throughout the primate order and how closely related they are. If this protein appears to be rapidly evolving, it may be of utility in analyzing interrelatedness of primate species. A preliminary account of this work has been presented (12).

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MATERIALS AND METHODS

Species and Cell Culture. The following species, number of individuals, and anatomical sites were sampled: chimpanzee (*Pan troglodytes*), 1 (vaginal); pygmy chimpanzee (*Pan paniscus*), 2 (vaginal); gorilla (*Gorilla gorilla*), 1 (vaginal); orangutan (*Pongo pygmaeus*), 2 (1 vaginal, 1 epidermal); gibbon (*Hylobates lar*), 1 (vaginal); cynomolgus monkey (*Macaca fascicularis*), 5 (5 vaginal, 1 epidermal, 1 bladder); rhesus monkey (*Macaca mulatta*), 1 (vaginal); owl monkey (*Aotes trivirgatus*), 2 (1 vaginal, 1 esophageal); cebus monkey (*Cebus albifrons*), 1 (esophageal); lemur (*Lemur catta*), 1 (esophageal); tree shrew (*Tupaia glis*), 2 (epidermal). Epithelial cells were cultivated from explants with feeder-layer support according to standard procedures (13) in Dulbecco–Vogt Eagle's and Ham's F-12 media (a 3:1 mixture) supplemented with fetal bovine serum (5%), hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), adenine (0.18 mM), triiodothyronine (20 pM), insulin (5 μg/ml), transferrin (5 μg/ml), penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml). When the explants were passaged, cholera toxin (9 ng/ml) was added to the medium upon inoculation (14) but not at subsequent feedings. Human foreskin and primate epithelial cell cultures were initiated as explants with 3T3 (murine fibroblast) feeder-layer support. Cells from the epithelial outgrowths were disaggregated by trypsinization and serially passaged, with periodic removal of fibroblasts as necessary by rinsing with 0.5 mM EDTA in isotonic saline (15).

Partial Purification of Involucrin. Cultures held at confluence for several days to a week were harvested by scraping and stored frozen. They were disrupted in a ground-glass homogenizer while thawing in the presence of an equal volume of 12% trichloroacetic acid. Insoluble material was recovered by centrifugation (14,000 × *g*, 5 min) and rinsed twice with 5% trichloroacetic acid. The contents of the drained tube were resuspended in water (1 ml per 10-cm culture) and adjusted to 2% in redistilled *N*-ethylmorpholine (final pH ≈ 8). The suspension was clarified by centrifugation, and to the clear supernatant were added 2 volumes of absolute ethanol. After standing for 20 min at 0°C, the cloudy suspension was clarified by centrifugation and the supernatant (which could be stored for months at −20°C) was sampled directly for gel electrophoresis or extracted twice with 1.5 volumes of amyl acetate, thereby reducing the volume. The aqueous (lower) phase was lyophilized and stored at −20°C. For use as a standard in immunoreactivity measurements, human involucrin was subsequently purified to homogeneity by DEAE-cellulose column chromatography (3).

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Immunoblots. Involucrin samples were separated by discontinuous $NaDodSO_4$/polyacrylamide gel electrophoresis (16), electrophoretically transferred to nitrocellulose, and tested for antigenic crossreactivity (17) with rabbit antiserum to human involucrin (3). After electrophoretic transfer, the nitrocellulose was blocked with 5% bovine serum albumin in 0.1 M Tris Cl buffer (pH 8.2) for 45 min at 37°C or overnight at 4°C and then incubated for 1 hr at room temperature with anti-involucrin antiserum diluted 1:5000 in 0.1% bovine serum albumin/0.1 M Tris Cl buffer (pH 8.2). The filter was washed with this albumin/Tris buffer and incubated overnight at room temperature with goat anti-rabbit IgG–colloidal gold (Janssen Life Science Products, Piscataway, NJ) diluted 1:100 in the albumin/Tris buffer containing 0.4% gelatin. The bound colloidal gold was enhanced by incubation in 0.2 M sodium citrate buffer (pH 3.85) containing 77 mM hydroquinone and 5.5 mM silver lactate and then was fixed with fixing solution (Janssen). Negative controls using preimmune serum were always run in parallel and showed no nonspecific binding.

Involucrin Immunoreactivity. Antigenic crossreactivity with anti-human involucrin antiserum was measured in cytosolic and partially purified extracts by a sensitive enzyme-linked immunoassay. The solid-phase antigen was prepared by distributing to each well of a Nunc 96-well plate 1 ng of human involucrin (74% purity by densitometry after electrophoresis) in 0.1 ml of 0.1 M sodium carbonate buffer (pH 9.6). After addition of 10 μg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 10 μl of water to each well, the plates were incubated overnight at 4°C and then washed with buffer, and remaining sites for adsorption were blocked with 0.1 M ammonium chloride for 30 min at room temperature. The amounts of involucrin protein in the partially purified primate samples assayed were quantitated by gel electrophoresis (16) and laser densitometry of the Coomassie blue R-250-stained involucrin bands in parallel with known amounts of the homogeneous, chromatographically purified human protein. Partially purified and standard involucrin preparations showed the same specific immunoreactivity. Samples (100 μl) of primate involucrins, appropriately diluted (1:10–1:10,000) in assay buffer [Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) containing 0.25% gelatin (type II, Sigma), 2 mM EDTA, 0.5% Tween 20, and 0.2% NaN_3], were incubated overnight in triplicate or quintuplicate at 4°C with rabbit anti-human involucrin antiserum (60 μl of a 1:1500 dilution) in a Linbro 96-well microtiter plate. Aliquots (100 μl) from each well were transferred to a Nunc plate containing adsorbed involucrin, incubated at room temperature for 30 min, and washed with assay buffer. The wells were then incubated for 1 hr with 0.1 ml of protein A-alkaline phosphatase conjugate (18) diluted 1:500, washed with buffer, and incubated with 0.1 ml of *p*-nitrophenyl phosphate (1 mg/ml) in 0.05 M sodium carbonate buffer (pH 9.8) containing 1 mM $MgCl_2$. After development of sufficient color (usually 1 hr at room temperature), absorbance values at 405 nm were recorded using a Titertek Multiskan (Flow Laboratories). In each experiment, standard curves were obtained in parallel from homogeneous, chromatographically purified involucrin (3). Assay sensitivity was approximately 0.025 ng of human involucrin. The human involucrin used as standard (chromatographically purified) and solid-phase antigen (partially purified) was from a single individual. The rabbit antiserum used in most experiments (including Fig. 2) was raised to chromatographically purified involucrin from a different individual. Antisera used in some experiments were raised in another rabbit to involucrin from this individual and in two other rabbits toward involucrin from a third individual.

Hybrid-Selection Translation. Poly(A)$^+$ RNA was isolated from cultured keratinocytes by centrifugation through a cesium chloride gradient (19) and chromatography on an oligo(dT)-cellulose column (20). Plasmid pλI-3H6B, which contains a 6-kilobase insert encoding the entire human involucrin gene (4), was immobilized on Biodyne A membrane (21) and incubated with poly(A)$^+$ mRNA at 140 μg/ml in standard buffer containing 50% formamide and 0.4 M NaCl (4, 22). The filters were then washed with 150 mM NaCl/15 mM sodium citrate, pH 7/0.5% $NaDodSO_4$ followed by 15 mM NaCl/1.5 mM sodium citrate, pH 7/1.5% $NaDodSO_4$ (both at 60°C), and bound RNA was eluted and translated in a reticulocyte lysate system in the presence of [^{35}S]methionine (23). The translation products were electrophoresed in an 8% acrylamide gel and visualized by fluorography.

RESULTS

Electrophoretic Mobility. Comparison of primate involucrins was facilitated by the observation that they could be substantially purified by a solvent-partitioning technique (11). The proteins were soluble in 67% (vol/vol) ethanol after aqueous extraction at pH 8 of trichloroacetic acid-precipitated crude homogenates. In six experiments, human involucrin was obtained in excellent yield with considerable purification (average 53% and 16-fold, respectively). As seen by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1), the involucrin was isolated nearly free of protein of similar molecular weight, although a major contaminant band containing comparable amounts of protein was often found migrating with the dye front (not shown).

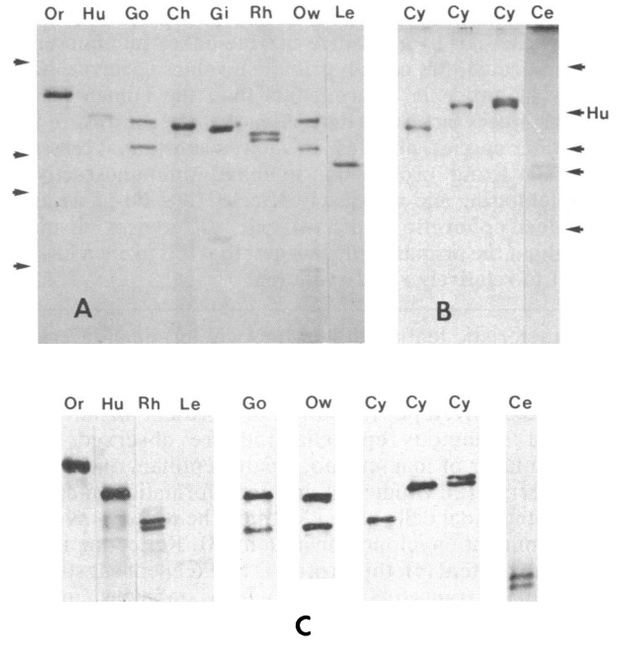

FIG. 1. Electrophoresis of partially purified involucrins. After treatment of crude extracts with trichloroacetic acid and ethanol, the soluble proteins were submitted to $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by Coomassie blue staining (*A*, 7% acrylamide; *B*, 8% acrylamide) or immunoblotting (*C*, 7% acrylamide). The groupings of lanes in *C* represent several independent experiments. Arrowheads in the margins of *A* and *B* show the positions of the standard proteins (top to bottom) myosin heavy chain (220 kDa), β-galactosidase (130 kDa), phosphorylase *a* (97 kDa), and bovine serum albumin (69 kDa) in parallel lanes. In the right margin of *B* the position of human involucrin (Hu) is also indicated. Species abbreviations are Or, orangutan; Hu, human; Go, gorilla; Ch, pygmy chimpanzee; Gi, gibbon; Rh, rhesus monkey; Ow, owl monkey; Le, lemur; Cy, cynomolgus monkey; Ce, cebus monkey. The mobility of chimpanzee involucrin (not shown) was identical to that of pygmy chimpanzee involucrin.

Using this solvent-extraction method of partial purification, we have examined extracts of cultured keratinocytes from a variety of species by $NaDodSO_4$/polyacrylamide gel electrophoresis. The involucrin-like proteins are illustrated in Fig. 1 for samples from the apes, macaques, New World monkeys, and a prosimian. From each species, one or two proteins were detected ranging in apparent molecular weight from 160,000 (orangutan) to 90,000 (cebus monkey). Chimpanzee and gibbon involucrins had mobilities quite close to that of the human protein, while samples from gorilla showed one band with similar mobility and a second band of comparable staining intensity migrating considerably more rapidly. A pattern similar to gorilla was obtained from owl monkey, and closely spaced doublets were also observed from rhesus and cebus samples. Extracts of keratinocytes cultured from the esophagus of the lemur *L. catta* contained a protein sharing this unusual solubility property and having an electrophoretic mobility nearly as great as that from cebus monkey. No such protein was seen in extracts of keratinocytes cultured from several rat stratified squamous epithelia (24) or from tree shrew epidermis.

Five individuals constituted a large enough sample size to observe involucrin polymorphism in cynomolgus monkeys. The three different electrophoretic patterns obtained are illustrated in Fig. 1*B*. Thus, samples from three monkeys appeared identical, giving a single band with mobility greater than that of human involucrin. Two monkeys gave bands with mobility slightly less than that of human—one individual a single band and the other clearly a doublet. From the latter individual, the patterns given by extracts of cultured epidermal, vaginal, and bladder epithelial cells were indistinguishable.

Antigenic Crossreactivity. With one exception, all the proteins putatively identified as involucrins by partial purification and gel electrophoresis (Fig. 1 *A* and *B*) were strongly positive in immunoblotting with rabbit anti-human involucrin antiserum. Fig. 1*C* illustrates the strong immunoreactivity obtained with the single bands of orangutan and human involucrin; the three cynomolgus monkey patterns; and the double bands from gorilla and rhesus, cebus, and owl monkey samples. In striking contrast, the lemur protein showed only faint immunoreactivity by the highly sensitive method of immunogold/silver enhancement, even when overloaded. Comparable amounts of human involucrin (overloaded) showed the very minor degradation products present in many such samples. Electrophoretic standard proteins, even at higher concentrations, in parallel lanes gave no detectable staining.

Since immunoblots provide only qualitative information, we used a quantitative immunoassay to compare the degree of antigenic crossreactivity among the various involucrins. This type of assay measures the amount of antibody available to react with human involucrin bound to a solid support after reaction with primate involucrin in solution. (For comparative purposes, the data in Fig. 2 were obtained using a single rabbit antiserum, although certain findings were confirmed, as indicated, with antisera from other rabbits.) Thus, as shown in Fig. 2, approximately 0.11 and 0.3 ng of soluble human involucrin removed 50% and 90%, respectively, of the antibody reactivity. The results presented for the hominoid primates show two distinctive features (Fig. 2 *A–C*). First, the gorilla and orangutan involucrins ($EC_{50} \leq 0.02$ ng) and pygmy chimp involucrin ($EC_{50} = 0.04$ ng) all reduced the antigenic reactivity more efficiently than seen in the standard curve for the human protein, indicating a higher density of determinants. This phenomenon was observed with antisera from three rabbits. Second, pygmy chimp involucrin was unable to absorb completely the anti-involucrin antibodies in two of three antisera tested, whereas both gorilla and orangutan involucrins completely absorbed the antibodies of

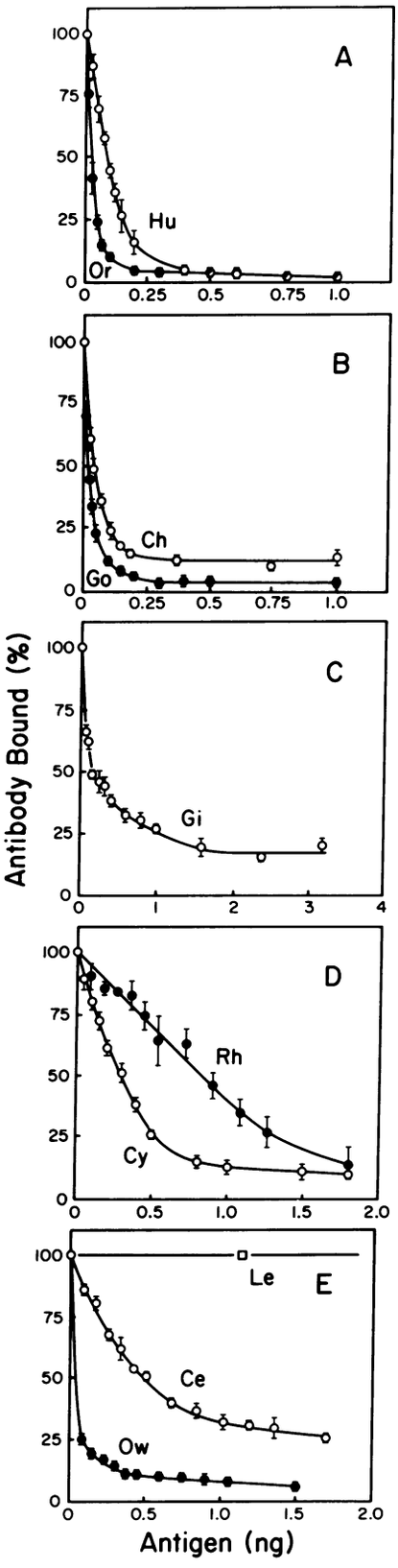

FIG. 2. Quantitation of involucrin crossreactivity. Involucrin samples partially purified from human and hominoid apes (*A–C*), macaques (*D*), and New World monkeys and a prosimian (*E*) were analyzed by enzyme-linked immunoassay. The high and low apparent molecular weight forms of the cynomolgus protein were indistinguishable in these assays. The error bars show the standard deviations about the mean.

all three antisera. Third, the gibbon protein removed much less of the reactivity, with 20% remaining even at the level of 1 ng (Fig. 2*C*). Examination of cynomolgus and rhesus monkey involucrins showed that both were less efficient in removing immunoreactivity than the human protein, with the rhesus monkey protein being the weaker of the two (Fig. 2*D*). Among the New World primates tested (Fig. 2*E*), the cebus monkey protein was a relatively weak antigen, leaving 25% of the reactivity even at the level of 1.7 ng. However, the owl monkey protein (Fig. 2*E*) was highly antigenic, approximating that of pygmy chimpanzee (Fig. 2*B*). Again, in striking contrast, the protein isolated from the lemur was virtually unreactive (Fig. 2*E*) with three antisera. This lack of detectable crossreactivity is compatible with estimates, by immunoblot titration, <1% of the antigenicity of the human protein. No antigenic crossreactivity was detected in extracts of cultured rat keratinocytes or cultured tree shrew epidermal cells (data not shown).

Cell-Free Translation. From confluent cultures of human, chimpanzee, gorilla, and rhesus monkey keratinocytes, poly(A)$^+$ RNA was isolated, hybrid-selected, and translated in a reticulocyte cell-free system. As shown in Fig. 3, the RNA from gorilla and rhesus monkey each directed the translation of two protein bands labeled with [^{35}S]methionine. Relative to the single bands of human and chimpanzee involucrin translated in parallel, the bands corresponded approximately in mobility to those observed by Coomassie blue staining of involucrins partially purified from the cultured cells by solvent extraction. This finding is compatible with the detection of two closely spaced bands by blot hybridization of electrophoretically fractionated poly(A)$^+$ RNA from rhesus monkey keratinocytes with a human involucrin probe (data not shown). The lack of any obvious hybrid-selected translation product from rat keratinocyte poly(A)$^+$ RNA parallels the lack of any distinctive protein isolable by the solvent-extraction method for involucrin and any antigenic crossreactivity detectable by immunoblot or solid-phase immunoassay techniques.

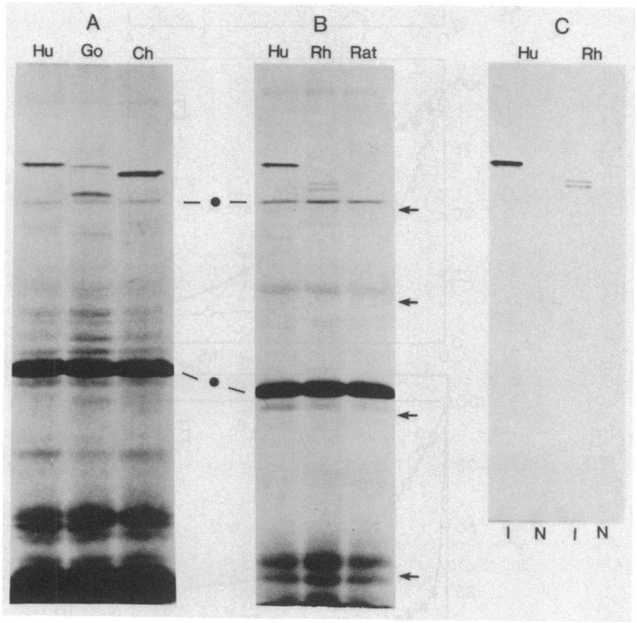

FIG. 3. Translation of hybrid-selected involucrin mRNA. [^{35}S]Methionine-labeled proteins directed by poly(A)$^+$ RNA from gorilla and chimpanzee (*A*), rat (*B*), and rhesus monkey keratinocytes (*B* and *C*) are compared with that from human. The major reticulocyte lysate translation artifacts are indicated by —●— connecting *A* and *B*. *C* shows an immunoprecipitation of the human and rhesus translation products with anti-human involucrin antiserum (I) or nonimmune serum (N) as indicated below each lane. Arrowheads to the right of *B* show the migration in a parallel lane of the standard proteins (top to bottom) phosphorylase *a* (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

DISCUSSION

The remarkable stability of human involucrin to conditions that ordinarily denature proteins presumably is a consequence of its peculiar structure. This protein features a highly elongated configuration and high net negative charge (3) bestowed by a high content of glutamic acid in a repeating configuration (4). That this protein does not adopt an insoluble "denatured" state upon boiling in neutral aqueous solution has also been demonstrated (25). The involucrin-like proteins from all the primate species examined share this novel solubility property and thus must share certain structural features. This conclusion is compatible with the considerable antigenic crossreactivity observed for all but the lemur protein. The specific structural features that the rabbit polyclonal antisera detect are uncertain, but the extremely weak crossreactivity apparent for the lemur protein suggests they are not required for the observed resistance to denaturation. That the lemur protein may function analogously to human involucrin is suggested by its lack of extractability when envelope crosslinking processes are induced by ionophore X537A permeabilization of the cultured lemur esophageal keratinocytes (unpublished observation). Preliminary efforts to show that the protein serves as a transglutaminase substrate by dansyl cadaverine labeling of cell extracts have been unsuccessful, possibly due to the limited amount of material available.

Upon gel electrophoresis, human involucrin migrates with considerably greater apparent molecular weight than expected for its size of 68 kDa (4). In line with the anomalous mobility observed previously for the human protein under different electrophoretic conditions (3, 5), we have noted small differences in relative mobility of primate involucrins depending upon specific parameters of given experiments such as acrylamide concentration in the gels. While the differences observed in electrophoretic mobility among species are clearly indicative of differences in primary structure, assignments of actual molecular weights will require sequence determinations.

Human keratinocytes ordinarily express only a single band of involucrin, judging by extracts of cells (or hybrid-selected translation of their mRNA) cultured from at least 10 individuals. The presence of different alleles in the human population has not been examined rigorously but has not been obvious in limited gel electrophoretic comparisons. Unlike those from human, keratinocytes from several primate species exhibit more than a single electrophoretic form of involucrin. The two forms observed from gorilla and several monkeys could logically arise (*i*) from different alleles in single individuals, (*ii*) at the level of mRNA transcription (alternative initiation, termination, or splicing), or (*iii*) from posttranslational processing. Our observation of two products in cell-free translation of mRNA rules out the last explanation for the two forms from the gorilla and rhesus monkey. Moreover, our finding that individual cynomolgus monkeys display different electrophoretic forms strongly suggests that different alleles are present in this monkey population.

The present results do not distinguish among the chimpanzee, gorilla, and orangutan as the human's closest relative, but involucrin nucleotide sequence information may prove more discriminating. This limited survey suggests that involucrin has been subject to considerable change during primate divergence. The data reveal that involucrin from a

New World monkey (owl) is antigenically more similar than those from certain Old World monkeys (rhesus, cynomolgus) and gibbon to human involucrin. This observation stands in contrast to the general congruence of protein and phylogenetic divergence, which occur at a near-uniform rate (26). However, species differences in the structure of involucrin, a highly repetitive protein, may not have arisen simply by drift (i.e., from accumulation of random, functionally neutral point mutations). Amplification of certain sequence elements in the various proteins after some previous duplication and drift would be compatible with the present findings and with the hypothesized origin of present-day human involucrin (4).

Elucidating the relation of the lemur protein to involucrins of other primates may be of assistance in searching for involucrin-like proteins beyond the primate order. That the lemur protein exhibits minimal antigenic crossreactivity compared to the rest of the primates examined provides a ready rationalization for the lack of immunochemically identifiable involucrin in more distantly related species. Thus, if the tree shrew (sometimes classified as a close relative of the primates by morphological criteria) expresses a related protein, it was not detectable in cultured epidermal cells by immunochemical methods or by the unusual solubility property noted even for the lemur protein.

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