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Expression of 4α-Carbinolamine Dehydratase in Human Epidermal Keratinocytes

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4α-Carbinolamine dehydratase is a bifunctional protein involved in the regeneration of tetrahydrobiopterin during the hydroxylation of the aromatic amino acids. It is also a dimerization cofactor of HNF1 and therefore is believed to function as part of the hepatic gene transcription system. In view of the recent discoveries that the distribution and developmental pattern of the dehydratase do not correlate strictly with those of the aromatic amino acid hydroxylases and HNF1, the hypothesis that the dehydratase may have other unknown functions has been put forward. In the present paper, we demonstrate unambiguously that human epidermal keratinocytes express detectable levels of this protein as indicated by enzyme assay, immunoprecipitation, Western blot, and RT-PCR. Its complete coding sequence has been cloned and was found to be identical with the human liver counterpart. The possible function of the dehydratase in skin is discussed.

4α-Carbinolamine dehydratase is an enzyme involved in aromatic amino acid hydroxylation. It was initially called phenylalanine hydroxylase stimulator (PHS) due to its ability to stimulate phenylalanine hydroxylase (PAH) activity in vitro at higher concentrations of PAH and higher pH (1). Subsequently, it was demonstrated that PHS is a 4α-carbinolamine dehydratase (2,3). It catalyzes the dehydration of 4α-carbinolamine to quinonoid dihydrobiopterin, which is then reduced back to tetrahydrobiopterin by the NADH-dependent dihydropteridine reductase. Because the dehydration reaction proceeds in vitro rapidly nonenzymatically at physiological pH (1,4), its physiological importance could not be assessed until it was demonstrated that certain hyperphenylalaninemic patients with a deficiency of this enzyme excrete large amounts of the biopterin analogue 7-biopterin (5,6), which is a potent inhibitor of phenylalanine hydroxylase (5,7). In vitro studies confirmed that the dehydratase can prevent the formation of 7-biopterin (8,9). Interestingly, the dehydratase was also shown to be identical to DCoH, the dimerization cofactor of HNF1 (10,11). In accord with these dual functions, it was expected that the expression pattern of the dehydratase would correlate with that of the aromatic amino acid hydroxylases or HNF1. However, our previous studies (12) showed that its distribution does not strictly parallel the distribution of aromatic amino acid hydroxylases; it also has a wider distribution compared to that of the transcription factor HNF1 (13). Moreover, in Xenopus it was shown that its developmental expression pattern differs from that of HNF1 (14). In Xenopus, the dehydratase is a maternal protein in the egg, whereas HNF1 expression starts during embryogenesis shortly after the mid-blastula transition. These findings indicate that the dehydratase may have other unknown functions. Regarding this point, more extensive investigation on the distribution of the dehydratase might be informative. Recent observations described by Schallreuter et al. (15) suggest a possible new function for the dehydratase. The authors reported that normal human skin has dehydratase activity and that the enzyme is missing in skin from patients with vitiligo. However, there were some potential problems in the assay that had been used to detect the dehydratase activity. First, this method measures the stimulated activity of phenylalanine hydroxylase (PAH) as dehydratase activity (1). Therefore, saturating amounts of PAH must be included in the assay. Second, 4α-carbinolamine is maximally stable at pH 8.2-8.4 (16). Accordingly, dehydratase activity is measured within this pH range. At lower pH such as pH 6.8, the condition used by Schallreuter et al. (15), the dehydration proceeds rapidly nonenzymatically (1,4). In view of these considerations, we decided to provide an unambiguous answer to the question of whether 4α-carbinolamine dehydratase is present in human skin, with the hope of shedding some light on the assumption of the involvement of the dehydratase in vitiligo.

MATERIALS AND METHODS

Cell culture. Keratinocyte cultures from normal adult human epidermis were purchased from Clonetics. Human foreskin keratino-

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cytes were isolated and cultured as described previously (17). All cells were used at passage three in these experiments. Keratinocytes were maintained in keratinocyte-SFM (Life Technologies, Gaithersburg, MD) to promote rapid growth. In some experiments, cultures were induced to undergo differentiation by washing them 3 times with phosphate-buffered saline and then maintaining the cells in basal keratinocyte-KSF lacking growth factors (bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin and triiodothyronine) and containing 2 mM calcium chloride. Undifferentiated cells were harvested when they were up to 70-80% confluent; differentiated cells were harvested 2 to 4 days after induction of differentiation.

Dehydratase assay. Our published PAH stimulation assay (18) was used to determine the specific activities of keratinocyte cell crude extracts. This method measures the formation of extra tyrosine caused by a dehydratase-mediated stimulation of PAH activity. The optimal amount of PAH to be used in the dehydratase assay was determined first. That is the amount of PAH required to saturate the system under dehydratase assay conditions but in the absence of dehydratase. A rat liver dehydratase standard was further used to determine the linear range. Both human foreskin and normal epidermal keratinocyte cell extracts were used for the dehydratase assay. Specific activity is expressed as nmol of increased formation of tyrosine per minute per mg crude cell extract.

Immunoprecipitation and Western blot analysis. 10 T75 flasks of undifferentiated or differentiated adult human normal epidermal keratinocytes were lysed and homogenized manually in 2 ml of lysis buffer (50mM Tris·HCl, pH8.0, 150mM NaCl, 0.5% Triton X-100, 100μg/ml phenylmethylsulfonyl fluoride (PMSF), 0.5μg/ml leupeptin, 1μg/ml aprotinin, 0.7μg/ml pepstatin). The protein inhibitors (Boehringer Mannheim) were added from stock solutions just before use. The homogenate was centrifuged at 13000rpm for 10 minutes at 4°C using a table microcentrifuge. The supernatant was used immediately or stored at −70°C. 700μg of crude cell extract was diluted to 1ml with lysis buffer and incubated with 4μl of rabbit dehydratase antibody for 3 hours at 4°C under gentle shaking. Subsequently, 15μl Protein A sepharose (Sigma) was added and shaking was continued at 4°C overnight. Protein A sepharose was spun down briefly and washed three times with lysis buffer. After the final wash, 10μl of lysis buffer without protease inhibitors and 15μl 2×SDS PAGE loading buffer were added. The mixture was heated at 85°C for 3min, then spun down and 20μl supernatant was loaded onto 16% SDS-PAGE (Novex). After electrophoretic separation, proteins were electrically transferred onto a PROTEIN nitrocellulose membrane (Schleicher & Schuell). Immunostaining was performed using the same polyclonal dehydratase antibody used for immunoprecipitation. Alkaline phosphatase conjugated anti-rabbit IgG(Fc) was purchased from Promega.

Isolation of total RNA. Total RNA was isolated from 6 T75 flasks of undifferentiated and differentiated human epidermal keratinocytes using TRIZOL reagent (Life Technologies) according to the manufacturer's protocol.

RT-PCR and cloning of dehydratase cDNA. The first strand of cDNA was synthesized from 5μg total RNA using poly(dT) as primer with SUPERSCRIPT II RNase H$^-$ reverse transcriptase (GIBCO BRL). PCR amplification of the dehydratase coding sequence was done for 30 cycles using the 5′ primer (5′-CGGAATTCATATGGCTGGCAAAGCACACAG-3′) and the 3′ primer (5′-CGGGATCCTATGTCATGGACACTGCTAC-3′) (19)(synthesized by Lofstrand Labs Limited). Denaturation was at 95°C for 40 sec, annealing at 55°C for 2 min, and extension at 72°C for 1.5 min. The PCR products were analyzed on a 1.5% agarose gel and purified using a PCR purification kit (QIAGEN). The purified PCR products were digested with EcoRI and BamHI and then subcloned into pUC18 for sequencing.

DNA sequence analysis. The complete insert was sequenced from both ends using pUC sequence primers 5′-GTAAAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGAC-3′ (New England Biolabs) with an automated laser fluorescence DNA sequencer (Pharmacia). Sequence data were analyzed using the Wisconsin Package Version 9.0, Genetics Computer Group (GCG).

TABLE 1

Specific Dehydratase Activity of Undifferentiated and Differentiated Human Keratinocytes

	Undifferentiated	Differentiated
Specific activity (nmol/min/mg)	6.24±1.40	4.95±1.72

RESULTS AND DISCUSSION

To answer the question of whether 4α-carbinolamine dehydratase is present in human skin, we first measured its enzyme activity. Table 1 shows the average activity of two batches (3 T75 flasks cells per batch) of human foreskin keratinocytes and one batch of human epidermal keratinocytes (10 T75 flasks). The activity in both undifferentiated and differentiated human keratinocytes was very low, albeit detectable. Because the dehydratase assay is based on the dehydratase stimulation of phenylalanine hydroxylase activity, there is an obligatory blank reaction due to the basal hydroxylase activity. When dehydratase activity is very low, this assay cannot unambiguously prove that human skin keratinocytes express 4α-carbinolamine dehydratase. To bolster the evidence in support of the presence of dehydratase, we carried out traditional Western blot assays. Loading 12.5 μg protein of a crude cell extract and extending the color development, we were not able to see any significant 4α-carbinolamine dehydratase band. To increase the sensitivity of the blot assays, we first performed immunoprecipitation to concentrate 4α-carbinolamine dehydratase from 700 μg protein of a crude cell extract, and then repeated the Western blot assay. The results are shown in Fig. 1. Both undifferentiated (lane 2) and differentiated keratinocytes (lane 3) showed a significant band at the same molecular weight position as that of a rat liver dehydratase standard (lane 4). To test the specificity of the rabbit anti-dehydratase antibody and the immunoprecipitation, several controls were included in the experiment. In lane 8, only BSA was loaded; in lane 5 and 6, no anti-dehydratase antibody was added to undifferentiated and differentiated cell extract, respectively, during the immunoprecipitation step. To exclude the possibility that the anti-dehydratase antibody was contaminated by dehydratase, 2μl of antibody was loaded onto the SDS-PAGE gel (lane 7). All controls were negative (the large bands in the high molecular region are dehydratase antibody bands), indicating that the antibody and the immunoprecipitation were specific. Therefore, the band seen in lane 2 (immunoprecipitated undifferenti-

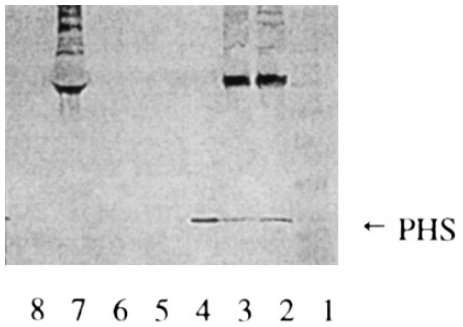

FIG. 1. Western blot analysis of immunoprecipitated protein. Immunoprecipitation was described in Materials and Methods. Immunoprecipitated protein from undifferentiated (lane 2) and differentiated (lane 3) human epidermal keratinocyte cell extracts was analyzed by Western blot. Lane 5 and 6 (dehydratase antibody was not included in the immunoprecipitation of undifferentiated and differentiated cell extracts) together with lane 7 and 8 (dehydratase antibody and BSA were loaded, respectively) were included as negative controls. A rat liver dehydratase standard was loaded as a positive control (lane 4). Lane 1 contains SeeBlue prestained standards (Novex).

ated cell protein) and lane 3 (immunoprecipitated differentiated cell protein) provides good evidence that the dehydratase protein is present in normal human skin keratinocytes.

Because only very low levels of dehydratase protein were detected in human skin by Western blot assays, additional evidence was needed to unequivocally demonstrate the presence of the dehydratase in this tissue. Accordingly, we carried out a comparison of the coding sequence of skin dehydratase with that of its liver counterpart. To make this comparison, we amplified the cDNA of human skin dehydratase by RT-PCR and cloned the complete coding region. Fig. 2 shows the RT-PCR results. A sharp cDNA band (about 330 bp) was elicited and amplified from both undifferentiated (lane 2) and differentiated keratinocyte (lane 3) total RNA. There was no apparent difference in the size and amount between these two bands. A control RT-PCR was carried out without reverse transcriptase. No product band was seen in the control, indicating that the total RNA was not contaminated with chromosomal DNA. Therefore, the band observed in lane 2 and 3 must be an amplified product from RNA. The band in lane 2 (undifferentiated keratinocytes) was purified and cloned into pUC18 (see MATERIALS AND METHODS). Sequencing the insert revealed that it has no introns, which are present in the dehydratase gene (19). That further confirmed that the RT-PCR products were amplified from RNA. Sequence comparison showed that the human skin keratinocyte 4α-carbinolamine dehydratase has the same coding sequence as the human liver counterpart (19). Interestingly, the codon for leucine 15 is also CTG, and has one base difference at the wobble position (CTA) compared to another published liver sequence (20). This result supports the assumption that a single genomic copy of the dehydratase gene appears to exist in the human genome (19). This gene is highly expressed in liver, moderately in kidney and at much lower level in other tissues such as brain, lung, heart, spleen, ovaries and hypothalamus. However, no tissue-specific response elements were found in its promoter and upstream region (19). Hence, the mechanism of differential expression of the dehydratase in different cells is still unknown. Due to their availability and ease of culture, keratinocytes could be developed as a good model for this purpose.

The above data unambiguously demonstrate that both undifferentiated and differentiated human epidermal keratinocytes express dehydratase, albeit at a low level. Our observation that the differentiated cells still have 80% of the dehydratase activity of undifferentiated cells is in contrast with the published observation that differentiated keratinocytes have no detectable dehydratase activity (21). A related question is what its function might be in keratinocytes. It has recently been reported (15) that in patients with vitiligo the dehydratase activity of the skin was extremely low or absent, and that the nonenzymatic by-product, 7-tetrahydrobiopterin (7-BH_4) accumulated up to the concentration that inhibited phenylalanine hydroxylase. The authors argued that in patients with vitiligo the accumulation of 7-BH_4 and the resulting inhibition of PAH may initiate the process of depigmentation by blocking the supply of L-tyrosine, either directly in the melanocytes or from the surrounding keratinocytes (15,21,22). In this context, it is instructive to compare this situation with that in classical phenylketonuric (PKU) patients where PAH mutations lead to a total or near-total loss of PAH activity. Indeed, reduced melanin synthesis was observed to a certain extend in hair follicles, pigmented areas of the brain, eyes and skin in some PKU patients

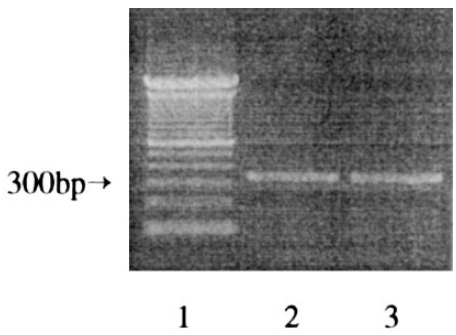

FIG. 2. Agarose gel analysis of RT-PCR products. 5 μg total RNA of undifferentiated (lane 2) and differentiated (lane 3) keratinocytes were reverse transcribed. The PCR amplification was performed using dehydratase specific primers. Aliquots of products were analyzed by 1.5% agarose gel electrophoresis. Lane 1 shows a 100bp DNA ladder (GIBCO BRL).

(23,24). But the mechanism for the reduced melanin synthesis in classical phenylketonuria and that proposed for vitiligo appear to be unrelated. The observed decrease in melanin synthesis in some PKU patients has been explained by inhibition of tyrosinase (25,26) or competitive transport of tyrosine by high concentration of phenylalanine (24,27) present in PKU patients. An additional observation that is inconsistent with the hypothesis that a loss of dehydratase activity is the cause of the decreased melanin levels seen in patients with vitiligo is the fact that patients with hyperphenylalaninemia caused by a defective dehydratase do not suffer from vitiligo (6). Furthermore, early studies made it clear that mammalian melanocytes do not use phenylalanine for melanin synthesis (28,29). Nevertheless, in view of the findings that high concentrations of phenylalanine inhibit tyrosinase and compete for the tyrosine transporter of melanosomes, if loss of dehydratase activity in vitiligo patients is confirmed, it would be worth determining the concentration of phenylalanine in epidermal keratinocytes or melanocytes of vitiligo patients.

In conclusion, it should be emphasized that our unequivocal demonstration that 4α-carbinolamine dehydratase is present in keratinocytes derived from human skin is a necessary but, by itself, an insufficient step in proving the postulated role for this enzyme in the development of the disease vitiligo (15). Many other observations, such as those outlined above, would need to be addressed before this postulate can be accepted.

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