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Desquamin Is an Epidermal Ribonuclease

Peter Selvanayagam,[1] Gang Lei,[2,3] Trace Bell,[2] Sandhya Ram,[2] Henry Brysk,[2] Srinivasan Rajaraman,[1] and Miriam M. Brysk[2,3,4*]

[1]Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555
[2]Department of Dermatology, University of Texas Medical Branch, Galveston, Texas 77555
[3]Department of Microbiology & Immunology, University of Texas Medical Branch, Galveston, Texas 77555
[4]Department of Human Biological Chemistry & Genetics, University of Texas Medical Branch, Galveston, Texas 77555

Abstract Desquamin is a glycoprotein that we have isolated from the upper granular layer and the stratum corneum of human epidermis; it is not ordinarily expressed in submerged cultures, whose terminal differentiation stops short of formation of these layers. The exogenous addition of desquamin to human cultured keratinocytes extended their maturation, and hematoxylin staining indicated a loss of cell nuclei. For confirmation, cultured cells were lysed in situ, and the nuclei were incubated with desquamin for several days, then stained with hematoxylin. Damage to the nuclei was evident: the nuclear inclusions remained intact, while the surrounding basophilic nuclear matrix was degraded. Desquamin was then tested directly for nuclease activity. Ribonuclease activity was determined by incubating desquamin with human epidermal total RNA and monitoring the dose-dependent disappearance of the 28S and 18S ribosomal RNA bands in an agarose/formaldehyde gel. On RNA-containing zymogels, we confirmed the RNase activity to be specific to desquamin. Using synthetic RNA homopolymers, we found the active RNase domains to be limited to cytosine residues. On the contrary, DNA was not degraded by an analogous procedure, even after strand-separation by denaturation. J. Cell. Biochem. 68:74–82, 1998.

Key words: cell culture; nuclei; nuclear degradation; endonucleases; polycytosine degradation; differentiation; cornification; stratum corneum

The epidermis undergoes many structural and functional changes during the process of terminal differentiation. In the outer epidermis, there is a definite transition zone between the granular layer and the stratum corneum. Many degradative events occur within this region. In particular, organelles (including mitochondria, lysosomes, cell membranes, keratohyalin granules, lamellar granules, and nuclei) are degraded, presumably by a variety of specific enzymes [Karásek, 1988]. Many macromolecules are catabolized to amino and nucleic acids. Among these degradation products are molecules that contribute to the structure and function of the stratum corneum. Epidermal differentiation culminates in cornification, the formation of large dead squames with disintegrated nuclei, ready for desquamation. The least understood process in this series of events is the disappearance of nuclei. While it is presumed that the nuclei are degraded by enzymes, no specific molecules synthesized within the epidermis have been linked to the process. Ribonuclease activity extends outward from the transition zone [Steigleder and Raab, 1962]. In freshly-isolated epidermal cells that had been fractionated on Percoll gradients, the nuclei of the granular cells showed clumping of the chromatin, which was further enhanced when these cells were incubated with serum [McCall and Cohen, 1991].

Abbreviations: DEPC, diethyl pyrocarbonate; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; KGM, keratinocyte growth medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Gang Lei's permanent address is Department of Laboratory Medicine, West China University of Medical Sciences, Chengdu, Sichuan, People's Republic of China.

*Correspondence to: Miriam M. Brysk, Department of Dermatology, University of Texas Medical Branch, Galveston, TX 77555-0783.

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Epidermal keratinocytes in culture do not complete a normal process of terminal differentiation. The extent of their maturation is dependent on the culture medium. Rheinwald and Green [1975] developed a method for culturing human epidermal keratinocytes that utilized irradiated 3T3 fibroblasts as a substratum, in a medium containing serum and epidermal growth factor. In this system, the outer cells were shed as either anucleated or nucleated squames. When the nucleated squames were incubated in a medium containing serum (or plasminogen, a serum component), their nuclei were degraded [Green, 1977].

Newer culture systems permit the proliferation and growth of keratinocytes by themselves (without fibroblasts) in more closely controlled media, in which the extent of terminal differentiation can be modulated by adjusting the composition. Epidermal keratinocytes grow as an undifferentiated monolayer in a low Ca^{2+} (~0.1 mM) medium, but can be induced to differentiate by elevating the extracellular Ca^{2+} concentration to physiological levels (~1.5 mM) [Hennings et al., 1980]. A serum-free and low-Ca^{2+} medium for the culture of human epidermal keratinocytes has been formulated by Boyce and Ham [1983]. Adding exogenous Ca^{2+} to this medium triggers suprabasal differentiation. Maturation progresses farther when the medium is also supplemented with serum, but still does not extend to the formation of distinct granular or stratum corneum cell layers, nor to either desquamation or nuclear degradation. The keratinocyte growth medium used in the present paper is a commercial version of Boyce and Ham's, which we have elevated to a Ca^{2+} concentration of 2 mM and augmented with 10% serum.

We have isolated and characterized desquamin, a glycoprotein expressed in the terminally differentiated epidermis [Brysk and Rajaraman, 1992]. A monospecific antibody to desquamin immunolocalizes to the stratum corneum and to the transition zone between the granular layer and the stratum corneum [Brysk et al., 1986]. Desquamin plays a role in cell adhesion and desquamation [Brysk et al., 1988]. It is not ordinarily found in cultured keratinocytes, with their more limited differentiation pattern (but the further addition of interferon-γ to the serum-enriched medium induces the expression of desquamin, as well as increased cell shedding) [Brysk et al., 1991]. The present study observed that the exogenous addition of desquamin to epidermal cells in culture (in the keratinocyte growth medium supplemented with Ca^{2+} and serum) extended their maturation to degradation of the nuclei. It also established that desquamin degraded human RNA (but not DNA), cleaving the RNA at the cytosine residues.

MATERIALS AND METHODS

Isolation of Desquamin

The procedure for the isolation of desquamin from human epidermis has been previously described [Brysk et al., 1994]. As a further precaution for the present set of experiments, all reagents and buffers were prepared with distilled water that was pretreated with diethyl pyrocarbonate (DEPC). Briefly, epidermis was homogenized and extracted in Dulbecco's phosphate-buffered saline (DPBS) at 4°C. The Concanavalin A positive glycoproteins were recovered by lectin-affinity chromatography, then fractionated by HPLC gel filtration chromatography. The fractions containing desquamin were pooled and further purified by ion-exchange chromatography on Q Sepharose (Pharmacia Biotech, Piscataway, NJ). The fraction eluting at 0.15 M NaCl was rerun on an analytical HPLC gel filtration column. The recovered desquamin was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel [Laemmli, 1970]. The desquamin band (35-38 kDa) was cut out from the SDS-PAGE gel, macerated in DEPC-treated water, and frozen at −70°C. The acrylamide in the freeze-thawed gel was removed by centrifugation at 15,000 rpm for 2 min. To document the purity of the isolated desquamin, the supernatant fluid was rerun on an SDS-PAGE gel and stained with Coomassie Blue. It was also subjected to two-dimensional SDS-PAGE gel electrophoresis [Anderson, 1991], over a pH range of 3–10 in one dimension and a 10–20% acrylamide gradient in the other, followed by silver staining (Anatek-EP, Portland, ME).

Cell Culture

The epidermis of fresh human foreskins was separated from the dermis by incubation with trypsin (0.25% of 1:250, in Hank's salt solution)

overnight at 4°C. Keratinocytes were cultured in keratinocyte growth medium (KGM; Clonetics, San Diego, CA) in 75 cm^2 flasks nearly to confluence, then detached from the culture flasks using 0.025% trypsin and 0.025% ethylenediamine tetraacetic acid in DPBS, and the trypsin neutralized with fetal calf serum (FCS). The cells were plated at high seeding densities (2 X 10^5 cells/cm^2) on collagen-coated 24-well culture plates and incubated in KGM overnight in order to achieve rapid cell attachment and contact inhibition of growth, then incubated in KGM augmented with 10% FCS (and adjusted to a Ca^{2+} concentration of 2 mM) with or without 20 µg of purified desquamin. After five days, the cells were photographed under a Leitz Diavert phase-contrast microscope. The same cells were then fixed for 1 min in ethanol, stained for 2 min with hematoxylin, destained with water, and photographed under a Zeiss Axiovert microscope.

Preparation of Nuclei

Cells grown as above (without desquamin) were rinsed twice in warm DPBS, then lysed in situ by incubation in buffer A (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 2 mM $MgCl_2$, 0.25% of the non-ionic detergent NP-40) for 1 min. The detergent was washed off with several rinses of DPBS. The nuclei in situ were then incubated in buffer B (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5% glycerol) supplemented with 2 mM Ca^{2+} and 10% FCS, with or without desquamin (20 µg/well), for 3 days in a 37°C incubator. To assess whether other standard enzymes would degrade nuclei in situ, we also tested RNase A (200 U/ml), RNase H (15 U/ml), DNase 1 (200 U/ml), S_1 nuclease (200 U/ml), and trypsin (10 U/ml). They were then washed with warm DPBS, fixed for 1 min in 100% ethanol, and visualized by staining with hematoxylin as described above.

RNase Assays

Total RNA was extracted from the skin and kidney tissues of a healthy human donor [method of Chomczynski and Sacchi, 1987]. Twelve µg of this total RNA was incubated at 42°C for 4 h with up to 50 ng of homogeneously pure desquamin that had been extracted from the SDS-PAGE gel, in a dose response in steps of a factor of 2. An equal aliquot of total RNA was exposed to buffer extracted from another part of the acrylamide gel (not containing the desquamin band), to rule out the possibility of RNA degradation by the contaminating presence of ubiquitous RNase in the reagents used. The mixtures were denatured with 50% formamide at 65°C for 5 min, size fractionated in 1.5% agarose/3% formaldehyde gel, then stained with 10 mM ethidium bromide. The resulting fluorescence intensity of the 28S and 18S ribosomal RNA bands was observed under UV light.

Zymogel assays were also done, using human epidermal RNA as a substrate enclosed in SDS-PAGE [method of Rosenthal and Lacks, 1977]. Briefly, the protein samples: desquamin (5 µg) and RNase A (10 pg/ml), were boiled for 5 min in a buffer of 50 mM Tris-HCl, pH 6.8, containing 2% SDS, 20% glycerol, 2 mM EDTA, and 0.01% bromphenol blue, then electrophoresed on a 12.5% polyacrylamide gel containing human epidermal RNA (25 µg/ml gel) and 2 mM EDTA, at room temperature. The gels were washed thrice for 20 min in a buffer of 50 mM Tris-HCl, pH 7.6, 2 mM $CaCl_2$, 0.02% NaN_3, and 1 µg/ml ethidium bromide. They were then incubated overnight in the same buffer at 37°C. The activity bands were visualized under UV light.

In order to determine base specificity, similar assays were performed using synthetic RNA polymers of varying lengths (Pharmacia, Gaithersburg, MD). Ten to 50 µg of poly A, poly U, poly G, and poly C were each incubated with 5 µg of desquamin purified from an SDS-PAGE gel or with a sham control buffer extracted from the same gel. The reactions were resolved in an agarose/formaldehyde gel, stained with ethidium bromide and observed and photographed under UV light.

DNase Assays

Amplified bacterial plasmid DNA was extracted [method of Birnboim and Doly, 1979]. One µg of plasmid DNA pBluescript (Stratagene, La Jolla, CA), previously linearized with the enzyme Eco RI, was subjected to digestion with 50 ng of desquamin, at 37°C for 16–20 h; DNA aliquots were also incubated with sham controls. The reactions were resolved in a 0.8% agarose gel which was stained with ethidium bromide and observed under UV light to ascertain degradative activities. In further search of DNase activity, the linear plasmid pBluescript DNA was strand-separated by denaturation with 0.2 N NaOH at 37°C for 30 min, neutral-

ized with 0.3 M sodium acetate, and purified by ethanol precipitation at 20°C for 16 h. The resulting single-stranded DNA was tested in the same manner as the original double-stranded DNA, by incubation with desquamin and with sham control buffer.

RESULTS

Isolation of Desquamin

Endonuclease assays require exacting care to avoid contamination of all reagents. The consequent added precautions in the course of isolating desquamin are described in the Materials and Methods section. The purity of desquamin is demonstrated in Figure 1. On an SDS-PAGE gel, desquamin was visualized with Coomassie Blue as a doublet (Fig. 1a); by two-dimensional gel electrophoresis and silver staining, the doublet remained unseparated (Fig. 1b).

Morphology of Keratinocytes

The epidermal cells cultured in the presence of desquamin (Fig. 2b) had larger outer squames than the controls (Fig. 2a). Staining with hematoxylin (a dye specific for the basophilic material in the nuclear matrix) revealed more fundamental morphological differences in these cells: In the absence of desquamin (Fig. 2c), the nuclei were small, darkly stained, and homogeneous in appearance, whereas cells incubated with desquamin (Fig. 2d) exhibited some large outer squames either without nuclei or with nuclei that were only lightly stained at the rims, if at all. Note that, although the micrographs are all at the same magnification, the cells were larger with desquamin and consequently the nuclei were further apart.

A more elegant demonstration of desquamin's ability to degrade nuclei was achieved by directly incubating isolated nuclei (obtained by lysing keratinocytes with detergent) with desquamin, followed by hematoxylin staining (Fig. 3). The hematoxylin stained the nuclear inclusions of micronucleoli or condensed chromatin that remained intact. On the other hand, the surrounding basophilic nuclear matrix failed to stain with hematoxylin; it was degraded by desquamin. As negative controls, we repeated the experiment with standard enzymes (trypsin, RNase A, RNase H, DNase 1, S_1 nuclease) substituted for desquamin; none of them caused any nuclear degradation.

RNase Assays

Total RNA extracted from human skin and kidney was incubated with desquamin and with

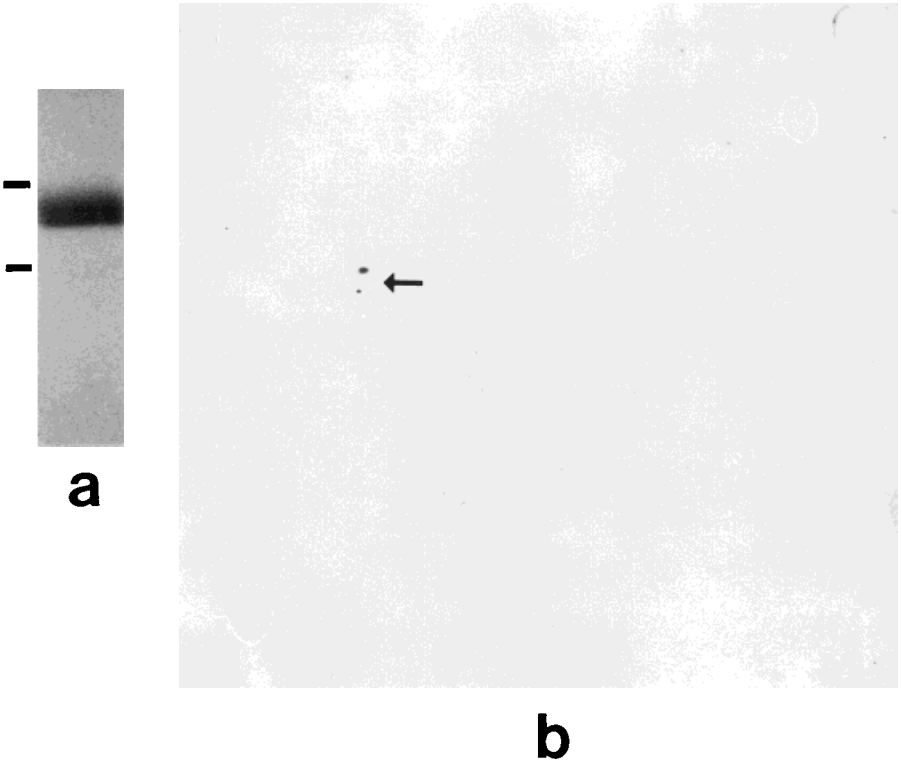

Fig. 1. a: Coomassie-Blue stained 10% SDS-PAGE gel, showing purified desquamin as a doublet, with standards indicated at 30 kDa (lower bar) and 46 kDa (upper bar). **b**: Silver-stained 2D gel (gradients of pH 3–10 and 10–20% acrylamide); desquamin doublet indicated by arrow.

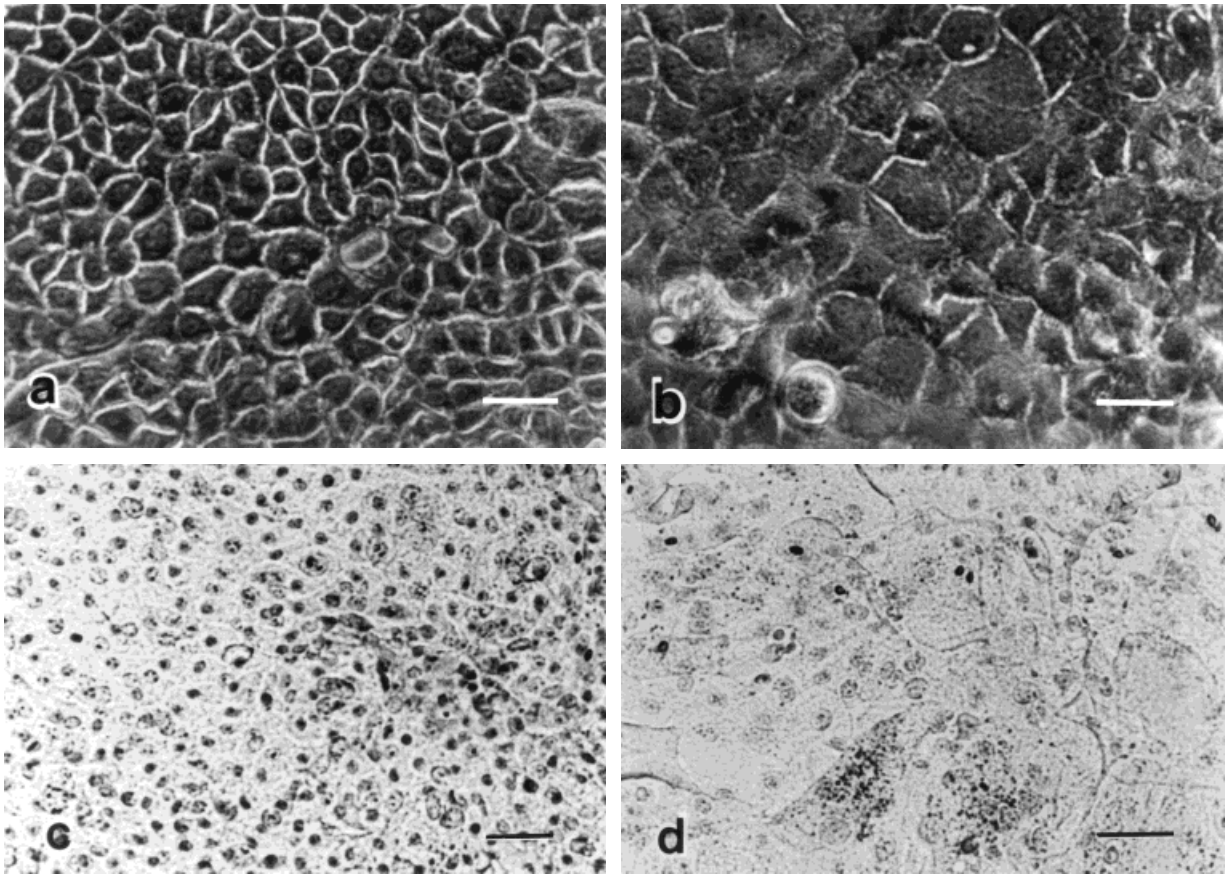

Fig. 2. Phase contrast micrographs of keratinocytes in primary culture, grown **(a**) without desquamin, **(b)** with 20 µg desquamin (scale bar = 10 µm). **(c,d)** Same as (a,b) except stained with hematoxylin (scale bar = 5 µm). Note larger squames in (b), nuclear degradation in (d).

controls. There was no breakdown of the 28S and 18S ribosomal RNA bands upon exposure to the DEPC-treated water, or to the buffer extracted from an area of the acrylamide gel, remote from the desquamin band, that served as sham control (Fig. 4). This indicates that the buffers and reagents that we used to purify desquamin were free of ubiquitous RNases [Holley et al., 1961]. The RNA was degraded by desquamin in a dose-dependent manner, starting with a concentration of 50 ng that was serially diluted. There was a progressive disappearance of the 28S and 18S ribosomal RNA bands and the concordant accumulation of breakdown products at the bottom of the gel.

RNase activity was also investigated by zymography, a technique that permits localization of the activity to specific proteins separated on SDS-PAGE gels. We incorporated human epidermal RNA as a substrate in polyacrylamide gels (Fig. 5). The RNase activity of desquamin is shown in lane 2, the positive control RNase A in lane 3. Our results clearly show colocalization of the RNase activity with the molecular weight of desquamin.

To ascertain whether the RNase activity was base-specific, we tested individual synthetic RNA homopolymers for a similar digestion (Fig. 6). Only poly C was degraded by desquamin (lane 6). Homopolymers of A, U, and G were unaffected.

DNase Assays

As cytosine is common to both DNA and RNA, it seemed plausible that desquamin might also degrade DNA. Desquamin, however, did not degrade plasmid DNA under the same experimental conditions (Fig. 7). No decrease was evident in the fluorescing 3 kb DNA strands, nor could any breakdown products be detected in the lower part of the agarose gel used to resolve the incubated molecules. There was some binding of the protein molecules to the target DNA, resulting in a slight retardation in

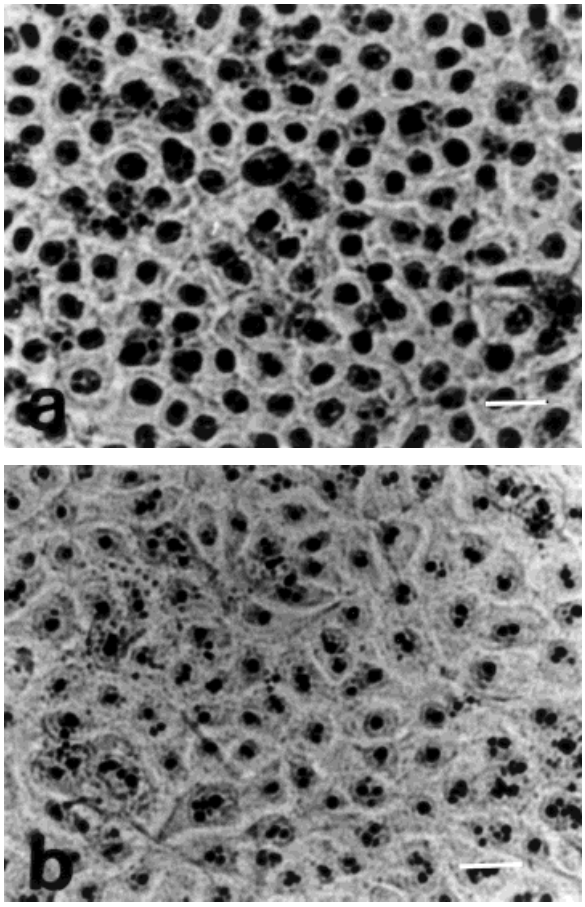

Fig. 3. Nuclei from lysed cultured keratinocytes, incubated with buffer **(a)** without desquamin, **(b)** with 20 µg desquamin, and stained with hematoxylin, showing nuclear degradation (scale bar = 7.5 µm).

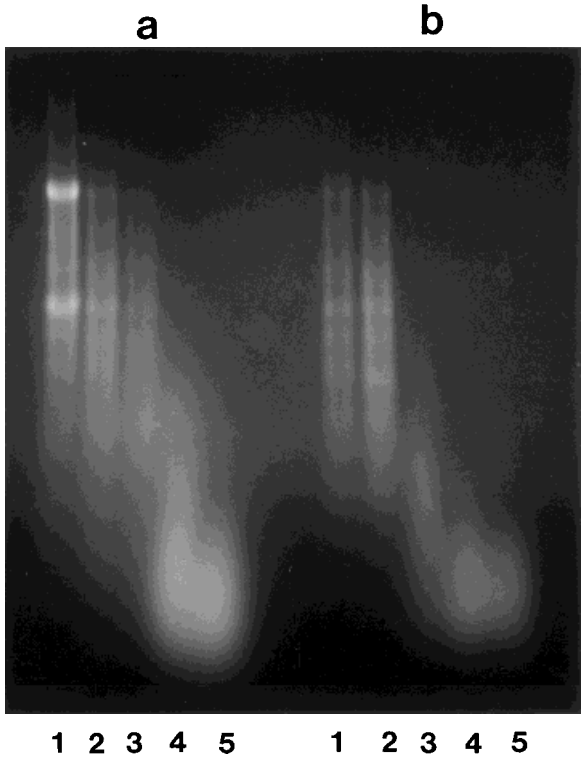

Fig. 4. Desquamin degrades human tissue RNA from **(a)** kidney and **(b)** skin. Dose-response for total RNA incubated with desquamin: y **(lane 1)** untreated control; **(lanes 2–5)** desquamin incremented in factors of 2 up to 50 ng. Note progressive disappearance of the ribosomal RNA bands and accumulation of degradation products at the bottom of the gel.

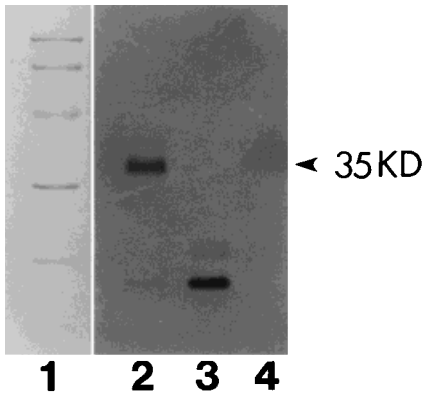

Fig. 5. Zymogel assay, using human epidermal RNA in SDS-PAGE as substrate, showing that RNase activity localizes to molecular weight of desquamin; **(lane 1)** molecular-weight standards, **(lane 2)** desquamin, **(lane 3)** RNase A, **(lane 4)** buffer control.

the electrophoretic mobility of the bound DNA and a consequent smear.

Noting that desquamin degraded RNA, which is single-stranded, but not DNA, which is double-stranded, we wondered whether this configurational difference might be pivotal. Accordingly, we incubated single-stranded DNA (obtained by denaturing native DNA) with desquamin under similar conditions for digestion. Single-stranded DNA was not degraded either (Fig. 7).

DISCUSSION

Terminal differentiation of cultured keratinocytes is substantially less advanced than in vivo, even in media that include serum. On the one hand, this restricts the value of such cultured cells as models for the maturation of the living organ. On the other hand, it provides an opportunity to gain insight into details of the mechanisms involved in the incomplete late stages of differentiation, particularly when the culture media are selectively supplemented with agents not present in vitro but that are available to the cells in vivo. We focus here on

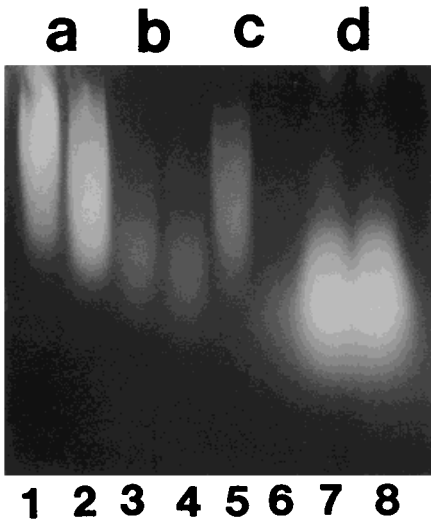

Fig. 6. Nucleotide-specific endonuclease activity of desquamin. Synthetic RNA homopolymers were incubated individually with desquamin purified from the gel, as well as with the gel buffer as sham control. **a**: Poly A incubated with **(lane 1)** the sham control buffer and **(lane 2)** desquamin; **b**: **(lanes 3,4)** corresponding results for poly U; **c**: **(lanes 5,6)** poly C; **d**: **(lanes 7,8)** poly G. Only poly C was digested by desquamin **(lane 6)**.

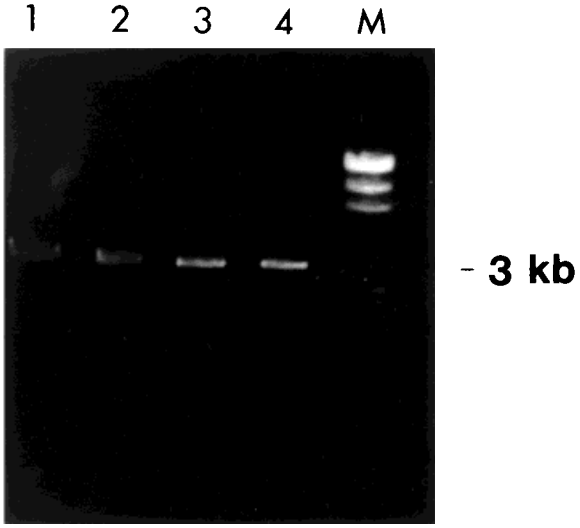

Fig. 7. Desquamin does not degrade DNA. Double-stranded native plasmid DNA pBluescript was linearized with the enzyme Eco RI, then incubated with **(lane 1)** sham control buffer or **(lane 2)** desquamin. The linearized plasmid DNA pBluescript was strand-separated, and then incubated with **(lane 3)** sham control buffer or **(lane 4)** desquamin. M denotes the size marker l DNA, digested with the enzyme Hind III. Persistence of the 3 kb DNA bands indicates lack of DNase activity.

desquamin, a glycoprotein first expressed in the transition zone between the granular layer and the stratum corneum [Brysk and Rajaraman, 1992]. It is in this transition zone that epidermal nuclei disappear during terminal differentiation [Karásek, 1988]. Keratinocytes in submerged cultures, in which the granular layer and the stratum corneum are not fully formed, do not ordinarily express desquamin. We observed that the exogenous addition of desquamin to keratinocytes in culture induced morphological changes which resulted in the formation of larger squames, more akin to those seen in vivo (Fig. 2). To verify that differentiation extended to anucleation, as in vivo, we stained the cells with hematoxylin; we saw, indeed, a loss of staining in the desquamin-treated cells. More directly, by lysing the cells in situ and incubating the nuclei with desquamin, we attained clear evidence that desquamin degraded the basophilic matrix of the nuclei (Fig. 3). The implication is that desquamin does so in vivo (where it is endogenous). The trypsin-like proteolytic activity of desquamin [Brysk et al., 1994] is not likely to be responsible, as serum (which is part of the culture medium) neutralizes trypsin. Indeed, when we used pancreatic trypsin as a control, the nuclei were not degraded.

The processes whereby cell nuclei disappear involve two complementary aspects: the degradation of the organelle itself as a structure, and the degradation of the DNA, RNA, and nucleoproteins that comprise the basophilic matter within the nucleus. Our morphological observations appeared to link desquamin to the latter mechanism, suggesting the presence of nuclease activity. As nucleases are frequent contaminants in protein preparations, negative controls were run by repeating the experiment with commercially available nucleases (RNase A, RNase H, DNase 1, S_1-nuclease) used in place of desquamin. None degraded the nuclei.

We proceeded to test desquamin for nuclease activity biochemically, by reacting it with extracted human RNA and DNA. As evidence that the ribonuclease activity was limited to desquamin and did not include a contaminant, we tested desquamin in a zymogel assay using human epidermal RNA as a substrate enclosed in SDS-PAGE. There was a clear area of RNA degradation at 35 kDa, the molecular weight of desquamin (Fig. 5). The purity of desquamin is documented in Figure 1.

Nucleases often exhibit substrate specificity: some cleave only RNA or DNA, some both; some cleave only single strands, others double strands. We found that desquamin degraded human epidermal and kidney RNA in a dose-

dependent fashion (Fig. 4). It did not degrade DNA, with or without strand-separation (Fig. 7).

Many degradative RNases display base specificity for the cleavage site of the RNA substrate: some act on pyrimidine residues while others cleave purine residues [Deutscher, 1993]. To assess the base specificity of the RNase activity, we exposed four synthetic RNA homopolymers to desquamin. The activity was limited to pyrimidines. Indeed, desquamin cleaved RNA only at the cytosine residues (Fig. 6).

Epidermal cell differentiation is a balanced system involving division of the basal cells and terminal differentiation of the outer cells. It is a well-regulated process that, in the course of the biogenesis of the stratum corneum, results in cell death. Epidermal RNA and DNA are known to be primarily degraded in the transition zone between the granular layer and the stratum corneum, although no specific endogenous nuclease has been identified heretofore [Santoianni and Rothman, 1961; Steigleder and Raab, 1962; McCall and Cohen, 1991]. Analysis of the nucleic acid content of human stratum corneum has shown that DNA and RNA are barely present there [Hodgson, 1962]. Most discussions of cell death and senescence have tended to focus on endonucleases that cleave DNA into defined smaller fragments, but specific RNases have also been linked to these processes. Onconase, a cytotoxic ribonuclease, kills tumor cells in vivo and in vitro [Wu et al., 1993]. Similarly, bovine seminal ribonuclease destroys thyroid epithelial tumors [Laccetti et al., 1992]. The association of RNase activity with senescence has been reported for the RNase RNS2 [Taylor et al., 1993]. The outer epidermis, in contact with the ambient environment, is sensitive to changes in temperature. The exposure of cells to high temperatures can activate latent RNases [Ito and Ohnishi, 1983] and inactivate other enzymes crucial to normal metabolic activities and survival. RNases can inhibit the cell's translational machinery, hence the biosynthesis of new proteins. This impairment can lead to organelle degradation and ultimate cell death. In the epidermis in vivo, the destruction of nuclei and other organelles occurs in the transition zone between the granular layer and the stratum corneum. As desquamin is first expressed in this region and has both RNase and protease activity, it can be presumed to have some connection to these processes (Indeed, our in vitro experiments demonstrate that desquamin is capable of degrading nuclei).

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