Monoclonal Antibodies to Human Epidermal Filaggrin, Some Not Recognizing Profilaggrin

Michel Simon, Mireille Sebbag, Marek Haftek,* Christian Vincent, Elisabeth Girbal-Neuhauser, Joël Rakotoarivony, Gérard Sommé,† Daniel Schmitt,* and Guy Serre

Department of Biology and Pathology of the Cell, Toulouse-Purpan School of Medicine, University of Toulouse III, Toulouse; *INSERM U346/CNRS, E. Herriot Hospital, Lyon; and †Clonatec-Biosoft S.A., Paris, France

To improve understanding of human profilaggrin processing to filaggrin, we produced seven monoclonal antibodies against epidermal filaggrin (AHF1–7). They were characterized on human epidermis by indirect immunofluorescence, immunogold labeling, and immunoblotting and found to be directed against seven different epitopes of (pro)filaggrin. AHF1–5 labeled the keratohyalin granules and the fibrous matrix of the lower corneocytes, and recognized filaggrin and profilaggrin. AHF6 also labeled the keratohyalin granules and the corneocyte matrix, but only recognized filaggrin. In addition to this reactivity within the upper epidermis, AHF4–6 stained the cytoplasm of the basal cells, and cross-reactivity of AHF5 and AHF6 with cytokeratin K14 was revealed on immunoblots. It is interesting that AHF7 recognized filaggrin, but not profilaggrin, and labeled only the corneocyte matrix and not the keratohyalin granules. This indicates that filaggrin and cytokeratins share several antigenic determinants and that filaggrin bears at least one epitope absent from its precursor. The original series of monoclonal antibodies described here appears to be a powerful tool for studying human profilaggrin processing in normal conditions and in the keratinization disorders in which processing is altered. *Key words: differentiation/human epidermis/keratohyalin granules/stratum corneum.*
J Invest Dermatol 105:432–437, 1995

Human filaggrin, a cationic protein of 37,000 apparent molecular weight, is synthesized in the granular cells of the epidermis as a large, highly phosphorylated precursor, profilaggrin, which accumulates in the keratohyalin granules [1–6]. Characterization of the gene encoding human profilaggrin [7–10] has shown that the protein consists of 10 to 12 filaggrin repeats, 317 amino acids long, separated by seven–amino-acid linker peptides. Neighboring repeats display up to 30% variation in amino acid sequences, inducing a large heterogeneity in their isoelectric points. The filaggrin repeats are flanked by truncated units fused to nonfilaggrin proteins. In particular, the amino terminal peptide is homologous to calcium-binding proteins [9,10]. During the transition from granular to cornified cell, profilaggrin is dephosphorylated, the linker peptides are proteolytically removed, and basic filaggrin units are produced [11–13]. Many questions concerning the molecular steps of profilaggrin processing into filaggrin remain unsolved, e.g., What are the enzymes involved in the process? Does human profilaggrin processing begin before or after the dispersion of keratohyalin granules? The basic filaggrin units apparently interact with and aggregate cytokeratin filaments, facilitating formation of intermolecular disulfide bonds to yield the intracellular fibrous matrix of the cornified cells [2,3,6,14,15]. After filament aggregation, basic arginine residues of filaggrin are converted to citrullines by a peptidylarginine deiminase; this results in a lower affinity of the molecule for cytokeratins [3]. Finally, filaggrin degradation into free amino acids in the upper stratum corneum seems to be necessary for the proper hydration of this protective layer [16,17]. In addition, it has been suggested that filaggrin might be cross-linked to, or at least associated with, the cornified cell envelopes [18–20].

A number of keratinization disorders [1,5,21] are associated with dysregulation in the formation of keratohyalin granules and in the processing of profilaggrin. In lamellar ichthyosis, for example, a large accumulation of profilaggrin and little processing to filaggrin are observed [1]. Immunologic probes able to differentiate filaggrin from its precursor directly on skin biopsy specimens would therefore be useful diagnostic tools. Moreover, we recently reported that human filaggrin is the target of rheumatoid-arthritis–specific autoantibodies [22]. Monoclonal antibodies (MoAbs) specific for filaggrin would also be useful to determine the epitopes recognized by these autoantibodies.

A number of antisera and a few MoAbs have been raised against human filaggrin [4,23,24]. However, all of them react with both the protein and its precursor, and the epitopes they recognize have not been identified. We report on the characterization of a series of MoAbs directed against purified human epidermal filaggrin, including two that recognize filaggrin but not profilaggrin.

MATERIALS AND METHODS

Protein Extraction and Purification Human filaggrin and profilaggrin were extracted from breast skin (obtained from patients undergoing plastic surgery) and partially purified by ion-exchange chromatography according to Lynley and Dale [2], as previously modified [22].

Manuscript received August 19, 1994; final revision received May 17, 1995; accepted for publication June 12, 1995.

Reprint requests to: Dr. Guy Serre, Laboratoire de Biologie Cellulaire et Cytologie, CHU Purpan, Place du Dr Baylac, 31059 Toulouse Cedex, France.

Abbreviations: AHF, anti-human filaggrin; IIF, indirect immunofluorescence.

0022-202X/95/$09.50 • SSDI0022-202X(95)00277-R

Table I. Summary of the Characteristics of Anti-Filaggrin MoAbs

	Immunofluorescence[a]					Immunoelectron Microscopy[b]		Immunoblotting[c]			
Name	SC	SG	SS	SB	Hair	Corneocyte Matrix	KHG	Fil	Pro	K14	ELISA[d]
AKH1	+	++	−	−	−	++	++	++	++	−	−
AHF1	+	++	−	−	$++_P$	++	++	++	++	−	−
AHF2[e]	+	++	−	−	−	++	++	++	++	−	−
AHF3[e]	+	++	−	−	−	++	++	++	++	−	−
AHF4	+	++	+/−	+	$+_{ORS}$	++	++	++	++	−	93–109
AHF5	+	++	−	+	$+_{ORS}$	++	++	++	++	+/−	−
AHF6	++	+	−	++	$++_{ORS}$	++	+	++	−	++	−
AHF7	++	−	−	−	−	+	−	++	−	−	−

[a] IIF reactivity to the various layers of epidermis, i.e., stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB), and to the components of the hair follicle (hair), i.e., outer root sheath (ORS) and papilla (P). Strong reactivity is indicated by (++), moderate reactivity by (+), weak reactivity by (+/−), and (−) indicates no detectable reactivity.

[b] Immunoelectron microscopy reactivity to corneocyte fibrous matrix and to keratohyalin granules (KHG).

[c] Immunoblotting reactivity to human epidermal filaggrin (fil), profilaggrin (pro), and cytokeratin K14.

[d] Position of the reactive peptides on the "consensus" sequence of human filaggrin [8].

[e] Their different migrations in nondenaturing gels clearly differentiated AHF2 and AHF3.

Production of MoAbs Balb/c mice were immunized by three injections of partially purified human filaggrin at 2-week intervals. Before fusion, performed as originally described by Köhler and Milstein [25], the presence of anti-filaggrin antibodies in the serum was confirmed by immunoblotting. Hybridoma supernatants were screened for antibody production by indirect immunofluorescence (IIF) on cryostat sections of human skin and by immunoblotting. Seven independent hybridoma isolates were cloned twice and then used to produce ascites fluids in Pristane-primed mice. Immunoglobulins (Igs) were purified by ammonium sulfate precipitation and chromatography on diethylaminoethyl Trisacryl LS (IBF, Villeneuve la Garenne, France). The MoAbs were all of the IgG1 isotype, as determined by MonoAb-ID (Tago, Burlingame, CA) enzyme-linked immunosorbent assay (ELISA).

Other Immunologic Reagents AKH1, a MoAb specific for human profilaggrin and filaggrin, was purchased from Biomedical Technologies, Inc. (Stoughton, MA). Anti-hK14, a rabbit antiserum specific for human cytokeratin K14 [26], was a generous gift from E. Fuchs (Howard Hughes Medical Institute, University of Chicago, Chicago, IL). BL6 MoAb (Immunotech, Marseille, France), specific for a CD1a antigen expressed by epidermal Lan- gerhans cells [27], was used as a negative control in immunoelectron microscopy.

IIF IIF was performed on 4-μm–thick unfixed cryostat sections and Carnoy's-fixed, paraffin-embedded sections of human skin, as described previously [28]. Control experiments omitting the primary antibody or using IgG from a nonimmune mouse were always negative.

Immunoelectron Microscopy Normal human skin was analyzed with post-embedding indirect immunogold labeling, as described earlier [20,28]. Fragments of abdominal skin were fixed in 3% paraformaldehyde, dehydrated at low temperature in ethanol, cryosubstituted, and embedded at −35°C in Lowicryl K4M (Chemische Werke Lowi GMBH, Waldkraiburg, Germany). Ultrathin sections were preincubated for 20 min with 2.5% fetal bovine serum in phosphate-buffered saline, then incubated for 1 h with MoAb-containing ascites fluids diluted to 1:50 in 0.1% fetal bovine serum. After washes, the sections were incubated for 1 h with goat Ig to mouse IgG G-10 colloidal gold conjugate, diluted to 1:10 (Amersham International, Aylesbury, UK), washed again, and counterstained with uranyl acetate in methanol for 5 min.

Protein Analysis Proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gel or by two-dimensional electrophoresis in the presence of urea, using the PhastSystem, as described by Pharmacia LKB (Uppsala, Sweden). Protein markers from Bio-Rad Laboratories (Richmond, CA) were used as molecular weight references, and the isoelectric point gradient profiles were obtained by the Broad pI Calibration Kit (Pharmacia LKB) run in parallel. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes and probed, as reported previously [20], with MoAbs diluted to 5 μg/ml. The MoAb-reactive proteins were visualized with peroxidase-conjugated sheep antibodies to mouse IgG (Zymed, San Francisco, CA).

ELISA Peptides were synthesized by Neosystem Laboratories (Strasbourg, France) using an optimized solid-phase procedure, and their purity was checked by reverse phase chromatography. Wells of polystyrene microtitration plates (Nunc, Copenhagen, Denmark) were coated overnight at 37°C with the peptides solubilized in 0.01 M acetate buffer, pH 5.6 (0–40 μg/ml). After rinsing with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (TBST), the plates were incubated with 1% gelatin in TBST for 0.5 h at 37°C and washed, and antibodies diluted in TBST were added for 2 h at 37°C. After further rinsing, appropriate anti-IgG conjugated with peroxidase and the substrate orthophenylene diamine dihydrochloride were added successively, as described previously [29]. The absorbance at 492 nm was measured with a Uniskan II photometer (Labsystems France, Les Ulis, France). All assays were performed in duplicate and the results averaged.

RESULTS

To produce anti-filaggrin MoAbs, we used basic filaggrin of human epidermis as an immunogen. The seven independent clones we describe here were derived from a single fusion and called AHF1 to AHF7 for their *a*nti-*h*uman *f*ilaggrin reactivity (initially designated 105e, 621b, 1105b, 812a, 1117a, 134j, and 711c, respectively). Their reactivities with human epidermis, epidermal proteins, and synthetic peptides are described in detail below and summarized in **Table I**.

AHF7 Does Not Label the Keratohyalin Granules of Human Epidermis The reactivity of the AHF MoAbs was first tested by IIF on cryosections of human skin. Their patterns of reactivity were compared with that produced by AKH1, a well-characterized and widely used anti-(pro)filaggrin MoAb **(Fig 1)**. Three of the AHFs (AHF1–3) showed strong granular cytoplasmic staining of the stratum granulosum and diffuse staining of the lower stratum corneum. The patterns of reactivity of these three MoAbs were indistinguishable from that of AKH1. AHF4 and AHF5 also showed strong granular staining of the stratum granulosum and diffuse staining of the lower stratum corneum, but they also weakly stained the cytoplasm of the basal cells. AHF4 was also observed to label the cytoplasm of the spinous cells irregularly from one skin fragment to another. AHF6 produced weaker granular staining of the stratum granulosum but intense staining throughout the entire stratum corneum up to the desquamating corneocytes. It also strongly labeled the cytoplasm of the basal cells. The staining of the stratum granulosum was consistent with the distribution of keratohyalin granules in every case. The seventh MoAb, AHF7, did not stain the viable cells, including the granular cells, but labeled all the cornified cell layers.

On sections of Carnoy's-fixed paraffin-embedded human skin, the AHF MoAbs showed the pattern of reactivity observed on cryosections, except that all of the antibodies labeled only the lower part of the stratum corneum, and AHF4 and AHF5 no longer labeled the cytoplasm of the basal cells. The absence of reactivity of AHF7 on the stratum granulosum was confirmed **(Fig 1)**.

No differences were observed in the patterns of reactivity between the interfollicular and intrafollicular epidermis on cryo-

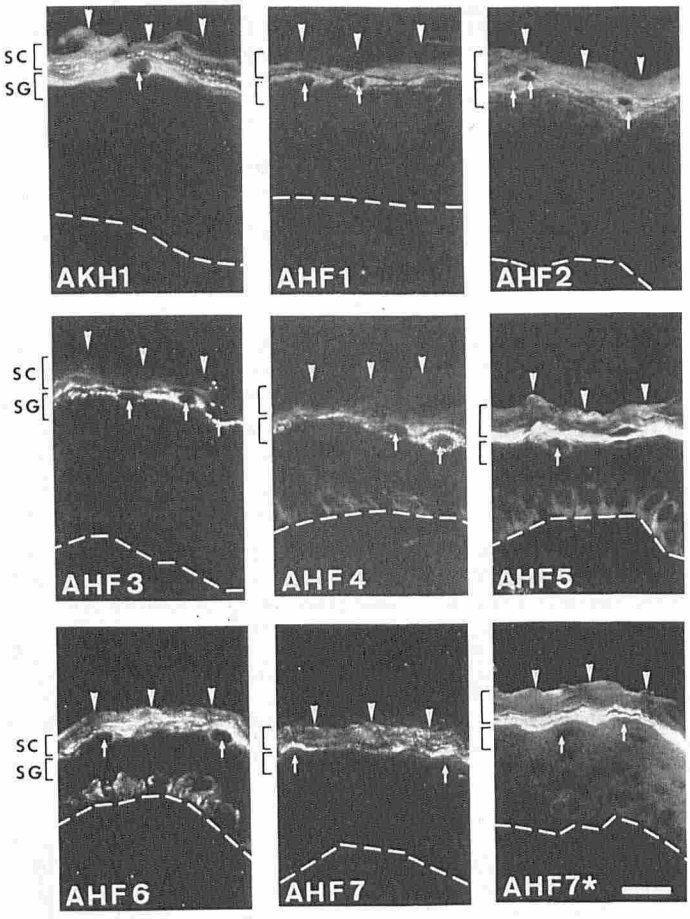

Figure 1. AHF1–6 label the stratum granulosum and stratum corneum of normal human epidermis, whereas AHF7 labels only the stratum corneum. AKH1 and the AHF MoAbs (as shown on the plates) were analyzed by IIF on cryosections of human skin and on sections of Carnoy's-fixed skin (*asterisk*). Note that AHF7 does not label the stratum granulosum but strongly labels the lower stratum corneum when tested on unfixed cryosections (AHF7) as well as on sections of Carnoy's-fixed skin (AHF7*). *Arrows* indicate some of the upper nucleated cells of the stratum granulosum; *arrowheads* show the surface of the epidermis. The stratum corneum (SC) and the stratum granulosum (SG) are indicated (*brackets*). *Dashed lines* denote the dermoepidermal junction. *Bar*, 50 μm.

sections of scalp skin. The three MoAbs (AHF4–6) that recognized the basal cells of the epidermis stained the cells of the outer root sheath of the hair follicles, and, curiously, AHF1 gave clear cytoplasmic labeling of cells of the hair follicle papilla (data not shown).

To determine the ultrastructural location of the antigen(s) recognized in the upper layers of human epidermis, we analyzed the AHF MoAbs by post-embedding immunoelectron microscopy after paraformaldehyde fixation **(Fig 2)**. In the stratum granulosum, keratohyalin granules were clearly visible as polygonal structures showing a microgranular texture typical of the embedding and counterstaining methods used. They showed typical reactivity with AKH1 **(Fig 2*a*)** and were also strongly labeled by five of the AHF MoAbs, namely AHF1–5 **(Fig 2*b*,*c*)**. With AHF6, the labeling intensity of the granules was weaker than that of the lower stratum corneum **(Fig 2*d*)**. In contrast, the keratohyalin granules were unlabeled by AHF7 **(Fig 2*e*)**. In the stratum corneum, immunogold labeling obtained with the seven AHF MoAbs was observed throughout the intracellular matrix of three to seven of the lower cornified cell layers; the labeling intensity decreased toward the surface and became undetectable in the upper corneocytes **(Fig 2*c*)**.

AHF6 and AHF-7 React With Filaggrin But Not With Profilaggrin on Immunoblots Analyzed by immunoblotting on epidermal proteins, all seven of the AHF MoAbs reacted with a doublet of approximate molecular weight 37,000 **(Fig 3)**. All but two (AHF6 and AHF7) also reacted with a high–molecular-weight protein. Because the same bands were also detected by AKH1, these proteins were identified as filaggrin and profilaggrin, respectively. In addition, AHF6 recognized a protein of 48 kD that was also weakly stained by AHF5 and comigrated with cytokeratin K14. The binding to the 48-kD protein was due to cross-reactivity, as it disappeared after preabsorption of the MoAbs on purified filaggrin together with the anti-filaggrin reactivity (not shown). To confirm these results, we analyzed filaggrin and profilaggrin by immunoblotting after partial purification by anion exchange chromatography. All MoAbs reacted with the basic filaggrin (flow-through fractions), and all but AHF6 and AHF7 also reacted with profilaggrin and its proteolyzed fragments (data not shown).

The identity of the recognized proteins was confirmed by two-dimensional gel analysis **(Fig 4)**. Once again, the seven MoAbs reacted with the cationic filaggrin. Five of them (AHF1–5) recognized the anionic profilaggrin, whereas AHF6 and AHF7 did not (or very faintly for AHF6). AHF6 also strongly stained the 48-kD protein that was identified as cytokeratin K14, as it reacted with an anti-human K14-specific antiserum. The cytokeratin K14 was also weakly stained by AHF5.

AHF4 Reacts With the Filaggrin Region 93–109 When Analyzed by ELISA To characterize further the AHF MoAbs as well as AKH1, we tested their reactivity by ELISA with 32 synthetic peptides 14 to 16 amino acids long, derived from the published "consensus" sequence of human filaggrin [8]. These peptides presented four– to five–amino-acid overlaps, and their combined sequence covered the entire sequence of one filaggrin unit without its seven–amino-acid linker. In a preliminary experiment, the antibodies have been shown to react in ELISA with purified human epidermal filaggrin. However, when the synthetic peptides were used, only AHF4 presented a significant reactivity to one of them (GTSGSRSASRQTRNQEQ), corresponding to the filaggrin region 93–109.

DISCUSSION

We have characterized seven AHF MoAbs produced against basic filaggrin of human epidermis. Various patterns of IIF staining, immunoblotting, and ELISA reactivities (summarized in **Table I**) indicate that they recognize different epitopes on human filaggrin. Even if AHF2 and AHF3 did not differ with respect to these characteristics and therefore might recognize the same or a closely related epitope, their migration in nondenaturing gels clearly differentiated them and confirmed that they do not originate from the same antibody-producing cell. Moreover, only AHF2 recognized a recombinant human filaggrin unit on immunoblots (preliminary result).

Six MoAbs (AHF1–6) labeled the keratohyalin granules of the granular cells and the fibrous matrix of the cornified cells. When analyzed by immunoblotting, all of these MoAbs except AHF6 recognized filaggrin and profilaggrin. Surprisingly, AHF6 recognized filaggrin but not (or very faintly) profilaggrin. It may therefore discriminate discrete steps in the maturation of profilaggrin to filaggrin that occur already in the keratohyalin granules. Alternatively, the profilaggrin epitope recognized by AHF6 may have been denatured after SDS treatment. In this event, because AHF6 labels basic filaggrin under the same immunoblotting conditions, the epitope it recognizes must be modified in some way and therefore less resistant to SDS denaturation in the precursor molecule. Consistent with this assumption is the fact that AHF6 reacted less strongly than the other five MoAbs with the keratohyalin granules and thus may show a lesser affinity for the precursor than for the mature protein. AHF7, which selectively recognized filaggrin but not profilaggrin on immunoblots, stained only the corneocyte fibrous matrix and did not label the keratohyalin granules. The epitope with which AHF7 reacts might be

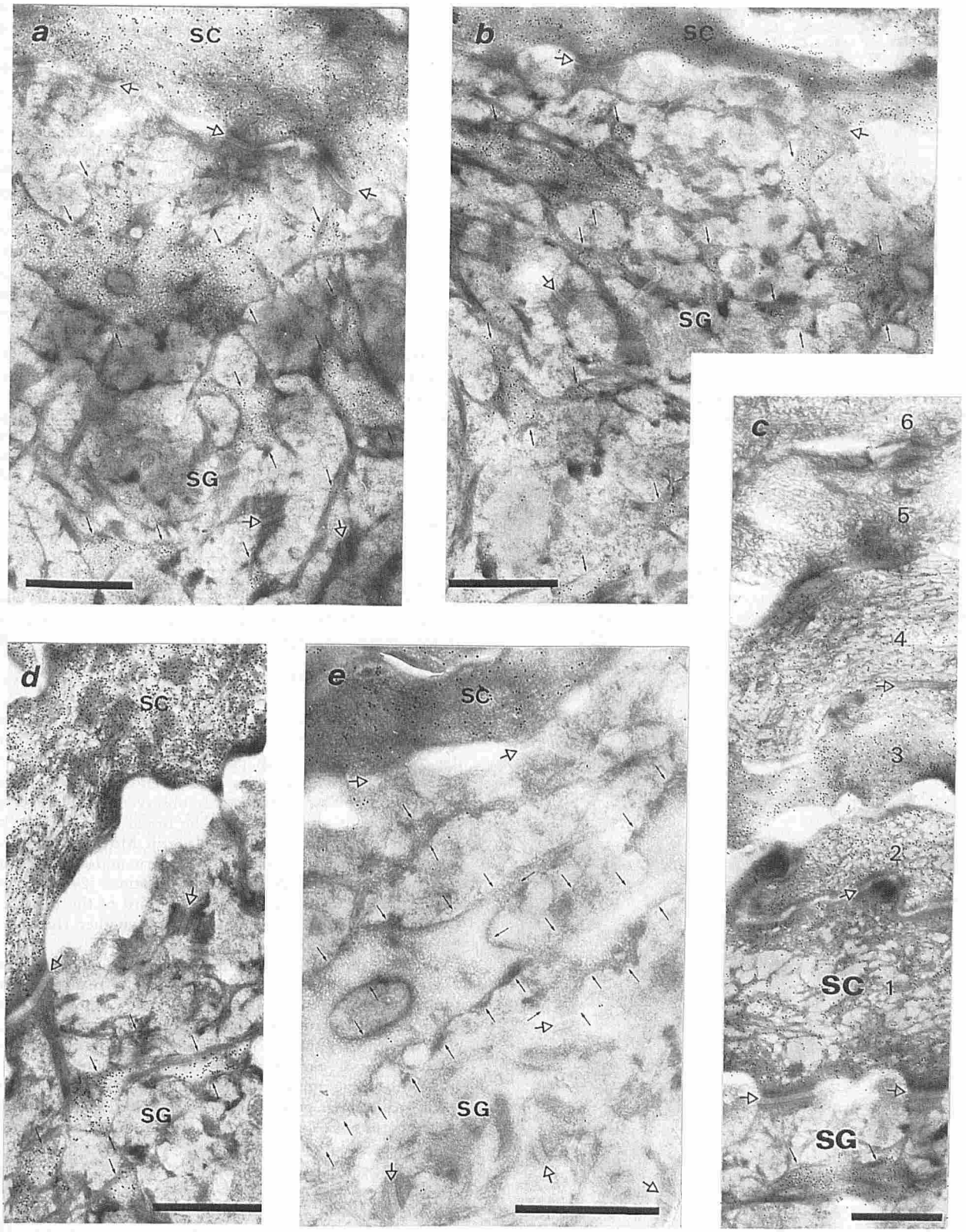

Figure 2. Immunoelectron microscopy analysis reveals the absence of reactivity of AHF7 with keratohyalin granules. Normal human skin was analyzed by post-embedding indirect immunogold labeling, as described in *Materials and Methods*. When compared with the reactivity of AKH1 (*a*), five of the AHF MoAbs (AHF1–5) showed a similar labeling pattern of the keratohyalin granules (*small arrows*) and of the fibrous intracellular matrix of the lower cornified cells. This typical pattern is shown for AHF2 (*b,c*). Note the disappearance of labeling in the upper cornified cell layers 5 and 6 (*c*). In contrast, AHF6 shows weaker reactivity on the keratohyalin granules than on the matrix of the lower corneocytes (*d*) and AHF7 proves unreactive on these granules, whereas it labels the corneocyte matrix (*e*). SC, stratum corneum; SG, stratum granulosum. Some desmosomes are indicated (*open arrows*). *Bars*, 1 μm.

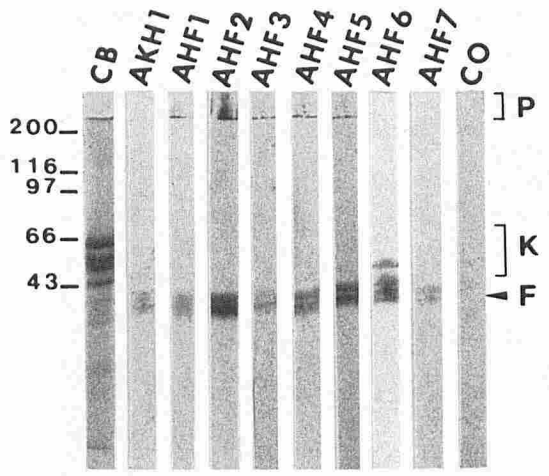

Figure 3. AHF1–5 recognize human epidermal filaggrin and profilaggrin, whereas AHF6 and AHF7 only recognize filaggrin. Proteins of human epidermis extracted with 6 M urea were separated by SDS-PAGE and stained with Coomassie blue (CB) or analyzed by immunoblotting with AKH1, with the AHF MoAbs, and with a control MoAb (CO), as indicated. The positions of molecular weight standards ($\times 10^{-3}$) are indicated (*left*), and proteins of interest are shown (*right*). P, profilaggrin; F, filaggrin; K, cytokeratins.

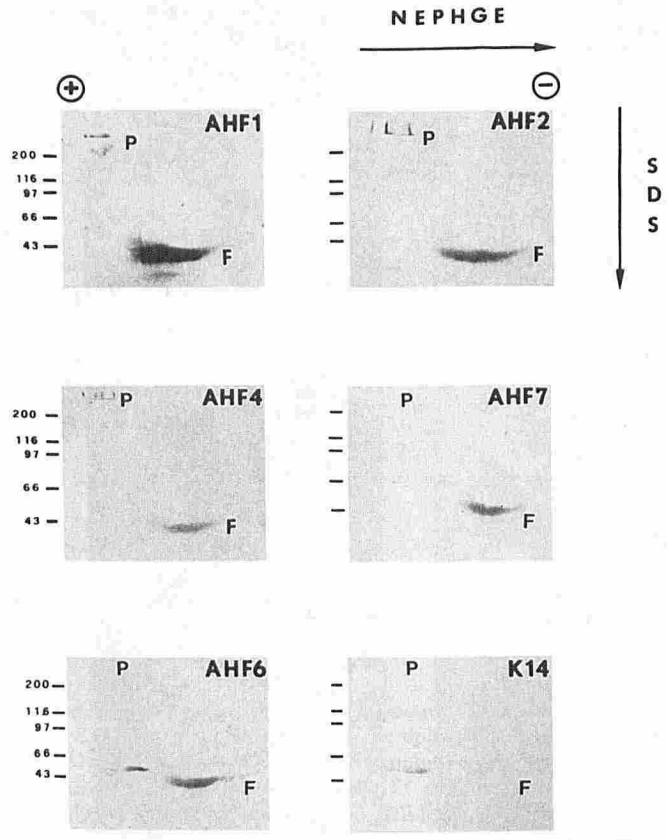

Figure 4. Immunoblotting analysis after two-dimensional gel electrophoresis confirms the specificity of the AHF MoAbs. Proteins of human epidermis extracted with 6 M urea were separated by nonequilibrium pH gel electrophoresis (NEPHGE) in the first dimension and by SDS-PAGE in the second dimension, and then analyzed by immunoblotting with AKH1, with the AHF MoAbs, and with an antiserum specific for human cytokeratin K14. Identical results were obtained with AKH1 and with AHF1–5, but only those corresponding to AHF1, AHF2, and AHF4 are shown. Nonimmune mouse IgG did not recognize any protein. The positions of molecular weight standards ($\times 10^{-3}$) are indicated (*left*). P, profilaggrin; F, filaggrin.

absent on profilaggrin because of the high degree of phosphorylation or because of a different conformation of this protein. Alternatively, AHF7 may react with a citrulline-containing epitope only present on the post-translationally modified filaggrin. Indeed, such a modification that results in subsequent degradation of filaggrin is known to occur after the aggregation of cytokeratin filaments. Similarly, the rheumatoid-arthritis–specific anti-filaggrin autoantibodies stain the corneocyte matrix but not the keratohyalin granules of human epidermal keratinocytes, and recognize filaggrin but not profilaggrin [22,30]. This result confirms the existence of filaggrin-specific epitopes absent from profilaggrin.

In addition to the staining of keratohyalin granules and corneocyte matrix, AHF4–6 reacted with the cytoplasm of the basal cells. In agreement, AHF5 and AHF6 cross-reacted with cytokeratin K14 on immunoblots. Although K14 is present throughout the living layers of epidermis [26,31], it was detected by AHF5 and AHF6 only in the basal cells. Such a result suggests that the antigenic sites recognized by these MoAbs on K14 are masked *in situ* in the suprabasal cells. This was previously shown to be the case for the monoclonal anti-type I cytokeratin antibody AE1 [31]. On the other hand, it is known that human filaggrin and the suprabasal cytokeratins K1, K2, and K10 share an epitope that is defined by AE2 MoAb. This antibody (raised against epidermal cytokeratins) labels the suprabasal cells of the epidermis and cross-reacts in immunoblotting with filaggrin.‡ Our results show that human filaggrin and K14, although very different from a biochemical point of view, nevertheless share two antigenic determinants. Whether this immunologic relationship is related to the filaggrin-cytokeratin interaction is not known. Although AHF4 did not detect cytokeratin on immunoblots, it is tempting to speculate about its labeling of the basal cells by IIF; it appears to cross-react with a conformational or discontinuous epitope of cytokeratin K14 (or K5) that is lost after SDS denaturation.

Among the eight MoAbs used in this study, AHF4 was the only one shown in ELISA to bind a synthetic peptide (93–109) derived from the "consensus" sequence of filaggrin. This result suggests that most of the epitopes become inaccessible after interaction of the relevant peptide with polystyrene, or, more likely, that the unreactive MoAbs recognize discontinuous or conformation-dependent epitopes, as is commonly observed [32]. Longer peptides or recombinant molecules must be tested to define the filaggrin region recognized by the anti-filaggrin MoAbs. Nevertheless, it should be noted that a rabbit anti-filaggrin antiserum we produced as a positive control also bound to peptides (27–46 and 62–76) corresponding to the amino terminal third of the filaggrin. More experiments will be necessary to test whether this part of the molecule is immunodominant.

Some of the MoAbs described here immunoprecipitate native filaggrin (M. Simon, unpublished data) and react with the denatured protein on immunoblots. They will therefore be useful tools to probe the structure and function of filaggrin. Moreover, four of the AHF MoAbs show no cross-reactivity with cytokeratins, label Carnoy's-fixed, paraffin-embedded tissues, and can be used in post-embedding immunoelectron microscopy studies. These properties make them potentially useful for histopathologic and diagnostic purposes, especially AHF7, which does not recognize keratohyalin granules and profilaggrin.

We thank Professor M. Costagliola (Service de Chirurgie Plastique, CHU Rangueil, Toulouse) for providing us with human skin. The technical assistance of M.-F. Isaïa, M.-P. Rué, and M.-H. Teillon is gratefully acknowledged. We are indebted to Dr. E. Fuchs (Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois) for her generous gift of anti-hK14 serum. Electron microscopy specimens were observed at C.M.E.A.B.G., Université C. Bernard, Lyon I. This study was

‡ Dale BA, Sun TT: Filaggrin, 56.5kd and 65–67kd keratins share an antigenic determinant as defined by AE2 monoclonal antibody (abstr). *J Cell Biol* 97:228a, 1983.

supported by grants from the INSERM (C.R.E. 93 0205), from the "Association pour la Recherche sur la Polyarthrite," and from the "Région Midi-Pyrénées." M. Simon was supported by a fellowship from the "Association de Secours des Amis des Sciences."

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