The Cytokeratin Filament-Aggregating Protein Filaggrin Is the Target of the So-called “Antikeratin Antibodies,” Autoantibodies Specific for Rheumatoid Arthritis

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Abstract
In rheumatoid arthritis (RA), the high diagnostic value of serum antibodies to the stratum corneum of rat esophagus epithelium has been widely reported. These so-called “antikeratin antibodies,” detected by indirect immunofluorescence, were found to be autoantibodies since they also labeled human epidermis. Despite their name, the actual target of these autoantibodies was not known. In this study, a 40-kD protein (designated as 40K), extracted from human epidermis and specifically immunodetected by 75% of RA sera, was purified and identified as a neutral/acidic isoform of basic filaggrin, a cyto-keratin filament-aggregating protein, by peptide mapping studies and by the following evidences: (a) mAbs specific for filaggrin reacted with the 40K protein; (b) the autoantibodies, affinity-purified from RA sera on the 40K protein, immunodetected purified filaggrin; (c) the reactivity of RA sera to the 40K protein was abolished after immunoadsorption with purified filaggrin; (d) the 40K protein and filaggrin had similar amino acid compositions. Furthermore, autoantibodies against the 40K protein and the so-called “antikeratin antibodies” were shown, by immunoadsorption experiments, to be largely the same. The identification of filaggrin as a RA-specific autoantigen could contribute to the understanding of the pathogenesis of this disease and, ultimately, to the development of methods for preventing the autoimmune response. (J. Clin. Invest. 1993. 92:1387–1393.) Key words: autoimmunity • autoantigen • diagnosis • intermediate filament-associated protein • epidermis

Introduction
RA is the most frequent (1–2% of the population worldwide) human systemic autoimmune disease. It is characterized by a mononuclear cell infiltration of the synovium and by proliferation of the synovial cells. This forms an invasive pannus and leads to the destruction of articular cartilage. Although the pathogenesis of RA remains unknown, both cellular and humoral autoimmune mechanisms have been implicated (1). The presence of a wide variety of circulating autoantibodies has been described, including rheumatoid factors and antibodies to nuclear or structural cellular components (1–3). In addition, antibodies against EBV proteins have been frequently observed (4, 5).

In 1979, Young et al. (6) showed, using indirect immunofluorescence, the presence, in rheumatoid sera, of IgG antibodies labeling the stratum corneum of rat esophagus epithelium. These antibodies are clearly autoantibodies since they also react with the stratum corneum of human epidermis (6–9). They have been found to be highly specific for the disease (6–15) and their detection is now widely used as a diagnostic test for RA. Although their role has not yet been defined, they are associated with more active and/or severe forms of RA (13–16) and they are detected at early stages (16) and even before the onset of joint symptoms (17), suggesting their involvement in the pathophysiology of the disease.

On the basis of their immunofluorescence pattern, these autoantibodies were thought to be directed to cytokeratins and were therefore called “antikeratin antibodies,” despite the absence of any immunochemical characterization of their targets. However, preadsorption of RA sera on purified human cytokeratins did not remove their reactivity to the stratum corneum of rat esophagus (7) and we recently showed by ELISA (15) and by Western blot (Simon, M., C. Vincent, E. Girbal, M. Sebbag, V. Gomès-Daudrix, M. Hałek, and G. Serre, manuscript submitted for publication) that these autoantibodies do not recognize cytokeratins either from human epidermis or from rat esophagus. Here we report the biochemical and immunochemical characterization of the human epidermal protein detected by these RA-specific autoantibodies and its identification as filaggrin, a well known cytokeratin filament-aggregating protein (18–21).

Methods
Patients and sera. Sera were obtained from 104 patients, including 48 with classical or definite RA according to the criteria of the American Rheumatism Association (22), 37 with various inflammatory rheumatic diseases (10 psoriatic arthritis, 9 systemic lupus erythematosus, 10 miscellaneous connective tissue diseases, 8 ankylosing spondylitis) and 19 with noninflammatory rheumatic diseases (9 Paget’s disease, 10 arthrosis or compressive neuralgia). Control sera were from 39 healthy adults.

Protein extraction and purification. Normal human breast epidermis was cleaved from dermis by heat treatment and homogenized in 0.2 ml/cm² of an ice-cold solution containing 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 15,000 g for 10 min to get a clear detergent extract of human epidermis. Proteins of this extract were precipitated with absolute ethanol, recovered by centrifugation at 15,000 g for 10 min, and resuspended in water after 20 min drying at 80°C. The cloudy suspension thus obtained was centrifuged to obtain a clear supernatant, the partially purified 40-kD protein, designated as the 40K protein throughout our discussion.

To further purify the 40k protein, this partially purified fraction was submitted to SDS-PAGE and electrotransferred to immobilon-PVDF membranes (Millipore Corp., Bedford, MA). The 40K protein
was eluted from the membranes with Triton X-100, as previously described (23). The detergent was then removed by chromatography on Extract-I-GET™ D (Pierce Chemical Co., Rockford, IL) as described by the manufacturer and the purified 40K protein finally desalted by gel filtration on a PD-10 column (Pharmacia LKB, Uppsala, Sweden). Human filaggrin was purified from epidermis as previously reported (23). Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany).

**Gel electrophoresis and Western blot.** Proteins were analyzed by SDS-PAGE on 12.5% acrylamide gel or by two-dimensional electrophoresis using the PhastSystem™ as described by Pharmacia LKB, the manufacturer. Protein markers from Bio-Rad Laboratories (Richmond, CA) were used as molecular weight references and the pl gradient profiles were indicated by the Broad pl Calibration Kit of Pharmacia LKB run in parallel.

After electrophoresis, proteins were electrotransferred to reinforced nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and probed, as reported (23), with various sera diluted to 1/50 and visualized by peroxidase-conjugated goat antibodies to human IgG, or with mAb ascites diluted to 1/200 and visualized with peroxidase-conjugated sheep antibodies to mouse IgG. AKH1, an IgG, mAb directed against human filaggrin, was purchased from Biomedical Technologies, Inc. (Stoughton, MA). Six other mAbs (IgG) to human epidermal filaggrin purified as described above were recently produced and characterized in our laboratory (Simon, M., C. Vincent, E. Girbal, M. Sebag, V. Gomès-Daudrix, and G. Serre, manuscript in preparation).

**Determination of the amino acid composition.** The amino acid composition of the purified 40K protein was determined in duplicate, after acid hydrolysis, using conventional methods (Neosystem, Strasbourg, France).

**Immunoprecipitation.** Before immunoprecipitation, the detergent extract of human epidermis was pre cleared with Protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) and made 1 M for NaCl; the test or control mAb was added and, after a 2-h incubation at 37°C, immune complexes were collected for 1 h with protein-A Sepharose and centrifuged for 2 min. The precipitates were washed twice with 10 mM Tris-HCl, pH 8, containing 0.5 M NaCl and 0.1% Nonidet P-40, and boiled in SDS-PAGE sample buffer.

**Affinity purification of the anti-40K autoantibodies.** The anti-40K autoantibodies were immunoaffinity-purified from RA sera on nitrocellulose-bound 40K antigen as previously reported (24) with the following modifications: the bound antibodies were eluted with 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid-NaOH, pH 12, containing 0.2% gelatin, neutralized by the addition of 0.01 vol of 2 M Tris-HCl, pH 6.8, and immediately used.

**Indirect immunofluorescence.** Indirect immunofluorescence analysis was done on rat esophagus cryosections, as previously described (14).

**Immunoadsorption experiments.** RA sera (2 μl) or the affinity-purified antibodies were preincubated for 2 h at 4°C in the presence of 6 μg of protein dissolved in water, before being used in Western blot or indirect immunofluorescence.

**Peptide mapping.** One-dimensional peptide maps of purified filaggrin and purified 40K antigen were obtained by the method described by Cleveland et al. (25) using 20% acrylamide gels. The digestions were carried out in the gel for 20 min with Staphyloccocus aureus V8 protease (Sigma Chemical Co.). The protein concentrations of filaggrin and the 40K antigen were approximately equal (3 μg per lane). After electrophoresis, digested proteins were transferred and analyzed by Western blot as described above.

**Results.**

**Specificity of RA sera for a 40K protein.** While searching, by Western blot, for RA antibody-reactive molecules, we noted the presence in human epidermis extracts of a diffuse band...
with an apparent molecular weight of 37–40,000 decorated by most RA sera. The molecule was confirmed to be a protein by digestion with proteinase K. It showed an unusual solubility property: when a detergent-containing lysate was precipitated with absolute ethanol, it selectively dissolved upon resuspension in water, whereas the other proteins were irreversibly precipitated (Fig. 1A). Taking advantage of this property of the 40K protein in order to partially purify it, we tested its reactivity, after SDS-PAGE, by Western blot analysis with a large panel of sera from patients with well-characterized rheumatic diseases and with normal human sera (Fig. 1B). Most of the RA sera (36/48 or 75%) reacted with the 40K protein. Moreover, this reactivity was correlated with their immunofluorescence intensity on the stratum corneum of the rat esophagus epithelium. However, 6 out of the 56 sera (11%) from patients with other rheumatic diseases (1 of 10 with miscellaneous connective tissue diseases, 2 of 10 with psoriatic arthritis, 1 of 8 with ankylosing spondylitis, and 2 of 19 with noninflammatory rheumatic diseases) and only 1 out of the 39 sera from healthy subjects (3%) weakly decorated this protein. Since it was recognized in a quite specific way by RA sera, the 40K protein was further characterized: its apparent molecular weight was not affected by electrophoresis under nonreducing conditions; analyzed by Western blot after PAGE or after isoelectric focusing under nondenaturing conditions, it exhibited extensive charge heterogeneity. This was confirmed by two-dimensional electrophoretic analysis: the 40K RA antigen presented a number of isoforms with pIs ranging from 5.8 to 7.4 (Fig. 1C). Moreover, there was a slight increase in apparent molecular weight of the more acidic isoforms.

Filaggrin and the 40K protein. Similar two-dimensional electrophoresis pattern and apparent molecular weight have been previously described for filaggrin (26), a basic histidine-rich marker of epidermal differentiation (21). Therefore, we investigated the relationships between the RA antigen and filaggrin. We first explored whether AKH1, a mAb specific for human filaggrin (27), could react with the RA antigen after two-dimensional gel electrophoresis. The characteristic neutral and "comma-shaped" protein revealed by the anti–40K RA sera was also detected by AKH1 (Fig. 2A). In addition, analyzed by Western blot after PAGE under nondenaturing conditions, the epidermis extract showed identical patterns whether the blots were probed with RA sera or with AKH1 (data not shown). We then analyzed whether AKH1 could immunoprecipitate the 40K antigen. A detergent extract was prepared from epidermis and immunoprecipitated, under conditions of high ionic strength preventing protein–protein association, with AKH1 and with a control mAb; the presence of the 40K protein in the immunoprecipitates was then analyzed by Western blot with 4 different anti–40K RA sera and 2 normal human sera (Fig. 2B). As expected, an immunoreactive band with an apparent molecular weight of 40,000 was detected with AKH1 in the immunoprecipitates obtained with the anti-filaggrin antibody but not in those obtained with the control mAb. In the former immunoprecipitates, a band of identical mobility was also specifically visualized with the RA sera, suggesting that AKH1 actually immunoprecipitated the 40K antigen. To confirm that the proteins recognized by AKH1 and the RA sera were the same, the supernatants resulting from immunoprecipitation with the anti-filaggrin mAb were subsequently analyzed by Western blot. Results (Fig. 2C) showed that AKH1

![Figure 2](image-url)

Figure 2. A mAb to human filaggrin and the anti–40K RA sera recognized the same protein of human epidermis. (A) Western blot of two-dimensional gels of the partially purified 40K protein (10 μg) probed with AKH1 and with an unrelated control mAb (Co). AKH1 immunostained the 40K protein. (B) The detergent extract of human epidermis (15 μl; 8.2 mg/ml) was immunoprecipitated with AKH1 or with the control mAb and the immunoprecipitates (IMP) were analyzed by Western blot with antibodies and sera as indicated at the top of each lane with the same code as in Fig. 1. The filaggrin–cross-reacting 40K protein (†), immunoprecipitated and immunostained by AKH1 (lane 1), was also immunostained by the anti–40K RA sera (lanes 3–6). (C) The supernatants (SUP) remaining after the immunoprecipitation of the detergent extract of human epidermis with AKH1 or with the control mAb were also analyzed by Western blot. Both AKH1 and the anti–40K RA sera strongly stained the 40K protein in supernatants recovered after immunoprecipitation with the control mAb (lanes 9, 11–14), but only weakly in supernatants recovered after immunoprecipitation with the anti–human filaggrin mAb (lanes 1, 3–6). The 50K band occasionally stained was probably due to the murine IgG, detected by the secondary antibody to mouse IgG (lanes 1, 2, 9, 10) or by cross-reacting rheumatoid factors (lanes 6, 14).
largely removed the protein recognized by the anti-40K RA sera, demonstrating complete cross-reactivity between the sera and the antifilaggrin mAb. The specificity of this experiment was established by the failure of an unrelated control mAb to deplete the 40K protein.

Analysed by one-dimensional Western blot, the 40K antigen was recognized by AKH1 and also by six different mAbs to human epidermal filaggrin recently produced in our laboratory, showing that filaggrin and the RA antigen present several common epitopes (Fig. 3 A). The close relationship between filaggrin and the 40K antigen was also confirmed by Cleveland peptide-mapping studies with S. aureus V8 protease digestion (Fig. 3 B).

Despite their net difference in pI values, filaggrin and the purified 40K RA antigen had similar amino acid compositions, both the proteins being rich in serine, glycine, histidine, and other basic amino acids, and lacking methionine but containing citrulline (Table I). This result confirms that the 40K protein was a modified form of filaggrin. Unfortunately, automated Edman degradation of the RA antigen failed to release any amino acid, as previously noticed for rat filaggrin (28).

Immunodetection of filaggrin by RA sera. We next investigated whether anti-40K RA sera really recognized mature human filaggrin. This protein, purified from human skin, was therefore analyzed by Western blot after SDS-PAGE. The purified filaggrin migrated with an approximate molecular weight of 37,000 as previously described (29), and was specifically immunostained with AKH1. All the anti-40K RA sera tested (10/10) also detected this protein, whereas control human sera did not (Fig. 4 A). Autoantibodies from RA sera were then affinity-purified on nitrocellulose-bound 40K protein and used to probe immunoblots of purified filaggrin. The affinity-purified anti-40K antibodies specifically bound onto filaggrin (Fig. 4 B). In addition, the reactivities towards the 40K antigen of both the RA sera and the affinity-purified immunoglobulins were not only specifically, but also completely abolished after immunoadsorption with filaggrin, confirming that the same autoantibodies recognized the 40K RA antigen and human filaggrin (Fig. 5). In a control test, filaggrin did not inhibit the binding to ovalbumin of a rabbit anti-ovalbumin serum (not shown).

![Figure 3](image1.png)

*Figure 3.* Relationship between the 40K protein and mature basic epidermal filaggrin. (A) The partially purified 40K protein (1.5 μg/ lane) was analyzed by Western blot with mAbs directed against six different epitopes of mature basic filaggrin (1–6), with a control mAb (7) or with AKH1 (8). All the antifilaggrin mAbs detected a protein with the same electrophoretic mobility as the 40K antigen (4). (B) The 40K protein (even numbers) and mature basic filaggrin (uneven numbers) were purified from human skin. Three μg of both proteins were digested with S. aureus V8 protease (5 to 500 ng) in the acrylamide gel and analyzed by Western blot with AKH1. Immunoreactive peptides with similar mobility were found in both digests (•). Essentially identical results were obtained with a RA serum, •, undigested proteins.

![Figure 4](image2.png)

*Figure 4.* The anti-40K RA sera immunostained mature filaggrin purified from human epidermis. (A) Basic epidermal filaggrin was purified, subjected to SDS-PAGE (0.5 μg per lane), electrotransferred to nitrocellulose and stained with Ponceau 5 (lane 1) or analyzed by Western blot with AKH1 (lane 2), with a control mAb (lane 3), with anti-40K RA sera (lanes 4–13) and with control sera (lanes 14–18). The sera used are coded at the top of each lane as in Fig. 1. The anti-40K RA sera specifically immunodetected the purified filaggrin. (B) Western blot on filaggrin was also performed with antibodies affinity-purified from a RA serum on nitrocellulose-bound 40K antigen (lane 1) or on control protein (lane 2). Antibodies were also affinity-purified from a control serum on the 40K antigen (lane 3). The affinity-purified anti-40K RA antibodies specifically reacted with filaggrin.

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<th>Table I. Amino Acid Compositions of the Purified 40K Protein and Human Epidermal Filaggrin (Residues per 100 Residues)</th>
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To demonstrate that the antifilaggrin/anti-40K protein autoantibodies were responsible for the specific immunofluorescence pattern observed with RA sera on rat esophagus epithelium, 2 anti-40K RA sera were analyzed by indirect immunofluorescence on this tissue before and after immunoabsorption on both filaggrin and the 40K protein. A significant inhibition of the fluorescence intensity on the stratum corneum was observed when the sera were preincubated with purified filaggrin or with the 40K antigen but no detectable change was observed after immunoabsorption with an equal amount of BSA (Fig. 6). These results indicate that both the 40K protein and filaggrin share some epitopes with the rat esophagus stratum corneum. In contrast to the complete adsorption of immunoblotting reactivity against the 40K protein or filaggrin, immunofluorescence reactivity against the stratum corneum was never completely abolished, even with a large excess of purified filaggrin, suggesting the existence of antibodies directed to other antigens of the stratum corneum of the rat esophagus. More probably however, this result indicates that discontinuous or conformational epitopes present on filaggrin may be lost during the purification steps.

**Discussion**

Using indirect immunofluorescence, antibodies of the IgG class directed against components of rat esophagus and human skin have been described as highly specific for RA (6–15). Until now, the presence of these autoantibodies, the so-called "antikeratin antibodies," in the serum of patients is the most specific serological criterion for the disease. Our study provides the first detailed biochemical characteristic of the human epidermal antigen recognized by the RA sera. We demonstrated that 75% of RA sera reacted in Western blot with a 40K protein of human epidermis that was weakly detected by only 7% of non-RA sera. Since the immunofluorescence intensity produced by the RA sera on rat esophagus correlated with their reactivity against the 40K protein in Western blot and was largely decreased by immunoabsorption with the 40K protein, the RA-specific labeling of esophagus epithelium was most probably due to these anti-40K antibodies. The 40K antigen was identified as a neutral/acidic modified form of human filaggrin and is probably identical to an AKH1-recognized protein recently described in epidermal extracts but not further characterized (30). The immunofluorescence labeled anti-40K antibodies were also shown to react with basic mature filaggrin, confirming this cytokeratin filament-aggregating protein as a major target of autoantibodies specific for RA. The immunoblotting detection of this autoantibody against filaggrin may be useful to the serological diagnosis of RA, in particular to differentiate RA from other arthritic diseases in the early stages of these disorders.

Filaggrin is a basic intermediate filament-associated protein that is involved in the aggregation of cytokeratin filaments during terminal differentiation in mammalian epidermis. It is synthesized as a large heavily phosphorylated, and therefore acidic, precursor (profilaggrin). During the late steps of normal differentiation, this precursor is dephosphorylated and cleaved to release functional filaggrin molecules (18–21). What is the origin of the 40K filaggrin isoform we have identified? It could be an undiscovered intermediate in the processing pathway from the acidic profilaggrin to the basic filaggrin, or a degradation product of profilaggrin that is known to be proteolytically labile; such a degradation polypeptide, with a pI of 6.9 and a slightly higher apparent molecular weight than mature filaggrin, has been described in the rat (31). However, a protein with a pI between those of profilaggrin and filaggrin has never been reported after 32P-phosphate labeling of human skin. Moreover, RA sera did not immunodetect profilaggrin on Western blot and did not decorate the keratohyalin granules (our unpublished observations) where profilaggrin is stored in human epidermis. Alternatively, the pI of this slightly slower migrating isoform may result from the extensive conversion of the amino acid arginine to citrulline, a reaction previously proposed by Harding and Scott to explain the characteristic "comma-shaped" electrophoretic migration of filaggrin (26).
Further characterization of the 40K antigen will be necessary to conclusively answer this question.

Another autoantibody, the antiperinuclear factor, directed against perinuclear granules of the epithelial cells of the human buccal mucosa, has also been described in RA (9, 32–35). Since it was found, by double immunofluorescence, that (pro)filaggrin colocalized with the perinuclear granules and since a correlation was found between the titer of antiperinuclear factors and the titer of the so-called “antikeratin antibodies” (35), it is tempting to speculate that these two autoantibodies are identical or at least closely related. However, most of the tested sera containing the antiperinuclear factor were found not to react or only to react weakly on Western blot with AKH1-reactive molecules from buccal cells (35). This apparent discrepancy with our results may simply reflect differences in sensitivity of the assays.

Although their importance in diagnosis has been established, the role as well as the origin of antifilaggrin autoantibodies in the serum of RA patients remain unclear. These autoantibodies may be derived from the destruction of synovial cells containing filaggrin, or more probably containing cross-reactive molecules, since the synovial lining cells are not considered to express filaggrin. In this context, it is interesting to note that filaggrin, like many other autoantigens (36, 37), displays unusual charge properties since it is highly basic, and is associated into macromolecular complexes with other autoantibody-reactive proteins, i.e., cytokeratins recognized by naturally occurring autoantibodies (38).

The importance of T cells in the pathogenesis of RA is becoming increasingly clearer. However, the antigenic specificity of the involved T cells is not known, even though a reactivity to heat shock proteins has been implicated (39–41). Therefore it will be important to determine whether filaggrin or filaggrin-related molecules are involved in this process.

The identification of the epitope(s) with which the antifilaggrin autoantibodies react will probably suggest possible mechanisms of their production, may provide more insight into the pathogenesis of RA, in particular in the event of molecular mimicry, and may even suggest new approaches to therapy, in a way similar to that successfully used in experimental allergic encephalomyelitis (42).

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References