Evidence for Specific Proteolytic Cleavage of the *N*-Terminal Domain of Human Profilaggrin During Epidermal Differentiation

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Profilaggrin is a large phosphoprotein that is expressed in the granular cells of epidermis where it is localized in keratohyalin. It consists of multiple copies of single filaggrin units plus *N*- and *C*-terminal sequences that differ from filaggrin. Profilaggrin is dephosphorylated and proteolytically processed during terminal differentiation to yield filaggrin, which associates with keratin intermediate filaments to form macrofibrils in the lower layers of the stratum corneum. The *N*-terminal sequence of human profilaggrin comprises two distinct domains; an acidic A domain of 81 amino acids that binds Ca^{2+}, and a cationic B domain of 212 residues. In this report, we further characterize the *N*-terminal domain by immunohistochemistry and immunoblot analysis using anti-peptide antibodies raised to the A and B regions. All of these antibodies (n = 4) immunostained keratohyalin in the granular layer of human epidermis and also showed some reaction with the lower stratum corneum. In immunoblot studies, the high molecular weight human profilaggrin reacted with both B domain antibodies whereas it showed a weak and variable reaction with A domain antibodies. In addition to profilaggrin, a cationic 32-kDa protein was detected with all *N*-terminal antibodies. A similar-sized *N*-terminal peptide was also produced by *in vitro* proteolysis of human profilaggrin with endoproteinase 1 (PEP1), a protease involved in processing of mouse profilaggrin, and in cultured rat epidermal keratinocytes transfected with a human profilaggrin cDNA construct. Evidence for at least one additional cleavage within the *N*-terminal domain is shown by immunoreactivity of smaller (16–20 kDa) acidic and basic proteins with A and B domain antibodies, respectively. These results demonstrate that the *N*-terminal domain is an integral part of profilaggrin in keratohyalin but is proteolytically cleaved from profilaggrin during the terminal differentiation of keratinocytes to yield a 32-kDa peptide. ***Key words: profilaggrin/epidermis/proteolytic processing. J Invest Dermatol 108:170–178, 1997***

The terminal differentiation of mammalian epidermal keratinocytes results in the formation of a stratum corneum that consists of dead cells (corneocytes) filled with cross-linked keratin proteins surrounded by a toughened cornified cell envelope. In order to form this protective sheath that envelops the organism, keratinocytes in the living layers of epidermis synthesize a series of structural proteins, including type I and II keratins, involucrin, profilaggrin, loricrin, and small proline-rich proteins (reviewed in Reichert *et al*, 1993; Fuchs and Byrne, 1994; Steinert and Marekov, 1995). Other changes include formation of lipid-laden lamellar granules that fuse with the plasma membrane of terminally differentiating keratinocytes, forming intercellular lamellae, which are important for formation of a normal permeability barrier and desquamation of cornified cells (Odland and Holbrook, 1981; Elias and Menon, 1991). One of the last proteins synthesized in differentiating cells is profilaggrin, a large, highly phosphorylated protein consisting mainly of filaggrin repeats. Profilaggrin is synthesized in granular cells and deposited in keratohyalin granules. As these cells undergo terminal differentiation to form corneocytes, profilaggrin is dephosphorylated and proteolytically processed to yield filaggrin, which functions in the lower layers of the stratum corneum to aggregate keratin intermediate filaments into dense macrofibrils (reviewed in Dale *et al*, 1993, 1994; Presland, 1996). This activity is thought to facilitate disulfide bond formation between keratin intermediate filament chains and may enable them to survive the massive remodeling that ensues with terminal differentiation. Finally, in the upper stratum corneum, filaggrin is mostly degraded to free amino acids that are thought to be important for normal epidermal osmolarity and flexibility of the epidermis (Scott *et al*, 1982; Scott and Harding, 1986; reviewed in Rawlings *et al*, 1994).

Extensive characterization of profilaggrin and filaggrin sequences obtained from analysis of cDNA and genomic clones, as well as purified protein preparations, has shown that filaggrin is a histidine-rich, highly cationic protein that varies in size and sequence

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Abbreviations: REKs, rat epidermal keratinocytes.

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between species (Rothnagel *et al*, 1987; McKinley-Grant *et al*, 1989; Haydock and Dale, 1990; Rothnagel and Steinert, 1990; Presland *et al*, 1992; Resing *et al*, 1993b; Thulin and Walsh, 1995). Human profilaggrin consists of 10–12 repeating units of filaggrin (324 amino acids each) that are flanked at either end by truncated filaggrin sequences and N- and C-terminal peptides that share little similarity with filaggrin (Gan *et al*, 1990; Presland *et al*, 1992; Markova *et al*, 1993). The *N*-terminal sequence of human profilaggrin, predicted from genomic sequence to be 293 amino acids in length, can be divided into two distinct domains (Presland *et al*, 1992). The first 81 amino acids (domain A) contains two calcium-binding motifs that are similar to the EF-hands of the S-100 family of calcium-binding proteins and is able to bind calcium *in vitro* (Presland *et al*, 1995). Profilaggrin isolated from mouse skin also binds calcium *in vitro* (Markova *et al*, 1993). The B domain is 212 amino acids in length, contains a high proportion of polar amino acids, and is predicted to have a cationic net charge.

The presence of an S-100–like calcium-binding domain in profilaggrin implicates calcium as a possible regulator of keratohyalin formation and/or processing of profilaggrin to filaggrin. It has previously been shown that relatively high concentrations of calcium are required for transcription of the profilaggrin gene and other late differentiation markers, such as loricrin, in cultured epidermal keratinocytes (Yuspa *et al*, 1989; Hohl *et al*, 1991). In addition, calcium is indirectly required for activation of the second stage protease, which processes profilaggrin intermediates to filaggrin (Resing *et al*, 1993a), as well as for the activity of epidermal transglutaminase, which cross-links loricrin, involucrin, and other proteins into the cornified cell envelope (Thacher and Rice, 1985; reviewed in Rice *et al*, 1994; Steinert and Marekov, 1995). These events are likely to be coordinated, at least in part, by calcium-binding proteins (Hardas *et al*, 1996) and a calcium gradient in epidermis, in which intracellular calcium concentrations are highest in granular cells, the site of profilaggrin expression (Menon *et al*, 1985; Menon and Elias, 1991). Thus, the A domain, by binding a proportion of the calcium in granular cells, could allow profilaggrin to regulate its own expression, formation of keratohyalin, or processing to filaggrin (Presland *et al*, 1995). At present, little is known about the B domain, except that it has a highly polar sequence and contains a number of potential phosphorylation sites.

In order to examine the possible role of the *N*-terminal domains of profilaggrin, we prepared several peptide antibodies directed to the A and B domains. We determined the location and fate of the *N*-terminal sequences during the terminal differentiation of epidermis and keratinizing oral epithelia. We show that the *N*-terminal peptide is cleaved from the filaggrin sequences during epidermal differentiation, which suggests that the A and B domains may have an independent fate and/or function from filaggrin.

MATERIALS AND METHODS

Antibodies The antibodies to the *N*-terminal A and B domains were elicited in rabbits by injection of synthetic peptides after conjugation via an added *C*-terminal cysteine residue to keyhole limpet hemocyanin. The peptides used were prepared based on the predicted amino acid sequence (Presland *et al*, 1992) as follows; peptide A1, KQY**SKKDKNTDTLSK** (amino acids 16–30); peptide A2, EKEFRQILKNPDDPD (amino acids 38–52); peptide B1, HEDNKQEENKENRKR (amino acids 110–124); peptide B2, PRETGGKRHESSSEKK (amino acids 144–159). Peptide A1 includes part of the first EF-hand (*boldface type*), whereas peptide A2 is located between the two EF-hands. Antibody titers were determined by enzyme-linked immunosorbent assay using the peptide as competitor (Genemed, San Francisco, CA; and Immunodynamics, La Jolla, CA). All rabbits used were screened for serum reactivity with epidermal keratins prior to injection of peptides. The antiserum was affinity purified using a peptide affinity column, and the affinity-purified antibodies were stored at −20°C. All studies reported here were performed with affinity-purified antibodies. The monoclonal (AKH1, Dale *et al*, 1987), and polyclonal (8959, Fleckman *et al*, 1985) antibodies, which react with both human profilaggrin and filaggrin, were developed previously by our laboratory.

Gel Electrophoresis and Immunoblot analysis Tris(hydroxymethyl) aminomethane (Tris)/urea extracts of human foreskin epidermis, cultured human keratinocytes, transfected rat epidermal keratinocytes (REKs) or COS-7 cells were prepared as described previously (Sybert *et al*, 1985; Haydock *et al*, 1993) except that the extraction buffer contained 8 M urea, 50 mM Tris, pH 7.6, and 100 μg phenylmethylsulfonylfluoride per ml (Sigma, St. Louis, MO). For immunoblotting, equal protein loadings were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), and blotted for 12 h at 100 mA onto nitrocellulose (0.45 μM, Schleicher & Schuell, Keene, NH). Immunoblots were calibrated with prestained protein standards (Life Technologies, Gaithersburg, MD). Immunoreactive proteins were visualized with the avidin-biotin peroxidase complex method using 4-chloro-1-napthol as substrate (Towbin *et al*, 1979; Haydock *et al*, 1993).

For two-dimensional (2-D) gels, Tris/urea extracts of foreskin epidermis were separated by charge in the first dimension using the non-equilibrium gradient system (NEpHGE, O'Farrell *et al*, 1977; Harding and Scott, 1983). Proteins were subsequently separated by molecular weight on gradient SDS-PAGE 7%–12% gels (Jule Inc., New Haven, CT). Samples were blotted as described for one-dimensional (1-D) gels, and proteins were detected with appropriate antibodies by the avidin-biotin peroxidase complex method.

***In Vitro* Endoproteinase 1 Assay** Mouse endoproteinase 1 (PEP1) was a gift of Drs. C. Thulin and K. Resing. Enzyme assays were performed essentially as described previously (Resing *et al*, 1995) using purified human profilaggrin isolated from a DE52 column. For endoproteinase assays, aliquots of human profilaggrin (~10 μg) were precipitated onto 1.5-ml Eppendorf tubes by adding 5 vol of methanol and incubating on ice for 30 min. The samples were centrifuged for 5 min in a microfuge, and the protein-containing pellets were air dried. Reactions were performed at 32°C for 0–4 h in 20 μl and contained 40 mM HEPES, pH 7.1, 12.5 mM putricine, 5 mM dithiothreitol, 2 mM $MgCl_2$, 1.7 mg aprotinin per ml, ~10 μg of profilaggrin, and 3 μl (~5 units) of the PEP1 enzyme preparation. Reactions were stopped by immediate freezing in a dry ice/ethanol bath and analyzed by SDS-PAGE and immunoblotting with profilaggrin antibodies as described above.

Constructs, Cell Culture, and Transfection Procedures The profilaggrin cDNA expression construct containing the *N*-terminal 467 amino acids of human profilaggrin (A + B + the truncated filaggrin unit, terminating at the beginning of the first filaggrin linker peptide) was prepared by a polymerase chain reaction approach followed by cloning into the expression vector pcDNA3-FLAG described previously (Dale *et al*, 1997). The vector comprises the pcDNA3 vector backbone (from Invitrogen, San Diego, CA) with the pcDNA3 polylinker being replaced by the FLAG epitope-containing polylinker from pFLAG-2 (IBI Kodak, New Haven,CT); the pcDNA3-FLAG construct was used to direct expression of the *N*-terminal 467 amino acids of profilaggrin in COS-7 cells and REKs. The construct (termed pFLAG467proF) contains a FLAG epitope at the *N* terminus.

The profilaggrin *N*-terminal construct was prepared by using the oligonucleotides 5′-CCCGAATTCTCTACTCTCCTGGAAAACATCTTT-GCC-3′ and 5′-CCCCTCGAGAGACCCTGAACGTCCAGACCGTT-CCCC-3′. The 5′- and 3′-oligonucleotides includes *Eco*RI and *Xho*I restriction sites for cloning, respectively (*underlined*). The polymerase chain reaction was performed for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C in a Amplitron I thermocycler (Barnstead Thermolyne, Dubuque, IA) using profilaggrin cDNA as template (Presland *et al*, 1992). The amplification product (~1.5 kb) was gel purified and digested with *Eco*RI and *Xho*I for directional cloning into pcDNA3-FLAG. Plasmid DNA was prepared by alkaline lysis and purified using Qiagen 500 columns (Qiagen, Chatsworth, CA) as directed by the manufacturer. The construct was verified by dideoxy sequencing using a Sequenase 2.0 kit (U.S. Biochemical Corporation, Cleveland, OH) and [α-^{35}S]dATP.

COS-7 African Green monkey kidney cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and were passaged weekly. REKs, a rat epidermal keratinocyte cell line (a generous gift from Dr. Howard Baden, Massachusetts General Hospital, Boston, MA) were grown as previously described (Baden and Kubilus, 1983; Haydock *et al*, 1993). COS-7 cells and REKs were transfected in 60-mm dishes at 60% confluence using LipofectAMINE reagent (Life Technologies) as described previously (Dale *et al*, 1997). Transfections were performed using 2.5 μg of pFLAG467proF DNA and 2 μg of pSV-βgal (Promega, Madison, WI). Cells were harvested 48 h after transfection and lysates prepared by extraction with urea/Tris buffer as described above. Profilaggrin expression and processing was analyzed by immunoblotting with profilaggrin antibodies.

Oral Keratinocyte Culture Lifted cultures of oral keratinocytes were grown as described (Kautsky *et al*, 1995). Second-passage keratinocytes

originating from cornified oral mucosa were grown on collagen lattices submerged in Dulbecco's modified Eagle's medium with 10% fetal bovine serum for 1 wk, then raised to the air-liquid interface and grown for 2 wk more. At the time of lattice elevation, the medium was switched to Dulbecco's modified Eagle's medium containing 10% delipidized fetal bovine serum, and all-trans retinoic acid (Sigma) was added to the medium to a final concentration of either 10^{-9} M or 10^{-7} M. Lattices were prepared for immunohistochemistry after fixation in methyl Carnoy's or extracted in urea/Tris buffer for immunoblots as detailed elsewhere (Kautsky *et al*, 1995; this paper).

Immunohistochemistry and Immunoelectron Microscopy Immunohistochemistry was performed on methyl Carnoy's fixed samples embedded in paraffin using the avidin-biotin peroxidase complex method as previously described (Hsu *et al*, 1981). Reaction was detected using diaminobenzidine. Controls were normally included where parallel sections were reacted only with secondary antibody; no significant immmunoreactivity was observed in the absence of primary antibody.

Immunoelectron microscopy was performed using the post-embedding method essentially as described previously (Manabe *et al*, 1991). Briefly, fresh tissue was placed in 2% paraformaldehyde and 0.02% picric acid[1] for 2 h on ice and then soaked in phosphate-buffered saline overnight. Samples were dehydrated in graded series of ethanol, incubated in mixtures of ethanol-LR White (Ted Pella, Redding, CA) with increasing concentration of LR White, then finally in 100% LR White overnight at room temperature. Samples were changed to fresh LR White, desiccated 1 h under vacuum, changed to fresh LR White, further evacuated, degassed in a vacuum chamber flushed twice with nitrogen gas, and placed in an oven for 24 h. Thin sections were cut, placed on parlodion-coated copper grids, and immunostained. Detection of primary antibody was with goat anti-rabbit, 20 nm gold-tagged antibody (Amersham, Arlington Heights, IL). Control immunostaining consisted of epidermal sections incubated with blocking buffer followed by secondary antibody alone. For contrast, the sections were stained by incubating in 0.1% phosphotungstic acid for 10 min, saturated uranyl acetate for 20 min, and finally in lead citrate solution for 20 min (Reynolds, 1963). Tissue was examined using a Philips 420 transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ) operated in the transmission mode at 60 kV.

RESULTS

Antibodies to Profilaggrin *N*-Terminal Domains Detect Profilaggrin and Peptides Derived from Profilaggrin To examine the fate and possible role of the *N*-terminal A and B domains in profilaggrin expression or processing, four peptide antibodies were developed to the *N* terminus of human profilaggrin: two that should specifically recognize the relatively acidic A domain and two that recognize the cationic B domain. Specificity of the antibody reactivity was investigated by immunoblot analysis of whole foreskin epidermal extracts on 1-D SDS/polyacrylamide gels **(Fig 1)** and on non-equilibrium 2-D gels (NEpHGE) **(Fig 2)**. The results are summarized in **Table I.** B domain antibodies react with profilaggrin on both 1-D and 2-D immunoblots, whereas the A domain reactivity with profilaggrin was variable, depending on protein load and immunoblot conditions. The reaction of A1 antibody with human profilaggrin (and the 32-kDa polypeptide discussed below) was enhanced by pre-treatment of the blot with 1 mM ethylenediamine tetraacetic acid **(Fig 1*B*)**, consistent with the possibility that Ca^{2+} ions may interfere with immunoreactivity by binding to, or altering the structure of, the EF-hand that contains the A1 epitope. The A domain antibodies also reacted with a 16-kDa protein **(Fig 1*A,B*)**. As expected, none of these antibodies reacted with human filaggrin **(Figs 1, 2)**, whereas all four detected the histidine-tagged profilaggrin *N*-terminal domain expressed in *Escherichia coli* (Presland *et al*, 1995, and data not shown).

In addition to profilaggrin, all four antibodies reacted with a 32-kDa polypeptide **(Fig 1*A,B*)**. Furthermore, the A2 antibody detected at least two polypeptides of 16 and 18 kDa **(Fig 1*A*)** that migrate in the acidic region of the 2-D gels **(Fig 2*B*)**, whereas the B domain antibodies reacted with one or more basic polypeptide(s) of 20 kDa on 2-D gels **(Fig 2*C,D*)**. All of these A- and B-reactive polypeptides were also observed on 1-D immunoblots of foreskin epidermal extracts fractionated by DE-52 ion exchange chromatography and eluted from the column in positions consistent with their cationic (32 kDa, A- and B-reactive; 20 kDa, B-reactive) or anionic (16, 18 kDa, A-reactive) nature. The results suggest that profilaggrin undergoes proteolytic processing *in vivo* to yield a cationic polypeptide of 32 kDa that contains all four *N*-terminal peptide epitopes **(Table I)**. Further, this *N*-terminal fragment may undergo additional proteolytic processing to yield a 20- kDa cationic polypeptide that is detected by only the B domain antibodies. The cationic nature of the 32- and 20-kDa proteins is consistent with the isoelectric point (pI) of these peptides predicted from the profilaggrin *N*-terminal amino acid sequence: the complete *N*-terminal domain has a predicted pI of 9.9, whereas the B domain sequence is even more cationic (pI = 10.2) (see **Fig 8**; *Discussion*).

Both A-domain antibodies reacted with a 16-kDa protein, and the A2 antibody also detected a slightly larger protein of 18 kDa, in addition to profilaggrin and the 32-kDa polypeptide **(Fig 1, Table I)**. These anionic polypeptides of 16 and 18 kDa may represent A-domain–containing peptides derived from the processing of the profilaggrin *N* terminus (the predicted pI of the 81-residue A domain of profilaggrin is 5.1). Alternatively, these immunoreactive peptides may represent antigenically related S-100–like EF-hand proteins expressed late in epidermal differentiation.

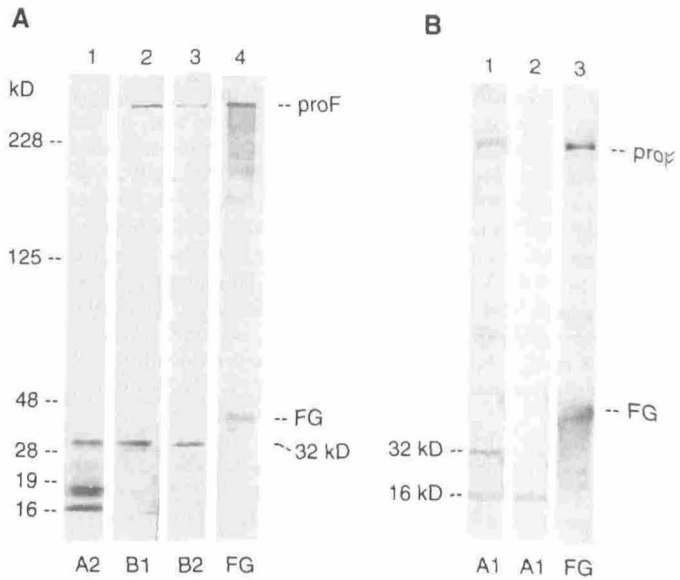

Figure 1. The *N*-terminal profilaggrin antibodies detect human profilaggrin and proteolytic processing products. Human epidermal foreskin extracts were separated on 4–15% SDS/polyacrylamide gels, transferred to nitrocellulose, and reacted with antibodies A2, B1, B2, and the polyclonal profilaggrin/filaggrin antibody 8959 (FG) *(A)* or A1 and 8959 *(B)*. In *(B)*, the antibody reaction protocol was done in the presence of either 1 mM ethylenediamine tetraacetic acid *(lane 1)* or 1 mM $CaCl_2$ *(lane 2)*. *Lane 3* represents a duplicate lane reacted with antibody 8959. Note that the reaction of A1 antibody *(B)* with profilaggrin and the 32-kDa protein is abrogated by the presence of Ca^{2+}, whereas immunoreactivity with a 16-kDa protein did not differ significantly between the two conditions *(lanes 1,2)*. Bands indicated are human profilaggrin (proF), filaggrin (FG), and the 32-kDa *N*-terminal processing product. Both A-domain antibodies also detect a 16-kDa polypeptide.

Expression of Profilaggrin cDNA in Epithelial Cells Results in Proteolytic Processing to Generate an *N*-Terminal Peptide To determine whether profilaggrin could be *N*-terminally processed in cultured epithelial cells, we transfected a human profilaggrin cDNA construct, encoding the *N*-terminal domain and some filaggrin sequences, into COS-7 cells and rat epidermal keratinocytes **(Fig 3)**. The results demonstrate that the full-length protein (~60 kDa) is proteolytically processed to smaller immunoreactive polypeptides in both keratinocytes *(lane 2)* and COS-7 cells

[1] Zamboni L, DeMartino C: Buffered picric acid-formaldehyde: a new rapid fixative for electron microscopy. *J Cell Biol* 35:148a, 1967 (abstr).

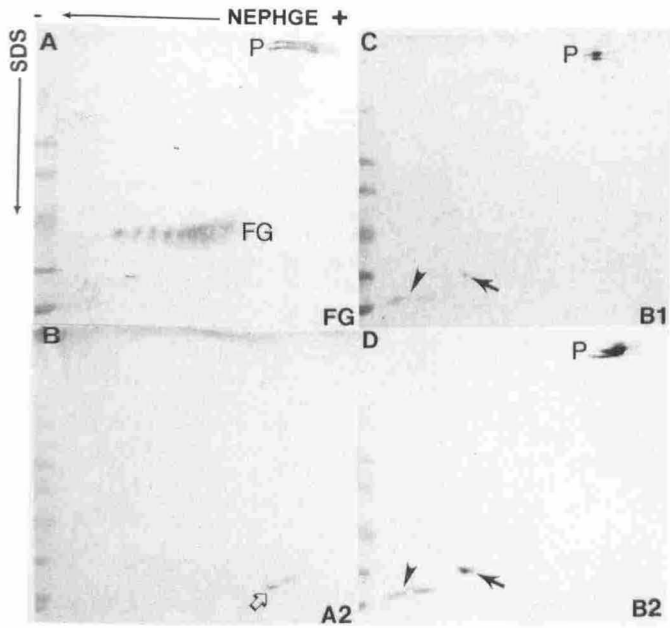

Figure 2. 2-D immunoblot analysis of human epidermal foreskin extracts with *N*-terminal profilaggrin antibodies. Epidermal proteins were separated in the first dimension using the non-equilibrium gradient system (NEpHGE) and in the second dimension by SDS-PAGE on 7–12% gels (see *Materials and Methods*). Duplicate blots were reacted with the polyclonal profilaggrin/filaggrin antibody 8959 *(A)*, and antibodies A2 *(B)*, B1 *(C)*, and B2 *(D)*. Note that both B-domain antibodies react with profilaggrin (P) and cationic polypeptides of 32 kDa (→) and 20 kDa (➤). The A2 antibody detects two acidic spots of 16 and 18 kDa (⇨); reaction with profilaggrin and the 32-kDa protein is not visible in this experiment. FG, filaggrin.

(lane 4); these polypeptides were not observed in mock-transfected cells *(lanes 3,5)*. Identically sized polypeptides were also detected in transfected cell extracts probed with B2 antibody (data not shown). The *N*-terminal processing product observed in transfected cells is slightly larger than the corresponding immunoreactive band in epidermal extracts because of the presence of a FLAG epitope at the *N* terminus of the expressed profilaggrin cDNA (compare *lanes 2,4* to *lane 1*). These data suggest that profilaggrin is proteolytically processed *in vivo* and *in vitro* (i.e., in transfected cells) to yield a similar-sized protein product.

The Profilaggrin Endoproteinase PEP1 Can Cleave Human Profilaggrin to Generate the *N*-Terminal 32-kDa Polypeptide Profilaggrin is processed to filaggrin by the action of two endoproteinases that have been isolated and characterized from rodent keratinocytes (Resing *et al*, 1989, 1993a, 1995). To determine whether the putative *N*-terminal 32-kDa profilaggrin peptide could be derived by *in vitro* proteolysis, we treated human profilaggrin (partially purified from foreskin epidermis by DE52 ion exchange chromatography) with the profilaggrin protease PEP1 isolated from mouse epidermis (Resing *et al*, 1995). The PEP1 preparation cleaved human profilaggrin to generate a 32-kDa polypeptide detected with A1 antibody **(Fig 4)**. Control incubations performed in the absence of enzyme gave little or no cleavage of this peptide.

Table I. Summary of Immunoblot Data with Profilaggrin Antibodies[a]

Antibody	Profilaggrin	Filaggrin	32 kDa[b]	20 kDa[b]	16, 18 kDa[c]
A1	+	–	+	–	+[d]
A2	+	–	+	–	+
B1	++	–	+	+	–
B2	++	–	+	+	–
8959[e]	++	++	–	–	–

[a] Results summarize polypeptides that are detected by the five profilaggrin antibodies on 1-D and 2-D immunoblots **(Figs 1,2)**. ++, strong reaction; +, some reaction; –, no reaction
[b] Cationic
[c] Anionic
[d] Only the 16-kDa protein was detected with antibody A1 **(Fig 1*B*)**.
[e] Polyclonal antibody that detects profilaggrin and filaggrin **(Fig 2*A*)**.

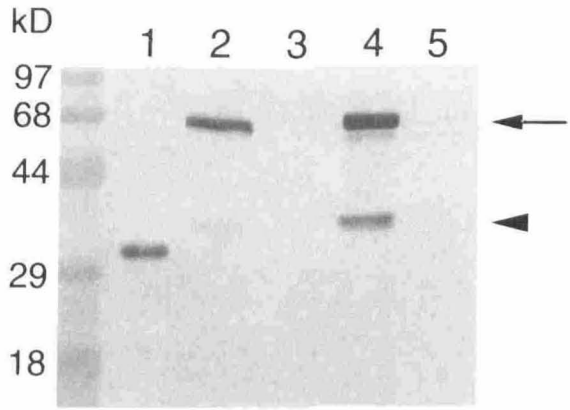

Figure 3. Expression of a profilaggrin cDNA construct in COS-7 cells and keratinocytes. Urea/Tris protein extracts of transfected cells were separated on 12–15% SDS/polyacrylamide gels, transferred to nitrocellulose, and reacted with antibody B1. Shown are REKs *(lanes 2,3)* and COS-7 cells *(lanes 4,5)* transfected with the profilaggrin cDNA construct pFLAG467proF *(lanes 2,4)*. *Lanes 3* and *5* represent mock-transfected cells, and lane 1 contains epidermal foreskin extract. Indicated are the unprocessed (→) and processed polypeptides (➤). Note that the processed polypeptide in REKs and COS-7 cells *(lanes 2,4)* is slightly larger than the corresponding 32-kDa band in foreskin *(lane 1)*.

Immunolocalization of the A and B Domains of Profilaggrin in the Upper Granular Layer and Terminally Differentiating 'Transition' Cells of Epidermis The studies reported here demonstrate that the *N*-terminal domain is cleaved from

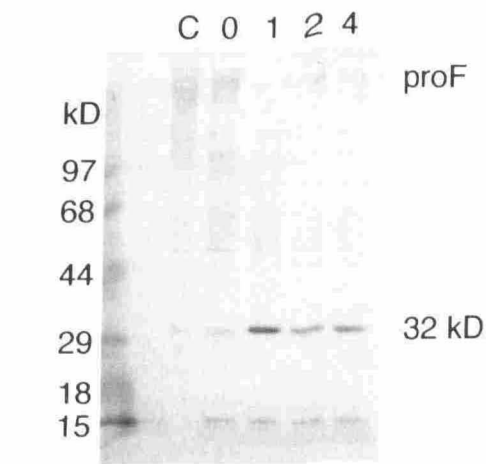

Figure 4. *In vitro* proteolysis of human profilaggrin by mouse endoproteinase PEP1 produces the 32-kDa polypeptide. Equal amounts (~10 μg) of human profilaggrin were incubated with PEP1 isolated from mouse epidermis for 0, 1, 2, and 4 h. Digested extracts were analyzed by SDS-PAGE on 7.5–12.5% gels, and immunoblotted polypeptides were detected with A1 antibody. The position of profilaggrin (proF) and the 32-kDa cleavage product are indicated. C indicates a control profilaggrin sample incubated only in enzyme buffer for 4 h.

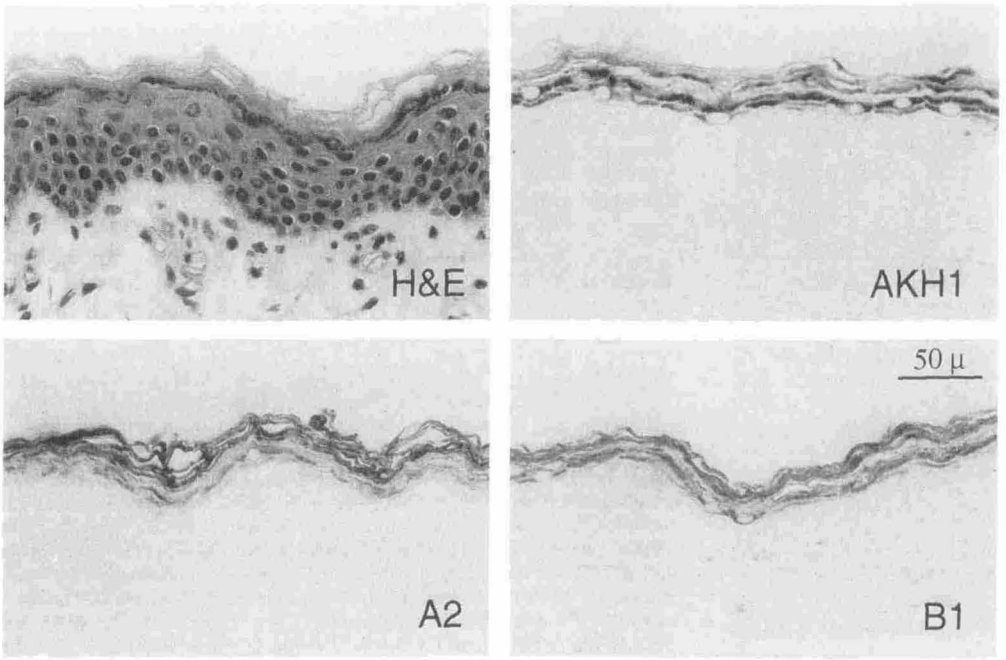

Figure 5. Immunohistochemical staining of human foreskin epidermis with profilaggrin antibodies shows immunoreactivity with granular and cornified cells. Sections fixed in methyl Carnoy's solution were stained with the monoclonal profilaggrin/filaggrin antibody AKH1 and the peptide antibodies A2 and B1. Each antibody stained the cytoplasm of granular cells and the lower cornified layers. Hematoxylin and eosin is included to show normal epidermal morphology. *Scale bar*, 50 μm.

profilaggrin during terminal differentiation. To determine whether the A and B domains of profilaggrin are localized in keratohyalin or whether they are cleaved prior to keratohyalin formation, immunohistochemical studies were performed using the *N*-terminal peptide antibodies. The results using antibodies A2 and B1 demonstrated an intense reaction in the granular layer of foreskin epidermis and a variable reaction with the stratum corneum **(Fig 5)**. A similar pattern of immunostaining was observed with adult human epidermis (data not shown). Parallel sections were stained using both monoclonal and polyclonal antibodies to human profilaggrin/filaggrin (Dale *et al*, 1987; Manabe *et al*, 1991); monoclonal antibody AKH1 showed typical particulate staining throughout the granular cells with some staining of the stratum corneum **(Fig 5)**. The B2 antibody worked poorly in immunostaining studies at both the light and ultrastructural levels and is therefore not shown, whereas the A1 antibody reacted with granular cells and the lower cornified layers and also weakly with basal and spinous layers (Presland, 1996).

Our observations made by immunohistochemistry were confirmed and extended using immunoelectron microscopy on human adult epidermis **(Fig 6)**. The A2 and B1 antibodies decorated keratohyalin in the granular layer, transition cells in focal areas where keratohyalin is in the process of dissolution, and the lower layers of the stratum corneum **(Fig 6*A,B,D,E*)**. Both A2 and B1 antibodies showed considerable labeling of the periphery in cornified cells **(Fig 6*F,G*)**, compared with a uniform cytoplasmic distribution of filaggrin **(Fig 6*C*)**. In general, however, the immunolabeling observed with the A2 and B1 antibodies is similar to that observed with the profilaggrin/filaggrin antibody 8959 in that all antibodies reacted with granular cells and the lower cornified layers **(Fig 6**; also see Manabe *et al*, 1991). No reaction was found in the basal or spinous cell layers, and little or no reaction was detected in the middle or outer cornified layers. These results demonstrate that the *N*-terminal domain of profilaggrin is localized in keratohyalin, as well as in transition cells where profilaggrin is being processed to filaggrin, and the inner layers of the stratum corneum.

Immunostaining of Raft Cultures Derived from Cornified Oral Mucosa with A- and B-Specific Antibodies In addition to epidermis, profilaggrin is also expressed in the differentiating layers of other keratinizing epithelia, including oral mucosa (Clausen *et al*, 1986; Smith and Dale, 1986; Dale *et al*, 1990; Kautsky *et al*, 1995). Cornified epithelia in the oral lining, as exemplified by gingiva, also express other markers of keratinization, including keratins K1 and K10, as well as K6, K16, and involucrin. In order to examine the immunostaining pattern of the A and B domain antibodies in a keratinizing epithelium other than skin, dermal equivalents of gingival keratinocytes grown on lattices containing oral fibroblasts from cornified mucosa were reacted with the A2 and B1 antibodies and the monoclonal anti-profilaggrin/filaggrin antibody AKH1 **(Fig 7)**. All three antibodies reacted similarly with the epithelial granular layer of dermal equivalent cultures grown in relatively low (10^{-9} M) retinoic acid **(Fig 7*A*)**, whereas no reaction was observed with keratinocytes grown in 100-fold higher retinoic acid (10^{-7} M) **(Fig 7*B*)**. These results demonstrate that these antibodies can detect profilaggrin expressed in differentiating oral keratinocytes and further show that the absence of profilaggrin in cultures grown in high retinoic acid concentrations is correlated with a lack of reactivity of A- and B-domain antibodies. This further supports our contention that these different peptides are derived from *N*-terminal processing of profilaggrin.

Immunoblot analysis of urea/Tris extracts of oral keratinocyte cultures demonstrated the presence of a 32-kDa protein that reacted with A and B antibodies (data not shown). Expression of both profilaggrin and the 32-kDa protein was not detectable in cultures grown in high (10^{-7} M) retinoic acid conditions, consistent with the immunohistochemical findings **(Fig 7)**. Therefore, it appears that human profilaggrin undergoes similar *N*-terminal processing in oral keratinocytes as observed in epidermis.

DISCUSSION

Profilaggrin is a large, insoluble protein that is a major component of the cytoplasmic keratohyalin granules of epidermal granular cells. Profilaggrin undergoes extensive post-translational processing in terminally differentiating cells. The fate and function of filaggrin, which aggregates keratin intermediate filaments, have been examined in a number of studies (Dale *et al*, 1978; Steinert *et al*, 1981; Manabe *et al*, 1991; Dale *et al*, 1997). In contrast, prior to this study, the fate of the non-filaggrin *N*- or *C*-terminal sequences had not been investigated. In the present report we utilized antibodies specific for the two distinct *N*-terminal A and B domains of human profilaggrin to follow the fate of this biochemically distinct region. We demonstrate that the *N*-terminal regions are present in granular and cornified cells in a pattern similar to that shown by reaction

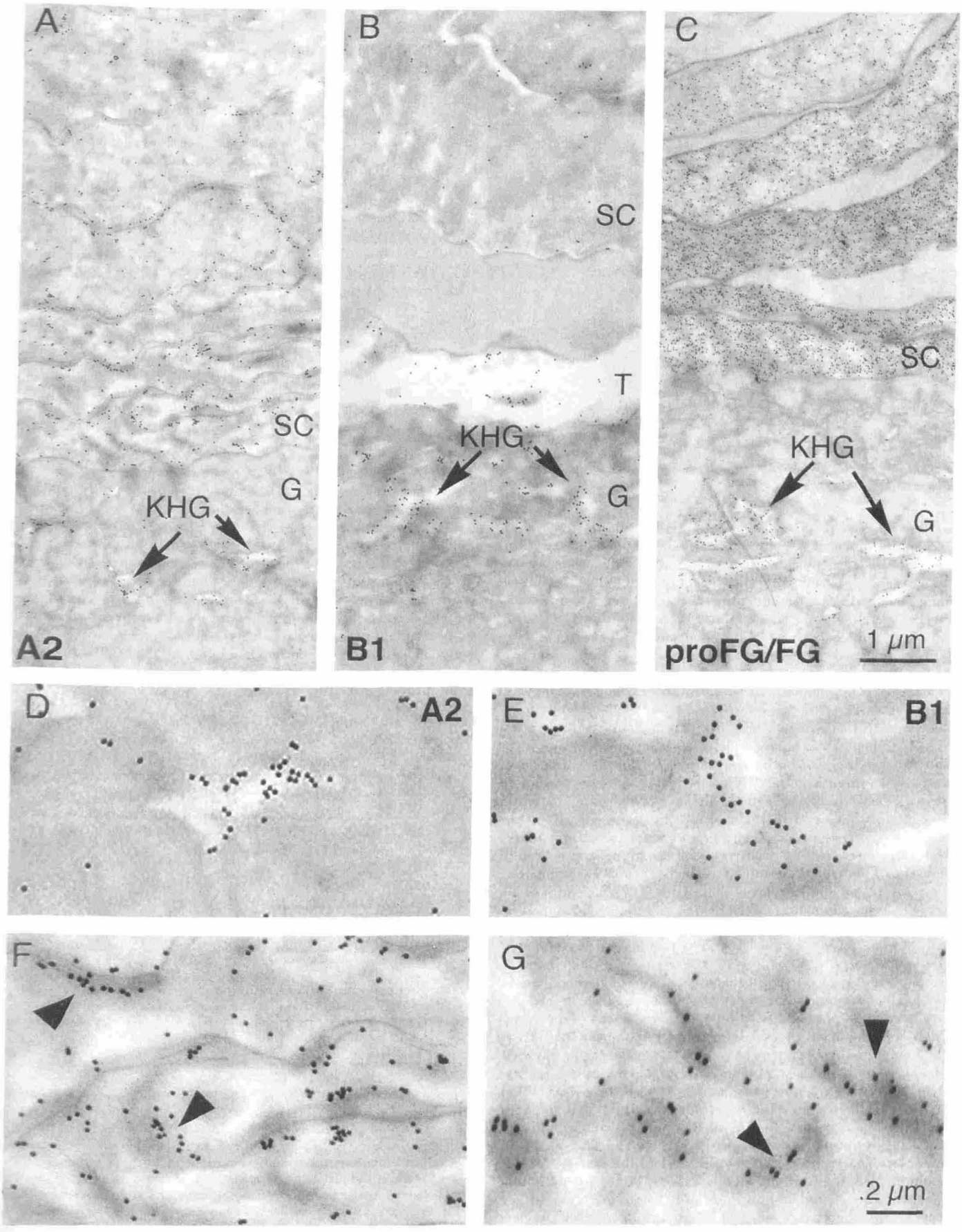

Figure 6. Immunoelectron microscopy of adult human epidermis with profilaggrin A and B domain antibodies shows immunoreactivity with keratohyalin and lower cornified layers. Shown are thin sections of adult human epidermis stained with profilaggrin antibodies A2 *(A)*, B1 *(B)*, and the profilaggrin/filaggrin (proFG/FG) antibody 8959 *(C)*. Indicated are keratohyalin granules (KHG) in the granular layer (→), transition cells (T, *panel B*), and the first layer of stratum corneum (SC). Insets show higher magnifications of keratohyalin in the granular layer *(D,E)*, and cornified cells *(F,G)*, that immunostain with A2 and B1 antibodies. Note the peripheral staining in cornified layers *(F, G,* ➤). *Scale bars*, 1 μm *(A-C)*, 2 μm *(D-G)*.

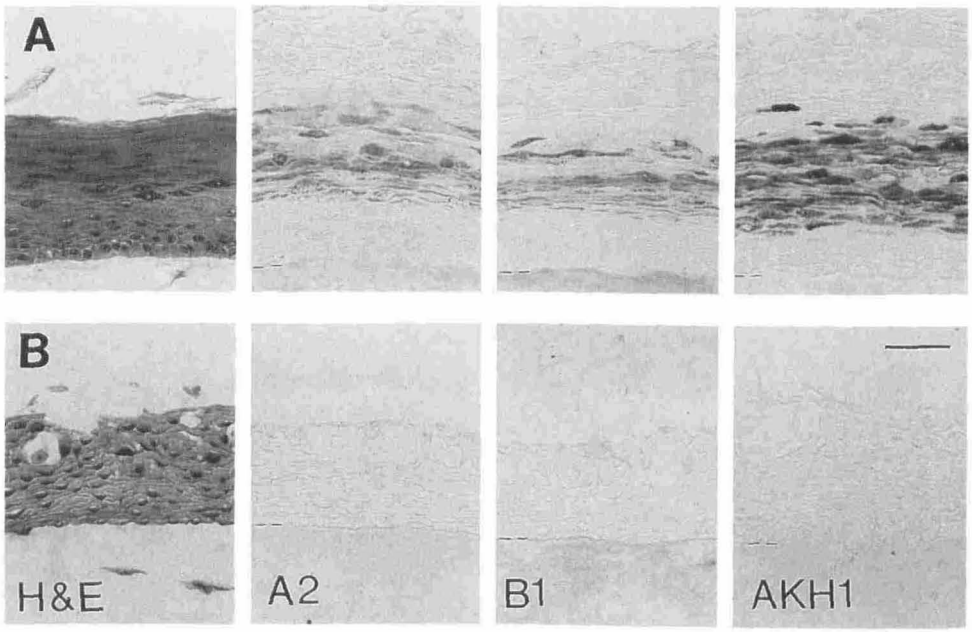

Figure 7. Immunohistochemical staining of human gingival keratinocyte cultures with profilaggrin antibodies show staining of granular and cornified layers, and downregulation by retinoic acid. Morphology (hematoxylin and eosin) and immunostaining with profilaggrin antibodies of normal human gingival keratinocytes derived from cornified oral cavity. Cells were grown at the air-liquid interface on lattices containing oral fibroblasts from cornified mucosa in delipidized medium that contains either 10^{-9} M *(A)* or 10^{-7} M *(B)* of retinoic acid added back (see *Materials and Methods*). Expression of profilaggrin was detected by immunostaining with the antibodies A2, B1, and AKH1. Note the loss of immunoreactivity with all three antibodies in cells cultured in high retinoic acid conditions. *Scale bar*, 50 μm.

with filaggrin domain antibodies. This localization to keratohyalin in the granular layer indicates that the A and B domains are a part of the insoluble, stored form of the protein **(Fig 6)**. The immunostaining reaction in the stratum corneum was both cytoplasmic and at the cell periphery and suggests that these *N*-terminal peptides could form a minor component of the cornified envelope. No significant immunostaining was observed with rodent epidermis using the B-domain antibodies, indicating that at least these peptide sequences are poorly conserved between mammalian profilaggrins (data not shown).

We have also shown in this paper that human profilaggrin is processed to generate a polypeptide of 32 kDa, which reacts with antibodies specific for both the A and B domains; this size corresponds to essentially the entire *N*-terminal region by predicted sequence **(Table I, Fig 8)**. Other, smaller polypeptides that react with either the A or B domain antibodies were also detected. Several lines of evidence suggest that these immunoreactive polypeptides are derived from proteolytic processing event(s) within the B domain of profilaggrin. First, the 32-kDa protein reacts with all four antibodies, suggesting that it contains most or all of the *N*-terminal domain **(Table I)**. Second, the acidic and basic nature of these various peptides, as shown by their elution profile from an ion exchange column (not shown) and their migration on 2-D immunoblots **(Fig 2)**, is consistent with the predicted charge of these peptides based on the profilaggrin cDNA sequence. Third, these small immunoreactive polypeptides were reproducibly detected in extracts prepared from human epidermis in the presence of high concentrations of urea and protease inhibitors, consistent with proteolysis occurring *in vivo*. Fourth, treatment of purified profilaggrin with partially purified PEP1, an endoproteinase involved in profilaggrin processing to filaggrin (Resing *et al*, 1989, 1995), also generated the 32-kDa peptide **(Fig 4)**. Fifth, a polypeptide similar in size to the 32-kDa protein was detected in cultured epithelial cells transfected with a profilaggrin cDNA **(Fig 3)**. Sixth, immunolocalization studies are consistent with the presence of this domain in profilaggrin in granular cells (i.e., in keratohyalin) and subsequent processing in transition cells, with retention in the lower stratum corneum **(Figs 5–7)**. These results support the view that these polypeptides are derived by cleavage of human profilaggrin, as illustrated in **Fig 8**. The 32-kDa polypeptide is produced by cleavage of the entire *N*-terminal region of profilaggrin; subsequently, one or more additional specific cleavages may occur to generate the smaller polypeptide(s) detectable with either the B- or A-domain antibodies at sufficiently high protein loads (e.g., **Fig 2**). We suggest that the initial cleavage is an early event of profilaggrin processing and may be important for keratohyalin dissolution and processing of profilaggrin to filaggrin. It should be noted that, although the B-domain antibodies are specific for profilaggrin (and its *N*-terminal processing products), the A-domain antibodies detected at least two polypeptides in addition to the 32-kDa protein in epidermal extracts. One (or more) of these are undoubtedly derived from profilaggrin **(Fig 1, Table I)**. Some of these additional

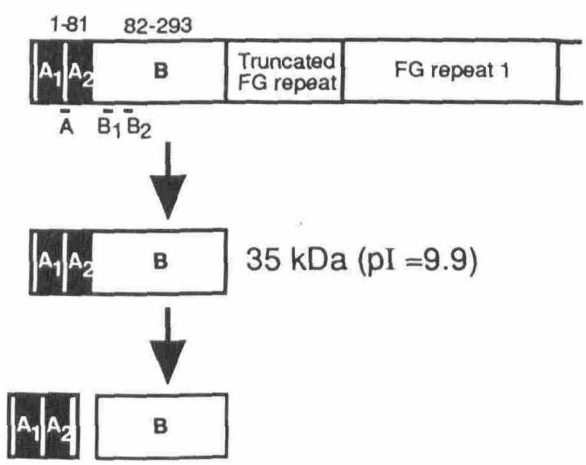

Figure 8. Model outlining probable *N*-terminal processing of profilaggrin. Schematic representation of *N*-terminal processing products detected *in vivo* and *in vitro* with A- and B-domain antibodies. The initial cleavage product (32 kDa by SDS-PAGE) is detected with both A- and B-domain antibodies, and comprises most, or all, of the *N*-terminal region. The *N*-terminal sequence has a *calculated* molecular mass of 35 kDa and is predicted to be cationic (pI = 9.9), consistent with its migration on 2-D gels **(Fig 2*C,D*)**. Additional smaller immunoreactive proteins suggest that the 32-kDa *N*-terminal protein undergoes internal cleavage to yield a cationic 20-kDa protein (predicted pI >10) that is detected with only the B-domain antibodies **(Fig 2*C,D*)**. A-reactive peptides of 16 and 18 kDa may or may not contain the S-100–like A domain of human profilaggrin. The position of the peptides used to raise antibodies A2, B1, and B2 are indicated below the unprocessed form of the profilaggrin protein.

polypeptides, however, may represent cross-reacting proteins, e.g., other S-100–like calcium-binding proteins that are expressed in differentiating keratinocytes (Hardas *et al*, 1996).

We do not yet know the nature of the endoproteinases that cleave profilaggrin within the *N*-terminal domain. We were, nevertheless, able to demonstrate that a partially purified preparation of mouse endoproteinase I, a chymotrypsin-like serine protease also known as PEP 1 (Resing *et al*, 1989, 1995), was able to cleave purified human profilaggrin *in vitro* to generate the 32-kDa polypeptide **(Fig 4)**. Recent studies have shown that the subtilisin-like serine endoproteinase, furin, can also cleave an *N*-terminal profilaggrin fragment expressed *in vitro* (Nirunsuksiri W, Rehemtulla A, Lewis SP, Presland RB, Dale BA: Evidence for involvement of furin in proteolytic processing of profilaggrin during epidermal differentiation, manuscript in preparation). Furin is a member of the proprotein convertase family of serine endoproteinases that cleaves known substrates such as hormone, growth factor, and neuropeptide precursors at dibasic amino acids (Barr, 1991; Rehemtulla and Kaufman, 1992). The variant *N*-terminal domain of profilaggrin ends with the sequence RKRR, which conforms to the recognition cleavage site for furin/PACE4 (Barr, 1991). *In toto*, these results suggest that both furin and the PEP1 preparation can cleave the profilaggrin *N* terminus.

In summary, we have prepared and characterized several new human profilaggrin-specific antibodies that have allowed us to demonstrate *N*-terminal processing during terminal differentiation of epidermal and oral keratinocytes. These results demonstrate that the *N*-terminal region is an integral part of profilaggrin in keratohyalin and that it is removed during terminal differentiation of keratinocytes. Further work is required to determine whether this peptide plays a specific role in regulating profilaggrin processing or is more generally involved in the terminal differentiation process in keratinizing epithelia.

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