Characterization of Two Distinct Calcium-Binding Sites in the Amino-Terminus of Human Profilaggrin

Richard B. Presland, James A. Bassuk,* Janet R. Kimball, and Beverly A. Dale†‡§

Laboratory of Epithelial Biology, Department of Oral Biology, and Departments of *Biological Structure, †Medicine (Dermatology), ‡Periodontics, and §Biochemistry, University of Washington, Seattle, Washington, U.S.A.

Profilaggrin is a large phosphorylated protein (approximately 400 kDa in humans) that is expressed in the granular cells of epidermis where it forms a major component of keratohyalin. It consists of multiple copies of similar filaggrin units plus amino- and carboxy-terminal domains that differ from filaggrin. Proteolytic processing of profilaggrin during terminal differentiation results in the removal of these domains and generation of monomeric filaggrin units, which associate with keratin intermediate filaments to form macrofibrils in the stratum corneum. The amino-terminal domain contains two calcium-binding motifs similar to the EF-hands found in the S-100 family of calcium-binding proteins. In this report, we expressed the 293-residue amino-terminal pro-domain of human profilaggrin as a polyhistidine fusion protein in *Escherichia coli,* and characterized calcium binding by a $^{45}Ca^{++}$ binding assay and fluorescence emission spectroscopy. Fluorescence measurements indicated that the profilaggrin polypeptide undergoes conformational changes upon the removal of Ca^{++} with ethylenediamine tetraacetic acid, demonstrating the presence of two calcium-binding sites with affinities for calcium that differ ninefold (1.4×10^{-4} M and 1.2×10^{-3} M). We suggest that this functional calcium-binding domain at the amino-terminus of human profilaggrin plays a role in profilaggrin processing and in other calcium-dependent processes during terminal differentiation of the epidermis. ***Key words: calcium-binding protein/epidermis/fluorescence spectroscopy. J Invest Dermatol 104:218–223, 1995***

The program of differentiation that occurs in mammalian epidermis involves the tightly controlled expression of a number of novel structural proteins in the spinous and granular cell layers before terminal differentiation and formation of the stratum corneum. These proteins include the differentiation-specific keratins K1 and K10, involucrin, and cornifin (small proline-rich proteins), which are synthesized in spinous cells; and profilaggrin and loricrin, which are expressed in granular cells and form the major components of keratohyalin [1–3]. Profilaggrin is a highly phosphorylated protein consisting largely of multiple filaggrin repeats. It is processed during terminal differentiation of epidermal cells to form filaggrin units, which function in the lower stratum corneum to aggregate keratin intermediate filaments into tightly aligned bundles called macrofibrils. The processing of profilaggrin involves several enzymes that dephosphorylate and proteolytically cleave the protein to yield filaggrin [4–7]. Finally, in the upper stratum corneum, filaggrin is degraded mostly into free amino acids, which are required for maintenance of epidermal osmolarity and flexibility [8,9].

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Reprint requests to: Dr. Richard Presland, Department of Oral Biology SB-22, University of Washington, Seattle, WA 98195.

Abbreviation: PVDF, polyvinylidene fluoride.

Sequencing of cDNA clones encoding mouse, rat, and human filaggrin repeats has shown that filaggrin is a histidine-rich, highly cationic protein that varies in size (e.g., mouse, 26 kDa; human, 37 kDa) and sequence among species [10–13]. However, the complete profilaggrin sequence derived from the human genomic DNA sequence revealed that the protein also contains sequences at the amino- and carboxy-termini of 293 and 157 amino acids, respectively, which share little similarity with filaggrin [14,15]. The amino-terminus can be divided into two distinct domains, A and B. The first group of 81 amino acids (domain A) is hydrophobic and contains two putative calcium-binding motifs similar to the EF-hands of the S-100 family of proteins. Domain B is 212 amino acids and is highly hydrophilic. Like other genes belonging to the S-100 family, profilaggrin contains two introns, one between the EF-hands and a second in the 5′ non-coding region. In addition, profilaggrin has been mapped to human chromosome 1q21 [13], where a number of other genes belonging to the S-100 EF-hand family also have been localized, including calcyclin, calgranulins A and B, and trichohyalin [16–19]. This region of chromosome 1q also contains a number of other epidermally expressed genes, including loricrin, involucrin, and the small proline-rich proteins ([17,19,20] and references therein). The domain-A protein sequences predicted from the cDNA sequence show greater than 90% identity among rat, mouse, and human profilaggrin (RBP, unpublished observations), in contrast to the amino acid sequence for the filaggrin repeat unit (42% to 60% identity [11]). This suggests that the EF-hand domain is functionally important.

Calcium is required at high intracellular levels (0.1–0.5 mM) for

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the expression of all known genes that encode differentiation-specific structural proteins, including keratins K1 and K10, profilaggrin, and loricrin [21–23]. Calcium also activates a number of enzymes in terminally differentiating epidermal cells, including a protease involved in processing profilaggrin to filaggrin [7] and epidermal transglutaminase(s), which cross-links a number of structural epidermal proteins into the cornified cell envelope [24–26]. This program of gene expression and some other events that occur during terminal differentiation are coordinated in part by a calcium gradient in the epidermis, whereby intracellular calcium concentrations increase from basal to granular cells [27,28]. The identification of a potential calcium-binding domain in profilaggrin suggests that calcium might regulate the expression of this protein or its processing in a calcium-dependent manner [7].

To determine whether the EF-hand domain of human profilaggrin binds calcium, as predicted from sequence comparisons with known S-100 proteins, we analyzed the calcium-binding properties of the amino-terminal domain expressed in *Escherichia coli* by both $^{45}Ca^{++}$ slot blot hybridization and fluorescence emission spectroscopy. We demonstrate that both EF-hands bind calcium with different affinities, in a manner analogous to that reported for other S-100 proteins.

MATERIALS AND METHODS

Construction of pET Expression Plasmid A polymerase chain reaction approach was used to generate a fragment containing the first 293 amino acids of human profilaggrin using the oligonucleotides 5′CCCCATATGTCTACTCTCCTGGAAAACATC 3′ and 5′CCCCTCGAGACGCTT-TCTTGTCCTGGACTCCTG 3′. The oligonucleotides include Nde I and Xho I restriction sites (underlined) for cloning; the Nde I site also includes a translation initiation codon. Polymerase chain reaction was performed for 30 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C in a Coy Tempcycler with a cDNA clone that contained the first 403 amino acids of human profilaggrin as template [14]. The PCR product (890 bp) was gel purified, digested with Nde I and Xho I, and cloned into pET22b (Novagen, Madison, WI), a plasmid that includes a stretch of six histidines at the carboxy-terminal end of the encoded recombinant protein. The DNA sequence was verified by dideoxy sequencing of both strands using a Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, OH) and [α-^{35}S]dATP.

Expression of the Amino-Terminal Domain of Human Profilaggrin in *E. coli* and Purification by Nickel-Chelate Affinity Chromatography The construct was transformed into *E. coli* BL21 (DE3), and a single colony was grown in 5 ml L-broth containing 50 μg/ml carbenicillin for 4–6 h. This culture was diluted 1:50 into fresh L-broth, and the bacteria were grown at 37°C until the A_{600} reached 0.6–0.8. Isopropyl-β-D-thiogalactopyranoside was added to 1 mM to induce expression of the profilaggrin-polyhistidine recombinant protein (denoted proFG293-his), and the bacteria were grown for a further 3 h. The cells were harvested by centrifugation, washed once in cold nickel column binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9), and resuspended in 2 ml binding buffer. The mixture was sonicated for 5 min on ice and then centrifuged at 27,000 $\times$ *g* for 45 min at 4°C. The pellet, containing the inclusion bodies, was resuspended in 2 ml binding buffer that contained 8.8 M urea and 10 μg/ml phenylmethylsulfonylfluoride. After homogenization by several strokes in a Duall ground glass homogenizer, the slurry was diluted with 2 ml of urea buffer and left at room temperature for 30 min. The insoluble material was removed by centrifugation. After overnight dialysis at 4°C against binding buffer that contained 6 M urea, the supernatant was passed through a 2-ml nickel-chelate affinity resin (Novagen), which had been prepared according to the manufacturer's directions. The protein was renatured on the column by stepwise reduction of the urea concentration from 6 M to 0 M in 1-M increments of 5 ml [29]. The bound protein then was eluted in buffer containing 100, 200, and 300 mM imidazole in 1-ml fractions (see **Fig 1**). Protein concentration was determined by absorbance at 280 nm and by densitometric scanning of a Coomassie Brilliant Blue R250-stained polyacrylamide gel. The signal obtained with the recombinant protein was compared to the signal of known concentrations of prestained standards (Life Technologies, Gaithersburg, MD). Typically, a 500-ml culture yielded 400–500 μg of the proFG293-his recombinant protein.

As a control for Ca^{++} binding, the his-lac Z (β-galactosidase-polyhistidine) protein was expressed from a pET-14b construct (provided by Novagen) and purified according to the manufacturer's directions.

Gel Electrophoresis and Immunoblotting Proteins were separated in 7.5% to 12.5% gradient slab sodium dodecylsulfate-polyacrylamide gels [30]. For immunoblotting, proteins were resolved on gels and transferred electrophoretically to nitrocellulose (0.45 μm; Schleicher & Schuell, Keene, NH) for 12 h at 100 mA. Immunoreactive proteins were visualized with the avidin-biotin-peroxidase method [31]. The proFG293-his protein was detected with a polyclonal antibody prepared against a synthetic peptide corresponding to amino acids 16–30 of the human profilaggrin sequence, which represents part of the first EF-hand (denoted EF-Ab). This antibody reacts with human profilaggrin on Western blots and stains granular cells of epidermis that express profilaggrin (RBP, JRK, and BAD, unpublished results).

$^{45}Ca^{++}$ Binding Assay Calcium-binding assays were performed on proteins bound to PVDF (polyvinylidene fluoride) membrane (Immobilon P, Millipore, Bedford, MA), as described [15]. Typically, the slot blots were incubated with 15 ml of 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 10 μM $CaCl_2$, and 10 μCi/ml of $^{45}CaCl_2$ (73 mBq/ml; Amersham, Arlington Heights, IL), for 1 h. The filters were washed as described and exposed to Hyperfilm x-ray film (Amersham) for 1–3 d with an intensifying screen. The radioactivity bound to the filters was quantified using a PhosphorImager Model 400S equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Fluorescence Emission Spectroscopy The proFG293-his protein (400 μg) was saturated with calcium by incubation with 20 mM $CaCl_2$ for 30 min at room temperature. Free and protein-bound calcium were separated by size exclusion chromatography on Bio-Gel P-6DG resin (Econo-Pac 10DG column, Bio-Rad Laboratories, Hercules, CA) using buffered Tris-saline (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl) as the eluent. The calcium-enriched recombinant protein then was aliquoted into tubes that contained varying concentrations of ethylenediamine tetraacetic acid (EDTA) in Tris-saline (see *Results*). This approach was used as an alternative to dialysis, as we found that the proFG293-his recombinant protein adhered tightly to dialysis tubing.

Intrinsic fluorescence measurements were performed at room temperature in a Perkin-Elmer LS-50B luminescence spectrometer controlled by FLDM software operating on a DEC 386SX computer. The emission spectra (330–500 nm) of protein or blank solutions were collected at 800 nm/min after excitation at 290 nm with a slit width of 20 nm. The excitation wavelength (290 nm) was chosen so as to give optimal separation between endogenous Rayleigh scattering and the emission spectra of the recombinant protein. The spectra of solutions that contained no peptide were subtracted from the spectra of solutions that contained peptide. The peak height at 390 nm of each spectrum was determined and used to calculate the percent change in fluorescence intensity (FI) from a control sample of protein that contained no EDTA, according to the following formula:

$$\frac{[(\text{FI sample} + \text{EDTA}) - (\text{FI sample} - \text{EDTA})]}{(\text{FI sample} - \text{EDTA})} \times 100$$

$$= \% \text{ change in fluorescence.}$$

Saturated solutions of tyrosine and tryptophan amino acids in Tris-saline were diluted with Tris-saline until the intensity of their fluorescence emission was within the scale observed for 40 μg of the proFG293-his protein. A saturated solution of phenylalanine in Tris-saline was used undiluted.

RESULTS

Expression of the Human Profilaggrin Amino-Terminal Domain in *E. coli* To determine whether the EF-hand region of profilaggrin binds calcium, we expressed the first 293 amino acids in *E. coli* as a profilaggrin-polyhistidine fusion protein. Both strands of the expression construct were sequenced to verify that the correct sequence, reported previously [14], had been cloned. Expression of the recombinant protein (denoted as proFG293-his) was induced with isopropyl-β-D-thiogalactopyranoside, and it was purified by nickel-chelate affinity chromatography. Purification of proFG293-his was followed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblot analysis with an antibody to part of the first EF-hand (EF-Ab) **(Fig 1)**. The results show that the recombinant protein displayed an electrophoretic mobility in agreement with the predicted molecular weight of 36 kDa. As expected, most of the endogenous bacterial proteins did not bind to the nickel-chelate resin or were eluted at low (20–100 mM)

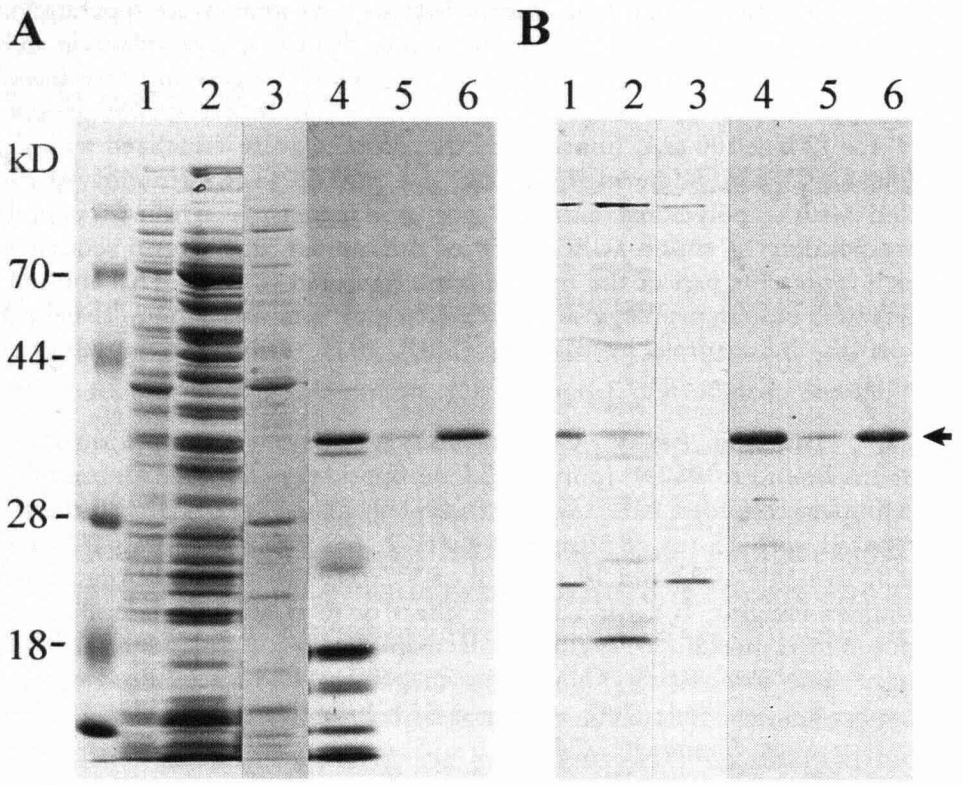

Figure 1. Purification of the proFG293-his fusion protein from *E. coli*. Expression of the proFG293-his protein (cloned into pET-22b and transformed into *E. coli* BL21 [DE3]) was induced with isopropyl-B-D-thiogalactopycanoside. The protein was extracted and affinity purified by nickel-chelate chromatography. *A*) Commassie-blue–stained 7.5% to 12.5% sodium dodecylsulfate-polyacrylamide gel of the purification. *B*) Immunoblot analysis using the amino-terminal EF-Ab antibody. *Lane 1,* total bacterial extract; *lane,* unbound (flow-through) fraction in 5 mM imidazole; *lane 3,* proteins eluted in 20 mM imidazole; *lane 4,* proteins eluted at 100 mM imidazole; *lanes 5,6,* proFG293-his fusion protein eluted at 200 mM imidazole (*arrow*). Note (in *B*) that some proFG293-his protein does not bind to the column (*lane 2*), and a significant amount of protein is eluted at 100 mM imidazole (*lane 4*) along with some lower–molecular-weight proteins. The fraction eluting at 200 mM imidazole is greater than 90% pure (*lane 6*) and was used for the calcium-binding studies.

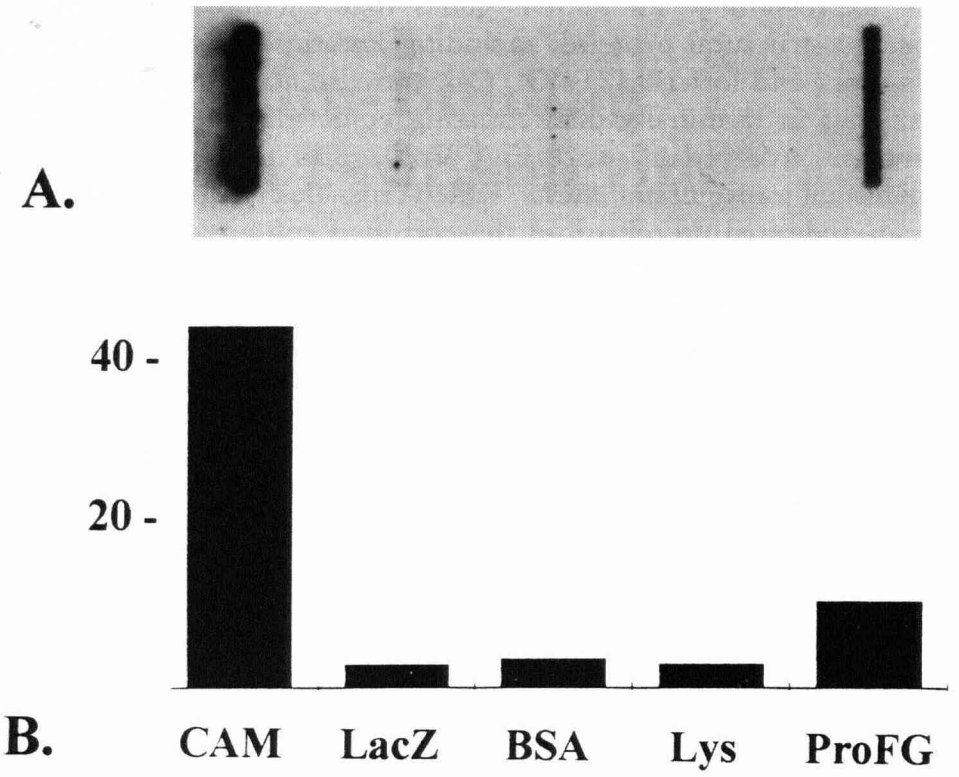

Figure 2. The proFG293-his fusion protein binds $^{45}Ca^{++}$. *A*) Thirty micrograms of each protein was immobilized onto PVDF membrane and incubated with $^{45}Ca^{++}$ as described in *Materials and Methods*. *B*) Quantitation of the amount of radioactivity bound to the membrane (shown in *A*) by PhosphorImager analysis. From the amounts of yeast calmodulin (1.9 pmol) and proFG293-his (0.9 pmol) bound to the membrane, it was calculated that, compared to yeast calmodulin, which binds 3 Ca^{++} per molecule [32] (22 relative PhosphorImager units/pmol), proFG293-his binds 1.5 Ca^{++} per molecule (11 relative PhosphorImager units/pmol). The proteins shown are CAM, yeast calmodulin; LacZ, β-galactosidase-polyhistidine protein; BSA, bovine serum albumin; Lys, lysozyme; proFG, the proFG293-his recombinant protein.

concentrations of imidazole (**Fig 1**, *lanes 2–4*). Some proFG293-his protein eluted from the column at 100 mM imidazole (**Fig 1*B***, lane 4). However, the bulk of the recombinant protein eluted at 200–300 mM imidazole and was estimated to be at least 90% pure (**Fig 1*A***, lanes 5,6, and data not shown). The proFG293-his protein eluted in buffer that contained 200 and 300 mM imidazole (**Fig 1*A***, lane 6) was used for the calcium-binding studies.

The Recombinant Amino-Terminal Fragment of Human Profilaggrin Binds $^{45}Ca^{++}$ For the slot blot analysis with $^{45}Ca^{++}$, equal amounts of the purified proFG293-his protein, yeast calmodulin (which has three functional EF-hands [32]), and the control proteins lysozyme, bovine serum albumin, and the β-galactosidase-polyhistidine fusion protein were bound to PVDF membrane and incubated with a solution containing $^{45}Ca^{++}$. The results **(Fig 2)** demonstrate that the proFG293-his recombinant protein binds calcium, as does yeast calmodulin, whereas the other proteins, including β-galactosidase-polyhistidine, do not bind Ca^{++}. Quantitation of the amount of $^{45}Ca^{++}$ bound **(Fig 2*B*)** indicated that compared to yeast calmodulin, which binds 3 mol of calcium ions to 1 mol of protein [32], the recombinant profilaggrin protein binds an average of 1.5 mol of calcium per mol of protein (average of two independent experiments). These results indicate that at least one of the two EF hands functions to bind calcium in this assay.

Analysis of Calcium Binding by Fluorescence Emission Spectroscopy The binding of calcium to the recombinant pro293-his protein was analyzed further by fluorescence emission spectroscopy [33]. This assay detects changes in the fluorescence of aromatic amino acids upon the removal of bound calcium and is therefore an indirect measure of change in the conformational state of the protein. The recombinant protein contains a total of 19 aromatic amino acids (11 tyrosines [Tyr], seven phenylalanines [Phe], and one tryptophan [Trp]) that are capable of fluorescence emission in response to ultraviolet light **(Fig 3)**. A number of these Tyr and Phe residues are located in and around the two EF-hand domains.

Intrinsic Fluorescence Emission Spectra of proFG293-his Are Due to a Number of Aromatic Amino Acids: We first compared the fluorescence spectra of the recombinant protein with solutions of the aromatic amino acids Trp, Tyr, and Phe after excitation at 290 nm. As shown in **Fig 4**, all three amino acids are capable of emission at wavelengths similar to that observed for the recombinant protein. A saturated solution of Phe was used to generate a signal of sufficient intensity for detection, whereas dilute solutions of Trp and Tyr were used. Phe is only weakly fluorescent compared to Trp or Tyr and is not detected readily in isolated protein preparations [34,35], a result confirmed in this study **(Fig 4)**. By comparing the

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MSTLLENIFA IINLFKQYSK KDKNTDTLSK KELKELLEKE    40
FRQILKNPDD PDMVDVFMDH LDIDHNKKID FTEFLLMVFK    80
LAQAYYESTR KENLPISGHK HRKHSHHDKH EDNKQEENKE   120
NRKRPSSLER RNNRKGNKGR SKSPRETGGK RHESSSEKKE   160
RKGYSPTHRE EEYGKNHHNS SKKEKNKTEN TRLGDNRKRL   200
SERLEEKEDN EEGVYDYENT GRMTQKWIQS GHIATYYTIQ   240
DEAYDTTDSL LEENKIYERS RSSDGKSSSQ VNRSRHENTS   280
QVPLQESRTR KRR hhhhhh                         299
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Figure 3. Sequence of the amino-terminal domain of human profilaggrin contained in the profilaggrin-polyhistidine recombinant protein. The two EF-hands are underlined, and the aromatic amino acids are shown in bold. The single tryptophan residue is located at residue 227. The polyhistidine tag at the carboxy-terminus is shown in lowercase. The sequence is described elsewhere [14].

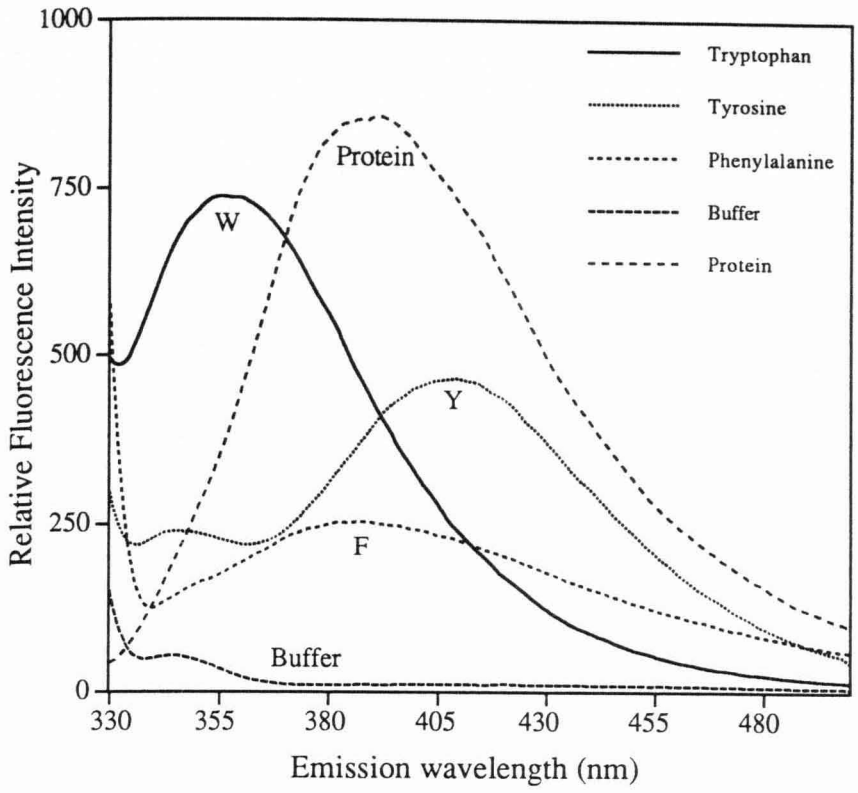

Figure 4. Emission spectra of aromatic amino acids and the proFG293-his recombinant protein. Measurements were performed in 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl at an excitation wavelength of 290 nm. Slit widths were 15 nm for excitation and 20 nm for emission. The raw spectra are shown and are not corrected for the contribution of the Raman spectra of water. Phenylalanine was used as a saturated solution.

emission intensities and maxima of the three aromatic amino acids with that of the proFG293-his recombinant protein **(Fig 4)**, we attributed the fluorescence emission spectra of the recombinant protein to a composite of signals from the Tyr and Trp residues in the protein, because these residues fluoresce 18- and 200-fold as much as Phe [34,35].

Intensity of Fluorescence of proFG293-his Is Concentration-Dependent: **Figure 5** shows the intrinsic fluorescence emission spectra of the fusion protein at 390 nm as a function of protein concentration, which demonstrates that the peak height is proportional to the amount of protein in solution. We therefore selected 15 μg/ml as the working concentration of protein for further characterization of calcium binding; this value is within the linear range of the assay.

ProFG293-his Undergoes Conformational Change(s) Upon Removal of Bound Ca^{++}: In preliminary experiments, we tested the recombinant protein for its ability to interact with Ca^{++} by measuring the change in intrinsic fluorescence upon addition of calcium. The addition of calcium to proFG293-his resulted in a substantial increase in fluorescence emission at 390 nm (data not shown). This indicated that the protein's calcium-binding sites were not occupied fully upon isolation from *E. coli*. We therefore saturated the recombinant protein with calcium, removed the free calcium by size exclusion chromatography, and then determined the change in fluorescence intensity that resulted from the addition of increasing concentrations of EDTA **(Fig 6)**. A dose-dependent decrease in peak height at 390 nm (represented in **Fig 6** by a percent change in fluorescence intensity) was observed between 12.5 μM and 20 mM EDTA (pEDTA 4.9–1.7). From these data, it is possible to identify the existence of two sets of calcium-binding sites on the protein at pH 8.0, as indicated by the two transitions in the EDTA titration curve. EDTA concentrations of 40 and 350 μM are required to produce 50% of the maximal change in fluorescence intensity for each of the two transitions **(Fig 6)**. From these data, we calculate that the K_ds of the two EF-hands for Ca^{++} are 1.2–1.6 $\times$ 10^{-4} M and 1.1–1.4 $\times$ 10^{-3} M, assuming that three to four molecules of Ca^{++} are removed by one molecule of EDTA.

DISCUSSION

Profilaggrin is a major protein component of the keratohyalin present in the granular layer of epidermis. It is a member of the S-100 family of calcium-binding proteins and contains two EF-hands at its amino terminus. In this paper, we show by two methods that the amino-terminal domain of human profilaggrin, expressed in *E. coli* and purified by nickel-chelate affinity chromatography, binds calcium. Furthermore, the fluorescence data demonstrate that both EF-hands can act as Ca^{++}-binding loops. This confirms and extends a previous report [15], which showed calcium binding to mouse profilaggrin immobilized on PVDF membrane.

Fluorescence emission spectroscopy allows detection of changes

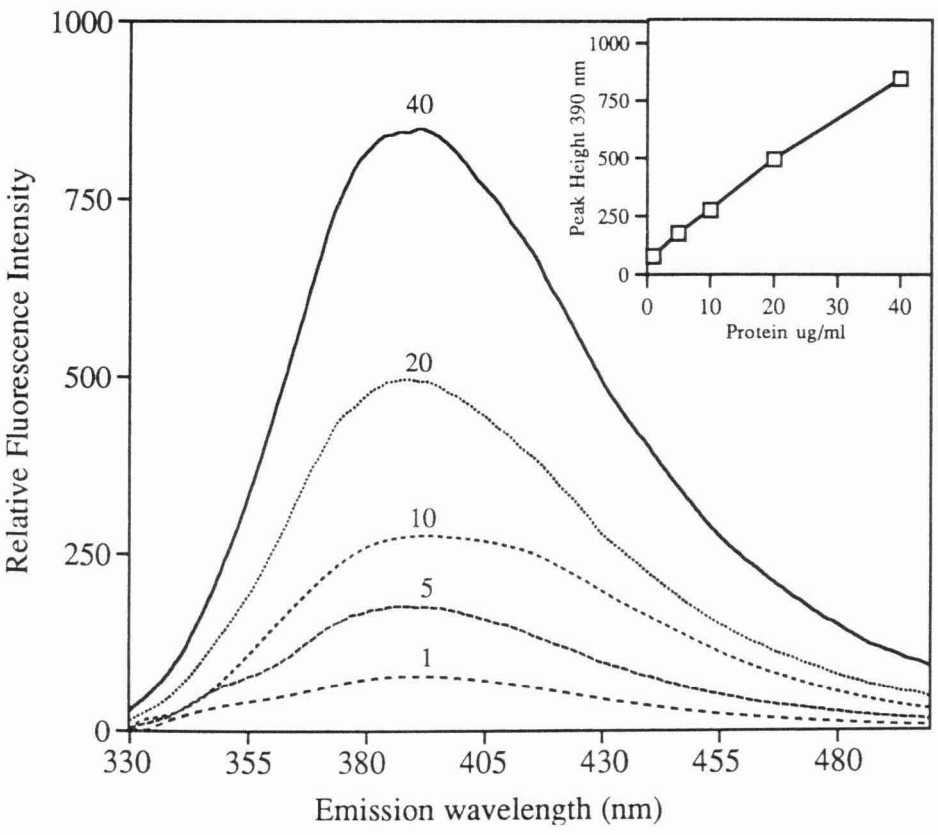

Figure 5. Intensity of fluorescence emission of proFG293-his is proportional to protein concentration. Measurements were performed after excitation at 290 nm as described in **Fig 4** (see also *Materials and Methods*). Shown are corrected spectra after subtraction of the spectrum contributed from buffer alone. The values above each spectrum represent the protein concentration in μg/ml. The peak height at 390 nm was determined at each protein concentration (see *inset*).

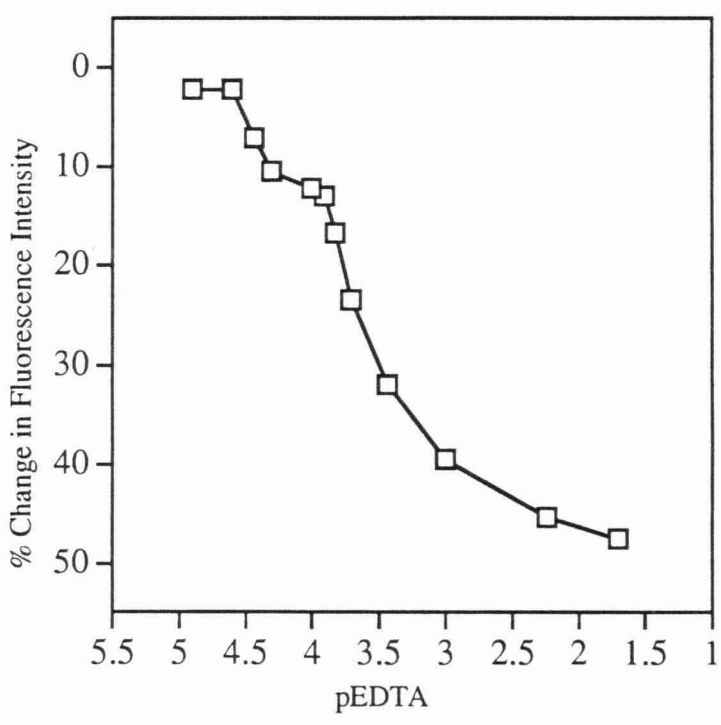

Figure 6. Loss of calcium from the proFG293-his fusion protein causes conformational changes. The figure shows the percent change in intrinsic fluorescence intensity at 390 nm of proFG293-his as a function of increasing EDTA concentration. The sample was prepared as described in *Materials and Methods*. Measurements were performed as in **Fig 4**. Note the two transitions in the fluorescence spectrum, indicative of two conformational changes in the protein.

in the fluorescence of aromatic amino acids in a protein and provides evidence of conformational changes. In this study, we demonstrated that the recombinant profilaggrin polypeptide undergoes conformational changes upon releasing calcium **(Fig 6)**. From the fluorescence emission wavelength, and because there is no shift in the emission maximum in response to addition of EDTA, we conclude that the contribution of Phe to the fluorescence emission signal of proFG293-his is negligible and that the fluorescence observed is due to multiple Trp and Tyr residues in the protein. The removal of bound calcium by titration with EDTA leads to a large reduction in fluorescence emission **(Fig 6)**, suggesting that the Trp (and at least some of the Tyr) residues in the protein subsequently become less accessible to the aqueous environment. The data demonstrate two distinct shifts in fluorescence, indicating loss of calcium from the high- and low-affinity calcium-binding sites in the profilaggrin amino-terminus, with estimated K_ds for Ca^{++} of 1.4×10^{-4} M and 1.2×10^{-3} M, respectively. Similar observations have been made with S-100a and S-100b proteins [36–38], whereby the S-100 proteins undergo conformational changes upon binding calcium.

We did not test the capacity of other divalent cations to bind to the recombinant profilaggrin protein. However, previous studies have shown that the affinity of EF-hand sites for magnesium is lower than for calcium by three to four orders of magnitude in the calcium-binding proteins S-100b [38], parvalbumin [39], and SPARC (osteonectin) [40]. Thus, these proteins do not bind magnesium at physiologic concentrations. Zinc also binds with high affinity to some S-100 proteins, but at a histidine-rich site distinct from the EF-hands that bind calcium [41].

Several lines of evidence have shown that calcium is a critical regulator of epidermal differentiation. It is required at levels above the concentrations present in serum for transcription of late differentiation markers such as profilaggrin and loricrin [21]. In addition, calcium regulates the activity of certain enzymes in cornifying cells, such as the second-stage protease, which processes profilaggrin intermediates to filaggrin [7]; and epidermal transglutaminase, which cross-links loricrin, involucrin, and other proteins into the cornified cell envelope [24–26]. The high concentration of calcium present in granular cells [27,28] suggests that, *in vivo,* the EF-hand domain of profilaggrin is likely to be saturated with calcium as keratohyalin accumulates in granular cells. Additional calcium may bind to profilaggrin deposited in keratohyalin via the many phosphoserine residues known to be present in each filaggrin repeat [42,43]. That profilaggrin does bind calcium *in vivo* is suggested by recent studies with the dye alizarin red S, a calcium-complexing dye, which showed a strong staining of the keratohyalin granules and adjacent most proximal layer of the stratum corneum of rat epidermis (R. Thieroff-Ekerdt, personal communication). This same localization is shown by both immunohistochemistry and immunoelectron microscopy with the profilaggrin EF-hand antibody, indicating that this domain is present in cells where free calcium is concentrated (our unpublished observations).

What is the function of the calcium-binding domain in profilaggrin? We have suggested previously [14] that it functions in transition cells to bind calcium that is necessary for profilaggrin processing during terminal differentiation, or it may regulate mRNA expression by feedback inhibition of profilaggrin transcription, or it may function in the granular layer to assist in the translation of profilaggrin and the formation of keratohyalin. During processing of profilaggrin to filaggrin, the calcium-binding domain apparently is cleaved from the filaggrin sequences and may function independently [6,7]. The high degree of sequence conservation of the calcium-binding domain between human, rat, and mouse profilaggrin (RBP, unpublished observations) and the conservation of this EF-hand motif in the related intermediate-filament–associated protein trichohyalin, a protein marker of the inner root sheath and medulla cells of hair follicles [44–46], suggest an important function for this motif. This similarity contrasts with the variability of filaggrin sequences between species, which differ in both length and amino acid sequence [11]. Our studies here characterize the calcium binding to this region and demonstrate that it has binding properties analogous to other S-100 proteins. These results suggest that this domain is significant in this biologic environment where calcium is essential. Future studies both *in vitro* and *in vivo* will be aimed at identifying the function of this important domain in profilaggrin.

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REFERENCES

1. Steinert PM, Roop DR: Cellular and molecular biology of intermediate filaments. *Ann Rev Biochem* 57:593–625, 1988
2. Marvin KW, George MD, Fujimoto W, Saunders NA, Bernacki SH, Jetten AM: Cornifin, a cross-linked envelope precursor in keratinocytes that is down-regulated by retinoids. *Proc Natl Acad Sci USA* 89:11026–11030, 1992
3. Dale BA, Presland RB, Fleckman P, Kam E, Resing KA: Phenotypic expression and processing of filaggrin in epidermal differentiation. In: Darmon M, Blumenberg M (eds.). *Molecular Biology of the Skin, Vol. 1. The Keratinocyte.* Academic Press, New York, 1993, pp 79–106
4. Haugen-Scofield J, Resing KA, Dale BA: Characterization of an epidermal phosphatase specific for filaggrin phosphorylated by casein kinase II. *J Invest Dermatol* 91:553–559, 1988
5. Kam E, Resing KA, Lim SK, Dale BA: Identification of rat epidermal profilaggrin phosphatase as a member of the protein phosphatase 2A family. *J Cell Sci* 106:219–226, 1993
6. Resing KA, Walsh KA, Haugen SJ, Dale BA: Identification of proteolytic cleavage sites in the conversion of profilaggrin to filaggrin in mammalian epidermis. *J Biol Chem* 264:1837–1845, 1989
7. Resing KA, Al-Alawi N, Fleckman P, Blomquist C, Dale BA: Independent regulation of two cytoplasmic processing stages of the intermediate filament-associated protein filaggrin and role of Ca^{2+} in the second stage. *J Biol Chem* 268:25139–25145, 1993
8. Scott IR, Harding CR, Barrett JG: Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. *Biochim Biophys Acta* 719:110–117, 1982
9. Scott IR, Harding CR: Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water environment. *Dev Biol* 115:84–92, 1986
10. Haydock PV, Dale BA: The repetitive structure of the profilaggrin gene as demonstrated using epidermal profilaggrin cDNA. *J Biol Chem* 261:12520–12525, 1986
11. Haydock PV, Dale BA: Filaggrin, an intermediate filament-associated protein: structural and functional implications from the sequence of a cDNA from rat. *DNA Cell Biol* 9:251–261, 1990
12. Rothnagel JA, Mehrel T, Idler WW, Roop DR, Steinert PM: The gene for mouse epidermal filaggrin precursor. Its partial characterization, expression, and sequence of a repeating filaggrin unit. *J Biol Chem* 262:15643–15648, 1987
13. McKinley-Grant LJ, Idler WW, Bernstein IA, Parry DA, Cannizzaro L, Croce CM, Huebner K, Lessin SR, Steinert PM: Characterization of a cDNA clone encoding human filaggrin and localization of the gene to chromosome region 1q21. *Proc Natl Acad Sci USA* 86:4848–4852, 1989
14. Presland RB, Haydock PV, Fleckman P, Nirunsuksiri W, Dale BA: Characterization of the human epidermal profilaggrin gene. Genomic organization and identification of an S-100-like calcium binding domain at the amino terminus. *J Biol Chem* 267:23772–23781, 1992
15. Markova NG, Marekov LN, Chipev DC, Gan S-Q, Idler WW, Steinert PM: Profilaggrin is a major epidermal calcium binding protein. *Mol Cell Biol* 13:613–625, 1993
16. Dorin JR, Emslie E, van Heyningen V: Related calcium-binding proteins map to the same subregion of chromosome 1q and to an extended region of synteny on mouse chromosome 3. *Genomics* 8:420–426, 1990
17. Fietz MJ, Rogers GE, Eyre HJ, Baker E, Callen DF, Sutherland GR: Mapping of the trichohyalin gene: co-localization with profilaggrin, involucrin, and loricrin genes. *J Invest Dermatol* 99:542–544, 1992
18. Lee S-C, Wang M, McBride OW, O'Keefe EF, Kim I-G, Steinert PM: The human trichohyalin gene is clustered with the genes for other epidermal structural proteins and calcium binding proteins at chromosome locus 1q21 *J Invest Dermatol* 100:65–69, 1993
19. Volz A, Korge BB, Compton JG, Ziegler A, Steinert PM, Mischke D: Physical mapping of a functional cluster of epidermal differentiation genes on chromosome 1q21. *Genomics* 18:92–99, 1993
20. Gibbs S, Fijneman R, Wiegant J, van Kessel AG, van de Putte P, Backendorf C Molecular characterization and evolution of the SPRR family of keratinocyte

differentiation markers encoding small proline rich proteins. *Genomics* 16:630–637, 1993
21. Yuspa SH, Kilkenny AE, Steinert PM, Roop DR: Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J Cell Biol* 109:1207–1217, 1989
22. Hohl D, Lichti U, Breitkreutz D, Steinert PM, Roop DR: Transcription of the human loricrin gene *in vitro* is induced by calcium and cell density and suppressed by retinoic acid. *J Invest Dermatol* 96:414–418, 1991
23. Rosenthal DS, Steinert PM, Chung S, Huff CA, Johnston J, Yuspa SH, Roop DR: A human epidermal differentiation-specific keratin gene is regulated by calcium but not negative modulators of differentiation in transgenic mouse keratinocytes. *Cell Growth Diff* 2:107–113, 1991
24. Rice RH, Green H: Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* 18:681–694, 1979
25. Thacher SM, Rice RH: Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell* 40:685–695, 1985
26. Hohl D, Mehrel T, Lichti U, Turner ML, Roop DR, Steinert PM: Characterization of human loricrin. *J Biol Chem* 266:6626–6636, 1991
27. Menon GK, Grayson S, Elias PM: Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. *J Invest Dermatol* 84:508–512, 1985
28. Menon GK, Elias PM: Ultrastructural localization of calcium in psoriatic and normal human epidermis. *Arch Dermatol* 127:57–63, 1991
29. Crowe J, Henco K. *The QIA Expressionist.* Qiagen Inc., Chatsworth, CA, 1992, p 39
30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
31. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979
32. Geiser JR, van Tuinen D, Brockerhoff SE, Neff MM, Davis TN: Can calmodulin function without binding calcium? *Cell* 65:949–959, 1991
33. Sage EH, Bassuk JA, Yost JC, Folkman MJ, Lane TF: Inhibition of endothelial cell proliferation by SPARC is mediated through a Ca^{2+}-binding EF-hand sequence. *J Cell Biochem* (in press)
34. Guilbault G: Assay of organic compounds. In: Guilbault G (ed.). *Practical Fluorescence.* 2nd ed. Marcel Dekker, New York, 1990, pp 281–286
35. Teale FWJ, Weber G: Ultraviolet fluorescence of the aromatic amino acids. *Biochem J* 65:476–482, 1957
36. Mani RS, Boyes BE, Kay CM: Physicochemical and optical studies on calcium- and potassium-induced conformational changes in bovine brain S-100b protein. *Biochemistry* 21:2607–2612, 1982
37. Mani RS, Kay CM: Isolation and spectral studies on the calcium binding properties of bovine brain S-100a protein. *Biochemistry* 22:3902–3907, 1983
38. Mani RS, Shelling JG, Sykes BD, Kay CM: Spectral studies on the calcium binding properties of bovine brain S-100b protein. *Biochemistry* 22:1734–1740, 1983
39. Rinaldi ML, Haiech J, Pavlovitch J, Rizk M, Ferraz C, Derancourt J, Demaille JG: Isolation and characterization of a rat skin parvalbumin-like calcium binding protein. *Biochemistry* 21:4805–4810, 1982
40. Pottgiesser J, Maurer P, Mayer U, Nischt R, Mann K, Timpl R, Krieg T, Engel J: Changes in calcium and collagen IV binding caused by mutations in the EF hand and other domains of extracellular matrix protein BM-40 (SPARC, osteonectin). *J Mol Biol* 238:563–574, 1994
41. Baudier J, Glasser N, Gerard D: Ions binding to S100 proteins. *J Biol Chem* 261:8192–8203, 1986
42. Resing KA, Dale BA, Walsh KA: Multiple copies of phosphorylated filaggrin in epidermal profilaggrin demonstrated by analysis of tryptic peptides. *Biochemistry* 24:4167–4175, 1985
43. Dale BA, Resing KA, Presland RB: Keratohyalin granule proteins. In: Leigh I, Lane B, Watt F (eds.). *The Keratinocyte Handbook.* Cambridge University Press, Cambridge, UK, 1994, pp 323–350
44. Rogers GE, Fietz MJ, Fratini A: Trichohyalin and matrix proteins. *Ann NY Acad Sci* 642:64–81, 1991
45. Fietz MJ, McLaughlan CJ, Campbell MT, Rogers GE: Analysis of the sheep trichohyalin gene: potential structural and calcium-binding roles of trichohyalin in the hair follicle. *J Cell Biol* 121:855–865, 1993
46. Lee S-C, Kim I-G, Marekov LN, O'Keefe EJ, Parry DAD, Steinert PM: The structure of human trichohyalin. *J Biol Chem* 268:12164–12176, 1993

ANNOUNCEMENT

The European Society of Dermatological Research Clinically Oriented Symposium, "Vitamin D: Actions and Applications in Dermatology" will be held April 27–29, 1995 in Aarhus, Denmark.

The international symposium is intended to bring together investigators interested in both basic science and clinical medicine. The program will include invited speakers for introductory lectures as well as free communications (oral and poster presentations). Selected papers from the symposium will be published in the *Journal of Investigative Dermatology.*

The program will include the biology of vitamin D; expression, regulation, and gene regulation of the vitamin D receptor; photobiology of Vitamin D, role of cytokines and growth factors; immunomodulatory role (lymphocytes, macrophages, Langerhans cells); cell differentiation and photobiology; clinical experience with vitamin D analogs; clinical tolerability and safety; and potential use in lymphomas and carcinomas.

The organizing secretariat includes Knud Kragballe (Denmark), Thomas Krieg (Germany), Karsten Fogh (Denmark), Christian Grønhøj Larsen (Denmark), John J. Voorhees (USA), Jean-Hilaire Saurat (Switzerland), Daniel Bikle (USA), Peter van de Kerkhof (The Netherlands), Michael Holick (USA), and Klaus Bendtsen (Denmark).

For information contact Knud Kragballe, MD, PhD, Department of Dermatology, Marselisborg Hospital, DK-8000 Aarhus C, Denmark. Tel. +45 89 49 18 56; Fax +45 89 49 18 70.