Identification and Cloning of a New Calmodulin-like Protein from Human Epidermis*

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After separating by two-dimensional gel electrophoresis an extract of total proteins from human stratum corneum, two spots were extracted and analyzed for their peptide sequence. The resulting internal protein sequences provided evidence for the identification of a new calcium-binding protein. Cloning of the corresponding full-length cDNA was achieved by reverse transcriptase-polymerase chain reaction using two keratinocyte libraries, one from proliferating cultured keratinocytes and one from differentiated keratinocytes of reconstructed human epidermis. The cDNA had an open reading frame encoding a new calcium-binding protein of 146 amino acids, a member of the calmodulin family. We named this new protein calmodulin-like skin protein (CLSP), since reverse transcriptase-polymerase chain reaction studies of CLSP expression in 10 different human tissues revealed that this protein was particularly abundant in the epidermis where its expression is directly related to keratinocyte differentiation. Expression of the cloned cDNA in Escherichia coli yielded a recombinant protein which allowed its further characterization. rCLSP is able to bind calcium, and similarly to calmodulin, exposes thereafter hydrophobic parts which most likely interact with target proteins. Epidermal proteins retained by CaM affinity column are quantitatively and qualitatively distinct from those of the rCLSP column. Sequencing of a rCLSP affinity purified protein revealed 100% identity with transglutaminase 3, a key enzyme in terminal differentiation, indicating an important role of CLSP in this process.

It is the uppermost layer of the human epidermis, the stratum corneum, that provides the vital barrier between the organism and its environment. The stratum corneum is generated by keratinocytes which migrate from the basal layer to the surface of the epidermis undergoing terminal differentiation, a process which is known to be calcium dependent (1). Low concentrations of calcium in the basal layer of the epidermis favor keratinocyte proliferation and an increasing calcium gradient toward the surface controls part of the complex differentiation program (2). This is why calcium-binding proteins, which mediate calcium signals by interacting with and modulating specific target proteins, are of particular interest to understand and eventually modulate epidermal differentiation.

Calmodulin (CaM), the major calcium-binding protein (CaBP) in non-muscular tissue is known to interact with at least 50 different target enzymes and structural proteins (3) including protein kinases, phosphatases, phosphodiesterases, ATPases, and NO-synthases, mediating many biological processes (4, 5). CaM, a small acidic protein (16.7 kDa), was discovered in 1970 (6). It is a member of the CaBP family that includes troponin C, calmodulin-like protein, squidulin, calcium-dependent protein-kinase, and caltractin. CaM is composed of 148 amino acid residues, two globular domains, and a single peptide chain containing 4 calcium-binding sites. The calcium-binding sites are helix-loop-helix domains, also named EF-hands, similar to those of other calcium-binding proteins. CaM is found in all eukaryotic cells and its amino acid sequence is highly conserved, in particular its primary amino acid sequence which is virtually identical in many different species. The binding of calcium induces large conformational changes of CaM which results in the exposure of hydrophobic regions, providing high affinity binding sites for target proteins.

CaM is reported to be implicated in the regulation of keratinocyte proliferation and differentiation. In highly proliferating in vitro cultures of keratinocytes, and in psoriasis, a hyperproliferative, epidermal skin disease, CaM levels are particularly elevated (7–9). Besides the identification of a CaM-binding protein in epidermal desmosomes, named desmocalm (10), and the CaM regulation of human platelet transglutaminase (factor XIII), no specific information is available on the mediation of calcium signals in the epidermis to control keratinocyte proliferation and differentiation. Thus, the discovery of new calcium-binding protein, specific for the epidermis, could help to better understand the mediation of calcium signals in the skin. Here we describe the isolation, characterization, and cloning of a new CaM-like protein of the human epidermis and discuss its possible involvement in epidermal differentiation.

EXPERIMENTAL PROCEDURES

Materials—Isopropyl-1-thio-β-D-galactopyranoside, phenylmethylsulfonyl fluoride, CHAPS, and glutathione-Sepharose matrix were purchased from Sigma. Immobilized pH gradient (IPG) solution, IPG strips, peroxidase, β-D-galactosidase, β-Galactosidase, phosphatase, alkaline phosphatase, and β-mercaptoethanol were from Pharmacia Biotech. RNase, DNase, thrombin, Tag polymerase, pfu polymerase, and restriction enzymes were from Promega. The RNA preparation kit RNasey was obtained from Qiagen. Oligonucleotides

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‡ The abbreviations used are: CaM, calmodulin; CaBP, calcium-binding protein; CHAPS, 3-[3-cholamidopropyl]dimethyammmonio]-1-propanesulfonate; CLP, calmodulin-like protein; CLSP, calmodulin-like skin protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIC, hydrophobic interaction chromatography; IPG, immobilized pH gradient; LB broth, Luria-Bertani broth; PSC, plantar stratum corneum; rCLSP, recombinant CLSP; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction.
were homogenized in 300 μl of lysis buffer (included in the kit) and the supernatant (2 ml) was recovered and processed through a 0.22-μm membrane filter. Proteins were precipitated by adding 10 μl of 1 M Tris-HCl, pH 8, containing 10 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 35 units of RNase, and 200 units of DNase and mixed for 30 min at 25 °C. Insoluble material was discarded by centrifugation at 10,000 × g for 20 min at 4 °C. The protein fraction containing supernatant was applied to a 15-ml glutathione S-transferase affinity column with a flow rate of 0.2 ml/min. Thereafter, the column was washed (0.5 ml/min) first with 2 bed volumes of PBS containing 1 mM EDTA, and 1% (v/v) Triton X-100, and then with 2 bed volumes of PBS. To liberate rCLSP, 42 units of thrombin in 5 ml of PBS was applied and the column kept at room temperature for 20 h. Thereafter, rCLSP was eluted by washing the column with 10 ml of PBS. Eluted fractions were pooled and kept at 4 °C. Analysis of CLSP expression in human skin was performed using PCR-ready cDNA kits were from Maxim Biotech. Calmodulin from human erythrocytes was obtained from Roche Molecular Biochemicals. Biomol Green®-Calcineurin assay kit number AK-804 was obtained from Biomol (Plymouth Meeting, PA).

Two-dimensional Gel Electrophoresis and Protein Sequencing—Stratum corneum samples of healthy normal human skin were obtained from Fluka, and calmodulin from hog brain was from Roche Molecular Biochemicals. Biomat Green®-Calcineurin assay kit number AK-804 was obtained from Biomol (Plymouth Meeting, PA).

Isoelectric focusing was performed using a home-made apparatus. One IPG strip, pH 3–10, was incubated for 20 min, at 4 °C. The pellet was resuspended in ice-cold 10 ml Tris-HCl, pH 8, containing 150 mM NaCl and pelleted by centrifugation. To lyse the cells, the pellet was resuspended in 75 ml of ice-cold 200 mM NaOH and stirred for 10 min at room temperature before genomic DNA was broken by repeated passages of the suspension through a tight syringe. The lysate was then neutralized by the addition of 75 ml of 1 M Tris-HCl, pH 9, containing 10 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 35 units of RNase, and 200 units of DNase and mixed for 30 min at 25 °C. Insoluble material was discarded by centrifugation at 10,000 × g for 20 min at 4 °C. The fusion protein containing supernatant was applied onto a 15-ml glutathione S-transferase affinity column with a flow rate of 0.2 ml/min. Thereafter, the column was washed (0.5 ml/min) first with 2 bed volumes of PBS containing 1 mM EDTA, and 1% (v/v) Triton X-100, and then with 2 bed volumes of PBS. To liberate rCLSP, 42 units of thrombin in 5 ml of PBS was applied and the column kept at room temperature for 20 h. Thereafter, rCLSP was eluted by washing the column with 10 ml of PBS. Eluted fractions were pooled and kept at 4 °C. Analysis of CLSP expression in human skin was performed using PCR-ready cDNA kits. PCR was performed using a Taq polymerase and different sets of primers (sc10 and sc29) (Table I), applying 1 cycle for 2 min at 95 °C, 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), and 1 cycle at 72 °C for 7 min. The resulting amplicon was used as template for a subsequent PCR with sc7 and sc21 (Table I) as primers applying the following conditions: 1 cycle for 2 min at 95 °C, 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), and 1 cycle at 72 °C for 7 min. The resulting amplicon was used as template for a subsequent PCR with sc7 and sc21 (Table I) as primers applying the following conditions: 1 cycle for 2 min at 95 °C, 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), and 1 cycle at 72 °C for 7 min. The resulting amplicon was used as template for a subsequent PCR with sc7 and sc21 (Table I) as primers applying the following conditions: 1 cycle for 2 min at 95 °C, 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min), and 1 cycle at 72 °C for 7 min.
different experiments were performed to elute the proteins. In a first experiment, a buffer B (25 mM Tris-HCl, pH 7.5, 5 mM EGTA, 1 mM MgCl2, 500 mM NaCl) gradient (0–100%) was used. In the second experiment, a buffer C (25 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 40% (v/v) ethylene glycol) gradient (0–100%) was used. All chromatographic experiments were performed at room temperature and protein elution was monitored by recording the A280 nm. Samples were analyzed by SDS-PAGE in a 15% (w/v) gel.

rCLSP and CaM Affinity Chromatography—Affinity columns with 1-ml bead volumes were used. Purified rCLSP was immobilized on a CNBr-activated Sepharose column from Amersham Pharmacia Biotech, following the manufacturer’s instructions, using 1.3 mg of rCLSP/ml of beads. The coupling efficiency was 0.95 mg of rCLSP/ml of beads, calculated from the ratio of rCLSP in solution before and after incubation with the activated matrix. For the CaM affinity chromatography, a commercially available CaM-agarose matrix (Sigma) was used. The initial concentration of 1.7 mg of CaM/ml of beads was adjusted with inactivated Sepharose 4B to match that of the rCLSP column. Human epidermis (0.83 g of abdominal skin from plastic surgery) and plantar stratum corneum (100 g) were homogenized with a Polytron in 10 and 120 ml, respectively, of 10 mM Hepes, pH 7.4, containing 150 mM NaCl, 0.1% (w/v) Triton X-100 and protease inhibitor mixture (Complete® EDTA-free from Roche Molecular, complemented with 1 μM pepstatin). The suspensions were centrifuged at 10,000 g for 10 min at 4 °C. The resulting supernatants were passed over 0.22-μm filters and adjusted to 2.5 mM CaCl2 before chromatographic separation at room temperature on CaM and rCLSP affinity columns. Thereafter, the columns were washed with 10 column volumes of sample buffer. Elution was performed using the same buffer containing 5 mM EDTA instead 2.5 mM CaCl2. The eluted proteins were submitted to SDS-PAGE electrophoresis on pre-fabricated gel bond PAG film-supported gels (12.5% homogenous gels) and silver stained according to the Amersham Pharmacia Biotech’s instructions.

**RESULTS**

Two-dimensional Electrophoresis and Protein Sequencing of Proteins Extracted from Human Stratum Corneum—A comparison of the protein pattern in the two-dimensional gels obtained after silver and Amido Black staining (Fig. 1) revealed that two acidic spots with 7,000 M₉ (noted 2D1 and 2D2) which appeared only after Amido Black staining and were not decorated by silver nitrate. The high performance liquid chromatography profiles of the tryptic digest of the two spots were essentially identical (data not shown). Analysis of the internal sequence (Table II) and a subsequent homology search, using the 2D1/pept1 (Table II) as a probe, revealed the highest score for a CaBP of the CaM family (data not shown). The protein was named calmodulin-like skin protein (CLSP).

Cloning of Human CLSP and Its Tissue-specific Expression—From the peptide sequence 2D1/pept1 (Table II) of CLSP, we designed a degenerated oligomer (seqc) and performed RT-PCR on a cDNA λgt11 library and on reconstructed human epidermis cDNA. The methionine at position 1 is the suggested start of the signal sequence. Double underlined amino acids indicate the four potential calcium-binding sites. Bold letters show the amino acid residues consistent with amino acid sequence of spots 2D1 and 2D2 in the two-dimensional gel. The single underlining at position 834–839 indicates the putative polyadenylation site.
acid sequence with those of other members of the CaM family (Fig. 3A) revealed an important homology among human CaM, *Chlamydomonas* CaM and human CLP, whereas the homology with CLSP was less important. A phylogenetic analysis of the CaM family and CLSP, presented in Fig. 3B, underlines the difference between CLSP and CaM.

RT-PCR was performed to study the expression of CLSP in different human tissues including heart, testis, skeletal muscle, pancreas, liver, small intestine, placenta, spleen, kidney, lung, and reconstructed human epidermis. Using different sets of primers coding for CLSP (Table I), abundant CLSP expression was only detected in reconstructed human epidermis (Fig. 4A). A very faint expression of CLSP was observed in lung tissue. On the contrary, CaM was expressed in all analyzed tissues, suggesting an important tissue specificity of CLSP.

To evaluate whether the expression of CLSP in the epidermis correlates with keratinocyte differentiation, RT-PCR experiments were performed in nonconfluent, proliferating human keratinocytes and in differentiating keratinocytes using reconstructed epidermis at different stages of culture. CLSP was not detected in proliferating cells and appeared only in differentiating keratinocytes between days 6 and 7, at an already advanced stage of keratinocyte differentiation, *i.e.* when other markers of keratinocyte differentiation like loricrin and filaggrin (17) are already expressed (Fig. 4, B and C). In contrast, CaM is expressed in both, proliferating and differentiating keratinocytes.

**Calcium Binding Properties of rCLSP**—Fig. 5 shows the electrophoretic mobility of rCLSP and CaM in the presence and absence of calcium. Both proteins exhibit the same calcium-dependent mobility. In the presence of calcium, CaM and rCLSP appeared as a single band with an apparent molecular weight of 12,700 and 12,900, respectively, whereas in the absence of calcium, the apparent molecular weight was 18,300 and 18,900, respectively. The calcium binding properties of CLSP were further studied using HIC. CLSP and CaM adsorbed to the phenyl-Superose matrix in the presence of calcium and were eluted in a sharp peak by an EGTA gradient (Fig. 6A). Subsequent SDS-PAGE analysis confirmed the recovery of all injected CaM and CLSP in three 0.2-ml fractions (Fig. 6B). To distinguish between the possibilities that the delayed elution of CLSP as compared with CaM (Fig. 6A) is due to a higher calcium affinity or a stronger hydrophobic interaction of CLSP, we eluted both proteins with an ethylene glycol gradient (Fig. 7). The result shows that the stronger hydrophobic interaction of CLSP is most likely responsible for its retarded elution from the HIC matrix.

**rCLSP and CaM Affinity Chromatography of Extracts from...**

![Fig. 3. Multiple amino acid sequence alignment of CLSP with five other EF-hand proteins (CaM from unicellular alga *Chlamydomonas*, human CaM, troponin C, caltractin isoform 1, and calmodulin-like protein). A, the amino acid sequences of known EF-hand proteins were extracted from the SwissProt data base, and multiple alignment was performed with theantheprot 4.3b program, selecting the multalin option. Common residues between EF-hand proteins and CLSP are shaded. Gaps are indicated by dashes. B, the alignment was used to establish a phylogenetic relationship between EF-hand proteins, calculated with the puzzle 3.1 program and represented by the treeview 1.5.2 program of T. M. Roderic.]

![Fig. 4. CLSP expression in various human tissues and keratinocytes. Panel A, agarose gel analysis of PCR products obtained with cDNA from reconstructed human epidermis after 13 days of culture (1) and from human tissues, spleen (2), testis (3), small intestine (4), placenta (5), heart (6), liver (7), skeletal muscle (8), pancreas (9), kidney (10), and lung (11). Panel B, expression of markers of epidermal differentiation, CaM, GAPDH, and CLSP in proliferating keratinocytes (K) and differentiating keratinocyte of reconstructed human epidermis at days 3, 6, and 13. Panel C, expression of filaggrin, CaM, GAPDH, and CLSP in differentiating keratinocyte of reconstructed human epidermis at days 1, 3, 6, 8, 10, and 13.]

Calmodulin-like Protein from Human Epidermis
Human Plantar Stratum Corneum and Total Epidermis—A comparison of the protein patterns, obtained after affinity chromatography of the two extracts on CaM and rCLSP columns, revealed striking differences (Fig. 8). Epidermal proteins eluted from the rCLSP column are quantitatively and qualitatively distinct from those recovered from the CaM column (lane E1 to be compared with lane E2). The same holds true for the stratum corneum extract (compare lanes PSC1 and PSC2), indicating a distinct target specificity for CaM and rCLSP.

DISCUSSION

The newly identified calcium-binding protein, which we named CLSP due to its abundant expression in human epidermis, has a calculated molecular mass of 15.9 kDa. The fragments of CLSP which were extracted from human stratum corneum exhibited an apparent molecular weight of 7,000, representing the NH₂-terminal part of the protein, including two of the four calcium-binding loops. Even though we do not have

Fig. 5. The effect of calcium on the electrophoretic mobility of human erythrocyte CaM and rCLSP. rCLSP (1) and CaM (2) (5 µg/each) were run on a 15% acrylamide SDS gel in the presence of calcium (A) and with EDTA (B) in the running and sample buffers. Proteins were stained with Coomassie Blue. Lane M, pre-stained low molecular weight Rainbow™ markers.

Fig. 6. Phenyl-Superose chromatography of rCLSP and CaM from hog brain eluted with an EGTA gradient. A, phenyl-Superose chromatography. 50 µg of rCLSP and CaM were loaded on a phenyl-Superose column. rCLSP (—) and CaM (○) were eluted with an EGTA gradient (0–5 mM). Inset represents the complete elution profile. B, SDS-PAGE analysis of fractions 30 to 40. Aliquots (7.5 µl) of each fraction were separated by 15% acrylamide SDS electrophoresis and stained with Coomassie Blue. Lane M contains the pre-stained low molecular weight Rainbow™ markers.

Fig. 7. Phenyl-Superose chromatography of rCLSP and CaM from hog brain, eluted with an ethylene glycol gradient. rCLSP (○) and CaM (—) (50 µg each) were eluted with an ethylene glycol gradient (0 to 40%).
Calmodulin-like Protein from Human Epidermis

A comparison of the amino acid composition of the calcium-binding loop in canonical EF-hand proteins and in CLSP

276 canonical calcium binding loops were examined and 165 unique sequences identified. The following table shows the frequency of each amino acid in the binding loop. This table was taken from Ref. 27. Amino acids which are present in one or more loops of CLSP are in bold and underlined. Amino acids at positions 1, 3, 5, 6, 8, and 12 are the most highly conserved. Amino acids 1, 3, 5, 7, 9, and 12 are at ligand positions. Most loops (106 out of 165) have four acidic residues (D, aspartic acid; or E, glutamic acid). The number of acidic residues in the ligand position of CLSP is: 3 in loop 1, 5 in loop 2, and 4 in loops 3 and 4 denoting a high calcium binding potential.

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**FIG. 8.** CaM and rCLSP affinity chromatography of extracts from human plantar stratum corneum and total epidermis. Extract of plantar stratum corneum (60 ml; 3.3 mg of protein/ml) and epidermis (4 ml; 2 mg of protein/ml) were loaded on CaM and rCLSP affinity columns. After washing with sample buffer, bound proteins were eluted with 2 ml of sample buffer containing 5 mM EDTA instead of 2.5 mM CaCl₂. The eluted proteins were separated by 12.5% SDS-PAGE and silver stained. E1, epidermal proteins eluted from the rCLSP column; E2, epidermal proteins eluted from the CaM column; PSC1, plantar stratum corneum proteins eluted from the rCLSP column; and PSC2 plantar stratum corneum proteins eluted from the CaM column.

data of the endogenous CLSP and its processing, the 7,000 fragments probably represent proteolytically degraded parts of CLSP generated to deactivate the protein. Like previously described for CaM (18), proteolysis of CLSP could result in the formation of two independent globular domains that still bind the target molecules, however, with reduced affinity, insufficient for their activation. Since CLSP contains two potential sites for N-glycosylation (amino acids 25 and 43), multiple sites for phosphorylation and one for myristoylation (amino acid 26), it is important to study its possible post-translational modifications which could affect and diversify CLSP activity. For CaM, several post-translational modifications such as acylation of the amino terminus, (tri-)methylation of Lys, and phosphorylation are described (19), the latter affecting its solubility and calcium affinity.

Compared with CaM, the CLSP has a distinct amino acid composition, in particular an increased number alanine and less methionine and threonine. As in CaM, cysteine and tryptophan are absent. The unusual high methionine content of CaM (6.1%), compared with a 1.5% average in proteins listed in the standard protein data base (20), has been suggested to be responsible for certain binding characteristics of CaM. The flexible and polarizable methionine side chains are responsible for the strong but nonspecific van der Waals interaction of CaM with a multitude of receptor molecules (21, 22). The considerably lower content in methionine residues in CLSP, compared with CaM, could indicate a more specific and restricted target interaction of CLSP. The lack of cysteine explains why CLSP fragments were not stained with silver nitrate in the two-dimensional gels (23).

A comparison of the amino acid composition of the calcium-binding loop in canonical EF-hand and in rCLSP shows a very good correlation, in particular amino acids located at positions 1, 3, 5, 6, 8, and 12 of rCLSP (Table III). An exception is the histidine in loop 3 of CLSP at ligand position 7. All of the above results indicate the presence of 4 calcium-binding sites in CLSP. The SDS-PAGE, as well as the HIC experiments performed in the presence and absence of calcium, clearly demonstrate the interaction of rCLSP with calcium. The binding of calcium modifies the electrophoretic mobility of rCLSP and induces conformational changes which results in the exposure of a hydrophobic region. This hydrophobic region binds more strongly to the HIC matrix than the hydrophobic region of CaM, as revealed by the elution profile with an ethylene glycol gradient and confirmed by its computer calculated hydrophobicity (data not shown) (26).

The results obtained so far are all in favor of a calmodulin-like functionality of CLSP, i.e. a conformational change upon calcium binding with subsequent exposition of a hydrophobic region, able to recognize and interact with specific target proteins. The different amino acid composition, as well as the localization of its hydrophobic regions indicate that CLSP may interact with target molecules distinct from CaM. Another argument for a distinct physiological role of CLSP is the fact that, unlike CaM, which has a ubiquitous tissue distribution, CLSP appears to be specific for the skin, where its expression is related to epidermal differentiation, a process known to be closely under the control of a calcium gradient.

To investigate whether CaM and rCLSP interact with distinct epidermal proteins, we performed several experiments. First we tested whether calcineurin, a known protein phosphatase activated by CaM, can also be activated by rCLSP. Unlike CaM, rCLSP was unable to activate calcineurin in a concentration range from 10 nM to 10 μM (data not shown) indicating that the two proteins are not interchangeable. Further evidence for a distinct reactivity of CaM compared with rCLSP was obtained from affinity chromatographic studies with protein ex-
tracts from human epidermis and stratum corneum. Proteins retained by the CaM affinity column are quantitatively and qualitatively distinct from those of the rCLSP column (Fig. 8).

Experiments are in progress to provide functional, physiological relevance of CLSP in the epidermis by characterizing the affinity purified proteins. Sequencing one of the purified proteins revealed a 100% homology with transglutaminase 3, a known calcium-dependent enzyme, expressed during a late stage of epidermal differentiation. Transglutaminases are key enzymes of epidermal differentiation (24, 28, 29) and are involved in the formation of the cornified envelope by cross-linking structural proteins via the formation of γ-glutamyl-lysine isopeptide bonds. This first result indicates that CLSP might play important role in the regulation of events related to late keratinocyte differentiation.

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