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The Novel Murine Ca^{2+}-binding Protein, Scarf, Is Differentially Expressed during Epidermal Differentiation

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Abstract

Calcium (Ca^{2+}) signaling-dependent systems, such as the epidermal differentiation process, must effectively respond to variations in Ca^{2+} concentration. Members of the Ca^{2+}-binding proteins play a central function in the transduction of Ca^{2+} signals, exerting their roles through a Ca^{2+}-dependent interaction with their target proteins, spatially and temporally. By performing a suppression subtractive hybridization screen we identified a novel mouse gene, *Scarf* (skin calmodulin-related factor), which has homology to calmodulin (CaM)-like Ca^{2+}-binding protein genes and is exclusively expressed in differentiating keratinocytes in the epidermis. The *Scarf* open reading frame encodes a 148-amino acid protein that contains four conserved EF-hand motifs (predicted to be Ca^{2+}-binding domains) and has homology to mouse CaM, human CaM-like protein, hClp, and human CaM-like skin protein, hClsp. The functionality of Scarf EF-hand domains was assayed with a radioactive Ca^{2+}-binding method. By Southern blot and computational genome sequence analysis, a highly related gene, *Scarf2*, was found 15 kb downstream of *Scarf* on mouse chromosome 13. The functional Scarf Ca^{2+}-binding domains suggest a role in the regulation of epidermal differentiation through the control of Ca^{2+}-mediated signaling.

Keratinocytes follow a complex program of differentiation from the basal layer to the spinous and granular layers of the epidermis, which ultimately leads to the formation of a water-impermeable barrier. The final phase in epidermal differentiation is characterized by granular cell death, destruction of organelles, covalent cross-linking of cornified envelope precursors by Ca^{2+}-dependent transglutaminases (TGases),[1] and attachment of lipid molecules to the cross-linked envelopes (1). This program is accomplished through specific transcriptional induction and repression of structural and enzymatic differentiation-specific markers (2). The differentiation process can be recapitulated partially in mouse keratinocytes cultivated *in vitro* by increasing the Ca^{2+} concentration in the culture medium (3) and is associated with the activation of protein kinase C (PKC) (4). This produces a situation that mimics the endogenous Ca^{2+} gradient present in the skin, with low extracellular levels surrounding the basal cells and increasing levels toward the upper granular layers (5). The ability to induce differentiation in primary cultured keratinocytes, along with the presence of a Ca^{2+} gradient, underscores the importance of this ion and the Ca^{2+}-dependent signaling pathways in the epidermis.

A crucial role for the transduction of the Ca^{2+} signal is accomplished by members of the Ca^{2+}-binding proteins, which are characterized by the presence of a common helix-loop-helix

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[1]The abbreviations used are: TGase, transglutaminase; PKC, protein kinase C; PKA, protein kinase A; CaM, calmodulin; hClp, human calmodulin-like protein; hClsp, human calmodulin-like skin protein; ORF, open reading frame; SSH, suppression subtractive hybridization; *LOR*, loricrin; *INV*, involucrin; *KI*, keratin 1; *K5*, keratin 5; rScarf, recombinant Scarf protein; RT-PCR, reverse transcription PCR; H89, *N*-[2–99p-bromocinnamyl-*O*-amino-*O*-ethyl]-5-isoquinolinesulfonamide; KN62, 1-[*N*,*O*-bis-5-isoquinolinesulfonyl-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; GF, GF109203X (bisindolylmaleimide I).

structural motif in their Ca^{2+}-binding domain, termed EF-hand (6). These proteins function by undergoing conformational changes upon binding of Ca^{2+}, allowing the association and regulation of activity of a range of specific target proteins. EF-hand-containing proteins have been described in epidermis; these include the large proteins profilaggrin and repetin (7,8), which present EF-hand motifs in the NH_2-terminal region followed by multiple tandem repeats and members of the dimeric EF-hand S100 multigenic family (9–11). The best studied of the Ca^{2+}-signaling proteins is CaM, a small, highly conserved, and ubiquitous protein that is crucial to many Ca^{2+}-dependent processes in eukaryotes (6,12). The functions of many proteins involved in cell signaling by phosphorylation/dephosphorylation or in the modulation of intracellular levels of second messengers are reportedly CaM-dependent (12). Recently, several Ca^{2+}-binding proteins with structural homology to CaM have been reported: calcium-binding proteins (CaBPs) (13,14), hClp (15), and the human skin-specific hClsp (16,17).

We present the cloning, genomic structure, expression, and functional assay for a novel mouse Ca^{2+}-binding protein that we have termed Scarf. The *Scarf* open reading frame (ORF) codes for a small protein with four EF-hand Ca^{2+}-binding domains. The Scarf gene and protein are differentially expressed *in vivo* in the spinous and granular layers of the epidermis. We also present the identification of a second highly homologous gene, *Scarf2*, which localizes 15 kb downstream from *Scarf* in the mouse chromosome 13. We propose that *Scarf* and *Scarf2* are new members of the CaM-like proteins with potential roles in the Ca^{2+}-dependent epidermal differentiation process.

EXPERIMENTAL PROCEDURES

Suppression Subtractive Hybridization (SSH)

An SSH screen was performed following the instructions of the manufacturer (Clontech) using basal cell RNA as a “driver” and suprabasal cell RNA as a “tester” (18,19). The primary mouse basal and suprabasal keratinocytes were obtained from neonatal skins that were trypsinized overnight at 4 °C and then separated by a discontinuous Percoll gradient (20). From this screen, we identified a partial cDNA sequence (231 bp), which corresponded to a partial coding region of a novel mouse gene we called *Scarf* (113–344 bp). The upstream sequence was obtained by performing rapid amplification of cDNA ends (5′-RACE, Clontech) utilizing a gene-specific oligonucleotide (5′-tgaacacagcccgcagctcccctgctctgtgccc-3′). The complete mRNA sequence was deposited in GenBank™ (accession no. AY293058).

Cloning and Genomic Sequences Analysis of Scarf and Scarf2

A genomic DNA region of ~120 kb was cloned through screening of a mouse VJ/129 BAC library (Genome Systems Inc.) using an 0.9-kb *Scarf* cDNA as a probe. Comparison of the genomic and cDNA sequences determined the 5′- and 3′-end boundaries, the absence of intronic regions in the *Scarf* gene, and the identification of a *Scarf*-related gene that we termed *Scarf2* (GenBank™ accession no. AY314008). For sequence homology searches we used a BLAST analysis (basic local alignment search tool), made available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

Cell Culture

Primary mouse keratinocytes were isolated from trypsinized newborn BALB/c mouse skins and cultured as reported previously (19). Keratinocytes were cultured in medium with 0.05 m_M Ca^{2+} to maintain a basal-like population of undifferentiated cells (3). Cells were treated with different inhibitors at 10 μ_M concentration: a PKC inhibitor, GF109203X; a PKA inhibitor, H89; and a CaM kinase II inhibitor, KN62. Differentiation was achieved by increasing the Ca^{2+} concentration in the medium to 0.12 m_M.

Northern Blots and Gene Expression

Total RNA was isolated from basal and suprabasal keratinocytes and mouse primary keratinocytes differentiated *in vitro* using TRIZOL (Invitrogen). The RNA samples (1–3 μg) were electrophoresed in 1.2% agarose-methymercuryhydroxide gels, electroblotted to nylon membranes, and hybridized according to Church and Gilbert (21). An mRNA dot blot and Northern blot for mouse tissues were purchased from Clontech and used according to the instructions of the manufacturer. The mouse-specific probes used were: the 3′-untranslated regions for loricrin (*LOR*), keratin 5 (*K5*), and keratin 1 (*K1*) (kindly provided by Dr. Yuspa and Dr. Compton). For the early differentiation marker involucrin (*INV*), the expressed sequence tag clone AA798100 was utilized. The coding region of *CaM* used as probe was obtained by PCR with the following gene-specific oligonucleotides: Forward, 5′-tccgttcttccttcttcgctcgcaccatggc-3′, and Reverse, 5′-gtgtgtggacagaggggcttctgacatcag-3′. The *Scarf* coding region was obtained by PCR using: Forward, 5′-gccggatccagatgtctcacgggtttactaaggag-3′, and Reverse, 5′-gccggatccttttaaatgtggaggcgcacaaactc-3′. A glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used to control for RNA integrity (22).

For the RT-PCR reactions, cDNA was synthesized by Superscript II kit (Invitrogen) using 0.2 μg of mRNA isolated from the total RNA of suprabasal keratinocytes with an Oligotex kit (Qiagen). The oligonucleotides used were: Forward *Scarf2*, 5′-tactaaggaggagaataaggatggcc-3′, and Reverse *Scarf2*, 5′-gtcagtcagatatgtggaccttgtat-3′. As a negative control, an RT-PCR reaction was performed with no cDNA input. PCR reactions were 40 cycles of denaturation at 94 °C for 30 s, annealing at 60°C for 30 s, and extension at 72 °C for 1 min.

Radioactive in Situ Hybridization

RNA probes corresponding to the sense and antisense strands of mouse *Scarf* partial cDNA (202–447 bp) were prepared using T7 and Sp6 RNA polymerase. *In situ* hybridization was performed at high stringency on consecutive sagittal sections of 8-, 9-, 15-, and 16-day mouse embryos and neonatal skin as described by Mackem and Mahon (23).

Protein Expression and Purification

Scarf coding sequence was obtained by PCR and subcloned into the *Bam*HI site of the pET-28c (+) vector (Novagen). The recombinant protein was expressed in BL21(DE3) and contained a His tag and a T7 tag at the NH_2 terminus of the protein. The soluble recombinant protein was purified over a nickel-nitrilotriacetic acid agarose column (Ni-NTA, Qiagen). We analyzed the predicted protein sequence using the Swiss Institute of Experimental Cancer Research tools (www.isrec.isb-sib.ch/), and Mac-Vector version 6.5.3. Mutations in the EF-hand motifs to generate the pET-28c/Scarf mutants were performed using the QuikChange site-directed mutagenesis kit (Stratagene). The conserved first Asp residue of each EF-hand motif (sequence GAC) was replaced with a Gly residue (GCC sequence). Mutant recombinant proteins were purified on nickel-nitrilotriacetic acid spin columns (Qiagen). mtEF1–4 represent the mutated forms of Scarf in each EF-hand 1–4 motif, respectively. Anti-Scarf antibody against the full-length protein was generated in hens (Aves Laboratories).

Recombinant hClsp was obtained by subcloning a PCR fragment containing the ORF into the BamHI site of pET-28a(+) vector (Novagen). The oligonucleotides used were: Forward, 5′-gccggatccatggccggtgagctgact-3′, and Reverse, 5′-cggggatcctcactcctgggcgagcat-3′. PCR reactions were performed for 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. Purification of the recombinant hClsp was performed as mentioned above. hClp recombinant protein was kindly provided by Dr. Strehler.

Immunohistochemistry and Western Blot Analysis

Immunohistochemical analysis was performed on paraffin-embedded 8-μm sections of murine neonatal skin. The deparaffinized sections were treated with a 1:2000 dilution of the anti-Scarf primary antibody overnight at 4 °C and a 1:200 dilution of anti-IgY fluorescein isothiocyanate-conjugated secondary antibody (Aves Laboratories) for 1 h at room temperature. Sections were visualized by fluorescence microscopy.

For Western blot analysis, basal and suprabasal keratinocytes were incubated separately in lysis buffer (10 mM Hepes (pH 7.9), 150 mM NaCl, 1% Triton X-100, 10 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 rpm for 20 min. Soluble fractions were collected, and insoluble pellets were resuspended in 6 M urea buffer (6 M urea, 50 mM Tris (pH 8.3), 5 mM EDTA, 0.05% SDS). 25 μg of total protein extract or 1 μg of recombinant CaM-like Ca^{2+}-binding proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed using anti-Scarf primary antibody (1:5000) and horseradish peroxidase-conjugated goat anti-chicken IgG or alkaline phosphatase-conjugated goat anti-chicken IgG secondary antibody (1:2000; Aves Laboratories).

Calcium Binding Assay

Calcium binding was assayed by the micro method described by Kawasaki *et al.* (24). Briefly, 3 μg of the recombinant proteins were blotted onto a polyvinylidene difluoride membrane and washed with two changes of methanol for 5 min, after which the membrane was soaked in 10 μM $CaCl_2$, 50 mM Tris-HCl (pH 7.5),100 mM NaCl, and incubated for 1 h with 10 μCi of $^{45}Ca^{2+}$/ml in the same buffer. The filter was washed twice for 30 s at room temperature in a solution containing 10 μM $CaCl_2$, 50 mM NaCl, 20 mM Tri-HCl (pH 7.5), and 10% methanol, dried, and exposed to an x-ray film. The autoradiograms were quantitated on a densitometer. The ability to bind Ca^{2+} by the recombinant proteins was assayed in three independent experiments.

RESULTS

Scarf Cloning and Genomic and Protein Characterization

A cDNA differentially expressed in suprabasal keratinocytes was obtained from an SSH screen. *Scarf* 899-bp cDNA (Fig. 1*A*) contains an ORF that encodes a protein of 148 amino acids with four EF-hand domains (Fig. 1*A*, *bold letters*) and a predicted molecular mass of 16.7 kDa. As is characteristic of other members of the CaM-like Ca^{2+}-binding proteins, Scarf is an acidic protein (pI = 4.59) with no cysteine or tryptophan residues. A particular difference with calcium-binding proteins (13,14) is that Scarf does not present putative sites for myristoylation. Comparison of the four EF-hand motifs of Scarf and other CaM-like proteins showed high homology in the EF-2, -3, and -4 motifs, with EF-1 being the most divergent (Fig. 1*B*). Protein sequence comparisons determined that Scarf presented 56.7% similarity to mouse CaM (mCaM), 64.2% to hClp, and 64.9% hClsp (Fig. 1*C*). A phylogenic analysis of several Ca^{2+}-binding proteins shows that Scarf and hClsp belong to the same subgroup and are more distantly related to CaM and hClp (data not shown).

A genomic DNA region of ~120 kb was cloned by screening of a mouse VJ/129 BAC library using an 0.9-kb *Scarf* cDNA probe. Comparison of the genomic and cDNA sequences determined the 5′- and 3′-end boundaries and the absence of intronic regions in the *Scarf* gene. Southern blot analysis of the BAC clone helped us identify the existence of a second gene with homology to *Scarf*. We termed this second gene *Scarf2*.

Computational analysis of the *Scarf2* genomic sequence proposed that a complete ORF would be derived after the splicing of a putative small intronic region. To determine whether the gene

was expressed in keratinocytes and whether the transcript was the product of a splicing event, *Scarf2*-specific oligonucleotides encoding sequences surrounding the putative intronic region (Fig. 2*A*) were utilized to perform RT-PCR with mouse keratinocytes mRNA. As shown in Fig. 2*A* (*lower panel*), a fragment was obtained, and after subcloning and subsequent sequencing we determined that the predicted intronic sequence had been spliced to generate the *Scarf2* mRNA transcript. As a control reaction, RT-PCR was performed with the same set of primers and no input cDNA. Therefore, *Scarf2* is expressed in keratinocytes; the mRNA sequence and putative ORF is presented in Fig. 1*A*.

The *Scarf2* gene localized 15.3 kb downstream of *Scarf* (Fig. 2*B*), and the high degree of homology between *Scarf* and *Scarf2* extended to regions upstream and downstream of the ORF for both genes. These regions contain potential proximal promoter and regulatory sequences (Fig. 2*B*). The upstream regions (Fig. 2*B*, *striped boxes*) are 1.6 kb in length and 96% homologous. The downstream regions (Fig. 2*B*, *gray boxes*) are 1.2 kb in length and 89.5% homologous.

A mouse gene with homology to *hClp* (*mClp*) localized ~33 kb upstream from the *Scarf* gene. Comparison of the genomic organization of the *CaM*-like genes in human chromosome 10 and the syntenic region on mouse chromosome 13 is shown in Fig. 2*B*. Human and mouse show similar genomic arrangement, with *Scarf* and *hClsp* being potential homologs and the differences being that *hClp* is in reverse transcriptional orientation and the apparent absence of a *Scarf2* homolog of human chromosome 10.

Scarf Expression during Development and in Cultured Primary Keratinocytes

Using Northern blot analysis and radioactive *in situ* hybridization, we examined the pattern of *Scarf* expression during development and in mouse primary keratinocytes. *In situ* hybridization of sagittal sections of 15-day (Fig. 3, *A* and *B*) and 16-day (Fig. 3, *C* and *D*) mouse embryos with antisense *Scarf* probes (Fig. 3, *A* and *C*), showed epidermis-specific expression at both developmental stages. The expression was not detected in the basal layer, but was clearly seen within the spinous and granular layers in the stratified epidermis of 16-day embryos (Fig. 3, *E* and *F*). Expression was also detected in the dorsum of the tongue (Fig. 3, *H* and *I*) and in the vibrissae of a 16-day mouse embryo (Fig. 3, *J* and *K*). *Scarf* expression was restricted to the differentiated layers of the stratified epidermis in neonatal skin (Fig. 3, *L* and *M*). No expression was detected with the use of *Scarf* sense probe (Fig. 3, *B*, *D*, *G*, and *N*).

Using a commercial Northern blot of total embryo mRNA at different developmental stages, *Scarf* was first detected at 15 days of embryogenesis and dramatically increased by day 17 (Fig. 4*A*). Sites of expression detected on a dot blot and Northern blot of different adult tissues (Clontech) were thyroid (data not shown) and skeletal muscle (Fig. 4*B*). Using the commercial blot, no expression was detected in heart, brain, spleen, lung, liver, kidney, or testis (Fig. 4*B*). With the hybridization of the commercial blot to a glyceraldehyde-3-phosphate dehydrogenase probe to determine loading amounts and integrity of the mRNA, it became apparent that there was less input from the spleen, lung, and testis samples. For this reason, it is not possible to clearly assess the expression level of *Scarf* in these tissues.

In the epidermis, *Scarf* expression was restricted to the suprabasal differentiated cells obtained by Percoll gradient of preparations from neonatal skins (Fig. 4*C*). Several epidermis-specific markers were utilized to validate the Percoll separation method for basal (*B*) and suprabasal (*SB*) cells (Fig. 4*C*). The *K5* marker is restricted to the basal fraction, whereas the markers *INV*, *LOR*, and *K1* are primarily in the suprabasal fraction. The ubiquitously expressed *CaM* mRNA was detected in both proliferating and differentiated keratinocytes (Fig. 4*C*).

The expression of *Scarf* was also studied in cultured primary keratinocytes induced to differentiate *in vitro. Scarf* expression was detectable in low Ca^{2+} (0.05 mM) and showed a 4-fold increase 24 h after induction of differentiation by raising the Ca^{2+} concentration to 0.12 mM and a further increase by 48 h (Fig. 4*D*). Northern blots were also hybridized with basal cell-specific marker (*K5*) and suprabasal differentiation-specific markers (*K1*, *LOR*, and *INV*). The *Scarf* expression pattern in cultured keratinocytes mimics that of early differentiation markers such as *INV*, whereas the late epidermal differentiation marker, *LOR*, was detected 48 h after Ca^{2+} switch.

Treatment of cultured cells with different kinase (PKC, PKA, CaM kinase II) inhibitors showed a specific down-regulation of *Scarf* expression by treatment with a PKC inhibitor, GF109203X (Fig. 4*E*). We also determined the down-regulation for other epidermal factors (*INV*, *KI*, and *LOR*), where expression is dependent on the PKC signaling pathway. No effect on *Scarf* expression was seen with treatment with PKA inhibitor H89 and CaM kinase II inhibitor KN62.

Scarf Ca^{2+}-binding Protein in Differentiated Keratinocytes

To assess the expression of Scarf protein in epidermis, we performed immunohistochemistry on paraffin sections of mouse neonatal skin with a polyclonal antibody generated in hens against the full-length recombinant protein (rScarf). Scarf protein was clearly detected from the spinous layers to anucleated cells of the stratum corneum in neonatal skin sections using a fluorescein isothiocyanate-conjugated secondary antibody against hen (shown in green, Fig. 5*A*). Propidium iodide counter-stain determined the localization of the nuclei (Fig. 5*A*, shown in *red*).

In Western blot assays, anti-Scarf polyclonal antibody detected rScarf and the endogenous Scarf protein in the soluble fraction of suprabasal cells (Fig. 5*B*). No Scarf was detected in the insoluble fractions of either basal or suprabasal keratinocytes. Using several recombinant CaM-like proteins in a Western blot analysis, Scarf antibody weakly detected recombinant hClsp but had no cross-reactivity to either hClp or CaM (Fig. 5*C*).

To investigate the capability of Scarf to bind Ca^{2+} and the functionality of each of the EF-hand motifs, we performed Ca^{2+} binding assays. Scarf mutants were obtained by substituting the conserved first Asp residue of each EF-hand motif (sequence GAC) with a Gly residue (GCC sequence) to generate mtEF1, mtEF2, mtEF3, and mtEF4. Parallel binding assays were performed with CaM as control, several CaM-like Ca^{2+}-binding proteins (CaM, hClsp, and hClp), rScarf, and each recombinant mutant. Quantitation of the autoradiograms with a densitometer show that Scarf is able to bind Ca^{2+} (Fig. 6*A*) and that mutations in each of the EF-hand motifs lead to a diminished ability to bind Ca^{2+}, with mutation of the EF-2 domain showing the highest effect (Fig. 6*B*). The data presented are the mean from three independent experiments ± S.D. (Fig. 6*B*).

DISCUSSION

Identification of differentially expressed proteins during epidermal stratification will help us elucidate the necessary precursors and the series of complex steps that are required for the formation of a functional water barrier in the skin. By performing an SSH screen between the undifferentiated and differentiated cells of the neonatal mouse epidermis, we identified a novel murine Ca^{2+}-binding protein gene we termed *Scarf*. Sequencing of the full-length cDNA determined that *Scarf* contained regions that would translate into putative EF-hand Ca^{2+}-binding motifs. The presence of four EF-hand motifs and the degree of homology to CaM indicate that Scarf belongs to the family of CaM-like Ca^{2+}-binding proteins.

In the *in situ* hybridization data, we showed that *Scarf* mRNA was expressed throughout the differentiated layers of the embryonic developing epidermis. In neonatal epidermis, the *Scarf* gene was also differentially expressed in the spinous and granular layers. An 0.9-kb mRNA *Scarf* transcript was detected in suprabasal cells obtained from neonatal epidermis by discontinuous Percoll gradients and in primary keratinocytes differentiated *in vitro*. *Scarf* expression was similar to that of other early differentiation markers such as *INV* and was dependent on PKC signaling.

The *Scarf* transcript encodes a small acidic protein of 148 amino acids detected with anti-Scarf-specific antibodies in the suprabasal layers of the epidermis. Scarf EF-hand motifs maintain the highly conserved residues present in the canonical 12 residue EF-hand motif (D*X*D*X*(D/N)G*X*(I/V)*XXX*E) (12). The Ca^{2+}-binding ability and functionality of each Scarf EF-motif were tested. Our results indicate that Scarf is able to bind Ca^{2+} and mutations in each EF-hand lead to a decreased ability to bind Ca^{2+}, with mutations in the EF-2 hand motif being the most effective. For the CaM-like protein hClsp, it has recently been reported that its four EF-hand motifs bind Ca^{2+}, but the motifs have been defined as two Ca^{2+}-Mg^{2+} high affinity binding motifs (EF-1 and EF-2) and two low Ca^{2+}-binding motifs (EF-3 and EF-4) (25). In CaM, mutations of the EF-2 motif decreased its affinity for several of its known target proteins: smooth and skeletal muscle myosin light chain kinases, adenyl cyclase, and plasma membrane Ca^{2+}-ATPase (26). It remains to be determined whether Scarf EF-hands have differential affinity for Ca^{2+}, whether the motifs are independent or mutually interactive, and what physiological relevance this has for its capacity to interact with its specific target proteins in the mouse epidermis. Targets for other members of the CaM-like binding-protein family have been reported, with hClp binding a human unconventional myosin X (27), CaBPs binding the inositol trisphosphate receptor (Ins-P_3-R) (28), and hClsp binding TGase3 (17). TGases are Ca^{2+}-dependent enzymes that catalyze the *N*-(γ-glutamyl)lysine isopeptide bonds that occur during the cross-linking of precursors to form the specialized cornified cell envelope in the epidermis (1). The Ca^{2+}-dependent binding of hClsp to TGase3 is a potential way of regulating epidermal differentiation through the modulation of TGase3 enzyme activity (16). Alternatively, members of the S100 EF-hand-containing protein family are TGase enzymatic targets (11,29), leading to a mechanism for regulating the S100 protein functions in the epidermis.

Based on sequence homology and genomic localization, we propose that *Scarf* is a potential mouse homolog of *hClsp*. However, certain relevant distinctions between *Scarf* and *hClsp* have been determined: (*a*) *hClsp* was reported as a late granular differentiation marker expressed only in the very late stages of human keratinocyte differentiation and detected in lung tissue (16,17), whereas *Scarf* is expressed early in the differentiation process throughout the spinous and granular layers in the stratified epidermis and is also detected in thyroid and skeletal muscle, and (*b*) Scarf has a slightly extended central helix when compared with hClsp (16), making it more similar to CaM. For CaM, the central helix serves a fundamental role, allowing the amino- and carboxyl-terminal domains to swing around and permitting interactions with specific target proteins (6,12). The focus of our future studies will be to establish whether the slightly extended central helix in Scarf allows for the binding to targets other than the factors that interact with hClsp and to determine the role of these interactions in the control of the Ca^{2+}-dependent epidermal differentiation.

In this study we have also identified a gene with high homology to *Scarf*, which we termed *Scarf2*, that localizes ~15 kb downstream on mouse chromosome 13. Sequence analysis of the genomic regions 5′ and 3′ to each gene showed that the homology extended to the putative proximal promoter and 3′-regions. We hypothesize that the bigenic cluster arose by recent duplication in the mouse, because we were unable to localize by genomic sequence scanning a counterpart to *Scarf2* in the human genome. We detected a transcript for *Scarf2* in mouse

keratinocytes by RT-PCR but not in Northern blots with samples of total RNA from basal and suprabasal cells isolated from neonatal skins or in commercial blots (data not shown), suggesting that *Scarf2* is expressed at lower levels than *Scarf*. Analysis of the *Scarf2* putative ORF indicated that it encodes a Ca^{2+}-binding protein, and based on a comparison with the canonical consensus sequence for the EF-hand motif and the critical role of the residues at positions 1 and 12, we hypothesize that the Scarf2 EF-1 and -4 motifs will be inactive or present altered Ca^{2+} binding. How these differences characterize Scarf2 function remains to be determined, as does the role of the highly homologous Scarf and Scarf2 and their interaction with target proteins in the transduction of the Ca^{2+}-dependent regulatory signals during the epidermal differentiation process.

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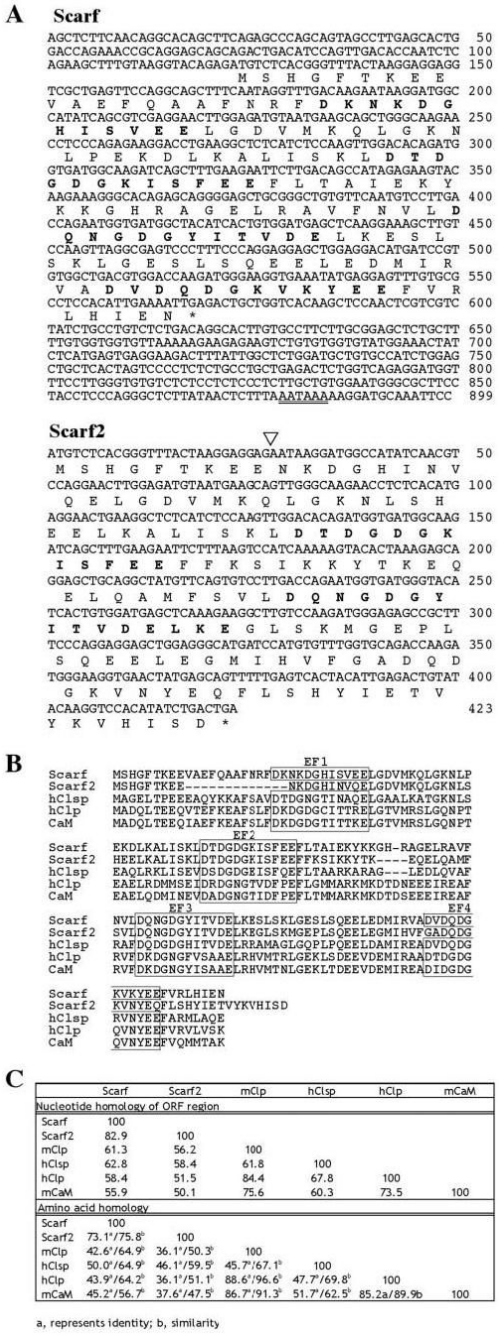

Fig. 1. Characterization of *Scarf* and *Scarf2*

A, nucleotide sequences of *Scarf* cDNA (899 bp) and *Scarf2* ORF (423 bp) were aligned with the predicted amino acid sequences. The *double underlined* nucleotides indicate the canonical poly(A) addition signal in *Scarf*. *Numbers* on the *right* indicate base pairs. Putative EF-hand motifs are in *bold* letters. The *open triangle* indicates the site of splicing (exon/intron boundary) in the *Scarf2* gene. *Asterisks* denote the termination codons (TGA) in the putative amino acid sequences of Scarf and Scarf2. *B* and *C*, comparison of the amino acid sequence (*B*) and percentage of homology (*C*) at the nucleotide and amino acid level among several CaM-like Ca^{2+}-binding proteins.

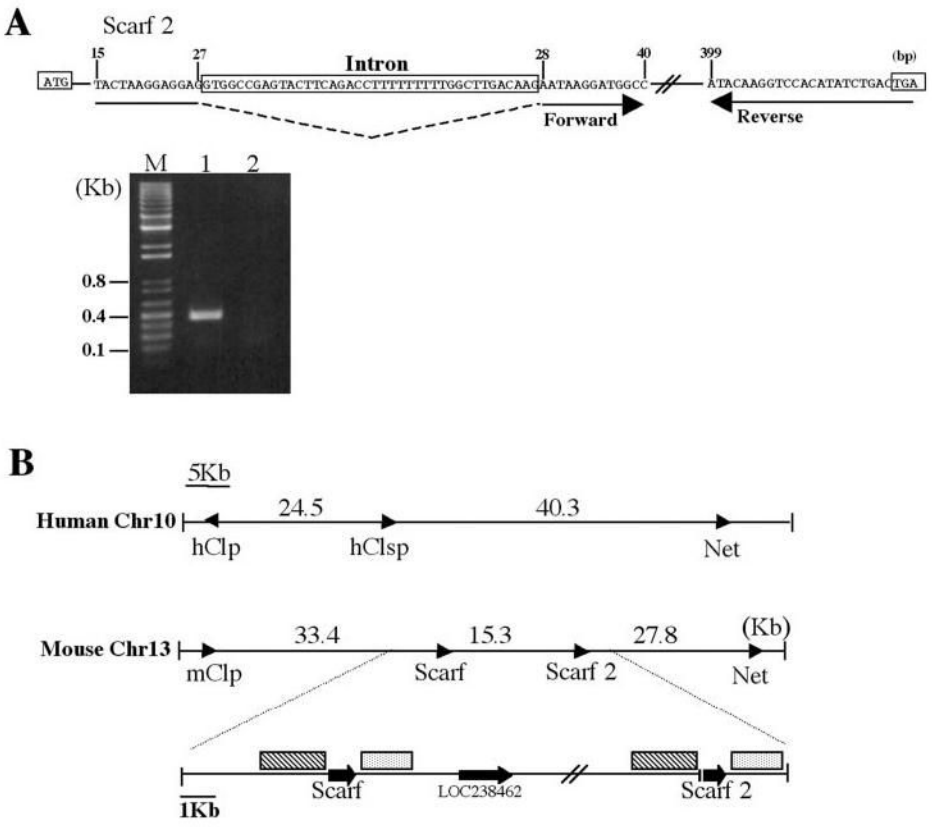

Fig. 2. Genomic localization of *Scarf* and *Scarf2* genes

A, *upper panel* shows partial sequence from the *Scarf2* gene, indicating the sequences that were used as primers in RT-PCR and the in-tronic sequence. *Lower panel* shows ethidium bromide-stained 1.5% agarose gel: *lane M*, molecular weight marker, 1-kb plus ladder (Invitrogen); *lane 1*, sample from RT-PCR performed with *Scarf2*-specific oligonucleotides; *lane 2*, RT-PCR performed with no cDNA input. *B*, genomic organization of human and mouse *CaM*-like protein genes on chromosome 10 and 13, respectively. *Numbers* indicate kilobases, and *arrows* show position of the genes and transcriptional orientation (direction of arrowheads). The *striped* and *gray boxes* indicate the regions of homology (*striped*, 1.6 kb in length and 96% homologous; *gray*, 1.2 kb in length and 89.5% homologous) between the *Scarf* and *Scarf2* genes.

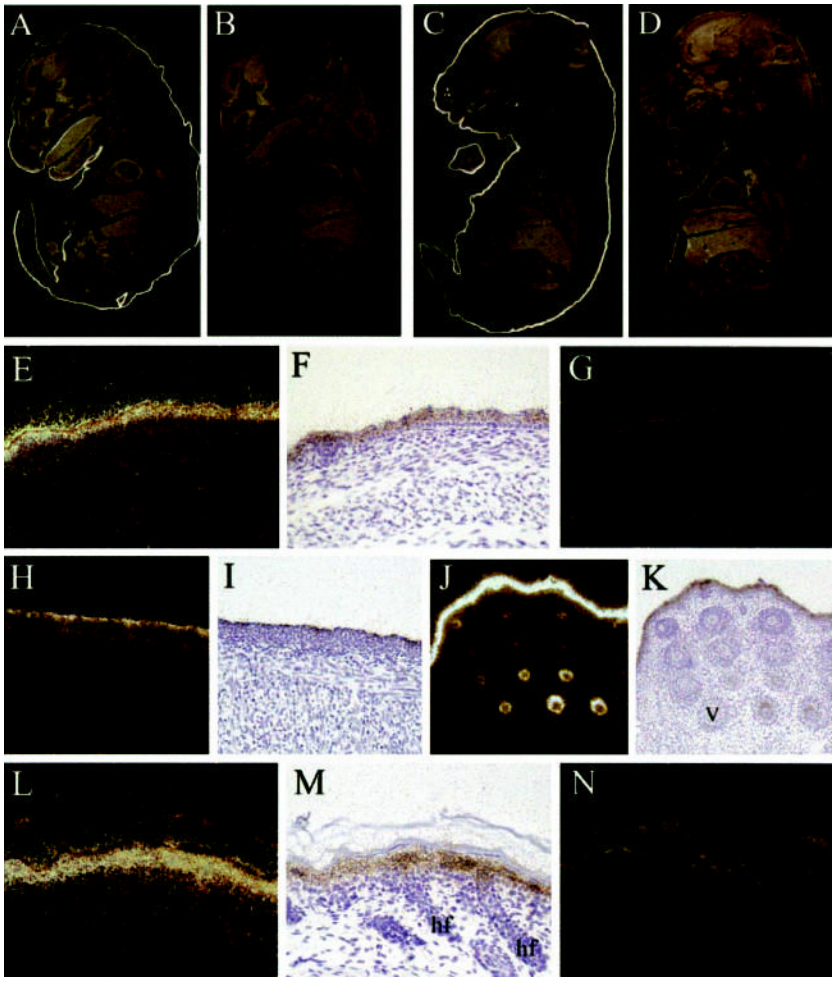

Fig. 3. Expression of *Scarf* during development

In situ hybridization was performed with antisense (*A* and *C*) and sense (*B* and *D*) *Scarf* probes on sagittal sections of day 15 (*A* and *B*) and day 16 (*C* and *D*) mouse embryos (magnification 6.5×). *E–K*, 20× magnification of hybridization of trunk skin (*E*), tongue palate (*H*), and vibrissae (*J*) from 16-day embryo with antisense Scarf probe and the corresponding hematoxylin staining (*F*, *I*, and *K*, respectively). *Panel G* shows hybridization with sense *Scarf* probe of trunk skin on a 16-day embryo. *L–N*, *in situ* hybridization with antisense (*L* and *M*) and sense (*N*) *Scarf* probes on sections of neonatal epidermis. *Panel M* is the corresponding hematoxylin staining. *hf*, hair follicle; *v*, vibrissae.

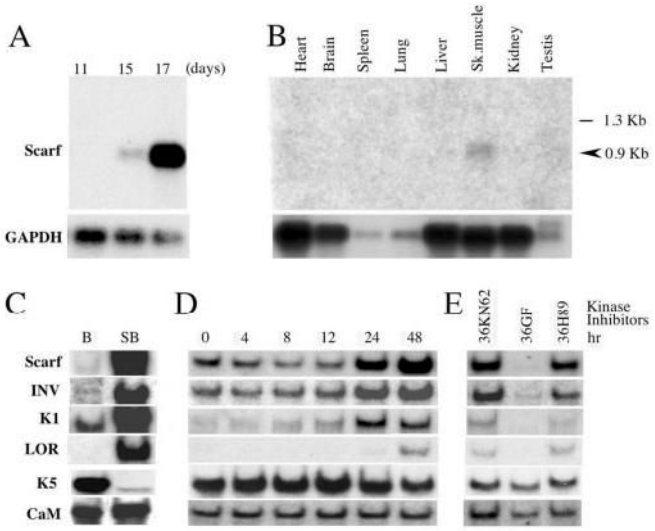

Fig. 4. Expression of *Scarf* in the stratified epidermis and cultured keratinocytes

A, expression of *Scarf* during mouse embryonic development (11, 15 and 17 day embryos). *B*, mouse adult tissue Northern blot (Clontech) panel shows 2 μg of poly(A)/lane of heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. The blots were hybridized with *Scarf* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The RNA size marker and *Scarf* (*arrowhead*) molecular weights are indicated on the *right. C* and *D*, Northern blots of total RNA from basal (*B*) and suprabasal (*SB*) cells isolated by discontinuous Percoll gradient from neonatal epidermis (*C*) and primary mouse keratinocytes differentiated *in vitro* by addition of Ca^{2+} (*D*). Time course of expression at specified lengths of time, 0, 4, 8, 12, 24, and 48 h and (*E*) after 36 h of treatment with different kinase inhibitors: KN62, a CaM kinase II inhibitor; GF109203X (*GF*), a PKC inhibitor; and H89, a PKA inhibitor. The blots (*C*, *D*, and *E*) were hybridized with *Scarf*, *INV*, *K1*, *LOR*, *K5*, and *CaM*.

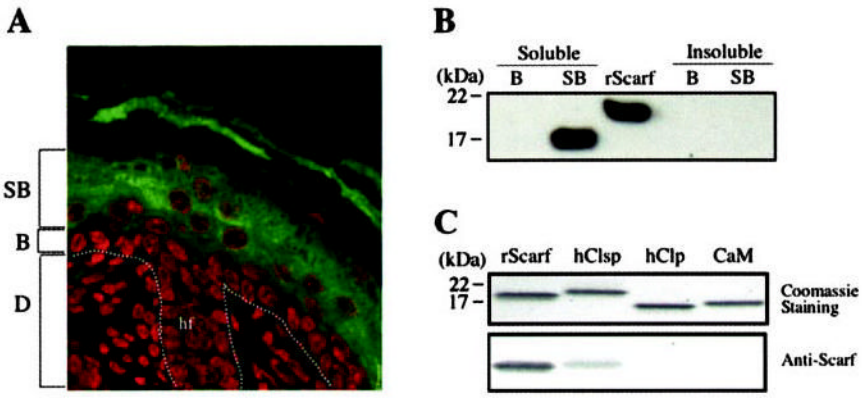

Fig. 5. Characterization of Scarf protein

A, immunohistochemistry was performed on paraffin sections of neonatal skin with anti-Scarf antibody. The nuclei were visualized using a propidium iodide counter-stain. Layers of the skin are indicated on the *left side* of the panel: dermis (*D*), basal cell layer (*B*), and suprabasal layers (*SB*). A *dotted line* demarcates the epidermis/dermis junction. *hf*, hair follicle. *B*, Western blot of total cell extracts of soluble and insoluble fractions from basal and suprabasal cells. The secondary antibody used was horseradish peroxidase-conjugated anti-chicken IgG. *C*, *upper panel*, Coomassie staining of acrylamide gel run of several recombinant CaM-like Ca^{2+}-binding proteins (rScarf, hClsp, hClp, and CaM). *Lower panel*, Western blot of recombinant proteins with anti-Scarf antibody and alkaline phosphatase-conjugated anti-chicken IgG as secondary antibody.

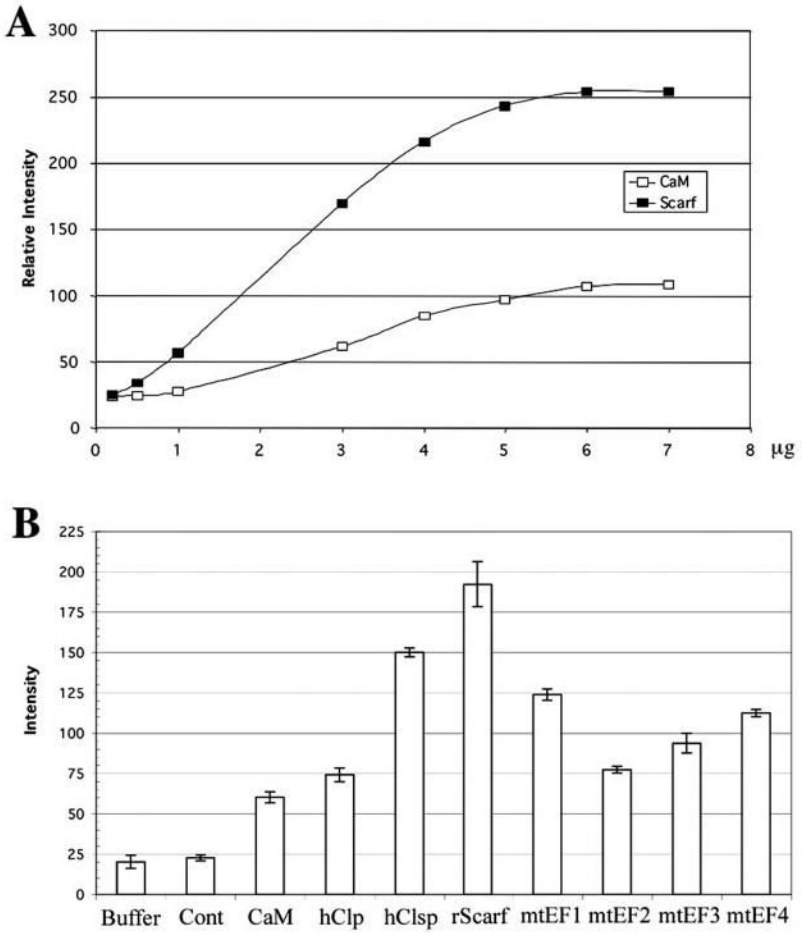

Fig. 6. Functional Ca^{2+} binding assay of CaM-like binding proteins

A, a Ca^{2+} binding assay was performed with rScarf and CaM with increasing amounts of protein (μg). The results were quantitated and plotted as concentration of protein (μg) *versus* relative intensity as determined by densitometer. *B*, Ca^{2+} binding assays were performed with wild-type and mutant proteins. The data presented are the mean from three independent experiments ± S.D. mtEF1–4 represent the mutated forms of Scarf in each EF-hand 1–4 motif, respectively.