Overexpression of Serpin Squamous Cell Carcinoma Antigens in Psoriatic Skin

Atsushi Takeda,*† Dousei Higuchi,‡ Tadahito Takahashi,§ Masashi Ogo,§ Peter Baciu,¶ Paul F. Goetinck,¶ and Toshihiko Hibino§¶

*Laboratory of Biochemistry, Faculty of Arts and Sciences, Sagami Women's University, Kanagawa, Japan; Departments of †Clinical Pathology and ‡Dermatology, Showa University Fujigaoka Hospital, Kanagawa, Japan; §Shiseido Research Center, Kanagawa, Japan; ¶MGH/Harvard Cutaneous Biology Research Center, Charlestown, Massachusetts, U.S.A.

Squamous cell carcinoma antigen belongs to the serpin family and is used for the diagnosis and management of squamous cell carcinoma. We investigated the involvement of squamous cell carcinoma antigen in psoriasis, as it is always detected in the sera of patients with psoriasis. Squamous cell carcinoma antigen localization in psoriatic epidermis varied depending on its concentration in the patient's sera. When its level was low in serum, weak and scattered staining was observed in the granular layer. With a high concentration of squamous cell carcinoma antigen, strong staining through the suprabasal to granular layer and condensed staining around the plasma membrane or intracellular space was detected in the affected epidermis. Interestingly, squamous cell carcinoma antigen was abundant in nuclei of the granular layer cells and elongated rete ridges. Immunoelectron microscopy confirmed the localization of squamous cell carcinoma antigen in the nuclei as well as in the periphery of the cell membrane. A cDNA library was constructed from psoriatic epidermis and both clones, SCCA1 and SCCA2, were obtained. Attempts to raise specific antibodies or to prepare cRNA probes for SCCA1 and SCCA2 were unsuccessful because of their nearly identical structures. A primer pair from each reactive site sequence enabled us to give a distinctive product for SCCA1 and SCCA2 by reverse transcription polymerase chain reaction. Analysis using these primers demonstrated that the SCCA2 transcript was specifically expressed in psoriatic skin tissues. Our results suggest that overexpression of squamous cell carcinoma antigens is associated with the disease activity of psoriasis. ***Key words: cross-class inhibitor/mRNA expression/protease inhibitor/RT-PCR. J Invest Dermatol 118:147–154, 2002***

Psoriasis is a common skin disease characterized by epidermal hyperproliferation, capillary dilation, and the presence of acute and chronic inflammatory cells in both the dermis and epidermis (Braun-Falco and Schmoeckel, 1977; Braverman and Sibley, 1982; Baker *et al*, 1984; Weinstein *et al*, 1985; Toruniowa and Jablonska, 1988; Bearskins *et al*, 1989). A variety of proteinases as well as their inhibitors that are not detected in normal skin have been shown in psoriasis (Fräki and Hopsu-Havu, 1976; Ohtani *et al*, 1982; Hopsu-Havu *et al*, 1983; Sumi *et al*, 1983; Järvinen *et al*, 1984; Hibino, 1985; Hibino *et al*, 1986, 1988a; Harvima *et al*, 1990; Kawada *et al*, 1997). Overexpression or activation of these proteinases and specific inhibitors are possibly responsible, at least in part, for the disease activity in psoriasis. Normal epidermis contains three detectable proteinase inhibitors: plasminogen activator inhibitor 2 (Hibino *et al*, 1988b), a trypsin inhibitor (Hibino *et al*, 1981), and a low molecular mass cysteine proteinase inhibitor (cystatin) (Järvinen, 1978; Takeda *et al*, 1986).

Manuscript received March 13, 2000; revised September 26, 2001; accepted for publication October 3, 2001.

Reprint requests to: Dr. Atsushi Takeda, Laboratory of Biochemistry, Faculty of Arts and Sciences, Sagami Women's University, 2-1-1 Bunkyo, Sagamihara-shi, Kanagawa 228-8533, Japan. Email: a-takeda_shokumotsu@star.sagami-wu.ac.jp

Abbreviation: SCCA, squamous cell carcinoma antigen.

We initiated investigation of the changes associated with inhibitory activities against proteinases in psoriatic skin tissues. We found increased inhibitory activity on cysteine proteinases, and purified a protein that strongly inhibited papain and cathepsin L without affecting cathepsins B or H (Takahashi *et al*, 1993). A partial amino acid sequence of this protein revealed similarities to squamous cell carcinoma antigen (SCCA). The gene for SCCA localized to human chromosome 18q21.3 with a tandem duplication of *SCCA1* and *SCCA2*, which encode proteins SCCA1 and SCCA2, respectively (Suminami *et al*, 1991; Barnes and Worrall, 1995; Schneider *et al*, 1995). These encode proteins that are members of the serine proteinase inhibitor family (serpin), whereas they inhibit papain-like cysteine proteinases (cathepsins K, L, or S) and chymotrypsin-like serine proteinases (cathepsin G and mast cell chymase), respectively. The levels of SCCAs are known to increase significantly in the sera of psoriatic patients (Duk *et al*, 1989; Hamanaka *et al*, 1997), indicating their use as possible serologic markers for the diagnosis and management of psoriasis. There is little information concerning the relationship of serum concentration and expression in psoriatic skin tissue, however. Furthermore, which SCCAs are expressed in the psoriatic skin tissues remains totally unknown.

In this study, we examined the precise distribution and localization of SCCA in psoriatic skin tissues during different clinical stages by light and electron microscopic immunohistochemistry. In addition, SCCA mRNA expression in psoriatic skin

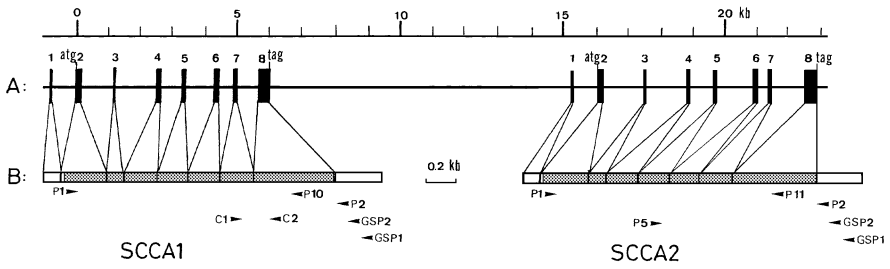


Figure 1. SCCA gene locus and cDNA structure. (*A*) Structure of the SCCA gene. The exons are indicated by solid boxes with the respective numbers above them; introns are indicated by thin lines. The translation start codon (atg) and translation stop codon (tag) are shown. The scale is shown at the top of the figure. (*B*) Structure of SCCA1 and SCCA2 cDNA. The shadowed boxes show the open reading frame regions. The open boxes at each 3′ and 5′ region show noncoding regions. The scale marker indicates 0.2 kb. *Arrowheads* below the cDNA show the position and direction of primers used in PCR.

tissues was analyzed by *in situ* hybridization and reverse transcription polymerase chain reaction (RT-PCR). Even though SCCA1 and SCCA2 are nearly identical in primary structure, we designed specific primers to distinguish each molecule by RT-PCR. We found, for the first time, that SCCA2 was expressed in psoriatic epidermis.

MATERIALS AND METHODS

Skin specimens Eighteen cases of psoriasis with typical clinical and histologic features were selected retrospectively. Histopathologic diagnoses were confirmed with sections stained by hematoxylin and eosin. All patients were between 38 and 67 y of age, were in an acute stage of the disease, and had not yet received treatment. The average SCCA concentration in sera from patients was 13.2 ng per ml (1.2–38.2 ng per ml), measured with an IMx-SCC test kit (DaiNabot, Tokyo, Japan). For RT-PCR, normal skin tissues excised from eight plastic surgery patients and uninvolved skin distal from nine psoriatic patients were obtained with informed consent. The tissues were immediately frozen with liquid nitrogen and stored at −80°C until use.

Cloning of SCCA1 and SCCA2 cDNA Psoriatic tissue specimens obtained by shave biopsy were immediately frozen in liquid nitrogen. Poly(A)$^+$ RNA was isolated from the frozen tissues using FastRNTrack mRNA isolation (Invitrogen, San Diego, CA). A psoriatic cDNA library was constructed using TrZAP Express cDNA Synthesis Kit (Stratagene, La Jolla, CA). Approximately 4 μg of poly(A)-rich RNA from psoriatic skin was reverse transcribed to cDNA using StrataScript RNaseH$^-$ reverse transcriptase primed with a hybrid oligo(dT) linker primer that contains the XhoI site. During the second DNA synthesis, EcoRI adaptors were ligated to the blunt ends. After the XhoI digestion, cDNA was size fractionated with a Sephacry S-400 spin column. The fraction with a molecular mass of around 2 kbp was recovered from the column, inserted into the ZAP expression vector, and packed with GigapackII Gold (Stratagene). The cDNA was cloned by mixed oligonucleotide primed amplification cloning (Lee *et al*, 1988). Two degenerate oligonucleotides, 5′-ATACACATCTTTTCATTTTG-3′ (primer C1) and 5′-GCCTGAGAGGTCTGCATCCC-3′ (primer C2), were synthesized based on the peptide sequences QYTSFHFAS and IFNGDADLSG, respectively, whose sequences were obtained by analysis of the tryptic peptides of the purified protein (C1 and C2 in **Fig 1**). When these nucleotides were used as PCR primers, the initial cDNA amplification from psoriatic skin revealed the presence of a 304 bp amplified product. The PCR product was sequenced and identified as a partial nucleotide sequence SCCA cDNA (Suminami *et al*, 1991). This specific oligonucleotide was used to screen the psoriasis cDNA library. Twenty-five positive clones were obtained by a tertiary screening with ^{32}P-labeled oligonucleotide; however, none contained the 5′ end. We employed the 5′ RACE system (Gibco-BRL, Rockville, MD) to obtain the 5′ end. Two gene-specific primers, 5′-AACAGGTCACAAACAG-AAT-3′ (primer GSP1) and 5′-GCAATCAGGTTTACCAGAA-3′ (primer GSP2), were synthesized based on the sequence of positive clones from the library, which contained the entire 3′-untranslated region (GSP1 and GSP2 in **Fig 1**). Approximately 1.2 kbp products were obtained, cloned into the pcDNAII vector (Invitrogen), and sequenced. The complete sequence of each clone was confirmed on both strands. DNA sequences were analyzed with GCG package and BLAST search programs.

Antigens and antibodies Five antigens were prepared for the antibody production. SCCA was purified from psoriatic scales as previously described (Takahashi *et al*, 1993). Histidine-tagged fusion SCCA1 and SCCA2 were purified from the homogenates of *Escherichia coli* transformed by expression vectors pQE-SCCA1 and pQE-SCCA2, respectively. Two peptides, AVVGFGSSPTSTNEE and AVVVVELSS-PSTNEE, corresponding to the respective amino acid residues 348–362 of SCCA1 and SCCA2, were synthesized. A cysteine residue was added to the NH_2-terminus of each peptide, and subsequently conjugated with keyhole limpet hemocyanin using 3-maleimidebenzoyl-N-hydroxy-succinimide ester (Kitagawa *et al*, 1982). Antisera against five antigens were raised in rabbits, and immunoreactivity was tested against all the antigens.

***In situ* hybridization** PCR products amplified with common primers 5′-ATGAATTCACTCAGTGAAGCC-3′ (primer P1) and 5′-GGA-GTGACAGACTAATTGCAT-3′ (primer P2) (P1 and P2 in **Fig 1**) were ligated into pGEM-T vector (Promega, Madison, WI) in order to construct pGEMT-SCCA1 and pGEMT-SCCA2 vectors. Sense and antisense RNA probes were prepared according to the manufacturer's manual (DIG RNA Labeling Kit SP6/T7; Boehringer Mannheim, Penzberg, Germany). Briefly, after linearization of pGEMT-SCCA1 and pGEMT-SCCA2 with NotI, the antisense or sense cRNA was transcribed *in vitro* using digoxigenin-labeled UTP T7 or SP6 RNA polymerase, respectively. Formalin-fixed and paraffin-embedded specimens were sectioned to 5 μm thickness, attached to glass slides coated with poly-L-lysine, and rapidly air-dried. Dewaxed paraffin sections were pretreated with 1 μg per ml of proteinase K for 15 min at 37°C, and fixed again with 4% paraformaldehyde. Following acetylation with acetic anhydride in 20 mM Tris-HCl (pH 7.5) and 1 mM ethylenediamine tetraacetic acid for 10 min, the sections were washed twice with phosphate-buffered saline (PBS) containing 0.1 M glycine for 15 min. The sections were hybridized with the SCCA1 or SCCA2 riboprobes (1 μg per ml each) in hybridization buffer (50% formamide/1 × Denhardt's solution/5 × sodium citrate/chloride buffer) for 16 h, and digested with 0.2 μg per ml RNase (DNase-free) in 0.5 M NaCl and 10 mM Tris-HCl (pH 8.0) for 30 min at 37°C. Sections were then incubated with 1% blocking reagent (Roche Diagnostics) and antidigoxigenin antibodies conjugated with alkaline phosphatase. Hybridized probe was visualized by incubation with enzyme substrate BM purple (Roche Diagnostics).

RT-PCR analysis of SCCA mRNA expression P1 was used as the forward primer for SCCA1 and 5′-CTGATGCATATGAGCTGA-AGATCG-3′ (primer P5) for SCCA2. The reverse primers were 5′-GTGTAGGACTCCAGATAGCAC-3′ (primer P10) and 5′-GTGTAG-GACTTTAGATACTGA-3′ (primer P11) for SCCA1 and SCCA2, respectively (GenBank Accession numbers U19559 for SCCA1 and U19569 for SCCA2) (P10 and P11 in **Fig 1**). Total RNA from skin tissue biopsies was isolated with Isogen (Nippon Gene, Tokyo, Japan) and genomic DNA was digested with RNase-free DNase I (Promega). The cDNA was synthesized using the murine moloney leukemia virus reverse transcriptase (Stratagene) and oligo(dT)$_{17}$ primer. The PCR mixture was composed of standard template DNA (pGEMT-SCCA1or pGEMT-SCCA2) or template cDNA reverse transcribed from 500 ng of total RNA, 0.2 mM dNTPs, 1.25 pmol primers, and 1 unit AmpliTaq DNA polymerase (Perkin Elmer, CA) in 20 μl of the reaction buffer. The endogenous marker, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was also amplified. Primers used were 5′-TGGCCAAGG-TCATCCATGAC-3′ and 5′-AGGCCATGCCAGTGAGCTTC-3′. PCR was performed by denaturation at 94°C for 1 min, annealing at 62°C (for SCCAs) or 58°C (for GAPDH) for 1 min, and extension at 72°C for 2 min with Iwaki Thermal Sequencer TSR-300 (Iwaki Glass,

Tokyo, Japan). The PCR-amplified products consisted of 990 and 705 bp for SCCA1and SCCA2, respectively. For semiquantitative RT-PCR, PCR conditions were carefully examined to stop the reaction during the exponential phase of amplification of each gene. PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed using a Polaroid 667 Instant Pack Film (Polaroid, UK). To quantify the PCR products, gels were photographed with Polaroid 665 Positive/Negative Film. The intensity of the DNA bands on the film was analyzed using densitometry (Densitograph 3.0, ATTO, Tokyo, Japan).

Immunohistochemistry Skin biopsy specimens were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 3 h. After overnight rinse with PBS, they were embedded in paraffin. Serial paraffin sections were mounted on glass slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin. For routine immunohistochemical examination, sections were incubated at room temperature for 60 min with 5% normal goat serum. After washing in PBS, the sections were incubated with 1:1000 diluted rabbit anti-SCCA IgG overnight and washed three times in PBS, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG(H + L). Color was developed on 0.04% diaminobenzidine and hydroxyperoxide in 50 mM Tris-HCl (pH 7.5). Control sections were incubated with preimmune rabbit serum.

Immunoelectron microscopy Skin specimens were fixed with 4% paraformaldehyde and 0.4% glutalaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h. The fixed tissues were washed three times in the above buffer, embedded in Lowicryl White (London Resin, UK), and thinly sectioned. The sections were mounted on 150 mesh nickel grids and incubated with anti-SCCA IgG at a dilution of 1:2000. Secondary incubation was performed with 15 min gold-particle-labeled goat antirabbit IgG (ZTMED Laboratory, CA). After immunostaining, the grids were postfixed with 1% glutaraldehyde solution followed by 1% uranyl acetate, and observed with a Hitachi H-7100 electron microscope at 75 kV. Control sections were incubated with preimmune rabbit serum.

RESULTS

Serum SCCA level correlates well with the intensity of protein expression in psoriatic epidermis The protein expression of SCCA in psoriatic skin tissues from patients with different concentrations in sera was studied immunohistochemically. No positive staining for SCCA was detected in normal skin tissues (**Fig 2*A***). SCCA staining patterns in psoriatic epidermis varied considerably with the stage of the lesion based on psoriasis area and severity index. In early psoriatic lesions showing the parakeratosis and moderate acanthosis of a low serum level of SCCA (under 10 ng per ml, 15 cases), relatively weak and scattered staining was observed in the uppermost portion of the spinous layer and the granular layer of the epidermis (**Fig 2*B***). Interestingly, some nuclei in this area were SCCA positive (**Fig 2*C***). Only a few cells were positive for SCCA in the dermis of psoriatic skin (**Fig 2*B***). The skin tissues from patients with high SCCA concentrations in serum (over 10 ng per ml, three cases), which presented active psoriatic lesions, showed a strong and diffuse staining from the spinous to granular layers (**Fig 2*D–I***), especially in active lesions with micropustules of Kogoj (**Fig 2*F***). Most of the nuclei in the lesion were SCCA positive except infiltrated neutrophils. In severe cases, as shown in **Fig 2(*G*)**, SCCA staining was detected from the suprabasal layer to the granular layer. SCCA-positive cells were also seen in the elongated rete ridges except basal cells (**Fig 2*H***). Condensed staining around the plasma membrane or intracellular space was frequently observed for these cells (**Fig 2*I***). The intensity of SCCA staining in psoriatic epidermis seemed to be correlated well with the SCCA concentrations in sera, indicating that the presence of SCCA in the sera of patients with severe psoriatic skin may be due to leakiness into the circulation.

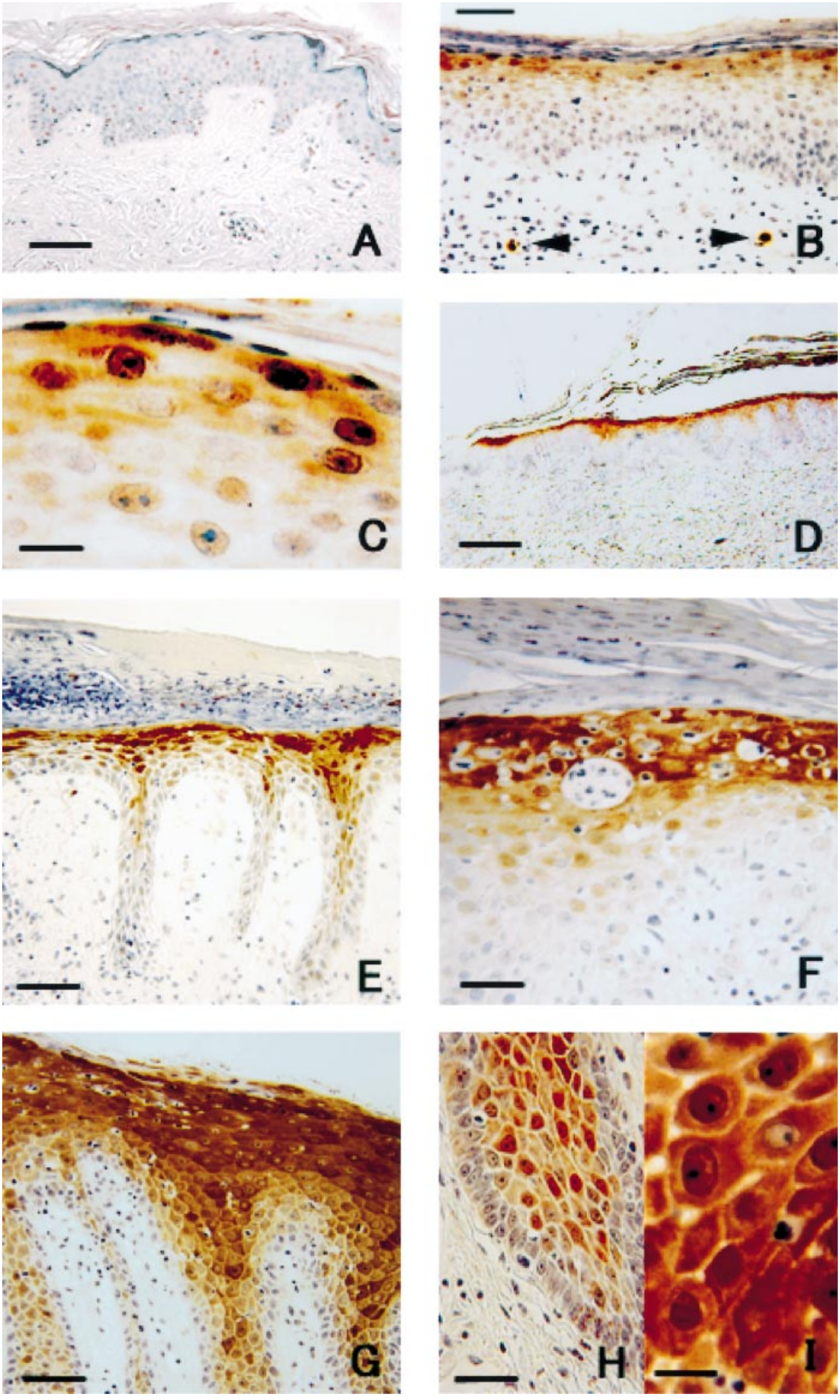
Immunoelectron microscopy confirmed localization of SCCA in nuclei Analysis by immunoelectron microscopy confirmed the results of the light microscopy study and further revealed the precise localization of SCCA in psoriatic epidermis.

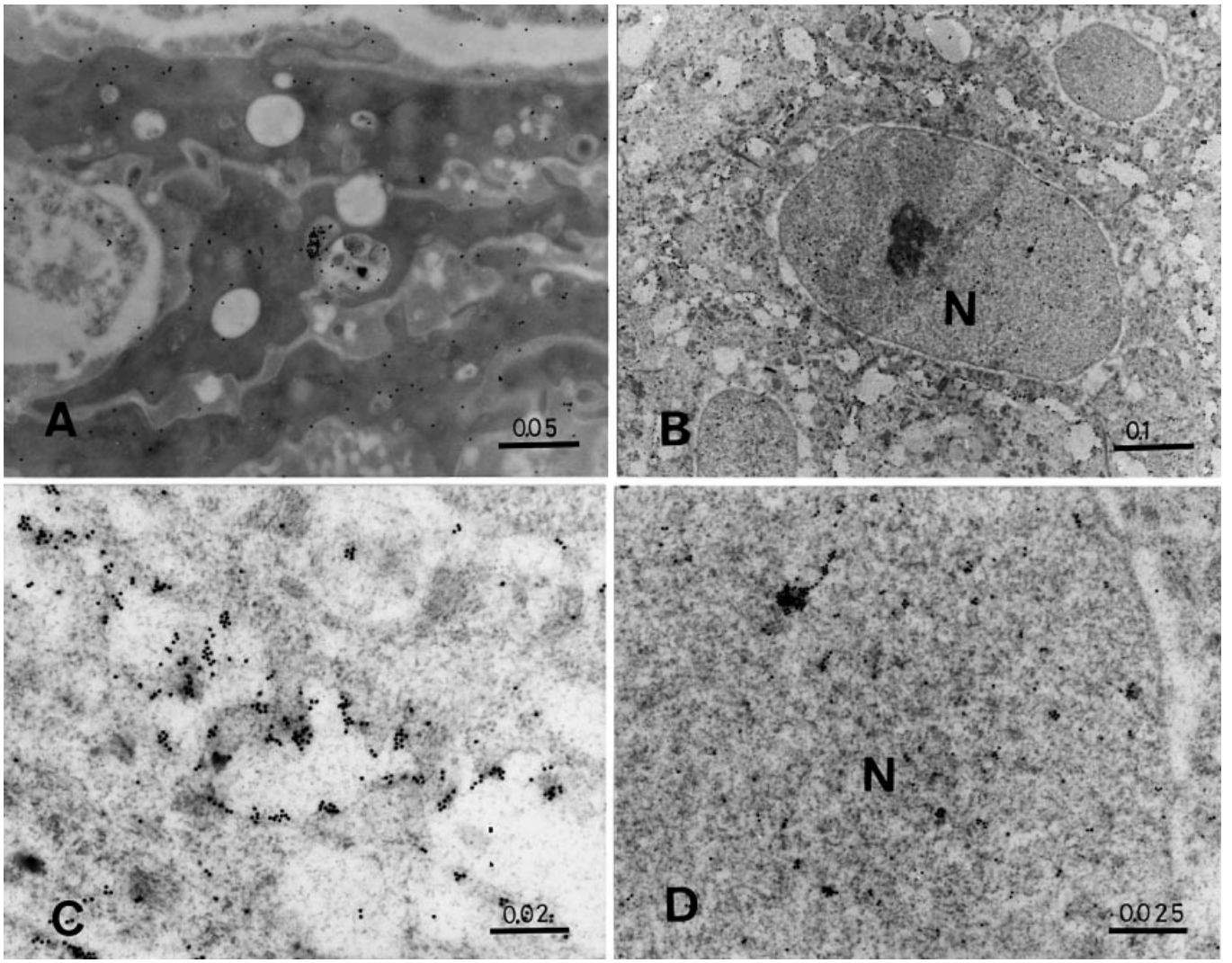
The labeling density was relatively low level and randomly scattered in the cornified cell layers, where SCCA-positive opaque and small round particles were detected in the cytoplasm (**Fig 3*A***). In the spinous cell layers, strong labeling was found in the cell–cell contact area (**Fig 3*B*, *C***). Furthermore, SCCA-positive labeling was consistently observed in the nucleus (**Fig 3*D***), but the specific nuclear components were not determined. Gold particles were also demonstrated at the papillary dermis and dermal–epidermal junction; in particular cytoplasmic granules in mast-cell-like cells and surrounding degranulated cells were also positive (**Fig 3*E*, *F***). No gold particles were detected in control sections.

Analysis of SCCA mRNA expression by *in situ* hybridization We isolated two independent clones from the cDNA expression library constructed with poly(A)$^+$ RNA from psoriatic skin. The nucleotide sequences revealed that both clones contained open reading frames for SCCA1 or SCCA2 cDNA. These clones can be distinguished by digestion with PstI (Schneider *et al*, 1995). Among 25 clones obtained, approximately half the clones (12 clones) contained a PstI site. We examined the localization of SCCA1 and SCCA2 mRNA in psoriatic skin tissues by means of *in situ* hybridization. Positive signals were localized in the suprabasal spinous layer and in the lower part of the elongated rete ridges of psoriatic epidermis, but not in the basal and corneal layers (**Fig 4*A*, *B***). No signals were detected in normal skin tissues. The same results were obtained using antisense SCCA1 cRNA or SCCA2 cRNA, indicating that neither probe could distinguish between SCCA1 and SCCA2 mRNA expression. We also tried northern blot analysis and found an identical hybridization pattern for the total RNAs from psoriatic skin tissues against both constructs (data not shown). We concluded that because of the nearly identical primary structures it would not be possible to separate each by immunohistochemistry, *in situ* hybridization, or northern blot analysis.

Establishment of a distinctive method for SCCA1 and SCCA2 We tried to establish a conventional method for discrimination of SCCA1 and SCCA2. Specific reverse primers P10 and P11 based on SCCA1 and SCCA2 cDNA were designed for the PCR analysis **(Fig 1)**. These sequences were chosen because of the difference in four successive nucleotides at each reactive site region. **Figure 5** shows PCR products of SCCA1 (990 bp) and SCCA2 (705 bp) independently amplified with the primer pairs P1/P10 and P5/P11 using SCCA1 and SCCA2 cDNA templates, respectively. Sequences of these products were confirmed as parts of SCCA1 and SCCA2 cDNA, respectively.

Figure 2. Immunohistochemical staining of SCCA in psoriatic skin tissues. (*A*) Normal skin. Serum SCCA concentration is < 5 ng per ml. SCCA staining was negative. (*B*)–(*I*) Psoriatic skin. The expression of SCCA in psoriasis was classified into two patterns: (i) diffuse staining in the cytoplasm and nuclei of epidermal cells (early psoriatic lesion, *B*) and (ii) condensed staining in the nuclei and plasma membrane/intercellular space (active psoriatic lesion, *D–I*). (*B*) Early psoriatic lesion (serum SCCA concentration 9.4 ng per ml). Upper spinous and granular cells are weakly positive. *Arrowheads* indicate SCCA-positive cells in the dermis. (*C*) High magnification of (*B*). Many nuclei are also positive for SCCA. (*D*)–(*F*) Active psoriatic skin (serum SCCA concentration 21.8 ng per ml). (*D*) Boundary lesion. Note that SCCA protein is found only in the psoriatic lesion. (*E*) High magnification of the center part, characterized by elongated rete ridges. (*F*) Micropustules of Kogoj. Strong SCCA staining is obvious in this area. Note that parakeratotic cells were SCCA negative. (*G*)–(*I*) Very active psoriatic skin (serum SCCA concentration 38.2 ng per ml). Most of the cells except basal cells are positive for SCCA. (*H*) High magnification of (*G*), elongated rete ridges. Most of the suprabasal cells are stained. (*I*) Higher magnification of (*G*), upper layer. SCCA-positive nuclei and condensed membrane staining are frequently seen. *Scale bars*: (*A*) 100 μm; (*D*) 200 μm; (*B*, *E*, *G*) 50 μm; (*F*, *H*) 20 μm; (*I*) 10 μm.


A
B
C
D
E
F
G
H
I



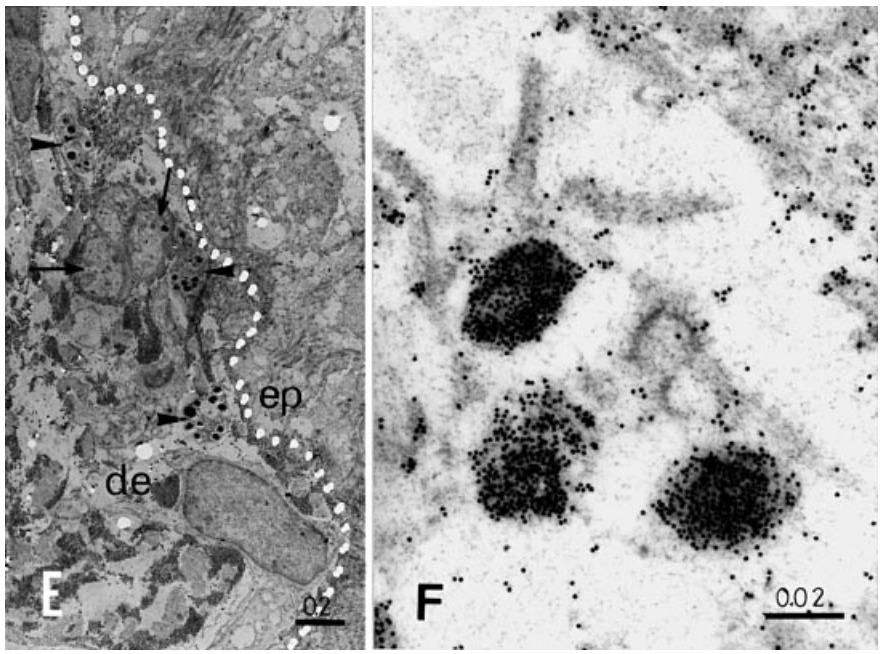


Figure 3. Electron micrographs of psoriatic skin sections treated with the antibody against SCCA. (*A*) Cornified cell layer. (*B*) Upper part of the spinous cell layer. (*C*) Higher magnification of (*B*). Gold particles were mainly localized along the periphery of the cell–cell contact area. (*D*) Higher magnification of (*B*). SCCA-positive particles are clearly shown in the nucleus designated N. (*E*) Filtrated-cell-rich region near the dermo-epidermal junction marked with dashes (ep, epidermis; de, dermis). Many immunoreactive particles were observed in the cytoplasmic granules of the filtrated cells (*arrowheads*) and in the surrounding degranulated cells (*arrows*). (*F*) Higher magnification of (*E*). Many immunoreactive gold particles were observed in the cytoplasmic granules of filtrated cells. Numbers on the scale bars indicate μm.

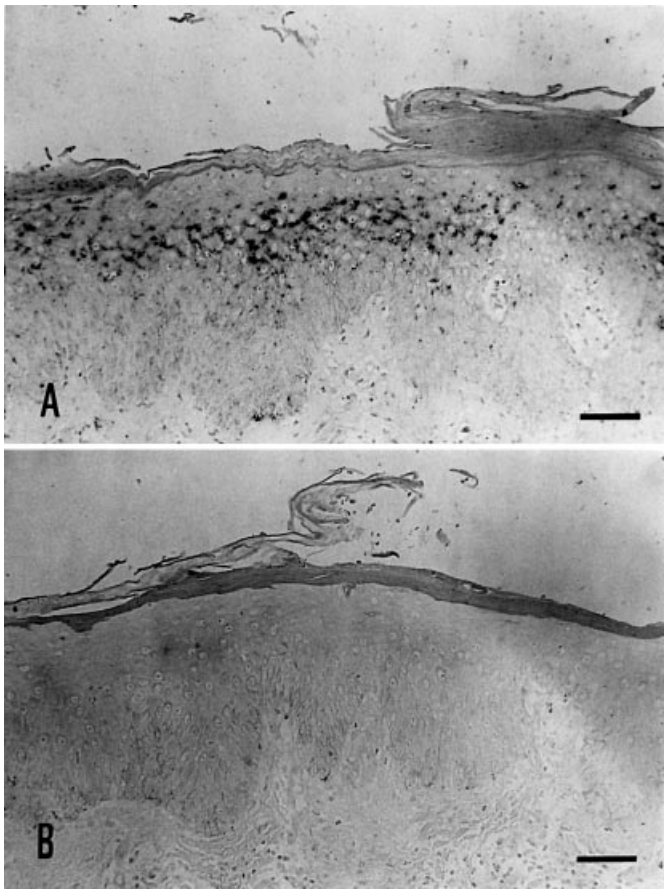


Figure 4. *In situ* hybridization of SCCA mRNAs in psoriatic epidermis. (*A*) *In situ* hybridization using the antisense SCCA1 cRNA probe. Positive signals were localized in the suprabasal spinous layer, but not in the supracorneal and basal layers. (*B*) *In situ* hybridization with a sense SCCA1 cRNA probe. No positive signal was observed. *Scale bars*: 100 μm.

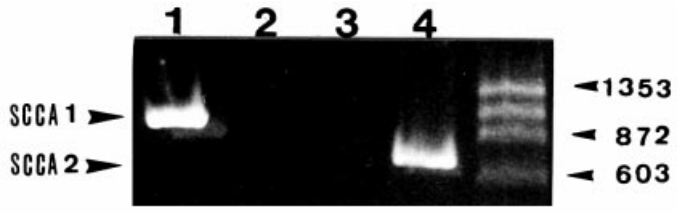


Figure 5. Distinctive amplification for SCCA1 and SCCA2. Specificity of cDNA amplification was tested for SCCA1 (*lanes 1* and *2*) and SCCA2 (*lanes 3* and *4*) by PCR. PCR was performed using P1/P10 (*lanes 1* and *3*) and P5/P11 (*lanes 2* and *4*) primer pairs, respectively. Each template concentration (*pGEMT-SCCA1* or *pGEMT-SCCA2*) was 2 ng. The right lane shows molecular weight markers (ϕX174RT DNA/*Hae*III fragments).

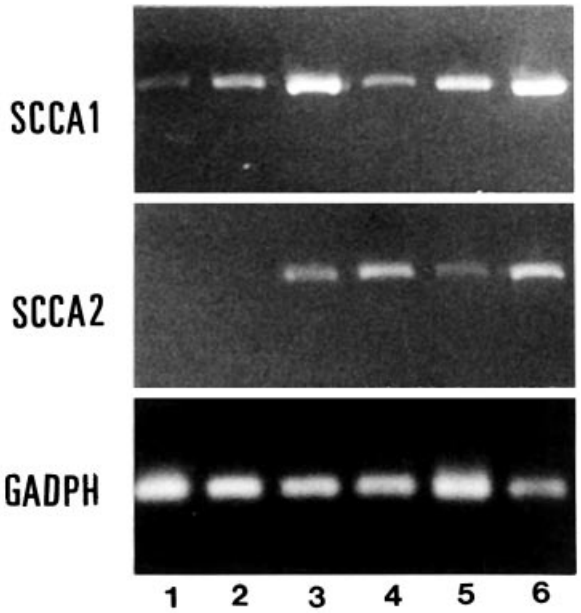


Figure 6. RT-PCR analysis of SCCA mRNA expression. Expression of SCCA1 and SCCA2 mRNA in psoriatic skin as well as normal tissues was analyzed by RT-PCR. *Lanes 1*, *2*, normal skin tissues; *lanes 3–6*, psoriatic skin tissues from four patients.

Comparison for tissue levels of mRNA expression between SCCA1 and SCCA2 We found a reliable method of distinguishing SCCA1 and SCCA2. Using this method, we tried to analyze tissue levels of mRNA expression in normal and psoriatic skin. mRNA concentrations were determined as follows. cDNA fragments diluted to various concentrations were resolved by electrophoresis on 1.5% agarose gel and photographed on Polaroid 665 Negative Film. The density measurement demonstrated a linear correlation for the amount of DNA loaded on the gel in the range between 10 and 200 ng. The results of RT-PCR analysis using total RNA obtained from skin tissues of normal and psoriatic patients are shown in **Fig 6**. SCCA1 and SCCA2 mRNA expression can be easily distinguished using these specific primer pairs. Semiquantitative assessment of amplified RT-PCR products of SCCA mRNA expression revealed that SCCA1 mRNA was ubiquitously expressed in all normal skin, and significantly overexpressed in psoriatic skin tissues (**Fig 7**). On the other hand, the expression of SCCA2 mRNA was specific to psoriatic skin tissues. None of the normal skin samples showed any positive band by RT-PCR. Although psoriatic epidermis showed different staining patterns depending on the serum SCCA levels, the ratio between SCCA1 and SCCA2 mRNA expression did not show significant changes.

DISCUSSION

SCCA contains two homologs, SCCA1 and SCCA2, which belong to the high molecular mass serine proteinase inhibitor superfamily, serpin (Suminami *et al*, 1991; Barnes and Worrall, 1995; Schneider *et al*, 1995). Although they are nearly identical (92% identical in nucleotide level), characteristic features of these molecules are unique inhibition spectra against proteolytic enzymes. SCCA1 is very different from other serpins and inhibits papain-like cysteine proteinases, cathepsins K, L, and S (Takeda *et al*, 1995; Schick *et al*, 1998). SCCA2 shows ordinary serpin-like properties and inhibits chymotrypsin-like serine proteinases, cathepsin G and mast cell chymase (Schick *et al*, 1997).

Cathepsin L is one of the lysosomal acid proteinases recently identified in psoriatic epidermis (Kawada *et al*, 1997). The active mature form of cathepsin L was confirmed by immunohistochemistry and Western blot analysis. Also, an increased number of mast cells was always noticed in the affected skin and various proteinases including tryptase, chymase, and cathepsin G are supposed to be released by degranulation (Harvima *et al*, 1990; Schechter *et al*, 1990). Indeed, chymase activity was detected in the papillary dermis of the psoriatic lesion (Harvima *et al*, 1993). Our immunoelectron microscopy study demonstrated that SCCAs were predominantly present along the periphery of the intercellular space in the upper spinous cell layer of psoriatic epidermis from patients with a high serum SCCA level. In addition, SCCA immunoreactivity was detected around the degranulated cells near the

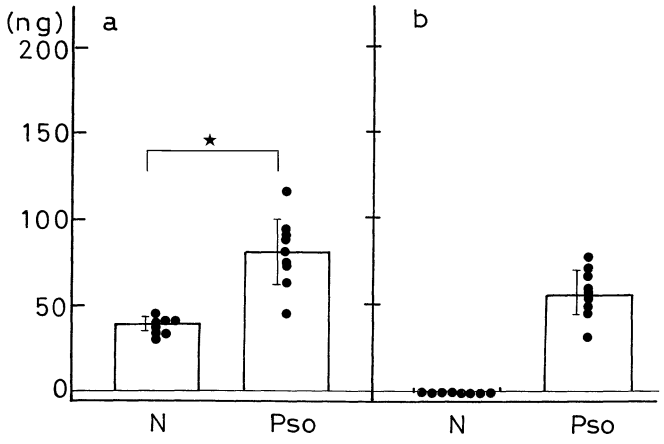


Figure 7. Semiquantitation of SCCA1 and SCCA2 mRNA expression in psoriatic skin tissues. The intensity of the DNA band was analyzed by densitometry. *Bar heights* and *error bars* depict means ± SEM. Individual values are shown as solid dots. (*a*) Comparison of SCCA1 mRNA expression between normal tissues (N, $n = 8$) and psoriatic tissues (Pso, $n = 9$) (*asterisk* indicates $p < 0.01$). (*b*) SCCA2 mRNA expression. Data are expressed as nanograms of DNA per 10 μl of reaction mixture.

dermo-epidermal junction as well as in the granules of filtrated cells. Collectively, these findings strongly suggest that SCCAs play a protective role against various proteinases in pathophysiologic events of psoriasis.

Strong positive staining for SCCAs was also found in nuclei of granular layer cells and a considerable number of cells in the elongated rete ridges, although we could not find any known nuclear localization signal in these molecules. The presence of SCCAs in nuclei was confirmed by immunoelectron microscopy. Nuclei for SCCA-positive cells were intact and did not show any morphologic changes. Although we tried to obtain specific antibodies against SCCA1 or SCCA2, none of the preparations contained monospecific antibody. Antibodies including peptide antibodies directed against the respective reactive site sequences also reacted with both SCCAs. Western blot analysis of the extracts from psoriatic skin tissues demonstrated a band of approximately M_r 45 kDa, which corresponds to the SCCA proteins (data not shown). It is unlikely that other proteins cross-reacting with antibodies against SCC in nuclei are present. At this point, we do not know which SCCA is localized in the nuclei of psoriatic epidermis. It is highly probable that SCCAs work as proteinase inhibitors in psoriatic skin. Localization of SCCA in nuclei seems to suggest a novel pathologic function in psoriatic keratinocytes, however. Interestingly, primary structures of SCCAs indicate that they are involved in the subfamily "ovserpin" (Schneider *et al*, 1995). Ovserpin includes ovalbumin, plasminogen activator inhibitor 2, maspin, and CrmA (Potempa *et al*, 1994). Of these, CrmA is known as the inhibitor of apoptosis (Miura *et al*, 1993). Although CrmA belongs to the ovserpin, it inhibits specific cysteine proteinases, caspases, executors of apoptosis (Ray *et al*, 1992; Komiyama *et al*, 1994). Recently, overexpression of SCCA1 in cancer cells contributed to their survival by mediating protection from apoptosis (Suminami *et al*, 2000). It would be interesting to see whether SCCAs play a role in apoptotic processes in skin.

In this study, SCCA mRNA was detected only in the spinous layer of psoriatic epidermis but not in normal epidermis by *in situ* hybridization. On the other hand, it has been reported that SCCA mRNA is localized in the basal and suprabasal layers of normal squamous epithelium in human gynecologic tissues (Takeshima *et al*, 1992). This discrepancy might be explained by the differences in environmental conditions in psoriatic epidermis. Numa *et al* (1996) showed that tumor necrosis factor α stimulated the production of SCCA in normal human epidermal keratinocytes. Tumor necrosis factor α has been considered to be one of the modulators related to the promotion of inflammatory cells in psoriatic skin (Kristensen *et al*, 1993). Overexpression of SCCA mRNAs would probably be due, at least in part, to the production of cytokines in psoriatic tissues.

By use of specific primer sets, we have succeeded in selectively amplifying SCCA1 and SCCA2. The SCCA1 transcript was ubiquitously expressed in all skin tissues tested, although we have not detected any immunoreactive SCCA in sera and skin tissues from normal individuals. This is probably due to the low expression level of SCCA1 in normal tissues, as an enhanced immunohistochemical method using True Blue® showed weak but considerable staining in the granular layer of normal epidermis (unpublished data). We observed considerable upregulation of SCCA1 mRNA in psoriatic skin compared to normal skin. In contrast, none of the normal skin tissues showed any expression of SCCA2 mRNA. SCCA2 mRNA was exclusively expressed in psoriatic skin. These pieces of evidence suggest that in psoriatic epidermis SCCA1 is overexpressed and SCCA2 is newly transcribed and is absent in normal epidermis.

In this study, we showed expression and localization of SCCAs in psoriatic skin by means of *in situ* hybridization, immunohistochemistry, and immunoelectron microscopy. In addition, RT-PCR analyses using specific primers revealed, for the first time, the presence of SCCA2 in psoriatic skin.

The authors thank Ms. T. Yamamoto, Mr. T. Suzuki, and Ms. M. Nakano for their technical assistance.

REFERENCES

Baker BS, Swain AF, Valdimarsson H, Fry L: T-cell subpopulations in the blood and skin of patients with psoriasis. *Br J Dermatol* 110:37–44, 1984

Barnes RC, Worrall DM: Identification of a novel human serpin gene: cloning, sequencing and expression of leupin. *FEBS Lett* 373:61–65, 1995

Beurskens T, Chang A, van Erp PEG, van de Kerkhof PCM: Epidermal proliferation and accumulation of polymorphonuclear leukocytes in the psoriatic lesion. *Dermatologica* 178:67–72, 1989

Braun-Falco O, Schmoeckel C: The dermal inflammatory reaction in initial psoriatic lesions. *Arch Dermatol Res* 258:9–16, 1977

Braverman IM, Sibley J: Roles of the microcirculation in the treatment and pathogenesis of psoriasis. *J Invest Dermatol* 78:12–17, 1982

Duk JM, von Voorst Vader PC, Ten Hoor KA, Hollema H, Doeglas HMG, de Bruijn HWA: Elevated levels of squamous cell carcinoma antigen in patients with a benign disease of the skin. *Cancer* 64:1652–1656, 1989

Fräki JE, Hopsu-Havu VK: Human skin proteases. Fractionation of psoriasis scale proteases and separation of a plasminogen activator and a histone hydrolyzing protease. *Arch Dermatol Res* 256:113–212, 1976

Hamanaka S, Ujihara M, Numa F, Kato H: Serum level of squamous cell carcinoma antigen as a new indicator of disease activity in patients with psoriasis. *Arch Dermatol* 133:393–395, 1997

Harvima IT, Naukkarinen A, Harvima RJ, Aalto ML, Neittaanmäki H, Horsmanheimo M: Quantitative enzyme histochemical analysis of tryptase- and chymase-containing mast cells in psoriatic skin. *Arch Dermatol Res* 282:428–433, 1990

Harvima IT, Schwartz LB, Horsmanheim M: Mast cell tryptase and chymase in developing and mature psoriatic lesions. *Arch Dermatol Res* 285:184–192, 1993

Hibino T: Purification and characterization of keratin hydrolase in psoriatic epidermis: application of keratin-agarose plate and keratin-polyacrylamide enzymography methods. *Anal Biochem* 147:342–356, 1985

Hibino T, Izaki S, Izaki M: Detection of serine proteinase inhibitors in human cornified cells. *Biochem Biophys Res Commun* 101:943–955, 1981

Hibino T, Izaki S, Izaki M: Keratin-hydrolyzing enzyme in psoriatic epidermis. *FEBS Lett* 208:273–277, 1986

Hibino T, Izaki S, Kimura H, Izaki M, Kon S: Partial purification of plasma and tissue kellikrein in psoriatic epidermis. *J Invest Dermatol* 90:505–510, 1988a

Hibino T, Izaki S, Ohkuma M, Kon S, Thörsen S, Åstedt B: Epidermal plasminogen activator inhibitor (API) is immunologically identical to placental-type PAI-2. *FEBS Lett* 231:202–206, 1988b

Hopsu-Havu VK, Jöronen IJ, Järvinen M, Rinne A: Cysteine proteinase inhibitors in psoriatic epidermis. *Arch Dermatol Res* 275:305–309, 1983

Järvinen M: Purification and some characterization of the human epidermal SH-protease inhibitor. *J Invest Dermatol* 71:114–118, 1978

Järvinen M, Rinne A, Hopsu-Havu VK: Partial purification and some properties of a new papain inhibitor from psoriatic scales. *J Invest Dermatol* 82:471–467, 1984

Kawada A, Hara K, Kominami E, Hiruma M, Noguchi H, Ishibashi A: Processing of cathepsin L, B and D in psoriatic epidermis. *Arch Dermatol Res* 289:87–93, 1997

Kitagawa T, Kawasaki T, Munechika H: Enzyme immunoassay of blasticidin S with high sensitivity: a new and convenient method for preparation of immunogenic (hapten-protein) conjugates. *J Tokyo (Tokyo)* 92:585–590, 1982

Komiyama T, Ray CA, Pickup DJ, Howard AD, Thornberry NA, Peterson EP, Salvesen G: Inhibition of interleukin-1β converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J Biol Chem* 269:19331–19337, 1994

Kristensen M, Chu CQ, Eedy DJ, Feldmann M, Brennan FM, Breathnach SM: Localization of tumor necrosis factor alpha (TNF-α) and its receptors in normal and psoriatic skin: epidermal cells express the 55-kD but not the 75-kD TNF receptor. *Clin Exp Immunol* 94:354–356, 1993

Lee CC, Wu X, Gibbs RA, Cook RG, Muzhy DM, Caskey CT: Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase. *Science* 239:1288–1291, 1988

Miura M, Zhu H, Rotello R, Hartwig EA, Yuan J: Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* 75:653–660, 1993

Numa F, Takeda O, Nakata M, *et al*: Tumor necrosis factor-α stimulates the production of squamous cell carcinoma antigen in normal squamous cells. *Tumor Biol* 17:97–1015, 1996

Ohtani O, Fukuyama K, Epstein WL: Biochemical properties of thiol proteinase inhibitor purified from psoriatic scales. *J Invest Dernatol* 82:280–284, 1982

Potempa J, Korzus E, Travis J: The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J Biol Chem* 269:15957–15960, 1994

Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ: Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β-converting enzyme. *Cell* 69:597–604, 1992

Scheher NM, Irani A-MA, Sprows JL, Abernethy J, Wintroub B, Schwartz LB: Identification of a cathepsin G-like proteinase in the MC_{TC} type of human mast cells. *J Immunol* 145:2652–266139, 1990

Schick C, Kamachi Y, Bartuski AJ, Cataltep S, Schecher NM, Pemberman PA, Silverman GA: Squamous cell carcinoma antigen 2 is a novel serpin that inhibits the chymotrypsin-like proteinases cathepsin G and mast cell chymase. *J Biol Chem* 272:1849–1855, 1997

Schick C, Pemberton PA, Shi GP, et al: Cross-class inhibition of the cysteine proteinases cathepsin K, L, and S by the serpin squamous cell carcinoma antigen 1: a kinetic analysis. *Biochemistry* 37:5258–5266, 1998

Schneider SS, Schick C, Fish E, *et al*: A serine proteinase inhibitor locus at 18q21.3 contains a tandem duplication of the human squamous cell carcinoma antigen gene. *Proc Natl Acad Sci USA* 92:3147–3151, 1995

Sumi H, Muramatsu M, Fujii S: Relation of C1s, a subunit of the activated first component, to other plasma enzymes. *Biochim Biophys Acta* 327:207–221, 1983

Suminami Y, Kishi F, Sekiguchi K, Kato H: Squamous cell carcinoma antigen is a new member of the serpin proteinase inhibitors. *Biochem Biophys Res Commun* 181:51–58, 1991

Suminami Y, Nagashima S, Vujanovic NL, Hirabayashi K, Kato H, Whiteside TL: Inhibition of apoptosis in human tumour cells by the tumour-associated serpin, SCC antigen-1. *Bri J Cancer* 82:981–989, 2000

Takahashi T, Hibino T, Horie I, Takeda A: Cathepsin L specific high molecular weight cysteine proteinase inhibitor (psoriastatin) from psoriatic scales. *J Dermatol Sci* 6:57, 1993

Takeda A, Kaji H, Nakaya K, Nakamura Y, Samejima T: Comparative studies on the primary structure of human cystatin As form epidermis, liver, and leukocytes. *J Biochem (Tokyo)* 105:986–991, 1986

Takeda A, Yamamoto T, Nakamura Y, Takahashi T, Hibino T: Squamous cell carcinoma antigen is a potent inhibitor of cysteine proteinase cathepsin L. *FEBS Lett* 359:78–80, 1995

Takeshima N, Suminami Y, Takeda O, Abe H, Okuno N, Kato H: Expression of mRNA of SCC antigen in squamous cells. *Tumor Biol* 13:338–342, 1992

Toruniowa B, Jablonska S: Mast cell in the initial stages of psoriasis. *Arch Dermatol Res* 280:189–198, 1988

Weinstein GD, McCullough JL, Ross PA: Cell kinetic basis for pathophysiology of psoriasis. *J Invest Dermatol* 85:579–583, 1985