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# Development and application of monoclonal antibodies against SKALP/elafin and other trappin family members

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*Abstract* **SKALP/elafin is an epithelial proteinase inhibitor with antimicrobial properties that is not normally expressed in human epidermis, but is induced under inflammatory conditions and in some types of skin cancer. SKALP is a member of the recently described trappin gene family, which encodes a new class of proteins, characterized by a four-disulphide core and a transglutaminase substrate domain. Polyclonal antisera against SKALP have been shown to be useful for monitoring disease activity in psoriasis and tumour differentiation in squamous cell carcinoma. We developed ten different mouse monoclonal antibodies (mAbs) against synthetic peptides corresponding to a hexapeptide epitope in the transglutaminase substrate domain and three mAbs recognizing an epitope in the proteinase-inhibiting domain. The antibodies could be used with high specificity by immunohistochemistry on formalin-fixed tissue, by affinity chromatography, by Western blotting, and by enzyme-linked immunoadsorbent assay (ELISA) for the detection of SKALP/elafin. These antibodies have several advantages over existing polyclonal antisera, such as a defined epitope, the detection of full-length SKALP/elafin and unlimited supply. An antibody against the hexapeptide epitope, which is common to all known human, simian, bovine and swine trappin family members, was used to immunolocalize bovine trappins expressed in trachea, that have recently been discovered. These mAbs will serve as important new tools to measure SKALP/elafin and trappin family members in research and diagnostics.**

**Keywords** Trappin-2 · Psoriasis · SKALP · Monoclonal antibody · Squamous cell carcinoma

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## Introduction

Skin-derived antileukoproteinase (SKALP) [1], otherwise known as elafin [2] or ESI (elastase-specific inhibitor) [3], is a proteinase inhibitor with specificity for elastase and proteinase-3 derived from polymorphonuclear leukocyte (PMN) [4]. SKALP is structurally related to secretory leukocyte proteinase inhibitor (SLPI) [5], also known as antileukoproteinase (ALP) [6], and both these proteins have recently been shown to possess antimicrobial activity [7, 8, 9]. SKALP is absent from normal skin whereas SLPI is only expressed in the stratum granulosum; however, both are expressed at high levels in psoriatic epidermis and several other inflammatory skin diseases [2, 7, 10, 11]. SKALP protein is found in psoriatic epidermis as a short polypeptide of 6 kDa originally described as elafin [2]. The full-length protein, as expressed in cultured epidermal keratinocytes, is translated as a 12.3-kDa protein of 117 amino acids termed pre-elafin or trappin-2 [12]. Cleavage of the signal peptide yields a mature protein of 95 amino acids with a calculated molecular mass of 9.9 kDa. In addition to the known proteinase inhibitor domain, the mature protein contains a domain with four repeats which are homologous with putative transglutaminase substrate motifs [13]. Recently several other proteins have been found with a transglutaminase substrate domain and a four-disulphide core and these are now known as the trappin gene family [14, 15]. The 9.9-kDa secreted protein is the major form found in cultured cells [12].

Once secreted in the epidermis, full-length SKALP is presumably crosslinked by transglutaminase to other proteins, and subsequently the 6-kDa form is clipped and reaches the circulation where it is cleared via the kidney [16]. SKALP is constitutively expressed by a number of epithelia [17] but is induced in skin upon inflammation. Therefore, SKALP is considered to be a clinically rele-

vant and useful marker for psoriasis and other inflammatory diseases [18]. Expression of this protein by keratinocytes in vitro has been shown to be inducible by TNF-alpha and serum, thereby transforming keratinocytes into a 'psoriasis-like' state [19]. The selection of drugs that attenuate or modulate this psoriatic-like transformation may be critical in psoriasis pharmacological research [20]. Therefore, antibodies to quantify SKALP in culture supernatants or in serum from psoriasis patients are useful tools to evaluate the potency of new antipsoriatic drugs. In addition to its use in psoriasis research, SKALP has been shown to be a useful marker in the histological grading of squamous cell carcinoma [21, 22]. So far, only polyclonal antibodies against the recombinant C-terminal 57 amino acids of SKALP, or synthetic peptides have been generated. We now describe the generation of monoclonal antibodies (mAbs) against different epitopes that will serve as an unlimited supply for research and diagnostics.

## Material and methods

### Chemicals

All reagents for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Novex (Invitrogen, Groningen, The Netherlands). Rabbit anti-mouse Ig conjugated with HRP was obtained from DakoPatts (Glostrup, Denmark). Columns and materials for chromatographic purification of anti-SKALP mAbs were obtained from Pharmacia (Uppsala, Sweden). Purified TRAB/2O mAb was biotinylated according to the manufacturer's instructions (Roche Diagnostics, Belgium). Synthetic peptides were obtained from Ansynth (Roosendaal, The Netherlands).

### Polyclonal antisera

The antisera were raised against the 6-kDa form of SKALP/elafin, which lacks the transglutaminase substrate domain. We used both recombinant elafin (a kind gift from Dr. N. Russell, ICI, UK) and SKALP purified from psoriatic scales. Protein (50 μg) was cross-linked with glutaraldehyde, dialysed against distilled water and emulsified with Freund's complete adjuvant. Rabbits were immunized intracutaneously in the back, followed by two subcutaneous boosters after 2 and 4 weeks. Preimmune serum was drawn before the experiments and immune serum was collected after 6 weeks.

### Generation of mAbs

Balb/c mice were primed with two different peptides in complete Freund's adjuvant. The first synthetic peptide comprised amino acids 66 to 82: AQEPVKGPVSTKPGSC (SKALP peptide-1) corresponding to the first amino acids of mature elafin. The second peptide for immunization contained a GQDPVK motif, which is common to all known trappin family members: GQDPVKGQDPVKGQDPVK (SKALP peptide-2). The peptides were prepared by coupling the peptides via a COOH-terminal cysteine residue to Keyhole Limpet Hemocyanin (KLH), using the sulphosuccinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (sulpho-SMCC) procedure according to the manufacturer's instructions (Pierce, Rockford, Ill.).

Mice were boosted every 3 weeks with peptides in incomplete Freund's adjuvant. On days 4 and 3 before fusion, mice were boosted intraperitoneally with 100 μg SKALP peptides in saline. Mouse spleen cells were fused with SP2/0 cells using the procedure of Kohler et al. [23] with modification. The hybridomas were seeded in 25 × 96-well plates and screened after 10 days in a direct ELISA on KLH-free SKALP peptides 1 and 2. Positive cells were immediately subcloned and frozen in liquid nitrogen.

All hybridomas were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, sodium pyruvate (1 m*M*), L-glutamine (2 m*M*), penicillin (100 U/ml) and streptomycin (50 μg/ml). All products were purchased from Life Technologies (Paisley, UK). Cells were incubated in an incubator containing a humidified atmosphere comprising 8% $CO_2$ in air.

### Antibody characterization

The screening ELISA used for the detection of anti-SKALP peptide-1 antibodies was a direct ELISA with 3 μg/ml SKALP peptide-1 coated overnight at 4 °C in NUNC (Life Technologies) U-bottomed high-binding 96-well microtitre plates in 50 μl/well coating buffer (10 m*M* Tris, 10 m*M* NaCl, and 10 m*M* $NaN_3$, pH 8.5). The next day, the plates were coated with 75 μl/well of 0.1% casein in PBS for 60 min at 37 °C to reduce nonspecific binding. Next, 50 μl hybridoma supernatant was added followed by incubation for 1 h at 37 °C. After washing, the bound mAbs were detected with 50 μl/well of rabbit HRP-conjugated anti-mouse Ig for 1 h at 37 °C. Both reagents were diluted in 0.1% casein/PBS. The plates were washed and 50 μl of a solution of 0.42 m*M* 3,5,3′,5′-tetramethyl-benzidine, 0.003% (v/v) $H_2O_2$ in 100 m*M* citric acid and 100 m*M* disodium hydrogen phosphate (pH 4.3) was added as the substrate. The reaction was allowed to proceed for maximum 15 min on a plate shaker at room temperature, after which the colour development was stopped with 2 *N* $H_2SO_4$, 50 μl/well and the plates were read on a Thermomax (Molecular Devices, Sunnyvale, Calif.) microtitre plate reader at 450 nm. The crossreactivity of the selected mAbs with recombinant SKALP was tested in a direct ELISA, identical to the screening assay, except that recombinant SKALP was used instead of peptide-1 SKALP. In a second step, the selected positive cultures were confirmed on Western blotting with recombinant SKALP and psoriatic scale extracts.

The screening ELISA used for the detection of anti-SKALP peptide-2 antibodies was a sandwich ELISA system with the anti-SKALP peptide-1 mAb TRAB/2F in the coating phase. This $IgG_{2b}$ mAb shows a high binding affinity for recombinant and native human SKALP in keratinocyte culture supernatant. TRAB/2F was coated overnight at 5 μg/ml, identical to the ELISA for the detection of antibodies against peptide-1 SKALP. After coating with casein to reduce nonspecific binding, 50 μl of native full-length SKALP was incubated for 2 h at 37 °C. Next, 50 μl hybridoma supernatant was added followed by incubation for 1 h at 37 °C. After washing, the bound mAbs were detected with a mixture of biotinylated goat anti-mouse $IgG_1$ and biotinylated goat anti-mouse $IgG_{2a}$ antibodies for 1 h at 37 °C. The plates were then washed five times and incubated for 30 min at 37 °C with HRP-conjugated streptavidin. Plates were further processed exactly as in the screening assay for SKALP peptide-1.

### Sandwich ELISA with two mAbs for SKALP detection

The ELISA for the measurement of SKALP levels in culture supernatants of keratinocyte monolayers, serum from psoriasis patients or control serum was performed as follows. Briefly, TRAB/2F antibody was coated at 5 μg/ml overnight at 4 °C in NUNC flat-bottomed high-binding 96-well microtitre plates in 100 μl/well coating buffer. The next day, plates were overcoated and incubated with 100 μl/well of sample for 2 h at 37 °C. The plates were washed and then incubated with 100 μl/well of biotinylated detecting antibody TRAB/2O at 2 μg/ml for 1 h at 37 °C. The plates were washed five times and incubated for 30 min at 37 °C with HRP-conjugated streptavidin. Plates were further processed exactly as in the screening assay for SKALP peptide-1.

### Extraction of SKALP from psoriatic scales

Scales from psoriatic patients were homogenized in a glass-glass grinder in distilled water. After centrifugation (15 min at 12000 *g*) the supernatant was boiled and again centrifuged. The supernatant was concentrated by vacuum evaporation and subjected to SDS-PAGE and immunoblotting as described below.

### Preparation of affinity-purified full-length human SKALP

TRAB/2F anti-SKALP mAb purified from serum-free culture supernatant on protein G (Pharmacia, Uppsala, Sweden) was coupled to cyanogen bromide-activated Sepharose (Pharmacia, Uppsala, Sweden) (5 mg antibody per 1 g Sepharose) using the method recommended by the manufacturer. Culture supernatant from normal human keratinocytes stimulated for 48 h with 5% fetal calf serum was diluted 1:2 in 0.1 *M* phosphate buffer (pH 8.5) and applied to the column. The column was washed with 0.1 *M* phosphate, and SKALP was eluted with 0.1 *M* glycine (pH 2.5) and neutralized immediately with concentrated Tris buffer. This yielded highly purified SKALP as shown by SDS-PAGE on 20% gels.

### SDS-PAGE and Western blotting

Psoriatic scale extract, affinity-purified normal human native SKALP and purified recombinant SKALP were boiled in reducing SDS sample buffer containing DTT. Psoriatic scale extract (10 μg per lane), purified recombinant SKALP (5 ng per lane) and affinity-purified normal human native SKALP (5 ng per lane) were run on precast 10–20% gradient gels (NOVEX, Invitrogen, Groningen, The Netherlands) and semidry blotted to 0.22 μm PVDF sheets for 30 min at 1 mA per $cm^2$. The blotting buffer composition was 1.3 m*M* SDS, 39 m*M* glycine, 48 m*M* Tris, pH 9.2, and 20% (v/v) methanol. A biotinylated low molecular weight protein ladder was used as molecular weight standard. The membranes were blocked with 5% (w/v) nonfat dried milk (Bio-Rad) in PBS for 1 h. Next they were incubated with the appropriate mAb at 1 μg/ml overnight at 4 °C. The membranes were then washed in PBS/0.1% Tween 20 for 5 min with five changes of buffer, incubated for 1 h with a HRP-conjugated goat anti-mouse antibody (New England Biolabs, Beverly, Mass.) at a dilution of 1 : 2000 for 1 h at room temperature. After washing, the bands of interest were visualized by chemiluminescence detection according to the manufacturer's instructions. Scans were taken with a Lumi-Imager (Roche, Boehringer Mannheim, Germany). Aurodye staining was performed using an AuroDye Forte Kit (RPN 490; Amersham Life Science, Little Chalfont, UK).

### Patients

The serum examined was from patients who had participated in a previously described clinical trial [24] in which a correlation was found between the psoriasis area and severity index (PASI) score and serum SKALP levels using a sandwich-type ELISA with polyclonal serum. Serum was collected from psoriasis patients before and after therapy with the retinoic acid analogue etretinate (two patients) or with liarozole, an inhibitor of endogenous retinoid metabolism (one patient). The PASI score was taken at the start and the end of treatment.

### Tissue samples and histology

Punch biopsies (4 mm) were taken from psoriatic patients under local anaesthesia, and were fixed in buffered formalin for 4 h and processed for routine histology. Permission of the local medical ethics committee was obtained. Bovine trachea was obtained from a local slaughterhouse and tissue samples were processed for histology as described above.

Paraffin sections (5 μm) were made and were used for immunostaining according to standard protocols, i.e. sections were deparaffinized, rehydrated, preincubated with 20% normal rabbit serum in PBS for 15 min, and incubated with the different anti-SKALP mAbs at a dilution of 1 : 10 to 1:100 for 60 min in PBS with 1% BSA. After incubation with HRP-labelled peroxidase-conjugated rabbit anti-mouse antibody, the sections were rinsed in PBS and sodium acetate buffer, and developed using aminoethyl carbazole as the chromogenic substrate. Counterstaining with haematoxylin was performed, and sections were dried and embedded in glycerol-gelatin solution.

## Results

### MAb production

#### *SKALP-specific peptide-1 antibodies*

A panel of six mice was immunized with 100 μg of KLH coupled SKALP-peptide-1. One mouse with a serum $IC_{50}$ titre of 1/10,000 was selected for fusion with SP2/0 myeloma cells, and 10 days after fusion, more than 6000 different hybridoma clones were screened in a SKALP peptide-1 solid-phase ELISA. Of these hybridomas, 73 culture wells initially showed a clear positive signal in a SKALP peptide-1 ELISA. These 73 cultures were tested in an IgG-specific recombinant SKALP ELISA and 21 cultures remained positive. Positive supernatants were checked by Western blotting on recombinant SKALP, and 11 wells with a clear positive signal were immediately cloned and recloned once. Finally, 11 stable hybridoma clones were selected, frozen in liquid nitrogen and named TRAB/2A to TRAB/2K (TRAB being an acronym for trappin antibody). The $IgG_{2b}$ mAb TRAB/2F showed the highest affinity for both recombinant and full-length SKALP (not shown) and this antibody was selected for further studies.

#### *GQDPVK motif peptide-2 antibodies*

Another six mice were immunized with 100 μg of KLH coupled SKALP peptide-2. This peptide is a trimer of the hexapeptide GQDPVK, a motif from the transglutaminase substrate domain that is common to all known trappin family members [13, 14]. The spleens from two mice, which showed high serum titres in a sandwich ELISA with native human full-length SKALP, were selected and pooled for fusion with SP2/0 myeloma cells. More than 12,000 different hybridoma clones were screened 10 days after fusion in a sandwich ELISA for the detection of human native full-length SKALP. After screening, 12 of 40 hybridomas which reacted clearly positive in the sandwich ELISA were selected. Of these antibodies, the $IgG_1$ mAb TRAB/2O showed the highest affinity for native human full-length SKALP, and was selected for further studies.

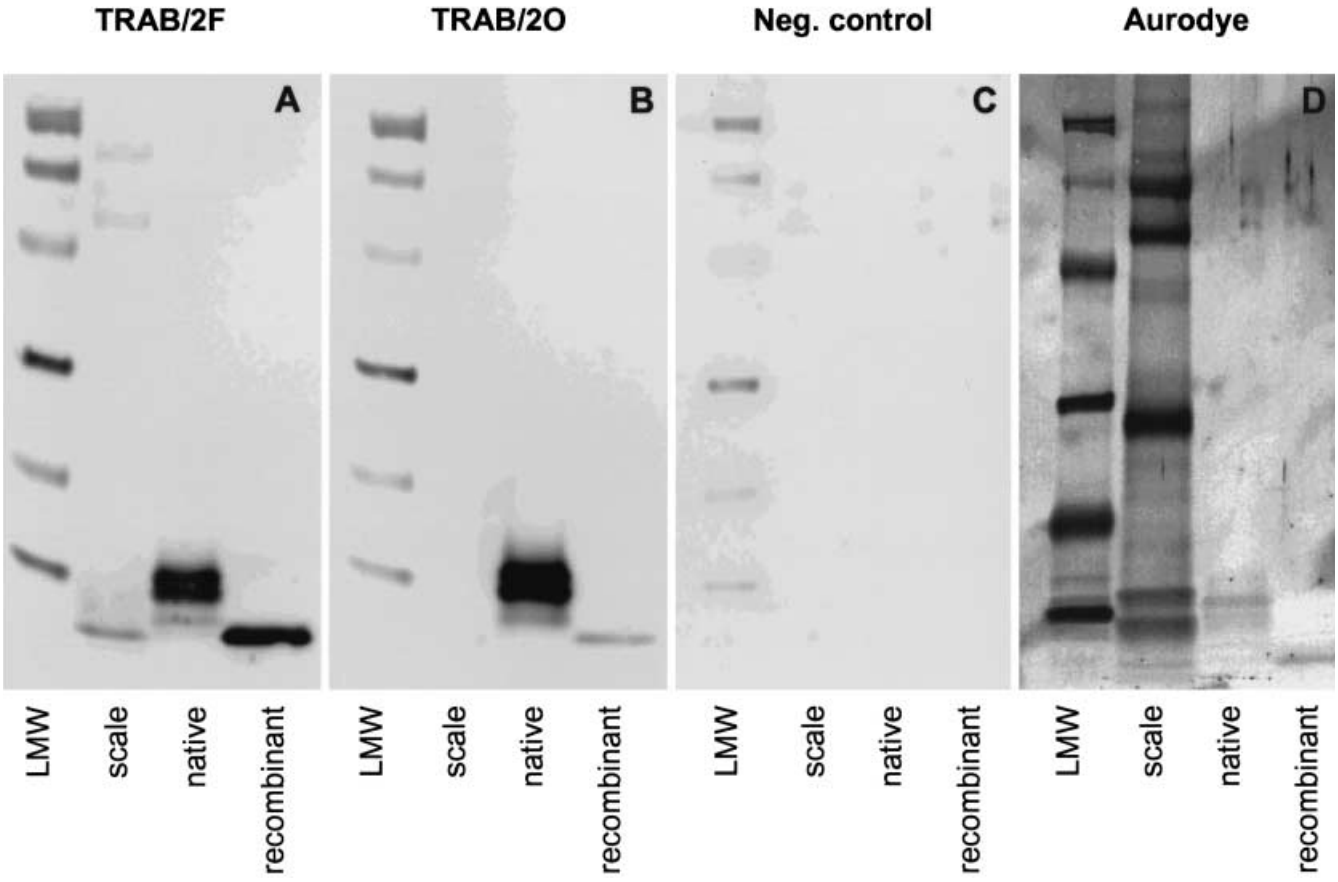


**Fig. 1 A–D** Immunoblots of SKALP preparations and scale extracts probed with mAbs TRAB/2F (**A**) and TRAB/2O (**B**). The immunoblot comprises a low molecular weight-biotinylated marker (*lane 1*), 10 µg/lane psoriatic scale extracts, containing multiple forms of SKALP/elafin (*lane 2*), 5 ng/lane purified human native full-length SKALP (*lane 3*) and 5 ng/lane recombinant SKALP (*lane 4*). The negative control of a duplicate blot of **A** and **B** was probed only with a goat anti-mouse HRP-labelled secondary antibody (**C**). Aurodye staining of a duplicate blot is shown in **D**

## Characterization and application of the mAbs

### *Western blotting*

Western blots of psoriatic scale extracts, recombinant SKALP and human native full-length SKALP were probed with purified TRAB mAbs (TRAB/2O and TRAB/2F) as shown in Fig. 1. Psoriatic scale extracts are known to contain predominantly the truncated forms (6 kDa and smaller) of SKALP in the soluble fraction, presumably leaving the crosslinked portion in the insoluble fraction. Native SKALP purified from cultured keratinocytes contains predominantly the 9.9 kDa form [12], whereas recombinant SKALP contains the C-terminal 6 kDa portion that lacks the transglutaminase substrate domain [25]. TRAB/2F showed a clear signal with recombinant SKALP and a moderate signal with psoriatic scale extracts at the expected molecular weight. This antibody also recognized human native full-length SKALP. In contrast, TRAB/2O mAb reacted strongly with full-length SKALP, but did not recognize the low molecular weight species present in scale extract. TRAB/2O, however, did show a minor reaction with recombinant SKALP, which was probably caused by a weak crossreaction of this mAb with the degenerate motif in the N-terminus of recombinant SKALP.

### *Sandwich ELISA with two mAbs*

Previous studies have used a competitive type ELISA [26] or a sandwich ELISA [24] with two polyclonal antisera to detect SKALP in biological samples such as serum, urine and culture supernatant. Here we used two of our mAbs (TRAB/2O and TRAB/2F) in an ELISA of serum samples from psoriasis patients before and after therapy. Figure 2 shows the calibration curve of the ELISA using purified full-length SKALP as a standard in the range of 100–6000 pg/ml. In this ELISA, SKALP concentrations in serum and culture supernatants can be read with sufficient sensitivity (more than 1 ng/ml) and greater accuracy than in previous assays. The correlation between SKALP levels in the old and new ELISA was good ($r^2 = 0.9$ and $P < 0.002$). Figure 3 confirms our previous finding [24] that serum SKALP levels strongly correlate with the clinical status of a patient (PASI score), and can be used as an objective measure of disease activity.

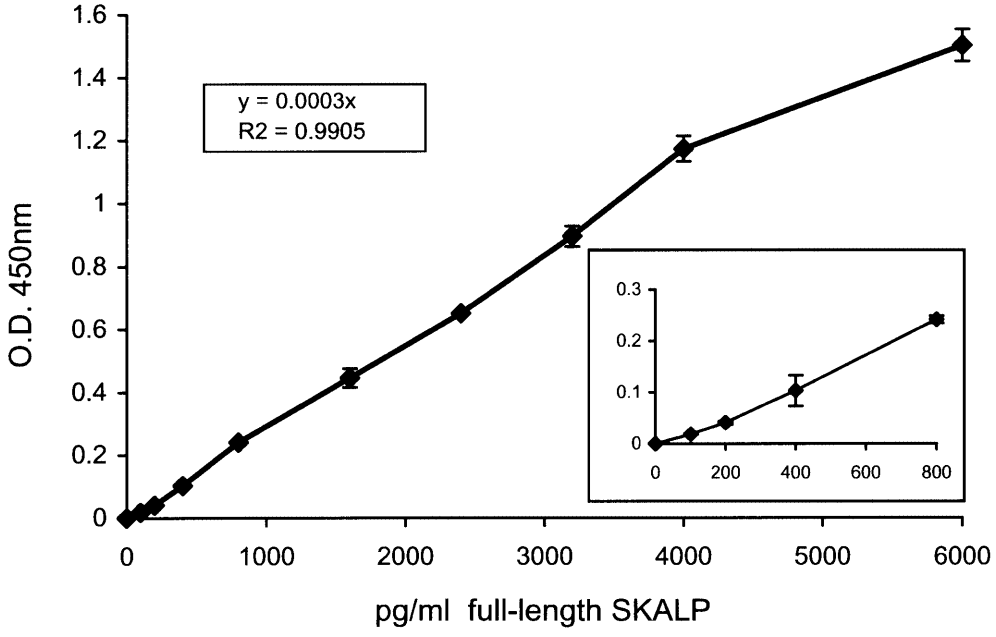


**Fig. 2** A calibration curve of the sandwich ELISA using purified full-length SKALP as a standard diluted in casein. Values are means±SD with $n = 3$

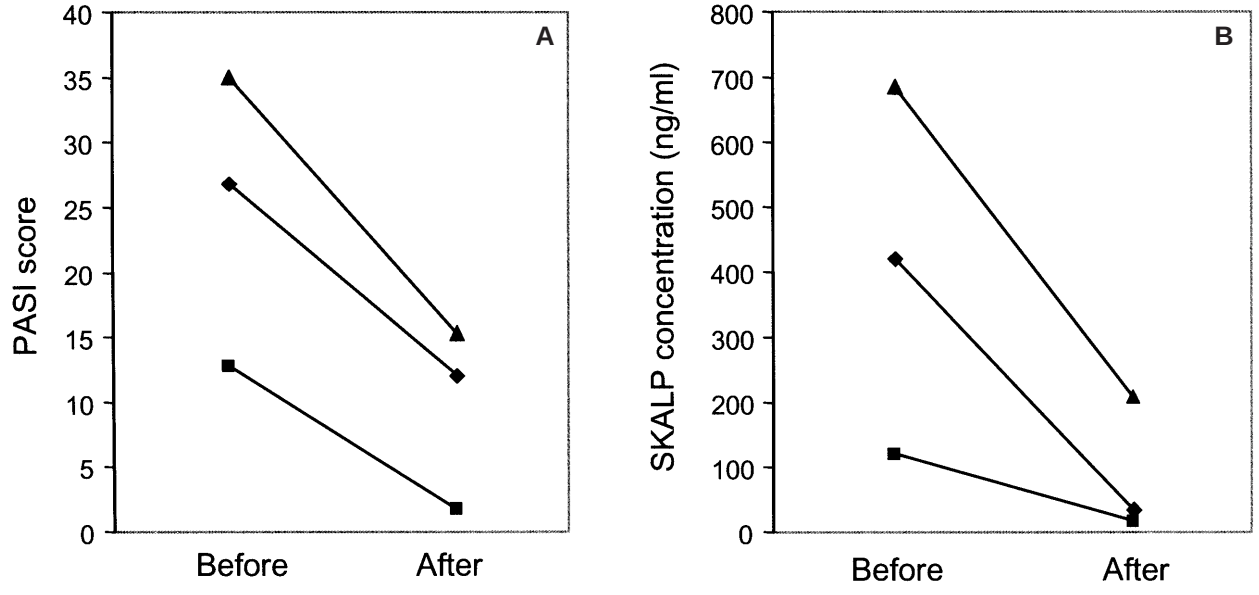


**Fig. 3 A, B** Three severely affected psoriasis patients were treated as indicated in the Materials and methods. Serum was collected before the start of treatment and after 20–24 weeks. Clinical improvement of the patients was scored using the PASI (**A**). Measurement of serum SKALP levels by ELISA (**B**) showed a concomitant decrease with clinical improvement. The same symbols refer to the same patient

*Immunohistochemistry*

All mAbs that showed a strong positive signal in the ELISA were tested on biopsies from psoriatic patients. TRAB/2C, 2E, 2F and 2O were all found to give an epidermal staining pattern that was indistinguishable from the known patterns obtained with polyclonal serum against recombinant material. The mAbs were found to give less background staining than polyclonal serum (not shown). Figure 4 shows the staining patterns obtained with TRAB/2F (Fig. 4 A–C) in human tissues. TRAB/2F is a SKALP-specific antibody and strongly stained the suprabasal layers of psoriatic epidermis (Fig. 4 A) and differentiated cells of a keratoacanthoma (Fig. 4 B). Figure 4C shows small areas of SKALP-positive cornified cells in a moderately differentiated squamous cell carcinoma. TRAB/2O, a mAb that recognizes all known trappins via the GQDPVK epitope was used to stain bovine trachea (Fig. 4 D-E). We have recently shown that in this tissue at least two new trappin family members are present at the mRNA level [14]. In this study glandular tissue (Fig. 4 E) and the ciliated cells of the epithelium (Fig. 4 D) were positive.

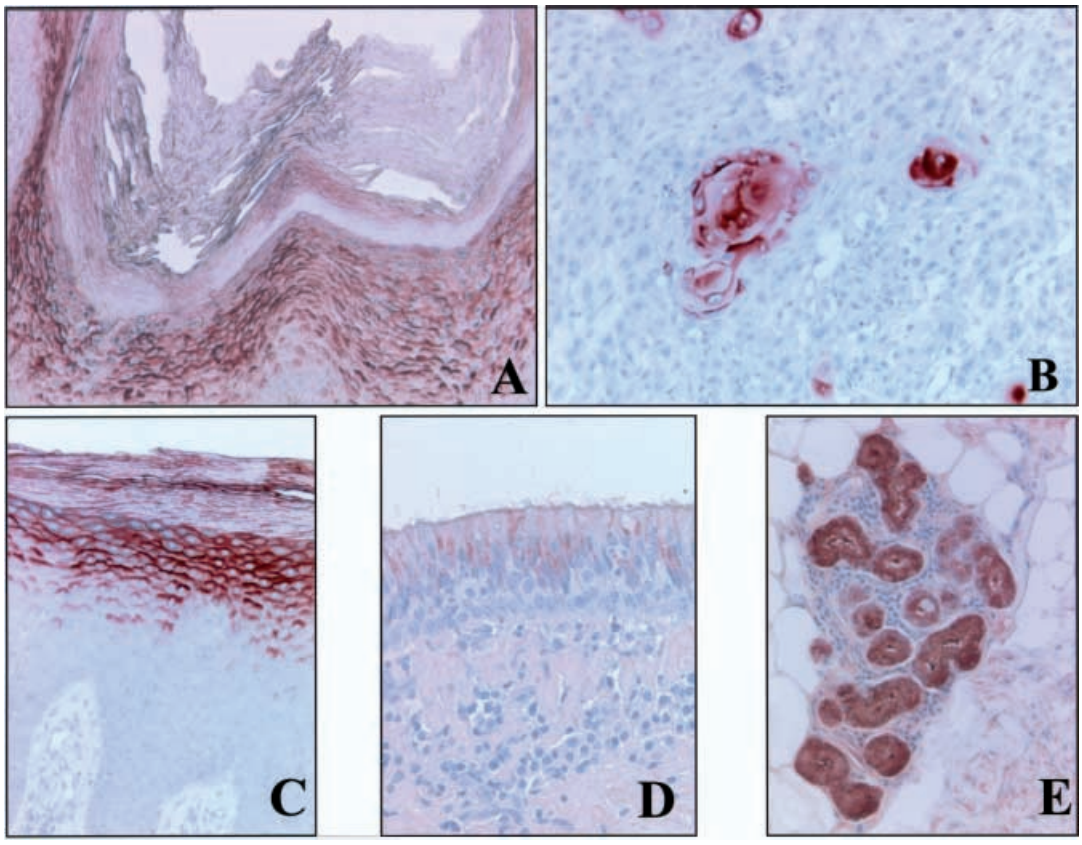


**Fig. 4 A–E** Immunohistochemical staining for SKALP in epithelia. Staining was performed on formalin-fixed paraffin sections using the TRAB2 mAbs. This figure shows the staining patterns obtained with TRAB/2F (**A–C**) in human tissues. TRAB/2F stains the differentiated cells of a keratoacanthoma (**A**) and small areas of cornified cells in a moderately differentiated squamous cell carcinoma (**B**). **C** shows staining of the suprabasal layers of psoriatic epidermis. TRAB/2O, a mAb that recognizes all known trappins via the GQDPVK epitope, was used to examine bovine trachea. Ciliated cells in the covering epithelium (**D**) and glandular cells (**E**) were found to be positive, presumably corresponding to the recently identified trappin-4 and trappin-5

## Discussion

The importance of SKALP/elafin as an epithelial protein implicated in host protection and defence is increasingly recognized [9, 26]. Therefore, antisera that can be used in research and diagnostics are important tools for further study of SKALP and other trappin family members. We have previously described polyclonal antisera that could be used for qualitative and quantitative assays [16, 18]. However, these sera have obvious disadvantages as (a) the epitopes are unknown, (b) they do not react with full-length SKALP, (c) availability is limited and (d) use in species other than humans is limited. Therefore, a strategy was developed to start the production of an unlimited supply of mAbs against SKALP/elafin and other trappin family members. Initial attempts to raise mAbs against recombinant protein failed, perhaps as a consequence of the low immunogenicity of the small 6-kDa protein. We therefore decided to use synthetic peptides coupled to KLH as an immunogen [13].

Because the NMR solution structure of recombinant SKALP/elafin (the 57 C-terminal amino acids comprising the WAP domain) had been published [27], we first focused for the immunization strategy on the most N-terminal sequence of the 57 amino acid form. This N-terminal part has a random conformation, probably because it is already part of the transglutaminase domain of SKALP. A N-terminal peptide (called peptide-1) was designed and applied as immunogen for fusion. MAbs isolated out of this fusion were superior to existing polyclonal sera with respect to specificity, affinity and detection of full-length SKALP/elafin by Western blotting (reducing and nonreducing conditions), ELISA (lower background) and immunocytochemistry (lower background). However, quantification of SKALP protein by ELISA was impossible with a combination of mAbs selected from the peptide-1 fusion. This sandwich ELISA failed probably because of steric hindrance of the antibodies for a random coil peptide that only comprises 16 amino acids that sticks out of the WAP domain. Therefore, a second peptide was chosen for an additional hybridoma fusion. This second peptide for immunization contained a trimer of the GQDPVK sequences, a motif common to all known trappin family members.

The screening strategy for the isolation of mAbs was specifically designed to pick up those that could recognize human native SKALP and its precursors in solution, i.e. as “presented” by a capturing mAb. Whereas antibody pairs from the pool of peptide-1 mAbs did not work in a

sandwich ELISA, this screening with peptide-2 hybridomas yielded clones that worked in combination with TRAB/2F (peptide-1 mAb) as capturing antibody. One of these mAbs called TRAB/20, was selected to develop a sandwich ELISA, based on the use of two mAbs. This ELISA was found to have several advantages over the existing assay based on polyclonal sera, such as reduced assay time, better reproducibility and detection of full-length SKALP. As such, this ELISA is a tool for drug screening with SKALP being a surrogate marker for psoriasis, as shown previously for retinoids [20] in psoriasis. Finally, the development of mAbs against peptide-2 (the common GQDPVK motif) could be useful in the detection of other members of the trappin gene family that has been shown to be extensively diversified in several mammalian species [14, 15]. In addition to their use in research and diagnostics in humans, these antibodies provide tools for further characterization of the new trappin gene family.

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