Identification and Sequence Analysis of Two New Members of the SKALP/elafin and SPAI-2 Gene Family

BIOCHEMICAL PROPERTIES OF THE TRANGLUTAMINASE SUBSTRATE MOTIF AND SUGGESTIONS FOR A NEW NOMENCLATURE

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From the Departments of Dermatology, Cell Biology and Histology, and Biochemistry, Institute of Cellular Signaling, University of Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands

Patrick L. J. M. Zeeuwen, Wiljan Hendriks, Wilfried W. de Jong, and Joost Schalkwijk

The human epithelial proteinase inhibitor SKALP/elafin and the porcine sodium-potassium ATPase inhibitor SPAI-2 are two highly homologous proteins that share an NH₂-terminal transglutaminase substrate domain and a COOH-terminal whey acidic protein (WAP) domain. Here we describe the bovine and simian orthologs of SKALP/elafin as well as two new bovine family members that are designated Trappin-4 and Trappin-5 on the basis of a new nomenclature that we propose (Trappin = TTransglutaminase substrate and WAP motif-containing Protein). Sequence analysis of Trappin-4 and Trappin-5 revealed a domain structure that is very similar to SPAI-2 (Trappin-1) and SKALP/elafin (Trappin-2). The transglutaminase substrate motifs are conserved although the number of repeats varies among species and among family members. The sequence of Trappin-4 and Trappin-5 diverges from Trappin-1 and Trappin-2 at the putative reactive site in the WAP domain. The bovine ortholog of Trappin-2 is expressed in tongue and snout epidermis; Trappin-4 is expressed in trachea, ileum, and tongue; and Trappin-5 is expressed at low levels in trachea, as determined by RNase protection and Northern blot analysis. Based on the analysis of 67 transglutaminase substrate repeats as present in all known Trappin gene family members from four different mammalian species a consensus sequence could be established: Gly-Gln-Asp-Pro-Val-Lys (GQDPVK). Using biotinylated hexapeptide probes we found that the GQDPVK sequence is a very efficient transglutaminase substrate both for guinea pig liver transglutaminase and for epidermal transglutaminase, and it acts as acyl donor as well as acceptor. We propose that the Trappin protein family forms a new group of enzyme inhibitors with various specificities of the WAP domain, which share transglutaminase substrate motifs that can act as an anchoring sequence.

Skin-derived antileukoproteinase (SKALP)¹ (1) and sodium-potassium ATPase inhibitor-2 (SPAI-2) (2) are two molecules that share an NH₂-terminal domain that functions as a transglutaminase (TGase) substrate, and a COOH-terminal whey acidic protein (WAP) domain that harbors an inhibitory activity toward at least two distinct enzymes. Porcine SPAI-2 was the first of these molecules to be described and is expressed mainly in the intestine (3). Human SKALP, otherwise known as elafin (4), or elastase-specific inhibitor (5), is a potent inhibitor of the leukocytic proteinases elastase and proteinase-3 (6, 7). We found that SKALP/elafin is expressed in several human stratifying squamous epithelia, except for epidermis where it is only expressed in the context of inflammation, such as psoriasis or wound healing (8–10). We mapped the genomic localization of human SKALP/elafin to chromosome 20q12–13 (11). Interestingly, this region contains various other genes involved in TGase-mediated cross-linking processes such as the tissue TGase gene (12), epidermal TGase (13, 14), and the genes coding for semenogelin I and semenogelin II (15), which are also epithelial TGase substrates. SKALP/elafin is distinct from SPAI-2 on the basis of its inhibitory activity, the amino acid sequence of the putative active site, and the epithelial expression pattern. The cDNAs and genes for porcine SPAI-2 (2, 3), porcine SKALP/elafin (16), human SKALP/elafin (7, 17), and a new porcine family member (16) that is more similar to SPAI-2 than to SKALP/elafin, have been cloned and revealed a high degree of conservation in the gene structure and the intronic sequences but a strong sequence divergence in the second exon.

The SKALP/SPAI-2 gene family members are composed of two evolutionary building blocks that are found in other proteins as well (Fig. 1). The COOH-terminal WAP domain is homologous to the second domain of secretory leukocyte proteinase inhibitor (SLPI), which inhibits elastase and cathepsin G (18). The NH₂-terminal domain, containing the TGase substrate motifs, is homologous to the guinea pig seminal vesicle protein-1 (19) and the human semenogelins (15). We showed that the NH₂-terminal TGase substrate domain, for which the name "cementoin" was coined by others (20), is actually used in vivo and in vitro for cross-linking to stratum corneum proteins. Recently, it was shown by direct sequencing of cross-linked peptides from human foreskin epidermis that SKALP/elafin is cross-linked in vivo to loricrin and cytokertatin-1 (21), which are structural proteins of the terminally differentiating keratinoocyte. The cross-linked SKALP/elafin is posttranslationally processed further by unidentified proteinases to yield low molecular weight COOH-terminal fragments containing the antiproteinase activity, starting at amino acid positions 149, 151 and 156 (numbering according to Fig. 2), as shown by NH₂-terminal sequencing of purified SKALP/elafin from epidermal scale extracts (7, 10). We have found that the COOH-terminal part of SKALP/elafin is cleared via the plasma and can be recovered from the urine (22, 23). This mechanism provides the epidermis with an anchored proteinase inhibitor.

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¹To whom correspondence should be addressed. Tel.: 31-24-3614094; Fax: 31-24-3541184.

²The abbreviations used are: SKALP, skin-derived antileukoproteinase; SPAI-2, sodium-potassium ATPase inhibitor-2; TGase, transglutaminase; WAP, whey acidic protein; SLPI, secretory leukocyte proteinase inhibitor; PCR, polymerase chain reaction; LI, lamellar ichthyosis; PAGE, polyacrylamide gel electrophoresis.

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which could protect the structural proteins of the stratum corneum against unwanted proteolysis; in addition it generates, after cleavage, a gradient of low molecular weight inhibitors from the epidermis to the dermis, thereby possibly interfering with polymorphonuclear leukocyte chemotaxis and polymorphonuclear leukocyte-induced degradation of extracellular matrix proteins such as elastin and basal membrane components.

To study the evolutionary divergence of SKALP/elafin in various species and to identify potential new members of this gene family we performed reverse transcription-polymerase chain reaction (PCR) on mRNA of epithelial tissues from various mammals, using degenerate primers that encompass the TGase substrate domain and the WAP domain. In this way we identified the putative simian and bovine orthologs of SKALP/elafin, and we have identified two new family members from bovine tissues.

## EXPERIMENTAL PROCEDURES

### Tissues

Bovine and simian (rhesus monkey) tissues were obtained from the central animal laboratory, University of Nijmegen, The Netherlands. Spontaneously shed scales from psoriatic, eczema, and lamellar ichthyosis (LI) patients were collected and stored at -20 °C.

### RNA Isolation and cDNA Synthesis

Total RNA from bovine tongue (10 mg) or to 10 μg of total RNA from human psoriatic skin was isolated with RNase-free DNase I, and the labeled RNA probe was transcribed from the XbaI-linearized SKALP/elafin (bovine Trappin-2) template. DNA template was degraded with RNase-free DNase I, and the labeled RNA probe was purified using Chroma spin+TE-100 columns (CLONTECH), acidic phenol/chloroform extraction, and ethanol precipitation. Using the RNA was subjected to electrophoresis on a 1% agarose gel and the radioactive labeled probe (1 × 10^6 cpm) was hybridized to 10 μg of total RNA from different bovine tissues or to 10 μg of total RNA from human psoriatic skin biopsies as a negative control. Samples were denatured for 10 min at 80 °C followed by an overnight incubation at 55 °C. Partial RNA:RNA hybrids were subjected to electrophoresis on a 6% denaturing polyacrylamide gel containing 7 M urea and transferred to nitrocellulose filters. The filters were probed with a radiolabeled PCR product encoding bovine Trappin-2.

### Northern Blot Analysis

For Northern blot analysis, 10 μg of total RNA from different bovine tissues was electrophoretically separated in a 1% agarose gel (dissolved in 10 mM sodium phosphate buffer, pH 7.0), and the RNA was transferred to a nitrocellulose membrane. The membrane was incubated with ethidium bromide (1 μg/ml) twice for 15 min at 60 °C using 125 mM sodium chloride, 0.15 mM sodium citrate. After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). The membrane was incubated with ethidium bromide (1 μg/ml) for 15 min prior to photograph. Hybridization was performed in 250 mM phosphate buffer at 60 °C according to Church and Gilbert (28), using a hexanucleotide probe for TGase 1 gene as determined by single strand conformation polymorphism analysis and sequence analysis. This mutation causes a deficiency for TGase type 1 as recently described (24).

### Biotylated Peptides

Six hexapeptides with an NH₂-terminal biotin following by a Cys spacer were synthesized: QEQDVQ, QEQDVF, QEDVFQ, GDVQEQ, GQEDVQ (Eurosequence Inc., Gendron, The Netherlands). 250 μg of a hexapeptide (TVQQEL) and a heptapeptide (PGQQQIV), two known acyl donor probes in the TGase assay (29), were biotinylated with 500 μg of NHS-LC-biotin (Pierce) for 2 h at 37 °C in 300 μl of 0.1 M NaClO₄ pH 8.0. The reaction was stopped by the addition of 20 μl of 1 M Tris, pH 8.0 (30).

### Cross-linking of the Biotylated Peptides to Stratum Corneum Proteins

Epidermal scales from a psoriatic patient (200 mg) were homogenized in 50 mM Tris-HCl, pH 7.8, 100 mM sodium chloride, and 1 mM phenylmethylsulfonyl fluoride (Sigma), and centrifuged for 30 min at 25,000 × g. The supernatants were used for scales from patients with eczema and LI. The supernatants were stored at -20 °C until further use. For cross-linking experiments 10 μl of scale extract was used with 5 μl (100 μg/ml) of biotinylated peptide. The following buffer conditions were used: 50 mM Tris-HCl, pH 7.8, 100 mM sodium chloride, and calcium chloride at a concentration of 2 mM, in a final reaction volume of 50 μl. In some experiments 2 μl (0.0313 unit/ml) of guinea pig liver TGase (Sigma) was added. After 60 min at 37 °C, the reaction was stopped by the addition of 5 μl of 100 mM EDTA, pH 7.8. Reaction mixtures containing 10 mM EDTA, pH 7.8, and heat-inactivated scale extracts were used as controls. The reaction mixtures were blotted directly onto polyvinylidene fluoride membrane (Boehringer Mannheim) using 10× SSC (1.5× sodium chloride, 0.15× sodium citrate). After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). The membrane was incubated with ethidium bromide (1 μg/ml) for 15 min prior to photograph. Hybridization was performed in 250 mM phosphate buffer at 60 °C according to Church and Gilbert (28), using a hexanucleotide probe for TGase 1 gene as determined by single strand conformation polymorphism analysis and sequence analysis. This mutation causes a deficiency for TGase type 1 as recently described (24).

## RESULTS

### Characterization of Proteins from TGase and/or TGase Substrate Motifs

The boxed S indicates the signal peptide. Panel A, protein structure of SPAI-2 and SKALP/elafin that share an NH₂-terminal TGase (TG) substrate domain and a COOH-terminal WAP domain. Panel B, protein structure of the COOH-terminal WAP domain of SLPI harbors protease inhibitory activity and is homologous to SKALP/elafin. Panel C, schematic structure of the seminal vesicle protein-1 and the human semenogelins. These proteins contain a domain that consists of TGase substrate motifs.
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| 10 | 20 | 30 | 40 | 50 |
|----|----|----|----|----|
| hTrappin-2 | MRRASSFLVL-- | ----- | ---> | VVYVLTA GTVLVAEAAT GV | ---- | ---- |
| sTrappin-2 | (ska1) | ----- | ---- | ---- | ---- | ---- |
| pTrappin-2 | MRRASSFLVL-- | ----- | ---- | ---- | ---- | ---- |
| bTrappin-2 | (skal)Q--- | ---- | ---- | ---- | ---- | ---- |
| bTrappin-4 | (skal)AA GKVYQGGQDC LTVQVLTVL- | ---- | ---- | ---- | ---- | ---- |
| pTrappin-1 | MRRASSFLVL-- | ----- | ---- | ---- | ---- | ---- |
| pTrappin-3 | MRRASSFLVL-- | ----- | ---- | ---- | ---- | ---- |

| 60 | 70 | 80 | 90 | 100 |
|----|----|----|----|----|
| hTrappin-2 | ----- | PVK QGQTVK GRVVPN QGQPVK QGQKVQ AGQPVK | ---- | ---- | ---- |
| sTrappin-2 | ----- | PVK QGQTVK GRVVPN QGQPVK QGQKVQ AGQPVK | ---- | ---- | ---- |
| pTrappin-2 | QGQTVK ACGEDK QGQDQK QGQPPK QGQPVK QGQPVK | ---- | ---- | ---- |
| bTrappin-2 | QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK | ---- | ---- | ---- |
| bTrappin-4 | QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK | ---- | ---- | ---- |
| pTrappin-1 | QGQPVK ACGEDK QGQPVK QGQPVK QGQPVK QGQPVK | ---- | ---- | ---- |
| pTrappin-3 | ----- | ---- | ---- | ---- | ---- | ---- |

| 110 | 120 | 130 | 140 | 150 | 160 |
|-----|-----|-----|-----|-----|-----|
| hTrappin-2 | ----- | ---- | ---- | ---- | ---- | ---- |
| sTrappin-2 | ----- | ---- | ---- | ---- | ---- | ---- |
| pTrappin-2 | QGQPVK ACGEDK QGQDQK QGQPPK QGQPVK QGQPVK | ---- | ---- | ---- |
| bTrappin-2 | QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK | ---- | ---- | ---- |
| bTrappin-4 | QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK QGQPVK | ---- | ---- | ---- |
| pTrappin-1 | QGQPVK ACGEDK QGQPVK QGQPVK QGQPVK QGQPVK | ---- | ---- | ---- |
| pTrappin-3 | ----- | ---- | ---- | ---- | ---- | ---- |

| 170 | 180 | 190 | 200 | 210 |
|-----|-----|-----|-----|-----|
| hTrappin-2 | GPVSTKPSGSC PILLRAML NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |
| sTrappin-2 | GPVSTKPSGSC PILLRAML NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |
| pTrappin-2 | LILLKPSGSC PILLRAML NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |
| bTrappin-2 | GFLPTKPSGSC PILLRAML NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |
| bTrappin-4 | GFLPTKPSGSC PILLRAML NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |
| pTrappin-1 | LPP-YLKLSC PILLRLCLDM NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |
| pTrappin-3 | LPP-YLKLSC PILLRLCLDM NPPPRLCQK DCPGKKGKE QGQCPPQF | ---- | ---- | ---- |

**Fig. 2. Alignment of amino acid sequences of members from the Trappin protein family.** The amino acid sequences (single letter code) of the novel bovine Trappin (bTrappin-2 and -5) and the bovine and stx2in orthologs of SKALP/elskin (hTrappin-2 and sTrappin-2) are deduced from the partial cDNA sequences obtained by reverse transcription-PCR (primer sequences are excluded). Comparisons with human SKALP/elskin (hTrappin-2), porcine SKALP/elskin (pTrappin-2), the porcine sodium-potassium ATPase inhibitor SPAI-2 (pTrappin-1), and a new porcine gene family member (pTrappin-3) are shown. The italicized amino acid residues (from position 176 to 182) correspond to the known active site of SLPI which is a protein that consists of two WAP motifs (18, 57). The binding site of SKALP/elskin with elastase is found at the amino-terminus of the common region of SLPI. The alignment was based on the fact that the amino acid sequence Leu(176)-Phe-Leu(177) (P1) of guinea pig TGase (36), which is marked by a vertical arrow (.), is conserved in the aligned members. The positions of the hydrophobic signal sequences of hTrappin-2, pTrappin-1, and pTrappin-3 are indicated by an arrow (4). The hydrophobic signal sequence of bTrappin-2 (7) and pTrappin-1 (2) are indicated in bold. A phase-1 intron (>) between the first and second nucleotides of the codon for the amino acid at position 42 separates the exon coding for the signal sequence from the TGase substrate domain. The hexapeptide repeats in the Trappin genes are grouped in the second and third blocks of the alignment. As pointed out by Tamechika et al. (16), expansion and contraction of the number of hexapeptides in this gene family occur readily, presumably as a result of gene conversions and DNA polymerase slippage. Some sequence similarities suggestive of conversions among the three porcine Trappin genes are underlined. Eight conserved cysteine residues involved in the formation of the WAP motif or four-disulfide core structure of the Trappin gene family are indicated (*). Amino acid residues with 100% conservation are indicated with **. The positions of the oligonucleotide forward primers (skal1 and skal2) and reverse primers (skal3 and oligo-dT) used in the PCR are indicated.

diffuse membranes (Millipore) by a slot-blot manifold or were subjected to SDS-PAGE (15% Ready gel, Bio-Rad) and blotted onto polynylidene difluoride membrane. Bioconjugated proteins were detected with the Western Light kit (Tropix) according to the manufacturer's instructions. This assay uses avidin-conjugated alkaline phosphatase and chemiluminescence of a sensitive alkaline phosphatase substrate. Positive bands were recorded on X-Omat S1 films (Kodak) and were processed with a maximum scanning OD range of 1.5 and quantitated using the ImageMaster™ data image system.

**Cross-linking of the Biotinylated Peptides to Purified Proteins by Exogenous TGase—** Purified bovine crystallins proteins αB, β-low, γ, and αd were prepared as described previously (15, 51). Recombinant human SKALP/elskin, containing amino acids 151–213 (numbering according to Fig. 2) of the complete amino acid sequence of SKALP as described by Mulhuisen et al. (7), was a kind gift of Dr. N. Russel, Zeneca, U.K. Bovine serum albumin was from Boehringer Mannheim. For cross-linking experiments 20 µl of protein (1 mg/ml) was used with 0.5 nmol of biotinylated QGDPVK peptide and 2 µl of guinea liver pig TGase (0.0013 units/ml) under reaction conditions as described in the previous paragraph.

**SDS-PAGE and Western Blotting—** Tissue assay reaction mixtures were diluted with SDS-sample buffer (containing dithiothreitol) and boiled for 2 min. These protein samples were separated by SDS-PAGE on a 12% Tris-glycine gel (Ready gels, Bio-Rad) using Tris-glycine as electrophoresis buffer (32). The broad range prestained marker from Bio-Rad was used as molecular marker (myosin (208 kDa), β-galactosidase (34 kDa), ovalbumin (49 kDa), carbonic anhydrase (34 kDa), ovalbumin (28 kDa), lysozyme (28 kDa), collagenase (28 kDa), and aprotinin (6.5 kDa)). Gels were electroblotted onto polyvinylidene difluoride membranes, and protein staining was performed with Amido Black according to standard procedures.

**Keratinocyte Secondary Culture—** Human primary keratinocytes, cultured according to the Rheinwald-Green system (33), were seeded at 10^6 cells in keratinocyte growth medium in 60-mm culture dishes as...
described by van Ruisson et al. (34). Keratinocyte growth medium was composed of KRB (Clonetics; 0.15 mM calcium) supplemented with ethanolamine (Sigma; 0.1 mM), phosphaethanolamine (Sigma; 0.1 mM), bovine pituitary extract (Collaborative; 0.4% v/v), epidermal growth factor (Sigma; 10 ng/ml), insulin (Sigma; 5 µg/ml), hydrocortisone (Collaborative bovine pituitary extract; Clonetics; 0.4% v/v), epidermal growth factor (Sigma; 10 ng/ml), insulin (Sigma; 5 µg/ml), hydrocortisone (Collaborative bovine pituitary extract; Clonetics; 0.4% v/v), epidermal growth factor

### RESULTS AND DISCUSSION

Cloning and Sequence Analysis of Bovine and Simian SKALP/Elafin Orthologs and Two New Bovine Members of the SKALP/Elafin Gene Family: Suggestions for a New Nomenclature—To isolate partial cDNAs of bovine and simian SKALP/elafin orthologs, three oligonucleotide primers (Fig. 2) were designed based on conserved sequences within human SKALP/elafin and porcine SPAI-2. Using total RNA derived from bovine tongue and rhesus monkey skin, first strand cDNA was generated in a reverse transcriptase reaction and amplified by PCR with the designed primers. PCR products were cloned and sequenced. Four clones were identified as members of the SKALP/elafin gene family based on the presence of sequences encoding an NH₂-terminal TCase substrate domain and a COOH-terminal WAP-domain. Computer-assisted comparison with published sequences of this gene family was performed to reveal the possible identity of the clones. Fig. 2 shows the alignment of the deduced amino acid sequences of the four clones with the currently known family members. Two sequences can be regarded as the simian and bovine orthologs of SKALP/elafin. Overall, simian SKALP/elafin is 98% identical to human SKALP/elafin, and bovine SKALP/elafin is 71% identical to porcine SKALP/elafin. Within a part of the WAP domain (amino acid residues at positions 163-194 in Fig. 2) these percentages are 97% and 81%, respectively (Table I). The sequences of the putative active sites (residues italicized in Fig. 2) of both simian and bovine SKALP/elafin closely correspond to the known protease binding site of SKALP/elafin (36) and the protease binding site of SLPI (36) which also belongs to the WAP protein superfamily. The homology in the putative active site of both simian and bovine SKALP/elafin suggests that these molecules could be elastase inhibitors, although we have no data from functional studies to substantiate this contention. It was, however, shown recently that the porcine SKALP/elafin ortholog is indeed an inhibitor of at least porcine pancreatic elastase (16).

At present, the nomenclature of the proteins containing an NH₂-terminal TCase substrate domain and a COOH-terminal WAP-domain is very confusing. Until now porcine SPAI-2 (2) and the proteinase inhibitor SKALP/elafin/elastase-specific inhibitor were described in the literature (1, 7, 17, 37). For the NH₂-terminal TCase substrate domain a separate name, ce-

| bTrappin-2 | sTrappin-2 | pTrappin-2 | bTrappin-3 | bTrappin-4 | bTrappin-5 | pTrappin-1 | pTrappin-3 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| bTrappin-2 | 97        | 69        | 76        | 76        | 56        | 52        | 47        |
| sTrappin-2 | 78        | 76        | 81        | 81        | 74        | 68        | 53        |
| pTrappin-2 | 91        | 91        | 88        | 88        | 74        | 68        | 47        |
| bTrappin-3 | 77        | 77        | 87        | 88        | 93        | 93        | 48        |
| sTrappin-3 | 74        | 74        | 81        | 81        | 93        | 93        | 39        |
| pTrappin-3 | 68        | 68        | 69        | 69        | 68        | 68        | 59        |
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Tissue Distribution of Trappin Family Members—The Trappin gene family members exhibit distinct tissue distributions. Porcine Trappin-1 (SPAI-2) was previously found to be abundantly expressed in the intestine, whereas porcine Trappin-3 is found in the intestine at relatively low levels (16). The mRNA for porcine Trappin-2 (SKALP/elafin) was mainly found in trachea and large intestine. This is different from human Trappin-2 which is expressed in several human stratifying squamous epithelia (9), except for epidermis where it is only expressed in the context of inflammation, such as psoriasis and wound healing (8, 10). The observed differences could be the result of different sensitivity of the detection methods or could represent real species differences.

To define tissue distribution patterns of bovine Trappin-2, expression of the Trappin-2 mRNA was studied by RNase protection analysis. Protected bands with the expected length (360 base pairs) were detected in ileum and tongue (Fig. 3B), whereas Trappin-5 was expressed at relatively low levels in trachea (Fig. 3C). Trappin-4 an -5 are very similar at the DNA level, and therefore it cannot be totally excluded that the signal of the Trappin-5 probe on the Northern blot is the result of cross-hybridization.

The high level of sequence divergence in the short amino acid stretch encoding the inhibitor domain of this gene family can be viewed as an example of accelerated evolution as described by Tamechika et al. (18). This is a process assumed to take place in genes after a duplication event (40) and has been interpreted as an effective mechanism to create new reactive site sequences with different substrate specificities. These amino acid changes are established by positive Darwinian selection as first reported by Hill and co-workers (41, 42), who described this phenomenon in the serine proteinase inhibitor (serpin) family.

Since the Trappin family members are all expressed in tissues that are exposed continuously to microbial stimuli (oral cavity, trachea, intestine) it raises the possibility that some of them are directed against bacterial proteinases rather than exclusively against self-proteases (as Trappin-2, which is directed against leukocyte elastase and proteinase-3). This contention is supported by the recent finding that the distant family member SLPI, which is homologous to Trappins in its COOH-terminal domain, possesses antiviral and antibacterial activity (43, 44).

The high substitution rates in the reactive center region of the Trappins could conceivably provide the host with a defense system against pathogens and parasites and give them the capacity to deal with an increasing number of attacking proteinases.

The TGase substrate motifs in the NH2-terminal part of the Trappins are conserved, although the number of amino acid repeats varies among species and among the Trappin gene family members. Porcine and bovine Trappin-2 display 11 and 12 repeats, respectively, whereas six repeats were found in human and simian Trappin-2. The deduced amino acid sequences of the novel members derived from bovine tongue, Trappin-4 and Trappin-5, both contained five repeats. Combined with 14 repeats found in the published porcine Trappin-1 protein and eight repeats in the new porcine family member Trappin-3, the frequency of amino acids at each position was calculated by comparing the total of 67 repeats in the putative TGase substrate domains of the currently known Trappin protein family members from four different mammalian species (Table II). In this way a consensus hexapeptide sequence of GQDPVK could be deduced, in agreement with earlier studies (2, 7).

Conservation of a TGase Substrate Motif in Members of the Trappin Gene Family—To characterize further the biochemical properties of the above mentioned TGase consensus substrate motif, the biotinylated hexapeptide GQDPVK was synthesized, and enzyme kinetic experiments were performed using a TGase assay (29) to determine optimal reaction conditions. The TGase cross-linking reaction is based on a Ca2+-dependent exchange of primary amines for ammonia at the ε-carboxamide group of glutamine residues. Peptide-bound lysine residues or polyamines serve as the primary amines to form either ε-(γ-glutamyllysine or (γ-glutamyl)polyamine bonds between proteins (45), which are highly resistant to chemical and enzymatic degradation (46). As human epidermis is known to contain both TGase activity (47, 48) and various substrate proteins (e.g. involucrin, loricrin, small proline-rich proteins) (21), an extract of porcine scales was used to study the incorporation of the biotinylated hexapeptide. A time course incubation at 37 °C of the biotinylated hexapeptide with scale extract showed that the reaction rate as measured by chemiluminescence detection was linear up to 3 h (data not shown). For further experiments a reaction time of 60 min was used for practical convenience. The effect of the peptide concentration on the rate of cross-linking to epidermal proteins by endogenous TGase is shown in Fig. 4. An apparent $K_m$ of 0.46 μM was found.

The protective callus layer resulting from terminal differentiation of the squamous epithelium is thought to be cross-linked by different TGase activities present in mammalian epidermis. These TGases are probably involved in the formation of the cornified cell envelope of terminally differentiating epidermis and of other stratified squamous epithelia. Three TGases are expressed in the epidermis, a ubiquitous tissue type TGase (TGc or TGase 2), a membrane-associated keratinocyte TGase (TGk or TGase 1) present in cultured epidermal keratinocytes and in many epithelial and nonepithelial tissues, and the zymogen epidermal TGase (TGz or TGase 3) known to

![Fig. 3. Tissue distribution of bovine Trappin family members by RNase protection and Northern blot analysis. Panel A, analysis of bovine Trappin-2 expression by RNase protection assay. Total RNA (10 μg) from different bovine tissues was incubated with a 32P-labeled cRNA probe specific for the bovine Trappin-2. After digestion with RNase A and RNase T, the protected fragments were subjected to electrophoresis on a 6% denaturing polyacrylamide gel containing 7 M urea. Lanes 1-9, respectively, represent the bovine tissues from kidney, liver, trachea, lung, ileum, epidermis from bovine snout, brain, esophagus, and tongue. Lane 10 is human psoriatic skin as a negative control. Panel B, analysis of Trappin-4 expression by Northern blot analysis. 10 μg of total RNA was loaded and electrophoretically separated. Hybridization was performed with a 32P-labeled bovine Trappin-4 probe. Panel C, analysis of Trappin-5 by Northern blot analysis. Hybridization was performed with a 32P-labeled bovine Trappin-5 probe.](image-url)
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**TABLE II**

Consensus amino acid sequence of the TGase substrate motif in various Trappin family members

| Position | A | B | C | D | E |
|----------|---|---|---|---|---|
| 1 | G | Q | D | P | D |
| 2 | Q | G | L | L | L |
| 3 | D | L | E | V | R |
| 4 | P | L | V | V | S |
| 5 | V | V | K | E | Q |
| 6 | K | E | Q | F | R |

The established hexapeptide is derived from the frequency of amino acids at each position by comparing 67 repeats in the putative TGase substrate domain within eight members of the Trappin family from four different species. Hexapeptide sequences that contain fewer than three residues identical to GQDPVK are excluded from the frequency calculation.

**Fig. 4.** Effect of peptide concentration on the rate of cross-linking to epidermal proteins by endogenous TGase. The biotinylated GQDPVK hexapeptide was incubated with scale extract from a psoriatic patient for 60 min at 37°C. The reaction mixture was blotted directly onto polyvinylidene difluoride membrane, and the biotinylated proteins were detected by chemiluminescence that was recorded on x-ray film and quantified by image analysis. An apparent $K_m$ of 0.46 $\mu$M was found.

Fig. 5. Chemiluminescent detection of slot-blotted biotinylated proteins demonstrating TGase-mediated cross-linking of biotinylated peptides to stratum corneum proteins and purified proteins. Panel A: 1, biotinylated GQDPVK hexapeptide incubated with psoriatic scale extract; 2, scale extract with exogenous type 2 TGase; 3, heat-inactivated scale extract with exogenous type 2 TGase; 4, heat-inactivated scale extract without exogenous TGase. Panel B, scale extract from a psoriatic patient incubated with different biotinylated peptides: GQDPVK (1), GQDPVR (2), GNDPVK (3), GNDPVR (4), KVPDQG (5), and GKDPVQ (6). Panel C, cross-linking of the biotinylated GQDPVK hexapeptide to different acceptor proteins by type 2 TGase: bovine serum albumin (1), recombinant COOH-terminal domain of SKALP/elafin (2), $\gamma$-crystallin (3), $\alpha$B-crystallin (4), $\alpha$A-crystallin (5), and $\beta$-low-crystallin (6). Panel D, cross-linking of different biotinylated peptides to stratum corneum proteins by endogenous TGases in a scale extract from a psoriatic patient: GQDPVK (1), TVQQL (2), and GGQTVQ (3). Panel E also shows cross-linking of these peptides to $\beta$-low-crystallin by type 2 TGase (4–6). Panel E, cross-linking of the biotinylated GQDPVK hexapeptide to stratum corneum proteins by endogenous TGase in scale extracts from three psoriatic patients (1–3), in scale extract from a patient with LI (4), and in scale extract from a patient with eczema (5).

be expressed in differentiated epidermal cells and hair follicles, but not in cultured epidermal keratinocytes (49–53). Incubation of the biotinylated GQDPVK hexapeptide with psoriatic scale extract leads to its cross-linking to stratum corneum proteins by Ca$^{2+}$-activated endogenous TGase (Fig. 5A, sample 1). Human epidermis and probably also stratum corneum are known to harbor TGases 1, 2, and 3 (21, 47, 49). From our data, however, it cannot be concluded which type is responsible for cross-linking the biotinylated GQDPVK hexapeptide to substrate proteins. Since TGase 1 and 3 are not available in purified form we only tested guinea pig liver TGase (type 2 TGase), and this was found to cause incorporation of the biotinylated hexapeptide into stratum corneum proteins of a native scale extract (sample 2) or scale extract that had been heat-inactivated to eliminate endogenous TGase activity (sample 3); so both the TGases present in scale extract and type 2 TGase catalyze the incorporation of biotinylated peptide into stratum corneum proteins. Specificity of the reaction was checked by the omission of guinea pig liver TGase (sample 4) or the addition of excess EDTA (not shown).

The biotinylated GQDPVK hexapeptide appeared to be an extremely efficient TGase substrate that acts both as an acyl donor and as an acyl acceptor probe (Fig. 5B). Substitution of the acyl acceptor residue lysine (K) for arginine (R) (sample 2) or substitution of the acyl donor residue glutamine (Q) for asparagine (N) (sample 3) showed no influence on cross-linking to stratum corneum proteins. Substitution of both the lysine and the glutamine residue for, respectively, arginine and asparagine, totally abolished cross-linking of the biotinylated hexapeptide (sample 4). The efficiency of cross-linking is sequence dependent as a hexapeptide in the reverse order (KVPDQG, sample 5) or the exchange of lysine and glutamine (GKDQV, sample 6) virtually eliminated cross-linking to stratum corneum proteins. Whether these negative effects on substrate reactivity are the result of changes in structural conformation of the peptide or are a consequence of changing the chemical nature of the side chains surrounding the substrate lysine and glutamine is not clear. Groothans et al. (29) showed that some residues directly preceding the substrate lysines have a negative effect on TGase activity. These residues, like Asp, Gly, Pro, His, and Trp, appeared to be largely avoided in a total of 30 characterized acyl acceptor (lysine) substrates. We would speculate that degenerate hexapeptide motifs could also be used in vivo. We have shown previously that a synthetic peptide comprising the NH$_2$-terminal 14 amino acids of processed SKALP/elafin, which contains the degenerate motif GQDVK, is also incorporated efficiently by TGase, suggesting that slight variations in the surrounding amino acids are tolerated. In the same study we used full-length SKALP/elafin purified from human keratinocytes and showed that the protein is cross-linked to an acyl acceptor probe by the action of type 2 TGase. A recent study by Steinert and Marekova (21) showed that the degenerate AQPVPK and GQDVKV sequences were used for cross-linking to loricrin and cytokeratin 1 in vivo, as deter-
mined by amino acid sequencing of purified peptides from human foreskin.

In addition to cross-linking of the GQDPVK hexapeptide to its natural substrate proteins in stratum corneum, we also used purified control proteins to investigate the substrate specificity of GQDPVK for these proteins. The biotinylated GQDPVK hexapeptide was found to be cross-linked efficiently by exogenous type 2 TGase to αB-crystallin and β-low-crystallin, structural proteins of the vertebrate eye lens (Fig. 5C). These proteins are known acyl acceptor substrates for type 2 TGase, as the COOH-terminal lysine residue of αB-crystallin was identified as the site of linkage (30), and a lysine residue in the NH₂-terminal extension acts as the sole acyl acceptor substrate in β3-crystallin, which is a component of a β-low-crystallin preparation (31). Two glutamine residues acting as acyl donor probes were characterized in the NH₂-terminal region of β3-crystallin by Berbers et al. (54). No cross-linking capacity was found using αA-crystallin and γ-crystallin as a substrate, which is in accordance with experiments described previously by Groenen et al. (30, 31). Recombinant SKALP/elafin, which only contains the 57 COOH-terminal amino acids of the full-length molecule (and thus lacking the TGase substrate domain), and bovine serum albumin did not show appreciable cross-linking to GQDPVK. To determine the specificity of the GQDPVK hexapeptide for cross-linking to stratum corneum proteins by endogenous TGase in psoriatic scale extracts, we introduced two other acyl donor probes: a biotinylated TVQQEL hexapeptide that is patterned on the NH₂-terminal extension of bovine β3-crystallin (30), and a biotinylated PGQQIV hexapeptide, patterned on the amine acceptor sequence in fibronectin (56). Fig. 5D shows that the GQDPVK hexapeptide reacts to a greater extent with stratum corneum proteins than the other acyl donor probes (samples 1–3), whereas they all show the same reactivity towards β-low-crystallin by the action of exogenous type 2 TGase (samples 4–6). This suggests that the GQDPVK motif is a preferred substrate for cross-linking to stratum corneum proteins by epidermal TGases.

TGase activity in stratum corneum extracts could be derived from TGase types 1, 2, and 3. Previous studies, however, have suggested that the bulk of the soluble TGase activity comes from TGase type 3 (49). To investigate the relative contribution of the various TGase types in stratum corneum extracts we used scale extracts from three psoriatic patients (Fig. 5E, samples 1–3), a patient with LI (sample 4), and a patient with eczema (sample 5). Surprisingly, the TGase activity measured in these five scale extracts was very similar. Assuming that TGase 1 would contribute significantly, a decreased TGase activity was expected in scale extract from the patient with LI, since this patient was homozygous for a splice mutation in intron 5 of the TGase type 1 gene as determined by single strand conformation polymorphism analysis and sequence analysis. This mutation causes a deficiency for TGase type 1 as described recently (24) and is probably the origin for disturbed formation of the cornified envelopes which may explain the phenotype of LI. Despite a defect TGase 1 gene in LI, our experiments showed that the GQDPVK hexapeptide is still cross-linked to stratum corneum proteins by endogenous TGases in scale extract from a LI patient. It is most likely that TGase type 2 or 3 is responsible for this phenomenon. In a recent publication TGases 1, 2, and 3 were shown to utilize loricrin in vitro as a complete substrate, but the types of cross-linking were different (56). TGase 1 mostly formed oligomeric complexes by interchain cross-links, whereas TGase 3 reactivity is involved in the formation of intrachain cross-links. The participation of TGase 2 in loricrin cross-linking was quite weak. It is therefore likely that TGase 3 is the active enzyme in the scale extract of the LI patient. Evidence that the GQDPVK motif can be used for cross-linking by TGase 1 was obtained by using cultured normal epidermal keratinocytes, which do not express TGase 3 (49), as a source of TGase type 1 and substrate (Fig. 6). Subsequent reaction with the biotinylated GQDPVK hexapeptide and analysis by SDS-PAGE show incorporation in proteins predominantly between 30 and 80 kDa (Fig. 6, lane 2). For comparison the incorporation pattern in stratum corneum proteins from psoriatic epidermis is shown (Fig. 6, lane 4). Cross-linking of GQDPVK to αB-crystallin by TGase 2 is demonstrated in lanes 5 and 6.

To conclude, we have identified novel Trappin family members, and we have characterized some of the biochemical properties of the GQDPVK motif with respect to TGase cross-linking in vitro. In addition to the TGase substrate domain, Trappin family members possess a COOH-terminal WAP motif that harbors putative proteinase inhibitory activity. The constitutive expression of Trappin gene family members in a number of normal epithelia which are subjected to continuous mechanical and microbial stress or inflammatory stimuli (e.g. oral epithelia, esophagus, trachea, ileum) is in line with a role for these molecules as proteinase inhibitors with different substrate specificities for self and (possibly) non-self proteases. Future research will be directed at investigation of the role of other Trappin family members in epithelial homeostasis and human diseases.

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