

Cloning and Regulation of Cornifin β , a New Member of the Cornifin/spr Family

SUPPRESSION BY RETINOIC ACID RECEPTOR-SELECTIVE RETINOIDS*

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Stephen J. Austin, Wataru Fujimoto[‡], Keith W. Marvin, Thomas M. Vollberg[§],
Laslo Lorand[¶], and Anton M. Jetten^{||}

From the Cell Biology Section, Laboratory of Pulmonary Pathobiology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

In this study, we describe the isolation and characterization of a cDNA clone C12 that encodes a new member of the cornifin/small proline-rich protein (spr) family, which we have named cornifin β . C12 encodes a 1.1-kilobase pair mRNA and a 24.3-kDa cytosolic protein with a high proline content (19%). Its total amino acid sequence exhibits a 37–66% identity while the first 30 amino acids at the amino terminus are 87% identical to that of members of the cornifin family. At its carboxyl terminus, cornifin β contains 21 tandem repeats of an octapeptide. Cornifin β expression is restricted to several squamous epithelia. It is highly expressed in esophagus, tongue, and oral mucosa but, in contrast to cornifin α , is not detectable in the epidermis. Both retinoic acid and a retinoid selective for the nuclear retinoic acid receptors were very potent suppressors of cornifin β expression while an analog selective for the nuclear retinoid X receptors was much less effective, suggesting that a specific retinoid signaling pathway is involved in this suppression. Cornifin β can function through some of its Gln residues as an amine acceptor in transglutaminase-catalyzed cross-linking reactions. These results indicate that cornifin β functions as a cross-linked envelope precursor.

Squamous differentiation is a multistage process that is accompanied by irreversible growth arrest and expression of squamous cell-specific genes (1–4). The formation of the cross-linked envelope is a characteristic feature of squamous differentiation in many tissues (5, 6). This structure consists of a layer of covalently cross-linked protein deposited just beneath the plasma membrane (6–9). These linkages are catalyzed by transglutaminases, enzymes that carry out the formation of ϵ -(γ -glutamyl)lysine bonds between precursor proteins (7–12). The formation of the cross-linked envelope is believed to occur in several stages and to involve multiple membrane-associated and cytosolic precursor proteins, including involucrin, cornifin/

small proline-rich protein (spr),¹ and loricrin (11). The first described envelope precursor, involucrin, is a glutamine-rich protein induced early during squamous differentiation (9, 13, 14). Loricrin, a glycine-rich precursor protein, is induced at later stages of differentiation than involucrin and cornifin and appears to be the major constituent of the mature cross-linked envelope (15, 16). Cornifins and sprs are a family of related envelope precursor proteins (17–22) which contain a highly repeated octapeptide (nonapeptide for spr2) at their carboxyl terminus and a high percentage of proline. Cornifin α has been reported to be an excellent substrate for epidermal (type I) transglutaminase and has also been shown to be assembled into the cross-linked envelope (20).

In this study, we describe the characterization of C12, a cDNA clone isolated from a library prepared from poly(A)⁺ RNA of squamous-differentiated rabbit tracheal epithelial (RbTE) cells. This clone represents an mRNA that is present at high abundance in squamous-differentiated cells but not in undifferentiated RbTE cells. Based on its amino acid sequence homology with the previously described cornifin/sprs, C12 is a new member of the cornifin/spr family (17–22). We have named the previously described cornifin (SQ37) (20), cornifin α , and the protein encoded by C12, cornifin β . We show that cornifin β functions as a substrate for transglutaminase type I indicating that it can also function as a cross-linked envelope precursor. The fact that the sequence of the tandem repeats and pattern of tissue-specific expression are different between the two cornifins may suggest distinct roles for specific cornifins, perhaps in determining the physical properties of the cross-linked envelope.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—RbTE cells were isolated and cultured as described previously (11, 23). Retinoic acid (RA) and the calcium ionophore Ro 2-2985 were obtained from Hoffmann-La Roche. The RAR-selective retinoid SRI-6751-84, RXR-selective retinoid SRI11217, and the analog SR11302, which does not activate transactivation by RARs or RXRs but exhibits anti-AP1 activity, were provided by Dr. Marcia Dawson, SRI International, Menlo Park, CA (24, 25).

Differential Screening of cDNA Library—A cDNA library was constructed in the vector λ Zap using poly(A)⁺ RNA from squamous-differentiated RbTE cells (12). The library was screened using ³²P-labeled cDNAs synthesized from poly(A)⁺ RNA isolated from undifferentiated and differentiated RbTE cells as reported previously (17). One of the differentially expressed clones, C12, was used for further analysis. The clone C12-3 was isolated after screening the cDNA library with the labeled insert of C12.

Sequencing—Sequencing of C12 was carried out in both directions by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) U40631.

[‡] Present address: Department of Dermatology, Okayama University Medical School, Okayama, Japan.

[§] Present address: Department of Medical Sciences, Creighton University School of Medicine, Omaha, NE 68178.

[¶] Present address: Department of Cell, Molecular, and Structural Biology, Northwestern University Medical School, Chicago, IL 60611.

^{||} To whom correspondence should be addressed. Tel.: 919-541-2768; Fax.: 919-541-4133; E-mail: jetten@niehs.nih.gov.

¹ The abbreviations used are: spr, small proline-rich protein; RbTE, rabbit tracheal epithelial; RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; ORF, open reading frame; kb, kilobase pair(s).

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1 .....ATAGCAGCTCTACACTCTTGAAGAATGAGCTCTACCAGCAGAAGCAACCT
1 M S S Y Q Q K Q P F F
55 TTACCCCAACCCCTCAGCCTCAACAGCAGGTAACAACCTGCCAGCTCCACCTC
11 T P P P Q Q Q Q H V K Q P Q C Q P P P Q
115 AAGATACGTTTGTTCCTACCAAGGACCCATGCCACCAATGTTCCAGTCCGGGA
31 D T F V P I T K D P C H P N V P S P G N
175 ACACCAACATTGCAGACGAAGGCTATGTCAAGATCCCTGAGCAAGGCTCCATCAAGTTC
51 T N I A E Q G Y V K I P E Q G S I K V P
235 CAGACACTGGCTACACCAAGTCCCTGACTCTGGCAACCAAGGTCCTGAGTCAGGAT
71 D T G Y T K I P D S G N T K V P E S G C
295 GCACCAAGTGTCCCTGGGTCAGGCTACTCCGTGGTTCCTCAGCTGGCTACACCAAGTTC
91 T S V P G S G Y S V V P Q P G Y T K V P
355 CTGACCAAGGCTACACCAAGTCCCTGAGTCAGGATGACCAAGTGTCCCTGGGTCAGGCT
111 D Q G Y T K V P E S G C T S V P G S G Y
415 ATTCTGTGGTTCCTCAGCTGGCTACACCAAGGTCCTGAGTCAGGATGACCAAGTGTCC
131 S V P P Q P G Y T K V P E S G C T S V P
475 CTGGGCCAGGCTACCCCAAGGTCCTCAGCTGGCTACACCAAGGTCCTGAGTCAGGAT
151 G P G Y P T V P Q P G Y T K V P E S G C
535 GCACCAAGTGTCCCTGGGTCAGGCTACTCCGTGATTCCTCAGCTAGCTACACCAAGTTC
171 T S V P G S G Y S V I P Q P S Y T K V P
595 CTGAGTCAGGATGCACCAAGTGTCCCTGGGCCAGGCTACCCACGGTCCCTCAGCTGGCT
191 E S G C T S V P P G P G Y P T V P Q P G Y
655 ACACCAAGGTTCAAGGACCAATCTCAATAGTCTCTGCGCTGTCTCAGAAGA
211 T K V P T E P V N P S I V T P G L S Q K K A
715 CCAAGTAGAAGTAATGTGCTGACATCCGTATCTTCAAGAAGCTGACCAACAGATGCTGAA
231 K
775 CACTCTCTTCCCATCTGCTCCCATGCTCAACTGCTCTATATCTGTAATCAGTATGTTT
835 GCACCCCGGTGCAATTTCTCTCTCTACTTGTACCGTGAAGCTATGTTTATGAATGT
895 CCTTGCATTCTAAAGTTCTCCCGCAGCTCTGCAATAAGCAGAAGCTTAGTGGCTTTGCT
955 GGCCCTCGGCTGCTAGGGTTTCACTGACAGAGGATTCCTGGATGAGAAGAAATGATTTCC
1015 TTATGACTTTCCTCCCAAATAAACCAGGCTCTT(A)n

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FIG. 1. Nucleotide and deduced amino acid sequence of C12 (cornifin β). The putative initiation codon (ATG), termination codon (TAG), and the polyadenylation signal (AATAAA) are indicated in *bold-face type*. The amino acid sequence is shown in the single-letter code. The underlined amino acid sequence represents the peptide C12-PEPB used to raise antibodies.

the dideoxynucleotide chain termination method with a Sequenase kit (U. S. Biochemical Corp.) (26). The DNA and deduced protein sequences were analyzed by the GCG sequence analysis software package (27).

Generation of Antiserum and Immunoblot Analysis—The peptide PPGGYTKVPESGCTSVPGSGYSVI (C12-PEPB) was cross-linked to bovine serum albumin with maleimide using a cross-linking kit from Pierce. Immunization of New Zealand White rabbits was carried out as described elsewhere (28). Immunoblot analysis was performed as described using an ECL kit (Amersham Corp.) (21).

Immunohistochemical Staining—Sections from rabbit tissue specimens were prepared and analyzed with C12-PEPB or SQ37B antisera as described previously using biotinylated goat-anti-rabbit IgG (Jackson Laboratories) and streptavidin-horseradish peroxidase (20, 21). Involucrin was detected using the involucrin immuno-kit from Biomedical Technologies (Stoughton, MA).

Transglutaminase Catalyzed Cross-linking—Total cell extracts were prepared in 50 mM Tris-HCl (pH 8.0) containing 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and leupeptin and aprotinin (1 μ g/ml each) and incubated at 37 °C with and without 1 mM dansylcadaverine (20, 29). The transglutaminase catalyzed cross-linking was initiated by the addition of CaCl_2 (10 mM final concentration). The reaction was stopped by the addition of 10 mM EDTA. Dansylated proteins were analyzed by Western blotting using a rabbit anti-dansyl polyclonal antiserum E7 (29).

Northern Blot Analysis—Samples of total RNA (30 μ g) were separated on a 1.2% agarose-formaldehyde gel, and transferred to Nytran⁺ maximum strength membrane (Schleicher & Schuell) (20). Northern blots were probed either with the *Eco*RI-excised, 0.9-kb insert of C12, *Hind*III/*Eco*RI-excised, 0.71-kb insert of SQ37₁₃ encoding cornifin α , or with a probe (pGAD28) encoding chicken glyceraldehyde-3-phosphate dehydrogenase (20, 21). Probes were labeled with [α -³²P]dCTP (3000 Ci/mmol; Amersham Corp.) using a random priming kit (Stratagene). Following hybridization (1–4 h at 68 °C) in Quikhyb (Stratagene), blots were washed (65 °C and 0.5 \times SSC for 30 min) as described previously (20).

In Situ Hybridization—Biopsy specimens of rabbit lip, tongue, and esophagus were fixed with 4% paraformaldehyde, dehydrated, and then embedded in paraffin. Ribonucleotide probes were synthesized with

A

Cornifin β : MSSYQQKQFPTPPPPQHQVQKPCQPPPPQ
 Cornifin α : MSSQQQKQFCTLPPQLQQHQVQKPCQPPPPQ
 SPR1: MNSQQQKQFCTPPPPQPPQQVQKPCQPPPPQ
 SPR2: M.SY.....QQQCKQPCQPPPPV
 SPR3: MSSYQQKQTFTPPPQLQQQVQKPSQPPPPQ

B

Cornifin α :		Cornifin β :				SPR3:	
Repeat:	A B	Repeat:	C D E F	Repeat:	G H	Repeat:	G H
QPCQPPQ		EQGYVKIP		EPCHSKVP			
EPCQPKTK		EQGSIKVP		QPGNTKIP			
EPCQPKVP	←	DTGYTKIP		EPGCTKVP	←		
EPCQPKVP	←	DSGNTKVP		EPGCTKVP	←		
EPCQPKVP	←	ESGCTSVV	←	EPGCTKVP	←		
EPCQPKVP	←	GSYSVVP	←	EPGCTKVP	←		
QPCQPKVP	←	QPGYTKVP	←	EPGCTKVP	←		
EPCQPKVP	←	DQGYTKVP	←	EPGCTKVP	←		
EPCQPKVP	←	ESGCTSVV	←	EPGYTKVP	←		
EPCQPKVP	←	GSYSVVP	←	EPGSIKVP	←		
EPCQSKVP	←	QPGYTKVP	←	DQGFIFKP			
QPCQPKVP	←	ESGCTSVV	←	EPGAIKVP	←		
EPCQTKQK		GPGYTVP	←	EQGYTKVP	←		
		QPGYTKVP	←	VPGYTKLP	←		
		ESGCTSVV	←				
		GSYSVVP	←				
		QPSYTKVP	←				
		ESGCTSVV	←				
		GPGYTVP	←				
		QPGYTKVP	←				
		EPNPSIVT					
CONSENSUS:						CONSENSUS:	
EPCQPKVP						EPG (C/Y) (T/I) KVP	

FIG. 2. Comparison of the amino acid sequence of cornifins and sprs. A, comparison of the first 30 amino acids at the amino terminus. The amino acids shown in bold vary from the amino acid sequence of cornifin β (C12). SPR1, 2, and 3 are the sequences of the human small proline-rich proteins (18, 19). B, comparison of the different octapeptides present in the tandem repeats of cornifins α and β , and spr3. The arrows line up the repeats that are identical or exhibit a high degree of homology. Two (A and B) such repeats can be identified in cornifin α , four (C, D, E, and F) in cornifin β and two in spr3 (G and H).

α -³⁵S-UTP (Amersham Corp.) from the full-length coding region of C12. *In situ* hybridization was performed using sense and antisense C12 probes as previously described (30).

RESULTS

Differential screening of a cDNA library prepared from poly(A)⁺ RNA isolated from squamous-differentiated RbTE cells yielded several cDNA clones encoding mRNAs that were abundantly expressed in squamous differentiated RbTE cells but not in undifferentiated cells (17). In this study, we describe the characterization of one of these cDNA clones named C12 and its derivative C12-3. These cDNAs contain inserts encoding overlapping 3'- and 5'-fragments of a novel squamous cell-specific mRNA. These inserts were sequenced in both directions; the cDNA sequence is shown in Fig. 1. A putative initiation codon was present 27 bases from the 5'-end of the cDNA. The open reading frame (ORF) terminates with a stop codon at nucleotide 720. A polyadenylation signal (AATAAA) was found 308 nucleotides further from the stop codon. Based on the deduced amino acid sequence, the mRNA encodes a hydrophilic 24.3-kDa protein with an estimated pI of 8 (Fig. 1).

Data base searching (GCG FastA on the combined nucleic acid data base) with the ORF of C12 revealed substantial similarity to sprs and cornifin α (18–20). The DNA coding sequence of C12 exhibited a 51% identity with that of cornifin α (20). The amino acid sequence of C12 was 49, 57, 37, and 66% identical to cornifin α , spr1, 2, and 3, respectively (18–20). The first 30 amino acids at the amino terminus were the most highly conserved (87%) between C12, cornifin α and the spr's (Fig. 2A). Like cornifin α /spr1 and spr3, C12 has a high proline content (19%) and contains a highly repeated octapeptide at the carboxyl terminus. However, these repeats deviate substantially from the consensus repeat sequences of cornifin α /spr1

and spr3 (Fig. 2B). Moreover, the octapeptide repeats in C12 were not as highly conserved between one another as those in cornifin α or the sprs. Four subclasses of octapeptides could be identified in C12 exhibiting a 40–60% identity between one another and a 25–50% and 50–87% identity with sequences in cornifin α and spr3, respectively (Fig. 2B). These octapeptides each were duplicated two to six times. These results indicate that C12 encodes a protein that is distinct but closely related to cornifin α and sprs. This protein was named cornifin β .

We next examined by Northern blot analysis and *in situ* hybridization the tissue-specific expression of cornifin β mRNA and its regulation during squamous differentiation. Northern blot analysis showed that C12 represents a 1.1-kb mRNA that is induced when RbTE cells undergo squamous cell differenti-

ation (Fig. 3A). Squamous differentiated RbTE cells express more than 50-fold higher levels of cornifin β mRNA than undifferentiated cells. Furthermore, Northern blot analysis of RNA from different rabbit tissues showed that cornifin β mRNA was highly expressed in esophagus and tongue and present at low levels in lip, but was not detectable in kidney, liver, brain, or testis (Fig. 3B). Although expression of cornifin β is highly restricted to several squamous differentiating tissues, it was, in contrast to cornifin α , undetectable in rabbit skin. Similar observations were obtained with human tissues. Cornifin β was highly expressed in human esophagus but was undetectable in epidermis and squamous-differentiated human epidermal keratinocytes in culture (not shown). Thus, cornifin β , rather than being a general marker for squamous differentiation, is expressed only in certain squamous tissues.

The conclusion that expression of cornifin β is induced during squamous cell differentiation was further confirmed in studies examining the localization of cornifin β transcripts by *in situ* hybridization. Expression of cornifin β mRNA was restricted to the suprabasal layers of the rabbit esophageal epithelium (Fig. 4) and other squamous epithelia such as the tongue and the oral mucosa (not shown). This pattern of expression is very similar to those reported previously for cornifin α and transglutaminase type I (21, 31) and confirms that cornifin β expression is associated with squamous differentiation. However, once again cornifin β mRNA was not detectable in the epidermis (not shown).

To analyze the expression of cornifin β at the protein level, an antiserum was raised against the synthetic peptide C12-PEPB (Fig. 1A). The antiserum recognized a major protein in total protein extracts of squamous differentiated RbTE cells that migrated at an apparent molecular mass of about 32 kDa (Fig. 5A). The latter is higher than the predicted molecular mass, as has also been observed for cornifin α (20). In several experiments the antiserum reacted weakly with another protein migrating at a slightly lower molecular mass (28 kDa). This smaller immunoreactive protein may have been derived from cornifin β by proteolytic digestion. The specificity of the immunoreactivity was shown by competitive blocking of the

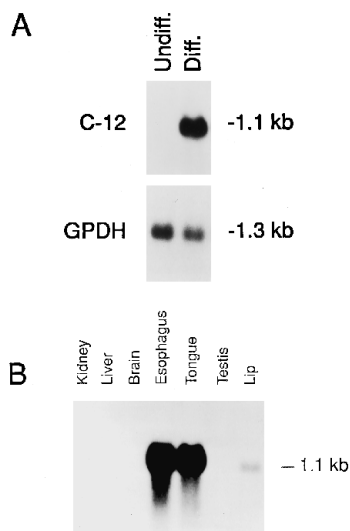


FIG. 3. **Expression of C12 mRNA in squamous differentiating cells.** Total RNA (30 μ g) prepared from RbTE cells and various rabbit tissues were fractionated, blotted to Nytran, and hybridized to 32 P-labeled probes for C12 or glyceraldehyde 3-phosphate dehydrogenase (GPDH). A, RNA from undifferentiated and squamous differentiated RbTE cells. B, RNA from various rabbit tissues.

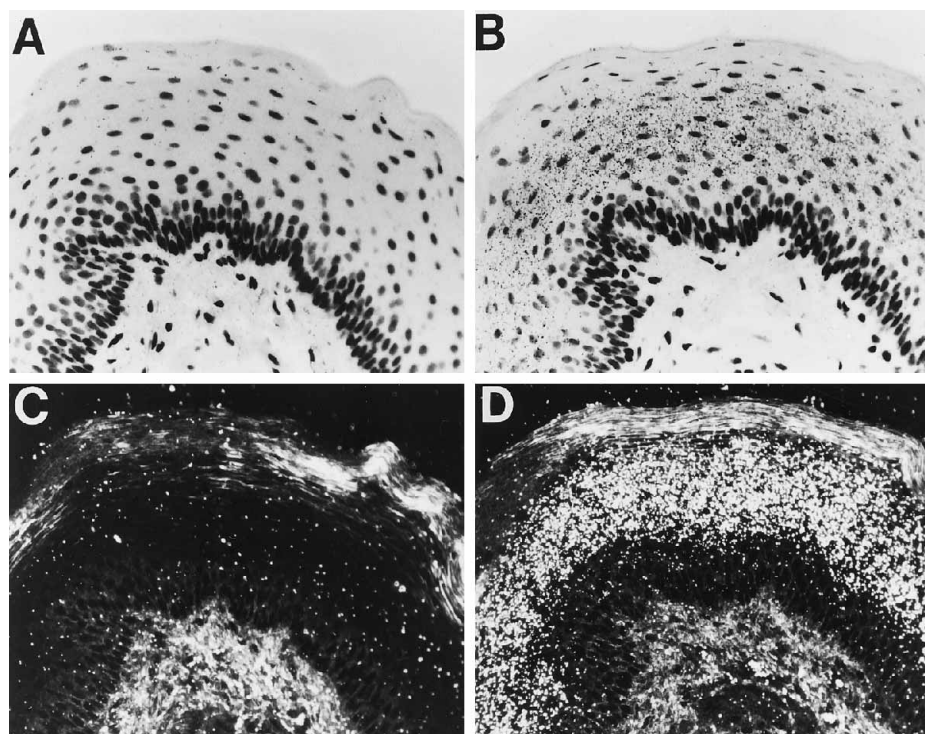


FIG. 4. **Localization of C12 mRNA in rabbit esophagus by *in situ* hybridization.** *In situ* hybridization on sections of rabbit esophagus was carried out as described under "Experimental Procedures" using 35 S-labeled sense (A and C) and antisense (B and D) C12 probes. A and B, bright field; C and D, dark field exposure.

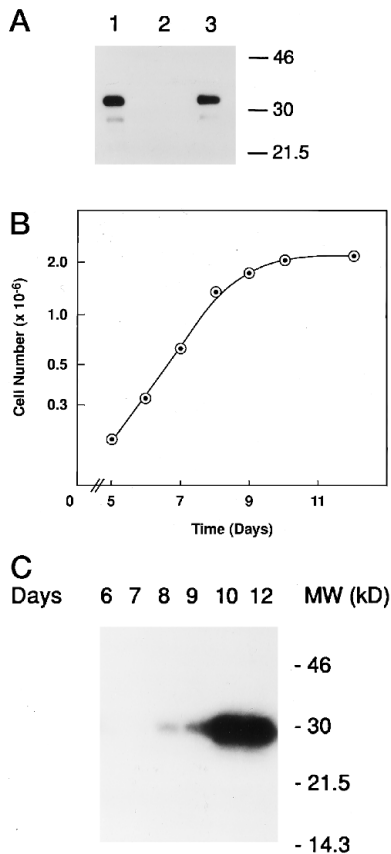


FIG. 5. Immunoblot analysis of cornifin β protein expression. Proteins from undifferentiated and squamous-differentiated RbTE cells were examined by immunoblot analysis using C12-PEPB-Ab antiserum. *A*, immunoblot analysis of total cellular protein from squamous-differentiated cells (lane 1), in the presence of the homologous peptide (lane 2), or heterologous peptide (lane 3). *B* and *C*, induction of cornifin β protein during differentiation of RbTE cells. RbTE cells were plated at 5×10^4 cells/60-mm dish and at the times indicated cells were collected for the determination of cell number (*B*) and cornifin β protein (*C*) by immunoblot analysis. The molecular mass of protein markers (kDa) is indicated on the right.

protein-antiserum interaction with the homologous peptide but not with a heterologous peptide (Fig. 5A). In addition, preimmune serum did not react with any protein in extracts from squamous differentiated RbTE cells (not shown). Fig. 5, *B* and *C*, shows the induction of cornifin β in relation to the onset of squamous differentiation in RbTE cells, which is induced when cultures reach confluence (at day 8 and 9) (1, 11). Cornifin β was detectable only in confluent, squamous-differentiated cultures but not in logarithmic cultures containing undifferentiated cells. Cornifin β was increased more than 50-fold when cultures of RbTE cells reached confluence.

In agreement with the results obtained by Northern blotting and *in situ* hybridization, immunoblot and immunohistochemical analysis indicated that cornifin β expression is associated with squamous differentiation in several, but not all, squamous tissues. Cornifin β was detectable in extracts from rabbit tongue, esophagus, and oral mucosa but undetectable in skin, trachea, muscle, and liver (Fig. 6). The localization of cornifin β was analyzed by immunohistochemical staining and compared with that of cornifin α and involucrin. In sections of esophageal epithelium, immunoreactivity with the C12-PEPB antiserum is restricted to the suprabasal layers (Fig. 7A). Although the staining pattern for cornifin β is very similar to that for involucrin, it appears that the induction of involucrin occurs somewhat earlier (Fig. 7C). The immunoreactivity for cornifin α occurs in layers that are closer to the lumen than those stained

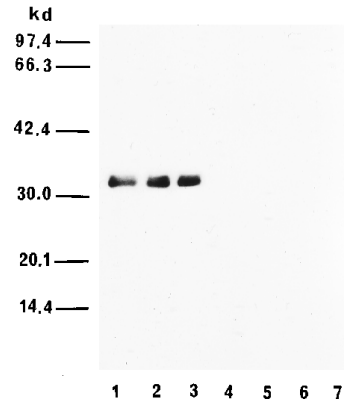


FIG. 6. Expression of cornifin β in different rabbit tissues. Protein extracts from the epithelium of the rabbit tongue (1), esophagus (2), oral mucosa (3), skin (4), trachea (5), muscle (6), and liver (7) were examined by immunoblot analysis using anti-C12-PEPB antiserum. The molecular mass (kDa) of protein markers is indicated on the left.

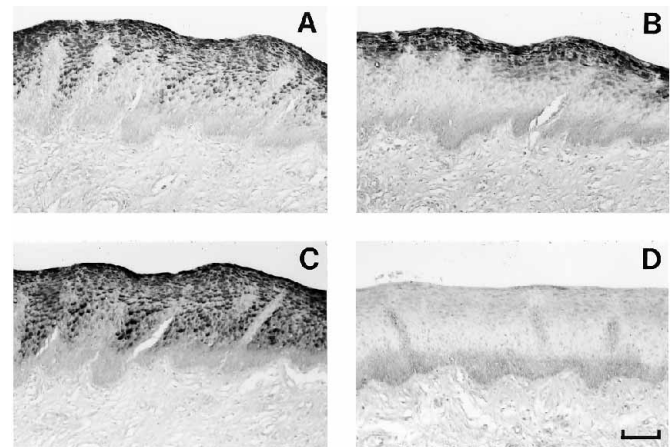


FIG. 7. Comparison of the expression of cornifin α and β and involucrin in the esophagus by immunohistochemistry. Sections of human esophagus were analyzed by immunohistochemistry using rabbit antisera against *A*, cornifin β ; *B*, cornifin α ; *C*, involucrin; and *D*, rabbit preimmune serum.

for involucrin and cornifin β . These results suggest that both cornifin β and involucrin are induced at an earlier stage of differentiation than cornifin α .

Fractionation of the cellular lysates showed that cornifin β was associated predominantly with the soluble fraction suggesting that it is a cytosolic protein (Fig. 8A). Since cornifin β is related to the cross-linked envelope precursor cornifin α (20), we determined whether it can also serve as a substrate for transglutaminase type I which catalyzes the formation of ϵ -(γ -glutamyl)lysine isopeptide bonds between cross-linked envelope precursors (7). We first examined whether cornifin β becomes cross-linked when cells are treated with calcium ionophore. Such an exposure increases the intracellular Ca^{2+} level which activates transglutaminase type I leading to subsequent cross-linking of envelope precursors (10, 20). Fig. 8A shows that the reactivity of cornifin β with the antibody against C12-PEPB is abrogated after the cells are treated with the calcium ionophore Ro 2-2985 in agreement with the concept that it becomes cross-linked into high molecular weight aggregates. Varying degrees of epitope masking have been observed previously during cross-linking of envelope precursors (20) and is probably responsible for the loss of immunoreactivity of the cross-linked C12 protein as well.

In order to confirm cross-linking of cornifin β , dansylcadaverine was supplied as an amine donor in *in vitro* cross-linking reactions (10, 20). As shown in Fig. 8B, two major proteins of 21

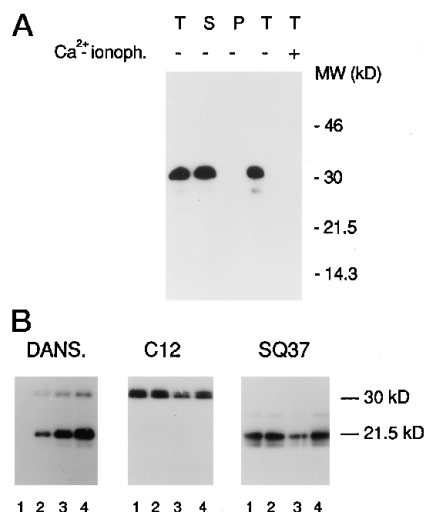


FIG. 8. Transglutaminase-induced cross-linking of cornifin β protein. A, total cellular protein (T), the soluble protein fraction (S) and the particulate protein fraction (P) from untreated and Ca^{2+} -ionophore-treated squamous differentiated RbTE cells were examined by immunoblot analysis with C12-PEPB-Ab. B, identification of proteins in differentiated RbTE cells that are covalently cross-linked with dansylcadaverine. Total cellular extracts were incubated in the presence of dansylcadaverine for 0, 2, 5, and 10 min (lanes 1, 2, 3, and 4). Samples were then examined by immunoblot analysis using E7 monoclonal antibody (DANS) (29) or antisera against C12-PEPB or SQ37A-Ab (20) for the presence of dansylated proteins, cornifins β and cornifin α , respectively.

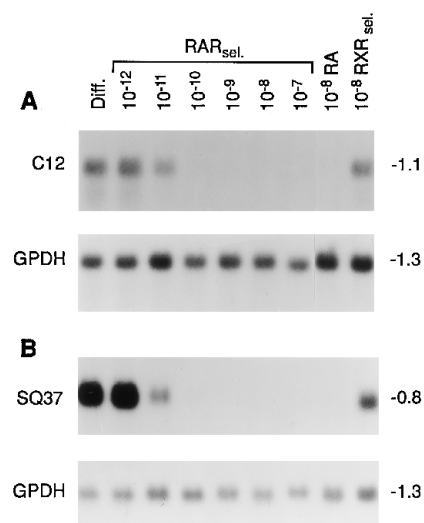


FIG. 9. Effect of RAR- and RXR-selective retinoids on cornifin α and β mRNA expression. RbTE cells were grown to confluence and then treated with RA, RAR-selective ($\text{RAR}_{\text{sel.}}$) or RXR-selective ($\text{RXR}_{\text{sel.}}$) retinoids at the concentrations indicated. Five days later cells were collected and total RNA isolated. RNA (30 μg) was analyzed by Northern blot analysis using ^{32}P -labeled probes for C12 (A), cornifin α (SQ37) (B), and GPDH.

and 32 kDa in crude extracts prepared from differentiated RbTE cells were covalently linked to dansylcadaverine in a time-dependent manner. The smaller protein comigrated with cornifin α (20), the larger one with cornifin β .

The expression of many squamous cell-specific genes have been reported to be down-regulated by retinoids (1, 2). Therefore, we examined the effect of several retinoids on the expression of cornifin β . As shown in Fig. 9A, 10^{-8} M retinoic acid totally suppressed the induction of cornifin β mRNA. To obtain more insight in the signaling pathway involved in this retinoid action, the effect of two retinoid receptor selective retinoids was determined. Nanomolar concentrations the RAR-selective ret-

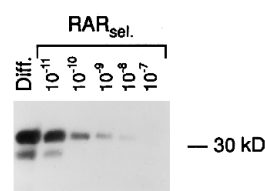


FIG. 10. Suppression of cornifin β protein by the RAR-selective retinoid SRI 6751-84. Subconfluent cultures of RbTE cells were treated with the RAR-selective retinoid SRI 6751-84 ($\text{RAR}_{\text{sel.}}$) at the indicated concentrations and four days later cells were collected and examined by immunoblot analysis using anti-C12-PEPB antiserum.

inoid SRI 6751-84 were able to suppress the expression of both cornifin β and α (SQ37) very effectively, whereas the RXR-selective retinoid SRI-11217 was much less potent. Cornifin β may be slightly less sensitive to retinoids than cornifin α . The repression of cornifin β by retinoids was also observed at the level of the protein (Fig. 10). The retinoid SR11302, which does not induce RAR- or RXR-dependent transactivation but inhibits AP1-dependent transactivation (24), had no effect on the expression of cornifin β (not shown).

DISCUSSION

In this study, we describe the isolation and characterization of a novel cross-linked envelope precursor which is a new member of the cornifin/spr family. This protein was named cornifin β . The lines of evidence supporting this classification include the presence of a highly conserved amino-terminal region characteristic of cross-linked envelope precursors and of a characteristic, highly repeated octapeptide. In addition, the strong association of its expression with squamous differentiation and its ability to serve as a substrate in transglutaminase-catalyzed cross-linking reactions. The predicted amino acid sequence of cornifin β is 49% identical to that of the previously reported cornifin, referred to now as cornifin α (20), and exhibits a 57, 37, and 66% identity to that of spr1, 2, and 3, respectively (18, 19). The sequence of the 30 amino acids at the amino-terminal region are remarkably well conserved, 87% between rabbit cornifin α and cornifin β . It is also well conserved across species (Fig. 2). Interestingly, this sequence also shows considerable homology to the amino-terminal region of two other cross-linked envelope precursors, involucrin and loricrin (13, 15, 16). Although one could expect this highly conserved region to be derived by the duplication of a single exon, no intron was found at the borders of this region (19).²

As for cornifins and sprs, cornifin β is rich in amino acids that can disrupt protein secondary structure. Cornifin β has a proline content of 19% compared to 31% for cornifin α and 22% for spr3 (19, 20) and a glycine content of 8% versus 0 and 9% for the other two, respectively. The percentage of glutamine, lysine, and cysteine in cornifin β (8.6, 6.9, and 3.0%, respectively) is much lower than that in cornifin α (20, 13, and 11%, respectively).

Cornifin β contains a highly repeated octapeptide at its carboxyl terminus as do cornifin α , spr1 and 3. Cornifin α contains 12 repetitions of the highly conserved consensus sequence EPCQPKVP, whereas cornifin β contains 21 octapeptide repeats. These sequences are not as highly conserved as those in cornifin α and spr3. The cornifin β repeat sequences fall into four subclasses: ESGCTSPV, QP(G/S)YTKVP, GPGYPTVP, and GSGYSV(V/I)P which are repeated, respectively, five, five, two, and three times (Fig. 2B). These octapeptides can be viewed as being organized in groups of three as repeats of a 24-amino acid sequence. During evolution, the cornifin β se-

² K. Marvin and A. M. Jetten, unpublished observations.

quence may have arisen from duplications of the octapeptide followed first by mutations leading to a diversion in amino acid composition between the octapeptides and subsequent duplications of the 24-amino acid sequence. The sequence and organization of the repeats in cornifin β deviate substantially from those found in cornifin α and spr3, yet still one-third of the amino acids in this region tend to disrupt conventional secondary structure. The repeat sequences exhibit a 25–50% and 50–87% identity with sequences in cornifin α and spr3. No similarity in the sequence of these repeats were observed with those found in other squamous cell marker genes such as involucrin, loricrin, and filaggrin (13, 15, 16, 31).

As cornifin α , cornifin β can function as a substrate for transglutaminase type I (20). This was indicated by treatment of RbTE cells with calcium ionophore Ro 2-2985 which results in the activation of transglutaminase type I and the disappearance of immunoreactive cornifin β when it becomes cross-linked and associated with the cross-linked envelope. In addition, labeling of proteins with dansylcadaverine revealed two major labeled proteins of 21 and 32 kDa. Previous studies identified the 21-kDa band as cornifin α and showed that the larger 32-kDa protein did not immunoprecipitate with anti-cornifin α antibodies (20). This protein comigrates at the same position as cornifin β , suggesting that this dansylated protein is cornifin β .

The expression of cornifin β is associated with squamous differentiation. This is demonstrated by Northern blot, *in situ* hybridization, and immunohistochemical analyses showing that the presence of cornifin β mRNA and protein was restricted to squamous epithelia and limited to the suprabasal layers of the squamous epithelium. The tissue-specific expression of cornifin β appears to be more restricted than that of cornifin α . In contrast to cornifin α , cornifin β was expressed in neither rabbit nor human skin nor in cultured NHEK cells; however, cornifin α and β each were abundantly expressed in both rabbit and human oral mucosa, esophagus, and tongue (Figs. 4 and 6) (20, 21).³ The differential expression of cornifin α and β may through alterations in the composition of the cross-linked envelope determine different physical properties of that structure as may be required in different tissues. Immunohistochemical analyses indicate that cornifin α and β , involucrin and loricrin are induced at different points during squamous differentiation (14, 15, 21). This sequential induction supports the hypothesis that the formation of the cross-linked envelope is a multistep process (20). As proposed previously, involucrin and cornifin may form a scaffold upon which loricrin and perhaps other cross-linked envelope precursors are assembled.

The induction of cornifin β protein and mRNA is suppressed by retinoic acid. Retinoids have been shown to mediate their action on gene expression through specific nuclear retinoid receptors, RARs and RXRs (reviewed in Giguere (32)). To ex-

amine what retinoic acid signaling pathway is involved in this suppression, the action of SRI 6751-84 and SRI-11217, an RAR- and an RXR-selective retinoid, respectively, and of SR11302, a retinoid that exhibits anti-AP-1 activity but that is unable to induce transactivation through the retinoid response elements, RARE or RXRE (25), were studied. In contrast to the RXR-selective retinoid, the RAR-selective retinoid was very effective in suppressing the expression of cornifin β mRNA while SR11302 had no effect. These observations suggest that the suppression of C12 is mediated through activation of RARs rather than RXRs and appears not to require the anti-AP1 activity of retinoids. Characterization of the DNA elements involved in the up-regulation and the retinoid-mediated repression of this gene has to await the isolation of the promoter region of cornifin β .

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