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

SUPPRESSION BY RETINOIC ACID RECEPTOR-SELECTIVE RETINOIDS*

(Received for publication, August 2, 1995, and in revised form, November 17, 1995)

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 epidermids. 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 transglutaminase and have 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 λZap 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

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

† Present address: Department of Dermatology, Okayama University Medical School, Okayama, J apan.
‡ 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).
Cloning and Regulation of Cornifin β

**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-kD protein with an estimated pI of 8 (Fig. 1).

The putative initiation codon (ATG), termination codon (TAG), and the polyadenylation signal (AATAAA) are indicated in Fig. 1. The amino acid sequence is shown in the single-letter code.

...
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 differentiation (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 then 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.](http://www.jbc.org/content/3739/23/3739/F1.large.jpg)

Total RNA (30 μg) prepared from RbTE cells and various rabbit tissues were fractionated, blotted to Nytran, and hybridized to 32P-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.](http://www.jbc.org/content/3739/23/3739/F2.large.jpg)

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

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 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 order to confirm cross-linking of cornifin β, dansylcadaverine was supplied as an amine donor in vitro cross-linking reactions (10, 20). As shown in Fig. 8B, two major proteins of 21 kDa became cross-linked when cells were treated with 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 vitro cross-linking reactions (10, 20). As shown in Fig. 8B, two major proteins of 21 kDa became cross-linked when cells were treated with 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.

**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.

**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.
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 Ca²⁺-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.

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 loricin (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).

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: ESGCTSVV, QP(G/S)YTIVP, GPGYTPP, 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-

DISCUSSION

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, loricin, 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 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 immunochemical 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)."
Cloning and Regulation of Cornifin, a New Member of the Cornifin/spr Family: SUPPRESSION BY RETINOIC ACID RECEPTOR-SELECTIVE RETINOIDS
Stephen J. Austin, Wataru Fujimoto, Keith W. Marvin, Thomas M. Vollberg, Laslo Lorand and Anton M. Jetten

J. Biol. Chem. 1996, 271:3737-3742.
doi: 10.1074/jbc.271.7.3737

Access the most updated version of this article at http://www.jbc.org/content/271/7/3737

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 15 of which can be accessed free at http://www.jbc.org/content/271/7/3737.full.html#ref-list-1