

An Involucrin-like Protein in Hepatocytes Serves as a Substrate for Tissue Transglutaminase during Apoptosis*

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Cornified envelopes and apoptotic bodies are transglutaminase-cross-linked end-products of physiological cell death pathways. The two structures have similar amino acid composition. Involucrin has been considered as a cornified envelope precursor protein expressed specifically in terminally differentiating keratinocytes and squamous epithelia. We report the presence in hepatocytes of an involucrin-like protein which could be purified from dog liver with procedures characteristic to involucrins. When compared to purified dog esophagus involucrin, the liver protein also reacts with anti-involucrin antibodies, has the same relative molecular mass, possesses similar amino acid composition, and shows almost identical peptide mapping pattern. The involucrin-like protein is detectable by immunohistochemistry in normal and apoptotic hepatocytes, is a substrate of tissue transglutaminase, and is incorporated into cross-linked apoptotic bodies. These results suggest that there are overlapping molecular components in the two characteristic forms (cornification and apoptosis) of naturally occurring cell death.

Transglutaminases (EC 2.3.2.13) are a family of calcium-dependent enzymes, which form $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ cross-links between polypeptide chains (1) giving them characteristic resistance against physicochemical, mechanical, and enzymatic actions (1, 2). Two of the transglutaminases are involved in biochemical events linked to cell death. The membrane-bound keratinocyte transglutaminase is induced during cornification and produces protein envelopes beneath the plasma membrane of terminally differentiating keratinocytes and squamous epithelial cells (3). The cytosolic tissue transglutaminase (4) participates in the biochemical pathway of apoptosis, which is the physiological cell death program in parenchymal tissues and non-squamous epithelia to eliminate individual cells as part of embryonic development and normal cell turnover (5). The dying cells preserve their integrity and form membrane-enclosed apoptotic bodies (6, 7). The prod-

ucts of these two processes are highly cross-linked protein scaffolds that are resistant to detergents and chaotropic agents (6, 8). Cornification has been extensively investigated (8), and a number of proteins have been identified as envelope precursors. However, nothing is known about the substrates of tissue transglutaminase in apoptosis. The first discovered and most extensively studied substrate of the keratinocyte transglutaminase is involucrin (9, 10), which is synthesized in the outer half of the spinous layer in epidermis, localized in the cytoplasm, then concentrated at the periphery of differentiating cells to be incorporated into the cornified envelope.

We report that the amino acid composition of cornified envelopes and apoptotic bodies are very similar and an involucrin-like protein can be isolated from hepatocytes which is incorporated into apoptotic bodies by tissue transglutaminase.

EXPERIMENTAL PROCEDURES

Materials

Rabbit anti-human-133 involucrin antibody (raised against residues 2–21 of the N-terminal ancestral segment of human involucrin) and a rabbit anti-dog involucrin antibody (11) were kind gifts of Dr. Marcia Simon (State University of New York, Stony Brook, New York). The rabbit anti-cornified envelope antibody (raised against cornified envelopes of SV-40 transformed human foreskin keratinocyte line SV-K14, Ref. 12) was a kind gift of Dr. Rainer Schmidt (Centre Internationale de Recherches Dermatologiques GALDERMA, France).

Preparation and Characterization of Cornified Envelopes and Apoptotic Bodies

Cornified envelopes of human foreskin and rat tail skin were purified with sodium dodecyl sulfate extraction as described by Rice and Green (2). Detergent-resistant apoptotic bodies from rat and dog liver were isolated as described earlier (6).

Amino Acid Analysis—The acid hydrolyzed samples were derivatized with phenylisothiocyanate (13) and separated by HPLC¹ on a C18 column (0.4 × 25 cm, 5 μm , BST, Budapest, Hungary). Detection was carried out at 254 nm, and the amount of the amino acids was determined with the help of standards.

Cyanogen Bromide Fragmentation—Samples were incubated with a 100-fold molar excess of CNBr for 24 h at room temperature in 70% formic acid. The fragments were dissolved in 10% trifluoroacetic acid for dot blot analysis or in denaturing solution for SDS-PAGE.

Digestion with Proteinase K—Samples were suspended in 25 mM Tris-HCl buffer, pH 7.6, containing 0.1% SDS and digested for 5 days. The fragments were separated by SDS-PAGE and transferred to nitrocellulose membrane. Western blot was performed using anti-dog involucrin antibody and detected with ECL (enhanced chemiluminescence detection system, Amersham).

Purification and Characterization of Involucrin and Involucrin-like Protein

Dog esophagus was kept in 1 M NaCl containing protease inhibitors and 1 mM EDTA for 3 days to remove mucous membrane. Dog liver slices were washed free of blood. Esophagus and liver samples were homogenized in isotonic phosphate buffer containing protease inhibitors and 20 mM EDTA. After ultracentrifugation the cytosol fraction was made 10% in glycerol and 62.5 mM in Tris-HCl, pH 6.8, heated for 10 min at 100 °C, and centrifuged (14). The proteins of the supernatant were precipitated in 12% trichloroacetic acid, centri-

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¹ The abbreviations used are: HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence detection system; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

fused, and redissolved at pH 8. Cold ethanol was added in a final concentration of 67%, and the precipitated proteins were removed by centrifugation. The supernatant was rotary evaporated and redissolved in water (15). The protein content of the fractions was determined by Bradford's method (16), separated on SDS-PAGE (17), and analyzed by Western blot technique. To determine the amino acid composition the proteins were transferred to polyvinylidene difluoride membranes (Millipore), and after staining with Ponceau S the appropriate bands were cut out, destained, and acid-hydrolyzed.

Peptide Mapping—The proteins were eluted from the gel and incubated with trypsin for 1 h. After the addition of 5 μ l of 50% trifluoroacetic acid the fragments were separated by HPLC on a C18 column.

Electrophoresis in Nondenaturing Conditions—Electrophoresis was carried out as described (14) omitting SDS from the gel and the running buffer.

Incorporation of Dansyl Cadaverine into Purified Proteins—Proteins separated by electrophoresis without SDS were transferred to nitrocellulose membrane, the membrane was blocked with polyvinylpyrrolidone-40 (18) and incubated with tissue transglutaminase (2.5 μ g/ml in 50 mM Tris-HCl buffer, pH 7.5, 2 mM dithiothreitol, 10 mM CaCl₂) in the presence of dansyl cadaverine (1 mM) at 37 °C, and the thoroughly washed membrane was observed under UV light.

Immunohistochemistry—Processing of dog liver samples for immunohistochemistry was carried out as described earlier (4), and the immunoreactivity was demonstrated with the peroxidase-anti-peroxidase method (19, 20) followed by slight counterstaining with Mayer's hemalum.

RESULTS

Comparison of Cornified Envelopes and Apoptotic Bodies—Comparing human and rat cornified envelopes with rat and dog apoptotic bodies purified by appropriate methods (2, 6), similarities in their morphological, physical, and chemical properties can be readily observed. They are polygonal, rigid structures with 10–30- μ m diameters and characteristic resistance to detergents and chaotropic agents. Acid or base hydrolysis and proteolytic digestion destroy them. The ϵ (γ -glutamyl)lysine cross-link content of cornified envelopes is 10–20 nmol/ μ mol amino acid with 15–20% of lysines engaged in this bond. In apoptotic bodies these numbers are somewhat lower, but still unusually high: 2–5 nmol of cross-link/ μ mol of amino acid involving 6–10% of lysine content (2, 6). After determining the amino acid composition of these structures, several common features could also be observed; the main components are Gly, Pro, Ser, and Glx, and the amount of Asx and Thr is the lowest in both (Table I). These results

have suggested to us that in spite of their very different origin the cornified envelopes and the apoptotic bodies may be built up from similar components.

Isolation and Characterization of an Involucrin-like Protein from Liver—To purify involucrin from dog liver homogenate proteins were separated using a combination of methods developed for the isolation of human and dog keratinocyte involucrin (14, 15, 21). Samples have been enriched mainly in two proteins (Fig. 1A), and one of them showed specific immunoreactivity using either anti-human-133 (Fig. 1B) or anti-dog (Fig. 1C) involucrin antibodies. Starting from 1 g of dog liver approximately 10 μ g of involucrin-like protein could be purified. When it was compared to dog involucrin isolated from squamous epithelia of the esophagus, the relative molecular mass and immunoreactivity of the two proteins were identical (Fig. 1D). The molecular mass is in close correlation with that reported for dog keratinocyte involucrin (11, 21).

The keratinocyte involucrin has an acidic isoelectric point and, when separated on polyacrylamide gel in the absence of SDS, migrates as a distinct band (14). Examining the liver protein under these circumstances, it showed this characteristic acidic property (Fig. 1E, lane 5) similarly to esophagus involucrin (Fig. 1E, lane 6). To prove that the purified proteins are substrates of tissue transglutaminase, the bands were transferred to nitrocellulose membrane and incubated with guinea pig liver enzyme in the presence of dansyl cadaverine, an established amine substrate of transglutaminases (22); intensive fluorescence appeared in the position of both esophagus and liver protein (Fig. 1F). When the incubation was carried out in solution and the products were separated on SDS-PAGE, the involucrin bands disappeared and an intensive fluorescent staining appeared on the top of the gel even after a very short incubation time (data not shown).

Comparing the amino acid composition of the two proteins, almost the same relative amounts of Thr, Arg, Val, and Leu were found in them, but differences in their Glx, Gly, and Ala content could be observed (Table I). However these data are not in complete agreement with the amino acid composition reported in the literature (Ref. 11; Table I). The difference may arise from the low extractability of dicarboxylic amino acids after acid hydrolysis in glass containers (23), from the difference in the purification and analytical methods applied or from the heterogeneity in dog involucrins.

TABLE I
Amino acid analysis

Amino acid (%)	Cornified envelopes		Apoptotic bodies		Involucrin-(like protein)		
	Human	Rat	Rat	Dog	Liver	Esophagus	Dog ^a
Asx	0.7	0.3	1.4	1.6	9.8	10.5	
Glx	7.1	4.8	9.3	9.6	15.7	16.6	48.6
Ser	9.7	8.9	8.3	8.2	4.5	4.8	
Gly	23.2	11.4	23.7	23.4	9.3	10.3	4.0
His	2.6	4.4	3.5	3.9	4.4	4.6	2.4
Thr	1.6	1.2	2.1	2.3	1.1	1.1	
Ala	4.1	7.4	5.9	13.7	9.4	10.5	
Arg	3.1	8.6	4.3	6.9	4.8	4.9	6.8
Pro	14.6	8.5	10.6	10.2	2.7	2.9	
Tyr	7.1	4.7	12.5	3.0	6.6	6.1	
Val	2.5	7.5	3.7	3.0	1.7	1.7	
Cys	1.4	1.6	0.1	0.8	ND ^b	ND	
Met	ND	ND	ND	ND	ND	ND	
Ile	4.6	8.0	2.0	2.0	2.5	2.9	
Leu	4.1	14.3	5.6	4.4	12.7	12.8	9.1
Phe	2.3	2.3	3.3	2.3	5.0	4.4	
Lys	11.3	6.1	3.7	3.3	7.4	7.8	6.6

^a See Ref. 11.

^b ND, not determined.

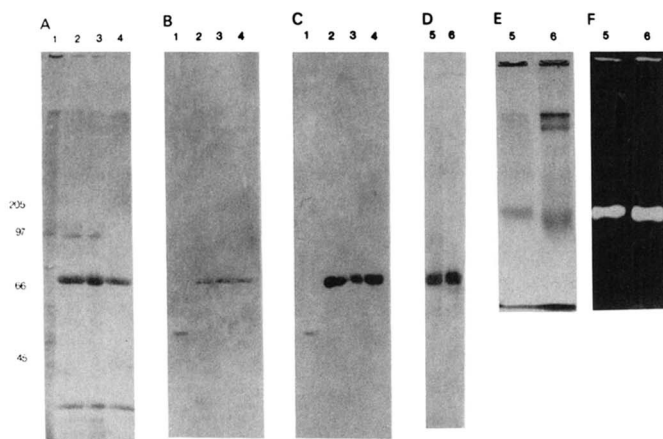


FIG. 1. Purification and characterization of involucrin-like protein from dog liver. Proteins separated either on SDS-PAGE (A–D) or in nondenaturing electrophoresis (E and F) on a 7.5% acrylamide gel, were stained with Coomassie Blue (A and E) or transferred to nitrocellulose membrane (B–D, F). Transblots were analyzed with anti-human-133 involucrin antibody (B) and with anti-dog involucrin antibody (C and D). In A–D each lane was loaded with 100 μ g of proteins: lane 1, cytosol fraction of dog liver; lane 2, heat-soluble protein fraction; lane 3, proteins redissolved at pH 8 after trichloroacetic acid precipitation; lane 4, proteins soluble in 67% ethanol; lane 5, purified involucrin of dog liver, as in lane 4; lane 6, involucrin isolated from dog esophagus. The position of the molecular weight standards is indicated on the left. E and F, lanes were loaded with 1 mg of the purified liver (lane 5) and esophagus (lane 6) protein; no molecular weight markers are included as they do not produce discrete band. F, after blocking the membrane was incubated with tissue transglutaminase in the presence of dansyl cadaverine. After 3 days of incubation at 37 $^{\circ}$ C, the membrane was washed and the reaction was detected under UV light.

The pattern of tryptic fragments obtained by HPLC separation was almost identical comparing the liver and esophagus protein (Fig. 2). The N-terminal residue of both proteins are blocked. Primary amino acid sequence (deduced from cDNA) of dog involucrin shows that only one internal methionine residue (the 47th amino acid from the N terminus) is present (24). When the involucrin-like protein of the liver was treated with cyanogen bromide and the digest was analyzed by SDS-PAGE, two fragments (66 and 17 kDa) appeared on the gel (data not shown).

Histological Localization of Involucrin-like Protein in Liver Cells—By anti-dog involucrin antibody immunostaining was observed in 20–30% of the hepatocytes showing a haphazard fashion (Fig. 3A, arrows). The immunoreactive hepatocytes tended to occur in clusters, without sign of zonality with respect to the hepatic lobules. The staining was diffuse cytoplasmic similar to that of involucrin observed in the spinous layer of keratinocytes. Epithelial cells of bile ducts were also decorated (data not shown). The strongest immunoreactivity was observed in intrasinusoidal cells (Fig. 3A, arrowheads). These cells represent most likely polymorphonuclear granulocytes but the intense cytoplasmic reaction precluded their identification with certainty as such. When anti-human-133 involucrin antibody was used as the primary antibody similar cytoplasmic reaction was observed in the hepatocytes (Fig. 3B, arrows) and duct epithelial cells. Intensive staining occurred in the apoptotic hepatocytes (Fig. 3C, arrows). Immunoreactivity was completely absent from sections treated with non-immune rabbit serum instead of the involucrin antibodies (Fig. 3D).

Detection of the Involucrin-like Protein in Apoptotic Bodies—To solubilize the cross-linked structures CNBr digests of the cornified envelopes and the apoptotic bodies were pre-

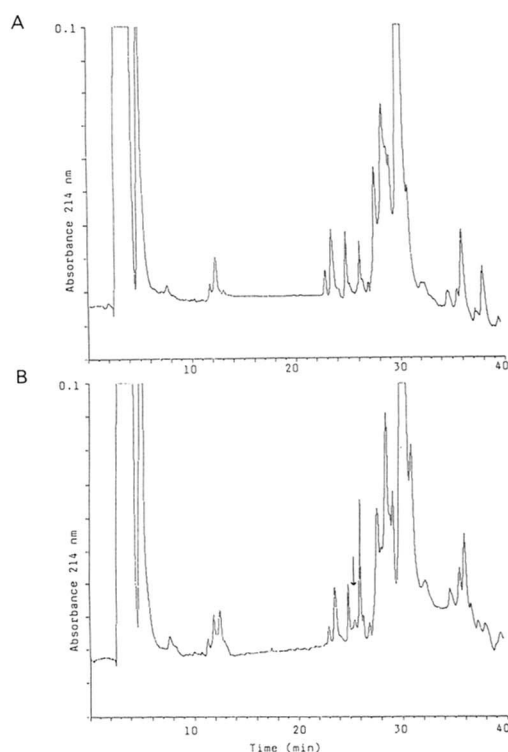


FIG. 2. Tryptic digestion pattern of involucrins. Involucrin containing bands of SDS gels were electro-eluted, and the proteins were incubated with trypsin for 200 min. The fragments were separated by HPLC on a C18 column (0.4 \times 25 cm, 5 μ m, BST Budapest). The eluents were as follows: A, 0.1% trifluoroacetic acid/water; B, 0.08% trifluoroacetic acid/acetonitrile. The separation was carried out after a 3-min wash in 100% A, with a linear gradient of 0–80% B in 32 min. The flow rate was 1 ml/min, with detection at 214 nm. A, fragments from liver; B, fragments from esophagus protein. The arrow indicates an extra peak of esophagus protein as a major difference.

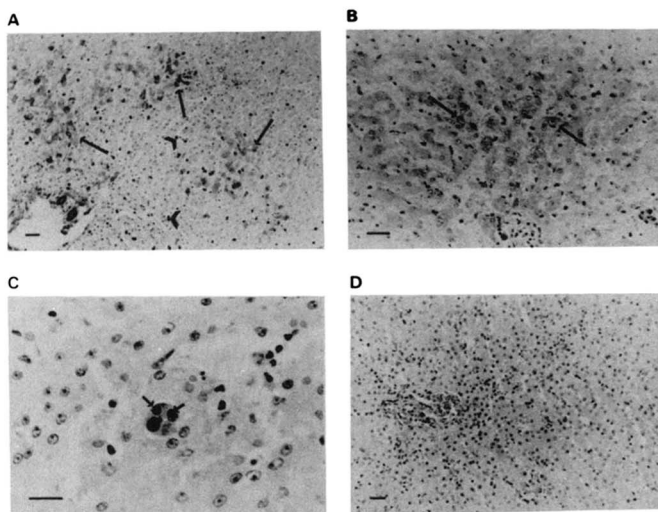


FIG. 3. Immunohistochemical detection of involucrin-like protein in dog liver cells. Staining pattern of formaldehyde fixed dog liver with anti-dog involucrin antibody (A), with anti-human-133 involucrin antibody (B and C), and using non-immune serum as the first antibody (D). Arrows indicate stained hepatocytes (A and B) and engulfed apoptotic bodies (C). Arrowheads on A indicate stained polymorphonuclear granulocytes. The bars represent 40 μ m.

pared. They were tested by dot blot analysis with anti-involucrin antibodies. Positive reactions could be detected either with anti-human-133 (Fig. 4A) or with anti-dog involucrin antibody (Fig. 4B) in case of both cornified envelopes and

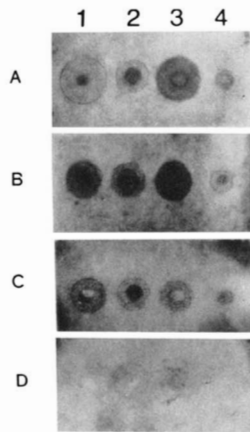


FIG. 4. Detection of involucrin-like protein in apoptotic bodies. Cornified envelopes and apoptotic bodies of different species were treated with CNBr and tested on dot blot with anti-human-133 involucrin antibody (A), with anti-dog involucrin antibody (B), with anti-human cornified envelope antibody (C), and with non-immune rabbit serum (D). Sample 1, human cornified envelope; sample 2, dog apoptotic body; sample 3: rat cornified envelope; sample 4: rat apoptotic body.

apoptotic bodies of various origin. An anti-cornified envelope antibody which does not recognize involucrin in cultured cells (12) also reacted with the apoptotic bodies (Fig. 4C). When the apoptotic bodies were digested with proteinase K and then tested with anti-dog involucrin antibody on Western blot with an ECL detection system, positive reaction could be observed on the top of the 4% stacking gel and diffuse staining close to the front of the 10% separating gel (data not shown).

DISCUSSION

Involucrin has been used as a marker of terminal differentiation in normal and neoplastic human skin (25) as well as in lung carcinomas (26). However, the correlation between involucrin expression and terminal differentiation is not general. Since involucrin is an envelope precursor, one may expect that its cellular appearance coincides with that of the envelope-producing keratinocyte transglutaminase. It has been demonstrated that the induction of the two proteins may differ in cultured squamous carcinoma cells and may be modulated differently by physiological and toxic agents (27). The diagnostic utility of involucrin in the typing of lung tumors also has been questioned (28), because tumors of nonepithelial origin showed involucrin expression.

Our results show that from dog liver an involucrin-like protein, which is either identical to or an isoform of keratinocyte involucrin, can be purified and both the liver and esophagus protein can serve as acceptor substrate for tissue transglutaminase *in vitro*. When Chinese hamster ovary cells, which contain exclusively tissue transglutaminase, were stably transfected with a constitutive expression plasmid of human involucrin, the synthesized protein became cross-linked following influx of Ca^{2+} into these cells (29). Based on these data we assume that involucrin may possess a wider function in living organisms than serving as one of the building blocks of the cornified envelope. Immunohistochemical studies on the liver sections clearly demonstrate the presence of involucrin-like immunoreactivity in hepatocytes. The island-like appearance and the absence of the protein in many hepatocytes suggest that its synthesis might be linked to a special phase of the cell life, very likely to the preliminary events of programmed cell death (apoptosis). This idea is

strongly supported by the presence of involucrin antigenicity in dying cells and in the isolated, cyanogen bromide-digested apoptotic bodies. The fact that the anti-human cornified envelope antibody also reacted with the apoptotic bodies on dot blots, although it does not recognize human involucrin (12), suggest that there might be even further precursor proteins mutually used for producing cornified and apoptotic envelopes. Furthermore, we could isolate the involucrin-like protein from other parenchymal tissues with significant apoptosis rate such as kidney and spleen (data not shown).

Terminal differentiation of stratifying epithelia and apoptosis of parenchymal cells are different forms of naturally occurring cell death. The two processes have distinct features (7, 8). The duration of terminal differentiation is 1 month, whereas apoptosis takes place within 4–18 h; the elimination of dead cells is desquamation in cornification and usually phagocytosis in apoptosis. However the common features seem to be more characteristic (7, 8). 1) Intrinsic control mechanisms utilize bcl-2 protein as a physiological blocker in both cases (7, 30). 2) Elimination of cells occurs without tissue destruction, inflammation, or scar formation. 3) Both processes are triggered by calcium ions and modulated by glucocorticoids as well as retinoids. 4) Disintegration of DNA into nucleosome-sized fragments by an endogenous endonuclease occurs (31). 5) Encapsulation of cells by a highly cross-linked protein envelope catalyzed by a transglutaminase. 6) Finally, our present results suggest that these transglutaminases may use the same or similar substrate proteins to form the cross-linked envelopes.

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