Involucrin Is a Covalently Crosslinked Constituent of Highly Purified Epidermal Corneocytes: Evidence for a Common Pattern of Involucrin Crosslinking *in Vivo* and *in Vitro*

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Involucrin (hINV) is an important structural component of the keratinocyte cornified envelope that is expressed early in the keratinocyte differentiation process and is thought to be a component of the initial envelope scaffolding. We have previously shown that cyanogen bromide (CNBr) cleavage of cornified envelopes isolated from cultured foreskin keratinocytes releases several discrete involucrin-immunoreactive peptides. In this study, we compare the pattern of release of immunoreactive hINV fragments from envelopes prepared from human breast skin and foreskin, and from spontaneous and induced envelopes prepared from cultured keratinocytes. We also identify one of the released products. Envelopes prepared from human breast skin or foreskin, or spontaneous or induced envelopes prepared from cultured cells differ significantly in structure. The envelopes isolated from epidermis appear to be structurally complete, whereas spontaneous envelopes appear less complete and the induced envelopes appear to be the least complete. In spite of these structural differences, CNBr cleavage releases an identical quartet of hINV-immunoreactive peptides migrating between 68 and 81 kDa from each preparation. Immunoblots indicate that the quantity of hINV-immunoreactive material released per μg of envelope protein is as follows: induced > spontaneous > foreskin > breast skin. The fastest migrating peptide (68 kDa) comigrates with a peptide that is released after CNBr cleavage of bacterially produced recombinant hINV. Amino-terminal amino acid sequencing of this peptide from recombinant hINV and from the cornified envelopes yields the sequence G-Q-L-K-H-L-E-Q-Q-E-G-Q-P-K-H. These results suggest that this fragment is the 275–amino acid segment of hINV beginning at G_{311} and extending to K_{585} and that this peptide is not crosslinked to another protein. These results indicate that a population of the envelope-associated hINV present in cultured and *in vivo* keratinocytes is crosslinked in the amino-terminal half. It is possible that this species represents an early intermediate in the involucrin crosslinking process.

Key words: *keratinocyte/epidermis/skin/epidermal/isopeptide bonds/transglutaminase/cornified envelope/cornification/differentiation/terminal differentiation. J Invest Dermatol 107:101–107, 1996*

The cornified envelope is a sheath of crosslinked protein adjacent to the interior surface of the plasma membrane (Matoltsy and Matoltsy, 1966; Rice and Green, 1977; Green, 1977; Green *et al*, 1982; Eckert, 1989). The molecular structure of the envelope has been difficult to study because of the nature of its construction. The envelope is assembled via formation of transglutaminase-catalyzed ϵ-(γ-glutamyl)lysine bonds between envelope precursor proteins (Ogawa and Goldsmith, 1976; Rice and Green, 1977; Rice and Green, 1979; Steinert and Marekov, 1995). These crosslinks render the envelope insoluble and resistant to extraction by detergent and reducing agents. Therefore, much of what is known has been deduced from studying envelope precursor proteins. A variety of precursor proteins have been described including involucrin (Rice and Green, 1977; Eckert *et al*, 1993; Murthy *et al*, 1993; Yaffe *et al*, 1993), loricrin (Mehrel *et al*, 1990; Hohl *et al*, 1991; Yoneda and Steinert, 1993), small proline-rich proteins (Kartasova and Van De Putte, 1988; Marvin *et al*, 1992; Gibbs *et al*, 1993; Greco *et al*, 1995), cystatin-α (Kartasova *et al*, 1987; Takahashi *et al*, 1992), and cysteine-rich protein, which may be elafin (Tezuka and Takahashi, 1987; Nonomura *et al*, 1994; Steinert and Marekov, 1995; Steinert, 1995).

The current model proposes that envelope assembly proceeds in two stages (Steinert, 1995; Eckert *et al*, 1993; Reichert *et al*, 1993). During the first stage, a scaffolding is assembled from soluble precursors such as involucrin and cystatin-α. In the second stage, other precursors are added to this scaffolding. Human foreskin

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Abbreviations: TCA, trichloroacetic acid; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% Tween-20; rhINV(1–585), human recombinant involucrin; CNBr, cyanogen bromide; PVDF, polyvinylidene difluoride; MS, mammary skin; FS, foreskin envelopes; SC, spontaneous cultured envelopes; IC, induced cultured envelopes.

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envelopes contain loricrin as the major component and lesser amounts of small proline-rich proteins, cystatin-α, and involucrin (Steven and Steinert, 1994). In contrast, envelopes isolated from cell culture contain small amounts of loricrin, but are rich in involucrin, cystatin-α, and cysteine-rich protein/elafin (Steven and Steinert, 1994). Thus, in general, cultured cells selectively make proteins that are part of the initial scaffolding. These results suggest that *in vitro* envelopes may be representative of the early phases of envelope assembly. We have previously noted several involucrin-immunoreactive protein fragments that are released after CNBr digestion of spontaneously formed envelopes prepared from cultured keratinocytes (Yaffe *et al*, 1993). Several of these fragments comigrate with fragments released after digestion of purified hINV. In the present study we extend these results and show that a quartet of hINV-immunoreactive fragments can be released from *in vivo* envelopes and envelopes prepared from cultured cells. Our results indicate that these crosslinked involucrin products are present in both *in vivo* and *in vitro* envelopes and suggest that a population of hINV molecules is linked to the envelope via a covalent linkage located in the amino-terminal half of the protein.

MATERIALS AND METHODS

Construction of hINV Expression Vector, Production of Recombinant Involucrin, and Production of An Anti-hINV Antibody A 2.1-kb cDNA fragment encoding the entire hINV coding sequence, 20 bp of 5′ noncoding sequence, and 325 bp of 3′ noncoding sequence was cloned into pRSET-B to yield expression vector phINV(1–585). This plasmid produces the complete recombinant hINV protein, rhINV(1–585), fused at the amino terminus to a short polyhistidine tract. phINV(1–585) was transferred to bacterial strain BL21, which carries the bacteriophage T7 RNA polymerase gene under *lacUV5* control. Production of rhINV(1–585) was induced with 2 mM isopropyl-β-thiogalactopyranoside. Lysates were prepared using standard methods, and the recombinant involucrin protein was purified using a nickel immobilized metal affinity chromatography column. The bacterially produced recombinant involucrin protein, rhINV(1–585), was used as a standard substrate for cyanogen bromide cleavage and to immunize rabbits to produce a polyclonal anti-hINV antibody.

Preparation of Intact Cornified Envelopes The epidermis and dermis of human breast skin were separated by heating for 1 min in phosphate-buffered saline at 65°C (Kvedar *et al*, 1992). The epidermis was collected, treated for 5 min at 95°C in TSBE buffer (50 mM Tris-HCl, pH 7.4, containing 2% sodium dodecyl sulfate, 1% β-mercaptoethanol and 5 mM ethylenediamine tetraacetic acid), cooled, and centrifuged at 20,000 $\times$ *g* for 10 min. The pellet was extracted an additional four times in fresh TSBE to yield a preparation of intact cornified envelopes. Envelopes were prepared from human neonatal foreskins by treating foreskins with Hanks' balanced salt solution containing 10 mg of dispase per ml and 5 μg of gentamicin per ml at 4°C overnight (Longley *et al*, 1991). The epidermis was peeled from the dermis, and the corneocytes were extracted five times with TSBE (1 foreskin per ml) as described above. For isolation of spontaneous cultured cornified envelopes, human epidermal foreskin keratinocytes were grown as previously described (Eckert and Green, 1984) using the 3T3 feeder system (Rheinwald and Green, 1975). At confluence, to promote differentiation, keratinocyte cultures were shifted to medium containing delipidized fetal calf serum, lacking epidermal growth factor (Yaffe *et al*, 1993). Cultures were scraped into TSBE, and intact envelopes were prepared by extracting five times in TSBE as outlined above. Cultured keratinocytes were induced to form envelopes by incubating 80–100% confluent cultures in serum-free growth medium supplemented with 0.75 M NaCl (Rice and Green, 1979). After 6 h at 37°C the floating sheets of cells were collected by centrifugation and extracted five times with TSBE. Cornified envelopes were counted using an hemocytometer. In some cases, intact envelopes were stored for several weeks at −20°C in TSBE containing 2 mM phenylmethyl sulfonyl fluoride, 20 μg of aprotinin per ml, and 5 mM benzamidine.

Preparation of Purified Cornified Envelope Fragments To generate envelope fragments, samples of intact envelopes were sonicated as previously described until >97% of the envelopes were fragmented (Yaffe *et al*, 1993). A portion of the sonicated sample was saved for protein estimation. The remainder of the sample was washed five times in 95°C TSBE buffer followed by centrifugation. The supernatant from the first wash step was saved (supernatant one, S1), as was the pellet from the last centrifugation, which yielded purified cornified envelope fragments. All samples were stored at −20°C in TSBE.

Estimation of Cornified Envelope Protein Concentration Envelope protein content was determined using a modified version of the solid phase densitometric method of Sportsman and Elder (1984). This method provides a rapid and simple method of estimating protein content in crosslinked and non-crosslinked structures. Envelopes or envelope equivalents (usually 1 $\times$ 10^5) were diluted into 1 ml of assay buffer (25 mM Tris/192 mM glycine, pH 8.3, containing 20% methanol) followed by addition of trichloroacetic acid (TCA) to a final concentration of 10% (w/v). Bovine serum albumin was prepared as a standard at 0–5 μg/300 μl in assay buffer and also treated with 10% TCA. The samples and standards were chilled on ice for 10 min prior to application to nitrocellulose. Nitrocellulose membrane (0.45 μM; Schleicher & Schuell, Keene, NH) was soaked in assay buffer for 10 min and placed on a piece of Whatman #1 filter paper (prewet with assay buffer) on a 96-well dot blot manifold. Assay buffer (300 μl) was added to the wells and drained prior to the addition of each sample. The TCA-treated bovine serum albumin standards were analyzed in triplicate. The envelope preparation fractions were typically analyzed at two concentrations (15,000 and 30,000 envelopes or envelope equivalents/well) in duplicate. After application of all samples, the membrane was removed from the manifold and placed in 100 ml of cold 7.5% TCA, which was then heated to 80°C for 30 min. The TCA was decanted and the membrane soaked for 1 min at room temperature in a solution of 0.1% amido black in 25% propanol (v/v) and 10% acetic acid (v/v) (Gershoni and Palade, 1982). Unbound dye was removed from the membrane by two quick rinses with 25% propanol and 10% acetic acid, followed by a 20-min wash in the same solution on a rotating platform. The background becomes white after this treatment. The thoroughly air-dried membrane was made translucent by immersion in heavy mineral oil, placed in a heat-sealed pouch, and scanned by transmission densitometry using a Sci Scan 5000 automated densitometer. The assay was linear between 0.5 and 5 μg of bovine serum albumin per 78.5-mm^2 dot (r^2 = 0.99). Moreover, a linear relationship between cornified envelope number (over the range from 5,000 to 50,000 envelopes per 78.5 mm^2 dot) and microgram equivalents of bovine serum albumin was observed (r^2 = 0.99). An essentially identical line was observed when sonicated envelope fragments were assayed. For cornified envelopes isolated from mammary skin, 1.0 $\pm$ 0.1 μg of protein per 10,000 intact envelopes was measured. As a comparison, the same preparation gave a value of 0.8 $\pm$ 0.1 μg of protein per 10,000 envelopes when it was assayed by the more tedious Bramhall method (Bramhall *et al*, 1969).

Gel Electrophoresis and Immunodetection of Involucrin Following electrophoresis through 6 or 8% polyacrylamide gels (Laemmli, 1970), proteins were detected by staining with Coomassie blue or were transblotted to nitrocellulose using Towbin transfer buffer (Towbin *et al*, 1979) in preparation for immunoblotting. For immunoblotting, the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2 h prior to incubation for 15 h with a rabbit polyclonal primary antibody raised against human involucrin (diluted 1:20,000 in TBS-T). The blot was washed with TBS-T followed by a 1-h incubation with horseradish peroxidase-linked goat anti-rabbit secondary antibody (diluted 1:20,000; Amersham Corp., Arlington Heights, IL) in TBS-T. Binding of the antibody complex was visualized using ECL detection reagents (Amersham).

Cyanogen Bromide Digestion of Envelope Fragments Purified envelope fragments (60 μg) were pelleted and suspended in 70% formic acid (0.1 ml) containing 12 mg of CNBr. Control samples were suspended in 70% formic acid. After 24 h at room temperature, the samples were diluted with four volumes of sterile distilled water and uncleaved material was removed by centrifugation. The supernatant was freeze-dried, and the dried residue was dissolved twice in 0.2 ml of sterile distilled water, dried by rotary evaporation, and finally dissolved in Laemmli sample buffer (Laemmli, 1970) for gel electrophoresis. Purified human recombinant involucrin, rhINV(1–585), isolated from *Escherichi coli*, was CNBr-digested in parallel.

Affinity Chromatographic Isolation of CNBr-Released hINV Peptides An anti-rhINV(1–585)–Sepharose affinity column was prepared by coupling 80 mg of affinity-purified anti-hINV IgG with 8 g of CNBr-activated Sepharose in 50 ml of coupling buffer using standard conditions (affinity-purified anti-hINV IgG was prepared by passing crude rabbit serum through a rhINV(1–585)–Sepharose column). The resin was washed extensively with phosphate-buffered saline and stored in phosphate-buffered saline containing 0.02% NaN_3. To enrich for CNBr-released hINV peptides, induced envelope fragments were purified from cultures. These fragments (9 mg) were washed three times with 8 ml of water, pelleted at 18,000 $\times$ *g*, and digested with CNBr as described above. After lyophilization, the dried residue was taken up in 5 ml of sterile water and insoluble material was removed by centrifugation. The sample was again lyophilized,

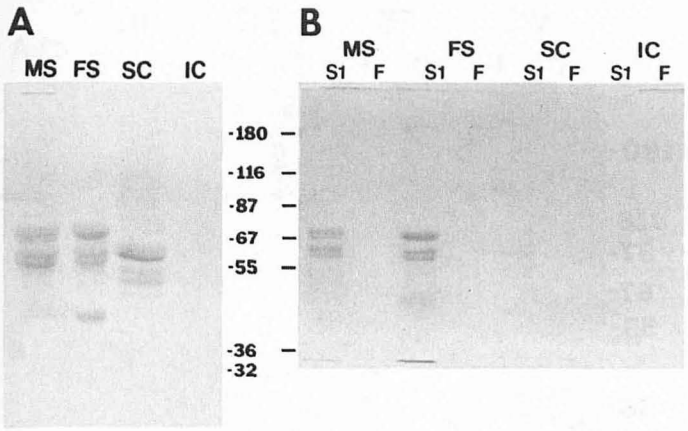

Figure 1. Electrophoretic detection of proteins present in fractions prepared from cornified envelopes. The S1 fraction (10 μg) from mammary skin (*MS*), foreskin (*FS*), spontaneous cultured (*SC*), and induced cultured (*IC*) envelopes were prepared, electrophoresed on an 8% acrylamide gel, and stained with Coomassie (*A*). In a parallel experiment (*B*), sonicated/washed envelope fragments (*F*) and S1 fraction (*S1*) derived from 100,000 intact envelopes were electrophoresed on a 8% acrylamide gel and stained with Coomassie. Migration of the molecular weight markers is indicated between the panels.

Table I. Comparison of Crosslinked and Non-crosslinked Proteins in Cornified Envelopes

Preparation	Micrograms Protein per 10,000 Envelopes[a]			
	Intact Envelopes[b]	Sonicated Envelopes[b]	S1 Fraction[b]	% S1[c]
Human Breast	1.0± 0.1	1.0± 0.1	0.80± 0.1	80
Human Foreskin	1.4± 0.5	1.5± 0.5	1.3± 0.5	87
Spontaneous Cell Culture	1.1	1.2	0.6	50
Induced Cell Culture	0.3± 0.1	0.4± 0.1	0.2± 0.1	50[d]

[a] Protein content per envelope was estimated by determining the quantity of protein per 10,000 envelope equivalents of intact envelopes, envelopes that had been sonicated, or S1 fraction as described in *Materials and Methods*.

[b] The intact and sonicated columns compare identical samples, before and after sonication. The sonicated sample includes both crosslinked and non-crosslinked material. The close agreement between the values in the intact and sonicated columns indicates that sonication, which physically improves accessibility to the envelope interior, does not significantly influence the estimate of protein present. The S1 column shows the amount of non-crosslinked protein (i.e, total envelope − envelope fragments = non-crosslinked (S1) fraction).

[c] Percent non-crosslinked protein (S1) was determined by dividing the mean quantity of released protein (S1 fraction) per 10,000 envelopes by the mean quantity of sonicated protein per 10,000 envelopes (i.e., non-crosslinked/[non-crosslinked + crosslinked] × 100).

[d] As explained in the text, this value is an overestimate. Only a trace amount of non-crosslinked, soluble protein is present in IC envelopes.

taken up in 2 ml of TBS, and incubated with 10 ml (packed volume) of anti-rhINV(1–585)–Sepharose for 16 h at 4°C. The mixture was poured into a 2.4- × 9-cm column, allowed to settle, washed with TBS to remove unbound protein, and bound protein was eluted with 3 M KSCN in TBS. Each 1.2-ml fraction was assayed (10 μl) by dot blot for reaction with involucrin antibody. The involucrin-positive fractions were pooled, and the protein was precipitated by the addition of 10% TCA. After 10 min on ice, the precipitate was collected by centrifugation at 16,000 × *g*. The pellet was washed once with iced 10% TCA and then taken up in Laemmli sample buffer. The sample was adjusted to pH 8 with sodium hydroxide prior to electrophoresis.

Protein Microsequencing CNBr-digested rhINV(1–585) (10 μg) and immunocolumn-purified, involucrin-positive CNBr fragments were electrophoresed on a 6% polyacrylamide gel. The separated peptide fragments were transferred onto a PVDF membrane (Transblot; 0.2 μm, Bio-Rad) using 10 mM 3-(cyclohexylamino)propanesulfonic acid buffer (pH 11) containing 10% methanol. The membrane was stained with Coomassie blue according to the manufacturer's instructions, and the membrane segment containing the peptide fragments of interest was excised. The peptide sequence was determined by sequencing the membrane-immobilized protein (LeGendre and Matsudaira, 1989) using a Procise 494 microsequenator.

RESULTS

Cell Culture Envelopes Contain Less Protein Than *in Vivo* Envelopes **Table I** compares the amount of soluble (non-crosslinked) and insoluble (crosslinked) protein present in cornified envelopes prepared from various sources. These results indicate that the amount of soluble and insoluble protein varies considerably in envelopes prepared from different sources. For envelopes prepared from *in vivo* sources, breast or foreskin, the soluble protein content measures around 80–90%. Envelopes formed by cultured cells, whether spontaneous or induced, contain about 50% soluble protein. In addition, the amount of total protein present per induced envelope is approximately three times lower than in the other preparations.

To further estimate the distribution of soluble *versus* non-soluble proteins, we electrophoresed 10 μg of S1 fraction from each of the four envelope preparations and stained with Coomassie blue. As shown in **Fig 1*A***, a large quantity of soluble protein is present in the S1 fraction prepared from mammary skin (MS) and foreskin (FS) envelopes and from spontaneous cultured (SC) envelopes. In contrast, very little non-crosslinked protein was detected in the S1 fraction from induced cultured (IC) envelopes. These results suggest that the protein assay estimate **(Table I)** showing that approximately 50% of IC envelope protein is soluble is an overestimate. The material measured as soluble protein in IC envelopes appears to consist of large fragments of cornified envelope that are not pelleted by low speed centrifugation, but cannot penetrate the acrylamide gel. We verified this by centrifuging the S1 fraction prepared from IC envelopes at 100,000 × *g* and measuring the protein concentration in the supernatant. This process yielded a glassy pellet that contained >95% of the protein. In contrast, when the S1 fraction from SC envelopes was centrifuged at 100,000 × *g*, no pellet formed, and >95% of the protein was recovered in the 100,000 × *g* supernatant. We conclude that approximately 80% of the protein measured in intact MS and FS envelopes and approximately 50% of the protein measured in intact SC envelopes is soluble, whereas intact IC envelopes contain little if any soluble protein.

To directly compare the amount of trapped protein per envelope, an equal number of envelope equivalents of S1 fraction (S1) or washed sonicated envelopes fragments (F) was electrophoresed per lane (i.e., 100,000 envelope equivalents/lane) of an acrylamide gel, and the gel was stained with Coomassie blue **(Fig 1*B*)**. Release of a similar quantity of protein per envelope was observed from the S1 fraction prepared from MS and FS envelopes, whereas less was released from SC envelopes. The cytokeratins were the most abundant soluble proteins in each preparation. No protein was detected in the S1 fraction from IC envelopes. Moreover, no protein was detected upon electrophoresis of the washed envelope fragments from any preparation. Thus, envelopes prepared from different sources contain different amounts of trapped non-crosslinked protein per envelope.

Envelopes Formed *in Vivo* and in Cell Culture Are Structurally Distinct Intact envelopes from the four sources were characterized by electron microscopy **(Fig 2)**. As expected from the biochemical analysis, purified intact envelopes from both mammary skin and foreskin epidermis contain large amounts of trapped protein. Intact envelopes formed spontaneously in culture contain much less nonenvelope protein, and intact induced envelopes formed in culture are free of trapped protein. In addition, the appearance of the intact envelopes differs for each preparation.

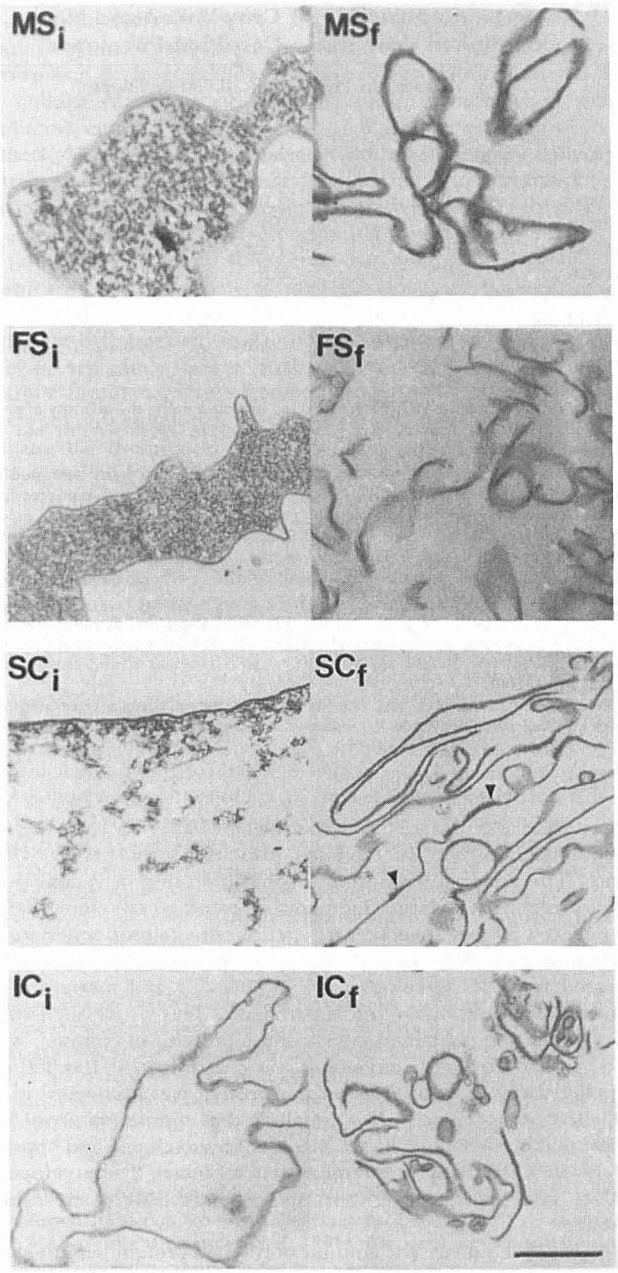

Figure 2. Electron microscopy of intact envelopes and envelope fragments. Intact envelopes (*i*) and envelope fragments (*f*) were isolated from mammary skin (*MS*) and foreskin (*FS*) and from spontaneous cultured (*SC*) and induced cultured (*IC*) envelopes. After repeated extraction in TSBE buffer, the preparations were washed and fixed for transmission electron microscopy. The *arrowheads* (▼) in the SC_f panel point to desmosomal plaques. *Scale bar* = 0.5 μm. Intact cornified envelopes or purified envelope fragments were processed for electron microscopy as previously described (Yaffe *et al*, 1993).

Intact human mammary envelopes have two electron-dense bands that sandwich a transparent layer. The foreskin and spontaneous cultured envelopes appear as a single thick band. The induced cultured envelopes display a "flimsy" appearance. The corresponding sonicated envelope fragments retain these structural characteristics. After sonication and washing, electron microscopy shows that all of the envelope fragments are free of soluble contaminants (**Fig 2**, *right panels*). As previously reported (Yaffe *et al*, 1993), the association of desmosomal plaques with the envelope fragments is readily apparent in the SC envelope fragments.

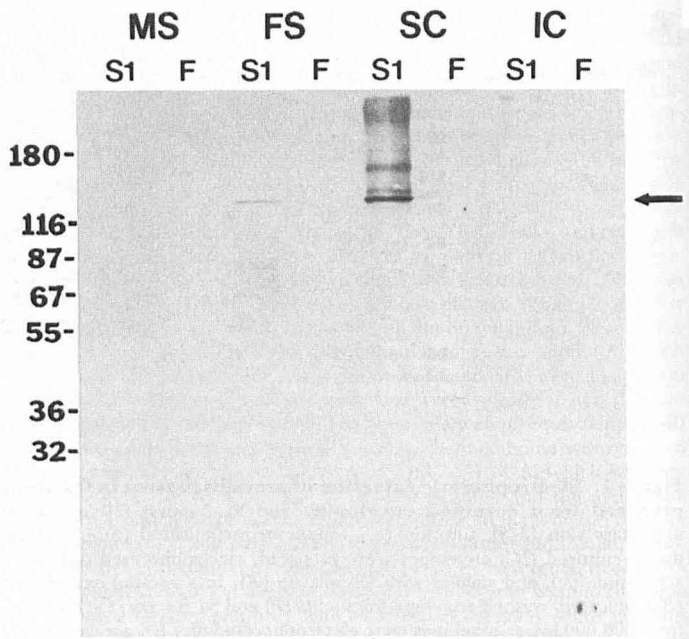

Figure 3. Absence of hINV release from purified cornified envelope fragments. Equal (100,000 envelope equivalents) numbers of envelope fragments (*F*) and S1 fraction (*S1*) from mammary skin (*MS*), foreskin (*FS*), spontaneous cultured (*SC*) and induced cultured (*IC*) envelopes were loaded per lane and electrophoresed on a 8% acrylamide gel. The separated proteins were transferred to nitrocellulose and the involucrin-immunoreactive material was detected by incubation with a primary anti-involucrin antibody followed by visualization with a secondary antibody linked to a horseradish-peroxidase. The *arrow* (→) indicates the migration of authentic intact involucrin, and molecular weight markers are indicated to the left of the panel.

CNBr Releases hINV-Immunoreactive Peptides from Envelopes Formed *in Vivo* and in Cell Culture To verify that the envelope fragments were free of soluble hINV prior to CNBr cleavage, we assayed the quantity of hINV-immunoreactive material present in the S1 fraction and present in purified envelope fragments. Equal envelope equivalents (equivalent to 100,000 envelopes) of S1 fraction and purified envelope fragments were electrophoresed on an acrylamide gel, transferred to nitrocellulose, and immunoblotted with the involucrin-specific antibody. As shown in **Fig 3**, the S1 fraction from human mammary skin envelopes (MS) contained no involucrin-immunoreactive material, whereas S1 fraction from foreskin envelopes (FS) contained full-length involucrin. The S1 fraction from spontaneous culture envelopes (SC) contains abundant quantities of immunoreactive hINV of varying sizes; however, only a small amount of hINV-immunoreactive material is present in the S1 fraction from induced envelopes (IC). No hINV-immunoreactive material was present in the purified sonicated envelope fragments from any source.

CNBr cleavage of the purified envelope fragments is shown in **Fig 4**. Envelope fragments from each preparation were incubated in the presence (+) or absence (−) of CNBr, and electrophoresed on an 8% acrylamide gel (*A*). The electrophoresed protein was transferred to nitrocellulose and incubated with anti-involucrin antibody. Three major bands of hINV-immunoreactive material were released from the cell culture envelope fragments (**Fig 4*A***, *left panel*). These migrated at apparent molecular masses of 68, 77, and 120 kDa. CNBr treatment of *in vivo* foreskin envelope fragments released the 68- and 77-kDa involucrin-reactive peptides, but the 120-kDa species was not detected (**Fig 4*A***, *left panel*). When the film was exposed for a short time, these bands could not be detected in MS envelope fragments, but, upon prolonged exposure

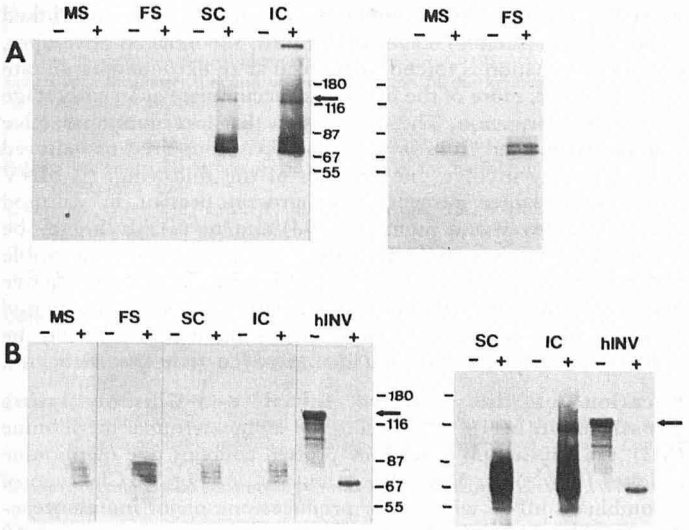

Figure 4. Cyanogen bromide releases a distinct set of hINV-immunoreactive peptides from purified envelope fragments. Purified envelope fragments from mammary skin (*MS*, 60 μg), foreskin (*FS*, 60 μg), cultured spontaneous (*SC*, 15 μg), and cultured induced (*IC*, 15 μg) envelopes were incubated in the absence (−) or presence (+) of CNBr and the products were fractionated on an 8% polyacrylamide gel. The fractionated proteins were transfer blotted to nitrocellulose, and hINV was detected by immunoblot (*A*). The right panel presents a longer exposure of MS and FS lanes from the left panel. In *B*, MS (60 μg), FS (60 μg), SC (3 μg), and IC (0.6 μg) envelope fragments were electrophoresed on a 6% acrylamide gel and immunoblotted for detection of involucrin fragments. The right panel presents a longer exposure of the SC and IC lanes from the left panel. The *arrows* (→) indicate migration of full length rhINV(1–585). Although rhINV(1–585) has a short amino-terminal poly-histidine tail, it comigrates with hINV extracted from cells (not shown). Molecular weight standards are shown between the panels.

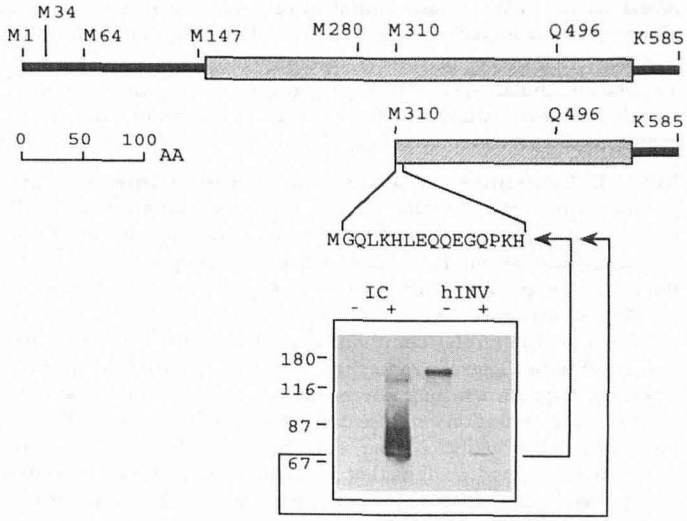

Figure 5. Microsequencing of CNBr-released 68 kDa hINV-immunoreactive fragment. Purified envelope fragments prepared from IC envelopes were incubated in the absence (*IC*, −) or presence (*IC*, +) of CNBr. The CNBr-released involucrin-immunoreactive fragments were enriched by immunoaffinity chromatography, electrophoresed on a 6% acrylamide gel, immobilized onto a PVDF membrane, and harvested for sequencing. In a parallel set of reactions rhINV(1–585) (10 μg) was incubated in the absence (*hINV*, −) or presence (*hINV*, +) of CNBr, electrophoresed an a 6% acrylamide gel and transferred to PVDF membrane. The PVDF-immobilized 68-kDa peptide was harvested and sequenced. The top line shows a map of the hINV protein showing the position of each of the six methionines (*M*) and K_{585} and Q_{496} (Eckert and Green, 1986). The second line shows the proposed cleavage product and the third line shows the amino acid sequence derived from the sequencing reaction. The *cross-hatched box* (▨) indicates highly conserved central involucrin segment (Eckert and Green, 1986). The bottom panel shows a representative immunoblot showing the peptides that were harvested for microsequencing. The scale indicates length in amino acid residues.

of the film, these bands were detected in MS envelope fragments (**Fig 4*A***, *right panel*).

To further compare the pattern and the quantity of CNBr-cleaved fragments released from each preparation, we adjusted the concentration of digest layered on the gel to give a comparable immunologic signal for each sample and electrophoresed the samples on a 6% acrylamide gel. This required 60, 60, 3, and 0.6 μg of protein, respectively, for MS, FS, SC, and IC envelope fragments. The doublet of involucrin-immunoreactive material (68 and 77 kDa) was resolved into four peptides of apparent molecular masses 68, 71, 77, and 81 kDa in the 6% gel. All four preparations yielded these same peptide fragments (**Fig 4*B***, *left panel*). Recombinant hINV migrates at approximately 148 kDa on 6% gels (hINV, −) and cleavage of recombinant hINV with CNBr releases a major peptide fragment migrating at 68 kDa and several minor higher molecular mass fragments (hINV, +). No immunoreactive material was detected released from envelopes that had not been treated with CNBr (−). The 68-kDa product released from each envelope preparation comigrates with the 68-kDa product released after CNBr treatment of rhINV(1–585). The relative amount of hINV-immunoreactive material contained in the quartet of peptides was estimated by densitometric scanning of the gels and then normalizing per microgram of digested protein. The relative amounts of these peptides released from MS, FS, SC, and IC envelopes were 1, 7, 100, and 480, respectively. In addition to these fragments, a fifth peptide (apparent size = 121 kDa) that migrated slightly faster than authentic hINV could be readily detected in CNBr digests of cell culture envelope samples when the film was exposed longer (**Fig 4*B***, *right panel*).

The 68-kDa CNBr-Released Immunoreactive Peptide Is the C-Terminal End of hINV CNBr cleavage of each preparation of envelope fragments resulted in the release of a peptide migrating at an apparent size of 68 kDa. To determine the identity of this peptide, we digested induced cultured envelope fragments with CNBr, purified the released involucrin-immunoreactive fragments using anti-hINV–Sepharose, and separated the products by electrophoresis on a 6% acrylamide gel. The electrophoresed material was electroblotted onto a PVDF membrane, and the area containing the 68-kDa peptide was removed for microsequencing **(Fig 5)**. This yielded the sequence G-Q-L-K-H-L-E-Q-Q-E-G-Q-P-K-H, indicating that this fragment was the result of CNBr cleavage downstream of M_{310} and that the peptide contained the 275–amino acid fragment extending from G_{311} to the carboxyl terminus at K_{585} (Eckert and Green, 1986). The sequence of the 68-kDa peptide matched that of the 68-kDa peptide derived from recombinant hINV (i.e., G_{311}-K_{585}). In addition, only a single sequence was detected, suggesting that this peptide was not covalently crosslinked to another protein.

DISCUSSION

Involucrin and the Cornified Envelope Our laboratory is interested in involucrin and its role as an envelope precursor protein (Eckert *et al*, 1993). Involucrin is a soluble precursor of the cornified envelope that possesses more than 100 potential glutamyl donor sites that could participate in isopeptide bond formation (Eckert and Green, 1986). Previous studies suggest that hINV is localized on the inner surface of the epidermal keratinocyte cornified envelope and is expressed in cells of the hair follicle inner root sheath (Rice and Green, 1979; Murthy *et al*, 1993). Recent transgenic studies indicate that overexpression of hINV in mouse epidermis is associated with a delay in hair ingrowth, alterations in hair appearance, and changes in the appearance of the epidermis

(Crish *et al*, 1993). Ultrastructural studies indicate that hINV is an elongated rod-shaped protein (Yaffe *et al*, 1992), and it has been suggested that hINV, because of its highly elongated rod-like shape and because glutamyl donor sites are uniformly distributed along its length, is ideally suited to function as an intermolecular cross-bridge in the cornified envelope (Yaffe *et al*, 1992).

hINV Is Crosslinked Via a Set of Common Intermediates In a previous study (Yaffe *et al*, 1993), we noted that CNBr cleavage of sonicated fragments of spontaneously formed envelopes prepared from cultured cells released several discrete hINV-immunoreactive peptides. Because we had only evaluated cultured envelopes, however, we could not be sure that these peptides represented structure(s) common to envelopes formed under natural conditions. Therefore, in the current study, we have purified envelope fragments from epidermis and cell culture and characterized these preparations ultrastructurally and biochemically. One interesting observation is that epidermal envelopes retain large amounts of trapped protein that is readily released after envelope disruption. Quantitative estimates suggest that >80% of the protein present in *in vivo* envelopes is non-crosslinked. In comparison to *in vivo* envelopes, spontaneous cell culture envelopes retain less trapped protein and induced cell culture envelopes do not trap any protein. This observation is supported by ultrastructural visualization of trapped protein within envelopes from each source, excepting induced envelopes. We interpret this difference in trapped protein content to result from differences in the crosslink density in envelopes from each source (i.e., *in vivo* envelopes have a higher number of crosslinks per unit envelope area, making it difficult to efficiently extract non-crosslinked proteins from these envelopes). Electrophoresis indicates that most of these proteins are keratins (Weiss *et al*, 1984).

Coomassie blue dye staining of gels containing CNBr-generated envelope cleavage products shows that the overall pattern of peptides released from *in vivo* and *in vitro* envelopes differs (Michel *et al*, 1988), a finding that we have verified (not shown). In spite of this difference in the pattern of CNBr-released Coomassie-stained peptides, however, immunoblot analysis with an hINV-specific antibody shows that CNBr cleavage releases four identical hINV-immunoreactive peptides from each preparation. This result suggests that identical sites on the involucrin protein are crosslinked during cornified envelope assembly both *in vivo* and *in vitro*. The finding that the hINV-immunoreactive peptides are discrete and limited in number suggests that the CNBr-released peptides represent a specific population of crosslinked hINV molecules in which one or a limited set of hINV glutamyl groups has formed crosslinks. Unpublished studies suggest that extensively crosslinked hINV may not be readily accessible to CNBr cleavage (LaCelle and Eckert, unpublished). Thus, we hypothesize that the released hINV peptides are derived from areas of the envelope that are actively being assembled and where hINV has not yet become extensively crosslinked.

CNBr Differentially Releases hINV from Envelopes Although the size and relative abundance of the four major hINV peptides released by CNBr appears generally similar for envelopes from each source, the total quantity of material released differs greatly. The largest amount of hINV-immunoreactive material is released from IC envelopes, less is released from SC envelopes, and very little is released from *in vivo* (MS and FS) envelopes. When normalized per μg of envelope protein, approximately 5 times less and 70–480 times less immunoreactive hINV is released from spontaneous cultured and *in vivo* corneocytes, respectively, compared to induced cultured envelopes.

For envelopes prepared from cultured keratinocytes (i.e., induced and spontaneous envelopes), this difference cannot be due to a difference in the level of the hINV precursor available for crosslinking, because soluble hINV content per cell is similar in each system (not shown). It is possible that less hINV is CNBr-released from spontaneous envelopes compared to induced envelopes because, in the spontaneous system, most of hINV has been moved from the initial crosslink stage to a more highly crosslinked (non-CNBr releasable) stage. In contrast, for induced envelopes, envelope formation is forced to proceed at an extremely rapid rate and, as a result, more of the hINV may accumulate at an early stage in crosslink formation. The observation that less immunoreactive material is released from *in vivo* envelopes compared to cultured envelopes may involve one or both of the following: (i) hINV represents a larger percentage of protein present in cultured envelopes (Steven and Steinert, 1994) and/or (ii) hINV may be more extensively crosslinked *in vivo* and as a result less susceptible to CNBr cleavage. It is interesting that less hINV-immunoreactive material is released from mammary epidermal envelopes compared to foreskin epidermal envelopes, suggesting that hINV may be more extensively crosslinked in mammary (i.e., adult) corneocytes.

Location of the Putative Initial ϵ-(γ-Glutamyl)lysine Crosslinks in hINV Excluding the amino-terminal methionine (M1), the 585–amino acid hINV protein contains five methionine residues (M_{34}, M_{64}, M_{147}, M_{280}, and M_{310}) **(Fig 5)**. Cleavage of recombinant hINV with CNBr produces one major immunoreactive peptide. This peptide has an apparent molecular mass of 68 kDa. Since involucrin fragments migrate at approximately twice their actual molecular mass (Simon and Green, 1984; LaCelle and Eckert-unpublished), the actual size of this fragment is around 34 kDa. Protein microsequencing shows that this 34-kDa peptide derived from rhINV is identical to smallest hINV-immunoreactive peptide released from cornified envelope fragments, indicating that each includes hINV amino acids G_{311} to K_{585}. The identical size of the envelope-derived and rhINV-derived CNBr peptide, and the fact that only one sequence is detected by microsequencing, suggests that that this peptide is free of crosslinks. Based on *in vitro* crosslinking studies, it has been suggested that Q_{496} may be the preferred site for formation of the first glutamyl crosslink in the hINV protein (Simon and Green, 1988). The observation that this segment is not crosslinked in the CNBr digested material suggests that hINV molecules exist within envelopes that are crosslinked via glutamine and/or lysine residue(s) that are located between M_1 and M_{310}.

hINV Crosslinking in Cornified Envelope Formation This report presents evidence that a quartet of discreet hINV-immunoreactive fragments can be released from envelopes formed by cultured keratinocytes and from envelopes formed *in vivo* (epidermal corneocytes). This result suggests the presence of a common pattern (or process) of involucrin crosslinking in both epidermal corneocytes and *in vitro* corneocytes. Involucrin, when extensively crosslinked by prolonged treatment with transglutaminase *in vitro*, cannot be released by cyanogen bromide (LaCelle and Eckert, unpublished). Thus, we speculate that the involucrin species that are released following CNBr cleavage of envelope preparations represent early intermediates in the crosslinking process. If this is the case, it would suggest a preference for formation of the initial crosslink in the amino-terminal half of the involucrin protein. As GLN_{496} has been reported to be a preferred site of crosslink formation *in vitro* (Simon and Green, 1988), it will be important, in future experiments, to determine whether other minimally crosslinked species of involucrin are present which are crosslinked via the carboxyl-terminal half of the protein.

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