Distinct Roles for Amino- and Carboxyl-terminal Sequences of SPRR1 Protein in the Formation of Cross-linked Envelopes of Conducting Airway Epithelial Cells*

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The small proline-rich protein, SPRR1, is a marker gene whose expression in conducting airway epithelium is elevated under a variety of conditions that enhance squamous differentiation. The purpose of this study is to elucidate the nature of the SPRR1 sequence involved in cross-linked envelope formation in a tissue/cell type, such as conducting airway epithelium, that normally does not express squamous function except after injury or maintenance in culture. For this, a Flag-SPRR1 fusion protein expression system has been developed. Using the liposome-mediated gene transfer technique on passage 1 culture of human tracheobronchial epithelial (TBE) cells, the Flag-SPRR1 fusion protein can be expressed and detected immunologically by both anti-Flag and anti-SPRR1 antibodies. The incorporation of Flag-SPRR1 fusion protein into cross-linked envelopes can be demonstrated when transfected human passage 1 TBE cultures are treated with phorbol 12-myristate 13-acetate and high calcium (1.5 mM). By deletion and site-directed mutagenesis, two distinct roles of the amino- and carboxyl-terminal sequences of SPRR1 have been demonstrated. First, we demonstrated that the amino-terminal sequence of SPRR1 protein is required for the incorporation of the fusion protein into cross-linked envelopes, whereas a deletion on the carboxyl-terminal region or on the middle repetitive unit has no effect. Interestingly, insertion of a 24-amino acid peptide of the monkey MUC2 repetitive sequence in the amino-terminal or on the middle repetitive unit has no effect. Secondly, we demonstrated that the carboxyl-terminal sequence of SPRR1 protein is required for the incorporation of Flag-SPRR1 fusion protein expressed in transfected cells. In contrast, there was only a slight decrease in the level of expression if the amino-terminus was deleted. Interestingly, the efficiency for fusion protein to incorporate into cross-linked envelopes was elevated by the mutation at the carboxyl end. These results suggest distinct roles, perhaps coordinately, for both amino- and carboxyl-terminal sequences in the regulation of the life cycle of SPRR1 protein in cultured TBE cells.

Squamous differentiation of tracheobronchial epithelial (TBE) cells is a multi-step process, just as it is in skin keratinocytes. The end stage of squamous differentiation is the formation of a cornified envelope (CE), which is a highly complex and insoluble structure adjacent to the inner leaflet of the plasma membrane. The CE is produced by a covalent fusion of both cytosolic (2–4) and particulate proteins (5–6) in a reaction catalyzed by transglutaminase. These proteins are cross-linked into an insoluble mesh by the formation of γ-(ε-glutamyl) lysine isopeptide and disulfide bonds (7). Several proteins, including involucrin, loricrin, annexin I, small proline-rich proteins (SPRRs), and others, have been implicated as CE precursor proteins (1–3, 8).

SPRR1, a small proline-rich protein, belongs to the SPRR gene family, which contains two SPRR1 genes, eight SPRR2 genes, and one SPRR3 gene. They are all localized in a 300-kilobase segment of human chromosome 1 area q21, a region referred to as the epidermal differentiation complex (9). Immunohistochemical studies, using a polyclonal antibody against carboxyl-terminal peptide (15 amino acid) of the human/monkey SPRR1 protein, demonstrated that SPRR1 is predominantly present in the suprabasal cell layer of various squamous tissues such as epidermis, oral mucosa, and esophagus (10). This tissue- and cell-type-specific distribution of SPRR1 gene expression suggests a useful marker associated with squamous differentiation. SPRR1 protein structure is characterized by the presence of internal repeats and conserved amino acid sequences of both amino- and carboxyl-terminal regions across species (11), including proteins isolated from monkey TBE cells (12). Furthermore, the amino- and carboxyl-terminal sequences contain domains similar to several cross-linked envelope precursors, such as loricrin and involucrin (13–14). Based on this structural similarity, Backendorf and Hohl (15) sug-

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The abbreviations used are: TBE, tracheobronchial epithelium; SPRR, small proline-rich protein; PMA, phorbol 12-myristate 13-acetate; CE, cross-linked envelope; EGF, epidermal growth factor; CMV, cytomegalovirus; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; BAP, bacterial alkaline phosphatase.

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2. G. An and R. Wu, unpublished data.
gested that SPRR1 protein is a potential substrate involved in squamous cell cornification. Recently, a series of studies of proteolytic digestion of cross-linked envelopes prepared from human skin have identified the SPRR1 protein as one of the CE components. Residue Lys at the amino-terminal region and residues Glu and Lys at the carboxyl end are thought to be the cross-linked sites with loricrin (16–17). A similar study using cultured human foreskin keratinocytes also demonstrated that the amino terminus (residues 1–23) and the carboxyl terminus (residues 86–89) of the SPRR1A and SPRR1B proteins are involved in cross-linked formation with involucrin. No loricrin cross-linked formation was detected in this study (8).

It is possible that the composition of cross-linked envelopes varies in different squamous tissues. Jarnik et al. (18) observed different protein components in various cross-linked envelope preparations from different cornified epithelia. Compositional analysis of fore-stomach epithelia and epidermis suggested a very high (about 65%) loricrin content in cross-linked envelope preparation. However, the levels of SPRRs varied. For fore-stomach epithelia, the level of SPRRs in cornified envelope preparation was 18%, whereas in epidermis it was only 8%. These results support the idea that cross-linked envelopes from different squamous epithelia have variable compositions. When TBE cells undergo terminal squamous differentiation, they also form cross-linked envelopes. Because almost all studies on cross-linked envelope composition are performed using epidermis, the composition of CE in TBE cells is still unknown. Furthermore, the functional role of SPRR1 in the terminal squamous differentiation of TBE cells remains unknown.

In this study, we focused on the contribution of SPRR1 protein to cross-linked envelope formation in passage 1 human TBE cultures. We have demonstrated that cross-linked envelope formation could be induced in the passage 1 culture after treatment with phorbol 12-myristate 13-acetate (PMA) and high calcium (1.5 mM). Using a Flag-SPRR1 fusion protein expression system, we examined whether SPRR1 is incorporated into the cross-linked envelope of TBE cells and what the molecular nature of the incorporation is. The Flag sequence in the fusion protein allows us to see the distinction between the transfected gene product and the endogenous SPRR1 protein because only the fusion protein in the transfected cells can be visualized by mono-specific anti-Flag antibody. Using this approach, the participation of SPRR1 protein in cross-linked envelope formation can be elucidated. By deletion, insertion, and site-directed mutagenesis, the distinct roles of the amino- and carboxyl-terminal peptides in the regulation of the life cycle of SPRR1 protein associated with the terminal squamous differentiation of TBE cells can be observed.

MATERIALS AND METHODS

Cell Culture Conditions—Human tracheobronchial tissues were obtained from the University of California at Davis Medical Center or the Anatomic Gift Foundation (Laurel, MD) with consent. The Human Subject Review Committee of the University of California at Davis approved all procedures involved in tissue procurement. Epithelial cell isolation and culture conditions were performed as described previously with some modification (19, 20). Briefly, protease-dissociated primary TBE cells were cultured in Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1) medium supplemented with six growth factors: insulin (5 μg/ml), transferrin (5 μg/ml), EGF (10 ng/ml), cholera toxin (10 ng/ml), dexamethasone (0.1 μM), and bovine hypothalamus extract (15 μg/ml), as well as 0.2% fetal bovine serum. Cultures were trypsinized until confluence and then plated in a low calcium (0.09 mM) keratinocyte basal medium (BioWhittaker Inc., Walkersville, MD) supplemented with these six factors. The low-calcium culture condition minimizes the expression of terminal squamous cell differentiation in passage 1 human TBE cultures. The addition of PMA and high calcium (1.5 mM) to this culture system could induce cross-linked envelope formation.

The immortalized normal human TBE cell line, HBE 1 clone (21), and BEAS-2B subclone S (22) were obtained from Drs. Jr. and J. M. onkas (University of North Carolina at Chapel Hill) and J. F. Lechner (Wayne State University, Detroit, MI). HBE 1 cell line was maintained in serum-free F-12 medium supplemented with six hormonal supplements as described before (19, 20). When appropriate conditions were applied, such as the addition of vitamin A or PMA/CaCl2 (1.5 mM), HBE 1 cell line could express mucin synthesis and secretion, and the formation of cross-linked envelopes, respectively.

Preparation of Flag-SPRR1 Fusion Protein Expression Constructs—Mammalian expression vector pFlag-CMV2 was obtained from Eastman Kodak Company (Rochester, NY). The vector carried cytomegalovirus (CMV) promoter, Flag sequence (nine amino acids), and human growth hormone poly(A) adenylation site and intron processing signal. Various monkey SPRR1 cDNA fragments (12) were cloned into the multiple cloning sites downstream from the Flag sequence, using the unique EcoRI and SalI cloning sites for directional cloning. The CMV promoter can mediate the gene expression of Flag-SPRR1 fusion protein in various mammalian cells.

The polymerase chain reaction (PCR) method was used to prepare full-length and different fragments of the monkey SPRR1 cDNA coding region, using the monkey cDNA clone as a template with two oligomers specific for SPRR1 cDNA. The primers were designed to amplify the restriction enzyme cloning sites. Amplified DNA fragments were ligated to vector pFlag-CMV2 according to the restriction enzyme cloning sites, and positive clones containing the appropriate inserts were screened by PCR and further confirmed by restriction enzymatic mapping and DNA sequencing. Precautions were taken to make sure that the SPRR1 coding region was in-frame with the Flag coding sequence. To verify the in-frame nature of the SPRR1 insert, the polyclonal antibody that is mono-specific to the carboxyl-terminal 15 amino acid sequence of human/monkey SPRR1 was used. This showed whether this fusion protein, expressed in culture, could be recognized by this antibody in addition to the monoclonal antibody (M5) mono-specific to the Flag amino sequence. For the site-directed mutagenesis, the pFlag-SPRR1 construct was used as a template, and primers carrying the specific mutation site sequences were used for PCR amplification. The amplified products were then cloned into the vector as described. DNA sequencing was carried out to verify the specific site of mutation in the construct.

The PCR reactions were carried out in a total volume of 100 μl, containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.1% gelatin, 10 μM of each primer, 250 μM each of dATP, dCTP, dGTP, and dTTP. Initial denaturation was at 95 °C for 5 min followed by 35 cycles at 94 °C for denaturation (1 min), 55 °C annealing (1 min), and 72 °C extension (2 min) and a final extension at 72 °C for 10 min in an automated thermal cycler (Perkin-Elmer).

DNA Transfection Study—DNA transfection was carried out by a Lipofectamine-mediated gene transfer technique, according to the instructions of the manufacturer (Life Technologies, Inc., Bethesda, MD). DNA and Lipofectin were mixed with keratinocyte basal medium without serum and antibiotics in two separate tubes and incubated at room temperature for 30 min. Then DNA and Lipofectin were mixed in a 1:4 ratio by weight and incubated at room temperature for another 30 min. Each culture dish with 80% confluence of TBE cells was washed with serum-free culture medium once and then transfected with 2 μg of pFlag-CMV2 plasmid DNA, carrying different SPRR1 inserts in 0.5 ml of culture medium. The transfected culture dishes were kept in an incubator at 37 °C with 5% CO2 for 4–6 h, and then 1 ml of fresh six factor-supplemented low calcium keratinocyte basal medium was added to each dish. Medium change was carried out the next day, and the dishes were continuously maintained in the same culture medium. For induction of cell cornification, cultures at day 2 of transfection were treated with PMA (5 ng/ml) and high calcium (1.5 mM) as described before. Cross-linked envelopes were harvested the next day after the PMA/CaCl2 (1.5 mM) treatment.

Western Blot Analysis—Cultured cells were lysed with ice-cold keratin extraction buffer (KEB: 20 mM Tris-Cl, pH 7.0, 0.8 M KCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 5000 rpm for 10 min (23). The supernatant was recovered, and the protein concentration was quantified by DC-protein assay kit (Bio-Rad).

3 J. Deng, Y. Chen, and R. Wu, unpublished data.

4 J. Deng, R. Fun, and R. Wu, unpublished observation.

5 J. Deng and R. Wu, submitted for publication.
To prepare a Western blot membrane, an equal amount of protein (20 μg) was loaded into each well and separated by SDS-PAGE electrophoresis and then blotted onto nitrocellulose membranes. The Flag-SPRR1 fusion proteins were detected by immunostaining with polyclonal anti-SPRR1 and M5 monoclonal anti-Flag (Kodak) antibodies. The immunoreactive bands were visualized by a Vectastain® ABC kit from the Vector Laboratoy (Burlingame, CA).

**Isolation and Characterization of Cross-linked Envelope—**One day after PMA/Ca^{2+} (1.5 mM) treatment, cultures were trypsinized, and the total cell number/dish was counted by a hemocytometer under a light microscope. Cross-linked envelope preparation was carried out as described before with a modified procedure to ensure the purity of cross-linked envelope preparation. Briefly, trypsinized cell suspensions were first treated with 1% SDS and centrifugation to reduce the viscous nature of the suspension. The pellets were then treated with 2% SDS and 2% β-mercaptoethanol and centrifugation. Pellets resistant to SDS and the reducing agent were spun down on a glass slide in a Cytospin Centrifuge from Shatspin Technologies (Norwood, MA). Glass slides were fixed in ice-cold methanol and processed for immunofluorescent staining based on instructions provided by the pCMV2-Flag vector supplier (Kodak). Briefly, glass slides were first reacted with primary monoclonal antibody against Flag sequence (M5) and/or against human/monkey C-terminal peptide polyclonal antibody. After extensive washing, glass slides were then reacted with an FITC-conjugated goat anti-mouse IgG antibody (Antibodies Inc., Davis, CA) and/or rhodamine-conjugated goat anti-rabbit antibody. After washing and air drying, one drop of mounting medium, Vectashield H-1000 (Vector Laboratory) was applied to each slide and covered with coverslip. For each immunostaining, a negative control was carried along to exclude the nonspecific binding of these antibodies. These slides were observed under a fluorescent microscope or a Bio-Rad scanning laser confocal microscope detected with two filter sets, excitation/emission = 494/530 nm and excitation/emission = 505/533 nm, for FITC and rhodamine fluorences, respectively.

**RESULTS**

**Characterization of Flag-SPRR1 Fusion Protein Expression in Transfected TBE Cells—**To verify whether the pFlag-SPRR1 construct expresses the correct fusion protein, the synthesis of the fusion protein was characterized in BEAS-2B (S) cell line by transfection. BEAS-2B cell line expressed a very low level of SPRR1, and this cell line was also deficient in forming a cross-linked envelope even after PMA/Ca^{2+} (1.5 mM) treatment. As shown in Fig. 1, in pFLAG-CMV2 vector transfected cells, both anti-Flag and anti-SPRR1 antibodies did not stain a protein band in the Western blot analysis. By contrast, if cells were transfected with pFlag-SPRR1 construct, a protein band at a molecular mass slightly larger than 20.4 kDa was stained by anti-Flag and anti-SPRR1 antibodies. This result supports the in-frame nature of the cloning of SPRR1 gene onto the pFlag-CMV2 vector.

Because Flag sequence is not a native peptide in most mammalian cells, the above study raises two concerns: can the Flag sequence be incorporated into cross-linked envelopes of TBE cells. Cross-linked envelope preparation was carried out as described before with a modified procedure to ensure the purity of cross-linked envelope preparation. Briefly, trypsinized cell suspensions were first treated with 1% SDS and centrifugation to reduce the viscous nature of the suspension. The pellets were then treated with 2% SDS and 2% β-mercaptoethanol and centrifugation. Pellets resistant to SDS and the reducing agent were spun down on a glass slide in a Cytospin Centrifuge from Shatspin Technologies (Norwood, MA). Glass slides were fixed in ice-cold methanol and processed for immunofluorescent staining based on instructions provided by the pCMV2-Flag vector supplier (Kodak). Briefly, glass slides were first reacted with primary monoclonal antibody against Flag sequence (M5) and/or against human/monkey C-terminal peptide polyclonal antibody. After extensive washing, glass slides were then reacted with an FITC-conjugated goat anti-mouse IgG antibody (Antibodies Inc., Davis, CA) and/or rhodamine-conjugated goat anti-rabbit antibody. After washing and air drying, one drop of mounting medium, Vectashield H-1000 (Vector Laboratory) was applied to each slide and covered with coverslip. For each immunostaining, a negative control was carried along to exclude the nonspecific binding of these antibodies. These slides were observed under a fluorescent microscope or a Bio-Rad scanning laser confocal microscope detected with two filter sets, excitation/emission = 494/530 nm and excitation/emission = 505/533 nm, for FITC and rhodamine fluorences, respectively.

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**RESULTS**

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Because Flag sequence is not a native peptide in most mammalian cells, the above study raises two concerns: can the fusion protein perform identically to the native one, and is the fusion protein just as stable. To address the first concern, we performed immunofluorescent staining on passage 1 human TBE cultures after transfection with pFlag-SPRR1 construct DNA. As shown in Fig. 2, A–C, prior to the induction of cell cornification by PMA/Ca^{2+} (1.5 mM), both anti-Flag (FITC) and anti-SPRR1 (rhodamine) antibody recognized a similar immunofluorescent stain pattern in transfected cells. A majority of anti-Flag and anti-SPRR1 stains were seen. A majority of anti-Flag and anti-SPRR1 stains were dense at the edge of the cross-linked envelope. For cultures transfected with vector pFlag-CMV2 only, no Flag-specific antigen could be seen. A majority of anti-Flag and anti-SPRR1 stains were dense at the edge of the cross-linked envelope. For cultures transfected with vector pFlag-CMV2 only, no Flag-specific antigen could be seen. A majority of anti-Flag and anti-SPRR1 stains were dense at the edge of the cross-linked envelope. For cultures transfected with vector pFlag-CMV2 only, no Flag-specific antigen could be seen. A majority of anti-Flag and anti-SPRR1 stains were dense at the edge of the cross-linked envelope.
SPRR1, D1-(31–89); a 16-amino acid sequence of carboxyl terminus of SPRR1, D2-(1–73); the middle repetitive unit, D3-(1–30/74–89); and both amino- and carboxyl-terminal peptides, D4-(31–73). Using these fusion expression constructs for transfection, we observed the synthesis of these fusion peptides in transfected cells (Fig. 4B). As shown in the Western blot, both wild-type and D1 transfected cells expressed higher levels of Flag-fusion proteins than those cells transfected with D2, D3, or D4. There was an additional protein band at a higher molecular weight than the predicted one in both wild-type (pFlag-SPRR1) and D1 transfected cells. The nature of these bands is unknown. However, it was previously observed that heat treatment could cause a change of molecular weight on purified human SPRR1 protein (24, 25). The other possibility is a simple overloading of the sample in the gel. In either case, the deletion construct of D1 resulted in a decrease of molecular weight, compared with the wild type fusion protein. Similarly, D2 and D3, under different deletions, had different molecular weights than those of the D1 and wild type fusion proteins. However, D4 fusion protein had a molecular mass around 30 kDa, which was much larger than the predicted mass. This was probably because of an unusually high proline content at the 37% level in the D4 fusion protein, which might influence the mobility of this fusion protein in gel. Interestingly, as compared with the amino terminus deletion, the carboxyl terminus deleted mutants D2 and D4 had very low levels of expression in transfected cells. For D4, as much as 100 μg of cell protein extract was needed for each Western blot to be detected by anti-Flag, as compared with the other transfection, in which only 20 μg was enough.

The incorporation of deleted fusion proteins into cross-linked envelopes was examined using these deletion-construct transfected cultures. As shown in Fig. 5, these transfected cultures could be induced to form a cross-linked envelope, and Flag-
specific antigen could be detected in these cross-linked envelope preparations. Both D2 and D3 fusion proteins were as effective as wild type Flag-SPRR1 transfected cultures in the incorporation. In contrast, the Flag antigen was greatly reduced in D1 transfected cultures (Fig. 5, B and B'). These results suggest that the amino-terminal region of SPRR1 is required for the formation of cross-linked envelope. For D4-transfected cultures, probably because of low abundance of D4 fusion protein in the culture, no anti-Flag antigen was found in cross-linked envelope preparation (data not shown).

In addition to deletion analysis, we examined the effect of insertion on Flag-SPRR1 incorporation into cross-linked envelopes. Because amino-terminal sequence is important to the incorporation, we inserted a DNA fragment with a sequence not found in any SPRRs-one corresponding to the 24-amino acid repetitive sequence of monkey MUC 2 (26), PTTSTPTTTTTATPTPTPTSTQT, between Flag and SPRR1 in pFlag-SPRR1 construct. When passage 1 TBE cells were transfected with this construct DNA, a protein with a molecular weight larger than Flag-SPRR1 fusion protein could be detected by both anti-Flag (Fig. 6) and anti-SPRR1 (data not included) antibodies. This indicates that the coding region of pFlag-MUC2-SPRR1 expression construct is in frame with the correct amino acid sequence. However, there was a persistent 2-fold decrease of fusion protein expressed in cells transfected with the insertion construct, DNA, a protein with a molecular weight larger than Flag-SPRR1 fusion protein could be detected by both anti-Flag (Fig. 6) and anti-SPRR1 (data not included) antibodies. This indicates that the coding region of pFlag-MUC2-SPRR1 expression construct is in frame with the correct amino acid sequence. However, there was a persistent 2-fold decrease of fusion protein expressed in cells transfected with the insertion construct, DNA, a protein with a molecular weight larger than Flag-SPRR1 fusion protein could be detected by both anti-Flag (Fig. 6) and anti-SPRR1 (data not included) antibodies. This indicates that the coding region of pFlag-MUC2-SPRR1 expression construct is in frame with the correct amino acid sequence. However, there was a persistent 2-fold decrease of fusion protein expressed in cells transfected with the insertion construct, DNA, a protein with a molecular weight larger than Flag-SPRR1 fusion protein could be detected by both anti-Flag (Fig. 6) and anti-SPRR1 (data not included) antibodies. This indicates that the coding region of pFlag-MUC2-SPRR1 expression construct is in frame with the correct amino acid sequence. However, there was a persistent 2-fold decrease of fusion protein expressed in cells transfected with the insertion construct.
1 human TBE cells varied (Fig. 8B). Despite these variations, all the mutated fusion proteins were able to incorporate into cross-linked envelopes (Fig. 9). These results suggest that the residues on Lys89, Gln88, Lys87, and Lys89 are not the only unique sites participating in the cross-linked envelope formation. One noticeable change was that there was a 5-fold decrease in the levels of M1, M4, and M5 fusion proteins as compared with the wild type and other mutated ones (Table II). These M1, M4, and M5 constructs, unlike the others, all had the mutation on Lys89 residue, and despite a low level of expression, they were more efficient than other Flag-fusion proteins in being incorporated into cross-linked envelopes (Fig. 9 and Table II). These experiments were repeated in a separate passage 1 TBE culture, derived from a different human donor, with similar results.

To further elucidate the nature of low level M1, M4, and M5 expression in transfected cells, RT-PCR was used to evaluate the message levels of these Flag-SPRR1 fusion genes. No difference was observed (data not shown), suggesting that the low levels of M1, M4, and M5 proteins are not because of a lack of message expression in these transfected cells. Subsequently, cycloheximide was used to inhibit new protein synthesis, and the turnover rates of these fusion proteins were assessed. As shown in Fig. 10, both Flag-SPRR1 wild type and M2 forms were quite stable in transfected culture cells, whereas M1 and M4 fusion proteins turned over rapidly, with a half-life of 24 h in culture. A similar result was observed with M5 protein (data not shown). One common mutation in M1, M4, and M5, but not in M2 and M3, was the mutation on the Lys89 of C-terminal region. These results further support the critical role of this Lys89 residue in the regulation of SPRR1 protein turnover in cultured cells.

Because there are clusters of Gln amino acid in the amino-terminal region which are potential substrates for cross-linked enzymes, we carried out mutation studies on these clusters to see whether they are involved in cornified envelope formation. As shown in Fig. 11, mutations on Gln4-Gln5-Gln6 and Gln22-Gln23-Gln24-Gln25 were performed. NM1 construct has mutations on the first Gln cluster (Gln4-Gln5-Gln6), and NM2 construct has mutations on the second Gln cluster (Gln22-Gln23-Gln24-Gln25), whereas NM3 has mutations on both Gln clusters (Fig. 11A). These mutations appeared to have no significant effect on the level of Flag-fusion protein expression (Fig. 11B); however, their incorporation seemed to be affected by these mutations. As shown in Table III, mutations on each Gln cluster reduced the incorporation of fusion protein into cornified envelope by half, and the incorporation was greatly reduced when both Gln clusters were mutated, suggesting the participation of these Gln clusters in the process of cell cornification.

TABLE I

| Construct               | CE/Fielda | Relative | Fusion protein expresseda | Relative (protein) expression | Relative incorporation efficiencya |
|-------------------------|-----------|----------|---------------------------|-------------------------------|----------------------------------|
| pFlag-SPRR1             | 26.7 ± 3.1| 1.0      | 48.0                      | 1.0                           | 1.0                              |
| pFlag-MUC2-SPRR1        | 36.4 ± 4.8| 1.36     | 23.9                      | 0.5                           | 2.72                             |

a The number of anti-Flag fluorescent positive cross-linked envelopes per optical field (CE/field) under a fluorescent microscope was based on ten randomly selected fields and expressed as mean ± SD.

b Arbitrary units from a densitometer measurement of these protein bands in Fig. 6 Western blot.

c Relative incorporation efficiency was calculated with the following equation: [relative cornification]/[Relative (protein) expression].

FIG. 6. Western blot and immunostaining analysis of Flag-SPRR1 and Flag-MUC2-SPRR1 expression in transient transfected human TBE cells. A, Flag-MUC2-SPRR1 construct; B, Western blot analysis. Flag-SPRR1 (lane 1) or Flag-MUC2-SPRR1 (lane 2) expression construct DNA was transfected into passage 1 human TBE cells. Seventy-two hours after transfection, proteins were harvested for Western blot and immunostaining with anti-Flag antibody. The density of the protein bands was quantified by a densitometer.

FIG. 7. Effects of N-terminal insertion of MUC2 sequence on the incorporation of Flag-SPRR1 into cross-linked envelopes. Cross-linked envelopes were purified from Flag-SPRR1 (A, A') or Flag-MUC2-SPRR1 (B, B') transfected cells and stained with mouse anti-Flag and goat anti-mouse IgG fluorescent antibodies. The pictures were taken under fluorescent (A, B) and phase-contrast (A', B') microscope. Bar = 10 microns.
It has been suggested before, based on the sequence similarity of amino and carboxyl termini between SPRRs and two other cornified envelope precursor proteins, involucrin and loricrin, that SPRR1 is also a precursor protein of cross-linked envelopes (9–10, 13). Direct evidence to support such a physical and biochemical link comes from two different approaches. One is by immunostaining with anti-SPRR1 antibody on cross-linked envelope preparation (18). The other is by proteolytic digestion of cross-linked envelopes, followed by amino acid sequence analysis of the isolated oligopeptide from digestion (8, 16). The current study, utilizing the transfection with Flag-SPRR1 fusion protein expression approach, further supports the theory that SPRR1 is involved in cross-linked envelope formation. Additionally, our study extends the observation, from those cell types that normally express squamous function, to those of an organ, such as in a conducting airway, which normally do not express squamous function except during injury and repair. We demonstrated that the incorporation of Flag-SPRR1 into cornified envelope is a sequence-dependent phenomenon, especially depending on the amino-terminal region of SPRR1 protein. This result suggests that SPRR1 is an active participant in cell cornification.

The other advantage of using the pFlag-SPRR1 transfection system is that it allows for sequence analysis of the functional role of the SPRR1 sequence at various regions and amino acid residues in the participation of cross-linked envelope formation. We have observed two distinct roles for the SPRR1 sequence in the regulation of cross-linked envelope formation. One, described above, is that the incorporation of SPRR1 into cross-linked envelopes of TBE cells depends on the amino-terminal sequence. By deletion, we have demonstrated that amino-terminal peptide is required for SPRR1 protein to participate in cross-linked envelope formation. Deletions in other portions of SPRR1 gene, such as the carboxyl-terminal region and the middle repetitive unit, had no effect. However, mutations on these residues, Lys7, Gln88, and Lys89, which had been shown to be involved in cross-linked envelope formation of skin epidermal cells, had no inhibitory effects. This finding is consistent with the theory that these residues are not the only sites involved in cross-linked formation. We further examined the potential role of Gln clusters of amino terminus in the cross-linked envelope formation. Mutations on one of the Gln clusters (NM1 and NM2 constructs) reduced the incorporation efficiency of fusion protein into cornified envelope preparation (Table III). However, mutations on all these Gln clusters, such as in the carboxyl-terminal end, had no inhibitory effects. This finding is consistent with the theory that these residues are not the only sites involved in cross-linked formation.
TABLE II

**Analysis of mutated Flag-SPRR1 expression and incorporation into cross-linked envelopes**

Experiments were carried out as described in Table I and Fig. 8. Data were obtained from one representative experiment. A duplicated experiment has been carried out in a separate passage TBE culture derived from a different human donor, with a similar result.

| Construct (pFlag-SPRR1) | CE/fields | Relative cornification | Fusion protein expressed | Relative (protein) expression | Relative incorporation efficiency |
|------------------------|-----------|-----------------------|-------------------------|------------------------------|----------------------------------|
| Wild type              | 26.7 ± 3.1| 1                     | 48.0                    | 1.00                         | 1.0                              |
| M1                     | 9.6 ± 2.6 | 0.36                  | 9.6                     | 0.20                         | 1.8                              |
| M2                     | 14.5 ± 3.2| 0.54                  | 36.1                    | 0.75                         | 0.7                              |
| M3                     | 19.0 ± 3.3| 0.71                  | 37.5                    | 0.78                         | 0.9                              |
| M4                     | 7.9 ± 1.2 | 0.30                  | 9.9                     | 0.21                         | 1.4                              |
| M5                     | 8.3 ± 2.3 | 0.31                  | 8.5                     | 0.18                         | 1.7                              |
| M6                     | 19.5 ± 2.4| 0.73                  | 29.8                    | 0.62                         | 1.2                              |

* Plasmid constructs used in this study were described in Fig. 8A.
* The number of anti-Flag fluorescent positive cross-linked envelopes per optical field (CE/field) under a fluorescent microscope was based on ten randomly selected fields and expressed as mean ± SD.
* Relative incorporation efficiency was calculated with the following equation: [relative cornification]/[relative (protein) expression].

**FIG. 10.** Effects of various mutations on the protein half-life of Flag-SPRR1 protein in transfected cells. Cultures were transfected as described in Fig. 9. Cycloheximide (10 μg/ml) was added to these cultures, and cellular proteins were isolated at various times as indicated after cycloheximide treatment. Western blot analysis, using anti-Flag antibody, was carried out on gel with an equal load of protein. A, composite blot of wild type (W), M1, M2, and M4 fusion protein at the time before cycloheximide treatment; B, time course study of W protein level in culture; C, time course study of M1 protein level in culture; D, time course study of M2 protein level in culture; E, time course study of M4 protein level in culture.

**TABLE III**

**Effects of mutations on the incorporation of Flag-SPRR1 protein into cross-linked envelopes**

Experiments were carried out as described in Table I and Fig. 11. Cross-linked envelopes were prepared as described under “Materials and Methods.” Total envelopes were counted under a light microscope on cytospin glass slides, while Flag-positive envelopes were counted under a fluorescent microscope on the same slides after immunostaining.

| Construct | Total envelope examined | Flag-positive envelope | Percent of incorporation |
|-----------|-------------------------|------------------------|-------------------------|
| Wild type | 5050                    | 436                    | 8.6                     |
| NM1       | 10200                   | 359                    | 3.5                     |
| NM2       | 8040                    | 257                    | 3.2                     |
| NM3       | 13300                   | 93                     | 0.7                     |

**FIG. 11.** A, site-directed mutations on Gln cluster of N-terminal region of Flag-SPRR1 protein. NM1 contained mutations on Gln4-Gln5-Gln6 cluster, and NM2 contained mutations on Gln23-Gln25-Gln26 cluster, whereas NM3 had mutations on both Gln clusters. B, Western blot analysis of these mutant fusion proteins in transfected human TBE cells, using anti-Flag antibody.
approaches on cornified envelopes of keratinocytes (8, 16), except in the carboxyl terminus. Steinert and Marekov (16) concluded that the three amino acid residues at Lys87, Gln88, and Lys89 are involved in the cross-linked envelope formation of keratinocytes. Utilizing a similar approach, Robinson et al. (8) were able to recover the central fragments of SPRR1 but not the fragments of Thr96-Lys89, Thr96-Lys87, Met-Lys7, and Gln9-23 Lys from trypsin-digested cross-linked envelope preparation of keratinocyte cultures. The recovery of the central fragments of SPRR1 from trypsin-digested envelope preparation is consistent with the current deletion analysis, which shows no effect on the incorporation of Flag fusion protein when the middle repetitive sequence of SPRR1 is deleted.

However, the no-effect phenomenon on the carboxyl-end deletion in the current study is difficult to understand. There are several possible explanations. One relates to the difference in the cell type and the culture condition used. Because TBE cells rarely form cross-linked envelopes except under cell culture conditions, the cornified envelopes formed may be different from those of keratinocytes in culture. Consistent with this notion, Jarnik et al. (18) have predicted differential incorporation of SPRR1 protein into cross-linked envelope in different tissues, based on compositional analysis. The other possibility is that the introduction of Flag sequence at the amino-end of SPRR1 gene product may negatively interfere with the accessibility of the carboxyl-end peptide for cross-linked enzyme. This possibility is less likely since Flag is introduced on the proximal end, rather than immediately adjacent to the carboxyl terminus; therefore, the interference should be more pronounced at the amino end. Furthermore, one would expect more interference to occur on the molecule if an unrelated amino acid sequence were added on next to this molecule. However, this study found no such interference on fusion protein incorporation when a 24-amino acid sequence of MUC2 repetitive unit was inserted on the amino-terminus of SPRR1. On the contrary, the efficiency was enhanced 3-fold in relation to this insertion. Therefore, the introduction of Flag and MUC2 sequences in the current study should not have any negative interference on the carboxyl end. Positive interference may occur on the amino end, which may explain an unusually higher efficiency of incorporation for Flag-MUC2-SPRR1 fusion protein. However, we cannot rule out this possibility based on the current study. To do so, both SPRR1 and Flag-SPRR1 fusion proteins need to be purified and used for the enzymatic kinetics analysis with isolated transglutaminase enzyme.

The second distinct role of the SPRR1 sequence is that the carboxyl-terminal sequence, especially the Lys89 residue, is involved in the regulation of the level of fusion protein expression in transfected culture. We noticed a great variation in the incorporation of various fusion proteins in transfected cultures. We noticed a great variation in the expression level. This phenomenon may suggest a coordinate regulation of the life cycle of SPRR1 in transfected TBE cells, an organ in which cells normally do not express squamous differentiation. Using the Flag-SPRR1 fusion protein expression approach, we observed two distinct roles for SPRR1 amino acid sequences in the regulation of the life cycle of SPRR1 in transfected TBE cells: the amino-terminal region of SPRR1 is essential for cross-linked envelope incorporation, whereas the carboxyl-end, especially the last lysine residue, is essential for the expression level of the fusion protein. Change of expression level in transfected cells can occur at the synthesis and degradation level. Further study is needed to determine the nature of the change of expression level. Interestingly, however, the incorporation of mutated Flag-SPRR1 fusion protein into cross-linked envelopes is generally enhanced despite low level of expression. This phenomenon may suggest a coordinate regulation of the life cycle of SPRR1 protein through the interactions between the SPRR1 amino acid sequences and the surrounding environment.

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Distinct Roles for Amino- and Carboxyl-terminal Sequences of SPRR1 Protein in the Formation of Cross-linked Envelopes of Conducting Airway Epithelial Cells

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