Transglutaminase Cross-linking Properties of the Small Proline-rich 1 Family of Cornified Cell Envelope Proteins

INTEGRATION WITH LORICIRIN

Small proline-rich 1 (SPR1) proteins are important for barrier function in stratified squamous epithelia. To explore their properties, we expressed in bacteria a recombinant human SPR1 protein and isolated native SPR1 proteins from cultured mouse keratinocytes. By circular dichroism, they possess no \( \alpha \) or \( \beta \) structure but have some organized structure associated with their central peptide repeat domain. The transglutaminase (TGase) 1 and 3 enzymes use the SPR1 proteins as complete substrates in vitro but in different ways: head domain A sequences at the amino terminus were used preferentially for cross-linking by TGase 3, whereas those in head domain B sequences were used for cross-linking by TGase 1. The TGase 2 enzyme cross-linked SPR1 proteins poorly. Together with our data base of 141 examples of in vitro cross-links between SPRs and loricrin, this means that both TGase 1 and 3 are required for cross-linking SPR1 proteins in epithelia in vivo. Double in vitro cross-linking experiments suggest that oligomerization of SPR1 into large polymers can occur only by further TGase 1 cross-linking of an initial TGase 3 reaction. Accordingly, we propose that TGase 3 first cross-links loricrin and SPRs together to form small interchain oligomers, which are then permanently affiliated to the developing CE by further cross-linking by the TGase 1 enzyme. This is consistent with the known consequences of diminished barrier function in TGase 1 deficiency models.

A large body of recent amino acid sequencing data has demonstrated that members of the three known classes of small proline-rich (SPR) proteins serve as constituents of the cornified cell envelope (CE) of stratified squamous epithelia (1–5), which is a specialized structure essential for barrier function (6–9). The SPR1 (two members), SPR2 (8–11 members) and SPR3 (one member) are assembled from a common plan (10–14). Their amino (head) and carboxyl (tail) domains are enriched in Gln and Lys residues and consist of sequences that have been conserved between each member of an SPR class but differ between classes. These flank a central domain consisting of a series of Pro-rich peptide repeats of sequences that likewise have been conserved between members of a class but vary between classes. The SPRs become cross-linked to themselves and other CE structural protein constituents by both disulfide bonds and \( N^\gamma-(\gamma-glutamyl)lysine \) and \( N^\epsilon, N^\epsilon-bis(\gamma-glutamyl)lysine \)-derivated isopeptide bonds formed by transglutaminases (TGases), resulting in an insoluble macromolecular protein complex ideal for barrier function (6–9). Moreover, for reasons that are not yet understood, individual SPR family members are differentially expressed in highly variable ways in many different types of epithelia (10–31).

Examination of the way in which the SPRs were cross-linked to protein partners in human and mouse CE preparations revealed several novel features of their properties and functions (1–5). First, only head and tail domain Gln and Lys residues were used for cross-linking. Indeed, multiple adjacent residues were often used simultaneously on the same protein to form a complex interchain, and perhaps intrachain, cross-linked network (3–5). Each SPR molecule participated in as many as four cross-links (4). Second, the SPRs were found to be cross-linked to many protein partners, and indeed, they were consistently found to function as cross-bridgers between themselves or between loricrin, involucrin, etc. (4, 5). Fourth, as initially suggested from expression studies by earlier investigators, our sequencing data directly revealed that the amounts of SPRs present in the CEs from various tissues varied widely; while human foreskin epidermal keratinocyte CEs contained about 5% SPRs (2), the CEs of the mouse stomach contained about 22% SPRs (5). Further modeling analyses revealed that the amounts of SPRs in the epidermal CEs from trunk, lip, and footpad correlate well with the degree of physical and mechanical trauma to which the tissues are normally subjected (5). Thus, we have proposed that cross-bridging SPRs play an important role as modulators of the biomechanical properties of the CEs and the entire epithelium in which they are expressed (5).

In order to further explore these functions of the SPRs, we need to be able to express large quantities of the proteins for study. Since their cross-bridging role is mediated to a substantial degree through TGase cross-linking, we also need to know which enzymes are responsible for their cross-linking in vivo and how this is done. In an initial study, we described the preparation and some properties of a member of the human SPR2 class, and showed that it is cross-linked almost entirely by the TGase 3 enzyme in vitro and in vivo (32). In this paper, we have extended this work to the study of a member of the human SPR1 class. We show here that its cross-linking is much more complex, since it requires at least two TGases (3 and 1) operating in a sequential manner using different Gln residues for cross-linking to its partners as seen in vivo.
**In Vitro Cross-linking of Small Proline-rich Protein 1**

---

**MATERIALS AND METHODS**

**Bacterial Expression and Purification of Human SPR1**—A full-length cDNA clone encoding human SPR1 (clone 15B of Ref. 10) was obtained as a generous gift from C. Backendorf. Following the addition of appropriate linkers, it was inserted into the pET-11a bacterial expression vector (Novagen, Madison, WI) and transformed into the host *Escherichia coli* B strain BL21 (DE3) (Novagen). Protein expression was induced in the presence or absence of IPTG (isopropyl-β-D-thiogalactoside (1 mM)) as described previously (32, 33). Fresh or previously frozen pellets of bacteria were lysed and dialyzed against several changes of 100 volumes of 25 mM sodium citrate (pH 3.6), 1 mM dithiothreitol, 1 mM EDTA, and a mixture of protease inhibitors as described (32, 33). While most of the bacterial proteins precipitated, the SPR1 protein remained soluble. Purification to homogeneity was achieved using an Amersham Pharmacia Biotek fast protein liquid chromatography system on a 0.5 × 5 cm Mono-S column equilibrated in the citrate buffer with a 0.1–0.4 M NaCl gradient and was eluted with 0.18 M salt.

The fractions were analyzed on 4–20% SDS-polyacrylamide gels (Novex, San Diego, CA) with Coomassie stain or by Western blotting using a polyclonal antibody broadly reactive against both mouse and human SPR1 proteins (14). The enhanced chemiluminescence detection system on 4–20% gradient gels, blotted onto nitrocellulose membranes, and analyzed by autoradiography. In preparative experiments, 100 μg of unlabelled recombinant human SPR1 were reacted in a volume 250 μl. In this case, we used the baculovirus membrane-bound full-length TGase 1 form and activated TGase 3 at concentrations of 700 nM for 18 h. Experiments showed that the cross-linking had proceeded to completion (see Fig. 3). We also performed a preparative experiment with the high specific activity keratinocyte membrane-bound 67/33/10-kDa isofrom at 50 nm enzyme concentration, a reaction that was complete in 2 h.

Kinetic constants of the three TGases were determined for the recombinant human SPR1 protein exactly as described before (32, 42).

**Protein Chemical Methods**—The concentrations and purity of the purified SPR1 proteins and TGase enzymes were determined by amino acid analysis. Uncross-linked or cross-linked SPR1 proteins were digested with trypsin (1:30 by weight; Sigma; sequencing grade) for 6 h at 37 °C, and the peptides were resolved on a Phenomenex ODS reverse phase HPLC column (2.1 × 250 mm) containing 0.8% TFA in acetonitrile and with a gradient of 5–65% acetonitrile over 70 min (32, 33). The peaks were collected and sequenced on a Poron LF3000 gas phase sequencer as described previously (32, 33, 42). In the case of the reactions with the TGase 3 enzyme, or TGase 3 followed by TGase 1, bacterial lysates were poorly resolved due to extensive cross-linking; in these experiments, 1-min fractions were collected across the peak for sequencing (32). Circular dichroism spectra on the intact SPR1 proteins or synthetic peptides (see below) were performed as before (32, 33, 42).

**Use of Synthetic Peptides**—The following peptides based on published human SPR1 sequences (13) were synthesized and purified by HPLC: human SPR1 head A domain, SSQQQKQPCIPPP; human SPR1 head B domain, PPQQLQQQQQKPC; human SPR1 head A + B domain, SSQQQKQPCIPPPQQLQQQKVQPCQ; human SPR1 tail domain, SIVTPPQAQKTQKQ; human SPR1 central domain repeat, (PKVPEFCQ)5, (PKVPEFCQ)6, and (PKVPEFCQ)6. Kinetic constants using the head and tail domain peptides as substrates for putrescine incorporation were determined exactly as described before (32, 33, 42).

**RESULTS AND DISCUSSION**

Recently, we described the preparation of a recombinant human SPR2 protein and demonstrated that it is a favored substrate of the TGase 1 enzyme in *vitro* and *in vivo* (32). In the present study, we have repeated these experiments using recombinant human and native mouse SPR1 proteins. We show here that both the TGase 3 and 1 enzymes are likely to be essential for SPR1 cross-linking *in vitro*, and in this consecutive order of reaction.

**Expression and Purification of the Recombinant Human SPR1 Protein**—Following expression in bacteria using the pET11a system, the human SPR1 protein was enriched from bacterial lysates by dialysis into 25 mM citrate buffer, pH 3.6 (in which it was very soluble), and purified by chromatography on a Mono-S fast protein liquid chromatography column (Fig. 1). The maximal yield was of the order of 2–5 mg/liter, typical of the CE buffer used in SPR1 purification (33) and SP12 (32) previously. As expected from its unusually basic pI value, the expressed SPR1 protein migrated on SDS gels with an apparent molecular mass of ~15 kDa, somewhat higher than the 10-kDa size calculated from its known sequence (Fig. 1, inset).

**Isolation of Native SPR1a and SPR1b from Cultured Mouse Epidermal Keratinocytes**—For the purposes of comparison of

---

The complete text is available in the provided image. The content is a detailed protocol for the expression, purification, and analysis of recombinant and native SPR1 proteins. It includes methods for bacterial expression, purification, and analysis, as well as experimental results and discussion.
biochemical and structural properties, we isolated native mouse SPR1 proteins from cultured keratinocytes. Under the conditions used, mouse keratinocytes express high levels of the SPR1 proteins, which consist of two gene products, termed SPR1a and SPR1b, that differ primarily by the presence of 13 or 14 central domain peptide repeats, respectively (14). By dialysis of phosphate-buffered saline extracts of keratinocytes into the pH 3.6 citrate buffer, the vast majority of epidermal proteins precipitated, thereby enriching for the SPRs, which were then purified to homogeneity on the Mono-S column. They were separated by cutting out bands from SDS gels. Their total yield was $\sim 1 \text{ mg/10}^8 \text{ confluent cells}$. These data indicate that a significant amount of the SPR1a/b proteins remain soluble in the keratinocytes cultured under these conditions.

Circular Dichroism Spectra of Native and Recombinant SPR1 Proteins—CD spectra were performed to evaluate the secondary structures of the recombinant human and native mouse SPR1 proteins. They have a limited degree of organized structure in phosphate-buffered saline (data not shown) or the TGase enzyme assay buffer at 20 °C, but there was essentially no $\alpha$ or $\beta$ structure present (Fig. 2A, lines 1 and 2, for recombinant and native proteins, respectively). However, the signal strength of native mouse SPR1a/b was reproducibly greater than that of recombinant human SPR1. Mouse and human SPR1 proteins have highly homologous head, tail, and central eight-residue peptide repeat sequences but differ by containing 13 versus 6 central repeats, respectively.

We performed additional experiments with synthetic peptides. First, peptides corresponding exactly to the head or tail domains of human SPR1 proteins generated only very weak CD signals, suggesting little significant structure (Fig. 2B, lines 1 and 2, respectively). On the other hand, peptides containing two (line 3), four (line 4), or six (line 5) peptide repeats characteristic of the eight-residue central domain of SPR1 proteins generated CD spectra that correlated with peptide size. Next, we assessed the overall structural properties of the SPR1 proteins as a function of temperature and guanidine hydrochloride. The recombinant human SPR1 (Fig. 2C, line 1) protein was at least partially unfolded by heating at 40 °C (line 2) and 60 °C (line 3), but the signals were normalized when returned to 20 °C (dotted line), indicating refolding of the protein structures. Similarly, recombinant human (Fig. 2D, solid line 1) or native mouse (dotted line 1) SPR1 proteins could be reversibly denatured upon the addition of lines 2) and then renatured upon the subsequent removal of (dashed lines) 4 M guanidine hydrochloride. Together, these data suggest that only the central peptide repeat domains of SPR1 proteins contain organized structures and that the signal strength of the CD spectra correlates to some degree with the numbers of peptide repeats. Moreover, the recombinant human protein possesses an organized structure similar to that of the native mouse SPR1a/b proteins of cultured keratinocytes. From this we conclude that the recombinant human SPR1 protein had folded into a native configuration upon isolation from the bacteria and is therefore appropriate for use in further biochemical experiments.

Epidermal TGases Use Recombinant Human and Native Mouse SPR1 Proteins as Complete Substrates but in Different Ways—Three TGase enzymes that are expressed in stratified squamous epithelia were used to cross-link in vitro the recombinant SPR1. We used the same molar amount (typically 500 nM) of each of the several isoforms of TGase 1, the only known active form of TGase 2, and inactive or activated forms of TGase 3, in order to make direct comparisons of their reactions (44, 45).

First, we measured the amount of isopeptide cross-link formed in the reactions and showed (Fig. 3) that most of the enzymes inserted measurable amounts into recombinant human SPR1, indicating that each enzyme uses this protein as a complete substrate. However, the extent and rates of reactions varied widely. The TGase 2 enzyme inserted relatively little (<0.2 mol/mol) cross-link (Fig. 3B); the activated TGase 3 enzyme inserted the most (3.1 mol/mol) (Fig. 3C); and, as expected, the proenzyme form of TGase 3 was inactive. A maximal amount of 1.1–1.2 mol/mol of cross-link was inserted by each of the five TGase 1 isoforms employed, but at different rates (Fig. 3A). The reaction was completed within 5 min with the highest specific activity form (67/33/10-kDa complex recovered from the membrane fraction of NHEK cells), but 15–18 h was required for the lowest specific activity form (full-length membrane-bound forms expressed in NHEK cells or baculovirus). Previous work has shown that there is about a 200-fold difference in specific activity between the intact and activated forms (38), which is reflected in these observed reaction rates. In general, the times required for reaction completion for each of the five isoforms mirror accurately their specific activities as previously measured against a succinylated casein substrate. These data support the conclusion that the various TGase 1 forms all use the SPR1 and casein substrates the same way.

Second, we resolved the cross-linking reactions by SDS-polyacrylamide gel electrophoresis and then performed autoradiography. By subsequent excision of the $^{35}$S-labeled bands from the SDS gels, we were able to obtain quantitative information.
With each of the TGase 1 isoforms, 85–92% of the SPR1 protein remained as a monomer (Fig. 4A for 67/33/10-kDa complex recovered from the NHEK membrane fraction, cytosolic 67-kDa form, and baculovirus intact form; data for other isoforms not shown); only traces of protein were oligomerized into apparent dimers or larger species trapped at the top of the gel. Similarly, only 8% of the SPR1 protein was oligomerized by the TGase 2 enzyme (Fig. 4A). However, Western blotting analyses with a specific TGase 2 antibody revealed that this was not due to SPR-SPR oligomerization but rather mostly due to autocatalytic cross-linking to TGase 2 itself (data not shown), a phenomenon described previously for this (32, 47, 48) and the related factor XIIIa TGase enzymes (49). On the other hand, the TGase 3 enzyme cross-linked the proteins, readily giving rise to short oligomeric products so that 88–98% of the SPR1 protein had been oligomerized (Fig. 4A). These data thus reveal fundamentally different reaction processes between the three TGases.

Third, we cross-linked in the same way samples of native mouse SPR1a/b proteins using the baculovirus full-length form of TGase 1, as well as TGases 2 and 3 (Fig. 4B). The pattern of cross-linking was essentially identical to that shown above.

FIG. 2. Circular dichroism spectra of SPR1 proteins. A, spectra of recombinant human SPR1 (line 1) and native mouse SPR1a/b proteins (line 2), measured at 20 °C. B, spectra of synthetic peptides corresponding in sequence to human SPR1 of the 25-residue head domain (line 1), the 15-residue tail domain (line 2), or of two (line 3), four (line 4), or six (line 5) repeats of the eight-residue central repeating motif, measured at 20 °C. C, spectra of recombinant human SPR1 measured at 20 °C (line 1), 40 °C (line 2), or 60 °C (line 3) or after a 20–60–20 °C temperature transition (dotted line). D, spectra of recombinant human SPR1 (solid lines) or mouse SPR1a/b (dotted lines) in the absence (lines 1) or presence of (lines 2) or after removal of (dashed lines) 4 M guanidine hydrochloride, measured at 20 °C.

FIG. 3. TGases cross-link recombinant SPR1 to varying degrees. Equimolar amounts (~500 nM) of five isoforms of TGase 1 (A), TGase 2 (B), and inactive proenzyme as well as dispase-activated TGase 3 (C) were used to cross-link 10 μg of SPR1 for varying times as shown. Aliquots were removed and digested to completion with proteases, and the products subjected to amino acid analysis to measure the amount of isodipeptide cross-link. The data are the averages of 2–4 separate experiments.
However, in these cases, the monomer and dimer bands of mouse SPR1a/b appeared to be broader, and some protein had migrated at a slightly faster rate than in the EDTA controls. Based on Coomassie staining, Western blots, and amino acid analyses (data not shown), this shift was probably due to intrachain cross-linking (i.e. some monomer and dimer SPR1 proteins had become more compact and migrated faster). A similar observation has been made previously in the in vitro cross-linking of loricrin (33). Moreover, these data mean that the ways in which the various TGases use the SPR1 proteins are independent of the numbers of repeating peptide motifs of the central domain.

Kinetics of TGase 1, 2, and 3 Cross-linking of Recombinant SPR1 Proteins—We determined the kinetic constants of the cross-linking of the recombinant human SPR1 protein with the baculovirus-expressed full-length TGase 1, TGase 2, and activated TGase 3 enzymes (Table I). By use of high concentrations of putrescine, we suppressed putrescine oligomerization of SPR1. Estimates of kinetic values are complicated by the fact that the SPR1 protein serves as a complete substrate and that multiple Gln residues are utilized (see below), so that the values obtained represent average data for the multiple Gln residues used. The data show that there are significant differences in the kinetic efficiencies ($k_{cat}/K_m$) of the reactions. As suggested by the autoradiograms of Fig. 4, the TGase 3 enzyme cross-linked SPR1 2–4 times more efficiently than TGases 1 or 2. These data are very similar to those for recombinant loricrin (33) and SPR2 (32). From the double displacement mechanisms involved, we also determined kinetic parameters for the TGase 1 and 3 enzymes using as substrates synthetic peptides of sequence corresponding to the head and tail domains of human SPR1 (Table I). While the kinetic efficiency values were all less than for the intact protein, the values for the tail domain peptide were only about 2-fold less. However, there were wide variations for the head domain sequences. We note that there are two Gln/Lys regions encompassing residues 1–13 and 14–25, which we have termed the head A and head B domains delineated by a triple Pro motif (see Fig. 5D). We found that the TGase 1 enzyme used the head B domain sequences 100 times more efficiently than the head A domain sequences, but the TGase 3 enzyme used the head A domain 18 times more efficiently than the head B domain. Thus, there are apparent sequence-specific preferences for these two TGases.

Amino Acid Sequencing Analyses of Cross-linking Reactions By The TGase 1 and 3 Enzymes in Vitro—Next, we obtained more specific information on the residues used for cross-linking in the recombinant SPR1 by the TGase 1 and 3 enzymes. Due to very low degrees of reaction (Figs. 3 and 4), we could not obtain useful information for TGase 2 cross-linking. Samples of uncross-linked or cross-linked protein were digested to completion with trypsin, and the peptides were separated by HPLC. Amino acid sequencing of shifted, reduced, or new peaks provided quantitative information on the utilization of every Gln and Lys residue of the protein.

In the case of the reaction with the baculovirus full-length TGase 1 (Fig. 5B), the peptide peaks eluted at 23 min (residues 38–43), 30 min (residues 44–51), 36 min (residues 52–67), and 37 min (residues 22–36) containing central domain repeats and were not visibly reduced. (Note that Lys$^59$ was reproducibly only partially cleaved by trypsin). However, the peptide eluted...
at 22 min containing residues 1–6 was reduced by about 20%;
the cases of 90% containing residues 68–84 and at 41
min containing residues 7–21 were reduced by about 60%.
Several smaller new peptides appeared late in the chromatogram
that contained cross-links involving Gln and Lys residues
of these peptides. Also, we observed in these peaks about a 5%
utilization of Lys residues, indicating a minor degree of cross-linking
of these peptides. Also, we observed in these peaks about a 5%
utilization of head (residues 1–6) domain sequences were quantitatively
shifted to an unresolved peak late in the chromatogram. By
68–84 at 39 min) domain sequences were quantitatively
used for cross-linking as summarized in Fig. 5. The total amounts used corresponded to the appearance
of equivalent amounts of isodipeptide. The total loss of these
residues suggested that the TGase 1 enzyme had inserted
about 1 mol/mol of cross-link, which is consistent with the
amount isolated directly.

Furthermore, samples that had been cross-linked by the high
specific activity 67/33/10 kDa TGase 1 form recovered from the
membrane fraction of NHEK cells gave similar data on residue
usage (not shown). Together with several foregoing data, it appears that the forms of TGase 1 all treat the SPR1 substrate
the same way and validate use of the baculovirus-expressed
full-length enzyme, which is more stable and available in
appropriate cross-linking of the SPR1 proteins

In the case of cross-linking with the TGase 3 enzyme (Fig.
5C), the peptides eluted at 23, 30, 36, and 37 min containing central domain sequences were reduced by about 10%, suggesting
a minor degree of usage of Lys residues, Lys, and Lys for
cross-linking. However, peptides containing head (residues 1–6
at 22 min and residues 7–21 at 41 min) and tail (residues
68–84 at 39 min) domain sequences were quantitatively
shifted to an unresolved peak late in the chromatogram. By
sequencing 1-min time aliquots across this peak, it was possible
to estimate the extents of usage of each Gln and Lys residue
of the end domains (Fig. 5D, lower numbers), although as many
as four sequences were running simultaneously. These accounted
for >95% of about 3.1 mol/mol of inserted isodipeptide.

Samples that had been cross-linked by these TGase 1 or
TGase 3 enzymes for only 6 h yielded nearly identical data on
Gln/Lys residue usage, except that the amounts used were less
because of incomplete reaction, indicating that there were no
time-dependent preferences for cross-linking.

Correlation of in Vitro and in Vivo Cross-linking of SPR1
Proteins—In Fig. 6, we have summarized the present in vitro
data for TGases 1 and 3, and compared them with the in vivo
utilization of Gln and Lys residues of SPR1 proteins observed
in our previous studies on CEs isolated from either human
foreskin (1, 2) and cultured epidermal keratinocytes (Fig. 6B)
(4) or mouse stomach epithelia (Fig. 6C) (5). Comparisons of
mouse and human data are relevant, since the numbers,
locations, and sequences around the head and tail domain Gln
and Lys residues are very similar (14). Several points emerge.
First, there were no cases of usage of central domain Gln or Lys
residues in the in vivo studies, which compares well with their
summed <5% utilization for cross-linking in vitro. Second,
only there are remarkable similarities in the patterns of
utilization of the head A and B and tail domain sequences: for
virtually every residue, the relative profiles were retained. The
most significant difference appears to be Gln of human SPR1,
which comparatively was used about twice more in vitro than
in vivo. Third, these data suggest that the recombinant SPR1
protein is treated in a generally very similar way by the TGase
1 and 3 enzymes cumulatively in vitro as occurs in vivo in the
foreskin epidermis or stomach epithelium and thereby offer
further evidence that the recombinant protein had adopted its
native configuration in vitro. Moreover, fourth, it becomes clear
that both the TGase 1 and 3 enzymes are required for the
appropriate cross-linking of the SPR1 proteins in vivo. In view
of the differences in the usage of some Gln residues of the head
domain in vivo compared with the summed in vitro data for
TGases 1 and 3, we cannot however exclude the possibility that
another TGase enzyme(s) may also contribute to the effective
cross-linking of the SPR1 proteins in vivo (50). We conclude
that the TGase 2 enzyme is either weakly involved, if at all (Figs.
3 and 4). Similar conclusions were made for another major CE
structural protein, loricrin (33), but these data differ significantly
from those of SPR2, for which essentially only the TGase
3 enzyme was used (32).

However, we observed some asymmetry in the distribution of
usage of head and tail domain sequences in vitro and in vivo
(Fig. 6). The majority of the Lys residues used are located on
the tail domain, while most Gln residues used are located on
the head domains. Since the first step of a TGase cross-linking
reaction involves recognition of a donor Gln residue (44–46),
our in vitro and in vivo observations strongly suggest that
TGase substrate specificities are governed primarily by the
head domain sequences of the SPRs. Consistent with this
conclusion, we observed that the patterns of in vitro cross-linking
of recombinant human SPR1 and native mouse SPR1a/b proteins,
which have highly homologous head domain sequences,

| Table I | Kinetic parameters of cross-linking of recombinant SPR1 and synthetic peptides by TGases |
|---------|-------------------------------------------------|
|         | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $V_{max}$ | $K_m$ of putrescine |
| TGase 1 | 6.2 ± 0.7 | 10.4 ± 1.1 | 0.6 ± 0.2 | 17.6 ± 1.8 | 83 ± 21 |
| TGase 2 | 5.9 ± 1.3 | 19.9 ± 1.9 | 0.3 ± 0.2 | 1.5 ± 0.5 | 77 ± 32 |
| TGase 3 | 6.5 ± 1.3 | 5.4 ± 0.4  | 1.1 ± 0.2 | 25.2 ± 4.2 | 357 ± 43 |

2 L. N. Marek and P. M. Steinert, unpublished observations.
were essentially identical (Fig. 4D), although they contain six or 13/14 central domain peptide repeats, respectively. Also, each of the Gln and Lys residues of the tail domain sequences was used relatively approximately equally by the TGase 1 and 3 enzymes, although 3-fold less by TGase 1 (Figs. 4 and 5).

Differential Functional Use of Head A and B Domains by TGases 3 and 1, Respectively—However, there were clear differences in utilization of different head domain sequences. Examination of the kinetic data for synthetic peptides of Table I and the cross-linking data of intact recombinant SPR1 of Figs. 5 and 6 reveal that the head A domain sequences were used almost exclusively (>90%) by TGase 3, and those of head B mostly (~85%) by TGase 1. Furthermore, in the case of the TGase 3 enzyme, more than 3 mol/mol of isopeptide cross-link were inserted, and ~90% of the protein was oligomerized into dimers and tetramers (Fig. 4). It is therefore likely that head A domain sequences were used by the TGase 3 enzyme in vitro largely for interchain head-to-head, or head-to-tail cross-linking. However, we cannot rule out the possibility of significant intrachain cross-linking as well. The reason for this uncertainty is that it was necessary to proteolyze the in vitro cross-linked products (Fig. 5) in order to elucidate sequencing information; thus, only fragments of sequences were obtained joined by isopeptide bonds, so that it remains unclear whether the cross-links were interchain or whether some were intrachain as well (4). On the other hand, the TGase 1 enzyme inserted 1.1–1.2 mol of isopeptide into the recombinant human and native mouse SPR1 proteins using almost exclusively the head B domain sequences (see Fig. 5B). Since only ~11% of the protein was oligomerized, it appears that most of the cross-linking in vitro involved intrachain links within individual SPR1 protein molecules, as inferred from the mobility shifts seen in Fig. 4. We note that there was a minor degree of overlap in these two enzyme functions, since 5–10% of TGase 3 cross-linked involved head B domain sequences, and ~15% of the TGase 1 reaction involved interchain cross-linking as well as head A domain sequences (Fig. 6).

The present conclusion that the head A and B domains are used in vitro for cross-linking by the TGase 3 and TGase 1 enzymes, respectively, is entirely consistent with our previous data for the recombinant human SPR2 protein (32). Only interchain cross-linking was observed with both enzymes, and moreover, TGase 3 inserted >10 times more cross-links than TGase 1 (32). In this regard, it is noteworthy that the SPR2 protein contains the equivalent of head A domain sequences only, since a second Gln/Lys-rich region following a triple Pro motif is absent (13, 32). These differences imply important structural-functional differences in their utilization in epithelia.

Analysis of in Vivo Cross-links—SPRs were found to be cross-linked in vivo to many other proteins, including to themselves. Our data base of in vivo cross-links is based on extensive proteolysis procedures in order to obtain informative peptides for sequencing and includes 234 occurrences of human (4) and 121 mouse (5) SPR proteins, of which 38 involved direct SPR-SPR linkages. Since the present work on SPR1 and the previous study on SPR2 proteins explored the cross-linking of the SPR proteins to themselves, it is now possible to predict which TGase enzymes may have formed the human and mouse cross-links in vivo (Table II). Some peptides clearly resulted from interchain cross-linking between separate SPR protein molecules, but in many cases, the short piece of SPR sequence recovered after proteolysis precluded assignment of the type of cross-linking. Of the 38 SPR-SPR cross-linked peptides, eight involved head A-head A sequences of which most were interchain, and 11 involved head A-tail linkages. We can now conclude that these were likely inserted by the TGase 3 enzyme. A group of seven involved head A-head B linkages and seven tail-tail linkages might have been inserted by either the TGase 1 or 3 enzymes. We predict that the TGase 1 enzyme could have inserted four head B-tail cross-links and one head B-head B linkage.

However, reconstruction of the potential TGase enzymes used for the 97 single SPR-protein (X) or 220 SPR-X-SPR linkages is less certain. We note that the head A domain Gln residues were used three times more than head B domain Gln residues (4), which based on the present observations, suggests that the TGase 3 and 1 enzymes preferentially use head A and head B domain sequences, respectively. Thus, we propose that 140 could have been inserted by TGase 3, 61 by TGase 1, and 116 by either enzyme (Table II).

SPR Cross-linking with Loricrin—Previously, we have demonstrated that loricrin is likewise cross-linked in vivo by both the TGase 1 and 3 enzymes using different Gln and Lys residues (33), but in this case, there was more overlap between the various residues used and enzymes (about one-third). We have documented 38 SPR1-loricrin and 19 SPR2-loricrin peptides in human epidermal CEs as well as 58 SPR1-loricrin and 26 SPR2-loricrin peptides from mouse forestomach CEs (4). Accordingly, now we have reexamined these 141 data elements to ascertain which Gln and Lys residues were joined in an attempt to correlate the TGase enzymes used (Table III). The analyses show that SPR1 head A domain or SPR2 head domain sequences, predicted to be used by the TGase 3 enzyme, were preferentially cross-linked to loricrin Gln and Lys residues that themselves were predicted to be utilized rather specifically by the TGase 3 enzyme. Likewise, we find a very high correlation of cross-linking of head B domain SPR1 sequences to loricrin at sites predicted to be used preferentially by the TGase 1 enzyme. These data strongly infer a common highly coordinated mechanism for the cross-linking of loricrin and SPRs together in vivo.

Recombinant SPR1 Is Cross-linked Sequentially by the TGase 3 Followed by the TGase 1 Enzymes to Form Very Large Oligomers—In view of the above considerations that both the TGase 1 and 3 enzymes are required for the correct cross-linking of SPR1 proteins in vivo using different Gln/Lys residues of the head B and A domains, respectively, we wondered whether the two enzymes operate simultaneously or at different times. To test this, we repeated the in vitro reactions using 35S-labeled recombinant human SPR1 in which we first separately cross-linked to completion with either TGase 1 or TGase 3 and then performed a second cross-linking reaction on the products with TGase 3 or TGase 1. When baculovirus-expressed full-length TGase 1 was used to cross-link the short oligomeric products of an initial complete TGase 3 reaction, there was a major shift to very large material that could not enter the gel: excision of 35S label revealed that within 12 h, >90% had been thus converted (Fig. 7A). Similar data were obtained when the high specific activity 67/33/10-kDa complex was used for 1 h (not shown). In both cases, a total of 4.2 mol of SPR proteins to themselves, it is now possible to predict which TGase enzymes may have formed the human and mouse cross-links in vivo (Table II). Some peptides clearly resulted from interchain cross-linking between separate SPR protein molecules, but in many cases, the short piece of SPR sequence recovered after proteolysis precluded assignment of the type of cross-linking. Of the 38 SPR-SPR cross-linked peptides, eight involved head A-head A sequences of which most were interchain, and 11 involved head A-tail linkages. We can now conclude that these were likely inserted by the TGase 3 enzyme. A group of seven involved head A-head B linkages and seven tail-tail linkages might have been inserted by either the TGase 1 or 3 enzymes. We predict that the TGase 1 enzyme could have inserted four head B-tail cross-links and one head B-head B linkage.

However, reconstruction of the potential TGase enzymes used for the 97 single SPR-protein (X) or 220 SPR-X-SPR linkages is less certain. We note that the head A domain Gln residues were used three times more than head B domain Gln residues (4), which based on the present observations, suggests that the TGase 3 and 1 enzymes preferentially use head A and head B domain sequences, respectively. Thus, we propose that 140 could have been inserted by TGase 3, 61 by TGase 1, and 116 by either enzyme (Table II).

**In Vitro Cross-linking of Small Proline-rich Protein 1**

Fig. 5. Cross-linking in vitro of recombinant human SPR1 by TGases 1 and 3. Tryptic digests of protein before (A) or after cross-linking by the TGase 1 (B) or TGase 3 (C) enzymes are shown. The products were resolved by HPLC as described under "Materials and Methods." D, sequence of human SPR1 listing the percentage of utilization of Gln and Lys residues; TGase 1 enzyme (upper row); TGase 3 enzyme (lower row). Similar experiments were done for double enzyme reactions of TGase 3 followed by TGase 1 (E) or TGase 1 followed by TGase 3 (F), together with the estimates of residue usage (G).
isodipeptide cross-link had been inserted per mol of SPR1 protein, or about 1 mol/mol more by TGase 1, thus implying that additional Gln/Lys residues had been recruited. Following trypsin digestion and HPLC fractionation, a very broad unresolved peak of highly cross-linked material was observed, not unlike that for TGase 3 enzyme alone (Fig. 5, compare E with C). Sequencing 1-min aliquots across the peak revealed significant increases in the amounts of head B Gln residues used for cross-linking, but those of head A domain sequences were essentially unchanged (Fig. 5G, upper numbers; compare with Fig. 5D, lower numbers). Thus, the additional cross-linking performed in the secondary TGase 1 reaction involved mainly head B sequences. We also repeated these experiments in the reverse order of cross-linking by TGase 3 following initial maximal TGase 1 cross-linking (Fig. 7B). In this case, however, there was not a major change in the cross-linking pattern. The secondary TGase 3 enzyme reaction transformed about 20% of the monomeric sized TGase 1 products into short oligomers and 5% into very large oligomers that could not enter the gel, but most remained as the monomer. Also, only about an additional 0.5 mol of cross-link/mol was inserted. Sequencing analyses revealed minor changes in the degrees of utilization of head A domain residues by the TGase 3 enzyme (Fig. 5, F and G, lower numbers).

Together, these in vitro data strongly imply an obligatory temporal order of the cross-linking of the SPR1 proteins in vivo by the TGase 3 enzyme first, followed later by TGase 1. For apparent spatial and/or structural reasons that are not clear, it appears the head A domain sites must be utilized first by the TGase 3 enzyme before TGase 1 can efficiently recognize the head B domain sites, whereas occupancy of head B sites first interferes with a subsequent TGase 3 reaction. Accordingly, further attempts to obtain detailed structural information on the SPR1 proteins seem desirable.

A Model for Cross-linking of SPRs and Loricrin Together in Vivo—Any model must account for several established aspects of SPR and loricrin expression in epithelia. First, when co-expressed in epithelia, SPR expression always precedes that of loricrin (21, 51). Second, we note that SPRs are very soluble and are deposited in both the nucleus and cytoplasm (21, 51), and antibodies elicited against tail domain epitopes used for cross-linking (Figs. 4 and 5; Table I) yield a bright cytoplasmic staining reaction in tissues such as footpad epidermis or rodent forestomach, where they are abundantly expressed (14, 21). Third, on the other hand, loricrin is insoluble in physiological conditions (33). Fourth, a peripheral staining by the SPR antibodies is observed in more differentiated cells coincidentally with initial loricrin expression (21, 51). Fifth, antibodies against both SPRs and loricrin no longer react in fully cornified epithelia, indicating lost epitopes due to cross-linking (14, 21, 41, 52). Sixth, the activated TGase 3 enzyme is soluble (cytosolic) (40, 53), whereas the most highly active forms of TGase 1 in keratinocytes are membrane-bound (37, 38, 54, 55). Finally, any model must conform to the phenotypic observations of the TGM1 gene/—/ mouse (56) and the natural human knockout of the TGM1 gene in lamellar ichthyosis.

Our data allow the following model, which is consistent with all of the extant observations. We propose that SPR proteins might be expressed early because more time is required to gather them together by an active (3) or passive transport system near the cell periphery than is required for loricrin. Then juxtaposed loricrin and SPRs at or near the cell periphery are first cross-linked together to form small oligomers by the cytosolic TGase 3 enzyme using most of the head A domain and some tail sequences of the SPR1 and -2 proteins and favored internal Gln and head/tail Gln/Lys residues of loricrin. Subsequently, the highly active TGase 1 enzyme form(s) anchored to the membrane attach this loricrin-SPR1/2 complex to the growing CE structure by further cross-linking using available SPR1 A, B, C, D, E.
head B domain and remaining tail sequences of SPR1/2 to the additional available head/tail sequences of loricrin. These temporal differences may be explained in part by TGase enzyme availability as well as in part by the particular conformation of the SPR head domain sequences. It is conceivable that the SPR1/2 head A-like domain sequences that are homologous to those of loricrin (57) evolved so as to favor initial cross-linking by TGase 3 because it is a cytosolic enzyme. Utilization of SPR1

**TABLE II**

**Predicted TGase enzymes used for SPR cross-linking in human and mouse CEs in vivo**

| Location of Gln residues | Total | SPR1-SPR1 | SPR1-SPR2 | SPR2-SPR2 | Predicted TGase |
|--------------------------|-------|-----------|-----------|-----------|-----------------|
| 38 SPR-SPR peptides      |       |           |           |           |                 |
| Head A-head A            | 8     | 4         | 3         | 1         | 3               |
| Head A-head B            | 7     | 4         | 1         | 2         | 1               |
| Head B-head B            | 1     | 1         | 3         | 1         |                 |
| Head A-tail              | 14    | 6         | 3         | 5         | 3               |
| Head B-tail              | 4     | 4         |           |           | 1               |
| Tail-tail                | 7     | 3         | 2         | 2         | 1 or 3          |
|                         |       |           |           |           |                 |
| 97 SPR-X (other protein) peptides |       |           |           |           |                 |
| Head A                   | 40    | 26        |           |           | 14              |
| Head B                   | 32    | 22        |           |           | 10              |
| Tail                     | 25    | 16        |           |           | 9               |
|                         |       |           |           |           | 1 or 3          |
| 220 SPR-X-SPR peptides   |       |           |           |           |                 |
| Head A-head A            | 54    | 28        | 15        | 11        | 3               |
| Head A-head B            | 34    | 16        | 11        | 7         | 1 or 3          |
| Head B-head B            | 10    | 4         | 4         | 2         | 1               |
| Head A-tail              | 46    | 19        | 14        | 13        | 3               |
| Head B-tail              | 19    | 8         | 6         | 5         | 1               |
| Tail-tail                | 57    | 21        | 22        | 14        | 1 or 3          |

**TABLE III**

**Cross-links between loricrin and SPRs: Predictions of TGases used**

Mouse data are from Ref. 5. Predictions of TGases used for cross-linking of human loricrin residue positions are from Ref. 33. For mouse loricrin, similar data are not known, but positions equivalent to human loricrin are marked by 1; nonequivalent positions are marked by 2. Numbers of occurrences are shown in parenthesis. Cross-links involving tail domain sequences are not listed, since there was no apparent TGase enzyme specificity.

| Predicted TGase | Predicted TGase |
|-----------------|-----------------|
| 1               | 3               |

**Human SPR proteins**

| Gln (11) | Lys (5) | Gln (6) | Lys (1) | Gln (8) | Lys (3) | Gln (16) | Lys (6) | Gln (12) | Lys (4) |
|----------|---------|---------|---------|---------|---------|----------|---------|----------|---------|
| SPR1 head domain A (16 of 38 cross-links) | SPR1 head domain B (7 of 38 cross-links) | SPR1 head domain A (11 of 19 cross-links) | SPR1 head domain A (22 of 58 cross-links) | SPR1 head domain B (10 of 58 cross-links) | SPR1 head domain A (16 of 26 cross-links) | SPR1 head domain A (16 of 26 cross-links) | SPR1 head domain A (16 of 26 cross-links) | SPR1 head domain A (16 of 26 cross-links) | SPR1 head domain A (16 of 26 cross-links) |
| Gln (11) | Lys (5) | Gln (6) | Lys (1) | Gln (8) | Lys (3) | Gln (16) | Lys (6) | Gln (12) | Lys (4) |
head B domain residues or other more internal loricrin residues by TGase 1 can occur only subsequently (Fig. 7). Because the vast majority of TGase 1 activity is membrane-bound presumably at or very near the site of final protein deposition of the CE, this enzyme is used for the final reinforcement stages of CE assembly.

Furthermore, this model is readily consistent with the pathophysiology of lamellar ichthyosis disease caused by absent TGase 1 activity. Because the TGase 3 enzyme cross-links the proteins with high specificity at specific sites different from those of the TGase 1 enzyme, the TGase 3 enzyme cannot adequately compensate or supplement for the lost TGase 1 enzyme activity. Thus, the reinforcement stage of CE assembly occurs only poorly, resulting in the devastating loss of barrier function seen in human lamellar ichthyosis patients. Similarly, this model is entirely consistent with the observed morphological and histological features and severe loss of barrier function in the mouse −/− model.

A similar scenario should be applicable to the CEs of those epithelia that express much less or no loricrin and where the SPRs instead are mostly cross-linked to themselves and other scaffold proteins. Furthermore, the second most frequent partner to which SPRs have been found cross-linked in vivo is involucrin (4). Again, both head A and head B domain sequences were commonly used, suggesting the involvement of both TGases 1 and 3 (4). However, comparable analyses of SPRs and involucrin cross-linking are not yet possible, since only limited data on favored residues used by TGases are available for involucrin (58).

Conclusions—Altogether, these studies indicate that the cross-linking of SPR proteins to the CE for effective barrier function is a complex process, involving multiple TGases operating through numerous Gln and Lys residues, and in multiple well-coordinated steps. The availability of recombinant loricrin and SPR proteins now will permit further experiments to explore how they function together to affect the biomechanical properties of the CE and the epithelium in which they are expressed (5). Such experiments may also shed light on the reason why there are multiple apparently functionally very similar yet differentially expressed SPR1 and SPR2 gene products.

Acknowledgments—We thank Dr. Claude Backendorf for providing cDNAs for human SPR proteins, Dr. Ulrike Lichti for expert advice, and Dr. Soo-Youl Kim for assistance in the initial construction of the expression vector.

REFERENCES
1. Steinert, P. M. & Marekov, L. N. (1995) J. Biol. Chem. 270, 17702–17711
2. Steinert, P. M. & Marekov, L. N. (1997) J. Biol. Chem. 272, 2021–2030
3. Robinson, N. A., Lapec, S., Welker, J. F. & Eckert, R. L. (1997) J. Biol. Chem. 272, 12035–12046
4. Steinert, P. M., Candi, E., Kartasova, T. & Marekov, L. N. (1998) J. Struct. Biol. 122, 76–85
5. Steinert, P. M., Kartasova, T. & Marekov, L. N. (1998) J. Biol. Chem. 273, 11758–11769
6. Hohl, D. (1990) Dermatologica 180, 201–211
7. Reichert, U., Michel, S. & Schmidt, R. (1993) in Molecular Biology of the Skin (Darmon, M. & Blumenberg, M., eds) pp. 107–150, Academic Press, Inc., New York
8. Simon, M. (1994) in The Keratinocyte Handbook (Leigh, I. M., Lane E. & Watt, F. M., eds) pp. 275–292, Cambridge University Press, Cambridge, United Kingdom
9. Martinet, N., Beninati, S., Nigra, T. P. & Folk, J. E. (1988) Biochem. J. 271, 115–120
10. Kartasova, T. & van de Putte, P. (1988) Mol. Cell. Biol. 8, 2195–2203
11. Kartasova, T., van Muijen, G. N., van Pelt-Heerschap, H. & van de Putte, P. (1988) Mol. Cell. Biol. 8, 2204–2210
12. Marvin, K. W., George, M. D., Fujimoto, W., Saunders, N. A., Bernacki, S. H. & Jetten, A. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11026–11030
13. Gibbs, S., Fijneman, R., Wiegant, J., van Kessel, A. G., van de Putte, P. & Backendorf, C. (1993) Genomics 16, 630–637
14. Kartasova, T., Darwiche, N., Kono, Y., Koizumi, H., Osaka, S.-I., Huh, N.-H., Steinert, P. M. & Kuroki, T. (1996) J. Invest. Dermatol. 106, 294–305
15. Phillips, S. B., Kobilus, J., Grassi, A. M., Goldaber, M. L. & Baden, H. P. (1990) Comp. Biochem. Physiol. 95B, 781–788
16. Gibbs, S., Lohnan, F., Teubel, W., van de Putte, P. & Backendorf, C. (1990) Nucleic Acids Res. 18, 4401–4407
17. An, G., Tesfaigzi, J., Chou, Y.-J. & Wu, R. (1993) J. Biol. Chem. 268, 10977–10982
18. Greco, M. A., Lorand, L., Lane, W. S., Baden, H. P., Parameswaran, N. P. & Kvedar, J. C. (1995) J. Invest. Dermatol. 104, 204–210
19. Hohl, D., de Viragh, P. A., Amiguet-Rangy, F., Gibbs, S., Backendorf, C. & Huber, M. (1995) J. Invest. Dermatol. 104, 902–909
20. Austin, S. J., Fujimoto, W., Marvin, K. W., Vollberg, T. M., Lorand, L. & Jetten, A. M. (1996) J. Biol. Chem. 271, 3737–3742
21. Jarnik, M., Kartasova, T., Steinert, P. M., Lichti, U. & Steven, A. C. (1996) J. Cell Sci. 119, 1381–1391
22. Fujimoto, W., Nakanishi, G., Arata, J. & Jetten, A. M. (1997) J. Invest. Dermatol. 108, 200–204
23. Koizumi, H., Kartasova, T., Tanaka, H., Okawara, A. & Kuroki, T. (1996) Br. J. Dermatol. 134, 686–692
24. An, G., Huang, T. H., Tesfaigzi, J., Garcia-Heras, J., Ledbetter, D. H., Carlson, D. M. & Wu, R. (1992) Am. J. Respir. Cell Mol. Biol. 7, 104–111
25. An, G., Tesfaigzi, J., Carlson, D. M. & Wu, R. (1993) J. Cell. Physiol. 157, 562–568
26. Saunders, N. A., Smith, R. J. & Jetten, A. M. (1993) Biochem. Biophys. Res. Commun. 197, 46–54
27. Owens, D. M., Zainal, T. A., Jetten, A. M. & Smart, R. C. (1996) J. Invest. Dermatol. 106, 647–654
28. Saunders, N. A. & Jetten, A. M. (1994) J. Biol. Chem. 269, 2016–2022
29. Yaar, M., Eller, M. S., Bhawan, J., Harkness, D. D., DiBenedetto, P. J. & Gilchrest, B. A. (1995) Exp. Cell Res. 217, 217–226
30. Tesfaigzi, J., Wright, P. S., Orefio, V., An, G., Wu, R. & Carlson, D. M. (1993) Am. J. Respir. Cell Mol. Biol. 9, 434–440
31. Gilchrest, B. A., Garmyn, M. & Yaar, M. (1984) Arch. Dermatol. 120, 82–86
32. Tarcea, E., Candi, E., Kartasova, T., Idler, W. W., Marekov, L. N. & Steinert, P. M. (1996) J. Biol. Chem. 271, 23297–23303
33. Candi, E., Melino, G., Mei, G., Tarcea, E., Chung, S.-I., Marekov, L. N. & Steinert, P. M. (1995) J. Biol. Chem. 270, 26382–26390
34. Stanwell, C., Denning, M. F., Rutberg, S. E., Cheng, C., Yuspa, S. H. & Dlugosz, A. A. (1996) J. Invest. Dermatol. 106, 482–489
35. Killackey, J. J. F., Bonaventura, B. J., Castellano, A. L., Billedeaux, R. J., Farmer, W., DeYoung, L., Kranz, A. & Plura, D. B. (1989) Mol. Pharma col. 35, 701–706
36. Candi, E., Melino, G., Lahm, A., Ceci, R., Rossi, A., Kim, I. G., Ciani, B. & Steinert, P. M. (1998) J. Biol. Chem. 273, 13693–13702
37. Kim, S.-Y., Chung, S.-I. & Steinert, P. M. (1995) J. Biol. Chem. 270, 18026–18035
38. Steinert, P. M., Chung, S.-I. & Kim, S.-Y. (1996) Biochem. Biophys. Res. Commun. 221, 101–106
39. Kim, S.-Y., Kim, I.-G., Chung, S.-I. & Steinert, P. M. (1994) J. Biol. Chem. 269, 27979–27986
40. Kim, H.-C., Lewis, M. S., Gorman, J. J., Park, S. C., Girard, J. E., Folk, J. E. & Chung, S. I. (1990) J. Biol. Chem. 265, 21971–21978
41. Hohl, D., Lichti, U., Mehrel, T., Turner, M. L., Roop, D. R. & Steinert, P. M. (1991) J. Biol. Chem. 266, 6626–6636
42. Tarcsa, E., Marekov, L. N., Andreoli, J., Idler, W. W., Candi, E., Chung, S.-I. & Steinert, P. M. (1997) J. Biol. Chem. 272, 27893–27901
43. Tarcsa, E., Marekov, L. N., Mei, G., Melino, G., Lee, S.-C. & Steinert, P. M. (1996) J. Biol. Chem. 271, 30709–30716
44. Folk, J. E. (1983) Adv. Enzymol Relat. Areas Mol. Biol. 54, 1–56
45. Lorand, L. & Conrad, S. M. (1984) Mol. Cell. Biochem. 58, 9–35
46. Folk, J. E. & Chung, S.-I. (1985) Methods Enzymol. 113, 358–375
47. Birkbichler, P. J., Orr, G. R., Carter, H. A. & Patterson, M. K. (1977) Biochem. Biophys. Res. Commun. 78, 1–7
48. Aeschlimann, D. & Paulson, M. (1991) J. Biol. Chem. 266, 15308–15317
49. Barry, E. L. & Mosher, D. F. (1990) J. Biol. Chem. 265, 9302–9307
50. Aeschlimann, D., Koeller, M. K., Allen-Hoffmann, B. L. & Mosher, D. F. (1998) J. Biol. Chem. 273, 3452–3460
51. Ishida-Yamamoto, A., Kajita, T., Matsuo, S., Kuroki, T. & Iizuka, H. (1991) J. Invest. Dermatol. 108, 12–16
52. Mehrel, T., Hohl, H., Rothnagel, J. A., Longley, M. A., Bundman, D., Cheng, C. K., Lichti, U., Steinert, P. M., Bisher, M. E., Steven, A. C., Yuspa, S. H. & Roop, D. R. (1990) Cell 60, 1103–1112
53. Kim, I.-G., Kim, J. J., Park, S.-C., Chung, S.-I. & Steinert, P. M. (1993) J. Biol. Chem. 268, 12682–12690
54. Rice, R. H. & Green, H. (1977) Cell 11, 417–422
55. Steinert, P. M., Kim, S.-Y., Chung, S.-I. & Marekov, L. N. (1996) J. Biol. Chem. 271, 32642–32650
56. Matsuki, M., Yamashita, F., Ishida-Yamamoto, A., Yamada, K., Kinoshita, C., Fushiki, S., Ueda, K., Morishima, Y., Tabata, K., Yasuno, H., Hasida, M., Iizuka, H., Ikawa, M., Okabe, M., Kondoh, G., Kinoshita, T., Takeda, J. & Yaminishi, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1044–1049
57. Backendorf, C. & Hohl, H. (1992) Nat. Genet. 2, 91
58. Simon, M. & Green, H. (1988) J. Biol. Chem. 263, 18093–18098