BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 39–44 (1997)
ARTICLE NO. RC976407

Novel Transglutaminase Inhibitors Reduce the Cornified Cell Envelope Formation

Soo-Youl Kim,[1] Won-Man Park, Sung-Won Jung, and Jinseon Lee
Laboratory of Skin Biology, Pacific Research and Development Center, Yongin, Kyonggi-do, Korea 449-900

Received February 26, 1997

Transglutaminase (TGase) is a calcium-dependent enzyme which catalyzes the iso-peptide cross-link between peptide-bound glutamine and lysine *in vivo*. Though the cross-link is developed as a barrier function in the skin system, overexpression of this could invoke skin hyperkeratosis in psoriasis and roughness in aged skin. In former research, many strong irreversible TGase inhibitors failed application because of high cytotoxicity. We selected one peptide after primary screening of six synthetic peptides designed from domains of known TGase substrates. Then we attempted to reduce the size and finally obtained two tetrameric peptides. When we treated keratinocyte with these TGase inhibitors under calcium-induced differentiation, the formation of a cornified cell envelope (CE) was decreased to the same level of CE under proliferating conditions without cytotoxic effect. Therefore, we propose that these TGase inhibitors may be useful for solving the physiological hypercross-linking problems for pharmaceutical or cosmetic purposes.

Transglutaminases (TGase) (EC 2. 1. 2. 13) are a class of calcium-dependent enzymes that catalyze the covalent coupling of the γ-carboxamide group of peptide-bound glutamine residues with an ϵ-amino group of peptide-bound lysine residues (1,2). The critical intermediate in the catalytic sequence is a thioester acyl-enzyme formed between a glutaminyl-peptide acyl donor and the active site cysteine residue, as illustrated in Fig. 1. In the many important physiological events, TGases have been implicated in a variety of conditions including acne (3), psoriasis (4), cataracts (5,6), scar formation (7), neuronal degeneration (8,9) and skin aging (10-14). Hence highly specific inactivators are required to clarify the role(s) of TGases in cellular phenomena.

Mechanistic features of the active site was determined by kinetic and inhibitor studies (1). On the basis of that study, it was revealed that cysteine residue of the active site was a key amino acid to catalyze cross-linking reaction. Like many enzymes with an active site thiol group, TGases are inactivated by alkylating agents such as halomethyl carbonyl derivatives (15,16). The intrinsically high reactivity of these agents toward thiol groups, however, limits their therapeutic utility. Alkyl isocyanates are covalent inhibitors of TGases but have the disadvantage of lacking specificity (17). Primary amines such as methylamine and dansylcadaverine, which act as competitive inhibitors, have been used to inhibit TGase activity (18). However, conditions are not ideal; they interfere with other enzymes; they are active only at high concentrations such as millimolar range; and they inhibit cell attachment (19). The mechanism-based TGase inhibitors by halodihydroisoxazoles have been described(20-23). These tyrosinamidomethyl dihydrohaloisoxazoles inhibit TGases irreversibly and thus reduce envelope formation in human keratinocytes *in vivo* and *in vitro* (24). As an alternative approach, it was demonstrated that selective inactivators of TGases can be prepared by replacing halo leaving group of halomethyl carbonyl compounds with a leaving group that lowers the intrinsic reactivity of the inhibitor as a nonspecific alkylating agent (25). The 2-[(2-oxopropyl)thio]-imidazolium derivatives inactivated factor XIIIa and TGase 2 irreversibly. Eventhough they developed many inhibitors with considerations, they could not rule out the side effect of irreversible reaction of thiol group in active sites of many other enzymes *in vivo* system.

In the different way, we had approched designing of inhibitors from substrate domains of TGases. We

[1] To whom correspondence and reprint requests should be addressed at Pacific R & D Center, 314-1 Bora-ri, Kiheung-eup, Yongin, Kyonggi-do, Korea 449-900. Fax: 82-331-285-0328.

Abbreviations used: CE, cornified cell envelope; NHEK, normal human epidermal keratinocytes; TGase(s), transglutaminase(s); TGase1, the membrane-associated TGase or TGase K or Type I keratinocyte TGase; TGase2, the tissue TGase or TGase C or Type II liver TGase; TGase3, pro-TGase E or epidermal TGase; Elafin, skin-derived antileukoproteinase, SCALP or elastase-specific inhibitor, ESI.

0006-291X/97 $25.00

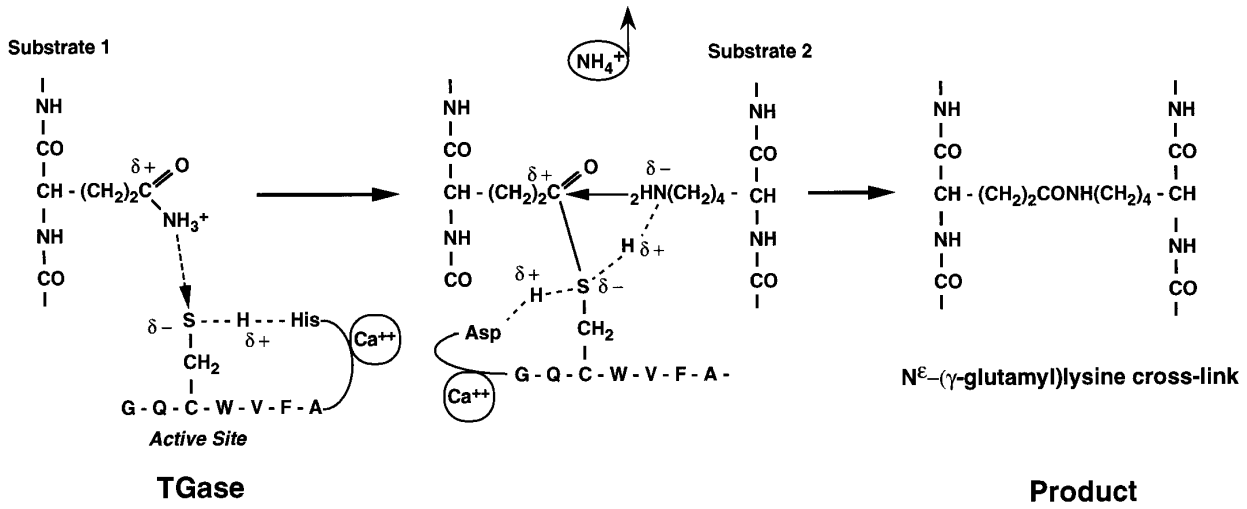

FIG. 1. A proposed catalytic mechanism of TGase. Substrate 1 is a peptide-bound glutamine residue as an amine donor and Substrate 2 is a peptide-bound lysine residue as an amine acceptor. GQCWVFA is a very well conserved active-site amino acid sequence.

expects peptide inhibitors designed from substrate domains for each TGases will give specificity and safety of inhibition based on reversible reaction and degradable peptide. Especially we have focused on TGase 1 enzyme. Because TGase 1 was proposed a key enzyme in the formation of cornified cell envelope (CE) during keratinocyte terminal differentiation among TGases (26,27). Recentely a model of structure for the human epidermal CE was suggested that should be consisted of loricrin(28,29), small proline rich proteins (SPR1,2) (30-32), elafin (32,33), involucrin (34,35) and cystatin A (36). And it was demonstrated that proteins including elafin, filaggrin (37), keratin intermediate filaments (38,39), loricrin and SPR 1,2 are isodipeptide crosslinked components of the human CE by TGases (40,41). We proposed that a certain component of CE must be very high specific substrates for TGase 1. By the series of kinetics of TGase 1 using synthetic peptides, we found highly specific peptide sequences as competitive inhibitors. These inhibitors prevented the formation of insoluble cell envelope proteins in *in vitro* keratinocyte culture system.

MATERIAL AND METHODS

Standard procedures. Isolation of recombinant TGase 1(ΔN62-816) from *E. coli* was followed by the established method (26). The purified active TGase 1 was used for kinetics. Procedures for growth of normal human epidermal keratinocytes(NHEK) in cell culture and assays for TGase activities were done as described (27).

Construction of synthetic peptide substrates. The two 23-mers and four 22-mers were synthesized, based on published sequences of loricrin (28,29) and elafin(skin-derived antileukoproteinase, SCALP or elastase-specific inhibitor, ESI) (32,33) cell envelope precursors, that have been shown to be involved in crosslinks in vivo. After primary screening, we have synthesized a series of tetrameric, trimeric, dimeric peptides which encodes putative TGase substrate domains, and performed kinetic assays (Table 1). All the peptides were synthesized, purified by HPLC, and the compositions and concentrations determined accurately by amino acid analysis (43).

Kinetic studies. Rate studies were carried out in 0.1 M Tris-acetate buffer (pH 8.0) containing 10 mM $CaCl_2$, 0.1 mM EDTA, 5 mM DTT, varying concentrations of succinylated Hammerstein casein or peptide inhibitors, five different levels of synthetic peptides (0, 1, 2, 5, 10 $\times 10^{-5}$M), 0.76 mM of C^{14} Putrescine (specific activity 110 Ci/mole) with an appropriate amount of enzyme at 37 °C. The reaction was terminated at varying incubation times (0, 10, 20, 30 and 60 min).

The preparation of CE from cultured human keratinocyte. We used a calcium-induced differentiation model of keratinocytes(NHEK) as described in (27). The peptides inhibitors were added into the media as several different concentrations from 1.13 μM to 1.13 mM. At the 5th day, the cultured keratinocytes were harvested by scrapper and CE was purified by established method (42). The purified CE was sonicated twice for 30 sec at 70-90 watts. Then we measured turbidity of the insoluble CE at 310 nm.

RESULTS

Six different peptides were synthesized to analyze the competitive inhibitory effect for substrate binding. The peptide sequences were from TGase substrate domains of loricrin and elafin (Table 1) because those are the most abundant components in cornified cell envelope as the best substrates for TGase 1 and 3 (40,41). In Fig. 2, we have fixed the sufficient concentrations of putrescine (0.76mM) as an amine donor, succinylated casein (1%,w/v) as an amine acceptor and synthetic inhibitors (100 μM) as a competitive inhibitor in the TGase catalytic reaction. And we used optimum amount of TGase 1 enzyme in the initial velocity range (20 nM/min). Fig. 2 A shows TGase inhibition as time dependant manner. Fig. 2 B shows the percent inhibition under the fixed concentrations and incubation time of 60 min. Among two 23-mers and four 22-mers, elafin peptide sequence (SY2001, SY2002) showed the best

TABLE 1

Sequence of Synthetic Peptides

Number	Sequence	*Km*, mM	IC_{50}, μM
Control		0.055 ± 0.0038	
SY1001[a]	SGKGVPICHQTQQKQAPTWPSK		
SY1002[b]	SSGGSGSGYVSSQQVTQTSCAPQ		
SY3001[c]	GLEPAVGTDPVKGQPAVRGFVG		
SY3002[d]	QVSGGRLPVSQQDPVKGHVPPK		
SY2001[e]	PVKGQDTVKGRVPINGQDPVKG		
SY2002[f]	QDTVKGRVPFNGQDPVKGQVSVK		
SY2003	GQDP	0.107 ± 0.0064	220 ± 3.5
SY2004	GQVS	0.088 ± 0.0054	410 ± 22.0
SY2005	QDTV	0.073 ± 0.0027	612 ± 7.5
SY2006	GQD	0.070 ± 0.0055	682 ± 19.0
SY2007	GQ	0.060 ± 0.0076	854 ± 2.2
SY2008		0.061 ± 0.0024	738 ± 12.3
SY2011	PVKG	0.128 ± 0.0035	165 ± 5.1
SY2012	VSVK	0.073 ± 0.0066	432 ± 11.0
SY2013	VKGR	0.134 ± 0.0091	144 ± 8.7
SY2021	KGQV	0.080 ± 0.0081	572 ± 34.0
SY2014	VKG	0.091 ± 0.0088	380 ± 10.2
SY2015	KG	0.086 ± 0.0022	425 ± 13.1
SY2016	VK	0.094 ± 0.0083	363 ± 23.0

Note. The synthetic peptides were made using the published method (43). Each peptide sequence was originated from (a) human loricrin C-terminus, (b) human loricrin N-terminus, and (e, f) human elafin N-terminus. (c, d) The artificial peptide sequences were generated for putative TGase substrate, based on consensus amino acid of TGase substrate domains of seminal vesicle protein-1 (44), involucrin (34, 35), nidogen (45), and plasminogen activator inhibitor-2 (46).

inhibitory effect. We have gradually reduced the peptide sequences and found tetrameric sequences presenting minimal structural size for specific inhibitory effect on TGase activity.

In Fig. 3, we used all the same kinetic conditions as above under varying inhibitor conditions from 0 to 0.1 mM for 30 min reaction time. Fig. 3 A showed the percent inhibition of TGase activity on the linear corelation with inhibitor concentration. Among seven different tetramers, two peptides were expected for good inhibitors (SY2011, SY2013). Fig. 3 B and 3 C showed the same experiments using further reduced peptide size as four different dimers and two different trimers respectively. One or two amino acid reduction from tetramer gave about 2 to 5 fold loss of inhibitory effect.

We measured the velocity versus [S] in the presence and in the absence of a competitive inhibitor (0.1 mM). In Table 1, Km values using casein were implied a competitive inhibition pattern to compare the control Km value with others. Among those values, Km of the SY2013 was increased up to 2.5 fold of control.

To investigate if those TGase inhibitors are useful for reducing the cornified cell envelope formation, we used the calcium-induced differentiation model of keratinocyte as described in method. Keratinocytes proliferate under low calcium condition and can switch to the differentiation state acompanying insoluble envelope formation under high calcium condition (34). We found 50 μM of inhibitor concentration in the cultured media was a optimum condition to observe the reduction of CE within 5 days. Fig. 4 showed a comparison of inhibitory effect of CE formation among synthetic inhibitors including negative control (High Ca^{++}). SY2003 and SY2011 gave the best inhibitory efficacy of CE formation to the almost same level of CE from keratinocyte grown under low calcium condition, although SY2013 showed a lower IC_{50} value (in vitro) than SY2003 (Table 1).

DISCUSSION

Earlier competitive TGase inhibitors such as primary amines have been used to inhibit TGase activity (18). However they interfere with other enzymes and inhibit cell attatchment (19). Hence we have developed competitive TGase inhibitor from putative TGase 1 substrate domains by competing acyl-enzyme interaction (Fig. 1). Through the various kinetics (Figs. 2,3) using different size of synthetic peptides, we have found that peptides from the TGase substrate domain could compete with TGase substrate as an inhibitory effect. Fig. 2 showed the pro-peptide sequence of preproelafin could be the best TGase substrate or competitor. The pro-sequence of the elafin precursor has 5 homologous repeats of a 6-amino acid unit containing lysine and glutamine (32). These repeats (V-**K**-G-**Q**-X-X) are very similar to those of the homologous repeats found in the major clotting protein SVP-1 from guinea

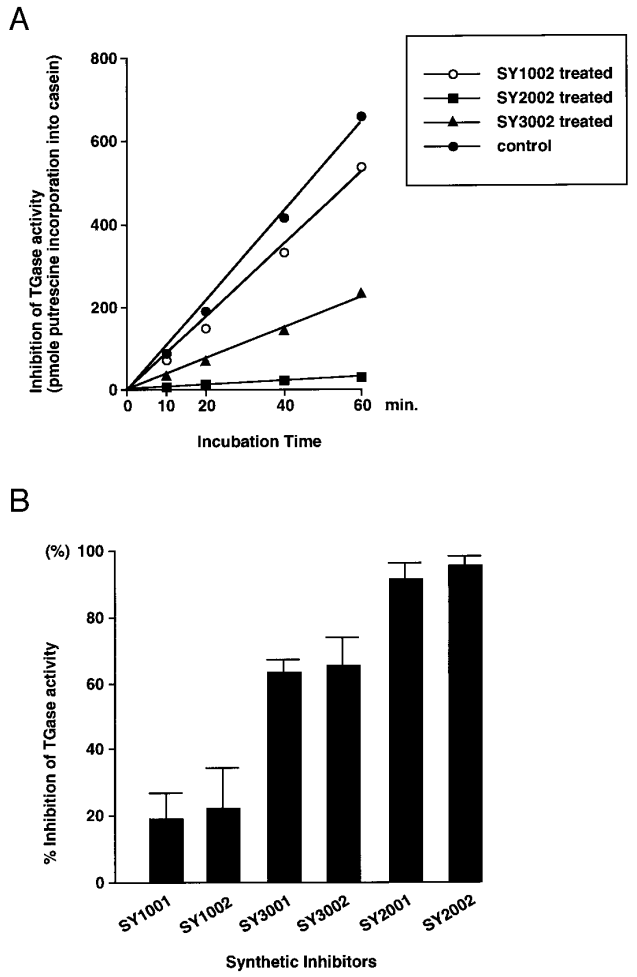

FIG. 2. Kinetics of TGase activity with synthetic inhibitors (four 22-mers and two 23-mers). (A) Kinetics of three different inhibitors as the concentration of 0.1 mM on the reaction of incorporating ^{14}C-putrescine (0.76 mM) into succinylated casein at various time. (B) The comparison of percentage inhibition of TGase activity was measured at the fixed incubation time (10 min) and inhibitor concentration (0.1 mM) within an initial velocity range.

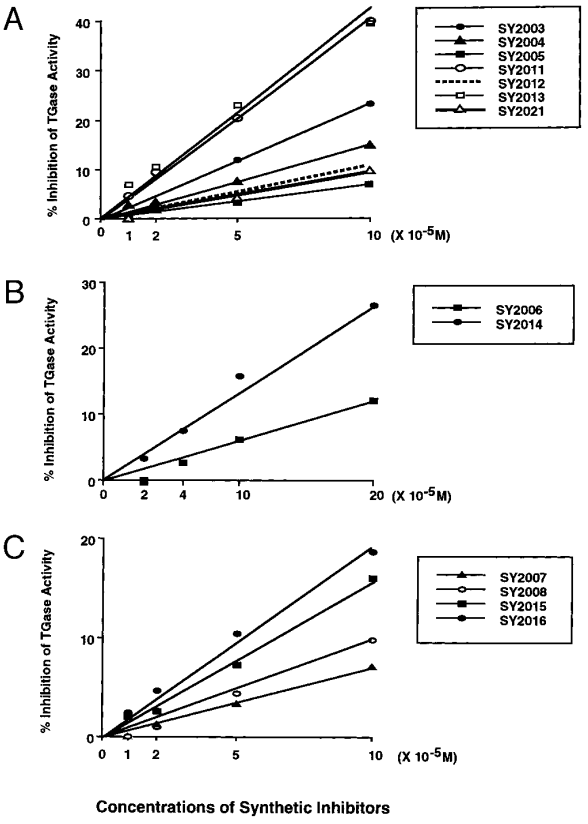

FIG. 3. Kinetics of TGase activity with synthetic inhibitors. (A) The comparison of percentage inhibition of TGase activity with seven tetrameric peptides was measured at the fixed incubation time (10 min) using various inhibitor concentration (10 to 100 μM) under an initial velocity range. (B) Two trimeric and (C) four dimeric peptides were measured using the same condition as in (A).

pig seminal vesicle which are covalentely cross-linked by TGase (44). We attempted to define the minimal structural conformation of substrate domain for the competition of TGase catalysis. Hence we reduced the peptide size from pro-sequence of preproelafin. *In vitro* kinetics revealed that series of tetramer such as SY2011 and SY2013 have the better inhibitory efficacy (Table 1). These two peptides share the three amino-acid sequence (V-K-G), which implies an adequate sequence for access to TGase 1 active site. Interestingly SY2003 and SY2011 showed the better inhibitory efficacy of the insoluble CE formation in NHEK culture system(Fig. 4). In this experiments, SY2011 was coincided with kinetics results, however SY2013 was not. Perhaps arginine residue of SY2013 might interfere transfering the peptide into the cell. The clear func-

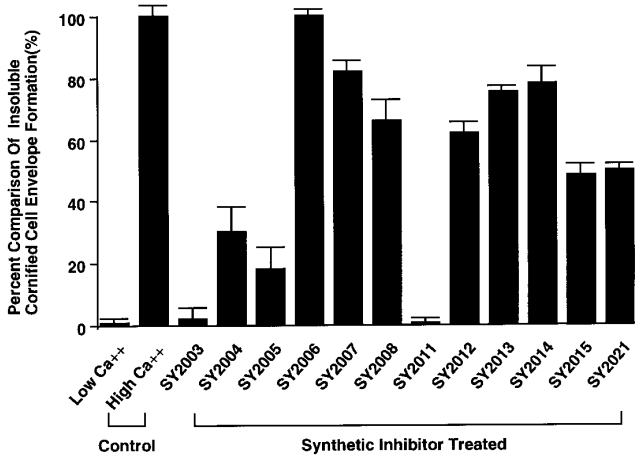

FIG. 4. The inhibitory effect of cornified cell envelope formation in a cultured human keratinocyte system by treatment of TGase inhibitors. Synthetic TGase inhibitors were added into culture media (to the concentration of 50 μM). The keratinocytes (NHEK) were harvested after 5 days under high calcium conditions and the CE was measured at 310 nm (details under Materials and Methods).

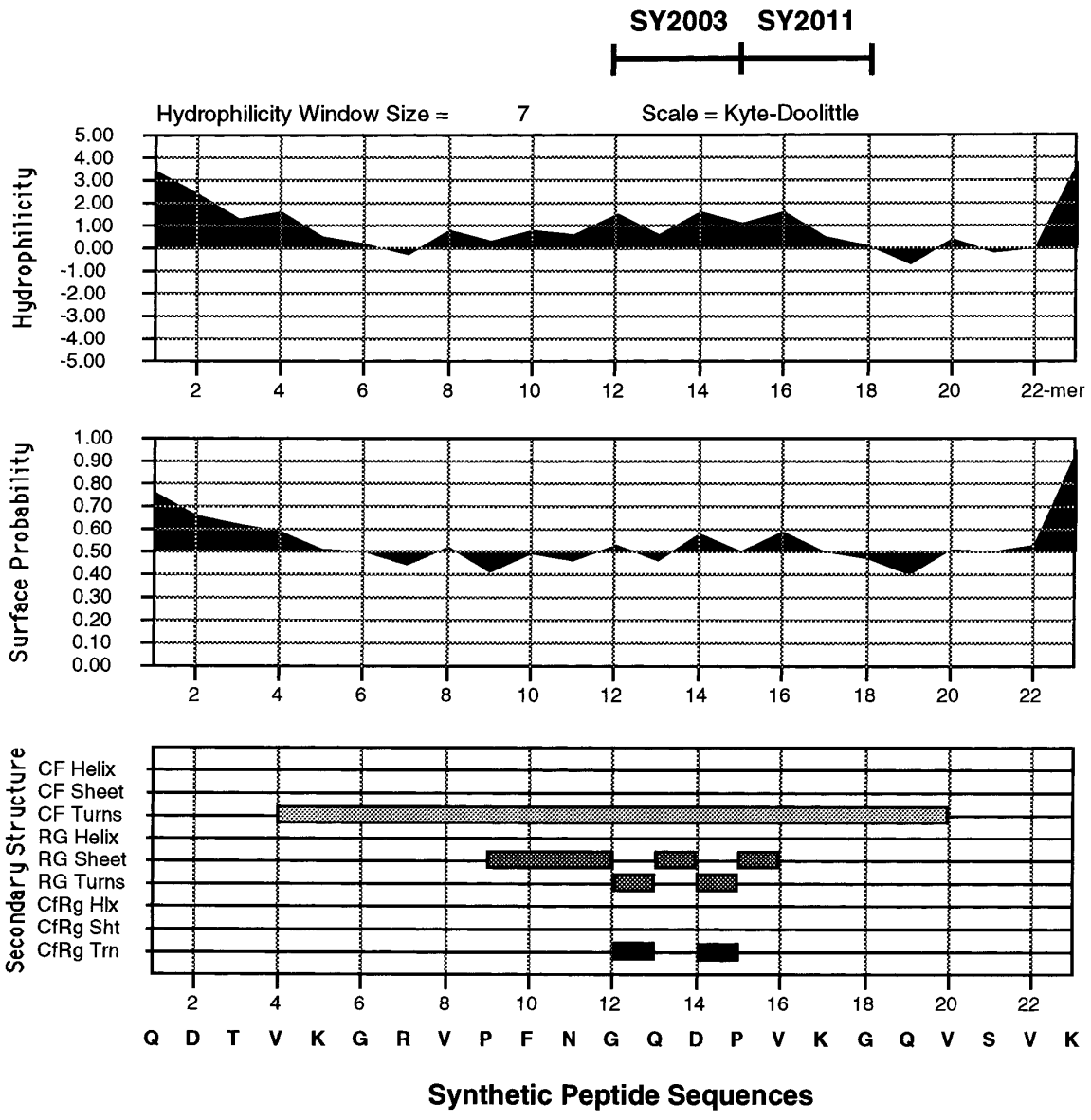

FIG. 5. Secondary structure characteristics of the 23-mer synthetic inhibitor sequence from pre-elafin sequence. These were performed using the IBI Pustell sequence software packages and are based on the analytical methods of Ref. 47. Only the hydrophilicity, surface probability, and secondary structure profiles are shown.

tion of proline in SY2003 and SY2011 was noticed to compare with SY2006 and SY2014 respectively. There were two to three fold differences of IC_{50} value between SY2003 and SY2006 or SY2011 and SY2014 in Table 1.

By the analysis of the secondary structure of SY2003 and SY2011 using IBI program, we found that these synthetic peptide inhibitors have four structural features: the first, glutamine or lysine group which could compete with second substrate or primary substrate respectively by means of nucleophilic substitution at the acyl-enzyme intermediate during catalysis; the second, short and fexible sequences to enhance affinity of inhibitor into active site pocket; the third, peptide structure retaining amino group, carboxyl group and side chains as glutamine or lysine to increase solubility in physiological media; and the last, flexible sequences to give turn structure which promote better diffusion into cells (Fig. 5).

In the inhibitor treatment on cultured cell, we have observed no side effect such as cell detachment or cell death. Interestingly there was an observation of increase in proliferation markers after TGase inhibition with cystamine in WI-38 human lung cells (48). Further study will be answered weather SY2003 and SY2011 have a growth-promoting effect or not.

ACKNOWLEDGMENTS

We thank Drs. Soo-Il Chung and Peter M. Steinert for encouraging advice during the course of this entire work.

REFERENCES

1. Folk, J. E., and Cole, P. W. (1966) *Biochim. Biophys. Acta* **122,** 244–264.
2. Folk, J. E., and Chung, S.-I. (1985) *Methods Enzymol.* **113,** 358–375.
3. Young, L. D., Ballaron, S., and Epstein, W. (1984) *J. Invest. Dermatol.* **82,** 275–279.
4. Schroeder, W. T., Thacher, S. M., Stewart-Galetka, S., Annarella, M., Chema, D., Siciliano, M. J., Davies, P. J. A., Tang, H.-Y., Sowa, B. A., and Duvic, M. (1992) *J. Invest. Dermatol.* **99,** 27–34.
5. Lorand, L., Hsu, L. K. H., Siefring, G. E., Jr., and Rafferty, N. S. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1356–1360.
6. Lorand, L., Velasco, P. T., Murthy, S. N. P., Wilson, J., and Parameswaran, K. N. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11161–11163.
7. Bowness, J. M., Tarr, A. H., and Wong, T. (1988) *Biochim. Biophys. Acta* **967,** 234–240.
8. Dudek, S. M., and Johnson, G. V. W. (1994) *Brain Res.* **651,** 129–133.
9. Rasmussen, L. K., Sorensen, E. S., Petersen, T. E., Gliemann, J., and Jensen, P. H. (1994) *FEBS Lett.* **338,** 161–166.
10. Flandin, F., Buffevant, C., and Herbage, D. (1984) *Biochim. Biophys. Acta* **791,** 205–211.
11. Rigby, B. J., Mitchell, T. W., and Robinson, M. S. (1977) *Biochem. Biophys. Res. Commun.* **79,** 400–405.
12. Selkoe, J. E., Abraham, C., and Ihara, Y. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6070–6074.
13. Sell, D. R., and Monnier, V. M. (1989) *J. Biol. Chem.* **264,** 21597–21602.
14. Yamauchi, M., Woodley, D. T., and Mechanic, G. L. (1988) *Biochem. Biophys. Res. Commun.* **152,** 898–903.
15. Holbrook, J. J., Cooke, R. D., and Kingston, I. B. (1973) *Biochem. J.* **135,** 901–903.
16. Chung, S.-I., Lewis, M. S., and Folk, J. E. (1974) *J. Biol. Chem.* **249,** 940–950.
17. Gross, M., Whetzel, N. K., and Folk, J. E. (1975) *J. Biol. Chem.* **250,** 7693–7699.
18. Chuang, D.-M. (1981) *J. Biol. Chem.* **256,** 8291–8293.
19. Cornwell, M. M., Juliano, R. L., and Davies, P. J. A. (1983) *Biochim. Biophys. Acta* **762,** 414–419.
20. Castelhano, A. L., Billedeau, R., Pliura, D. H., Bonaventura, B. J., and Krantz, A. (1988) *Bioorg. Chem.* **16,** 335–340.
21. Killackey, J. J. F., Bonaventura, B. J., Castelhano, A. L., Billedeau, R. J., Farmer, W., DeYoung, L., Krantz, A., and Pliura, D. H. (1989) *Mol. Pharmacol.* **35,** 701–706.
22. Auger, M., McDermott, A. E., Robinson, V., Castelhano, A. L., Billedeau, R. J., Pliura, D. H., Krantz, A., and Griffin, R. G. (1993) *Biochemistry* **32,** 3930–3934.
23. Tso, J. Y., Bower, S. G., and Zalkin, H. (1980) *J. Biol. Chem.* **255,** 6734–6738.
24. Goldsmith, L. A., DeYoung, L. M., Falciano, V., Ballaron, S. J., and Akers, W. (1991) *J. Invest. Dermatol.* **97,** 156–158.
25. Freund, K. F., Doshi, K. P., Gaul, S. L., Claremon, D. A., Remy, D. C., Baldwin, J. J., Pitzenberger, S. M., and Stern, A. M. (1994) *Biochemistry* **33,** 10109–10119.
26. Kim, S.-Y., Kim, I.-G., Chung, S.-I., and Steinert, P. M. (1994) *J. Biol. Chem.* **269,** 27979–27986.
27. Kim, S.-Y., Chung, S.-I., and Steinert, P. M. (1995) *J. Biol. Chem.* **270,** 18026–18035.
28. Mehrel, T., Hohl, D., Rothnagel, J. A., Longley, M. A., Bundman, D., Cheng, C., Lichti, U., Bisher, M. E., Steven, A. C., Steinert, P. M., Yuspa, S. H., and Roop, D. R. (1990) *Cell* **61,** 1103–1112.
29. Hohl, D., Mehrel, T., Lichti, U., Turner, M. L., Roop, D. R., and Steinert, P. M. (1991) *J. Biol. Chem.* **266,** 6626–6636.
30. Kartasova, T., and Van de Putte, P. (1988) *Mol. Cell. Biol.* **8,** 2195–2203.
31. Gibbs, S., Fijneman, R., Wiegant, J., VanKessel, A. G., Van de Putte, P., and Backendorf, C. (1993) *Genomics* **16,** 630–637.
32. Saheki, T., Ito, F., Hagiwara, H., Saito, Y., Kuriki, J., Tachibana, S., and Hirose, S. (1992) *Biochem. Biophys. Res. Commun.* **185,** 240–245.
33. Molhuizen, H. O. F., Alkemade, H. A. C., Zeeuwen, P. L. J. M., DeJongh, G. J., Wieringa, B., and Schalkwijk, J. (1993) *J. Biol. Chem.* **268,** 12028–12032.
34. Rice, R. H., and Green, H. (1977) *Cell* **11,** 417–422.
35. Yaffe, M. B., Beegen, H., and Eckert, R. L. (1992) *J. Biol. Chem.* **267,** 12233–12238.
36. Kartasova, T., Cornelissen, B. J. C., Belt, P., and Van de Putte, P. (1987) *Nucleic Acids Res.* **15,** 5945–5962.
37. Richards, S., Scott, I. R., Harding, C. R., Liddell, J. E., Powell, G. M., and Curtis, C. G. (1988) *Biochem. J.* **253,** 153–160.
38. Abernethy, J. L., Hill, R. L., and Goldsmith, L. A. (1977) *J. Biol. Chem.* **252,** 1837–1839.
39. Steven, A. C., and Steinert, P. M. (1994) *J. Cell Sci.* **107,** 693–700.
40. Candi, E., Melino, G., Mei, G., Tarcsa, E., Chung, S.-I., Marekov, L. N., and Steinert, P. M. (1995) *J. Biol. Chem.* **270,** 26382–26390.
41. Steinert, P. M., and Marekov, L. N. (1995) *J. Biol. Chem.* **270,** 17702–17711.
42. Schmidt, R., Michel, S., Shroot, B., and Reichert, U. (1988) *FEBS Lett.* **229,** 193–196.
43. Steinert, P. M., and Parry, D. A. D. (1993) *J. Biol. Chem.* **268,** 2878–2887.
44. Moore, J. T., Hagstrom, J., McCormick, D. J., Harvey, S., Madden, B., Holicky, E., Standford, D. R., and Wieben, E. D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6712–6714.
45. Aeschlimann, D., Paulsson, M., and Mann, K. (1992) *J. Biol. Chem.* **267,** 11316–11321.
46. Jensen, P. H., Schueler, E., Woodrow, G., Richardson, M., Goss, N., Hojrup, P., Petersen, T. E., and Rasmussen, L. K. (1994) *J. Biol. Chem.* **269,** 15394–15398.
47. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
48. Birckbichler, P. J., Orr, G. R., Patterson, M. K., Jr., Conway, E., and Carter, H. A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5005–5008.