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Facile synthesis of membrane-embedded peptides utilizing lipid bilayer-assisted chemical ligation†

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Received (in Cambridge, UK) 17th March 2004, Accepted 28th May 2004
First published as an Advance Article on the web 28th June 2004

Lipid bilayer-assisted chemical ligation between thiolester and N-terminal cysteine peptides has been developed with successful application to the synthesis of membrane protein segments possessing both two transmembrane and one extracellular regions.

A wide variety of membrane proteins including 7-transmembrane G-protein-coupled receptors (7TM-GPCR) are receiving increasing attention as potential drug targets because of their crucial function in converting extracellular stimuli to intracellular signals.[1] These functions are achieved through stimulus-induced structural changes of membrane proteins; however, little is known about the structural basis of this process. Since chemically synthesized proteins incorporating informative modifications have served as tools for elucidating the functions of proteins,[2] chemical synthesis of membrane proteins should represent a potential means for studying these proteins.[3,4] However, membrane proteins have remained synthetic targets of great challenge due to the fact that peptide segments corresponding to their transmembrane domains (TMDs) are sparingly soluble in media commonly used in peptide synthesis.

In this study, we report methodology for the synthesis of membrane-embedded peptides utilizing lipid bilayer-assisted chemical ligation (Fig. 1). The presented protocol features native chemical ligation[5] between two TMDs within lipid bilayers. The ligation site is situated in the hydrophilic extracellular loop (ECL) region. Formation of membrane-embedded peptide loops derived from CXCR4-chemokine receptor (7TM-GPCR)[6] by this approach was achieved.

Native chemical ligation allows unprotected peptides to be chemoselectively coupled between C-terminal thiolesters and N-terminal Cys residues, giving peptides possessing an SH-free Cys residue at the ligation site. Since CXCR4 has one Cys residue in each of the three plausible ECLs, these positions (Leu–Cys: TMDs 2–3; Ile–Cys: TMDs 4–5 and Gly–Cys: TMDs 6–7) ‡ were selected as the ligation sites. Chemical ligation between Ile and Cys, as required for the coupling of TMD 4 with TMD 5, has been reported to proceed with a slow reaction rate.[7] This prompted us to first optimize coupling conditions using a model Ile-thiolester peptide§. Fmoc- or Boc-based solid-phase protocols followed by HPLC-purification of sparingly soluble TMDs using solvent systems consisting of HCO_2H–2-propanol–H_2O yielded the desired Cys-peptides (TMD 3, 5, and 7) or peptide thiolesters (TMD 2, 4, 6, and model Ile-thiolester peptide), respectively.[8] Optimization of chemical ligation of the model thiolester with cysteine revealed that reaction in the presence of thiophenol (2%, v/v) and tris(carboxyethyl)phosphine[9] (TCEP, 2%, w/v) in phosphate buffer (pH 7.8) containing EDTA (0.1%, w/v) at 37 °C for 54 h gave the coupled product in 88% yield without any accompanying epimerized product (see ESI†).

Next these optimized ligation conditions were applied to the coupling of membrane-embedded TMDs (Table 1, entries 1–3). Incorporation of TMDs into lipid bilayer was accomplished by a mixed film method.[10] A mixture of a TMD peptide pair for ligation and palmitoyloleoyl phosphatidylcholine (POPC) (peptide : lipid = 1 : 100, molar ratio) in 1,1,1,3,3,3-hexafluoro-2-propanol:$CHCl_3$ (1 : 4) or in TFA was taken to dryness to yield the mixed lipid/peptide film. The resulting film was hydrated with phosphate buffer containing TCEP (2%, w/v) and vortex-mixed followed by 10 freeze-thaw cycles to produce multilamellar vesicles (MLVs). Native chemical ligation was initiated by the addition of thiophenol (2%, v/v) to the above TMDs-MLVs suspensions. Progress of chemical ligation was monitored by solubilizing the peptide-lipid samples with TFA followed by HPLC analysis using a HCO_2H–2-propanol–H_2O elution system. The lipid bilayer-assisted chemical ligation between TMD 4 and TMD 5 proceeded efficiently within 24 h to yield the desired peptide loops possessing the second ECL of CXCR4 in 83% yield as indicated by HPLC analysis followed by ion-spray MS (ISMS) measurement of eluted peaks (Table 1, entry 2, Fig. 2). Similarly, ligation of TMD 2 + 3 or TMD 6 + 7 afforded the desired membrane-incorporated loop sequences, including the first or third ECL, respectively (Table 1, entries 1 and 3).

Next, we ascertained whether literature procedures were applicable to the chemical ligation of TMD peptides. The use of guanidine hydrochloride (GdnHCl) under conventional ligation conditions[5]

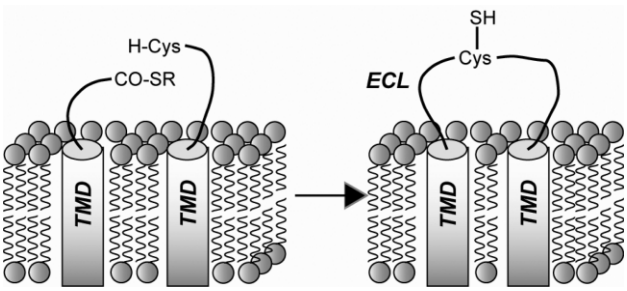

Fig. 1 Synthetic strategy for membrane-embedded peptides.

Table 1 Results of chemical ligation of membrane-embedded peptides under several coupling conditions

Entry	Peptides (ligation site)	Conditions[a] (peptide, mmol dm^{-3})	Yield[b] (%)
1	TMD 2 + 3 (Leu-Cys)	POPC vesicle, 24 h (0.05)	85
2	TMD 4 + 5 (Ile-Cys)	POPC vesicle, 24 h (0.05)	83
3	TMD 6 + 7 (Gly-Cys)	POPC vesicle, 12 h (0.05)	88
4	TMD 2 + 3 (Leu-Cys)	6 M GdnHCl, 48 h (1.0)	0
5	TMD 4 + 5 (Ile-Cys)	6 M GdnHCl, 48 h (1.0)	0
6	TMD 6 + 7 (Gly-Cys)	6 M GdnHCl, 48 h (1.0)	0
7	TMD 2 + 3 (Leu-Cys)	1% SDS, 48 h (1.0)	< 10
8	TMD 4 + 5 (Ile-Cys)	1% SDS, 48 h (1.0)	< 10
9	TMD 6 + 7 (Gly-Cys)	1% SDS, 24 h (1.0)	81
10	TMD 6 + 7 (Gly-Cys)	1% SDS, 24 h (0.05)	52

[a] In the presence of PhSH (2%, v/v) and TCEP (2%, w/v) in phosphate buffer (pH 7.8) containing EDTA (0.1%, w/v) at 37 °C. [b] Yields are estimated from HPLC peak areas.

† Electronic supplementary information (ESI) available: results of optimization of Ile-Cys ligation. Representative experimental procedure for the ligation. HPLC analyses of ligations of TMD 2 + 3 and TMD 6 + 7, and ISMS data of the resulting peptides. See http://www.rsc.org/suppdata/cc/b4/b404008b/

DOI: 10.1039/b404008b

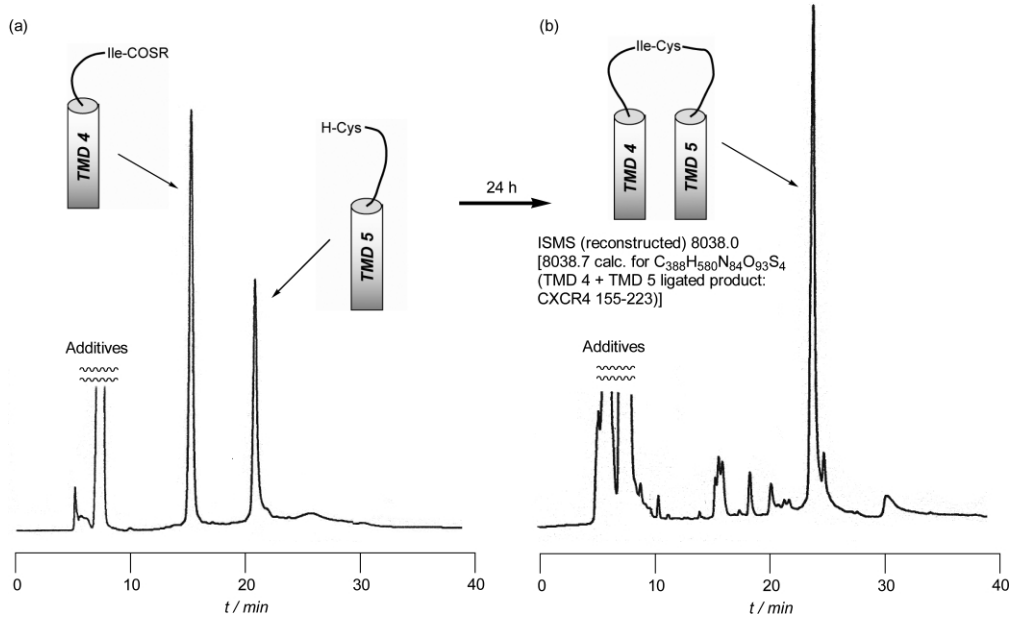

Fig. 2 HPLC analysis of ligation progress in the reaction of TMD 4 with TMD 5 under lipid bilayer-assisted conditions: (a) t = 0 h (reaction time). (b) t = 24 h. Column: Cosmosil 5C_4 (4.6 x 150 mm); buffer A: H_2O–HCO_2H (3 : 2); B: 2-propanol–HCO_2H (1 : 4); linear gradient 60–100% B in A over 30 min; flow rate 0.65 cm^3 min^{-1}; detect 280 nm.

resulted in the formation of insoluble material without any ligation product (Table 1, entries 4–6). Alternatively, application of ligation in the presence of SDS, which has been reported to be useful for the preparation of a membrane protein,[4] to the coupling of TMD 6 with TMD 7 (Gly-Cys site) yielded the desired peptide in 81% yield (Table 1, entry 9). However, use of this SDS-mediated protocol for the coupling of TMD 2 + 3 or TMD 4 + 5 resulted in failure (< 10%), with recovery of starting materials and/or accumulation of thiolester-exchange intermediates (thiophenyl ester) being observed (Table 1, entries 7 and 8). Chemical ligation under lipid bilayer-assisted conditions proceeded efficiently, in spite of the fact that the apparent concentration of the segments is 20 times lower (0.05 mmol dm^{-3}) than under other conventional conditions (1 mmol dm^{-3}). This result can probably be attributed to a "concentration effect" of sparingly soluble TMD peptides into the lipid bilayer.

In this study, we have developed a lipid bilayer-assisted ligation protocol that is applicable to the synthesis of membrane proteins. This newly developed methodology consists of native cysteine-dependent chemical ligation of the lipid bilayer-embedded TMD peptide segments. TMD peptide segments derived from the 7TM-GPCR protein CXCR4 were subjected to this new protocol to yield the desired peptides, which corresponded to polytopic membrane protein sequences possessing single CXCR4 ECL. When used in connection with a tandem ligation strategy, this methodology could provide a promising avenue for the preparation of polytopic membrane proteins. The direction of the peptides has yet to be controlled. In terms of this issue, innovative methods including transfer of peptides to other vesicles have to be developed.[11]

We thank Dr. Terrence R. Burke Jr., NCI, NIH, Frederick, MD., USA, for proofreading this manuscript. This research was supported in part by 21th Century COE Program "Knowledge Information Infrastructure for Genome Science", a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the Japan Society for the Promotion of Science, the Japan Health Science Foundation, and Takeda Science Foundation.

Notes and references

‡ TMD 2 (CXCR4 76–108 Ac-YRLHLSVADLLFVITLPFWAVDA-VANWYFGNFL-thiolester): Ion-spray MS (ISMS) (reconstructed) 4075.0 (4073.8 calc. for $C_{200}H_{283}N_{43}O_{47}S$); TMD 4 (CXCR4 155–185 Ac-VVYVGVWIPALLLTIPDFIFANVSEADDRYI-thiolester): ISMS (reconstructed) 3774.0 (3373.5 calc. for $C_{182}H_{267}N_{37}O_{38}S$); TMD 6 (CXCR4 240–273 Ac-TTVILILAFFACWLPYYIGISIDSFILLEIIKQG-thiolester): ISMS (reconstructed) 4078.0 (4076.0 calc. for $C_{200}H_{283}N_{43}O_{47}S$, 4076.0). thiolester = $S(CH_2)_2CO$-Ala-OH.

TMD 3 (CXCR4 109–132 H-CKAVHVIYTVNLYSSVLILAFISL-NH_2): ISMS (reconstructed) 2667.0 (2666.3 calc. for $C_{127}H_{205}N_{29}O_{31}S$); TMD 5 (CXCR4 186–223 H-CDRFYPNDLWVVVFQFQHIMVGLILP-GIVIL-SCYCIII-NH_2): ISMS (reconstructed) 4444.0 (4441.5 calc. for $C_{212}H_{324}N_{48}O_{48}S_4$); TMD 7 (CXCR4 274–308 H-CEFESVVHKWISITE-ALAFFHCCLNPILYAFLGA-NH_2): ISMS (reconstructed) 4006.0 (4004.9 calc. for $C_{185}H_{272}N_{44}O_{48}S_3$).

§ Model peptide: H_2N-NVSEADDRYI-$S(CH_2)_2CO$-Ala-OH

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