Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 261–266
Published online 20 September 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.916

JOURNAL OF PEPTIDE SCIENCE

Fmoc-based solid phase chemical synthesis of 71-meric neuregulin 1-β1, an epidermal growth factor-like domain

TAEKO KAKIZAWA,[a] SHIZUYO KOIDE-YOSHIDA,[b] TOORU KIMURA,[a] HIROMASA UCHIMURA,[b] YOSHIO HAYASHI,[a,c] KAZUKI SAITO[b] and YOSHIAKI KISO[a]*

[a] Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan
[b] Laboratory of Proteomic Sciences, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan
[c] Department of Medicinal Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

Received 6 July 2007; *Accepted 12 July 2007*

Abstract: The human neuregulin 1-β1 (NRG1-β1, amino acid residues 176–246) was chemically synthesized by Fmoc-based solid phase peptide synthesis (SPPS) followed by folding in a redox buffer. The biological activity of the synthesized NRG1-β1 was confirmed by ligand-induced tyrosine phosphorylation on Chinese hamster ovary (CHO) cells expressing ErbB-4. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: neuregulin 1-β1; solid phase peptide synthesis; fluorenylmethoxycarbonyl group; ErbB-4

INTRODUCTION

Synthesis of proteins by chemical means has enabled the preparation of high-purity proteins in milligram scale [1–4]. Numerous synthetic approaches have been carried out, so that the obtained proteins would provide further wide-ranging studies such as NMR and crystallography, as well as various explorations of biological functions [5–10].

In chemical protein synthesis, elongation of the sequence and experimental folding studies have not received the attention they deserve [11–19]. Epidermal growth factor (EGF) and its protein family are widely used for synthetic studies, and many approaches have been reported [13–19]. However, these proteins still remain as challenges to peptide chemists because they have moderately long sequences and three disulfide bonds within a molecule. The NRG (an EGF-like motif of neuregulin, also known as HRG, NDF, and by various other names) family has also been used as target proteins for chemical synthesis [9,10,15,19]. In addition, the biological functions of NRGs are of interest because the overexpression of their receptors (ErbB families) is sometimes observed on the surface of human cancer cells [20,21]. Chemical synthesis of a 67-amino-acid protein comprising of residues 175–241 of NRG1-α was performed by Nagata *et al.* based on Boc-strategy in 1993 [19]. A 65-residue NRG1-β1 in the 177–241 region and the related chimeric NRG/EGF proteins were also synthesized by Barbacci *et al.* in 1995 using standard Boc protocols on an ABI 430A peptide synthesizer [9]. However, the synthetic details of Barbacci's work were not described in their publication because the biological functions of NRGs were their main investigation. We could not find descriptions on the synthetic yield, purity, or obtained amounts of the target compounds. Considering the importance of well-documented chemical protein synthesis, the reaction details of NRGs are necessary for future research. Here we report the detailed synthesis of NRG1-β1 as a 71-amino-acid region encompassing residues 176–246, using ABI 431A peptide synthesizer based on Fmoc chemistry. The sequence and disulfide bond pattern of target NRG1-β1 (residues 176–246, **1**) and related molecules are shown in Figure 1.

In previous studies, NRG1-β1 was synthesized by Barbacci *et al.* as a 65-amino-acid region (residues 177–241) [9]; chemical syntheses of much shorter domains (52 amino acids) were also reported by Zamborelli *et al.* [15]. However, in our case, we preferred a 71-amino-acid NRG1-β1, because its sequence is consistent with the current recombinant NRG1-β1 (R&D System Inc. (Minneapolis, MN, USA), etc.) used in various biological studies [22–25]. We obtained the desired molecules in high purity by Fmoc-based solid phase peptide synthesis (SPPS), folding techniques, and HPLC purification. In this study, we examined tyrosine phosphorylation assay on the target protein **1** using Chinese hamster ovary (CHO) cells expressing ErbB-4.

MATERIALS AND METHODS

General

All protected amino acids were purchased from Applied Biosystems (Forester City, CA, USA). Resins were purchased

*Correspondence to: Yoshiaki Kiso, Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan; e-mail: kiso@mb.kyoto-phu.ac.jp

Figure 1 Sequence and disulfide bond pattern of NRG1-β1 (**1**) and related molecules.

from Calbiochem–Novabiochem Japan Ltd (Tokyo). Other chemicals were mostly purchased from commercial suppliers, Wako Pure Chemical Ind. Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI, USA) and were used without further purification. MALDI-TOF mass spectra were recorded on a Voyager DE-RP instrument using α-cyano-4-hydroxycinnamic acid as matrix. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm $\times$ 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse-phase column (20 mm $\times$ 250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 ml min^{-1} (40 °C), detected at 230 nm. Solvents used for HPLC were of HPLC grade. CHO cells were donated by Cell Resource Center for Biomedical Research, Tohoku University. ErbB-4 expression vector pcDNA3.1-ErbB-4 was generously donated by Professor Shigeyuki Yokoyama [25]. Transfection of ErbB-4 gene into CHO cells was carried out using Lipofectoamine 2000 transfection reagent (Invitorgen) according to the manufacturer's instruction [25]. Anti-[phospho-p44/42 MAP kinese (Tyr202/Tyr204)] antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-ERK2 antibody (sc-153) and anti-ErbB-4 antibody (sc-283) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant NRG1-β1 in the 176–246 region was purchased from R&D System Inc. (Minneapolis, MN, USA).

Solid Phase Peptide Synthesis

Fmoc-amino acid side-chain protections were selected as follows: *t*Bu (Ser, Thr, Tyr), O*t*Bu (Asp, Glu), Boc (Lys), Pmc (Arg), Trt (Asn, Gln, His, Cys). Peptide chain was elongated using automated standard Fmoc cycles with HOBt/DCC activation on ABI 431A peptide synthesizer. Each coupling consisted of (i) removal of the N^{α}-Fmoc protection by piperidine (20% v/v in NMP) and (ii) a single 20 min coupling of an HOBt ester of an N^{α}-Fmoc-amino acid (1 mmol) in NMP. From Fmoc-Lys(Boc)-*O*-chlorotrityl resin (**2**, 1.071 g, 93.14 µmol), side-chain-protected NRG1-β1-*O*-chlorotrityl resin (**3**, 1.950 g) was obtained.

Peptides were cleaved from the resin with TFA in the presence of 1,2-ethanedithiol, triisopropylsilane, and distilled water (94 : 2.5 : 1 : 2.5, v/v/v/v, 22 µl per 1 mg resin) for 90 min at room temperature (rt), concentrated *in vacuo*, and precipitated with diethyl ether at 0 °C followed by centrifugation at 3000 rpm for 5 min (3×). The resultant peptide was dissolved or suspended in water and lyophilized for at least 12 h. Finally, a crude [$Cys^{182,190,196,210,212,221}$] NRG1-$\beta$1 (**4**, 660.6 mg) was obtained.

Crude **4** was dissolved and reduced in a buffer (2 mg ml^{-1}, rt) consisting of GuHCl (8 M), Tris-HCl (100 mM, pH 8.0), and DTT (150 mM). After 1 h, the reduced products were purified by preparative reverse-phase HPLC with a 0.1% aqueous TFA–CH_3CN system and immediately frozen at −78 °C and lyophilized for at least 12 h to give a reduced peptide **4** (123.2 mg, 14.95 µmol, 16.05% yield calculated from **2**). MALDI-MS (TOF): M_{calcd}: 8241.5 Da; $[M+H]_{found}$: 8242.8 Da; HPLC analysis at 230 nm: purity was >95%.

Folding

Purified [$Cys^{182,190,196,210,212,221}$] NRG1-$\beta$1 (**4**) was dissolved in a redox buffer consisting of Tris-HCl (50 mM, pH 8.0), EDTA (1 mM), GSH (1 mM), and GSSG (1 mM) to a concentration 0.3 mg ml^{-1} (0.04 mM). The solution was stirred overnight at rt, and filtered using a 0.46 µm filter unit. NRG1-β1 (**1**) was obtained and purified by preparative reverse-phase HPLC. After 12 h of lyophilization, folded **1** was elucidated as a white powder. The folding reactions were performed at the 0.12–0.36 µmol scale with an average yield of 37% (typically, obtained **1** was 0.37 mg (0.045 µmol) from 1.0 mg (0.12 µmol) of **4**). MALDI-MS (TOF): M_{calcd}: 8235.4 Da; $[M+H]_{found}$: 8235.7 Da; HPLC analysis at 230 nm: purity >95%. The retention time on HPLC (0–100% CH_3CN for 40 min) of **1** was identical to that of the purchased recombinant NRG1-β1.

Stimulation, Lysate Preparation, Immunoprecipitation, and Immunoblotting

Transfected CHO cells were starved in a serum-free medium containing bovine serum albumin (BSA) (1 mg ml^{-1}) for 24 h, and then recombinant or synthesized NRG1-β1 (20 ng ml^{-1}) was added to stimulate the cells for 5 min. The cells were washed with ice-cold, phosphate-buffered saline and lysed in a buffer containing Tris-HCl (30 mM, pH 7.4), NaCl (150 mM), EDTA (5 mM), 2-glycerophosphate (40 mM), glycerol (10%, v/v), Triton X-100 (1%, v/v), phenylmethanesulfonyl fluoride (1 mM), sodium orthovanadate (1 mM), aprotinin (10 µg ml^{-1}), and leupeptin (10 µg ml^{-1}). Cell debris was removed by microcentrifugation at 4 °C for 10 min, and protein concentrations of the cell lysates were determined by a protein assay kit (Bio-Rad). Lysates were resolved by SDS/PAGE, and then transferred to a nitrocellulose membrane for immunoblotting with an appropriate antibody. Protein bands were visualized by an electrochemiluminescence (ECL) system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

The synthesis of NRG1-β1 (**1**) is shown in Scheme 1. Protected 71-aa peptide resin **3** was prepared by automated standard Fmoc-based SPPS methodology using HOBt/DCC activation on an automated ABI 431A

DOI: 10.1002/psc

Scheme 1 Reagents and conditions: (i) Fmoc protocol on automated peptide synthesizer (ABI 431A); (ii) TFA/EDT/TIS/H_2O (94 : 2.5 : 1.0 : 2.5), 90 min; (iii) preparative HPLC (a linear gradient of CH_3CN in 0.1% aqueous TFA); (iv) overnight stirring in a buffer (Tris-HCl (50 mM, pH 8.0), EDTA (1 mM), GSH (1 mM), GSSG (1 mM)) at rt; (v) preparative HPLC (a linear gradient of CH_3CN in 0.1% aqueous TFA).

peptide synthesizer. Side-chain-protected NRG1-β1-*O*-chlorotrityl resin **3** (1.950 g) was obtained from Fmoc-Lys(Boc)-*O*-chlorotrityl resin **2** (1.071 g, 93.14 µmol). Deprotection of **3** and cleavage from the resin were performed by a treatment of TFA in the presence of 1,2-ethanedithiol, triisopropylsilane, and distilled water (94 : 2.5 : 1.0 : 2.5, v/v/v/v) for 90 min at rt to afford crude [$Cys^{182,190,196,210,212,221}$] NRG1-$\beta$1 **4** (660.6 mg).

The HPLC profile of crude **4** shows a main broad signal as illustrated in Figure 2(A). To obtain the purified **4**, the crude peptides were dissolved and stirred in a buffer consisting of GuHCl (8 M), Tris-HCl (100 mM, pH 8.0), and DTT (150 mM). After reducing the disulfide bonds, the reaction mixture was subjected to purification on preparative HPLC, using a 0.1% aqueous TFA–CH_3CN system. Purification of crude materials afforded purified **4** (123.2 mg, 14.95 µmol) in 16% yield calculated from **2**. The HPLC profile of purified **4** is given in Figure 2(B). Purified **4** shows a single sharp signal with a purity of >95%. MALDI-TOF MS analysis gave an observed mass (MS: M_{calc}: 8241.5 Da; $[M + H]_{found}$: 8242.8 Da) that was consistent with the desired **4** as a 71-aa residue target sequence (Figure 2(C)). In previous NRG synthetic studies by other research groups, the linear sequences were often elongated using Boc-cycles and a peptide synthesizer [9,19]. Their protocols involved the following steps: (i) all the Boc amino acids were coupled twice and (ii) unreacted NH_2 groups were capped using acetic anhydride. Compared to their studies, our desired sequence was efficiently produced by ordinary Fmoc-based protocols consisting of Fmoc-deprotecting and single-coupling cycles, without any difficulties.

As previously mentioned, NRG1-β1 has three disulfide bonds per molecule. The oxidation in glutathione buffer has been performed in earlier attempts as a common method. Zamborelli *et al.* carefully examined the folding of their 52-residue NRG α/β chimera, and reported several strategies to overcome difficulties arising from the oxidation processes [15]. In their typical procedure, oxidation was performed by 24-h stirring in a buffer consisting of Tris-HCl (50 mM, pH 8.0), EDTA (1 mM), GSH (1 mM), and GSSG (1 mM) at 20 °C. We essentially applied a similar procedure to **4** to form intramolecular disulfide bonds. Purified **4** was dissolved and stirred in a glutathione redox buffer (0.3 mg ml^{-1}) consisting of Tris-HCl (50 mM, pH 8.0), EDTA (1 mM), GSH (1 mM), and GSSG (1 mM) at ambient temperature. The oxidation process, which was monitored by HPLC (0–100% CH_3CN for 40 min), is presented in Figure 3. Retention time of **1** in Figure 3 was authenticated with that of recombinant NRG1-β1. Within 5 min

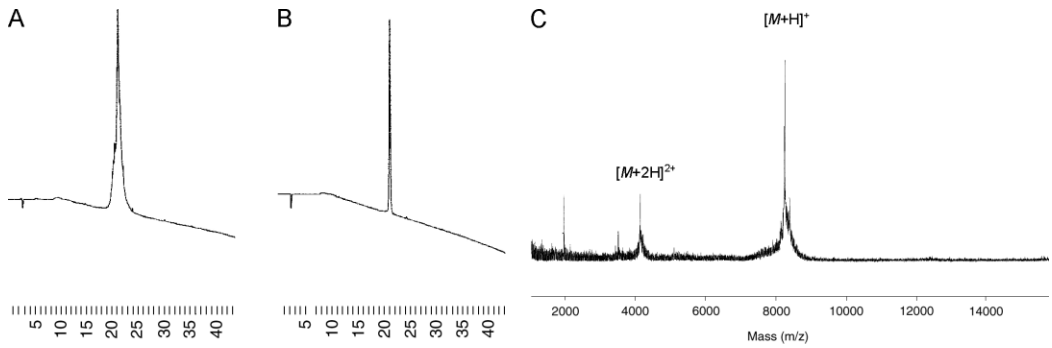

Figure 2 HPLC profiles of (A) crude and (B) purified [$Cys^{182,190,196,210,212,221}$] NRG1-$\beta$1 (**4**). Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm. (C) MALDI-TOF mass spectrum of purified **4**.

DOI: 10.1002/psc

of stirring, formation of disulfide bonds began to form **1**. Being consistent with typical findings [15], folded **1** eluted faster than the linear product **4** in the HPLC profile. After 12 h of stirring, oxidation seemed to be almost complete with a slight remnant of a white precipitate. The HPLC profile did not show notable changes after being stirred for 12 h, up to 24 h. Upon filtering the solution mixture, folded **1** was collected by preparative HPLC. The analytical HPLC profile of synthesized **1** is depicted in Figure 4(A). Obtained **1** shows a single sharp signal with a purity of >95%. Mass spectrometry analysis of lyophilized powder **1** was in agreement with expected values (MALDI-MS (TOF): M_{calc}: 8235.4 Da; $[M+H]_{found}$: 8235.7 Da), i.e. an almost 6 Da decrease from **4**, which corresponds to the loss of six protons (Figure 4(B)). In a typical procedure, folded **1** was recovered by preparative HPLC with an average 37% yield from **4**. Thus, if all of **4** were successfully subjected to folding and purification, it would be possible to obtain *ca* 45 mg (5.5 μmol) of high-purity **1** from a single-scale synthesis. Hence, the overall yield based on the resin (93.14 μmol) would be 6%. Zamborelli *et al.* reported that the overall yields of their 52-aa NRGs were typically 2–12% [15]. In the case of 67-aa region of NRG1-α, the overall yield was only <1% (From 0.25 mmol of resin, 10.2 mg (1.3 μmol) of NRG1-α could be obtained) [19]. In contrast to these results, our synthetic route could give the desired molecule in modest yield, in spite of its long sequence.

In previous studies, NRG1-β1 was proven as a potent inducer of tyrosine phosphorylation of ErbB-4 (a homologue of ErbB-1/EGF receptor) [25–29]. To confirm the biological activity of synthesized **1**, we examined NRG1-β1-dependent phosphorylation of extracellular signal-regulated kinases (ERKs) as a probe for the downstream signaling pathway of ErbB-4. In this study, CHO cells were transfected with human ErbB-4 gene. Forty-eight hours after transfection the cells were stimulated by NRG1-β1 (20 ng ml^{-1}), and phosphorylation of the receptors was visualized by immunoblotting with an anti-phosphorylation antibody. The assay results are shown in Figure 5. In lanes 1 and 2 of Figure 5, results of the CHO cells assay without ErbB-4 overexpression are shown as controls. Among them, faint bands of the anti-phospho-p44/42 MAPK antibody were observed with recombinant NRG1-β1 treated (lane 2) and untreated (lane 1). Similarly, a faint band was found, when nothing was added to ErbB-4-expressing cells (lane 3). These bands are thought to be basal tyrosine phosphorylations that respond to other endogenous ErbB family on CHO cells. On the other hand, ligand-induced phosphorylation of ErbB-4 clearly exhibited a strong response to the treatment of recombinant NRG1-β1 (lane 4), in agreement with literature reports [25–29]. When the synthesized **1** was examined (lane 6), an intense response was obviously found in a manner analogous to that of lane 4. These observations suggest that the biological activity of synthesized **1** for ErbB-4 is similar to the recombinant NRG1-β1. In addition, we could also prove that ErbB-4 was not phosphorylated by a linear product **4** (lane 5). In lane 5, the lower intensity of the response was almost the same as that of lanes 1–3.

In summary, we synthesized NRG1-β1 (amino acid residues 176–246) on standard Fmoc-based SPPS using an automated peptide synthesizer and followed by folding in a glutathione redox buffer. In a single-scale synthesis, the desired protein could be obtained

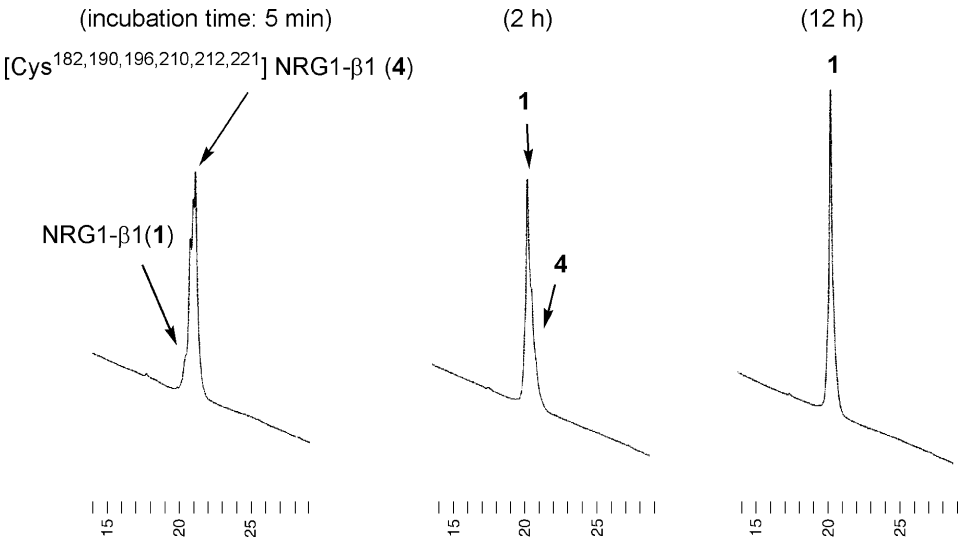

Figure 3 HPLC profiles of conversion of [$Cys^{182,190,196,210,212,221}$] NRG1-$\beta$1 (**4**) to NRG1-$\beta$1 (**1**) in a buffer (Tris-HCl (50 mM, pH 8.0), EDTA (1 mM), GSH (1 mM), and GSSG (1 mM)) at rt. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm.

DOI: 10.1002/psc

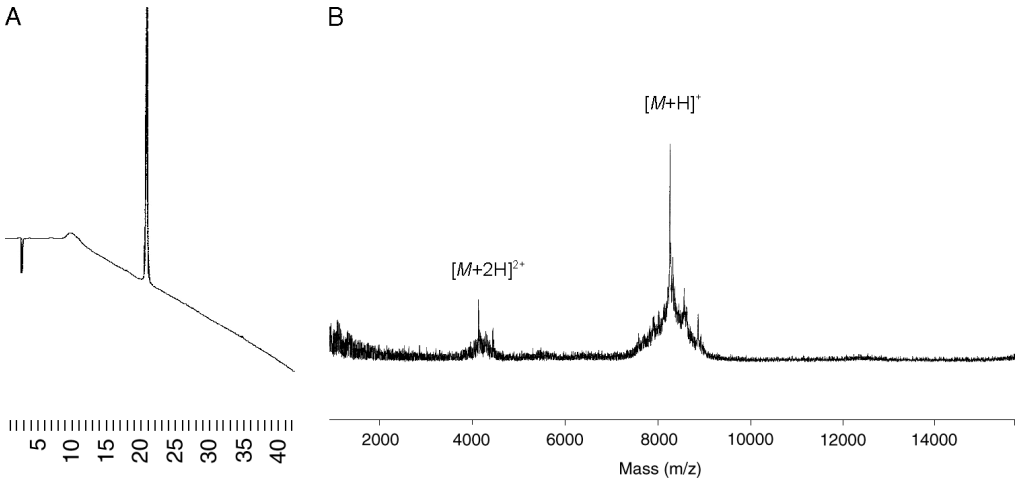

Figure 4 (A) HPLC profiles of NRG1-β1 (**1**). Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm. (B) MALDI-TOF mass spectrum of **1**.

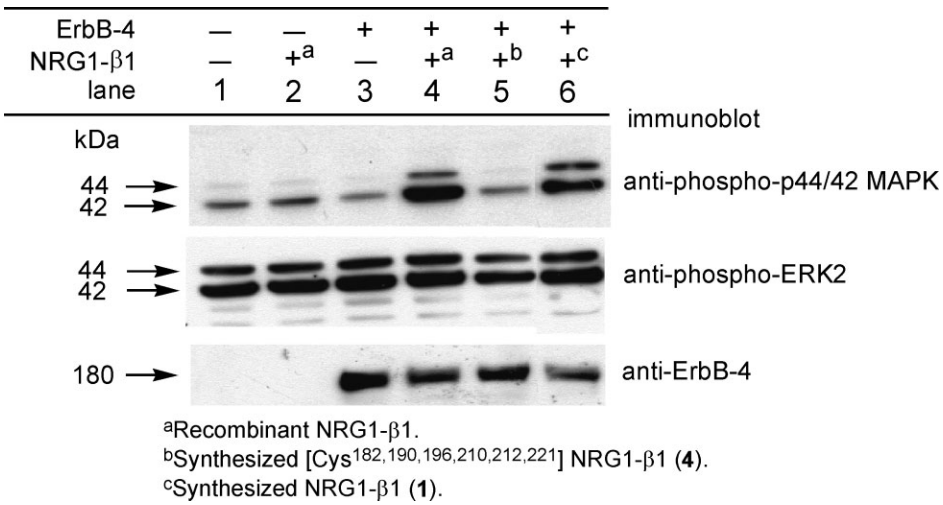

Figure 5 Ligand-induced tyrosine phosphorylation of ErbB-4. CHO cells were transfected with an expression vector for pcDNA3.1-ErbB-4. The cells were serum-starved for 24 h in serum-free medium containing BSA (1 mg ml^{-1}). After the starvation, the cells were treated (+) or untreated (–) with the NRG1-β1 (20 ng ml^{-1}) for 5 min. Whole-cell lysates were resolved by 4–12% gradient gel, transferred to a nitrocellulose membrane, and visualized by immunoblotting with anti-phospho-p44/42 MAPK (Cell Signaling Technology), anti-ERK2 (Santa Cruz), and anti-ErbB-4 (Santa Cruz) antibodies.

in high purity with improved synthetic yield over that of literature reports. We also demonstrated that ErbB-4 stimulation was induced by our synthesized NRG1-β1, in an analogous manner with that of the recombinant.

Acknowledgements

This research was supported in part by the 'Academic Frontier' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), and the 21st Century Centre of Excellence Programme 'Development of Drug Discovery Frontier Integrated from Tradition to Proteome' from MEXT. We are grateful to Cell Resource Centre for Biomedical Research, Tohoku University for CHO cells, and Professor Shigeyuki Yokoyama for cDNA of ErbB-4. Mass spectra measurements were performed by H–O. Kumada and T. Hamada. This manuscript was revised by Dr. J.-T. Nguyen.

REFERENCES

1. Johnson ECB, Kent SBH. Towards the total chemical synthesis of integral membrane proteins: a general method for the synthesis of hydrophobic peptide-$^{\alpha}$thioester building blocks. *Tetrahedron Lett.* 2007; **48**: 1795–1799.
2. Sohma Y, Taniguchi A, Yoshiya T, Chiyomori Y, Fukao F, Nakamura S, Skwarczynski M, Okada T, Ikeda K, Hayashi Y, Kimura T, Hirota S, Matsuzaki K, Kiso Y. 'Click peptide': a novel '*O*-acyl isopeptide method' for peptide synthesis and chemical

biology-oriented synthesis of amyloid β peptide analogues. *J. Pept. Sci.* 2006; **12**: 823–828.
3. Sohma Y, Chiyomori Y, Kimura M, Fukao F, Taniguchi A, Hayashi Y, Kimura T, Kiso Y. '*O*-acyl isopeptide method' for the efficient preparation of amyloid β peptide 1–42 mutants. *Bioorg. Med. Chem.* 2005; **13**: 6167–6174.
4. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Novel and efficient synthesis of difficult sequence-containing peptides through *O–N* intramolecular acyl migration reaction of *O*-acyl isopeptides. *Chem. Commun.* 2004; 124–125.
5. Torbeev VY, Kent SBH. Convergent chemical synthesis and crystal structure of a 203 amino acid "Covalent Dimer" HIV-1 protease enzyme molecule. *Angew. Chem. Int. Ed.* 2007; **46**: 1667–1670.
6. Taniguchi A, Sohma Y, Kimura M, Okada T, Ikeda K, Hayashi Y, Kimura T, Hirota S, Matsuzaki K, Kiso Y. "Click Peptide" based on the "*O*-Acyl Isopeptide Method": control of Aβ 1–42 production from a photo-triggered Aβ 1–42 analogue. *J. Am. Chem. Soc.* 2006; **128**: 696–697.
7. Johnson ECB, Durek T, Kent SBH. Total chemical synthesis, folding, and assay of a small protein on a water-compatible solid support. *Angew. Chem. Int. Ed.* 2006; **45**: 3283–3287.
8. Bang D, Makhatadze GI, Tereshko V, Kossiakoff AA, Kent SB. Total chemical synthesis and x-ray crystal structure of a protein diastereomer: [D-Gln 35] Ubiquitin. *Angew. Chem. Int. Ed.* 2005; **44**: 3852–3856.
9. Barbacci EG, Guarino BC, Stroh JG, Singleton DH, Rosnack KJ, Moyer JD, Andrews GC. The structural basis for the specificity of epidermal growth factor and Heregulin binding. *J. Biol. Chem* 1995; **270**: 9585–9589.
10. Nagata K, Kohda D, Hatanaka H, Ichikawa S, Matsuda S, Yamamoto T, Suzuki A, Inagaki F. Solution structure of the epidermal growth factor-like domain of heregulin-α, a ligand for p180^{erbB-4}. *EMBO J.* 1994; **13**: 3517–3523.
11. Bang D, Chopra N, Kent SBH. Total chemical synthesis of Crambin. *J. Am. Chem. Soc.* 2004; **126**: 1377–1383.
12. Bang D, Kent SBH. A one-pot total synthesis of Crambin. *Angew. Chem. Int. Ed.* 2004; **43**: 2534–2538.
13. Shin SY, Yokoyama T, Takenouchi T, Munekata E. The chemical synthesis and binding affinity to the EGF receptor of the EGF-like domain of heparin-binding EGF-like growth factor (HB-EGF). *J. Pept. Sci.* 2003; **9**: 244–250.
14. Husbyn M, Cuthbertson A. A novel approach to the synthesis of EGF-like domains: a method for the one-pot regioselective formation of the three disulfide bonds of a human blood coagulation factor VII EGF$_{-1}$ analogue. *J. Pept. Res.* 2002; **60**: 121–127.
15. Zamborelli TJ, Dodson WS, Harding BJ, Zhang J, Bennett BD, Lenz DM, Young Y, Haniu M, Liu CendashF, Jones T, Jarosinski MA. A comparison of folding techniques in the chemical synthesis of the epidermal growth factor-like domain in neu differentiation factor α/β. *J. Pept. Res.* 2000; **55**: 359–371, references therein.
16. Hackeng TM, Dawson PE, Kent SBH, Griffin JH. Chemical synthesis of human protein S thrombin-sensitive module and first epidermal growth factor module. *Biopolymers* 1998; **46**: 53–63.
17. Yang Y, Sweeney WV, Schneider K, Chait BT, Tam JP. Two-step selective formation of three disulfide bridges in the synthesis of the C-terminal epidermal growth factor-like domain in human blood coagulation factor IX. *Protein Sci.* 1994; **3**: 1267–1275.
18. Shin SY, Shimizu M, Munekata E. Synthesis of N-terminal truncated peptides of human epidermal growth factor (h-EGF). *Chem. Lett.* 1994; **23**: 1997–2000.
19. Nagata K, Kohda D, Hatanaka H, Ichikawa S, Matsuda S, Yamamoto T, Suzuki A, Inagaki F. Synthesis and solution structure analysis of the EGF-like domain of heregulin-α, a ligand for p185^{erbB-2}. *Pept. Chem.* 1993; **31**: 421–424.
20. Holbro T, Civenni G, Hynes NE. The ErbB receptors and their roles in cancer progression. *Exp. Cell Res.* 2003; **284**: 99–110.
21. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* 2001; **2**: 127–137.
22. Warren CM, Kani K, Landgraf R. The N-terminal domains of neuregulin 1 confer signal attenuation. *J. Biol. Chem.* 2006; **281**: 27306–27316.
23. Jacobson C, Duggan D, Fischbach G. Neuregulin induces the expression of transcription factors and myosin heavy chains typical of muscle spindles in cultured human muscle. *Proc. Natl. Acad. Sci. U.S.A.* 2004; **101**: 12218–12223.
24. Ferguson KM, Darling PJ, Mohan MJ, Macatee TL, Lemmon MA. Extracellular domains drive homo- but not hetero-dimerization of ErbB receptors. *EMBO J.* 2000; **19**: 4632–4643.
25. Kim J-H, Saito K, Yokoyama S. Chimeric receptor analyses of the interactions of the ectodomains of ErbB-1 epidermal growth factor and of those of ErbB-4 with neuregulin. *Eur. J. Biochem.* 2002; **269**: 2323–2329.
26. Riese DJ II, van Raaij TM, Plowman GD, Andrews GC, Stern DF. The cellular response to neuregulin is governed by complex interactions of the ErbB receptor family. *Mol. Cell. Biol.* 1995; **15**: 5770–5776.
27. Tzahar E, Levkowitz G, Karunagaran D, Yi L, Peles E, Lavi S, Chang D, Liu N, Yayon A, Wen D, Yarden Y. ErbB-3 and ErbB-4 functions as the respective low and high affinity receptors of all neu differentiation factor/heregulin isoforms. *J. Biol. Chem.* 1994; **269**: 25226–25233.
28. Culouscou J-M, Plowman GD, Carlton GW, Green JM, Shoyab M. Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180^{erbB4} receptor. *J. Biol. Chem.* 1993; **268**: 18407–18410.
29. Plowman GD, Green JM, Culouscou J-M, Carlton GW, Rothwell VM, Buckley S. Heregulin induces tyrosine phosphorylation HER4/p180^{erbB4}. *Nature* 1993; **366**: 473–475.