Mol. Cells, Vol. 21, No. 2, pp. 244-250

Molecules and Cells

Identification of a Peptide Enhancing Mucosal and Systemic Immune Responses against EGFP after Oral Administration in Mice

Sae-Hae Kim[1], Kyung-Yeol Lee[2], Ju Kim[1,3], Seung-Moon Park[4], Bong Kyun Park[5], and Yong-Suk Jang[1,3,*]

[1] *Devision of Biological Sciences and Institute for Molecular Biology and Genetics, Chonbuk National University, Chonju 561-756, Korea;*

[2] *Department of Microbiology and Institute of Oral Bioscience, Chonbuk National University, School of Dentistry, Chonju 561-756, Korea;*

[3] *Bank for Cytokine Research, Chonbuk National University, Chonju 561-756, Korea;*

[4] *Institute of Basic Science, Chonbuk National University, Chonju 561-756, Korea;*

[5] *Department of Veterinary Virology, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea.*

(Received December 12, 2005; Accepted January 11, 2006)

Gangliosides are receptors for various peptides and proteins including neuropeptides, β-amyloid proteins, and prions. Recently, the role of gangliosides in mucosal immunization has attracted attention due to the emerging interest in oral vaccination. Ganglioside GM1 exists in abundance on the surface of the M cells of Peyer's patch, a well-known mucosal immunity induction site. In the present study we identified a peptide ligand for GM1 and tested whether it played a role in immune induction. GM1-binding peptides were selected from a phage-displayed dodecapeptide library and one peptide motif, GWKERLSSWNRF, was fused to the C-terminus of enhanced green fluorescent protein (EGFP). The fusion protein, but not EGFP fused with a control peptide, was concentrated around Peyer's patch after incubation in the lumen of the intestine *ex vivo*. Furthermore, oral feeding of the fusion protein but not control EGFP induced mucosal and systemic immune responses against EGFP resembling Th2-type immune responses.

Keywords: Adjuvant; Ganglioside; Mucosal Immunization; Phage Display Library.

* To whom correspondence should be addressed.
Tel: 82-63-270-3343; Fax: 82-63-270-4312
E-mail: yongsuk@chonbuk.ac.kr.

Introduction

Interactions between carbohydrates and proteins are important in many cellular processes and are potential targets for therapeutic and preventive measures against various diseases (Bergelson *et al*., 1982; Fishman, 1982; Markwell *et al*., 1981). Gangliosides, as cell-surface glycolipids, are involved in signal transduction and also act as receptors for cell-to-cell interactions, viruses, and toxins (Kurganov *et al*., 2004; Mattei *et al*., 2004; Valdes-Gonzalez *et al*., 2001). In particular, GM1-ganglioside, a glycolipid with a terminal sialic acid, is a receptor for subunit B of cholera toxin (CTB), and disruption of this interaction could be an approach to preventing CT-induced diarrhea.

Recently, the role of gangliosides in mucosal immunization has received attention due to emerging interest in oral and intranasal vaccination (Bae *et al*., 2003; de Haan *et al*., 1998; Gardby *et al*., 2003; Kang *et al*., 2004; Kawamura *et al*., 2003). CT and its close relative, *E. coli* heat-labile toxin (LT), bind to GM1 and these proteins are well-known adjuvants (Ogushi *et al*., 2004). Therefore, CT, LT, and their derivatives have been tested as mucosal adjuvants, but the results were unsatisfactory because the entire CT molecule was found to accumulate in the nervous system after intranasal administration (van Ginkel *et al*., 2000) and a flu vaccine containing LT adjuvant was withdrawn from the market for a similar reason. Later, the non-toxic B subunit of CT was tested as an adjuvant, although is adjuvant effects are controversial (Fujihashi *et al*., 2002).

Another strategy for identifying mucosa-targeting peptides, phage display, has been used to screen peptides or proteins with high affinity for specific ligands; for example antibodies for lipopolysaccharide, and peptides for GM1 (Matsubara *et al*., 1999; Miura *et al*., 2004). In a previous report, specific peptides binding to the carbohydrate moiety of GM1 glycolipid were isolated by biopanning through a GM1 monolayer on a water-air interface, but their biological roles were unclear (Matsubara *et al*., 1999). It seemed possible that different methods of preparing GM1 ligands for phage display yield quite different peptides and that a combination of phage display and bioassay could yield peptides of biological significance. We therefore isolated GM1-specific peptides from a phage display library, analyzed their homology to CTB, and then tested the biological activities of antigens containing these motifs. We report that oral feeding of a fusion of the peptide, GWKERLSSWNRF, with EGFP induced mucosal and systemic immune responses against the fusion antigen in mice.

Materials and Methods

Chemicals and Laboratory ware Unless otherwise specified, chemicals and laboratory ware were obtained from Sigma Chemical Co. (USA) and Falcon Labware (Becton-Dickinson, USA), respectively.

Biopanning A phage display library of 10^{15} dodecapeptides (New England Biolabs, Inc., USA) was used to screen peptides binding to GM1 ganglioside according to the manufacturer's protocol. In brief, a 60 mm dish was coated with 150 μg GM1 overnight at 4°C, blocked with 2 ml of 1% BSA for 2 h at 4°C, and then washed 6 times with TBST (TBS, 0.01% Tween-20). About 1.5×10^{10} peptides were added to the dish which was incubated for 1 h at 4°C. After 3 washes with TBST, the bound peptides were eluted with 1 ml of elution buffer (0.1 M glycine, pH 2.0). Panning was repeated twice with the successively eluted and amplified phages.

Production of recombinant EGFP-peptide fusion protein In order to generate an EGFP-peptide fusion vector, referred to as EGFP-CL3, EGFP was amplified by PCR from pEGFP-1 (Clontech, USA) using forward and reverse primers: 5′-GAT CGG ATC CAC CGG TCG CCA CCA TGG TGA GC-3′ and 5′-AGC TGA GCT CCT AAT ACA GAG TAG TCG TCT GAC TAT TCT TAT GAA ACG CCT TGT ACA GCT CGT CCA T-3′. The amplified product was digested with BamHI and SacI and ligated into the same cloning site of pQE31 (Qiagen, Germany), yielding pEGFP-CL3. The integrity of the product was confirmed by nucleotide sequencing. In a similar way, a control expression vector, pEGFP-C, which contains the sequence of an unrelated dodecapeptide, was prepared using the same forward primer and a different reverse primer: 5′-AGC TGA GCT CCT AGT GAT TAC TAA TTG TCT GCA GAA TTG TTC GGG TGT TCA TCT TCT TGT ACA GCT CGT CCA T-3′. The fusion proteins were expressed and purified, and quantified by Bradford assay and Coomassie staining (Bradford, 1976). About 1 mg of nearly homogeneous recombinant protein was obtained per 1 L culture. Purified EGFP-CL3 and EGFP-C were dialyzed against PBS and diluted to 1 mg/ml.

Mouse gut loop experiments Mouse intestinal loops were prepared as described previously (Foster *et al*., 1998; Jang *et al*., 2004). Briefly, gut loops were created by appropriate ligature of small pieces of gut intestine, 2–3 cm long with one or more Peyer's patch. They were filled with PBS containing purified EGFP-CL3 or EGFP-C and incubated for 1 h. After washing the specimen five times with PBS, bound protein was examined with UV illumination or with a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

Mucosal immunization BALB/c mice (Charles River Technologies, Orient Inc., Korea) were maintained by feeding sterile food and water *ad libitum*. Groups of five mice between 5 and 12 weeks of age were immunized by oral administration. Briefly, the mice were deprived of food for 2 h prior to oral immunization, and 30 min before antigen administration each mouse was gavaged to reduce stomach acidity with 0.5 ml of neutralization buffer (8 parts Hanks' balanced salt solution and 2 parts 7.5% sodium bicarbonate). Then 100 μg of EGFP-CL3 or EGFP-C in phosphate-buffered saline was orally administrated on day 0, 7, and 14.

Serum and feces collection Serum samples from the immunized mice were collected and analyzed to monitor the presence of anti-EGFP IgG. Fecal extracts were also prepared as described (Hino *et al.*, 2005; Jang *et al*., 2004) and used to detect anti-EGFP IgA. Briefly, 0.1 g of fecal pellet was mixed with 1 ml of PBS containing NaN_3 and vortexed for 5–10 min. After centrifugation, supernatants were collected and stored at −70°C.

ELISAs for antigen-specific fecal IgA or serum IgG from immunized mice Levels of antibodies in serum and fecal extracts were titrated by ELISA as described previously (Okahashi *et al*., 1996; Shin *et al*., 2005). Briefly, ELISA plates were coated with 100 ng of recombinant EGFP and blocked with nonfat dry milk. After washing, serial twofold dilutions of the serum or fecal samples were added to individual wells. After incubation, HRP-conjugated goat anti-mouse γ or α heavy chain-specific antisera (Southern Biotechnology Associates, Inc., USA) were added and developed to measure titers of IgG or IgA. Data are presented as the reciprocal $\log_2$ titers from titrations in which samples were first diluted 15-fold and subsequently by successive 2-fold dilutions. The minimum value for a positive result was set at 0.1 because the value without serum was below 0.1. The reciprocal $\log_2$ titers are given as $-\log_2$ (dilution). The same method was used to titer IgG isotypes in immunized serum and cytokines secreted from immunized splenocytes as a result

of activation by the protein antigen.

Isotyping of serum IgG from immunized mice In order to determine IgG subclass titers, 100 μl of biotin-conjugated rat monoclonal anti-mouse γ1 (G1-7.3, 2 μg/ml), γ2a (R19-15; 1 μg/ml), γ2b (R12-3; 0.5 μg/ml), or γ3 (R40-82; 1 μg/ml) heavy chain-specific antibodies were used as described previously (Okahashi *et al*., 1996). After incubation and washing, 100 μl of HRP-conjugated streptavidin (Life Technologies, Gaithersburg, MD, USA) was added and developed with 3,3′,5,5′-tetramethylbenzidin (TMB) (MOSS Inc., USA).

Cytokine analysis of activated T cells from immunized mice Splenocytes were isolated from the spleens of immunized mice by passing the tissue through a mesh screen as described previously (Kim *et al*., 2004; Yamamoto *et al*., 2000). After stimulation of the splenocytes with EGFP, the levels of diverse cytokines in the culture supernatant were measured by cytokine-specific ELISAs as described previously. Briefly, Maxisorp immunoplates (NUNC, Napeville, USA) were coated with monoclonal anti-IFN-γ, and anti-IL-4 antibodies (BD Biosciences Pharmingen, USA). After blocking, samples and serial dilutions of standards were added to duplicate wells and incubated overnight for 4°C. The wells were washed and further incubated with biotinylated anti IFN-γ and anti-IL-4 antibodies (BD Biosciences Pharmingen). After washing, peroxidase-labeled anti-biotin antibody (Vector Laboratories, USA) was added and developed with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)-containing H_2O_2.

Proliferation and cytokine assays About 10 d after the last feed, spleens were removed and splenocytes were prepared under sterile conditions (Son *et al*., 2004). After lysis of erythrocytes, the cells were washed and plated at 5×10^5 cells per well. They were then further stimulated with 100 μg/well EGFP or medium alone for 4 d at 37°C. Culture supernatants were collected for cytokine analysis and the cells were pulsed with 0.5 μCi of [^{3}H]-TdR (Amersham Life Science, Buckinghamshire, UK) per well for 18 h to measure the extent of proliferation. The cells were then harvested with a 96-well cell harvester (Inotech, Switzerland) and tritium contents were measured with a liquid scintillation counter (Packard Instrument Co., USA).

Statistical analyses The results are expressed as means ± standard errors of the means (SEM) using SPSS ver. 12.0 software and at least three independent experiments were performed unless otherwise stated. A value of $P < 0.05$ by Student's *t*-test was considered significant.

Results

Selection of GM1-specific peptides using a phage library In order to select high-affinity ligands for GM1 ganglioside, three rounds of panning were performed using a phage library containing 15 billion dodecapeptides (Wu *et al*., 1998). Out of 40 ligands sequenced after the final panning, three ligands were chosen for further experiments based on the high frequencies with which they were selected by panning (Table 1). Each of these ligands was then compared with the amino acid sequence of CTB, revealing that two of them, CL1 and CL3, had limited homology to CTB. Interestingly the CL3 motif, GWK-ERLSSWNRF, partially overlapped with the GM1 binding region of CTB (Merritt *et al*., 1994; Verlinde *et al*., 1994). These three peptides were further tested for activity in mucosal immunization.

Table 1. GM1 ganglioside-targeting peptide motifs.

Peptide	Sequence	Repeat
CL1	A F H K N S Q T T T L Y	4
CL2	T N C L Q N C S G V H L	4
CL3	G W K E R L S S W N R F	4
C	K M N T R T I L Q T I S	

Association of a fusion protein between EGFP and a GM1-specific peptide with the mucosal surface of the intestine To test whether the selected ligands enhance the immune responses of mice after feeding foreign proteins fused with them, we generated constructs containing fusions of the peptides with the C-terminus of EGFP. Only one fusion protein, EGFP-CL3 was well-expressed in the bacterial expression system, while the other two fusion proteins strongly reduced bacterial growth. We also generated a control protein named EGFP-C, containing a nonspecific peptide whose amino acid sequence is shown in Table 1. The fusion proteins had N-terminal His-tags and were purified near to homogeneity by a Ni-NTA affinity column as shown in Fig. 1.

The purified recombinant proteins were incubated in the mouse gut *ex vivo* to check their binding affinity for gut epithelium or M cells in Peyer's patches, a main portal for foreign materials in the mouse small intestine (Neutra *et al*., 1996). UV illumination revealed that EGFP-CL3 was well retained after extensive washing but EGFP-C was not (Fig. 2).

Enhancement of antigen-specific mucosal immunity using the GM1-specific peptide We also tested the role of the selected ligand in mucosal immune induction in the mouse model. After feeding recombinant EGFP-CL3, the levels of antigen-specific IgA monitored by ELISA showed that EGFP-specific IgA was more efficiently induced by EGFP-CL3 than by EGFP-C (Fig. 3). For example, in the fifth week after feeding, EGFP-specific IgA production by EGFP-CL3 was more than 2-fold higher than by EGFP-C.

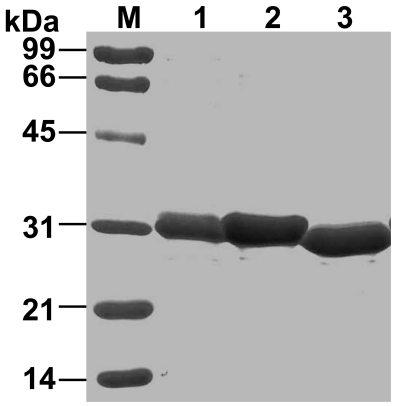

Fig. 1. Purification of recombinant proteins. His-tagged recombinant EGFP-CL3 (lane 1), EGFP-C (lane 2), and EGFP (lane 3) were expressed in *E. coli* and purified per the manufacturer's protocol. Recombinant proteins were separated by SDS-PAGE followed by Coomassie staining. Lane M is a molecular weight size marker.

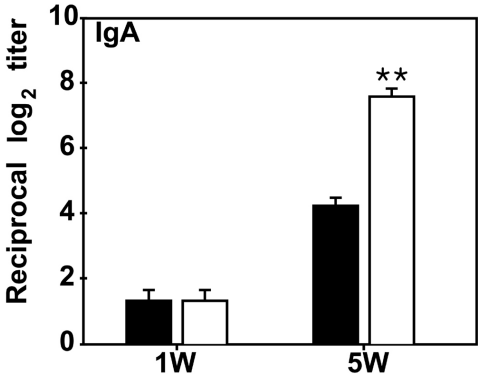

Fig. 3. Induction of mucosal immunity in mice fed EGFP-CL3. ELISAs with fecal IgA revealed that after 5 weeks more EGFP-specific IgA was produced by mice fed EGFP-CL3 (white bar) than those fed EGFP-C (black bar). Data are given as reciprocal $\log_2$ titers and represent the means ± SEM of five mice per group. They are typical of three independent experiments. Stars denote significant differences; ** $P < 0.01$.

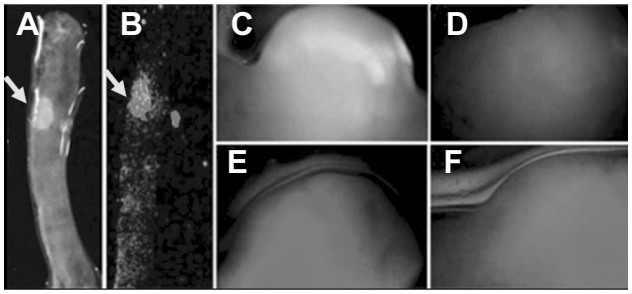

Fig. 2. Binding of EGFP fusion proteins around mouse Peyer's patch. EGFP fused with a control peptide (EGFP-C) or the GM1-specific peptide (EGFP-CL3) was incubated in mouse gut *ex vivo*. After several washes, fusion proteins were revealed by UV illumination (B). EGFP-CL3 was concentrated around mouse Peyer's patch (A and B). Enlarged images around Peyer's patch show that green fluorescence was much stronger with EGFP-CL3 (E and F) than with EGFP-C (C and D). Arrow indicates Peyer's patch.

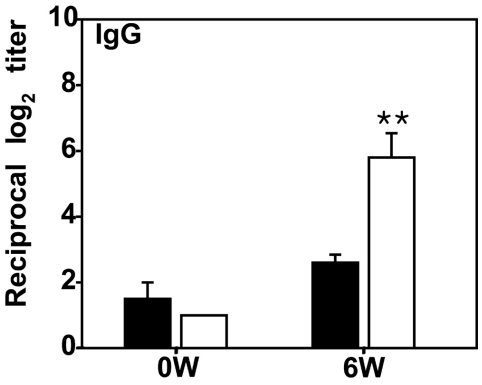

Fig. 4. Induction of systemic immunity in mice fed EGFP-CL3. ELISAs with serum IgG showed that after 5 weeks EGFP-specific IgG was higher in mice fed EGFP-CL3 (white bar) than in those fed EGFP-C control protein (black bar). Data are given as reciprocal $\log_2$ titers and represent the means ± SEM of five mice per group. They are typical of three independent experiments. Stars denote significant differences; ** $P < 0.01$.

Enhancement of antigen-specific systemic immunity using the GM-1 specific peptide After determining that feeding of the CL3-fused EGFP induced mucosal IgA, we tested whether systemic immunity could also be enhanced by oral administration of EGFP-CL3. Indeed, feeding with EGFP-CL3 generated about 1.5-fold higher EGFP-specific antibody titers than feeding with EGFP-C (Fig. 4). Levels of EGFP-specific IgG1 and IgG2a, in particular, were high 6 weeks after EGFP-CL3 administration, while only a slight increase in IgG1 and IgG2a concentrations were detected in the serum from mice fed the EGFP-C control protein (Fig. 5).

IL-4 secretion by splenocytes of mice orally immunized with EGFP-CL3 Next, we tested which T helper cells were involved in EGFP-CL3 immunization. As shown in Fig. 6, EGFP-CL3 induced both IFN-γ and IL-4 secretion from spleen cells but the increase in cytokine secretion relative to that observed with EGFP-C was higher for IL-4. Since IgG1 production and IL-4 secretion are indicators of Th2 type immunization (Carter and Dutton, 1996; Parronchi *et al*., 1992; Spellberg and Edwards, 2001), this result indicates that a Th2 type response plays a major role in EGFP-CL3 immunization.

Induction of splenocyte proliferation by EGFP-CL3 In order to test whether spleen cells from immunized mice

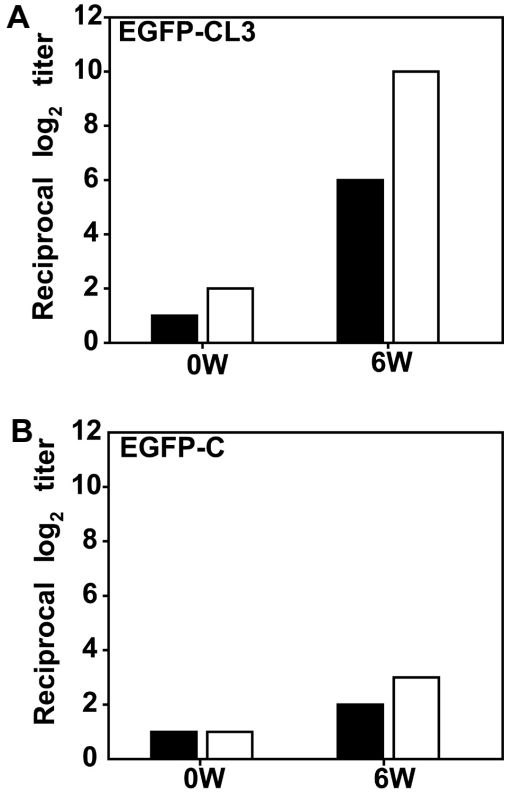

Fig. 5. IgG1 and IgG2a in mouse sera after oral administration of recombinant proteins. six weeks after feeding with EGFP-C (A) or EGFP-CL3 (B), serum IgG subtypes were measured by sandwich ELISAs for IgG1 (white bar) or IgG2a (black bar). More of both isotypes were generated in mice fed EGFP-CL3 than in those fed EGFP-C. Assays were done with the combined sera of five mice, and data are reciprocal $\log_2$ titers.

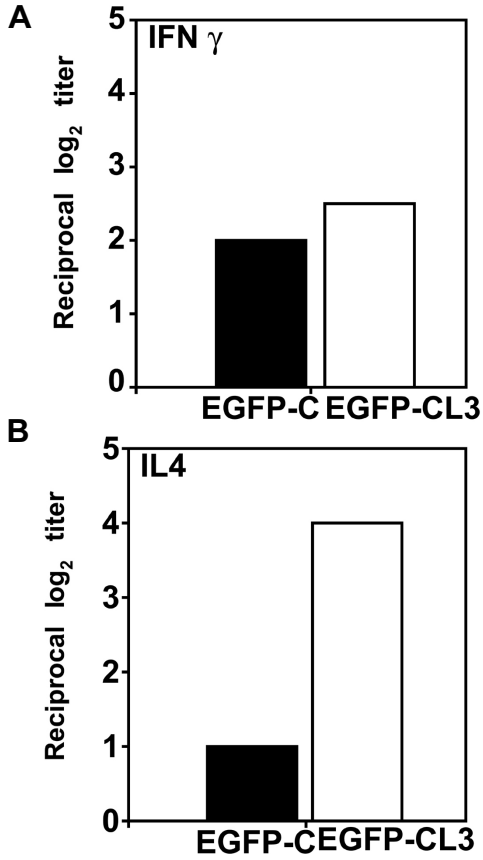

Fig. 6. Analysis of cytokines secreted from splenocytes of immunized mice after restimulation by antigen. Spleen cells were isolated from mice immunized with EGFP-C (white bar) or EGFP-CL3 (closed bar) after 6 weeks feeding. They were restimulated by EGFP-C or EGFP-CL3 and secreted cytokines were measured by sandwich ELISAs for IFNγ (A) or IL-4 (B). Assays were done with the combined sera of five mice and data are reciprocal $\log_2$ titers. Similar results were obtained in two independent experiments and a representative figure is shown.

could be activated by the EGFP fusion protein, splenocytes were isolated 10 d after the last feed and restimulated with the same antigen. Although some cell proliferation was detected in mice administered EGFP-C, stimulation was much greater in mice administered EGFP-CL3 (Fig. 7).

Discussion

A vital need for the development of effective vaccines is to enhance the low immunogenic of antigens on their own. Newton *et al*. (1989) reported that an antigen epitope was active when inserted into *Salmonella* flagellin and induced epitope-specific immune responses. However this procedure could not be generalized, especially for large antigens, because of interference with flagellin expression. Recently, flagellin-EGFP protein was tested for ability to induce specific immune responses (Cuadros *et al*., 2004). This new approach can accommodate larger epitopes, or even multiple epitopes, and avoids the unknown risks of live vaccination. In the present study we also aimed to develop a mucosal carrier of small size which would be useful for various sizes of epitopes. Thus, we identified a dodecapeptide among fifteen billion peptides with affinity for GM1 ganglioside and tested whether it enhanced epitope-specific immune responses using recombinant peptide-EGFP protein.

Among the three most often selected peptides obtained after three rounds of panning against GM1 ganglioside, only one was capable of yielding a fusion protein with EGFP in *E. coli*. We are not sure why the other two motifs affected bacterial growth, but we assume that they have high affinity for a hydrophobic region of GM1 ganglioside and may interfere with cytoplasmic membrane function. The motif of CL3, GWKERLSSWNR, has limited but significant homology to the GM1 binding motif of CTB protein (Verlinde *et al*., 1994; Merritt *et al*., 1994). The sequence ERLssWN of CL3 is very similar to the EKLcvWN

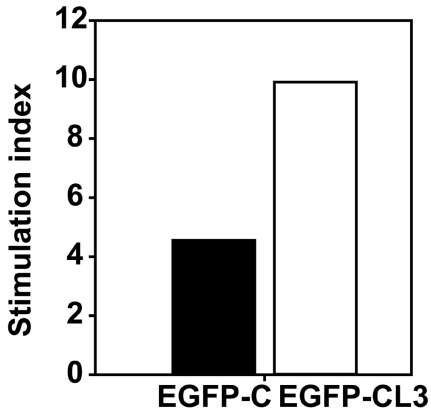

Fig. 7. Induction of splenocyte proliferation by antigen. After a last feed, splenocytes were isolated and restimulated with EGFP-C or EGFP-CL3, and cell proliferation was measured by [^{3}H]-thymidine uptake. The stimulation index was calculated as the [^{3}H]-thymidine incorporation by cells receiving antigen divided by the [^{3}H]-thymidine incorporation of unstimulated cells. The black bar indicates the stimulation index for EGFP-C, and the white bar that for EGFP-CL3. Assays were done with the combined splenocytes of five mice. Similar results were obtained in two independent experiments and a representative figure is shown.

sequence of residues 90 to 96 of CTB near its GM1 binding domain. Interestingly, it also resembles the sequences of some viral proteins, for example WnEtRLSnWN (aa 398-407) of human immunodeficiency virus (HIV) gp160, and GWsERLaSWrR (aa 134-144) of hepatitis C virus (HCV) E2 (Daniels *et al*., 2003; Wu *et al*., 2001). This suggests possible roles of the CL3 motif. Thus, HIV gp160 is known to induce strong mucosal and systemic immune responses and has been proposed as a mucosal adjuvant (Sakaue *et al*., 2003). Also, HCV E2 is a component of the viral envelope protein complex consisting of E1 and E2, a ligand for the cellular receptor CD31.

Although we showed that EGFP-CL3 was retained around Peyer's patch after incubation in the mouse intestine (Fig. 2), we cannot exclude the possibility that the induction of immunity was caused by an effect on cells outside Peyer's patch. This could involve the villous M cell (Jang *et al*., 2004), a recently identified immune induction site, a possibility strengthened by reports that Peyer's patch is more relevant to immune tolerance (Fujihashi *et al*., 2001; Kato *et al*., 2003).

Although the antigen-specific immune responses induced by EGFP-CL3 were much higher than those induced by the EGFP-C control, continuous feeding of EGFP-C also seemed to induce an EGFP-specific immune response (Figs. 3–5). We assume that even the control ligand, which was selected at random from the primers, can enhance the delivery of fused EGFP protein to mucosal immune induction sites, since we did not detect immune induction by feeding EGFP alone (data not shown). Currently, we are preparing several dodecapeptide-conjugated EGFPs to clarify the effects of control peptides.

In conclusion, we have isolated a peptide motif from a 15 billion peptide library and generated a fusion protein between it and EGFP. EGFP with this motif was more efficient than a fusion of EGFP with a control peptide in inducing mucosal and systemic immune responses. Based on cytokine secretion, IgG subtype, and splenocyte proliferation, we believe that the peptide induces Th2-dependent immune responses. We are currently testing the efficacy of this ligand in inducing mucosal and systemic immune responses with antigens isolated from pathogenic bacteria and viruses.

Acknowledgments This work was supported by a Korean Research Foundation Grant (KRF-2002-070-C00069) and, in part, by a grant from the Plant Diversity Research Center of the 21st Century Frontier Research Program (PF0330301-00) funded by the Ministry of Science and Technology of Korea.

References

Bae, J. L., Lee, J. G., Kang, T. J., Jang, H. S., Jang, Y. S., *et al*. (2003) Induction of antigen-specific systemic and mucosal immune responses by feeding animals transgenic plants expressing the antigen. *Vaccine* **21**, 4052–4058.

Bergelson, L. D., Bukrinskaya, A. G., Prokazova, N. V., Shaposhnikova, G. I., Kocharov, S. L., *et al*. (1982) Role of gangliosides in reception of influenza virus. *Eur. J. Biochem.* **128**, 467–474.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Carter, L. L. and Dutton, R. W. (1996) Type 1 and type 2: a fundamental dichotomy for all T cell subsets. *Curr. Opin. Immunol.* **8**, 336–342.

Cuadros, C., Lopez-Hernandez, F. J., Dominguez, A. L., McClelland, M., and Lustgarten, J. (2004) Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. *Infect. Immun.* **72**, 2810–2816.

Daniels, R. S., Kang, C., Patel, D., Xiang, Z., Douglas, N. W., *et al*. (2003) An HIV type 1 subtype B founder effect in Korea: gp160 signature patterns infer circulation of CTL-escape strains at the population level. *AIDS Res. Hum. Retroviruses* **19**, 631–641.

de, Haan, L., Verweij, W. R., Feil, I. K., Holtrop, M., Hol, W. G., *et al*. (1998) Role of GM1 binding in the mucosal immunogenicity and adjuvant activity of the Escherichia coli heat-labile enterotoxin and its B subunit. *Immunology* **94**, 424–430.

Fishman, P. H. (1982) Role of membrane gangliosides in the binding and action of bacterial toxins. *J. Membr. Biol.* **69**, 85–97.

Foster, N., Clark, M. A., Jepson, M. A., and Hirst, B. H. (1998)

Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells *in vivo*. *Vaccine* **16**, 536–541.

Fujihashi, K., Dohi, T., Rennert, P. D., Yamamoto, M., Koga, T., *et al*. (2001) Peyer's patches are required for oral tolerance to proteins. *Proc. Natl. Acad. Sci. USA* **98**, 3310–3315.

Fujihashi, K., Koga, T., van Ginkel, F. E., Hagiwara, Y., and McGhee, J. R. (2002) A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. *Vaccine* **20**, 2431–2438.

Gardby, E., Wrammert, J., Schon, K., Ekman, L., Leanderson, T., *et al*. (2003) Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant. *J. Immunol.* **170**, 55–63.

Hino, A., Fukuyama, S., Kataoka, K., Kweon, M.-N., Fujihashi, K., *et al*. (2005) Nasal IL-12p70 DNA prevents and treats intestinal allergic diarrhea. *J. Immunol.* **174**, 7423–7432.

Jang, M. H., Kweon, M. N., Iwatani, K., Yamamoto, M., Terahara, K., *et al*. (2004) Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc. Natl. Acad. Sci. USA* **101**, 6110–6115.

Kang, T. J., Han, S. C., Jang, M. O., Kang, K. H., Jang, Y. S., *et al*. (2004) Enhanced expression of B-subunit of *Escherichia coli* heat-labile enterotoxin in tobacco by optimization of coding sequence. *Appl. Biochem. Biotechnol.* **117**, 175–187.

Kato, H., Fujihashi, K., Kato, R., Dohi, T., Fujihashi, K., *et al*. (2003) Lack of oral tolerance in aging is due to sequential loss of Peyer's patch cell interactions. *Int. Immunol.* **15**, 145–158.

Kawamura, Y. I., Kawashima, R., Shirai, Y., Kato, R., Hamabata, T., *et al*. (2003) Cholera toxin activates dendritic cells through dependence on GM1-ganglioside which is mediated by NF-kappaB translocation. *Eur. J. Immunol.* **33**, 3205–3212.

Kim, A., Noh, Y. W., Kim, K. D., Jang, Y. S., Choe, Y. K., *et al*. (2004) Activated natural killer cell-mediated immunity is required for the inhibition of tumor metastasis by dendritic cell vaccination. *Exp. Mol. Med.* **36**, 428–443.

Kurganov, B., Doh, M., and Arispe, N. (2004) Aggregation of liposomes induced by the toxic peptides Alzheimer's Abetas, human amylin and prion (106-126): facilitation by membrane-bound GM1 ganglioside. *Peptides* **25**, 217–232.

Markwell, M. A., Svennerholm, L., and Paulson, J. C. (1981) Specific gangliosides function as host cell receptors for Sendai virus. *Proc. Natl. Acad. Sci. USA* **78**, 5406–5410.

Matsubara, T., Ishikawa, D., Taki, T., Okahata, Y., and Sato, T. (1999) Selection of ganglioside GM1-binding peptides by using a phage library. *FEBS Lett.* **456**, 253–256.

Mattei, V., Garofalo, T., Misasi, R., Circella, A., Manganelli, V., *et al*. (2004) Prion protein is a component of the multimolecular signaling complex involved in T cell activation. *FEBS Lett.* **560**, 14–18.

Merritt, E. A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J. A., *et al.* (1994) Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci.* **3**, 166–175.

Miura, Y., Sasao, Y., Kamihira, M., Sakaki, A., Iijima, S., *et al*. (2004) Peptides binding to a Gb3 mimic selected from a phage library. *Biochim. Biophys. Acta* **1673**, 131–138.

Neutra, M. R., Frey, A., and Kraehenbuhl, J. P. (1996) Epithelial M cells: gateways for mucosal infection and immunization. *Cell* **86**, 345–348.

Newton, S. M., Jacob, C. O., and Stocker, B. A. (1989) Immune response to cholera toxin epitope inserted in *Salmonella flagellin*. *Science* **244**, 70–72.

Ogushi, K., Wada, A., Niidome, T., Okuda, T., Llanes, R., *et al*. (2004) Gangliosides act as co-receptors for Salmonella enteritidis FliC and promote FliC induction of human beta-defensin-2 expression in Caco-2 cells. *J. Biol. Chem.* **279**, 12213–12219.

Okahashi, N., Yamamoto, M., Vancott, J. L., Chatfield, S. N., Roberts, M., *et al*. (1996) Oral immunization of interleukin-4 (IL-4) knockout mice with a recombinant Salmonella strain or cholera toxin reveals that CD4+ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses. *Infect. Immun.* **64**, 1516–1525.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Sampognaro, S., *et al*. (1992) IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cutolytic potential by Th1 or Th2 human T cell clones. *J. Immunol.* **149**, 2977–2983.

Sakaue, G., Hiroi, T., Nakagawa, Y., Someya, K., Iwatani, K., *et al*. (2003) HIV mucosal vaccine: nasal immunization with gp160-encapsulated hemagglutinating virus of Japan-liposome induces antigen-specific CTLs and neutralizing antibody responses. *J. Immunol*. **170**, 495–502.

Shin, S. J., Bae, J. L., Cho, Y. W., Lee, D. Y., Kim, D. H., *et al*. (2005) Induction of antigen-specific immune responses by oral vaccination with *Saccharomyces cerevisiae* expressing Actinobacillus pleuropneumoniae ApxIIA. *FEMS Immunol. Med. Microbiol.* **43**, 155–164.

Son, Y. O., Lee, K. Y., Choi, K. C., Chung, Y., Kim, J. G., *et al*. (2004) Inhibitory effects of glycoprotein-120 (G-120) from Ulmus davidiana Nakai on cell growth and activation of matrix metalloproteinases. *Mol. Cells* **18**, 163–170.

Spellberg, B. and Edwards, J. E. (2001) Type 1/type 2 immunity in infectious diseases. *Clin. Infect. Dis.* **32**, 76–102.

Valdes-Gonzalez, T., Inagawa, J., and Ido, T. (2001) Neuropeptides interact with glycolipid receptors: a surface plasmon resonance study. *Peptides* **22**, 1099–1106.

van Ginkel, F. W., Jackson, R. J., Yuki, Y., and McGhee, J. R. (2000) Cutting edge: The mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J. Immunol.* **165**, 4778–4782.

Verlinde, C. L., Merritt, E. A., Van den Akker, F., Kim, H., Feil, I., *et al.* (1994) Protein crystallography and infectious disease. *Protein Sci*. **3**, 1670–1686.

Wu, H., Goud, G. N., and Sierks, M. R. (1998) Artificial antibodies for affinity chromatography of homologous proteins: application to blood clotting proteins. *Biotechnol. Prog.* **14**, 496–499.

Wu, C., Zeng, Z., and Wang, Q. (2001) Experimental study of inhibition of hepatitis B by dual-target antisense RNA. *Zhonghua Yi Xue Za Zhi.* **81**, 605–608.

Yamamoto, M., Rennert, P., McGhee, J. R., Kweon, M. N., Yamamoto, S., *et al*. (2000) Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. *J. Immunol.* **164**, 5184–5191.