Engineering of cholera toxin A-subunit for carriage of epitopes at its amino end

J. Sanchez*, R. Argotte, A. Buelna

Centro de Investigación sobre Enfermedades Infecciosas, Instituto Nacional de Salud Publica, Av Universidad 655, Col. Sta. Maria Ahuacatitlan, Cuernavaca, Morelos 62508, Mexico

Received 31 October 1996

Abstract The cholera toxin A-subunit (CTA) was genetically engineered at its amino end and tested for carriage of epitopes by fusion of the STa heat-stable enterotoxin analogue CAELCCNPAC. Efficient holotoxin formation by complementation in trans with cholera toxin B-subunit (CTB) indicated no decrease in affinity for CTB but evidence of reduced toxicity suggests steric interference by the decapeptide with the active site. The holotoxin was stable, able to bind to GM1 and was recognized by anti-STa and anti-CTA antibodies. The use of a full-length CTA might have been a key step for successful genetic fusions. Based on these findings, it seems worthwhile pursue the development of CTA for construction of recombinant mucosal immunoadjuvants.

Key words: Fusion protein; Heat-stable enterotoxin, *E. coli*; Mucosal immunoadjuvant; Toxin assembly; Toxin active site

1. Introduction

Vibrio cholerae, the causative agent of cholera, induces intestinal fluid secretion by releasing several active virulence factors including the powerful cholera toxin (CT) [1]. This protein is a prototype enterotoxin made up of five copies of a B-subunit (CTB), required for binding to GM1 ganglioside [2] in the intestinal cell and one copy of an A-subunit (CTA) [3] which catalyzes the ADP-ribosylation of the Gs component of adenylate cyclase in the target cell [4]. In the CT holotoxin the homopentameric CTB holds CTA in its center by non-covalent interactions [3]. CT has been crystallized and its three-dimensional model has been worked out [5]. The model for CT closely resembles that of its structural and functional homologue, the heat-labile enterotoxin (LT) of *Escherichia coli* [6]. The A-subunit of CT is synthesized as a single polypeptide chain post-translationally cleaved at or near residue 192 to give fragments designated CTA1 (1–192) and CTA2 (192–240) [7]. Fragment CTA1 is the enzymatically active portion of the A-subunit while CTA2 mediates association between CTA and pentameric CTB [8]. By mechanisms that have yet to be clarified, CT is a potent immunoadjuvant which stimulates mucosal immune responses against co-administered or coupled antigens [9,10]. Although CTB boosts immune reactions it seems that much of the immunoadjuvanticity of CT is due to CTA [10–13]. This work explored whether CTA could be made amenable to genetic fusion of peptides to eventually harness its natural immunoadjuvanticity. By genetically engineering the amino end of CTA, carriage of an enterotoxin antigen [14] was accomplished. The results presented here may provide an approach to the development of CTA for the production of recombinant mucosal immunoadjuvants.

2. Materials and methods

2.1. Bacterial strains and cultures

Competent DH5α or electrocompetent DH10B *E. coli* were purchased commercially (Gibco BRL). When required, strains were made competent and transformed via CaCl/RbCl procedures [15]. *E. coli* S17-1 [16] was used to conjugate plasmids derived from pJS162 [17]. All strains were kept in 20% glycerol at −70°C and thawed the day before use. Liquid LB [15] cultures were carried out overnight at 37°C with continuous orbital shaking. Recombinant clones were selected on ampicillin at 100 μg/ml and/or chloramphenicol at 25 μg/ml. When required X-gal (Boehringer-Mannheim) at 125 μg/ml was included in the plates. For expression by *tac*P, IPTG at 100 μg/ml was added to cultures.

2.2. DNA manipulations

Restriction and ligation enzymes and Taq Polymerase were purchased from Gibco-BRL, New England Biolabs, Promega, Boehringer-Mannheim or Perkin Elmer and used according to the manufacturers' instructions. Plasmid was isolated using a kit (Wizard miniprep, Promega) or by CsCl/EtBr gradients [15]. Plasmid electroporation (Cell-Porator, Gibco BRL) was performed following the manufacturer's instructions. Amplifications were in a PTC-1 cycler (Gibco-BRL). Synthetic primers were obtained from Genosys, Inc. Plasmid pVCD15 [18] used to amplify the CTA gene was generously provided by Dr. Jim Kaper (CVD, University of Maryland). The 5′ end primer, 5′GTCGAAGAATTCACGGAGCTCCCGGGCAGATTCCAGACCTCCTGATGAAATAAAG3′, eliminated the *Xba*I site in the CTA gene [18] and incorporated *Eco*RI, *Sac*I and *Xma*I sites. The 3′ end primer, 5′GTAAAAAAAACACCAAAGCTTAATTTAATCATAATTCATCC3′, added a *Hin*dIII site after the stop codon (TGA). The amplicon was digested with *Eco*RI and *Hin*dIII and cloned into pUC19. Oligonucleotides to restore the first 6 amino acids in CTA were joined at the *Sac*I and *Xma*I sites for subcloning and subsequent insertion into *Sac*I-*Hin*dIII cut pJS162 [17] to give plasmid pRA003. Plasmid pJS384, for complementation by CTB, was created by insertion of a pJS752-3 [19] *Bam*HI-*Hin*dIII fragment, carrying the CTB gene and *tac*P, into plasmid pACYC184 [20]. Fusion of decapeptide to CTA was carried out by ligation of synthetic oligonucleotides (Fig. 1B) to *Sac*I restricted pRA003 to obtain plasmid pRA003-STd. The latter was introduced into *E. coli* S17-1 and conjugally transferred to *E. coli* DH5α carrying pJS384.

2.3. Immunological techniques

Culture supernatants of *E. coli* carrying pJS384 plus pRA003-STd were 20-fold concentrated by ultrafiltration on YM-10 Amicon membranes (Amicon Inc.). 50-μl aliquots or 5 μg of purified CTA (Calbiochem) were boiled and run on SDS-PAGE [14]. Proteins were electrotransferred (Bio-Rad, MiniProtean System) to nitrocellulose membranes (Bio-Rad) for incubation with anti-CTA [21] or anti-STa [22] monoclonal antibodies followed by goat anti-mouse IgG-peroxidase conjugate (Gibco-BRL) and development with α-chloronaphthol substrate. GM1 ELISA [14] was used to assay recombinant CT or holotoxin with the decapeptide-CTA.

2.4. Ileal loop experiments

E. coli culture supernatants containing the decapeptide-CTA holotoxin were investigated by using the ileal loop assay [23]. 4–5 cm ileal

*Corresponding author. Fax: (52) (73) 11 24 69.
E-mail: jsanchez@insp3.insp.mx

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(96)01445-7

loops were prepared in two 2–3 kg adult male albino rabbits. Supernatants were injected in 1 ml volumes. After 16 h the fluid inside the loops was recovered and measured. The ratio of the collected volume (ml) over the loop length (cm) was used to assess toxicity. Cholera toxin (List Biologicals) at 0.1 μg/ml in PBS was used as a positive control.

3. Results and discussion

3.1. Engineering of CTA

Amplification of the CTA gene and cloning under *tac*P in plasmid pJS162 [17] joined full-length CTA to LTB leader peptide and incorporated a *Sac*I site at the junction (Fig. 1A) for genetic fusions. The cloned CTA assembled into holotoxin (not shown) when complemented by CTB encoded by plasmid PJS384. In contrast, a holotoxin where CTA had its first 6 amino acids replaced by LQSSRA was not obtained despite numerous genetic engineering strategies. The presence of free CTA antigen in whole cell sonicates shown by immunoblot assays (not shown) indicated failure to associate with CTB as seen for mutations in residue 114 in CTA [24]. Since none of the two regions interacts with CTA2 in the final toxin [5], one can postulate that replacements have an effect on either periplasmic holotoxin assembly [25,26] or periplasmic translocation as observed after deletion of CTA residues 6–13 [27].

3.2. Genetic fusion of decapeptide to CTA

Fusions to CTA are stimulated by the potential to develop mucosal antigens where its immunoadjuvanticity would be harnessed [28]. The peptide CAELCCNPAC is a non-toxic analogue of the sequence CCELCCNPAC found within the 13 amino acid toxic moiety of STa heat-stable enterotoxins [29]. This decapeptide has turned out to be immunogenic after its genetic fusion to CTB [19] and was thus deemed to be a suitable candidate for testing peptide carriage by CTA. Since substitution of residues 1–6 in CTA hampered holotoxin recovery, fusion was performed on the full-length CTA gene described (Section 3.1). After fusion CTA had an amino end

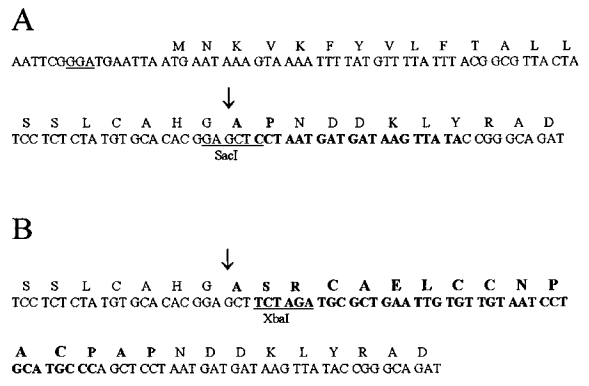


Fig. 1. (A) Sequence of junction in cloned CTA after fusion to LTB leader peptide. Single-letter coded amino acids are shown above the DNA sequence. The arrow shows the native leader peptide cleavage site (14). Amino acids encoded by the *Sac*I site (underlined) are in bold-face type. Synthetic oligonucleotides added to restitute amino acids in CTA are in bold. (B) Sequence of junction after fusion of decapeptide to CTA. The decapeptide is shown in bold type and larger font. Flanking amino acids which do not form part of CTA or decapeptide are in bold type and smaller font. The introduced *Xba*I site is underlined as is the proposed S/D. Synthetic oligonucleotides are distinguished in bold from the cloned CTA sequence.

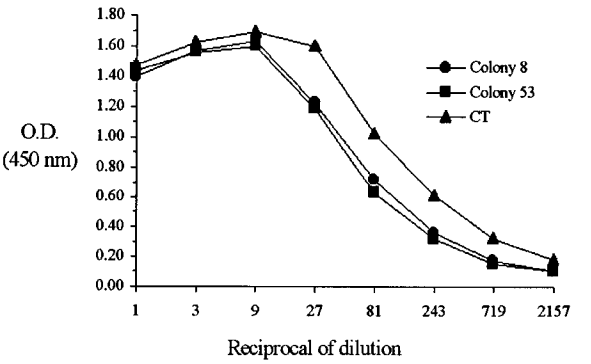


Fig. 2. Titration of CTA antigen in decapeptide-CTA holotoxin by GM1 ELISA [22]. Curves were constructed plotting optical density values (ordinate axis) obtained in a microplate reader against the reciprocal of each 3-fold serial dilution (abscissa). Two independently isolated and cultured colonies expressing the decapeptide-CTA holotoxin (colonies 8 and 53) were tested. Cholera toxin at 10 μg/ml was used as a standard. The calculated concentration of decapeptide-CTA holotoxin using anti-CTA is 6–7 μg/ml of holotoxin per ml of culture.

tail of 16 amino acids which included the decapeptide (Fig. 1B). This protein was designated decapeptide-CTA. After coexpression with CTB holotoxin in *E. coli* supernatants ranged from 5 to 10 μg/ml (Fig. 2). Sonicated cell pellets had about 5 times more holotoxin (not shown) due to poor excretion of CT by *E. coli* [30]. The hybrid nature of decapeptide-CTA was demonstrated by development of a protein band of approx. 30 kDa in immunoblots with either anti-STa or anti-CTA (Fig. 3). This immunoreactive band was invariant in number upon reduction (not shown), indicating that the decapeptide-CTA was not cleaved at the CTA1-CTA2 junction as observed for CTA [31]. Moreover, this indicated no degradation of the

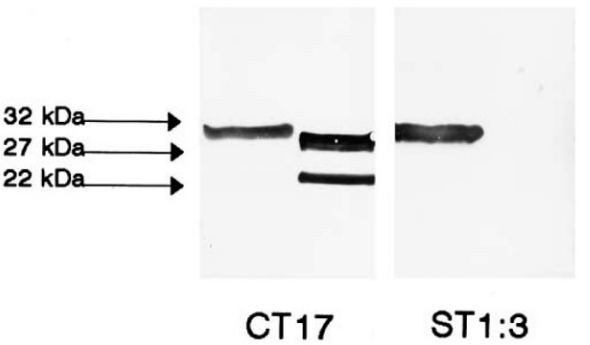


Fig. 3. Immunoblot of decapeptide-CTA in concentrated *E. coli* culture supernatants. A concentrated supernatant with decapeptide-CTA holotoxin was electrophoresed and proteins electroblotted. Although CTB was present, membranes were reacted only with anti-CTA or anti-STa to demonstrate the hybrid nature of the decapeptide-CTA. A strong protein band for pentameric CTB was obtained (not shown) with anti-CTB when non-boiled samples [17] were run. Arrows (kDa) indicate positions of reference proteins in a pre-stained molecular marker (Gibco-BRL). Identical aliquots were transferred to both membranes. Supernatant was loaded on the left and purified CTA (Calbiochem) on the right. The left membrane (CT17) was reacted with anti-CTA. The position of the left band, slightly larger than CTA on the right, is consistent with it being the decapeptide-CTA. The band below CTA is most probably residual CTA1 in the standard. The right membrane (ST1:3) was reacted with anti-STa. The single band developed comigrated with the protein developed by anti-CTA as expected for decapeptide-CTA. No reaction by CTA and CTA1 was observed here.

fusion protein perhaps due to protection by CTB [32]. Toxicity estimates in rabbit ileal loops using 0.5–1 μg/ml of decapeptide-CTA holotoxin gave negative 0.1 ml/cm ratios while 0.1 μg/ml of pure CT gave positive 0.69 and 0.78 ml/cm ratios. This indicates 5–10-fold lower toxicity. Reduction in toxicity because of an unsplit decapeptide-CTA chain (above) seems unlikely, since this model is not dependent upon prior toxin nicking [33]. Instead, reduced toxicity could be due to steric interference with the spatially close CTA catalytic residues [5]. Immunological assays will help to determine whether the immunoadjuvant capacity has been preserved as in detoxified LTA mutants [34].

In conclusion, engineering at the amino end of CTA lead to the successful carriage of a 16 amino acid heterologous peptide which included the enterotoxin analogue CAELCCN-PAC. Our results suggest that the use of a full-length protein may be a key step when trying to achieve peptide carriage by CTA. The CTA fusion protein was able to associate with CTB to form a stable holotoxin. Epitope addition leads to a CTA with reduced toxicity, suggesting that the added epitope interferes with the active site in CTA. Based on these findings, it would seem rewarding to proceed further with developing the cholera toxin A-subunit as a molecular carrier to induce mucosal immunity against bacterial and viral infections.

Acknowledgements: This work was supported by grant SPE-VACC-HN-01 (SAREC, Sweden). We extend our appreciation to Ann Mari Svennerholm for monoclonals used in this study. We are grateful to Jan Holmgren for the facilities to undertake part of this work and to Gudrun Wiklund for technical support in ileal loop experiments. J.S. gratefully acknowledges a stipend by the Sistema Nacional de Investigadores (SNI), Mexico. A.B. receives a scholarship for M.Sc. studies from CONACYT, Mexico.

References

[1] De, S.N. and Chatterjee. (1953) J. Pathol. Bacteriol. 66, 559–562.
[2] Van Heyningen, S. (1971) Science 183, 656–657.
[3] Gill, D.M. (1976) Biochemistry 15, 1242–1248.
[4] Gill, D.M. and Meren R. (1978) Proc. Natl. Acad. Sci. USA 75, 3050–3054.
[5] Zhang, R-G., Scott, D.L., Westbrook, M., L., Nance, S., Spangler, B., Shipley, G.G. and Westbrook, E.M. (1995) J. Mol. Biol. 251, 563–573.
[6] Sixma, T.K., Pronk, S.E., Kalk, K.H., Wartna, E.S., Van Zanten, B.A.M., Witholt, B. and Hol, W.G.J. (1991) Nature 351, 371–378.
[7] Gill, D.M. and Rappaport, R.S. (1979) J. Infect. Dis. 139, 674–680.
[8] Mekalanos, J.J., Collier, R.J. and Romig, W.R. (1979) J. Biol. Chem. 254, 5855–5861.
[9] Elson, C.O. (1989) Curr. Top. Microbiol. Immunol. 146, 29–33.
[10] Holmgren, J., Lycke, N. and Czerkinsky, C. (1993) Vaccine 11, 1179–1184.
[11] Holmgren, J., Czerkinsky, C., Lycke, N. and Svennerholm, A.-M. (1994) Am. J. Trop. Med. Hyg. 50 (Suppl. 5), 42–54.
[12] Tamura, S., Ishihara, K., Miyata, K., Aizawa, C. and Kurata, T. (1995) Vaccine 13, 339–341.
[13] Vadolas, J., Davies, J.K. Wright, P.J. and Strugnell, R.A. (1995) Eur. J. Immunol. 25, 969–975.
[14] Sanchez, J., Svennerholm, A.M. and Holmgren, J. (1988) FEBS Lett. 241, 110–114
[15] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[16] Simon, R., Priefer, U. and Pühler, A. (1983) Bio/Technology 1, 784–791.
[17] Sanchez, J. and Holmgren, J. (1989) Proc. Natl. Acad. Sci. USA 86, 481–485.
[18] Kaper, J.B., Lockman, H., Baldini, M.M. and Levine, M.M. (1984) Nature 308, 655–658.
[19] Sanchez, J., Johansson, S., Lowenadler, B., Svennerholm, A.M. and Holmgren J. (1990) Res. Microbiol. 141, 971–979
[20] Chang, A.C. and Cohen, S.N. (1978) J. Bacteriol. 134, 1141–56.
[21] Svennerholm, A.M., Wikström, M., Lindholm, L. and Holmgren, J. (1986) in: Monoclonal Antibodies Against Bacteria, vol III (Macario, A.J. and Conway de Macario, E. eds.) pp. 77–85, Academic Press, New York.
[22] Svennerholm, A.M., Wikstrom, M., Lindblad, M. and Holmgren, J. (1986) J. Clin. Microbiol. 24, 585–590.
[23] De, S.N., Ghose, M.L. and Sen, A. (1960) J. Pathol. Bacteriol. 79, 373–380
[24] Fontana, M.R., Manetti, R., Giannelli, V., Magagnoli, C., Marchini, A., Olivieri, R., Domenghini, M., Rappuoli, R. and Pizza, M. (1995) Infect. Immun. 63, 2356–2360.
[25] Okamoto, K., Takatori, R. and Okamoto, K. (1995) Microbiol. Immunol. 39, 193–200.
[26] Streatfield, S.J., Sandkvist, M., Sixma, T.K., Bagdasarian, M., Hol, W.G. and Hirst, T.R. (1992) Proc. Natl. Acad. Sci. USA 89, 12410–12144.
[27] Vadheim, K.L., Singh, Y. and Keith, J.M. (1994) Microb. Pathog. 17, 339–346.
[28] Lobet, Y., Cluff, C.W. and Cieplak, W. (1991) Infect. Immun. 59, 2870–2879.
[29] Takeda, T., Nair, B., Susuki, K., Zhe, H.Q., Yokoo, Y., De Mol, P., Hemelhof, W., Butzler, J.P., Takeda, Y. and Shimonishi, Y. (1993) Infect. Immun. 61, 289–294.
[30] Gennaro, M.L., Greenaway, P.J. and Broadbent, D.A. (1982) Nucleic Acids Res. 10, 4883–4890.
[31] Pearson, G.D.N. and Mekalanos, J.J (1982) Proc. Natl. Acad. Sci. USA 79, 2976–2980.
[32] Sanchez, J., Hirst, T.R. and Uhlin, B.E. (1988) Gene 64, 265–275.
[33] Rappaport, R.S., Sagin, J.F., Pierzchala, W.A. Bonde, G., Rubin, B.A. and Tint, H. (1976) J. Infect. Dis. 133, S41–S54.
[34] Di Tommaso, Saletti, G., Pizza, M., Rappuoli, R., Dougan, G., Abrignani, S., Douce, G. and De Magistris, T. (1996) Infect. Immun. 64, 974–979.