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NICKING SITES IN A SUBUNIT OF CHOLERA TOXIN AND *ESCHERICHIA COLI* HEAT-LABILE ENTEROTOXIN FOR *VIBRIO CHOLERAE* HEMAGGLUTININ/PROTEASE

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A. Naka, T. Iida, T. Ohara, K. Yamamoto, T. Miwatani and T. Honda. Nicking sites in a subunit of cholera toxin and *Escherichia coli* heat-labile enterotoxin for *Vibrio cholerae* hemagglutinin/protease. *Toxicon* **36,** 1001–1005, 1998.—We analyzed the nicking site of the A subunit of *Escherichia coli* heat-labile enterotoxin for hemagglutinin/protease produced by *Vibrio cholerae* non-O1 (NAG-HA/P). The determined nicking site was the Thr193-Ile194 junction, which was distinct from that for a protease of *V. cholerae* (Ichinose *et al*., *European Journal of Epidemiology* 8, 743–747, 1992). We further analyzed proteolytic cleavage by NAG-HA/P of a synthetic peptide corresponding to the nicking region of cholera toxin A subunit and determined the cleavage site to be preferentially between Ser194 and Met195, and in addition between Ser193 and Ser194. © 1998 Elsevier Science Ltd. All rights reserved

Vibrio cholerae non-O1, which closely resembles *V. cholerae* O1 biologically, morphologically and biochemically, but dose not agglutinate with *V. cholerae* O1 antiserum (non-agglutinable: NAG), produces a protease named hemagglutinin/protease (NAG-HA/P). We previously reported the purification and characterization of the NAG-HA/P from a *V. cholerae* non-O1, strain TH81, and revealed that the NAG-HA/P is physicochemically and immunologically indistinguishable from the HA/P of *V. cholerae* O1 (Vc-HA/P) (Honda *et al*., 1989).

Cholera enterotoxin (CT) produced by *V. cholerae* O1 consists of two subunits, A and B. In usual culture conditions, the A subunit of CT (CT-A) is produced from *V. cholerae* as a nicked form composed of two fragments, A1 and A2, linked by a disulfide

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bond. It was reported that the Vc-HA/P is an endogenous enzyme that nicks the CT-A (Booth *et al.*, 1984).

Enterotoxigenic *Escherichia coli* produces a heat-labile enterotoxin (LT) which consists of two subunits, A and B. LT is structurally, functionally and immunologically closely related to CT. The A subunit of LT (LT-A) is exclusively produced as an unnicked form from the *E. coli* (Clement and Finkelstein, 1979). As the nicking of LT by trypsin resulted in activation of LT (Rappaport *et al.*, 1976), proteolytic activation in host guts may play a role in the pathogenesis of LT-producing *E. coli*.

Some *V. cholerae* non-O1 strains are known to produce CT, and the CT is secreted as a nicked form (Yamamoto *et al.*, 1984). In this study we examined whether NAG-HA/P is also involved in the nicking of CT-A in *V. cholerae* non-O1, and in activation of the toxin. It is, however, difficult to obtain an unnicked form of CT, because CT-A is detected as a nicked form under usual culture conditions of isolation of CT from *V. cholerae*. Thus we firstly conducted experiments using LT isolated from enterotoxigenic *E. coli*, which is unnicked. We also performed experiments using a synthetic peptide corresponding to nicking region of CT-A, as a substrate for the nicking by NAG-HA/P.

E. coli strain 240 producing LT (Tsuji *et al.*, 1987) was cultured in 1 l CAYE medium (Takeda *et al.*, 1981) in a 5-l flask with shaking at 37°C for 20 h. LT having unnicked A subunit was purified by the method of Clement and Finkelstein (1979) with slight modification. Briefly, whole-cell lysate was precipitated with 70%-saturated ammonium sulfate. The precipitate was harvested and dialyzed against TEAN buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM NaN3 and 0.2 M NaCl, pH 7.4). The dialyzed sample was applied and LT was absorbed to a column of Bio-Gel A5m (Bio-Rad Laboratories, Richmond, CA) (2×60 cm) equilibrated with TEAN buffer at 4°C. LT was eluted from the gel with TEAN buffer containing 0.3 M galactose (Wako Pure Chemical Industries. Ltd, Osaka, Japan). The A subunit of LT thus purified was confirmed to be unnicked on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

NAG-HA/P was purified from *V. cholerae* non-O1 strain TH81 cultured at 30°C for 20 h as described previously (Honda *et al.*, 1989). Culture supernatant was fractionated with ammonium sulfate (40–55%) and NAG-HA/P was purified by immunoaffinity chromatography as described previously (Naka *et al.*, 1992).

The purified LT with unnicked A subunit was incubated for 5, 10, 30 and 60 min at 37°C with NAG-HA/P at an enzyme to substrate molar ratio of 1:40 in 10 mM phosphate buffer (pH 7.0). The A subunit of the LT was converted to apparent molecular mass consistent with that of nicked form by 5 min or longer treatment with NAG-HA/P (Fig. 1). Once LT-A was nicked, further cleavage did not occur by prolonged treatment with NAG-HA/P. We examined if the relative biological activity of the nicked LT is increased or not by the Chinese hamster ovary (CHO) cell assay (Tsuji *et al.*, 1984). Elongation of 50% of the cells was observed after 4 h of incubation with the nicked toxin and after 7 h of incubation with the same dose (0.27 ng ml^{-1}) of unnicked toxin (Fig. 2). No spontaneous morphological change was observed during the observation period (12 h). After treating LT with NAG-HA/P, the unreduced peptides were separated with SDS-PAGE. Proteins in the gel were transferred to PVDF (polyvinylidene difluoride) membranes (ProBlott™, Applied Biosystems, CA), then the portions containing A fragment were cut out and loaded to an amino acid sequencer (model 473A, Applied Biosystems) to determine the N-terminal amino acid sequences. Since the N-terminal of LT-A1 fragment is masked naturally, only the N-terminal sequence of A2 fragment appeared as Ile-Thr-Gly-Asp in accord with 194-197 of the previously reported

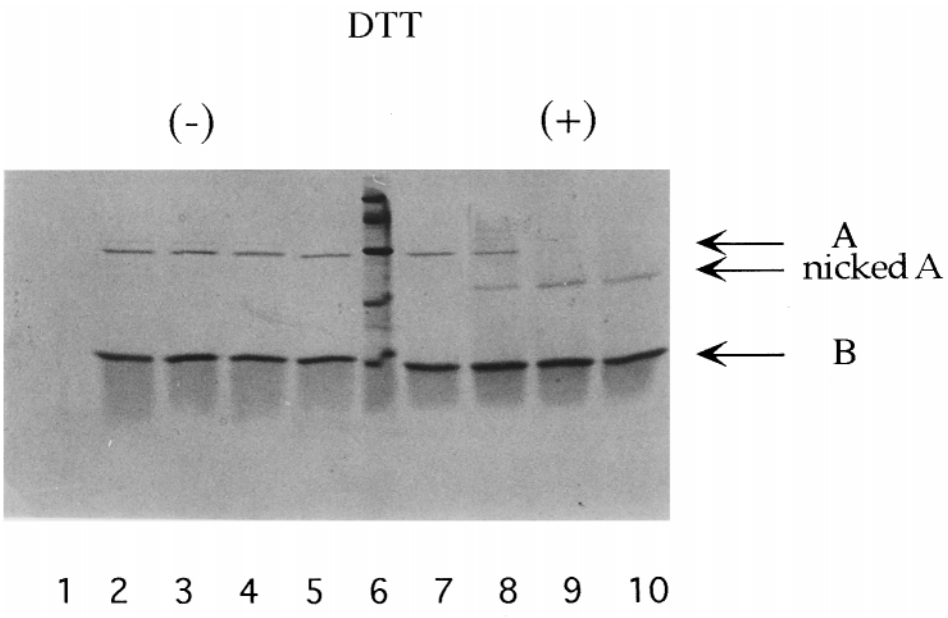

Fig. 1. SDS-PAGE profile of LT treated by NAG-HA/P. SDS-PAGE was carried out on 12% polyacrylamide gel. Lane 1, NAG-HA/P; L2, 3, 4, LT incubated with NAG-HA/P at 37°C for 10, 5, 1 min, respectively, were applied to the gel on SDS-PAGE without dithiothreitol treatment; L5 and 7, LT without NAG-HA/P treatment; L6, Molecular weight markers (66.3, 42.4, 30.0, 20.1, 14.4 kDa); L8, 9, 10, LT incubated with NAG-HA/P at 37°C for 1, 5, 10 min, respectively, were applied to the gel of SDS-PAGE with dithiothreitol treatment. A and B denote the respective subunits.

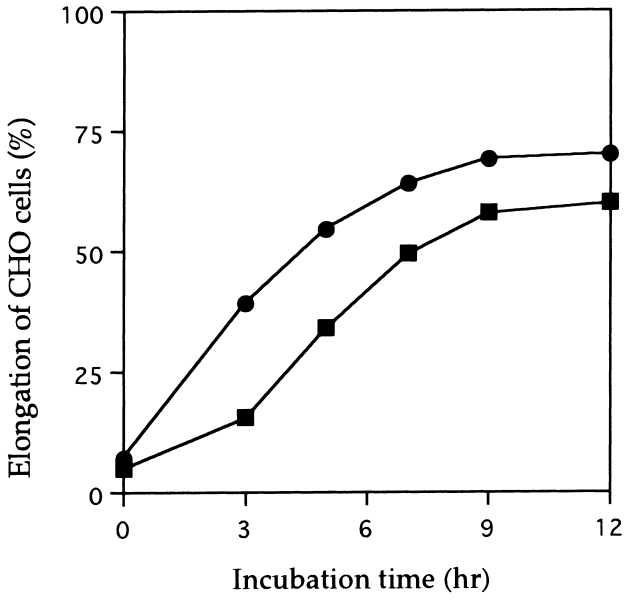

Fig. 2. Time course of elongation of CHO cells induced by nicked or unnicked NAG-HA/P. CHO cells (3×10^8 well^{-1}) were incubated with nicked (●) or unnicked (■) LT (50 ml, 0.27 mg ml^{-1}). Then each mixture was incubated further in a CO_2 incubator at 37°C for the indicated period. To obtain nicked toxin, LT had been pretreated with NAG-HA/P at an enzyme to substrate molar ratio of 1:40 in 10 mM phosphate buffer (pH 7.0) for 10 min.

LT (Yamamoto *et al.*, 1984b). Thus, the nicking site of LT-A for NAG-HA/P is located between the preceding Thr193 and Ile194.

To analyze whether the cleavage site is identical in the case of CT-A, we did experiments using a synthesized peptide (pep-$CT_{188\text{-}198}$, a kind gift from Dr. Y. Kiso and Dr. T. Kimura, Kyoto Pharmaceutical University) corresponding to Gly188-Thr198 of the CT-A sequence (Mekalanos *et al.*, 1983). The pep-$CT_{188\text{-}198}$ was incubated with NAG-HA/P at 37°C for 15 min at enzyme-to-substrate of 1:45 in 10 mM phosphate buffer (pH 7.0), then the digests were applied to μ-Bondasphere HPLC column (Nihon Millipore Ltd, Tokyo, Japan). A linear gradient of 0 to 60% 2-propanol/acetonitrile (7:3) in 0.1% TFA was used for elution. Four cleavage products [designated as RT 7.01, 12.40, 17.49 and 18.26, respectively, based on their retention time (min)] were separated and then the N-terminal amino acid sequences analyzed by Edman degradation. The N-terminal of two of the four cleavage products (RT 7.01 and 12.40) was Met-Ser-Asn-Thr and Ser-Met-Ser-Asn-Thr, respectively, which corresponded to the positions Met195-Thr198 and Ser194-Thr198 in the amino acid sequence previously reported (Mekalanos *et al.*, 1983). Another two products (RT 17.49 and 18.26) could not be sequenced by this method. These fragments seemed to be N-terminal region of the synthetic peptide, since the N-terminal of the synthetic peptide is masked by acylamino acid. To determine the sequence of the unidentified products, we analyzed the amino acid composition and mass spectra of these peptides. Amino acid analysis was performed with an Hitachi L-8500 amino acid analyzer. Hydrolysis was performed in 6 M hydrochloric acid at 110°C for 24 h. Mass spectrometry was done on a Kompact Maldi III (Shimazu/KRATOS) and dihydro-benzoic acid (DHB) served as matrix. Taking together the results from amino acid composition analysis and mass spectrometry, the two peptides RT 17.49 and 18.26 were found to be Gly-Asn-Ala-Pro-Arg-Ser-Ser and Gly-Asn-Ala-Pro-Arg-Ser, which correspond to Gly188-Ser194 and Gly188-Ser193, respectively. Calculated molar ratio of RT 7.01:RT 12.40 and RT 17.49:RT 18.26 were both 2.4:1. These results suggested that the nicking sites of the pep-$CT_{188\text{-}198}$ for NAG-HA/P were mainly the Ser194-Met195 junction, and in addition Ser193-Ser194.

We have localized the nicking site of LT-A for NAG-HA/P between Thr193 and Ile194. On the other hand the nicking site of LT-A by a *V. cholerae* O1 protease (Ichinose *et al.*, 1992) was reported to be the carboxyl-terminal of Arg at position 192 of LT-A. Thus, the nicking site we identified as a target for NAG-HA/P was not the same position, although the nicking at Thr193-Ile194 junction of LT was also accompanied with activation of LT. We can not explain this discrepancy, but the *V. cholerae* protease isolated from a strain (K23) of *V. cholerae* O1 and used by Ichinose *et al.* (1992) may be distinct from HA/P.

Klapper *et al.* (1976) and Kurosky and Markel (1976) reported that the N-terminal of CT-A2 was Met195 by amino acid sequencing of the CT-A2. Our present results are consistent with these previous reports. They support the notion that NAG-HA/P is the endogenous protease of *V. cholerae* non-O1 that nicks CT.

Taking the present data and our previous report (Nagamune *et al.*, 1996) together, the major recognition sites of NAG-HA/P are likely to be the N-terminal side of hydrophobic amino acids. Thus, NAG-HA/P is an endopeptidase with rather broad target specificity like the well known metalloprotease thermolysin (Titani *et al.*, 1972).

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