Volume 280, number 2, 207–210 FEBS 09490 March 1991

ADONIS 0014579391002000Q

Combination of Trp and Glu residues for recognition of mRNA cap structure

Analysis of m^{7}G base recognition site of human cap binding protein (IF-4E) by site-directed mutagenesis

Hitoshi Ueda[1], Hiromi Iyo[1], Mitsunobu Doi[1], Masatoshi Inoue[1], Toshimasa Ishida[1], Hiroshi Morioka[2], Toshiki Tanaka[3], Satoshi Nishikawa[4] and Seiichi Uesugi[5]

[1]*Osaka University of Pharmaceutical Sciences, 2-10-65 Kawai, Matsubara, Osaka 580, Japan,* [2]*Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12 Nishi 6 Kitaku, Sapporo 060, Japan,* [3]*Protein Engineering Research Institute, 6-2-1 Furuedai, Suita, Japan,* [4]*Fermentation Research Institute, Agency of Industrial Science and Technology, MITI, 1-1-3 Higashi, Tsukuba 305, Japan* and [5]*Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan*

Received 10 January 1991; revised version received 16 January 1991

Four mutants of the human cap binding protein (hCBP), in which Trp-102, Glu-103, Asp-104 or Glu-105 was changed to the aliphatic Leu or Ala, were prepared, and their cap binding abilities were examined. Cap binding abilities of two mutants, W102L (Trp-102→Leu) and E105A (Glu-105→Ala), were significantly decreased in comparison with the wild-type hCBP. This result suggests that Trp-102 and Glu-105 are both necessary for the cap binding, and the most probable binding mode with the m^{7}G of cap structure is the combination of the stacking by Trp-102 and the hydrogen-bond pairing by Glu-105, as was already proposed from the model studies.

Human cap binding protein; Cassette mutagenesis; Cap binding activity

1. INTRODUCTION

The 5′-terminal portions of most eukaryotic mRNA consist of a 'cap' structure [1]. This structure plays important roles in the biological function of mRNA [2]. Several studies have been conducted to elucidate the function of the cap structure in different physiological processes in which it is involved. The results so far clarified the existence of an initiation factor for the protein biosynthesis, termed eIF-4E (eukaryotic initiation factor-4E) or CBP (cap binding protein); this initiation factor plays an important role in the translation initiation through the specific binding to the mRNA cap structure [3–7].

The amino acid sequence of CBP was deduced from the cDNA or genomic sequence in yeast [8], mouse [9] and human [10] (see Fig. 1), and cDNA expression of yeast CBP in *E. coli* was also reported [8]. Recently, we succeeded in the large-scale expression of a synthetic gene coding hCBP in *E. coli*, as the first step to investigating the recognition mechanism of the mRNA cap structure at the atomic level (Ueda, H., Maruyama, H., Doi, M., Inoue, M., Ishida, T., Morioka, H., Tanaka, T., Nishikawa, S. and Uesugi, S., manuscript submitted). From a series of interaction studies using model complexes [11–14], on the other hand, we reported that the cooperation of the stacking interaction with aromatic amino acids such as Trp and the hydrogen-bond pairing with acidic amino acids such as Glu is important for the specific m^{7}G recognition. This insight suggests that the sequence containing the Trp and Glu residues is the most probably binding site for the mRNA cap structure. Therefore, four residues of sequence 102–105 (Trp-Glu-Asp-Glu) in hCBP was selected as the most probable candidate, and each residue was changed to aliphatic Leu or Ala residues by the site-directed mutagenesis of the native hCBP gene. In this paper, we present clear-cut evidence that both the Trp-102 and Glu-105 residues are necessary for the recognition of mRNA cap structure.

Correspondence address: T. Ishida, Osaka university of Pharmaceutical Sciences, 2-10-65 Kawai, Matsubara, Osaka 580, Japan

Abbreviations: hCBP, human cap binding protein; hGH, human growth hormone; IAA, 3-indoleacrylic acid

2. MATERIALS AND METHODS

2.1. *Materials*

T4 DNA ligase, Klenow enzyme and restriction endonucleases were purchased from Takara Shuzo (Kyoto, Japan). Polynucleotide kinase and α-thrombin were purchased from New England Biolabs (MA, USA) and Mochida Seiyaku (Tokyo, Japan), respectively. The Q-Sepharose, Sephadex G-75 and m^{7}GTP-Sepharose were obtained from Pharmacia LKB Biotechnology (Tokyo, Japan). GMP and IMP were purchased from Sigma Chemical (MO, USA). The methylations

of GMP and IMP were carried out by the direct methylation according to Griffin and Reese [15].

2.2. *Cassette mutagenesis*

Oligodeoxyribonucleotides were designed typically as 30 nucleotides in length ML10, MU11 and ML11 (Fig. 2A), and were synthesized by the phosphite triester method with a DNA synthesizer (Applied Biosystem 380A) in 0.5 μmol scale. The purification of these oligodeoxyribonucleotides and synthesis of mutation cassette were carried out by according to the method of Kim et al. [16].

The high-level expression plasmid of a synthetic hGH-hCBP fusion protein gene, pFUCBP, was digested by *Sma*I and *Sal*I, and the large fragment was isolated by 1% agarose gel electrophoresis. To construct the mutant protein expression plasmid (pFMCBP), the synthetic mutation cassette was religated with the recovered vector by T4 DNA ligase (Fig. 2B). The DNA sequence of the synthetic gene was confirmed by the dideoxy method [17].

2.3. *Expression of the mutant gene and purification of the mutant protein*

The *E. coli* HB101 transformants harboring the plasmid that encodes the hCBP mutant gene were grown at 37°C in M9 liquid media containing 0.2% casamino acids and ampicillin. The gene expression was induced by addition of 3-indoleacrylic acid (IAA). The *E. coli* HB101 transformant cells cultured for 24 h after induction with IAA were harvested and disrupted by sonication. The pellets were collected by the centrifugation and resuspended in 5 ml of 20 mM Tris-HCl (pH 8.0) containing 7 M urea. After the centrifugation, the combined supernatants were dialyzed against 20 mM Tris-HCl (pH 8.0) containing 5 M urea. The urea concentration was gradually reduced to 3 M, 1 M and then 0 M. This partially purified fusion protein was then treated with α-thrombin in 50 mM Tris-HCl (pH 8.0). After the proteolysis, the sample was fractionated by column chromatography on Q-Sepharose with a linear gradient of NaCl (0–0.5 M) in 20 mM Tris-HCl (pH 7.5) and by a Sephadex G-75 column with 20 mM ammonium bicarbonate. The purity of the recombinant hCBP mutant was checked by 15% SDS-PAGE.

2.4. *Fluorescence studies*

Fluorescence spectra were measured on a Jasco FP-770F with a 10-mm cell. Titration measurement and determination of association constant was carried out according to the method of Carberry et al. [18].

2.5. *Binding assay using a cap affinity column*

The binding abilities of wild-type and mutant hCBPs to a m^7GTP-Sepharose column (0.5 × 10 cm) were investigated under the elution condition of a linear gradient of m^7GMP (0–500 μM) in 20 mM Tris-HCl and 500 mM NaCl (pH 7.6) at a flow rate of 0.5 ml/min. The detection was carried out by the fluorescence intensity.

3. RESULTS AND DISCUSSION

Fig. 1 shows the comparison of the amino acid sequence of hCBP with those of mouse and yeast CBPs. Although the sequences are different from one another (about 33% homology between human and yeast CBPs), eight Trp residues are all conserved. The high homology is observed at the following two sequences, i.e. sequence 66–74: Phe-Paa-Thr-Val-Glu-Aaa-Phe-Trp-Ala (where Paa and Aaa represent the hydrophilic and acidic amino acids, respectively) and sequence 100–107: Pro-Xaa-Trp-Glu-Asp-Glu-Xaa-Asn (Xaa represents any amino acid).

Based on a series of interaction studies between the nucleic acid base and amino acid or peptide [11–14], on

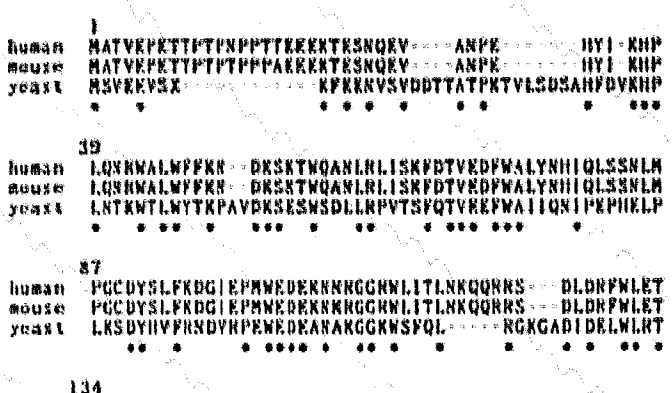

Fig. 1. Comparison of the amino acid sequences of human, mouse and yeast CBPs. The residues conserved among those three CBPs are indicated by asterisks.

the other hand, it was proposed that the cooperation of the stacking interaction of Trp indole ring and the hydrogen-bond pairing of Glu carboxyl side chain is important for the specific recognition of m^7G base. Therefore, the latter Trp-Glu-Asp-Glu sequence was firstly selected the most probable binding site for the mRNA cap structure, and decided to examine the possibility by the site-directed mutagenesis. Fig. 2A shows the sequence of mutation cassettes designed for four different mutants. The change of Trp-102 to Leu (W102L) was intended to clarify the role of the aromatic ring for the binding with m^7G base. Three kinds of mutants, E103A, D104A and E105A, were also prepared to investigate the effect of acidic side chain on the cap binding. A *Sac*II site (Fig. 2A) was prepared in each mutation cassette to facilitate mutation screening. The plasmids which contain the mutant gene were constructed as shown in Fig. 2B. All mutant fusion genes showed the expression levels as high as those of wild type gene. The purities of mutant hCBP proteins were checked by the SDS-PAGE. The CD spectra of these four mutants were very similar to that of the wild-type hCBP, suggesting no dramatic changes in their secondary structures (data are not shown).

Association constants between the cap analogues and the native or mutant hCBPs were determined by the fluorescence titration experiments, and results are given in Table I. The K_a values for m^7GMP indicate that E103A and D104A mutants behave exactly like the wild-type hCBP. On the other hand, the W102L and E105A mutants show noticeable decreases in their K_a values. It is of special importance to note that the K_a of W102L mutant is almost the same as that of wild-type

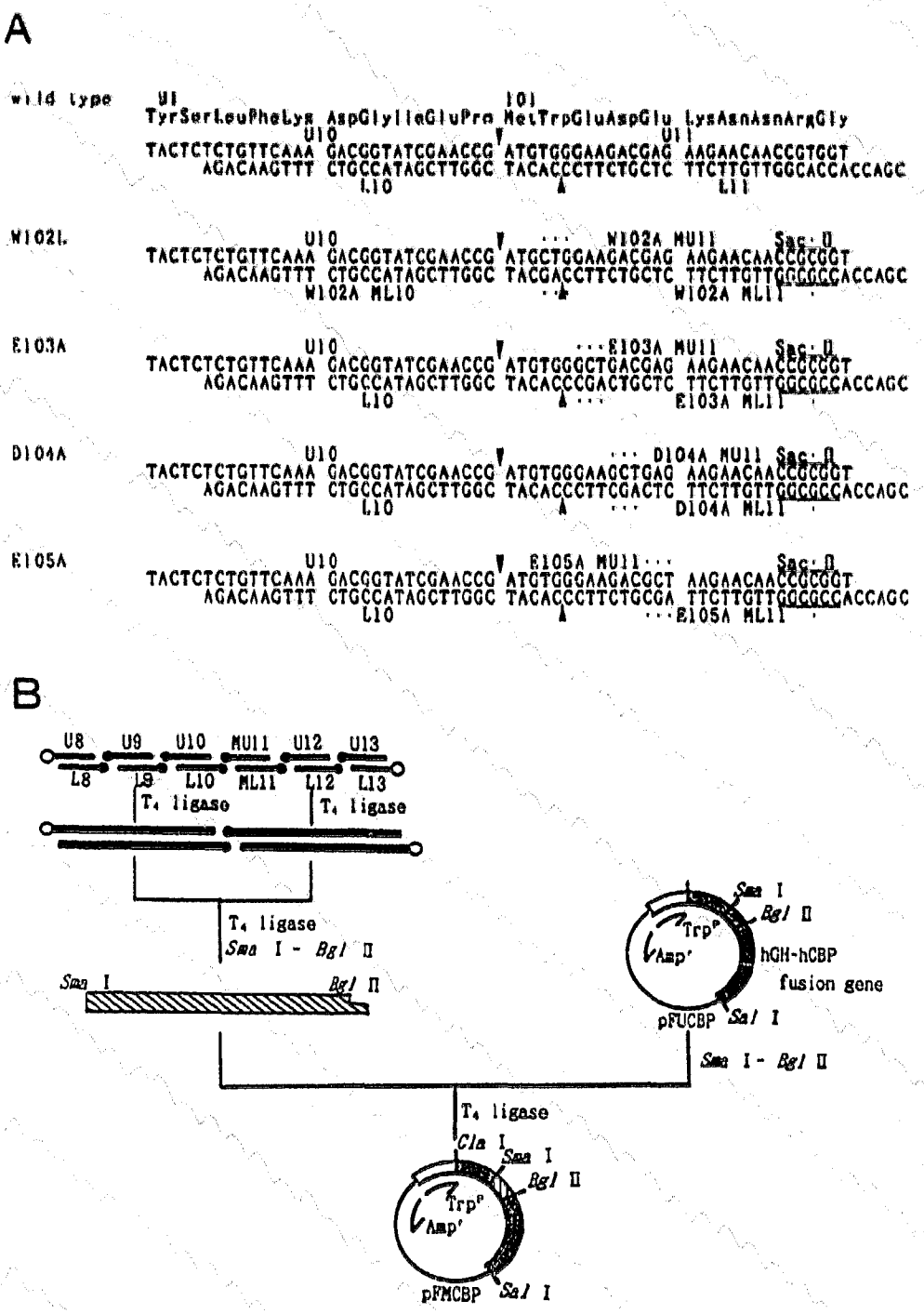

Fig. 2. (A) The nucleotide sequences of the mutation cassettes, together with that of the wild-type. The enzymic jointing points are shown by arrowheads. (B) Synthesis of the mutation cassette by segment ligation, and the construction of a mutant expression plasmid. Filled circles indicate 5′-phosphoryl groups and open circles indicate free 5′-hydroxyl groups.

hCBP-GMP, and the K_a value of E105A is similar to that of wild-type hCBP-m^{7}IMP. These results are consistent with the notion that the cooperation of the stacking interaction of Trp-102 indole ring and the hydrogen-bond pairing of Glu-105 carboxyl side chain is important for m^{7}G binding. It has been experimentally shown [11–14] that: (i) the methylation (*N*-quarternization) of guanine base strengthens the stacking interaction with the aromatic amino acid; and (ii) the hydrogen-bond pairing of the carboxyl group of acidic amino acid through guanine N2 amino groups is specific.

Table 1
Association constants between hCBP or its mutants and cap analogues at 20°C

hCBP	Cap analogue	$K_a(M^{-1})$
wild-type	m^7GMP	1.97×10^5
W102L	m^7GMP	3.34×10^4
E103A	m^7GMP	1.87×10^5
D104A	m^7GMP	1.97×10^5
E105A	m^7GMP	6.84×10^4
wild-type	GMP	3.32×10^4
wild-type	m^7IMP	6.25×10^4

Temperature accuracy was ±0.2°C. The mean error was less than 10%.

In order to further validate these results, the cap binding abilities of the wild-type and mutant hCBPs were examined using a cap affinity column. The hCBP elution was carried out by a linear gradient of m^7GMP in the 20 mM Tris-HCl (pH 7.6) containing 500 mM NaCl and the profile was monitored by the fluorescence measurement at 353 nm; the salt was added in order to make it more sensitive to the difference of cap binding ability. The results are shown in Fig. 3, where the ascent of baseline is due to the m^7GMP containing in the elution solvent. These chromatographic patterns indicate that while the E103A and D104A mutants have specific cap binding abilities as high as the wild-type hCBP, the W102L and E105E mutants show much weaker abilities. This results clearly supports the results of association constants by the fluorescence titration experiments.

In conclusion, it became obvious that the Trp-102 and Glu-105 in hCBP are required for the specific recognition of mRNA cap structure. Altmann et al. reported the site-directed mutagenesis of all eight of the Trp residues in yeast CBP to Phe [19]. They showed that the W104F mutant, in which Trp-104 corresponds to Trp-102 in hCBP, had 40–70% of the ability for the cap binding in comparison with the wild-type CBP. The difference between the binding abilities of W104F (yeast) and W102L (human) mutants may be due to the difference in the stacking abilities of Phe and Leu side chains; the prominent stacking interaction between the Phe and m^7G base was shown by X-ray crystallographic analysis [13]. Therefore, it is imaginable that the Trp-102 is situated above the m^7G base in the cap binding pocket of hCBP. On the other hand, the participation of Glu-105 in the m^7G binding was shown for the first time by the present study. It is interesting to note that the acidic amino acid such as Glu, which is situated at the carboxyl terminal side of Trp residues, increases the stacking ability of Trp with m^7G base as a result of the base fixation through the hydrogen-bond pairing of the carboxyl side chain [12,14]. The present result suggests the importance of cooperation of Trp and Glu residues for the specific recognition of mRNA cap structure. In the near future, X-ray crystallographic analysis will enable the elucidation of all of the cap recognition mechanisms at the atomic level.

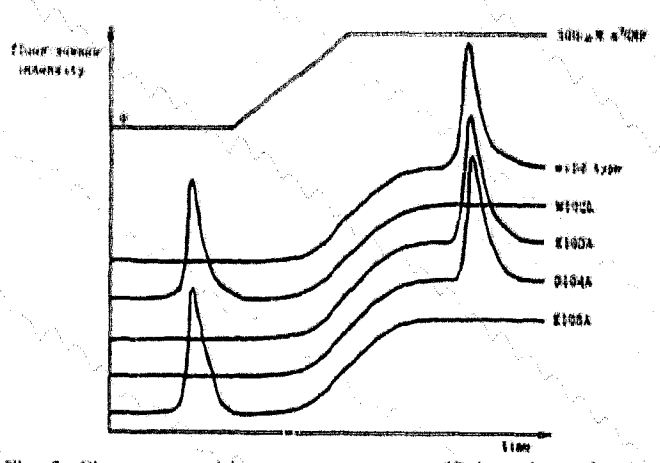

Fig. 3. Chromatographic patterns on a cap affinity column for the wild-type and four mutant hCBPs.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (No. 02453146).

REFERENCES

[1] Furuichi, Y. and Miura, K. (1975) Nature 253, 374–375.
[2] Miura, K. and Shinozaki, K. (1985) Protein Nucleic Acid and Enzyme 30, 711–721.
[3] Furuichi, Y., LaFlandra, A. and Shatkin, A.J. (1977) Nature 266, 235–239.
[4] Green, M., Melton, D. and Maniatis, T. (1983) Cell 32, 681–694.
[5] Edery, I., Pelletier, J. and Sonenberg, N. (1987) in: Translational Regulation of Gene Expression (Ilan, I. ed.) pp. 335–366, Plenum Press, New York.
[6] Sonenberg, N., Mogan, M.A., Merrick, W.C. and Shatkin, A.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4343–4349.
[7] Altmann, M., Edery, I., Sonenberg, N. and Trachsel, H. (1985) Biochemistry 24, 6085–6089.
[8] Altmann, M., Handschin, C. and Trachsel, H. (1987) Mol. Cell. Biol. 7, 998–1003.
[9] McCubbin, W.D., Edery, I., Altmann, M., Sonenberg, N. and Kay, C.M. (1988) J. Biol. Chem. 25, 17663–17671.
[10] Rychlik, W., Domier, L.L., Gardner, P.R., Helman, G.M. and Rhoads, R.E. (1987) Proc. Natl. Acad. Sci. USA 84, 945–949.
[11] Ishida, T., Doi, M., Ueda, H., Inoue, M. and Sheldrick, G.M. (1988) J. Am. Chem. Soc. 110, 2286–2294.
[12] Ueda, H., Doi, M., Inoue, M., Ishida, T., Tanaka, T. and Uesugi, S. (1988) Biochem. Biophys. Res. Commun. 154, 199–204.
[13] Ishida, T., Doi, M. and Inoue, M. (1988) Nucleic Acids Res. 16, 6175–6190.
[14] Ishida, T., Iyo, H., Ueda, H., Doi, M. and Inoue, M. (1990) J. Chem. Soc. Chem. Commun. 217–218.
[15] Griffin, B.E. and Reese, C.B. (1963) Biochem. Biophys. Acta 68, 185–188.
[16] Kim, H.J., Nishikawa, S., Tanaka, T., Uesugi, S., Takenaka, H., Hamada, M. and Kuby, S.A. (1989) Protein Eng. 2, 379–386.
[17] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[18] Caberry, S.E., Rhoads, R.E. and Goss, D.J. (1989) Biochemistry 28, 8078–8083.
[19] Altmann, M., Edery, I., Trachsel, H. and Sonenberg, N. (1988) J. Biol. Chem. 25, 17229–17232.