FEBS Letters 374 (1995) 262–264 FEBS 16194

Construction of a divalent cell adhesive lysozyme by introducing the Arg-Gly-Asp sequence at two sites

Takao Yamada*, Yoshimi Shimada, Atsuko Uyeda, Shigeru Sugiyama, Masakazu Kikuchi

Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan

Received 11 September 1995

Abstract To increase the cell adhesion activity of 74RGD4, an RGDS-inserted mutant between Val[74] and Asn[75] of human lysozyme, one more site for the RGD introduction was investigated in the lysozyme molecule. We found that 47RGD4 with RGDS in place of AGDR (residues 47 to 50) in a β-turn region possesses the same level of adhesion activity as that of 74RGD4. The acceptance of the RGD introduction in the β-turn region of human lysozyme is in good agreement with recent studies on the functional conformation of RGD. We constructed (47,74)RGD4, a mutant containing RGD at two sites, by combining the N-terminal domain of 47RGD4 and the C-terminal domain of 74RGD4. The (47,74)RGD4 lysozyme, with two functional RGD sequences, exhibits even higher cell adhesion activity than that of 74RGD4 or 47RGD4.

Key words: Human lysozyme; Arg-Gly-Asp introduction; Cell adhesion activity; β-Turn conformation; Divalent adhesive protein

1. Introduction

The Arg-Gly-Asp (RGD) sequence is well-known as a site in cell adhesive proteins such as fibronectin [1], vitronectin [2], and fibrinogen [3] for binding to their receptors, the integrins [4]. We previously constructed a mutant, 74RGD4, by inserting RGDS of human fibronectin between Val[74] and Asn[75] in a flexible loop region of human lysozyme, using a yeast expression system [5]. To increase the cell adhesion activity of the 74RGD4 mutant, we constructed another mutant, Cys-RGD4, by inserting RGDS flanked by two Cys residues at the same site in the lysozyme molecule [6]. This design is based on the fact that the cyclic form of an RGD-containing peptide has much higher affinity to the integrins than the linear counterpart [7]. For both mutants, we have already reported the structural and functional analyses, and discussed the functional conformation of the RGD sequence [5,8].

In parallel with the construction of Cys-RGD4, we have tried another design to tailor the 74RGD4 mutant to possess higher adhesion activity. In this study, we have searched for one more RGD introduction site in the lysozyme molecule. Here we report the construction of (47,74)RGD4, a mutant lysozyme containing RGD at two sites, and its functional evaluation. The structural feature of the possible introduction site of RGD in human lysozyme is discussed, in connection with the functional conformation of RGD.

*Corresponding author. Fax: (81) (6) 872 8210.

Abbreviations: RGD, Arg-Gly-Asp; BHK, baby hamster kidney.

2. Materials and methods

2.1. Vector constructions

Oligonucleotides were chemically synthesized using an automated DNA synthesizer (Model 380B, Applied Biosystems). The mutation was performed in M13mp19XhLZM using the site-directed mutagenesis system Mutan-K (Takara Shuzo). The primer used for the construction of 47RGD4, for example, was 5′-AGTCTGTAGA-ACTGTCGCCACGGTTGTAATT-3′. The underlined bases indicate mismatches. The sequence of the mutated gene was confirmed by dideoxy sequencing. The genes encoding the signal sequence and the mutated human lysozyme were combined with an *Xho*I- *Sma*I large fragment from pERI8602 [9] to construct the expression plasmid.

The M13mp19XhLZM plasmid possesses an *Xba*I restriction site between codons corresponding to Ser[61] and Arg[62] of human lysozyme [9]. To construct the (47,74)RGD4 expression plasmid, the following three fragments were combined: an *Xho*I-*Xba*I fragment encoding the signal sequence and the N-terminal domain of 47RGD4, an *Xba*I-*Sma*I fragment encoding the C-terminal domain of 74RGD4 [5], and an *Xho*I-*Sma*I large fragment from pERI8602.

2.2. Expression and purification of mutant human lysozymes

Mutant human lysozymes were expressed in yeast as described previously [10]. Secreted mutant lysozymes were purified essentially as described [9]. HPLC was performed using a cation-exchange column (Asahipak ES-502C; Asahikasei, Japan) and a hydroxyapatite column (TAPS-020810, Tonen K.K., Japan).

2.3. Measurement of activity

Lytic activity was measured using *Micrococcus lysodeikticus* cells as a substrate [9]. Protein was determined by measuring the weight in the freeze-dried form of each mutant lysozyme.

Cell adhesion activity was determined using baby hamster kidney (BHK) cells as described [6,11]. The amount of lysozyme adsorbed onto a plate was estimated by subtracting the unadsorbed amount from the added amount of lysozyme in the assay. The unadsorbed amount was determined based on the lytic activity remaining in the sample solution after binding to the plate. The results indicated that the adsorption efficiency was in the range of 60–80% at the concentrations shown in Fig. 2, for both native and mutant lysozymes.

3. Results and discussion

We previously constructed a cell adhesive protein, 74RGD4, by inserting the RGDS sequence between Val[74] and Asn[75] in a long loop consisting of residues of Cys[65] to Cys[81] in human lysozyme [5]. The backbone structure of the 74RGD4 lysozyme refined by X-ray crystallography is shown in Fig. 1. To find an RGD introduction site in a different region than the long loop of the lysozyme molecule, we tried to construct three lysozyme variants by means of site-directed mutagenesis, namely 14RGD4 with RGDS in place of RLGM (residues 14 to 17) in an α-helix, 41RGD4 with RGDS in place of RATN (residues 41 to 44) in a β-sheet, and 47RGD4 with RGDS in place of AGDR (residues 47 to 50) in a β-turn. The 47RGD4 mutant was secreted with high efficiency in our yeast expression system (Table 1), while the 14RGD4 and 41RGD4 mutants failed to

SSDI 0014-5793(95)01123-4

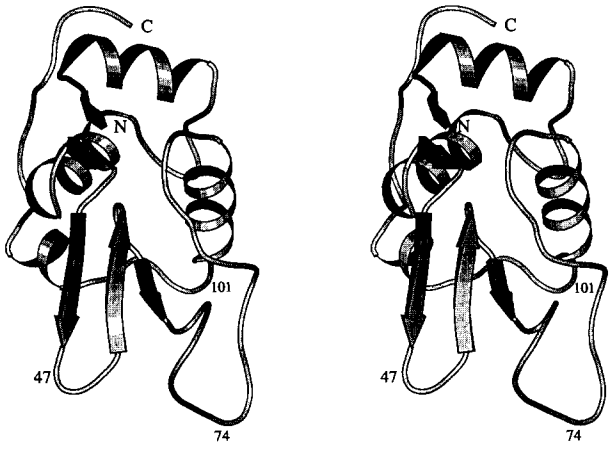

Fig. 1. Stereo drawing of the backbone structure of the 74RGD4 lysozyme. The picture was produced with the program MOLSCRIPT [20] using the atomic coordinates of 74RGD4 [5]. The α-helix and β-strand parts are shown as a ribbon model. The RGD introduction sites in this study are labeled.

be folded correctly, and were not secreted from the yeast cells. The lytic activity of 47RGD4 as well as 74RGD4 was nearly the same as that of the native form (Table 1), indicating that these RGD introductions had no effect on the active cleft of human lysozyme. The successful folding of these fully active mutants is probably due to the distant location of the introduction sites from the active cleft [12].

Using baby hamster kidney (BHK) cells, 47RGD4 was shown to possess the same level of adhesion activity as that of 74RGD4 (Fig. 2). We also produced another mutant, 101RGD4, by substituting RGDS for RDPQ (residues 101 to 104) in a turn-like region of the lysozyme molecule. In the cell adhesion assay, however, the 101RGD4 mutant was inactive. This is probably because the RGD region of the 101RGD4 lysozyme fails to have a β-turn structure owing to the location in the bottom of a loop consisting of residues of Arg^{101} to Val^{110} [12] (Fig. 1). On the other hand, the RGD sequence of the 47RGD4 mutant is destined to assume a stable turn conformation, since the two β-strands, Ala^{42}-Asn^{46} and Ser^{51}-Gly^{55}, connect by the RGDS (residues 47 to 50) to form a rigid β-sheet (Fig. 1). Thus, these results suggest that the introduction of a functional RGD sequence is acceptable only in a certain β-turn region of human lysozyme.

This structural information about the RGD introduction site is in good accordance with the experimental studies on the functional conformation of the RGD region in several proteins. We have recently solved the X-ray crystal structure of the CRGDSC-inserted lysozyme, Cys-RGD4. The RGD sequence in this protein resides within a stable type II′ β-turn, with Gly and Asp in positions 2 and 3 of the turn [8]. The fibronectin type III domain from human tenascin [13], and the leech protein decorsin [14] also have type II′ β-turn structures in their RGD regions. In addition, a flexible conformation of RGD has been reported in the 74RGD4 lysozyme [5], the tenth type III module of human fibronectin [15], and the disintegrins, a family of integrin antagonists from snake venoms [16,17]. In these cases, the RGD region, which is highly flexible by nature, could assume a rigid turn conformation when it binds to integrins to form a ligand–receptor complex. It is also possible to consider that the RGD region has a type II′ β-turn by itself, and that it was ill-defined because of its location at the apex of a flexible, long loop.

We finally constructed (47,74)RGD4, a mutant containing RGD at two sites, by combining the N-terminal domain of 47RGD4 and the C-terminal domain of 74RGD4, as described in section 2. The (47,74)RGD4 lysozyme was successfully secreted with full lytic activity (Table 1). As shown in Fig. 2, the (47,74)RGD4 lysozyme exhibited even higher adhesion activity to BHK cells than that of 74RGD4 or 47RGD4. It was also confirmed that this activity is nearly equal to 10% of human vitronectin activity (Fig. 2). The difference might be reasonable, if we consider that the natural cell adhesive proteins including vitronectin, possess a second binding site besides the RGD region to exhibit the optimum affinity for the integrins [17–19]. The cell adhesion activities of the three mutants, 74RGD4, 47RGD4, and (47,74)RGD4, were completely inhibited by the addition of either GRGDSP peptide or polyclonal antibody against vitronectin receptor, as was the case for the vitronectin activity (data not shown). The results indicate that the cell adhesion signals in these mutant lysozymes are transduced to BHK cells through the interaction with the vitronectin receptor, the integrin $\alpha_v\beta_3$.

The high cell adhesion activity of the (47,74)RGD4 mutant suggests that both of the introduced RGD sequences are functional. This speculation was further supported by additional experiments, in which each substitution of Glu for Asp in the

Table 1
Production and relative lytic activity of each mutant lysozyme

Lysozyme	Productivity[a] (mg/l)	Relative lytic activity (%)
Native	5.3	100
74RGD4	6.3	109
47RGD4	4.5	92
(47,74)RGD4	6.7	83

[a] Based on lytic activity in the culture supernatant.

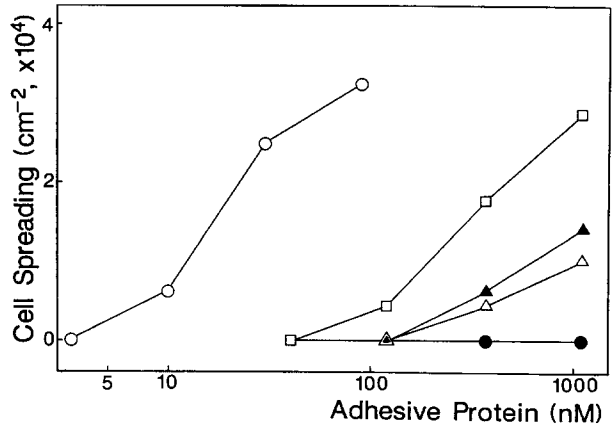

Fig. 2. Cell adhesion assay on the substrates coated with each mutant lysozyme. The plastic substrates were coated with different concentrations of vitronectin (○), native lysozyme (●), 74RGD4 (△), 47RGD4 (▲), and (47,74)RGD4 (□). BHK cells were incubated on the substrates for 60 min at 37°C in a CO_2 incubator. The cell adhesion activity was expressed as the number of cells adhering to unit surface area (cm^2).

two RGD-containing regions of (47,74)RGD4 results in a significant decrease in the cell adhesion activity (data not shown). In addition, adhesion activity in a mixture of 74RGD4/47RGD4 (1:1) was similar to that of (47,74)RGD4, when expressed against their RGD concentrations (data not shown). These results suggest that the higher activity of (47,74)RGD4 is due to the increased concentration of the RGD sites, namely the additive effect of 74RGD4 and 47RGD4.

We have reported the successful construction of (47,74)RGD4, a cell adhesive lysozyme with two functional RGD sequences, and discussed the structural feature of the possible introduction site of RGD in human lysozyme. According to the model structure of the lysozyme molecule (Fig. 1), the two RGD introduction sites are located at a considerable distance (19.4 Å). Thus, the (47,74)RGD4 mutant may be useful as a functional cross-linker in RGD-dependent cell-to-cell interactions, differently from 74RGD4 or 47RGD4.

Acknowledgements: We thank Dr. M. Ikehara for his interest and encouragement throughout this work. We also thank Dr. M. Matsushima for helpful discussions.

References

[1] Pierschbacher, M.D. and Ruoslahti, E. (1984) Nature 309, 30–33.
[2] Suzuki, S., Oldberg, A., Hayman, E.G., Pierschbacher, M.D. and Ruoslahti, E. (1985) EMBO J. 4, 2519–2524.
[3] Watt, K.W.K., Cottrall, B.A., Strong, D.D. and Doolittle, R.F. (1979) Biochemistry 18, 5410–5416.
[4] Hynes, R.O. (1987) Cell 48, 549–554.
[5] Yamada, T., Matsushima, M., Inaka, K., Ohkubo, T., Uyeda, A., Maeda, T., Titani, K., Sekiguchi, K. and Kikuchi, M. (1993) J. Biol. Chem. 268, 10588–10592.
[6] Yamada, T., Uyeda, A., Kidera, A. and Kikuchi, M. (1994) Biochemistry 33, 11678–11683.
[7] Pierschbacher, M.D. and Ruoslahti, E. (1987) J. Biol. Chem. 262, 17294–17298.
[8] Yamada, T., Song, H., Inaka, K., Shimada, Y., Kikuchi, M. and Matsushima, M. (1995) J. Biol. Chem. 270, 5687–5690.
[9] Taniyama, Y., Yamamoto, Y., Kuroki, R. and Kikuchi, M. (1990) J. Biol. Chem. 265, 7570–7575.
[10] Yoshimura, K., Toibana, A., Kikuchi, K., Kobayashi, M., Hayakawa, T., Nakahama, K., Kikuchi, M. and Ikehara, M. (1987) Biochem. Biophys. Res. Commun. 145, 712–718.
[11] Maeda, T., Oyama, R., Ichihara-Tanaka, K., Kimizuka, F., Kato, I., Titani, K. and Sekiguchi, K. (1989) J. Biol. Chem. 264, 15165–15168.
[12] Inaka, K., Taniyama, Y., Kikuchi, M., Morikawa, K. and Matsushima, M. (1991) J. Biol. Chem. 266, 12599–12603.
[13] Leahy, D.J., Hendrickson, W.A., Aukhil, I. and Erickson, H.P. (1992) Science 258, 987–991.
[14] Krezel, A.M., Wagner, G., Seymour-Ulmer, J. and Lazarus, R.A. (1994) Science 264, 1944–1947.
[15] Main, A.L., Harvey, T.S., Baron, M., Boyd, J. and Campbell, I.D. (1992) Cell 71, 671–678.
[16] Adler, M., Lazarus, R.A., Dennis, M.S. and Wagner, G. (1991) Science 253, 445–448.
[17] Saudek, V., Atkinson, R.A. and Pelton, J.T. (1991) Biochemistry 30, 7369–7372.
[18] Obara, M., Kang, M.S. and Yamada, K.M. (1988) Cell 53, 649–657.
[19] Healy, J., Murayama, O., Maeda, T., Yoshino, K., Sekiguchi, K. and Kikuchi, M. (1995) Biochemistry 34, 3948–3955.
[20] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.