Identification of Functionally Important Residues of Human Thrombopoietin

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Thrombopoietin (TPO) is a megakaryocyte growth and differentiation factor. It consists of a characteristic two domain structure. The amino-terminal domain of TPO has a sequence homology with erythropoietin and is required for the binding and activation of its receptor c-Mpl. To determine the functionally important regions interacting with its receptor, a series of site-directed mutants of TPO were constructed based on a three-dimensional model of the amino-terminal domain. Two strategies of mutagenesis were employed: 1) nonnative N-linked glycosylation scan of 12 residues predicted to be on the surface, and 2) alanine replacement scan of mostly charged 44 amino acid residues. Each TPO mutein was transiently expressed in COS7 cells, and the specific bioactivity of the TPO protein secreted into the culture medium was measured using a recombinant BaF3 cell line expressing human c-Mpl. Four alanine substitutions at Arg10, Pro42, Glu50, and Lys138 nearly or completely abolished the activity, whereas the mutation at Arg14 slightly decreased the activity, suggesting that these residues are functionally important in interacting with its receptor. These residues mapped to helix A, loop AB, and helix D. Sequence comparison between human TPO and other mammalian TPO showed that the identified residues are completely conserved among the species. However, unlike the recent report on the mutational analysis of TPO, alanine substitutions at Lys29, Lys59, Arg136, and Arg140 did not affect the TPO activity significantly in our system. The identified receptor binding regions of TPO are analogous to those of human growth hormone and erythropoietin. Based on the similarity of these three cytokines, we propose that Lys138 of helix D and Pro42 and Glu50 of loop AB may constitute one binding region, whereas Arg136 and Lys44 of helix A may constitute the other binding region to dimerize the receptors.

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1 The abbreviations used are: TPO, thrombopoietin; EPO, erythropoietin; hGH, human growth hormone; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Thrombopoietin (TPO)1 is a cytokine that specifically stimulates the proliferation and differentiation of megakaryocytes and thus leads to the production of platelets in vitro and in vivo

(1–5). TPO exerts its action by binding to a specific cell surface receptor encoded by the proto-oncogene c-mpl (1–5). The human TPO cDNA encodes 353 amino acids including a 21-amino acid signal peptide. The human TPO protein can be divided into an amino-terminal domain of 153 amino acids and a carboxyl-terminal domain of 179 amino acids containing six potential N-linked glycosylation sites. The amino-terminal domain itself can stimulate human megakaryopoiesis in vitro and is highly conserved among mice (4), rats (6), dogs (2), pigs (7), and humans (1), whereas the carboxyl-terminal domain is not required for the binding of its receptor and displays a wide species divergence (1). Sequence analysis showed that the amino-terminal domain of TPO has a homology with erythropoietin (EPO) that is a member of four-helix bundle cytokine family, but its structure has not been determined either by x-ray diffraction or by NMR.

Ligand-induced receptor oligomerization is widely believed to initiate signal transduction for all of the cytokine receptors (8). Cytokine receptors share a conserved extracellular cytokine receptor superfamily module comprising two immunoglobulin-like domains and the characteristic WSXWS box (9–11). Some members of this receptor family, such as the human growth hormone (hGH) receptor and EPO receptor, seem to be homodimerized upon ligand stimulation, with each of the two different domains of the cytokine molecule bound to each receptor molecule (12–14). In the case of the TPO receptor, c-Mpl, the extracellular domain has the two cytokine receptor modules (15), and the activation may also occur through receptor homodimerization (16). However, the precise mechanism and the structural information of how the TPO molecule interacts with the receptor molecules is not known.

In this study, as a step toward a better understanding of the molecular basis of TPO function, we have identified functionally important regions of TPO by employing two strategies of site-directed mutagenesis based on a three-dimensional model of the amino-terminal domain of TPO and measuring the specific bioactivities of the TPO muteins. First, a limited number of TPO muteins with nonnative N-linked glycosylation sites on the surface region of the amino-terminal domain were prepared and tested, which enabled us to test the TPO model and roughly identify the regions important in interacting with the receptor. Then an alanine replacement scan of the mostly charged residues of the initially identified important regions was performed. We describe here the identification of the functionally important residues in interacting with the receptor. Our result is different from that of the recent report on the mutational analysis of TPO (17) that showed that alanine substitutions at Asp8, Lys14, Lys52, Arg136, Lys138, and Arg140 caused the greatest reduction in receptor binding affinity. The possible reasons for the discrepancies between our result and their result are discussed.
EXPERIMENTAL PROCEDURES

Cloning of TPO cDNA and Construction of TPO Expression Plasmid—A 1.06-kilobase cDNA corresponding to the complete coding sequence of TPO was synthesized by PCR from human liver cDNA library (Stratagene) using 5' and 3' primers containing HindIII and EcoRV sites at each end, respectively. The resulting cDNA was subcloned into HindIII and EcoRV sites of mammalian expression plasmid pcDNA3 (Invitrogen) to yield TPO expression plasmid. The nucleotide sequence of the TPO cDNA cloned into the plasmid was completely sequenced using Sequenase (U. S. Biochemical Corp.) according to the protocol suggested by the supplier.

Site-directed Mutagenesis—Mutations were created by an overlap extention PCR method (18). Briefly, two partial TPO cDNAs encoding each TPO mutein were separately synthesized by PCR from the wild type TPO cDNA using overlapping antisense or sense oligonucleotides and the 5' or 3' primer. The two PCR products were mixed, then the second PCR was performed using the 5' and 3' primers. The product was digested with HindIII and EcoRV and subcloned into the HindIII and EcoRV sites of pcDNA3. Each mutant cDNA was completely sequenced to confirm the intended mutation.

Transient Expression of TPO—COS7 cells were grown to 70–80% confluence before transfection. Five μg of each plasmid DNA in 300 μl of Opti-MEMI medium (Life Technologies, Inc.) and 10 μl of LipofectAMINE (Life Technologies) in 300 μl of Opti-MEMI medium were mixed and added to the cells. After a 6–12-h incubation at 37 °C, the transfection medium was replaced with 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After an additional 48-h culture, the supernatant was removed for the quantitation of TPO.

FIG. 1. Primary and secondary structure of human TPO. The amino acid sequence of the mature exported protein is shown by the single letter code. Amino acid residues that create a consensus sequence of N-linked glycosylation by single replacement with asparagine are designated by an asterisk. The predicted amphipathic α helices are enclosed in rectangular boxes. Residues predicted to be on the surface are underlined. The asparagine replacement muteins are shown above the primary sequence, and the alanine replacement muteins are shown below.

FIG. 2. Dose-dependent proliferation of N-linked glycosylation muteins showing decreased bioactivity. Recombinant BaF3 cells expressing human c-Mpl were incubated with increasing amounts (0.1–100 ng/ml) of wild type (WT) or mutant TPO, and cell growth was determined by a colorimetric MTT assay. The optical density was measured at 540 nm in an ELISA reader. The x and y axes indicate the TPO concentration (ng/ml) and the optical density at 540 nm, respectively.
and cell proliferation assay.

Quantitation of TPO Proteins—The amount of TPO secreted into the culture supernatant of the transfected COS7 cells was determined by a sandwich ELISA using goat anti-human TPO polyclonal antibody (R & D Systems) and the biotinylated anti-human TPO polyclonal antibody. Biotinylation of the polyclonal antibody was performed using an American biotinylation kit. To detect the TPO proteins present in the culture medium, the supernatant was diluted 1:100 and added to each well (which had been coated with 1 μg of anti-human TPO polyclonal antibody). After washing, 100 μl of the biotinylated anti-human TPO polyclonal antibody (1:300 v/v) and streptavidin horseshadish peroxidase (1:1,000 v/v; Sigma) were sequentially added to each well and incubated at 37°C for 1 h. Finally, 100 μl of 0.2 M citrate-PO4 buffer (pH 5.0) containing 0.04% o-phenylenediamine (Life Technologies) and 0.03% H2SO4 was added and incubated for 10 min. The reaction was stopped by the addition of 50 μl of 2.5 M H2SO4, and the optical density was measured at 492 nm on a TiterTek ELISA reader. To determine the concentration of secreted TPO, the purified recombinant human TPO (R & D Systems) was used to generate a standard curve. The quantity of wild type or mutant TPO was determined as the mean optical density of triplicates.

Cell Proliferation Assay—The cell proliferation activity of wild type or mutant TPO was assayed using a recombinant murine BaF3 cell line expressing human c-Mpl (BaF3-Mpl) (1, 24). The BaF3-mpl cells were plated at a density of 2 × 105 cells/well, and after 22 h of incubation, the cell growth was determined by a colorimetric MTT assay according to Mosmann (19), with some modification (24). Briefly, MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in phosphate-buffered saline at a concentration of 5 mg/ml and filtered to sterilize. At the indicated times, the MTT solution (10 μl of medium) was added to each well, and the plates were incubated at 37°C for 4 h. The dark blue crystals of MTT formazan formed in the presence of living cells were solubilized by adding 100 μl of dimethyl sulfoxide (Pierce). After mixing at room temperature for 10 min, the optical density was measured at 540 nm in an ELISA reader. The optical density at 540 nm in the absence of any source of TPO (nonspecific background) was subtracted from each experimental point. Any nonspecific effect of other substances in the COS7 cell supernatants on the growth of cells has not been identified in the bioassay. The activity of each mutein was determined as the mean optical density of triplicates.

RESULTS

Strategies of Mutagenesis—Since the three-dimensional structure of TPO is not known, site-directed mutagenesis of TPO was based on the model of the three-dimensional structure of the amino-terminal domain (2). The structural model of the amino-terminal domain shows that the domain is comprised of four α-helices arranged with an up-up-down-down topology, and the four helices are located at residues 9-29 (helix A), 56-77 (helix B), 85-107 (helix C), and 127-147 (helix D). To identify the functionally important regions of TPO, a comprehensive alanine replacement scan of TPO would be appropriate, but it would require the preparation and testing of a large number of muteins, which are labor intensive and time consuming. Therefore, to reduce the number of required replacements and also to test the TPO model, 12 novel N-linked glycosylation sites, predicted to be on the external surface of the molecule and scattered almost evenly on the four helices and loops AB and CD, were separately introduced into the amino-terminal domain of the molecule. This was based on the assumption that sugar chains protruding on the surface of the molecule would prevent the adjacent amino acids in primary and/or tertiary structure from interacting with their receptor, and thus this mutation would have a more broad and powerful effect than alanine substitution. Next, the alanine replacement scan was focused on the regions that had been identified to be functionally important by the N-glycosylation replacement scan. Forty-four alanine replacements at mostly surface residues were constructed. All the replacements are presented in Fig. 1.

Expression of Wild Type and Mutant TPO in Mammalian Cells and Determination of Their Specific Bioactivities—A whole molecule of wild type or mutant TPO was transiently expressed in COS7 cells. The TPO protein secreted into the culture medium by the cells was analyzed for the specific bioactivity, i.e., biological activity/unit mass of protein. The amount of the secreted TPO was determined by a sandwich ELISA using anti-human TPO polyclonal antibody and the biotinylated polyclonal antibody, as described under “Experimental Procedures” (data not shown). For the measurement of the biological activity, a stable recombinant BaF3 cell line expressing human c-Mpl was constructed, and the cell proliferation in response to each mutein as well as wild type TPO was measured by colorimetric MTT assay. The bioactivities of the wild type or mutant TPO at different concentrations (0.1-100 ng/ml) are shown in Figs. 2 and 3. The cell proliferation stimulated by wild type TPO increased with the increase in the TPO concentration, and thus, it was almost saturated at the highest tested concentration (100 ng/ml). However, some muteins showed moderate or basal activities at the tested TPO concentrations. The other muteins with normal activity showed the same dose-dependent curve as displayed by wild type TPO (data not shown). The specific bioactivity of the wild type or

FIG. 3. Dose-dependent proliferation of alanine replacement muteins showing the decreased bioactivity. Dose-dependent proliferation assay was carried out as described in the legend to Fig. 2. WT, wild type.
Bioactivity of TPO muteins with novel N-linked glycosylation

The amino acid of wild type human TPO, its position from the N terminus, and its replacement according to the single-letter amino acid code are listed under the mutein column. Specific bioactivity of each mutein was expressed as a percentage of wild type human TPO bioactivity, with the background COST supernatant alone subtracted from the value. Each was induced from the mean of triplicate samples. Muteins and control muteins with low specific activity are bold face. Specific bioactivities of control muteins replaced by Ala will be described in Table II.

Table I

### Mutational Analysis of Human Thrombopoietin

| Domain | Mutein | Specific bioactivity | Control mutein | Specific bioactivity |
|--------|--------|----------------------|----------------|---------------------|
| Helix A | R17N  | 60.1 | R17A | 99.7 |
| | R25N  | 92  | R25A | 96  |
| Loop AB | D45N  | 45.6 | D45A | 90.8 |
| | W51N  | 14  |       |       |
| Helix B | E56N  | 97.3 | E56A | 93.6 |
| | A66N  | 70.2 |       |       |
| Helix C | Q92N  | 104.6 |       |       |
| | R98N<sup>a</sup> | 12.0 | R98A | 98.0 |
| |       |       | L100T | 57.6 |
| Loop CD | L108N | 113 |       |       |
| | R136N/K138T | 100.5 | R17A | 96.9 |
| Helix D | F128N | 2.8  | F128A | 8.6  |
| | R136N<sup>a</sup> | 0 | R136A | 97.5 |
| |       |       | K138T | 1.8  |

<sup>a</sup> Double mutation (R98N, R98N/L100T, R136N, R136N/K138T).

*mutant TPO was calculated as the biological activity at 100 ng/ml TPO concentration.

**N-linked Glycosylation Scan of TPO**—The N-linked glycosylation machinery of mammalian cells recognizes Asn-X-Ser/Thr to add carbohydrates to the side chain of asparagine (20). In choosing the sites to introduce N-linked glycosylations, we searched for the amino acid residues that are exposed to the surface and upstream of a serine or threonine residue by two amino acids to create the consensus sequence for N-linked glycosylation without disrupting much of the structure. Two sites per each helix or loop AB or CD were mutated, and thus, a total of 12 muteins, each with a single novel N-linked glycosylation site, were prepared, as shown in Fig. 1. Control muteins were prepared when charged residues were replaced by asparagine or when double mutations (R98N/L100T and R136N/K138T) were required to create the consensus sequence. The specific bioactivity of each mutein was compared with that of wild type TPO and expressed as a percentage of wild type TPO-specific bioactivity. The data are summarized in Table I.

(a) In predicted helix A, replacement of Arg<sup>17</sup> by asparagine (R17N) resulted in a considerably impaired specific bioactivity (60.1%), but the control mutein R17A had normal bioactivity, suggesting that the amino acid residues near the Arg<sup>17</sup>, but not the Arg<sup>17</sup> itself, may be involved in receptor binding. In contrast, R25N and R25A muteins retained almost normal activity.

(b) In predicted loop AB, D45N had decreased activity (45.6%), whereas D45A had nearly normal activity. Also, mutein W51N had abolished activity (14.0%). These results suggest that areas nearby these residues are important in the receptor binding.

(c) In predicted helix B, A66N showed somewhat impaired bioactivity (70.2%), but E56N and E56A had normal activity.

(d) In predicted helix C, mutein R98N/L100T had abolished bioactivity (12.0%), whereas control muteins R98A and L100T had normal and considerably impaired activity (57.6%), respectively. These data suggest that Arg<sup>98</sup> is not involved in the receptor binding, but nearby residues in the primary or tertiary structure may be involved in the binding. As far as the L100T mutatein is concerned, Leu<sup>100</sup> is predicted to reside internally on this helix. Therefore, it is presumed that the replacement of Leu<sup>100</sup> by threonine may partially disrupt the hydrophobic interaction of the helices and affected the activity.

(e) In predicted loop CD, two muteins, L108N and R117N, had normal activity.

(f) In predicted helix D, R136N/K138T and K138T showed complete loss of bioactivity, whereas R136A as a control mutein had normal bioactivity, suggesting that Lys<sup>138</sup> but not Arg<sup>136</sup> is directly involved in the receptor binding. Also two muteins, F128N and F128A, had impaired activity. In the model, Phe<sup>128</sup> is predicted to be inside this helix, suggesting that Phe<sup>128</sup> is very important for the hydrophobic interaction to stabilize its structure.

The bioactivities of the tested muteins at a wide (0.1–100 ng/ml) range of TPO concentration are shown in Fig. 2. The four muteins (R17N, D45N, A66N, L100T) showed moderate activities and five muteins (W51N, R98N/L100T, F128N, R136N/K138T, K138T) did not show any activity at the tested concentration.

Taken together, mutations at the five surface residues (R17N, D45N, A66N, R98N and K138T) significantly reduced the activity. These residues mapped to helix A, loop AB, helix B, helix C, and helix D, suggesting that these regions may be involved in receptor binding.

**Alanine Replacement Scan of TPO**—An alanine replacement scan of surface residues on the four helices and loop AB was performed. A total of 44 alanine replacement muteins, including the previous eight control muteins for the N-linked glycosylation scan, were prepared as shown in Fig. 1. The specific bioactivities of the muteins are summarized in Table II.

(a) In predicted helix A, seven muteins were tested. Among them R10A was nearly inactive (11.3%), and K14A was somewhat inactive (78.9%), whereas the others were active, indicating that the positively charged residues Arg<sup>10</sup> and Lys<sup>14</sup> may be important for the function.

(b) In predicted loop AB, 13 muteins were tested. P42A, F46A, and E50A had completely or considerably decreased bioactivity, whereas the other muteins retained the full activities. According to the TPO model, Phe<sup>46</sup> is not predicted to be exposed to the surface. Therefore, Pro<sup>42</sup> and Glu<sup>50</sup> may be important for the function.

(c) In predicted helix B, eight muteins were tested. None of them affected the activity, suggesting that helix B may not be directly involved in the receptor binding. The reduced bioactivity of A66N may be due to the steric hindrance or indirect effect induced by the protruding N-linked sugar.

(d) In predicted helix C, there is only one charged residue (Arg<sup>136</sup>). Alanine replacement of the residue was shown to have no effect on the activity, and the mutein Q105A also had normal bioactivity. The other surface residues in helix C may need to be tested. However, considering that R98N had impaired bioactivity, helix C may be slightly or indirectly involved in receptor binding.

(e) In predicted helix D, out of the tested eight muteins, the K138A and F128A showed almost completely abolished activity, and the other mutations did not affect the activity significantly. In the case of the Lys<sup>138</sup> replacement, by threonine (K138T) also abolished the activity in the N-linked glycosylation scan analysis (Table I). Therefore, Lys<sup>138</sup> seems to be very important in receptor binding. In the case of the Phe<sup>128</sup>, since this residue is predicted to be buried in helix D, it is presumed that this mutation affects backbone conformation of the helices of the amino-terminal domain and thus resulted in the loss of activity.

In the case of other regions (loops BC and CD), alanine
replacement of charged residues Arg\(^{78}\), Arg\(^{117}\), His\(^{121}\), Lys\(^{122}\), and Asp\(^{123}\) had no significant effect on the bioactivity. E72A and F131A were not secreted well by COS7 cells, as determined by ELISA and therefore could not be assayed for the bioactivity.

The six alanine replacement muteins (R10A, P42A, F46A, E50A, F128A, and K138A) that showed nearly abolished bioactivity were assayed using a wide range of TPO concentrations. As shown in Fig. 3, the result was in good agreement with the previous result.

Overall, we concluded that Arg\(^{10}\) and Lys\(^{14}\) of helix A, Pro\(^{42}\) and Glu\(^{50}\) of loop AB, and Lys\(^{138}\) of helix D are important functionally in receptor binding.

**DISCUSSION**

TPO is a recently identified cytokine that specifically stimulates the proliferation and differentiation of megakaryocytes (1–5). The amino-terminal domain of TPO was reported to bind to the receptor and stimulate human megakaryopoiesis in vitro and was predicted to have a four-helix bundle structure like EPO (1), but the precise mechanism and detailed structural information of how the TPO molecule interacts with its receptor are unknown. In this study, to identify functionally important residues that are involved in receptor binding, we constructed a series of site-directed mutants of TPO in which amino-terminal domains had single N-linked glycosylation additions or alanine substitutions based on the three-dimensional model of the amino-terminal domain, and the specific bioactivity of each TPO mutein in the whole molecule was analyzed. Our study identified that Arg\(^{10}\) and Lys\(^{14}\) of helix A, Pro\(^{42}\) and Glu\(^{50}\) of loop AB, and Lys\(^{138}\) of helix D are functionally important in the receptor binding. Since these residues are predicted to be on the surface of the molecule, alanine substitutions at these residues are not likely to affect the stability or folding. The functional residues are presented on the three-dimensional model of TPO, as shown in Fig. 4. Sequence comparison between human TPO and other mammalian TPO showed that the identified residues (Arg\(^{10}\), Lys\(^{14}\), Pro\(^{42}\), Glu\(^{50}\), and Lys\(^{138}\)) are identical among species, suggesting that the critical residues may be conserved evolutionally.

The functionally important residues of TPO are analogous to the previously identified receptor binding sites of hGH (22) and EPO (23). hGH and EPO have two separated regions that interact with two identical receptor molecules. The interaction site I resides in helix D and loop AB, whereas the interaction site II resides in helix A and helix C. The receptor-ligand interactions of both hGH and EPO are mediated mainly by the charged residues in helices A and D. In the case of helix A, Arg\(^{16}\) and Arg\(^{19}\) of hGH and Arg\(^{10}\) and Arg\(^{14}\) of EPO are essential for the interaction with their receptors. In case of helix D, Arg\(^{167}\), Lys\(^{168}\), and Asp\(^{171}\) of hGH and Arg\(^{143}\), Arg\(^{150}\), and Lys\(^{154}\) of EPO were shown to be in contact with their receptors. An analogy is also observed in TPO. The Arg\(^{10}\) and Lys\(^{14}\) of helix A and Lys\(^{138}\) of helix D of TPO were shown to be involved in the receptor binding (Table II). On considering that the receptors for hGH, EPO and TPO are homodimerized upon ligand stimulation and all the three cytokines have the functionally important positively charged amino acids in their helices A and D, the interaction of TPO with its receptor is highly likely to occur in the same fashion as hGH and EPO. Taken together, we propose that Lys\(^{138}\) of helix D and Pro\(^{42}\) and Glu\(^{50}\) of loop AB may constitute one functional region, whereas Arg\(^{10}\) and Lys\(^{14}\) of helix A may constitute the other functional region.
in interacting with two identical receptor molecules.

Very recently it was reported by Pearce et al. (17) that alanine substitutions of TPO at Asp8, Lys14, Lys59, Arg136, Lys138, and Arg140 caused the greatest reduction in receptor binding affinity. On comparing their data with ours, the localized overall functional regions (helices A and D and loop AB) are the same as our result, but the functionally important residues identified are different, except Lys14 and Lys138. Unlike their results, the specific bioactivities of alanine replacement mutants at Lys52, Lys59, Arg136, and Arg140 were all active in our system. Also, the receptor binding affinity (about 16%) of their K14A mutant is different from the specific bioactivity (78%) of ours. We did not test alanine substitution at Asp8. This discrepancy may be due to the differences in the forms of TPO molecule and the assay systems used in these two studies. They expressed the amino-terminal domain of TPO fused to the gene III coat protein of M13 bacteriophage in *Escherichia coli*, and the resulting TPO phage was directly used to bind to recombinant Mpl-IgG in an indirect ELISA. The binding affinities of the TPO mutants for the Mpl-IgG were determined by competitive ELISA using soluble TPO receptor as a competitor. We used the whole molecule of wild type or mutant TPO that had been expressed in mammalian cells and secreted by the cells to stimulate the c-Mpl-expressing responsive cells for the determination of the specific bioactivity.

In conclusion, we identified the functionally important regions and residues of human TPO that are involved in receptor binding and found that the receptor binding regions are analogous to those of the same cytokine family such as hGH and EPO. This information provides a better understanding of the molecular basis of TPO-receptor interaction. Also, our analysis system will be useful in elucidating a more detailed mechanism of the molecular interaction. Moreover, since TPO has great potential of treating thrombocytopenia that results from chemotherapy and bone marrow transplantation, this information will be useful in the development of more effective thrombopoietic agents.

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