Ligand Binding Characteristics of the Carboxyl-terminal Domain of the Cytokine Receptor Homologous Region of the Granulocyte Colony-stimulating Factor Receptor*

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Hiroyuki Anaguchi, Osamu Hiraoka, Kazuhiko Yamasaki, Shoko Naito, and Yoshimi Ota‡
From the Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan

The carboxyl-terminal domain (BC domain, roughly 100 amino acid residues) of the cytokine receptor homologous region in the receptor for murine granulocyte colony-stimulating factor was secreted as a maltose binding protein fusion into the Escherichia coli periplasm. The murine BC domain was prepared from the fusion protein by restriction protease factor Xa digestion and was purified to homogeneity. The purified BC domain specifically and stoichiometrically bound granulocyte colony-stimulating factor. This result indicates that the BC domain is also critical for ligand binding, as shown for the amino-terminal domain of the cytokine receptor homologous region (Hiraoka, O., Anaguchi, H., Yamasaki, K., Fukunaga, R., Nagata, S., and Ota, Y. (1994) J. Biol. Chem. 269, 22412–22419). The tertiary folding and the β-sheet structure of the BC domain were confirmed by NMR spectroscopy. The disulfide bond pattern suggested from peptide mapping was Cys224-Cys271 and Cys242-Cys285. Disruption of the disulfide bonds suggested that both bonds are critical for maintaining the folding of the BC domain, although a BC domain lacking the second bond still retained ligand binding activity. Mutational analysis of the WSXWS sequence conserved in the cytokine receptor family suggested that this motif is critical for protein folding rather than for ligand binding.

Granulocyte colony-stimulating factor (G-CSF)1 is a cytokine that plays an essential role in maintaining the number of neutrophilic granulocytes in peripheral blood and that is responsible for granulocytosis during inflammation (Nagata, 1990; Nicola, 1989; Demetri and Griffin, 1991). Since the administration of G-CSF causes granulopaesia, it is used to treat patients suffering from granulopenia (Lieschke and Burgess, 1992a, 1992b). Investigations of the ability of ligand binding to the G-CSF receptor to mediate a specific signal inside the cell may promote clinical applications of G-CSF. The cDNAs for the murine and human G-CSF receptors (800 amino acid residues) have been isolated (Fukunaga et al., 1990a, 1990b). The extracellular region of the G-CSF receptor (600 amino acid residues) has a composite structure containing an immunoglobulin-like domain, a cytokine receptor homologous (CRH) region, and three fibronectin type III domains (Fukunaga et al., 1990a, 1990b). Among them, the CRH region (200 amino acid residues) was predicted to be the ligand binding region (Bazan, 1990). Thus, knowledge of the CRH region is crucial to understanding the binding mechanism of the G-CSF receptor.

The CRH region was predicted to be cysteine-rich β-structure, which was defined by its striking homology to receptors for various cytokines, such as interleukins 2–7, erythropoietin, growth hormone (GH), and prolactin (Bazan, 1990). The CRH region shares two distinctive features in the extracellular region: four conserved cysteine residues, known as the "cysteine motif," are located in the amino-terminal domain of the CRH region (BN domain), and its carboxyl-terminal domain (BC domain) contains a "WSXWS" motif (Bazan, 1990). It is very likely that the cysteine motif constitutes a domain for the interaction of the receptor with its cognate ligand (Ullrich and Schlessinger, 1990). Deletion analysis of the G-CSF receptor cDNA indicated that the deletion of the BN domain completely abolished ligand binding (Fukunaga et al., 1991). Recently, Hiraoka et al. (1994), using an Escherichia coli maltose binding protein (MBP, maLE gene product) fusion system, showed that the BN domain of the G-CSF receptor was a discrete folding unit that could bind ligand. On the other hand, the G-CSF receptor with a deletion of the BC domain still retained the ligand binding activity, although the Kd value was increased as much as 50-fold, as compared with the intact molecule (Fukunaga et al., 1991). It was reported that the WSXWS motifs of the interleukin 2 (Miyazaki et al., 1991) and erythropoietin (Yoshimura et al., 1992) receptors were essential for ligand binding and signal transduction. These data indicated that the function of the G-CSF receptor BC domain in ligand binding is still unclear and suggested that the expression and purification of the BC domain in quantities suitable for biochemical and structural analyses would be essential for understanding the ligand binding mechanism of this receptor.

In the present study, we expressed the BC domain of the G-CSF receptor, using the E. coli MBP fusion system, and purified the product. The purified BC domain specifically bound ligand. Furthermore, we characterized the functions of the WSXWS motif and the cysteine residues.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids for the BC Domain and Its Mutants—The murine BC (mBC) domain corresponds to exons 7 and 8 of the murine G-CSF (mg-CSF) receptor (Seto et al., 1992) and contains 4 cysteine residues (Fukunaga et al., 1990a). Since we had developed an MBP fusion protein secretion system for the expression of the murine BN (mBN) domain, which contains many cysteine residues (Hiraoka et al., 1994), we directed the mBC domain fusion protein into the E. coli...
The term Ig-like in the mG-CSF receptor indicates the immunoglobulin-like domain. Fn3 indicates the fibronectin type III region, and TM indicates the transmembrane region. The thin bars in the mBN domain represent conserved cysteine residues. The thick bar in the mBC domain represents the WXSWS motif. Numbers correspond to the amino acid numbering of the mG-CSF receptor (Fukunaga et al., 1990a).

The far UV CD spectrum of the mBC domain is shown in Fig. 6. It has positive ellipticity around 198 °C-proton and negative ellipticity around 213 nm (Fig. 6, left panel). Expression and purification of the plasmid pMALp-mBC was introduced into E. coli strain K5474 (Strauch et al., 1989). Transformed E. coli cells were grown in M9 medium (usually a 4-l. culture) containing 0.8% glucose at 37 °C to an A500 of 0.6, and the expression of the MBP-mBC fusion protein was induced by adding isopropyl-1-thio-b-D-galactopyranoside to a final concentration of 1 mM. After incubation for 18 h, the cells were pelleted, and the periplasmic fraction was prepared as described (Hirakado et al., 1994). After the addition of ammonium sulfate with stirring to 20% saturation (11.4 g/100 ml), the solution was applied to a butyl-Toyopearl 650M (2.5 cm, 6 cm; Tosoh), equilibrated with 0.1% trifluoroacetic acid, and eluted with a linear gradient of acetonitrile from 0 to 40%. The fractions were collected manually and lyophilized. The amino acid sequence was determined by a protein sequencer (Applied Biosystems 477A sequencer equipped with a model 120A PTH analyzer). Disulfide bonds were identified by detecting diphénylthiodantoin (PTH)-cystine in the corresponding cycle (Marti et al., 1987).

RESULTS

Initial Characterization of the Purified mBC Domain—The MBP-mBC fusion protein (54 kDa) from the E. coli periplasmic fraction was digested with restriction protease factor Xa to separate the mBC domain from the MBP, and the mBC domain was purified to homogeneity as described under “Experimental Procedures” (Fig. 1). The molecular mass of the mBC domain was estimated to be 12.5 kDa by gel filtration HPLC and by automated sequencer, indicated that the purified mBC domain suffered no degradation of the purified mBC domain, using a gas-phase automated sequencer, indicated that the purified mBC domain has the expected amino-terminal sequence of GSSLEP. The amin proton shift was performed for 3 h at 40 °C in 50 mM Pipes, pH 6.5, containing 10 mM CaCl2. Cyanogen bromide (1% w/v) treatment in 70% formic acid was performed for 24 h at room temperature. Thereafter, pepsi n (Sigma) (1/100 (w/v)) digestion was performed for 8 h at 30 °C in 5% formic acid. The periplasmic fluid was separated by C18 reverse phase high performance liquid chromatography (HPLC) (TSK gel ODS-120T; 4.6 mm, inner diameter, × 250 mm; Tosoh), equilibrated with 0.1% trifluoroacetic acid, and eluted with a linear gradient of acetonitrile from 0 to 40%. The fractions were collected manually and lyophilized. The amino acid sequence was determined by a protein sequencer (Applied Biosystems 477A sequencer equipped with a model 120A PTH analyzer). Disulfide bonds were identified by detecting diphénylthiodantoin (PTH)-cystine in the corresponding cycle (Marti et al., 1987).
The purified mBC domain retained its activity after storage at 4°C for over 2 months.

Competitive Ligand Binding Assay—To measure the ligand binding activity of the mBC domain, we performed a competitive ligand binding analysis using chemical cross-linking. As shown in Fig. 4A, when 125I-G-CSF was incubated with the mBC domain and cross-linked, an extra band migrating at 30 kDa was revealed by SDS-PAGE. The size of the 30-kDa band corresponds to that of the receptor-ligand complex, which consists of one molecule of G-CSF (19 kDa) per molecule of mBC (12.5 kDa). As a control, a 125I-labeled 30-kDa complex was also formed with the BN domain as described (Hiraoka et al., 1994). Both of the 125I-labeled 30-kDa bands formed with either the BN or the BC domain were competed away by almost the same concentration of unlabeled G-CSF. These results suggest that the BC domain specifically and stoichiometrically bound to G-CSF and that its binding affinity is similar to that of the BN domain. The apparent $K_d$, derived from several repeated competitive ligand binding analyses using chemical cross-linking and obtained with different preparations of the mBC domains, was $4 \times 10^{-8} M$. The specificity of the ligand binding activity of the mBC domain was further studied. As shown in Fig. 4B, the presence of 1 μM (about 1000-fold excess to the labeled G-CSF) of the human GH receptor BC domain, as well as either RNase A or bovine serum albumin in the assay mixture as a competitor, did not inhibit the binding of 125I-G-CSF to the purified mBC domain, whereas the same amount of unlabeled G-CSF competed with 125I-G-CSF for binding to the purified mBC domain.

Limited Proteolysis and Identification of the Disulfides of the mBC Domain—The mBC domain encodes four cysteine residues (Cys224, Cys242, Cys271, and Cys285). Titration of the mBC domain with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of 6 M guanidine hydrochloride did not reveal any free thiols (data not shown), suggesting that all of the cysteine residues in the mBC domain formed disulfide bonds. To assign the individual disulfide bonds in the mBC domain, cystine-containing fragments of the mBC domain were isolated by HPLC after proteolytic digestion. Cystine residues were directly identified as di-PTH-cystine after release in the corresponding cycle during Edman degradation, as reported (Marti et al., 1987; Hiraoka et al., 1994). The mBC domain was digested with thermolysin in the absence of reductants, and a fragment (TL11) was separated by C18 reverse phase HPLC. Sequencing revealed that the TL11 fragment consisted of two major, equal quantities of regions, which were interpreted as being MEQEC242E (residues 238–243) and LQMRC285 (residues 281–285) (Fig. 5A), in which the C in the sequence denotes a cystine directly identified as a di-PTH-cystine in cycle 5. Digestion with pepsin was also performed after the treatment with cyanogen bromide, and two fragments (BP17 and BP22)

**Fig. 2. Purification of the mBC domain.** Non-reducing 0.1% SDS, 15% PAGE analysis of proteins present in various stages of the purification is shown. The gel was stained with Coomassie Brilliant Blue. Lane 1, the eluate from the butyl-Toyopearl column; lane 2, the eluate from the S-Sepharose column; lane 3, the eluate from the O-Sepharose column (before factor Xa digestion); lane 4, the factor Xa digest; lane 5, the eluate from the S-Sepharose column. Lane M shows the molecular weight standards. The numbers show the sizes (kDa) of the marker proteins. The faint band above the main 12.5-kDa band in lanes 4 and 5 disappeared in the electrophoresis under reducing conditions. This faint band should be derived from a protein that formed an unexpected disulfide bond during the electrophoresis.

**Fig. 3. NMR of the mBC domain.** The 1H-NMR spectrum was measured on a 500-MHz spectrometer (Bruker AM-500) at 20°C. The purified mBC domain was dissolved in 20 mM sodium phosphate buffer, pH 5.5, at a concentration of 12.5 mg/ml (1 mM). The arrow a indicates the extremely high field-shifted methyl proton resonances. The arrow b indicates the down field-shifted α-proton resonances. The protein concentration of the purified mBC domain was calculated from the absorption at 280 nm (a value of 2.9). This value was calculated using 1,576 M⁻¹-cm⁻¹ for tyrosine and 5,225 M⁻¹-cm⁻¹ for tryptophan at 280 nm (Goodwin and Morton, 1946).
were separated by C18 reverse phase HPLC. Sequencing of the BP17 fragment revealed two major, equal quantities of sequences, which were interpreted as being EGE{C24{E}ELRYQ-PQLKGAN (residues 239-254) and RX{P}281{S}SSLPGF (residues 284-293) (Fig. 5A). The X in the sequence denotes a Cys residue predicted from the known sequence but which was not evident during the Edman degradation. These data indicated that these two sets of sequences were connected by a disulfide bond between Cys{C}24{C}2 and Cys{C}285{C}2 and suggested that the Cys{C}24{C}2 residues should be connected by a disulfide bond (Fig. 5B). The fragment BP22 contains two equal quantities of sequences, interpreted as being LD{G}{P}D{V}SH{Q}{P}{G} (residues 211-223) and ELX{G}{L}{H}{Q}{A}{P}{V}{Y}{T} (residues 269-280) (Fig. 5A). The di-PTH-cystine should be identified at the carboxyl terminus of the former fragment (residue 211-223) as residue 224 but was not analyzed because of the low purification yield of the fragment. However, these data are consistent with the disulfide pattern suggested above (Fig. 5B).

Cysteine Mutants—To determine the effect of each of the disulfide bonds of the mBC domain on the structure and the ligand binding activity of the receptor, we mutated each pair of cysteine residues to alanine (C224A/C271A and C242A/C285A), thereby disrupting these disulfide bonds. Among them, only the second disulfide bond mutant (C242A/C285A) was purified to homogeneity. The far UV CD spectrum of the mutant was similar to that of the wild-type mBC domain (Fig. 6, left and middle panels, at 20 °C). To compare the stabilities of the mutant and wild-type proteins, their apparent denaturations were measured by comparison of the CD spectra at various temperatures. The apparent thermal denaturation of C242A/C285A began around 35 °C because the positive and negative peaks of the CD spectra started to decay around this temperature (Fig. 6, middle panel). In contrast, the apparent denaturation of the wild-type mBC domain began at 50 °C (Fig. 6, left panel). This indicates that the second disulfide mutant (C242A/C285A) was more labile than the wild type. The second disulfide mutant (C242A/C285A) still retained the ligand binding activity. Its K{D} appears to increase more than 10-fold compared to the wild-type mBC domain, but we could not determine it accurately because of the lability of the protein. The 54-kDa band, which corresponds to the first disulfide bond mutant (C224A/C271A) fused to the MBP, was detected in whole cell extracts by SDS-PAGE and Western blotting. The intensities of the 54-kDa bands of the native and mutant proteins were similar, suggesting similar expression levels. However, a reduced level of the mutant 54-kDa protein was detected in the periplasm. The mutant 54-kDa protein eluted as a broad peak from the S-Sepharose column, whereas the wild-type mBC domain was obtained as a sharp peak. The mutant C224A/C271A mBC product was precipitated immediately after the restriction protease factor Xa digestion. It appears that both disulfide bonds are critical for the maintenance of the stably folded protein.

WSXWS Motif Mutants—To determine the function of the WSXWS motif, we constructed four WSXWS motif mutants (W294A, S295A, W297A, S298A). The expression levels of the native and the four mutant proteins were similar. Among them, the W294A and the S298A mutants were purified to homogeneity. The mutant W294A retained ligand binding activity (Fig. 4A). It appeared that its apparent K{D} obtained by several experiments, is somewhat higher than that of the wild-type mBC domain, but the difference is not remarkable. The far UV CD spectrum of the mutant W294A is similar to that of the wild type (Fig. 6). The beginning of the apparent thermal denaturation of the mutant W294A, determined by measuring the CD spectra at various temperatures, appears around 50 °C (Fig. 6, right panel), indicating that the stability of the mutant W294A is similar to that of the wild-type mBC domain. The mutant S298A also retained ligand binding activity. However, we could not compare the ligand binding activity and the CD spectrum of the mutant S298A to those of the wild-type mBC domain because of its low purification yield and stability, such as precipitation during concentration. It appeared that the expression levels of the fused proteins (54 kDa) corresponding...
to two other mutants (S295A, W297A) were similar to the wild type, but the amounts of these bands were reduced in the periplasmic fraction, as determined by SDS-PAGE and Western blotting. These two fused mutants were obtained as broad peaks by S-Sepharose chromatography, whereas the wild type was obtained as a sharp peak, and the final products were precipitated by factor Xa digestion. Thus, it appears that the disruption of the WSXW motif did not affect the ligand binding activity remarkably but instead affected the stable folding of the mBC domain, although the W294A substitution still allowed stable folding of the mBC domain.

**DISCUSSION**

The BC domain specifically and stoichiometrically bound to G-CSF. The tertiary folding of the BC domain was confirmed by NMR spectroscopy. These data indicated that this domain (approximately 100 residues) folds discretely. NMR and far UV CD spectra indicated that the BC domain has β-structure. Analysis of the disulfide bonds of the mBC domain of the G-CSF receptor
showed that the 4 cysteine residues of this domain formed two disulfide bonds. The structure of the CRH region of the cytokine receptor was predicted to be β-strands held together by disulfide bonds (Bazan, 1990). These two disulfide bonds (Cys224-Cys271, Cys242-Cys285) were mapped between the B and E β-strands and between the C and F β-strands, respectively, on the predicted topology (Fig. 7). These BC domain disulfide bonds are not conserved in the cytokine receptor family (Bazan, 1990). However, two disulfide bonds are essential for proper folding of the BC domain of the G-CSF receptor. Miyazaki et al. (1991) determined that the two Trp residues in the WSXS motif are critical for proper ligand binding to the interleukin-2 receptor β-chain, with the use of a mutant cDNA transfection system in lymphoma cells. Rozakis-Adcock and Kelly (1992) reported that substitution of the WSXS motif precluded high affinity ligand binding by the prolactin receptor. However, our data suggest that the WSXS motif may play an important role in the proper folding of the BC domain rather than in the formation of the ligand binding site. It is likely that the disruption of the WSXS motif leads to the formation of an abnormally folded receptor and to a reduction in the ligand binding activity. Recent x-ray crystallographic analyses of the GH and prolactin receptors showed that the WSXS motif (which is not completely conserved in the GH receptor) is an irregular, extended chain immediately preceding the following short β-strand and is quite removed from the interfaces that contact ligand (de Vos et al., 1992; Somers et al., 1994). Interestingly, it was reported that the WSXS box of the prolactin receptor is part of a much larger, highly organized pattern, and the side chains of the two Ser residues form hydrogen bonds to the main-chain atoms of a neighboring β-strand (Somers et al., 1994). In the present work, the removal of the side chains of the Ser residues in the two mutants S295A and S298A caused liability of the mBC domain. Thus, it can be speculated that these Ser side chains may form hydrogen bonds that anchor the strands preceding the β-structure and stabilize the BC domain. Yashimura et al. (1992) suggested that the WSXS motif of the erythropoietin receptor is critical not only for ligand binding but also for the ability of the receptor to exit from the endoplasmic reticulum. Abundant work on secretory proteins suggests that there is selective retention and/or degradation of unfolded, misfolded, or aggregated proteins in the endoplasmic reticulum (Pelham, 1989). These results are also consistent with our results. The reduction of the yield of our precipitable mutants (S295A and W297A) of the BC domain from the periplasmic fraction is compatible with these results.

The apparent Kd of 4–10 × 10⁻⁸ M indicates that the BC domain plays a critical role in the recognition and the binding of the ligand. This Kd value is similar to that of 3–8 × 10⁻⁹ M exhibited by the purified mBN domain of the G-CSF receptor (Hiraoka et al., 1994). Both of the Kd values of the BN and the BC domains are relatively high as compared to the Kd of 3–4 × 10⁻¹⁰ M exhibited by the deletion mutant of the G-CSF receptor expressed on the surface of murine myeloid cells, which contains both the BN and the BC domains (Fukunaga et al., 1991). Our previous study indicated that the BN domain also folds discretely (Hiraoka et al., 1994). These results indicate that the CRH region of the G-CSF receptor is composed of two small BN and BC domains, as predicted (Bazan, 1990), and both domains are required for high affinity ligand binding, such as −10⁻⁹ M. Probably, G-CSF recognition occurs via a generic binding trough within the CRH region formed by the BN and BC domains. Mutational and x-ray crystallographic analyses of the human GH receptor indicated that two amino acid residues (Trp¹⁰⁴ and Pro¹⁰⁶) in its BN domain are critical for human GH binding (Bass et al., 1991; de Vos et al., 1992). Such important amino acid residues for ligand binding were not detected in the BC domain of the human GH receptor, although a substantial contact surface with the ligand exists in the BC domain. However, most of the residues important for ligand binding in the GH receptor and the interleukin-6 receptor could be located in or near the hinge region of the two terminal domains (Bass et al., 1991; Yawata et al., 1993).

In earlier work, Fukunaga et al. (1991) reported the absence of detectable affinity of the deletion mutants (YΔ(5–195)) of the mG-CSF receptor, which contains the BC domain but lacks the immunoglobulin-like and the BN domains, at the cell surface. Probably, the Kd value of the mutant YΔ(5–195) was below the level of detection among the series of mutants analyzed with their system. Recent analyses suggest that receptor oligomerization is the major consequence of ligand binding, and a 2:1 receptor-ligand complex was detected with the GH receptor (Cunningham et al., 1991; Fuhr et al., 1991; de Vos et al., 1992). However, we did not detect a 2:1 mBC domain-G-CSF complex by SDS-PAGE using chemical cross-linking. This is reasonable, because our recent study indicated that both the BC and the immunoglobulin-like domains, in addition to the BN domain, would aid further investigations of the mechanism and function of this receptor. The size of the purified BC domain (12.5 kDa) is sufficiently small for NMR analysis.

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