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Evidence for the ligand-induced conversion from a dimer to a tetramer of the granulocyte colony-stimulating factor receptor

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Abstract An extracellular portion of granulocyte colony-stimulating factor (G-CSF) receptor, which contains an immunoglobulin-like (Ig) domain and cytokine receptor homologous (CRH) region, was secreted into the medium using *Trichoplusia ni-Autographa californica* nuclear polyhedrosis virus system. The gene product was purified to homogeneity mainly as a dimer (85 kDa) using G-CSF affinity column chromatography and gel filtration HPLC, although the product existed as a monomer (45 kDa) in the medium. Scatchard analyses suggested that only the dimer had high affinity ligand binding (K_d = about 100 pM), which is comparable with the K_d value of the cell surface receptor. The binding of G-CSF to Ig-CRH induced its tetramerization (200–250 kDa). The molecular composition of the tetrameric complex showed a stoichiometry of four ligands bound to four Ig-CRH. These results suggested that the oligomeric mechanism of the G-CSF receptor differs from that reported for growth hormone (GH) receptor, although CD spectrum spectroscopy suggested that the Ig-CRH has a GH receptor-like structure.

Key words: Extracellular portion; Granulocyte colony-stimulating factor receptor; Insect cell; Tetramer

1. Introduction

Ligand induced receptor oligomerization has been proposed as the key mechanism of signal transduction for a family of single transmembrane receptors such as cytokine receptors and tyrosine kinase-type receptors [1,2]. In models, oligomerization of extracellular portions induced by their ligand binding are followed by activation of their cytoplasmic regions. For detailed studies of the oligomerization mechanism of receptors, it is essential to analyze the molecular basis of their extracellular portion in solution.

Granulocyte colony-stimulating factor (G-CSF) receptor is a member of a family of receptors for various cytokines, such as interleukins 2–7, erythropoietin, growth hormone (GH), interferons α, β, γ and prolactin [3]. A requirement for dimerization of the cytoplasmic region of the G-CSF receptor for the signal transduction has been suggested using a chimera with the GH receptor [4,5]. The extracellular region (~600 amino acid residues) of the G-CSF receptor has a composite structure containing an immunoglobulin-like (Ig) domain, a cytokine receptor homologous (CRH) region, and three fibronectin type III-like domains [6, 7]. The amino terminal domain (~100 amino acid residues) of CRH has been expressed as a minimun ligand binding unit using *E. coli* system [8]. The purified domain exhibited ligand binding activity, but it did not form an oligomeric structure [8]. Deletion analyses using the cDNA of G-CSF receptor have indicated that the cDNA encoding the Ig domain and the CRH region (Ig-CRH; ~300 amino acid residues) as the ligand binding unit had almost the same the ligand binding activity as the wild type cDNA [9]. Thus, we expressed Ig-CRH to analyze the oligomerization.

Here, we describe the expression of a gene encoding the Ig-CRH of the G-CSF receptor extracellular portion using a *Trichoplusia ni-Autographa californica* nuclear polyhedrosis virus system to analyze the oligomerization mechanism of the G-CSF receptor. The expressed Ig-CRH was purified mainly as a dimer. Furthermore, the Ig-CRH was converted to a tetramer by G-CSF.

2. Materials and methods

2.1. Production of recombinant baculovirus carrying the gene encoding Ig-CRH of G-CSF receptor

The DNA encoding the murine Ig-CRH of G-CSF receptor and its signal sequence was amplified by the polymerase chain reaction from plasmid pBLJ17 coding the murine G-CSF receptor (Fig. 1A) [6]. The 5′ sense primer had the sequence 5′-CGGGATCCATGGTAGGGC-TGGGAGCCTG-3′, which contains the *Bam*HI site at the amino terminus, followed by the coding sequence for the murine G-CSF receptor starting from Met^{-25}. The 3′-antisense primer has the sequence 5′-GCGGATCCTTAGGCCTTCATGGTAGGCCTCA-3′, which contains the coding sequence for Ala^{309} of the G-CSF receptor, followed by a stop codon and the *Bam*HI site. The transfer vector pAcIg-CRH was obtained by the insertion of the amplified DNA into a *Bam*HI site of a plasmid pAcYM1[10], which carries part of the genome of *Autographa californica* nuclear polyhedrosis virus. The Bam HI site in pAcYM1 had been inserted downstream of the polyhedrin promoter. A recombinant virus *Ac*Ig-CRH carrying the Ig-CRH gene was produced by in vivo homologous DNA recombination using the transfer vector pAcIg-CRH, as described [11,12].

2.2. Expression and purification of the Ig-CRH

Trichoplusia ni cells (Invitrogen; High Five Cells) cultured to confluence were diluted 10-fold with Sf-900 II SFM medium (GIBCO) and incubated at 27°C for one day in a 225-cm^2 flask (Costar). Culture cells were infected with the recombinant virus *Ac*Ig-CRH and cultured for 9 days at 18°C. The culture supernatant was passed over a G-CSF affinity column (1 × 5 cm; prepared as described by Fukunaga et al.) [13]. then washed thoroughly with phosphate buffered saline. The protein was eluted in a stepwise manner, with 0.1 M glycine-HCl buffer pH 2.0 containing 0.2 M NaCl. The eluted protein was immediately neutralized with 2 M Trizma base and applied to a gel filtration HPLC column (7.6-mm, inner diameter, ×60 cm; TSKgel G3000 SW HPLC column; TOSO Co., Ltd) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. The sample was eluted with the same solution at a flow rate of 0.5 ml/min, and the fractions containing the Ig-CRH were collected.

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Abbreviations: CRH, cytokine receptor homologous region; G-CSF, granulocyte colony-stimulating factor; GH, growth hormone; HPLC, high performance liquid chromatography; Ig, immunoglobulin-like; kDa, kilodalton; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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2.3. G-CSF Binding to Ig-CRH

A ^{125}I-G-CSF binding assay was performed by means of gel filtration. The Ig-CRH was mixed with ^{125}I-G-CSF (up to 200,000 cpm) in 50 μl of 50 mM sodium phosphate buffer (pH 7.0). The binding reaction proceeded for 90 min at room temperature. Thereafter, the mixture was applied to TSKgel G3000SW HPLC equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl and eluted with the same buffer at 0.5 ml/min. The Ig-CRH·^{125}I-G-CSF complex was analyzed by the tracing the radioactivity using a γ-counter (Packard, AUTO-GAMMA model A5002 COBRA). The assay used for Scatchard analysis of the Ig-CRH was essentially that as described by Fukunaga et al. [13]. The Ig-CRH was mixed with various concentrations (10 pM to 1 nM) of ^{125}I-G-CSF (Amersham International, plc.) in 50 μl of phosphate buffered saline containing 10% fetal calf serum and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesufonic acid in the presence or absence of 500 nM unlabeled G-CSF. The binding reaction proceeded for 90 min at room temperature. After incubation, the Ig-CRH·G-CSF complex was precipitated with carrier protein (γ-globulin) by 15% (w/v) polyethylene glycol 6000. The radioactivity in the precipitate was measured using the γ-counter as described above.

3. Results and discussion

3.1. Expression and Purification of Ig-CRH using insect baculovirus secretion system

Culture fluid of *Trichoplusia ni* cells infected with the recombinant virus *Ac*Ig-CRH was applied to a G-CSF affinity column and the G-CSF binding activity was eluted by the glycine-HCl buffer, pH 2.0. When the eluted material was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), an homogenous 45 kDa band was identified (Fig. 1B). Western blotting using anti-M1 serum specific for the CRH of the G-CSF receptor [9] identified the same 45 kDa band (Fig. 1B). The 45 kDa band identified by Western blotting in the culture fluid disappeared upon adding tunicamycin, a glycosylated inhibitor, to the culture medium after infection and there was a corresponding increase in the 40–42 kDa band in the cell extract. The molecular mass of 40–42 kDa is similar to the molecular weight of the monomeric form of the Ig-CRH deduced from the DNA sequence [6]. These results indicated that the 45 kDa band was the glycosylated Ig-CRH, because the Ig-CRH has five potential Asn-linked glycosylation sites [6].

The affinity purified protein was further chromatographed by gel filtration HPLC. As shown in Fig. 1C, the protein was eluted mainly at a position of a molecular mass of 85 kDa as well as two minor proteins having a molecular mass corresponding to 45 and 200–250 kDa (Fig. 1C). The positions of the eluted peaks suggested that the purified Ig-CRH existed as monomer, dimer and tetramer. SDS-PAGE and Western blotting confirmed that all three peaks identified as the homogenous 45 kDa band. Contamination of the G-CSF into all three peaks during the G-CSF affinity chromatography was not detected. The product in the culture fluid on the gel filtration HPLC was eluted at the position of 45 kDa, suggesting that Ig-CRH was secreted as a monomer (identified by the Western blotting and ^{125}I-G-CSF binding activity). Thus, the dimeric and tetrameric forms were generated by the G-CSF affinity column chromatography. The dimer as the main product was re-chromatographed by gel filtration and used for further experiments. About 0.2–0.5 mg of purified dimeric Ig-CRH was constantly obtained per liter of culture.

3.2. Initial characterization of the dimeric Ig-CRH

Only one amino-terminal amino acid sequence detected in purified protein was identified as CGHISPP by direct amino acid sequencing on an Applied Biosystems automated gas-phase sequencer. This sequence was the same as that of the amino-terminus of the receptor for G-CSF deduced from the murine G-CSF receptor cDNA sequence [6], confirming that the purified protein was the Ig-CRH. The results also indicated that *Trichoplusia ni* cells recognized and correctly cleaved the mammalian signal sequence. The far UV CD spectrum of the Ig-CRH exhibited positive ellipticity at 230 nm and negative ellipticity around 210 nm (data not shown). This was basically similar to the spectrum of the intact extracellular region of the human GH receptor [14], although the latter is composed of a CRH region and lacks the Ig-like region. The extracellular region of the human GH receptor is composed of β-sheets and turns that are held together by disulfide bonds, and which lack an α-helix [15]. A similar CD spectrum has also been found for the amino-terminal domain of the CRH region of the G-CSF receptor [8]. These results suggested that the Ig-CRH has a GH receptor-like structure.

Scatchard analysis of ^{125}I-G-CSF binding data showed that the dimeric Ig-CRH has two classes of affinity, termed 'high' (K_d = approximately 100 pM), and 'low' (K_d = approximately 2.5 nM). In contrast, the gene product secreted into the culture medium seemed to be a monomer (45 kDa; detected by gel filtration HPLC, Western blotting and G-CSF binding activity), and exhibited low affinity ligand binding. These results suggested that the high affinity ligand binding was induced by the dimerization of the purified protein. The G-CSF binds on the cell surface only with high ligand affinity [13]. Thus, it appeared that the G-CSF receptor on cells are not monomers and ligand binding is not required for the dimerization of the G-CSF receptor in vivo. In solution, the dimer may exist as two forms, because of the low ligand affinity found in the purified dimer. These data were consistent with the results of the native receptor purified from the mouse myeloid leukemia cell line NSF-60, indicating that the monomer (100–130 kDa) exhibited low affinity ligand binding, whereas oligomeric forms (200 kDa and larger) have the low and the high affinity ligand binding [13]. As described above, we purified a trace amount of tetrameric Ig-CRH as well as the monomer. This tetrameric Ig-CRH retained the G-CSF binding activity. This tetramer may correspond with the larger native oligomeric form having a molecular mass above 200 kDa. However, we could not characterize the purified tetrameric Ig-CRH precisely, because of the low yield of the purification.

3.3. Tetramerization of Ig-CRH induced by ligand

To analyze the effect of the ligand G-CSF upon the oligomerization of the Ig-CRH, the size of the complex of purified dimeric Ig-CRH (85 kDa) and the G-CSF (19 kDa) was established by separating mixtures of them (in ratios of 1:10, 1:1, 1:0.5, 1:0.1 and 1:0) by gel filtration on TSK gel G3000 SW HPLC (Fig. 2A). At a 1:1 ratio of Ig-CRH to G-CSF, virtually all the protein chromatographed at the position of a molecular mass of 200–250 kDa, which corresponds to that of a complex composed of tetrameric Ig-CRH. When the ratio of Ig-CRH to G-CSF is 1:10, excess free G-CSF (19 kDa) peak was present. Dissociation of the 200–250 kDa peak and appearance of

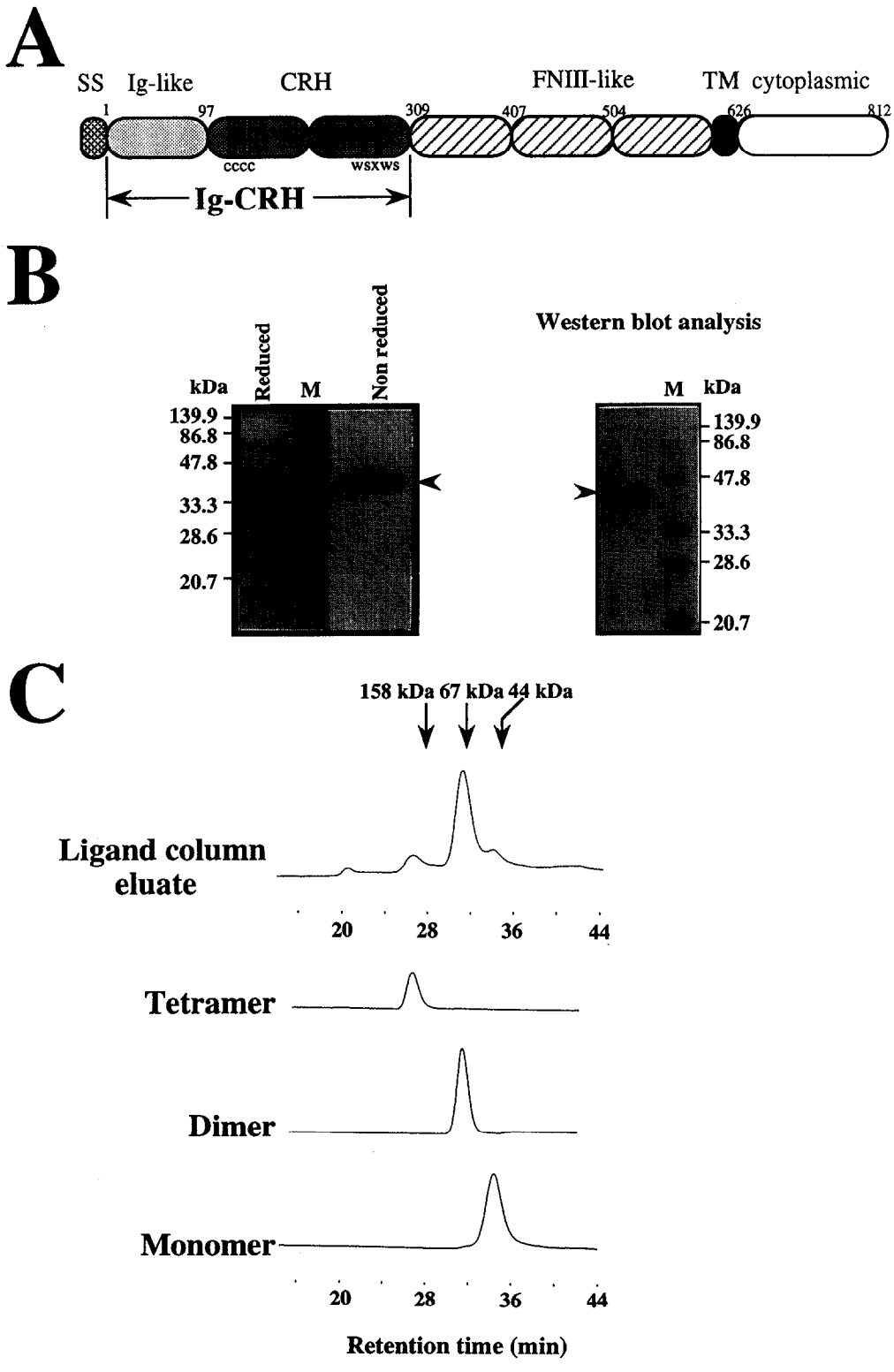

Fig. 1. Purification of Ig-CRH of G-CSF receptor. Panel A shows a schematic representation of the Ig-CRH of the murine G-CSF receptor [6]. SS, FNIII-like and TM indicate a signal sequence, fibronectin type III-like domain and a transmembrane domain, respectively. The thin bars in the G-CSF receptor represent conserved cysteine residues; the thick bar represents conserved WSXWS motif. Numbers indicate the amino acid number of the G-CSF receptor. Panel B shows the 0.1% SDS-12.5% PAGE (reducing and non-reducing conditions) and Western blotting (reducing condition) of the eluate from the G-CSF affinity column chromatography. The SDS-PAGE gels were stained with Coomassie brilliant blue. Western blotting was performed as described [8]. Arrow heads indicate the position of the Ig-CRH. The numbers show the sizes (kDa) of marker proteins in lane M. Panel C shows the TSK gel G3000 SW gel filtration HPLC profile of the G-CSF affinity column eluate. Arrows show molecular size markers (kDa).

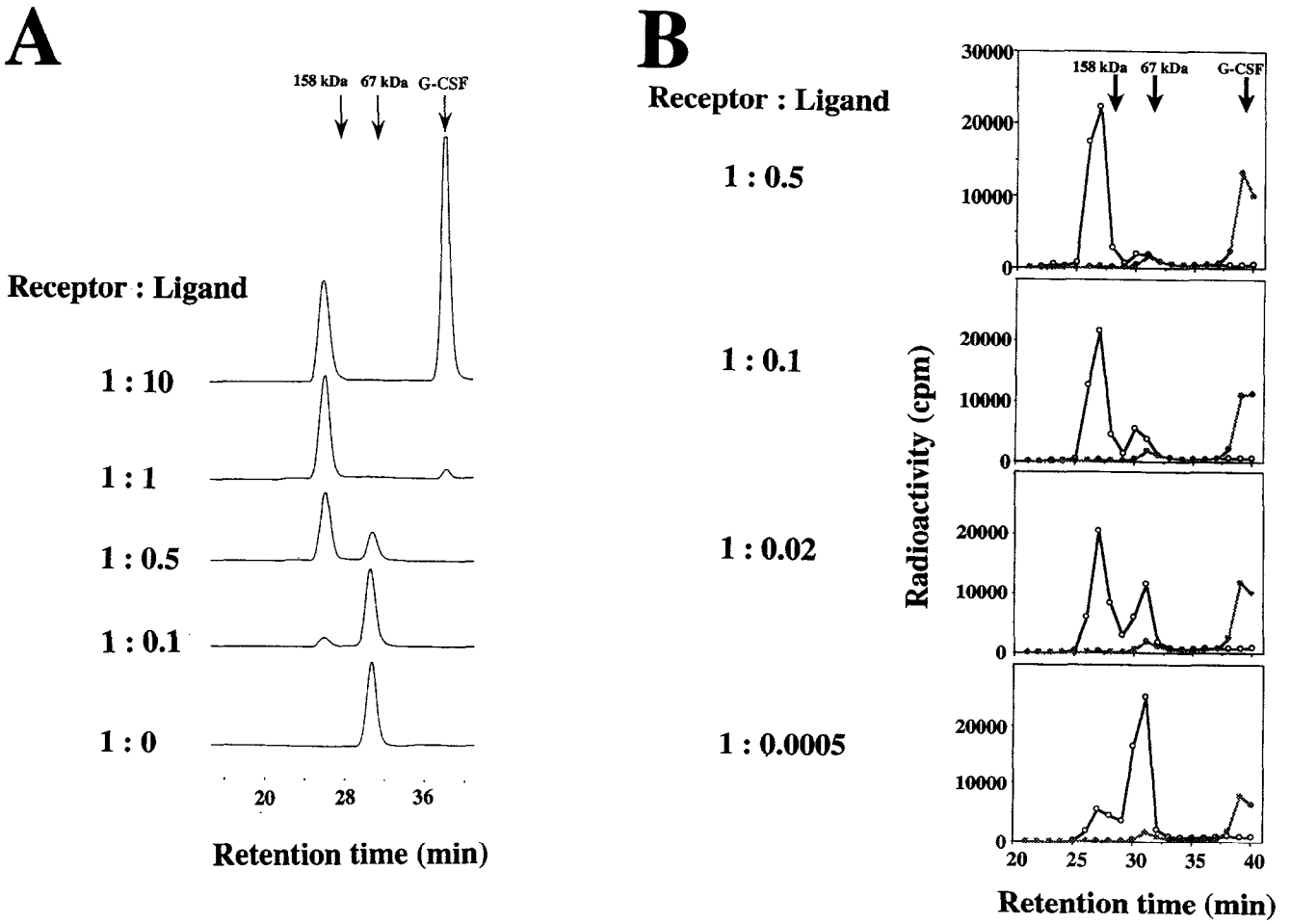

Fig. 2. Gel filtration of Ig-CRH·G-CSF complexes. Panel A shows the gel filtration profile of various ratios of Ig-CRH to G-CSF corresponding to 1:10, 1:1, 1:0.5, 1:0.1, 1:0 (top to bottom tracing). The protein concentrations of Ig-CRH (fixed at 1.6 μM) and G-CSF were calculated from the absorption at 280 nm (an $A_{280}^{0.1\%}$ value of 1.83 and 0.74, respectively). This value was calculated using 1,576 $M^{-1} \cdot cm^{-1}$ for tyrosine and 5,225 $M^{-1} \cdot cm^{-1}$ for tryptophan at 280 nm [21]. Protein mixtures were equilibrated for 90 min at 20°C in 20 mM sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl. Samples (80 μl) were applied to a TSKgel G3000SW gel filtration HPLC and eluted with the same buffer at 0.5 ml/min. Peaks were monitored for absorbance at 280 nm. The elution positions of the molecular size markers (kDa) are indicated by arrows. Panel B shows the gel filtration profile of Ig-CRH·G-CSF complex at a low concentration of the G-CSF containing ^{125}I-G-CSF. Ratios of Ig-CRH to G-CSF were corresponded to 1:0.5, 1:0.1, 1:0.02, 1:0.0005 (top to bottom tracing). The protein mixtures (50 μl) of the Ig-CRH (fixed at 2.2 μM) and various concentrations of the G-CSF (mixed with ^{125}I-G-CSF fixed at 1.1 nM; approximately 200,000 cpm) were eluted by gel filtration as shown in Panel A and counted for ^{125}I-G-CSF using a γ counter (open circles; solid lines). The control elution profile of ^{125}I-G-CSF without Ig-CRH is also shown (shaded circles; shaded lines).

that having a higher molecular mass did not occur. When the ratio was below 1:1, a dimeric Ig-CRH (85 kDa) peak was present, whereas free G-CSF was absent. SDS-PAGE confirmed that the 200–250 kDa peak was a complex between Ig-CRH and G-CSF. The composition of the peaks was analyzed by molar ratio of the released amino-terminus amino acid of the ligand and the Ig-CRH using an automated gas-phase amino acid sequencer. From three separate 200–250 kDa peaks under various conditions (in the ratios 1:10, 1:1 and 1:0.1), the molar ratio of the amino-termini of the Ig-CRH and the G-CSF in the ligand-induced tetramer was equal. The equal molar ratio of the Ig-CRH and the G-CSF in these 200–250 kDa peaks was also confirmed by a reverse phase HPLC analysis (data not shown). These data indicated that its stoichiometry was 4 to 4.

At a ratio of Ig-CRH to G-CSF below 1:0.1, the tetrameric complex was not the preferred protein product (Fig. 2A). Gel filtration of the Ig-CRH·G-CSF complex using ^{125}I-G-CSF showed that most of the ^{125}I-G-CSF chromatographed at the position of 94 kDa at an extremely low concentration of the ligand (1:0.0005 ratio of Ig-CRH to G-CSF) (Fig. 2B). When the ratio of Ig-CRH to G-CSF was increased, the position of the chromatographed ^{125}I-G-CSF migrated from 94 to 200–250 kDa. The molecular mass of 94 kDa almost corresponded with that of dimeric Ig-CRH·G-CSF complex, such as 2:1 or 2:2. These results indicated that the preferred Ig-CRH·G-CSF complex was the dimeric form at the extremely low concentration of G-CSF and converted to tetrameric form by the addition of G-CSF. The 2:1 GH receptor·GH complex induced at the appropriate concentration of GH is reportedly the active form, whereas the 1:1 complex induced at high concentration

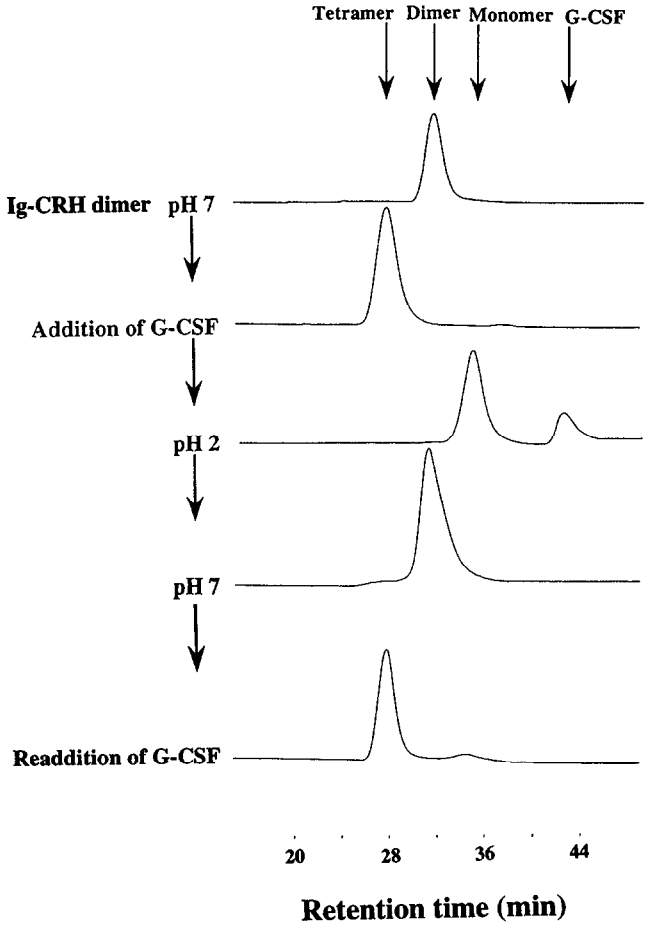

Fig. 3. Reversibility of dimerization and tetramerization of Ig-CRH. The purified Ig-CRH was chromatographed as the dimer (85 kDa) by gel filtration using the TSKgel G3000SW HPLC at pH 7.0 (top chart; Dimer pH 7.0). Tetramerization of the Ig-CRH was observed by addition of an equal amount of G-CSF at pH 7.0 (second chart; addition of G-CSF). Dissociation of the Ig-CRH·G-CSF complex was performed in the incubation with 0.1 M Glycine-HCl buffer pH 2.0 at 25°C for 1 h. Resulting monomeric Ig-CRH (45 kDa) and G-CSF (19 kDa) were analyzed using the gel filtration HPLC at pH 2.0 (third chart; pH 2.0). Re-dimerization of the Ig-CRH was observed by the gel filtration at pH 7.0, after the eluted monomeric Ig-CRH was incubated in 20 mM sodium phosphate buffer pH 7.0 containing 0.2 M NaCl.(fourth chart, pH 7.0). Re-tetramerization of the Ig-CRH was produced by the addition of G-CSF. (bottom chart readdition of G-CSF). Flow rate was performed at 0.5 ml/min and peaks were monitored for absorbance at 280 nm.

of GH is the form inactive for signal transduction [4,15,16]. Similar 2:1 receptor·ligand complexes were also obtained using receptors for interferon-g and prolactin [17,18]. Our data indicated the G-CSF induced oligomerization of Ig-CRH, like these receptors. However, the stoichiometry of the Ig-CRH·G-CSF complex was different from those reported. If the G-CSF receptor is inactivated at a high concentration of the ligand as found in the GH receptor [4], we might propose that the 4:4 G-CSF receptor·G-CSF complex is the inactive form. Certainly the size and the stoichiometry of the active form of the G-CSF receptor is still an open question.

It has been reported that binding of interleukin-6 to its receptor induced the disulfide-linked homo-dimerization of gp130 protein, which is a second chain of the interleukin-6 receptor [19]. The Ig-CRH contains 14 cysteine residues. However, all dimeric and tetrameric forms of the Ig-CRH with and without the ligand were found to be the 45 kDa by SDS-PAGE under nonreducing conditions, suggesting that these oligomerizations are not caused by covalent bonding, such as that of disulfide bonds. When the tetrameric Ig-CRH·ligand complex was exposed to an acidic buffer of pH 2.0, the induced tetramer dissociated into monomeric Ig-CRH and G-CSF (Fig. 3). We confirmed that the purified dimer also dissociated into monomers at pH 2.0 (data not shown). These results confirmed that the formation of dimers and tetramers is not mediated by covalent interaction. The noncovalent nature of oligomerization is further demonstrated by the reversibility of the oligomerization process. The dissociated monomeric Ig-CRH was re-dimerized quantitatively by neutralization with the buffer (Fig. 3). Moreover, re-dimerized Ig-CRH was tetramerized by adding G-CSF. An additional control experiment showed that the unpurified monomeric Ig-CRH induced by adding excess G-CSF was the tetramer, according to the gel filtration HPLC profile (detected by a ^{125}I-G-CSF binding assay and Western blotting). This excluded the notion that an unknown modification of Ig-CRH during purification might cause the tetramerization. It appears that the homo-dimer without the ligand and homotetramer in the presence of excess G-CSF is an intrinsic property of the receptor molecule in solution. This is different from the property of the GH receptor [16], although the Ig-CRH has a GH receptor-like structure.

Many oligomeric proteins contain identical subunit control metabolism and signal transduction. During these events, intrinsic properties such as homo-oligomeric structures should play a crucial role for the regulation of activities of these proteins. A detailed biochemical explanation of the molecular basis of the homo-oligomeric structure has been proposed for allosteric proteins and only a few receptors [1,2,20]. Thus, our finding of homo-oligomeric Ig-CRHs and the study of their mechanism in solution should be of interest. The Ig-CRH of the G-CSF receptor contains an Ig domain (Fig. 1A), whereas extracellular regions of receptors for GH, interferon-γ and prolactin are composed of only the CRH region [3]. The effect of the Ig domain might be important for the homodimerization and homotetramerization of the G-CSF receptor.

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