Molecular Characterization of the Haptoglobin-Hemoglobin Receptor CD163

LIGAND BINDING PROPERTIES OF THE SCAVENGER RECEPTOR CYSTEINE-RICH DOMAIN REGION*

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CD163 is the macrophage receptor for endocytosis of haptoglobin-hemoglobin complexes. The extracellular region consisting of nine scavenger receptor cysteine-rich (SRCR) domains also circulates in plasma as a soluble protein. By ligand binding analysis of a broad spectrum of soluble CD163 truncation variants, the amino-terminal third of the SRCR region was shown to be crucial for the binding of haptoglobin-hemoglobin complexes. By Western blotting of the CD163 variants, a panel of ten monoclonal antibodies was mapped to SRCR domains 1, 3, 4, 6, 7, and 9, respectively. Only the two antibodies binding to SRCR domain 3 exhibited effective inhibition of ligand binding. Furthermore, analysis of purified native CD163 revealed that proteolytic cleavage in SRCR domain 3 inactivates ligand binding. Calcium protects against cleavage in this domain. Analysis of the calcium sensitivity of ligand binding to CD163 demonstrated that optimal ligand binding requires physiological plasma calcium concentrations. And an immediate ligand release occurs at the low calcium concentrations measured in acidifying endosomes. In conclusion, SRCR domain 3 of CD163 is an exposed domain and a critical determinant for the calcium-sensitive coupling of haptoglobin-hemoglobin complexes.

The haptoglobin (Hp)-hemoglobin (Hb) receptor CD163 is a 130-kDa macrophage protein belonging to a group of proteins constituting a subfamily of scavenger receptor cysteine-rich (SRCR) domain proteins (1–3). These proteins, including CD55, CD6, CD163, CD163b, and WC1, are membrane proteins with a short cytoplasmic tail, a transmembrane segment, and an extracellular domain consisting exclusively of a variant number of consecutive class B SRCR domains (3, 4). The overall structure of the ~100-aa class B SRCR domain is suggested to resemble the class A SRCR domain, which based on its crystal structure (5, 6) is described as a six-stranded β-sheet cradling an α-helix. The class B domains differ from the class A domains by having four disulfide bridges instead of three. Furthermore, the class B domains are most common in multidomain mosaic proteins containing single SRCR domains associated to other functional domains, whereas the class A domains are most common in proteins exclusively composed of tandem repeats of SRCR domains. The SRCR domain structure is compatible with a role in molecular recognition, as shown for the membrane-proximal region of the three SRCR domains of CD6, which interacts with the activated leukocyte cell adhesion molecule (CD166) (7), and for two of the SRCR domains in bovine WC1 that bind to an unknown counter receptor (8).

CD163 is the only protein of the class B SRCR domain protein subfamily identified as a receptor for an extracellular ligand. It binds with high affinity to the Hp-Hb complex (1) that instantly forms in plasma when Hb is released from ruptured erythrocytes and is exposed to plasma Hp (0.3–2 g/liter) (9). This captor-receptor system controlling the metabolic route of Hb during limited hemolysis thereby protects against hemolyzed oxidative damages, in particular in the kidneys, that readily filterate non-complexed Hb and take up Hb in the proximal tubules (10). CD163 has no measurable affinity for non-complexed Hp or Hb (1). This Hp-Hb complex-specific recognition by CD163 explains the decrease in Hp concentration in plasma during accelerated hemolysis (11).

Besides having a role in clearance of Hb from plasma, CD163 is suggested to have an immunomodulatory role (12–15). Two distinct mechanisms may account for this. First, clearance of the hemoglobin results in conversion of the heme molecule to CO, bilirubin, and Fe, which overall are suggested to have an anti-inflammatory effect (15, 16). Second, ligand binding to CD163 is reported to induce tyrosine kinase- and calcium-dependent signaling and increased secretion of IL-6 and IL-10 (12, 15).

CD163 also circulates in plasma (~1–3 mg/liter) as a soluble protein with a size identical to that of the nine extracellular SRCR domains (17, 18). During sepsis and other conditions affecting macrophage activity, the level of soluble CD163 may raise many-fold (19, 20). The function of the extracellular domain is unknown, but it has been claimed to have an anti-inflammatory role (21). It is not reported whether soluble CD163 binds any ligands.

To delineate ligand binding properties of the membrane-associated and soluble CD163 protein and to identify the SRCR domains involved in ligand and antibody binding leading to endocytosis and signaling, we have performed a comprehensive analysis of a panel of recombinant CD163 variants and proteolytic fragments of CD163, including soluble CD163 circulating in plasma.
EXPERIMENTAL PROCEDURES

Purified CD163, Ligand, and Antibodies—Full-length CD163 was purified from solubilized human spleen membranes as previously described (1). Amino-terminal sequencing of CD163 fragments was carried out by the Edman degradation procedure as previously described (22). Human Hp (Hp1–1 and Hp2–2 phenotypes) and Hb were purchased from Sigma. Hp/Hb complexes were made by mixing equimolar concentrations of Hp and Hb in phosphate-buffered saline. The following anti-CD163 monoclonal antibodies were used: Ber-MAC3 (DakoCytomation, Copenhagen, Denmark), 5C6-FAT (BMA Biomedicals AG, Augst, Switzerland), EDhu1 (12), Ki-M8 (BMA Biomedicals AG), D11 (kind gifts from Dr. N. Petrovichev, Moscow), Mac2–48, Mac2–158, and R-20 (a kind gift from Dr. B. Davis, Trillium Diagnostics, Scarborough, ME), GHI/61 (BD PharMingen), and RM 3/1 (BMA Biomedicals AG). A polyclonal rabbit antibody has previously been described (1).

Construction of Plasmids for Expression of Recombinant Human CD163 Fragments—CD163 cDNA fragments extended with enzyme restriction sites were amplified by polymerase chain reaction (PCR) with the Pfu turbo polymerase (Stratagene, La Jolla, CA) and purified with the QIAEX II gel extraction kit (Qiagen). The PCR products were subcloned into the expression vector (pcDNA5/FRT or pSecTag2B from Invitrogen) by use of the appropriate restriction enzymes (HindIII and XhoI, New England BioLabs, Beverly, MA) and the T4 DNA ligase (New England BioLabs). Plasmids were transformed using DH5α (Clontech, Palo Alto, CA), and plasmid DNA was isolated by the Qiagen Maxiprep method (Qiagen) and sequenced before transfection. The following eleven constructs designated according to their SRCR domain composition were subcloned and expressed: r-CD163SRCR1–3 (aa 1–362); r-CD163SRCR1–4 (aa 1–478); r-CD163SRCR1–5 (aa 1–574); r-CD163SRCR1–6 (aa 1–679); r-CD163SRCR1–7 (aa 1–815); r-CD163SRCR1–8 (aa 1–922); r-CD163SRCR1–9 (aa 1–1025); r-CD163SRCR2–9 (aa 149–1025); r-CD163SRCR3–9 (aa 256–1025); r-CD163SRCR4–9 (aa 363–1025); and r-CD163SRCR5–9 (aa 470–1025).

Expression of Recombinant Human CD163 Fragments—Chinese hamster ovary (CHO) K1 cells (BioWhittaker) were transfected using...
TABLE I
Analysis of a panel of monoclonal anti-CD163 antibodies

| Antibody | r-CD163SRCR1–3 | r-CD163SRCR1–4 | r-CD163SRCR1–5 | r-CD163SRCR1–6 | r-CD163SRCR1–7 | r-CD163SRCR1–8 | r-CD163SRCR1–9 | r-CD163SRCR2–9 | r-CD163SRCR3–9 | r-CD163SRCR4–9 | r-CD163SRCR5–9 | r-CD163 SRCR3–9 | r-CD163 amino-terminal fragment* | r-CD163 carboxyl-terminal fragment* | Inhibition of HpHb binding |
|----------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Mac2-48  | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| Mac2-158 | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| Ber-MAC3 | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| MAC3    | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| 5C6-FAT | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| Edhul1  | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| KL-M8   | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| R-20    | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| D11     | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| GHI/61  | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |
| RM 3/1  | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              | +              |

* The fragments seen after cleavage of CD163 in SRCR domain 3. Major cleavage site at Asp-265.

RESULTS
Identification of HpHb Binding SRCR Domains in CD163—A panel of truncation variants of the SRCR domain region of CD163 was expressed (Fig. 1A) in stably transfected CHO cells. Fig. 1B shows Western blotting of the constructs expressed. Except for four of the constructs, the truncation variants were effectively secreted (lanes 3–7 and 10–11) to the cell culture medium. The non-secreted variants, which included the amino-terminal r-CD163SRCR1–3 and r-CD163SRCR1–4 constructs (Fig. 1B, lanes 1–2) and the constructs truncated in the amino-terminal part (r-CD163SRCR2–9 and r-CD163SRCR3–9) (lanes 8–9), accumulated in the cells, indicating abnormal folding and/or incomplete processing of these recombinant products.

Binding of the secreted CD163 truncation variants to HpHb as tested by precipitation with HpHb-Sepharose beads revealed binding activity in the r-CD163SRCR1–5 variant and all larger variants (CD163SRCR1–6, CD163SRCR1–7, CD163SRCR1–8, and CD163SRCR1–9) encompassing the SRCR1–5 region. Accordingly, the variants were readily purified from conditioned growth media by HpHb-affinity chromatography. Fig. 2 shows SDS-PAGE of the purified r-CD163SRCR1–5 and r-CD163SRCR1–9 variants and their high affinity binding (Kd = 1–5 nm) to HpHb as measured by SPR analyses. No binding to the r-CD163SRCR4–9 or r-CD163SRCR4–9 variants was seen (Fig. 1B), indicating that the amino-terminal third (SRCR1–3) of the...
extracellular CD163 domain is essential for ligand binding. Hp/Hb precipitation of the four non-secreted constructs (r-CD163 SRCR1–3, r-CD163 SRCR1–4, r-CD163 SRCR2–9, and r-CD163 SRCR3–9) accumulating in the cells did not reveal any binding activity (not shown). However, this finding might be due to an incomplete processing of these amino-terminal truncation variants; the finding therefore does not exclude that the corresponding parts of full-length native CD163 contain a binding site for Hp/Hb.

Further information on the location of the ligand binding site was obtained by mapping the binding sites and analyzing ligand-inhibiting effects of a panel of ten monoclonal antibodies (Table I). Western blotting of the recombinant CD163 expression products and the proteolytic fragments revealed that four antibodies (Ber-MAC3, 5C6-FAT, Mac2–48, Mac2–158) bind within SRCR domain 1 and two antibodies (EDhu1 and Ki-M8) bind to SRCR domain 3, whereas R-20, D11, GHI/61, and RM 3/1 bind to SRCR domains 4, 6, 7, and 9, respectively (Fig. 3, Table I). The SPR data revealed that only the antibodies EDhu1 and the Ki-M8 with binding epitopes in SRCR domain 3 of CD163 effectively compete for binding of Hp/Hb complexes (Fig. 3, Table I). A partial inhibition was seen by the antibodies binding to domain 1, 4, and 9, suggesting that these antibodies may cause some steric hindrance or conformational change but not a direct blocking of ligand binding (Table I).

The indicated role of SRCR domain 3 in the Hp/Hb binding was further verified by analyzing cleavage products of affinity-purified native CD163 from spleen membranes. Limited proteolytic cleavage occurred spontaneously after exposure of native receptor to calcium-free condition, as apparent by SDS-PAGE showing the appearance of two major proteins bands of ~90–95 kDa and ~35–40 kDa (Fig. 4, lane 1). No cleavage was seen when 5 mM calcium or 1 mM serine proteinase inhibitor (phenylmethyl-sulfonylfluoride) was added (not shown), indicating that calcium protects against proteinase activity. Western blotting using the mapped monoclonal antibodies (Table I), Triton X-114 precipitation (precipitates integral membrane proteins), and amino-terminal sequencing (data not shown) revealed that...
the known calcium dependence of the Hp phenotypes were analyzed, Hp (2–2) phenotype, Hp (1–1) phenotype. No change in elution was seen when Hb was absent from the samples. With the addition of Hb (lysed erythrocytes) to the serum samples, the CD163 reactivity eluted as a much larger protein (a1), indicating that the structural integrity of domain SRCR 3 suggests that the soluble form of CD163 is essential for the ligand receptor interaction. Furthermore, the cleavage virtually abolished recognition of CD163 by the EDhu1 and Ki-M8 antibodies (Table I). This further indicates that the epitopes of these antibodies overlap with the Hp-Hb binding region of CD163.

The ligand binding activity of the recombinant CD163 products encompassing SRCR 3 suggests that the soluble form of CD163 circulating in plasma is active in terms of ligand binding. To verify that soluble CD163 binds Hp-Hb complexes formed during hemolysis, we added Hb (lysed erythrocytes) to serum and measured the elution volume for plasma CD163. As seen in Fig. 5, the addition of Hb changed the elution volume of Cd163 toward that of a protein of substantially larger size. The change was, as expected, more pronounced using serum from a person with the oligomeric high molecular mass Hp 2–2 and Hp 2–1 phenotypes compared with serum with the lower molecular mass Hp 1–1 phenotype. In accordance with the known calcium dependence of the Hp-Hb binding to CD163, no change in elution was seen when calcium was complexed to EDTA.

Finally, we used the purified soluble ligand-binding r-CD163SRCR1–5 protein to characterize the calcium and pH sensitivity of Hp-Hb binding to the ligand binding site of CD163. As shown by SPR analysis (Fig. 6A), concentrations of calcium lower than the plasma concentration of calcium (~2.2–2.5 mM) resulted in a dose-dependent decrease in affinity. No binding was measured in calcium concentrations below 0.2 mM. Accordingly, low calcium concentrations strongly accelerated dissociation of Hp-Hb bound to CD163 at high calcium concentrations (Fig. 6B). An accelerated dissociation of the ligand was also seen when pH was lowered to 6.0–6.5. (Fig. 6C).

**DISCUSSION**

In the present study we have analyzed a large spectrum of CD163 truncation variants and used various approaches to identify and characterize the ligand binding site of the CD163 molecule. Fig. 7 summarizes the mapping of the calcium-sensitive ligand binding site and the antibody epitopes. The blocking effect of the EDhu-1 and Ki-M8 antibodies as well as the inactivation of ligand binding by cleavage in SRCR domain 3 pinpointed SRCR domain 3 as crucial for the CD163 recognition of Hp-Hb complexes. The position of the Hp-Hb site in the amino-terminal third of the receptor appears biologically meaningful in the sense that this part of the molecule may be spaced a favorable distance from the membrane, thereby facilitating binding of the ligand complexes, which have a size substantially larger than the extracellular part of CD163. The data presented here do not exclude involvement in ligand binding of adjacent regions to SRCR 3, such as SRCR domain 2. However, the fact that there is no blocking of ligand binding of the antibodies binding to domain 1 and 4 suggest that these domains are not parts of the ligand binding site, although they might contribute to the receptor folding and accessibility of the ligand.

In addition to identifying the ligand binding region, the present study delineated the CD163 epitopes of a broad spectrum of CD163 antibodies, including the ligand-blocking EDhu-1 and Ki-M8 antibodies, which have been widely described in the literature in studies of the function of CD163 (12, 15). Interestingly, the EDhu-1 antibody has been shown to induce a protein tyrosine kinase-dependent signaling, slow-type calcium mobilization, inositol triphosphate production, and secretion of IL-6 and granulocyte-monocyte colony-stimulating factor (12), whereas the Ki-M8 antibody has been shown to induce IL-10 secretion (15). Uptake of Hp-Hb also induces IL-10 secretion (15), but it is unknown whether it is because of a direct receptor-mediated signal or because of the hemoglobin uptake leading to a heme-mediated cellular response. The effects of EDhu-1 and Ki-M8 antibodies are suggested to involve cross-linkage of CD163 in the membrane. It is therefore tempting to believe that the Hp-Hb complex, which is di- or multivalent in terms of CD163 recognition (depending on the Hp phenotype), also is able to cross-link CD163. In vitro binding of
Hp\textsubscript{a}/H18528Hb complexes to purified CD163 does, in fact, support that CD163 cross-linkage can occur (1). The Hp\textsubscript{a}/H18528Hb complex is the first soluble ligand identified for SRCR domains in a membrane receptor. The known ligands to the canonic type A scavenger receptor bind to the collagenous part of this receptor and not to the SRCR domain (24). However, the CD6 protein, which has an extracellular domain consisting of three class B SRCR domains, is known to bind an integral membrane protein, the activated leukocyte adhesion protein, CD166 (7). Interestingly, the SRCR domain 3 of CD6 has also been identified as the ligand-binding domain, and site-directed mutagenesis has shown that a highly variable SRCR region of this domain is important for the CD166 interaction (7).

In the present study we further explored the previous observation (1) that calcium is essential for binding Hp\textsubscript{a}/H18528Hb to CD163. This is, to our knowledge, the only SRCR interaction reported to depend on calcium. However, calcium binding to many other endocytic receptors, including the low density lipoprotein receptor family proteins (25–27), is a well known prerequisite for ligand binding. Interestingly, the present data revealed that a much higher calcium concentration is required to elicit ligand binding activity of CD163 compared with the low density lipoprotein receptor family proteins that bind calcium with very high affinity. It is therefore tempting to speculate that release of calcium from a low affinity calcium binding site in CD163 may cause ligand-receptor segregation in acidifying endosomes (28). The SPR analysis in the present study showed that Hp\textsubscript{a}/H18528Hb bound to CD163 immediately dissociated when the calcium concentration was lowered to 0.2 mM. Decreasing pH to 6.5 also increased dissociation of Hp\textsubscript{a}/H18528Hb from CD163, although at a slower rate. In cell cultures (28), the calcium concentration in endosomes has been measured to be less than 10 µM when pH is 6.5. Under these conditions, the Hp\textsubscript{a}/H18528Hb complex may immediately dissociate from CD163. Such a calcium- and pH-dependent mechanism is different from the biological events causing ligand segregation from the low density lipoprotein receptor. This receptor uncouples its ligand by means of the YWTD propeller repeats that at low pH bind with affinity to ligand binding repeat (25).

In accordance with the mapping of the Hp\textsubscript{a}/H18528Hb binding to the CD163 SRCR domain 3 region, the present data showed that the circulating soluble form of CD163 representing the extracellular domain of CD163 (17) is able to bind Hp\textsubscript{a}/H18528Hb complexes. The physiological implication of this interaction is not known. How-

FIG. 6. Hp\textsubscript{a}/H18528Hb dissociation from CD163 at various calcium concentrations and pH values. A, SPR analysis of the binding of Hp\textsubscript{a}/H18528Hb complex (40 µg/ml) at various calcium concentrations. B and C, SPR analysis of dissociation of Hp\textsubscript{a}/H18528Hb complex from immobilized CD163 at various calcium concentrations and pH values. The arrows indicate the time point for buffer change and recording of the dissociation phase. The association phase prior to that was recorded with 1 mM CaCl\textsubscript{2} in the flow buffer (pH 7.4).

FIG. 7. Summary of the CD163 SRCR domains responsible for binding Hp\textsubscript{a}/H18528Hb and monoclonal antibodies (mAbs). The red sub-units of the Hp\textsubscript{a}/H18528Hb complex represent the Hb α subunits (small orange spheres) and the larger Hb β subunits (orange circles) that bind the Hb subunits. The asterisk symbols (****) below the SRCR domain of CD163 indicate the SRCR domain responsible for CD163 recognition by the mAbs listed.
ever, it is possible that it further prevent toxic effects of Hb or it may influence cellular signal pathways as previously proposed for soluble CD163 (21). Probably, soluble CD163 has little effect on the overall clearance of Hb during accelerated hemolysis because soluble CD163 has a ~1000-fold lower plasma concentration than Hp, which therefore immediately may saturate soluble CD163 when high amounts of Hb are released into plasma from erythrocytes.

In conclusion, the present study has provided new insight into the Hp-Hb-CD163 interaction and the role of the SRCR domains in calcium-sensitive ligand coupling and uncoupling. Future studies may define the structural basis of the calcium dependence and the structural change leading to formation of a receptor binding epitope when Hp and Hb join in a complex. Furthermore, the homologous structure of the SRCR domains in CD163 and related proteins suggests that a number of ligand-receptor interactions are yet to be defined in the family of SRCR receptors.

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