Partial characterization of natural and recombinant human soluble CD23

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The purification to homogeneity of an active soluble 25 kDa fragment of CD23, produced in insect cells using the baculovirus expression system, is described. Peptide mapping and analysis by Edman degradation and mass spectrometry permitted partial characterization of the protein. A total of 165 out of 172 residues, including *N*-terminal and *C*-terminal regions, were mapped. The positions of the two disulphide bonds in the IgE-binding region were also determined: residue 110 is joined to residue 124, and residue 42 to residue 133. Natural CD23 25 kDa fragment was also analysed and found to possess the same disulphide bond arrangement. These results extend the previously noted sequence similarity with lectins to elements of secondary structure.

INTRODUCTION

The low-affinity receptor for IgE (FcεRII), which is identical to CD23 (Yukawa *et al.*, 1987; Bonnefoy *et al.*, 1987) is a 45 kDa glycoprotein containing one potential N-linked carbohydrate chain and several O-linked chains (Letellier *et al.*, 1988). This receptor is a type II integral membrane protein (i.e. its *C*-terminus is outside the cell). It displays sequence similarity to animal C-type lectins (Kikutani *et al.*, 1986; Ikuta *et al.*, 1987; Lüdin *et al.*, 1987) and to a new family of adhesion molecules (Bevilacqua *et al.*, 1989).

The known soluble fragments of CD23, with the exception of the 12 kDa species, all bind to IgE (Letellier *et al.*, 1989). Soluble CD23 fragments have been reported to have pleiotropic activities *in vitro*, including (a) the regulation of human IgE synthesis (Sarfati *et al.*, 1984; Sarfati & Delespesse, 1988; Pène *et al.*, 1988); (b) the proliferation of B and T cells (Swendeman & Thorley-Lawson, 1987; Gordon *et al.*, 1988; Armitage *et al.*, 1989); (c) the inhibition of monocyte migration (Flores-Romo *et al.*, 1989); and, in synergy with interleukin 1, the following: (d) the induction of the differentiation of early thymocytes (Mossalayi *et al.*, 1990a); (e) the proliferation of early myeloid precursors (Mossalayi *et al.*, 1990b), and (f) the survival and differentiation of germinal centre B cells (Liu *et al.*, 1991).

We have produced active recombinant CD23 fragments (Jansen *et al.*, 1991; Gordon *et al.*, 1991; Liu *et al.*, 1991) in insect cells using the baculovirus expression system. In the present paper, we have purified to homogeneity recombinant 25 kDa sCD23, active in preventing germinal centre B-cell apoptosis, and assigned the important disulphide bonds in the IgE-binding domain. For comparison, we also determined these linkages for the CD23 25 kDa fragment isolated from the culture supernatant of a CD23-positive Epstein–Barr virus-infected cell line, RPMI 8866. The bonding patterns determined experimentally were identical and are in agreement with the theoretical structure proposed by Delespesse *et al.* (1989).

MATERIALS AND METHODS

Protein purification

Human recombinant 25 kDa sCD23 expressed in insect cells (Jansen *et al.*, 1991) was purified as follows.

Unless otherwise specified, all steps were carried out at 4 °C. The purification was monitored throughout on salt-free (dialysed) samples by SDS/PAGE; the 25 kDa sCD23 was detected by Western blotting with anti-CD23 antibody Mab25 (Immunotech, Luminy, France).

Ammonium sulphate (1768 g) was added slowly to 4 litres of the supernatant of infected insect cells (obtained by centrifugation at 1500 *g* for 10 min) and stirred for 1 h. The precipitate was removed by centrifugation at 10000 *g* for 30 min using a GSA rotor (Sorvall) and then taken up in 110 ml of phosphate-buffered saline (PBS; 140 mM-NaCl, 3 mM-KCl, 8 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄) supplemented with NaCl to a final concentration of 240 mM, pH 7.1, and also containing 2 mM-phenylmethanesulphonyl fluoride (PMSF), 0.4 mM-7-amino-1-chloro-3-L-tosylamidoheptan-2-one (Tos-Lys-CH₂Cl; 'TLCK'), 40 mM-iodoacetate, 10 mM-benzamidine hydrochloride, 2 mM-CaCl₂ and 2 mM-MgCl₂, pH 7.1. After sonication for 10 s, the sample was centrifuged at 130000 *g* for 110 min using a Beckman Ti 50.2 rotor. The supernatant (100 ml) was filtered through a GS 0.22 µm filter (Millipore, Bedford, MA, U.S.A.) to remove non-pelleted particulate material and then applied in portions of 25 ml to an Ultrogel AcA44 column (95 cm × 2.5 cm diam.) equilibrated and eluted at 1 ml/min with PBS supplemented with NaCl to a final concentration of 240 mM, pH 7.1.

The 25 kDa sCD23 was identified by Western blotting as the fraction eluting in the tail of the albumin peak. Positive fractions were pooled (250 ml) and made 0.5 mM in Tos-Lys-CH₂Cl, 10 mM in iodoacetate, 1 mM in PMSF and 1 mM in benzamidine hydrochloride. An immunoaffinity column was prepared by coupling the anti-CD23 antibody Mab25 to an Affi-gel 10 resin

Abbreviations used: 25 kDa sCD23, soluble 25 kDa fragment of CD23; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; f.a.b.-m.s., fast-atom bombardment m.s.; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK').

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(20 ml; Bio-Rad) according to the manufacturer's instructions. The pooled fractions from the AcA44 column were applied at 50 ml/h with constant recycling for 15 h to this immunoaffinity column, previously washed with 0.2 M-glycine/HCl, pH 3.0, and equilibrated in PBS supplemented with NaCl to a final concentration of 240 mM, pH 7.1. The immunoaffinity resin was washed first with 40 ml of PBS/240 mM-NaCl, followed by 600 ml PBS/640 mM-NaCl, pH 7.1, to remove material which had bound non-specifically. The 25 kDa sCD23 was eluted with 50 ml of PBS/240 mM-NaCl/3.0 M-ammonium thiocyanate, pH 6.5. The eluate was concentrated to 3 ml on a Centrprep 10 concentrator (Amicon) and applied to a Superdex 75 16/60 gel filtration column (Pharmacia), equilibrated in PBS, pH 7.1, run at 1 ml/min and monitored at 280 nm.

Prior to physico-chemical analysis, portions of 50 µg of protein were further purified by reversed-phase h.p.l.c., as described below.

Natural material was obtained from the RPMI 8866 cell supernatant using the same methodology. The purification of human 25 kDa sCD23 expressed in *Escherichia coli* will be described elsewhere.

Activity

Activity was tested in the apoptosis assay of purified germinal centre B-cells as described elsewhere (Liu *et al.*, 1991; Graber *et al.*, 1992). An optimal concentration of 100 ng of sCD23/ml was used as previously determined (Liu *et al.*, 1991).

N-Terminal sequencing

N-Terminal amino acid sequence analysis was carried out on a liquid pulsed sequencer model 477 A (Applied Biosystems). Samples were loaded on filters precycled with polybrene. Phenylhydantoin-amino acid derivatives were analysed on-line by a model 120 PTH analyser (Applied Biosystems).

Digestion with trypsin

To 300 µg of recombinant-derived CD23 25 kDa fragment in 2.2 ml of 0.1 M-phosphate buffer (pH 7.0; counterion Na⁺) was added 22 µl of iodoacetic acid (1 M, adjusted to pH 5 with NaOH) followed by 30 µl of trypsin (Tos-Phe-CH₂Cl-treated bovine trypsin; Worthington; 1 mg/ml in water). After incubation in the dark at 37 °C for 2 h, the solution was acidified with 100 µl of acetic acid and injected on to h.p.l.c. (see below).

Two similar digestions were performed with 150 µg of protein (one with natural and one with recombinant-derived 25 kDa fragment). For these digestions the incubation volume was 150 µl and the reaction time was restricted to 1 h. These two digests were also acidified and applied to h.p.l.c.

Reversed-phase h.p.l.c.

Intact 25 kDa sCD23 was purified as follows. Solvent A was prepared by adding 1 g of trifluoroacetic acid (TFA) (h.p.l.c. grade; Applied Biosystems) to 1000 ml of h.p.l.c.-grade water (Milli-Q system) followed by vacuum filtration. Solvent B was prepared by adding 1 g of TFA to 100 ml of h.p.l.c.-grade water (previously degassed by vacuum filtration) and then adding 900 ml of acetonitrile (gradient grade; Merck; previously degassed by vacuum filtration). After dilution of the Superdex eluate with 2 vol. of solvent A, sample was applied to an Aquapore RP-300 7 µm C4 column (250 mm × 4.6 mm; Applied Biosystems, Foster City, CA, U.S.A.) equilibrated with solvent A. Protein was eluted with a gradient of 0–80 % (v/v) solvent B over 60 min at 1 ml/min.

Tryptic peptides were separated on a Waters h.p.l.c. system, monitoring at 214 nm and 280 nm. Solvents A and B were prepared as described above. A column (250 mm × 4 mm internal diam.) of Nucleosil (30 nm pore size; 5 µm-diam. particles) C8 (Machery Nagel) was used at a flow rate of 0.6 ml/min. Samples were injected on to the column equilibrated with solvent A. After 5 min a linear gradient of 2 % solvent B/min was applied for 5 min, then a linear gradient of 0.2 % solvent B/min was applied for 125 min. The column was then washed with 100 % solvent B for 5 min prior to re-equilibration with solvent A. Fractions were collected manually, portions set aside, and solvent then removed in a vacuum centrifuge (SpeedVac, Savant). A portion of each fraction was treated under reducing conditions (dithiothreitol 50 mM, ammonium bicarbonate, 1 %, at 37 °C for 1 h), acidified with acetic acid and then re-analysed by h.p.l.c.

Mass spectrometry

M_r values were measured either in fast-atom bombardment mode or using electrospray ionization. Fast-atom bombardment (f.a.b.) m.s. was performed using equipment and operating conditions as previously described (Savoy *et al.*, 1988). Electrospray ionization m.s. was performed on a Trio 2 machine equipped with a 3000 amu rf generator and Lab-Base software (VG Biotech, Manchester, U.K.).

RESULTS AND DISCUSSION

Protein purification

The recombinant 25 kDa sCD23 was purified (Table 1) from the conditioned medium of Sf9 insect cells infected with the recombinant baculovirus clone H₂ (Jansen *et al.*, 1991). A cocktail of proteinase inhibitors was used to prevent protein degradation when dissolving the precipitate after the ammonium sulphate precipitation step. Gel filtration on AcA44 allowed the isolation of a monomeric protein which was identified by Western blotting as eluting in the tail of the albumin peak. Most of the contaminants were then removed by immunoaffinity chromatography. The final gel filtration on Superdex 75 gave a protein judged 97 % pure by densitometric scanning of silver-stained SDS/PAGE gels and by reversed-phase h.p.l.c. (Fig. 1). The yields obtained throughout this purification procedure are shown in Table 1. Similar results were obtained with the natural material using the same procedure (Table 1).

Biological activity

Both recombinant and natural 25 kDa sCD23 were shown to prevent apoptosis of germinal centre B-cells in the presence of IL-1 (Table 2).

Determination of disulphide bonds

The amino acid sequence of recombinant 25 kDa sCD23, as deduced from the DNA sequence (Kikutani *et al.*, 1986), is shown in Fig. 2. In an attempt to simplify the task of determining the disulphide bond arrangement, we first treated the recombinant protein with cyanogen bromide (3-fold weight excess in 70 % formic acid for 20 h at room temperature; results not shown). The protein peak broadened and moved to a slightly later retention time on reversed-phase h.p.l.c., but no free fragments were visible. The most reasonable interpretation of this result is that CNBr cleaved the polypeptide chain at Met residues but the fragments formed were all held together by disulphide bonds (except for free homoserine lactone released by cleavage between residues 113–114 and 114–115; this would elute in the front of the chromatogram).

The eight Cys residues in both recombinant and natural

Table 1. Summary of the purification of recombinant and natural 25 kDa CD23

The 25 kDa CD23 content (%) was estimated by densitometric scanning of the silver-stained SDS/polyacrylamide gels.

Purification step	Recombinant 25 kDa CD23 from Sf9/BV				Natural 25 kDa CD23 from RPMI 8866			
	Total protein (mg)	CD23 content (%)	25 kDa amount (mg)	Yield (%)	Total protein (mg)	CD23 content (%)	25 kDa amount (mg)	Yield (%)
Culture medium	4930	1.9	93.7	100	1140	0.2	2.28	100
Ultrogel AcA 44 (pooled fractions)	1960	2.3	45.1	48.1	260.5	0.5	1.30	57.0
Mab 25-affinity (pooled fractions)	3.40	62.0	2.1	2.2	0.25	31.0	0.08	3.5
Superdex 75 (pooled fractions)	1.90	97.0	1.8	1.9	0.06	97.0	0.06	2.6

Fig. 1. SDS/PAGE analysis of recombinant (a) and natural (b) 25 kDa sCD23

Aliquots from the different steps of the purification were subjected to electrophoresis on a 15% (w/v) polyacrylamide gel and stained with Coomassie Blue. Lane a, standards; lane b, culture medium; lane c, pooled fractions from Ultrogel AcA44; lane d, pooled fractions from Mab25 affinity column; lane e, pooled fractions from Superdex 75. All the samples were prepared with dithiothreitol alone prior to analysis.

Table 2. Activity of purified recombinant and natural 25 kDa CD23 on germinal centre B-cell apoptosis in the presence of interleukin-1

Crude or purified sCD23 was tested at 100 ng/ml; interleukin-1 (IL-1) was added at 1 ng/ml to all assays.

Protein	Stimulus	Rescue from apoptosis* (%)
Recombinant	Conditioned medium of insect cells (H2)	35
	Purified recombinant 25 kDa sCD23	40
Natural	Conditioned medium of RPMI 8866 cells	32
	Purified natural 25 kDa sCD23	36
None	PBS	0

* Apoptosis in the absence of rescue factor was 60%.

proteins (Fig. 2) were known to be all occupied in disulphide bond formation: there was no reaction with Ellman's reagent (Ellman, 1950) even under denaturing conditions [1% (w/v) SDS; P. Graber, unpublished work]. Nevertheless, in order to avoid any possibility of rearrangement, we performed tryptic digestion for a short period (1–2 h) and in the presence of iodoacetate. The resulting separation of the tryptic peptides of the recombinant material is shown in Fig. 3(a). Analysis of

Fig. 2. Amino acid sequence of 25 kDa sCD23 as deduced from the DNA sequence (Kikutani *et al.*, 1986)

Peptides identified in the present article are underlined: — for recombinant material, — for natural material. The natural sequence was found to begin at Leu-3; its C-terminus was not positively identified, however (see Table 4).

fractions by f.a.b.-m.s. and Edman degradation led to the assignments shown in Table 3.

Peptides that were identified are aligned under the sequence of the protein in Fig. 2. The finding of both N-terminal and C-terminal tryptic fragments confirms the integrity of the preparation. The initiating Met residue was found still attached to the N-terminus of the recombinant protein (peaks r8 and r9, Table 3). Disulphide bonds were found to be present between residues 110 and 124 (established by r6 and r7), and between residues 42 and 133 (established by r10, r12 and r13). Two other bridges remained undefined, the formal possibilities being 11–14/25–139; 11–25/14–139; and 11–139/14–25. However, the combination 11–14/25–139 is inconsistent with the following data. The peptide containing residues 1–17 would be unconnected to any other if Cys-11 were to be joined to Cys-14, and so should not elute in both peaks r8 and r9. (The existence of peaks r8 and r9 is

Fig. 3. Reversed-phase h.p.l.c. of the tryptic digest of (a) recombinant and (b) natural 25 kDa sCD23

Conditions are given in the Materials and methods section. The absorbance trace shown is that recorded at 214 nm, 1.0 absorbance unit full-scale. A total of 150 µg of protein was used in each case.

explained by the presence of the extra hydrophilic Lys residue in the earlier-eluting peak.) We may thus exclude possibility 11–14/25–139.

Having established two of the disulphide linkages in the recombinant protein, we turned to the natural protein. The natural protein gave a chromatogram which was generally similar to that of the recombinant protein, but with some differences (Fig. 3b). Fractions were analysed, and from the results shown in Table 4, the natural material clearly has one disulphide bond between Cys-110 and Cys-124 (supported by peaks n6 and n7), and another between Cys-42 and Cys-133 (supported by peak n13). As was found for the recombinant material, the other two disulphide bonds tie up three peptides (found in peaks n8 and n9, which correspond to recombinant peaks r8 and r9 respectively).

The results discussed above show that both recombinant and natural CD23 have disulphide bonds between residues 110 and 124, and between residues 42 and 133. It is most likely, in view of the similarity of the results obtained, that the other two bridges are also in the same positions in the two proteins. For the natural material, we can rule out the formal possibility 11–14/25–139 for the same reasons given for the recombinant material. Two possibilities thus exist for the two remaining bridges in both proteins. Successful attempts to determine these rely on cleavage between Cys-11 and Cys-14 in the sequence -Cys-Asn-Thr-Cys-without disrupting the existing disulphide bonding arrangement. Using recombinant material (peaks r8 and r9), we were able to effect cleavage with asparaginyl-endopeptidase (Pierce). However, this enzyme requires reducing conditions, and control incubations showed that chain separation occurred rapidly under the conditions used (results not shown), so this approach was unsuccessful. A further attempt to cleave between Cys-11 and

Table 3. Tryptic peptides of recombinant 25 kDa sCD23

Peaks refer to fractions collected during the chromatography shown in Fig. 3(a), or to corresponding fractions collected either during the run with 300 µg of protein or from a similar digest of recombinant material produced in *E. coli*. The *E. coli*-derived material was used to obtain the mass spectrometric data on peaks r8, r9, r11, r12 and r13, which confirmed and extended the Edman-derived data obtained using material from insect cells. ‘Reduces’ refers to whether there was a change in retention characteristics upon treatment with dithiothreitol; Amino acid sequences are shown in the one-letter code, residues identified by Edman degradation are underlined, and peptides in bold type were identified by mass spectrometry.

Peak	Reduces	Peptide
r1	No	QWVHAR
r2	No	NLDLK
r3	No	LPTPSAPLHS
r4	No	HASHTGSWIGLR
r5	No	WINFQR
r6	Yes	<u>SQGEDCVMMR</u> WNDAF-DRK
r7	Yes	<u>SQGEDCVMMR</u> WNDAF-DR
r8	Yes	<u>MELQVSSGFVCNTCPEK</u> KCYFVGK LATCTPPASEGSAESMGPDSPDPDGR
r9	Yes	<u>MELQVSSGFVCNTCPEK</u> CYFVGK LATCTPPASEGSAESMGPDSPDPDGR
r10	Yes	<u>LGAWVCDR</u> YA-DDMEGQLVSIHSPEEQDFLTK...*
r11	No	GEFIWVDGSHVDYDYSNWAPEPTSR
r12	Yes	<u>KLGAWVCDR</u> YACDDMEGQLVSIHSPEEQDFLTK
r13	Yes	<u>LGAWVCDR</u> YACDDMEGQLVSIHSPEEQDFLTK

* Degradation which did not necessarily terminate at the last residue identified.

Cys-14 was made with remaining material (peak r9). An attempt was made to deamidate Asn-12 by incubation at 45 °C for 66 h in 0.1% TFA. The resulting material was dried, taken up in phosphate buffer and treated with endoproteinase Asp-N according to the manufacturer’s recommendations (Boehringer). After separation of the digest by h.p.l.c., Edman analysis showed presence of the original sequence, still with Asn at position 12, and two fragments, Asp-Ser-Arg-Pro and Asp-Ser-Arg-Pro-Asp-Pro, produced by expected cleavage on the *N*-terminal side of Asp residues. In order to determine the final pair of bridges, much more material would be required.

Nevertheless, the two bridges which have been determined permit testing of the model of Delespesse *et al.* (1989) (Fig. 4). In this prediction, based on sequence similarity with animal lectins, disulphide bonds are predicted between Cys-191 and Cys-282, and Cys-259 and Cys-273, of FcεRII. This corresponds to residues 42/133 and 110/124 on the 25 kDa CD23 numbering system (Fig. 2), and is exactly the pattern determined experimentally for both recombinant and natural 25 kDa fragments (Fig. 4). The third bond of the model, that between Cys-160 and Cys-174 (residues 11 and 25 on the fragment numbering system) corresponds to one of the two allowed possibilities (Fig. 4b). The fourth bond does not form part of the model, but must be present since no free thiol was detected. Since submission of the original version of this paper, an article has appeared (Bettler *et al.*, 1992) which proposes, on the basis of mutagenesis analysis, the bonding arrangement shown in Fig. 4(a).

Fig. 4. Disulphide bonding in 25 kDa sCD23

Cys (C) residue positions are shown numbered according to the FcεRII system; numbers in parentheses refer to the 25 kDa fragment numbering system used in the present paper. The N- and C-termini of the polypeptide chain are represented by NH₂ and OH respectively. The polypeptide chain is represented by a single line, and disulphide bonds by double lines. (a) and (b) represent alternative models (see the text for details).

Conclusion

Data on the structure of CD23 are important because the molecule is involved in the regulation of IgE synthesis (Pène *et al.*, 1988; Delespesse *et al.*, 1989; Bonnefoy *et al.*, 1990) and in the control of immune responses (Gordon *et al.*, 1989). Since the protein exhibits several different bioactivities, it is reasonable to expect that these activities are exhibited by different domains of the molecule, as is the case for many other multi-domain proteins (e.g. urokinase). In this context, the assignment of the disulphide bridges of the CD23 molecule is a key step in identifying the different domains in order to engineer the corresponding fragments. We have determined experimentally the two disulphide bonds in the IgE-binding domain of both natural and recombinant 25 kDa sCD23.

Table 4. Tryptic peptides of natural 25 kDa sCD23

Peaks refer to fractions collected during the chromatography shown in Fig. 3(b). Sequences were determined by Edman degradation. Positions of Cys residues, which do not yield a clearly identifiable phenylhydantoin derivative, are indicated by -.

Peak	Peptides
n6	SQGED-VMMR WDAF-DRK
n7	SQGED-VMMR WDAF-DR
n8	LQVSSGFV-NT-PEK LAT-TPPASEGSAESMGPD...*
n9	LQVSSGFV-NT-PEK LAT-TPPASEGSAESMGPD...*
n10	LGAWV-DR YA-DDMEGQLVSIHSPEEQDFL...*
n11	GEFIWVDGSHVDYSNWAPGPTSR
n13	LGAWV-DR YA-DDMEGQLVSIHSPEEQDFLTK...*

* Degradation which did not necessarily terminate at the last residue identified.

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