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CD23/FcεRII and its soluble fragments can form oligomers on the cell surface and in solution

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SUMMARY

Human CD23 (also known as FcεRII) is a 45 000 MW glycoprotein with homology to C-type animal lectins. It is involved in B-cell differentiation and IgE regulation, and is naturally cleaved to give soluble products of 37 000, 33 000, 29 000, 25 000 and 16 000 MW. Previous work has suggested that the region between the transmembrane sequence and the extracellular lectin head is capable of forming an α-helical coiled coil, one of the main consequences of which would be formation of dimers or trimers. Here we present protein–protein cross-linking data showing that CD23 forms trimers on the cell surface and hexamers in solution, and we use several different fragments to determine the regions of the protein involved in this self-association. The region of the putative coiled coil is indeed responsible for trimerization, with additional interactions between the lectin heads resulting in the formation of hexamers observed in solution.

INTRODUCTION

CD23 is found on a variety of cells, including B cells, T cells, natural killer (NK) cells, eosinophils, platelets, macrophages, follicular dendritic cells, Langerhans' cells and some epithelial cells, and has two known ligands: IgE, which it binds with an affinity of 10^7 M, and CD21, a complement receptor.[1,2] It is a 45 000 MW protein, with two forms, differing only in the N-terminal six or seven amino acids.[3] the *a* form which is constitutively expressed on B cells and the *b* form, expression of which is induced by interleukin-4 (IL-4) on a variety of cell types. The extracellular portion contains a domain homologous to C-type (Ca^{2+} dependent) animal lectins,[4–6] with the human protein having an extended C-terminal tail containing a reverse RGD sequence that may act in cell adhesion. Although the binding site for IgE is entirely in the lectin domain, and the interaction is Ca^{2+} dependent (for review see ref. 7), it is known that CD23 does not bind to the extensive carbohydrate on IgE.[8] However the interaction between CD23 and CD21 requires glycosylation of the latter protein[9] and is thus a more typical lectin interaction.

The region between the lectin domain and the membrane is predicted using sequence analysis to form an α-helical coiled coil,[10] and the result of this should be that the molecule forms either dimers or trimers, although it is not possible to distinguish between these two possibilities by sequence analysis alone. Data obtained on the human[11] and mouse receptors[12,13] suggest the majority of the protein exists in an oligomeric state, although the molecular weight of this has not been well defined, and also suggests that oligomer formation of mouse CD23 is required for binding to IgE with the measured affinity of 10^8 M.[14]

The receptor undergoes natural proteolytic cleavage to yield fragments of 37 000, 33 000, 29 000, 25 000 and 16 000 MW,[15] all of which contain the lectin domain. Both the 37 000 and 33 000 MW have significant lengths of potential coiled coil, but the 25 000 and the 16 000 MW lack this, and the 16 000 MW is also thought to lack the C-terminal tail. It was therefore expected that only the intact protein and the 37 000 and 33 000 MW fragments would be capable of forming oligomers, at least via a coiled coil. All fragments bind to IgE, although the 25 000 MW appears to have an affinity for IgE lower than the larger fragments. Fragments larger than 25 000 MW promote IgE synthesis whereas the 16 000 MW inhibits IgE synthesis.[16] All the fragments are thought to have other cytokine activities.

We have investigated the capacity of recombinant intact human CD23 and the 37 000, 25 000 and 16 000 MW soluble fragments to form oligomers in solution, using chemical cross-linking, and we have also investigated the association of the native molecule on the surface of the transformed B-cell line RPMI-8866.

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MATERIALS AND METHODS

Cross-linking of purified recombinant CD23

Recombinant 45 000, 37 000 and 25 000 MW CD23 were purified at Glaxo Institute for Molecular Biology (Geneva, Switzerland) along with the rabbit polyclonal anti-CD23 antibody Rb55.[17–19] The 16 000 MW was isolated by fast-performance liquid chromatography (FPLC) on a Superose 12

column as a natural cleavage product of the 25000 MW fragment. The proteins were cross-linked using either 25 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in 25 mM sodium acetate pH 5·0 or with 4 mM 1,5-difluoro-2,4-dinitrobenzene (DFDNB) in 100 mM sodium carbonate pH 10·0. Samples were then run on either 5–15% gradient or 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels[20] under non-reducing conditions, and the gels either silver stained[21] or Western blotted. Western blotting involved transfer of the gel to nitrocellulose filter using a semi-dry method.[22] The resulting filter was then blocked for 1 hr with 2% milk powder in 25 mM Tris pH 8·4, 125 mM sodium chloride (TS), washed twice for 5 min with TS, and incubated for 2 hr at room temperature with Rb55 diluted 1 : 200 in TS with 1·5% bovine serum albumin (BSA) and 0·1% sodium azide. The filter was washed twice with TS, then incubated for 1 hr at room temperature in donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham, International, Amersham, UK) diluted 1 : 1000 in 2% milk powder, TS. The filter was then thoroughly washed with TS (5 × 5 min), then the bands visualized using the Amersham ECL system, with exposure to Blue X-ray film (Xograph, Ltd, Malmesbury, UK) for a period of 10 seconds to 5 min.

Tissue culture and preparation of affinity matrix

RPMI-8866 cells were grown in RPMI-1640 medium (Flow Laboratories, Irvine, UK), supplemented with 10% fetal calf serum (FCS; Advanced Protein Products, Brierley Hill, UK), 2 mM glutamine and 10 U penicillin/streptomycin per 500 ml. The antibodies used for purification were the mouse monoclonals MHM6 for the native protein and BU38 for the cross-linked protein, both kindly provided by Dr J. Gordon (Birmingham, UK). These, and BSA (Sigma, Poole, UK), were coupled to Affigel-10 (Bio-Rad, Watford, UK) at a concentration of 1–2 mg/ml, according to the manufacturer's instructions.

Cell surface cross-linking

Approximately 3×10^7 cells were harvested and washed three times in phosphate-buffered saline (PBS). They were resuspended in 500 μl PBS (0·14 M sodium chloride, 2·7 mM potassium chloride, 1·5 mM potassium dihydrogen orthophosphate, 8·1 mM disodium hydrogen orthophosphate) containing 4 mM DFDNB was rotated at 4° for 2 hr. The cells were then washed twice with PBS and lysed with 500 μl lysis buffer containing protease inhibitors [0·5% Nonidet P-40 (NP-40), 0·2 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 10 mM iodoacetamide, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF) in PBS], then spun in a microfuge for 5 min and the supernatant transferred to 150 μl BSA–affigel. The samples were rotated at 4° for 1 hr to absorb any non-specific interactions of proteins with the affigel, the supernatant was then transferred to 150 μl BU38–affigel and rotated at 4° overnight. The gel was then washed thoroughly with PBS + 0·1% NP-40 for 5 min, high salt buffer (PBS, 0·5 M sodium chloride, 0·1% NP-40) twice for 10 min, stripping buffer (10 mM Tris pH 7·4, 150 mM sodium chloride, 0·1% SDS, 0·5% NP-40, 1 mg/ml BSA) twice for 10 min, and PBS twice for 5 min, and the samples were incubated at 4° for the required time on a rotating wheel. As much liquid as possible was removed and then the bound protein was eluted by the addition of 40 μl non-reducing SDS sample buffer (10% glycerol, 3% SDS, 62·5 mM Tris–HCl pH 6·8, 0·001 Bromophenol Blue), and boiled for 5 min. The samples were subjected to electrophoresis on a 10% SDS gel, and Western blotted, as for the pure proteins.

RESULTS

The prediction of the presence of a coiled coil stalk in CD23 implies that the protein should form dimers or trimers. Chemical cross-linking of 37000 MW fragments with EDC (Fig. 1) produces a major band corresponding to the position of a trimer, with a minor dimer band, but there is proportionately little cross-linking of the 25000 and 16000 MW (not shown) under the same conditions, despite the high sensitivity of the detection techniques used. As EDC forms a zero length covalent linkage between lysine residues and aspartic or glutamic acid residues,[23] this suggests that part of the protein contained in the 37000 MW fragment (and indeed in the 45000 MW intact protein) but not in the smaller fragments contains at least one of these interactions. This is consistent with this region forming a coiled coil, as the presence of stabilizing salt bridges between strands[24] and ensures that many additional potential cross-linking sites were available.

When cross-linked with DFDNB however, a different pattern arises. Figure 2 shows the results obtained for 16000 and 25000 MW fragments; similar results for 37000 and 45000 MW are not shown. A series of at least six bands of

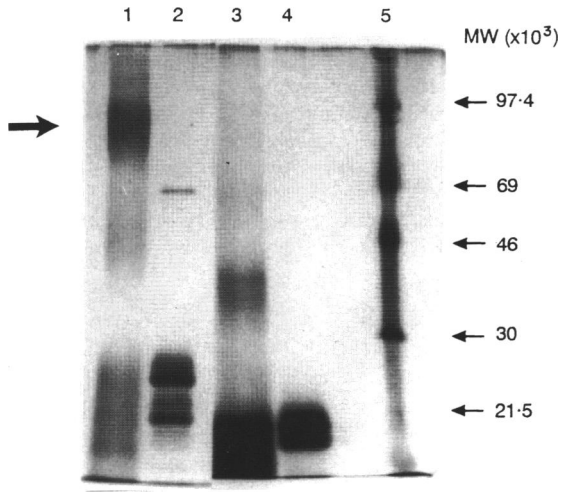

Figure 1. Cross-linking of pure, recombinant CD23 with EDC. CD23 was cross-linked with EDC and analysed by non-reducing SDS–PAGE and silver staining or Western blotting (results not shown). Lane 1, 37000 MW + EDC; lane 2 37000 MW uncross-linked; lane 3, 25000 MW + EDC; lane 4, 25000 MW uncross-linked; lane 5, Amersham high molecular weight prestained rainbow markers, which enable easy comparison of silver staining and Western blots but run anomolously high. When compared with unstained markers (results not shown), the fragments appear as previously observed.[17] Cross-linking resulted in the formation of trimer mainly for the 37000 MW (arrow), with some dimer being visible, whereas under the same conditions, only a very small proportion of the 25000 MW cross-linked to dimer. These results represent the optimal cross-linking conditions for both fragments.

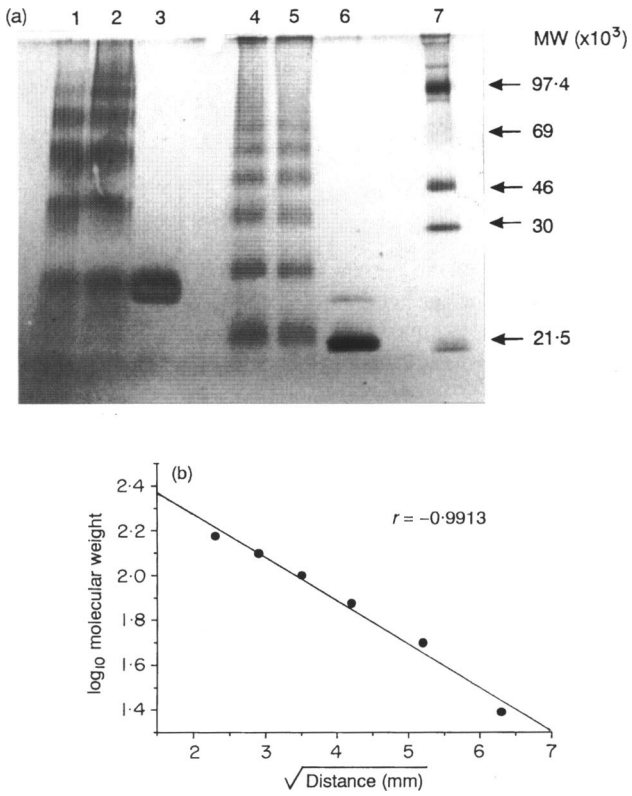

Figure 2. Cross-linking of pure, recombinant CD23 with DFDNB. Samples were cross-linked with DFDNB and analysed by SDS–PAGE and silver staining with duplicates shown in each case. (a) Results for the 25 000 and 16 000 MW fragments: Lanes 1 and 2, 25 000 MW + DFDNB; lane 3, 25 000 MW uncross-linked; lanes 4 and 5, 16 000 MW + DFDNB; lane 6, 15 000 MW uncross-linked; lane 7, markers. (b) The mobility of each was then measured to the nearest 0·5 mm and a graph plotted for the 25 000 MW fragment of $\log_{10}$ of the predicted molecular weight (assuming the lowest band to be monomer, the remainder dimer, trimer, tetramer, pentamer and hexamer, respectively) against the square root of the migration distance. The linear regression is shown (———) with the regression coefficient R indicated. These results clearly indicate a linear relationship.

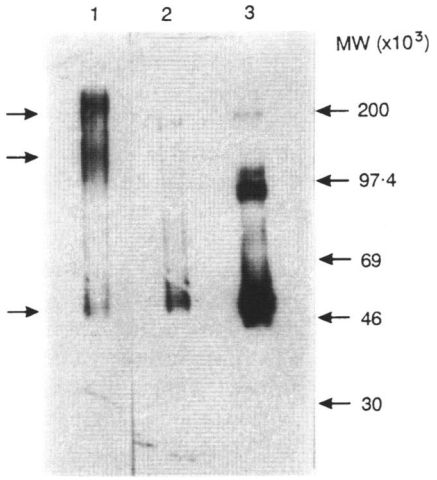

Figure 3. Cross-linking of CD23 on the cell surface. Lane 1, cell surface proteins of RPMI-8866 cells were cross-linked with DFDNB, then the CD23 affinity purified on BU38–affigel, eluted with non-reducing SDS sample buffer and analysed by Western blotting; lane 2, a sample treated as above but uncross-linked; lane 3, control lane containing pure, recombinant 45 000 MW CD23.

increasing size are visible, and we have assumed that the series of bands correspond to monomer, dimer, trimer, tetramer, pentamer and hexamer, as they all cross-react with anti-CD23 antibody on a Western blot and gave the expected molecular weights. If this relationship between the bands is true, then a plot of $\log_{10}$ molecular weight against the square root of the migration distance should give a straight line for a linear gradient gel.[25] Figure 2b shows this plot for the 25 000 MW with the calculated linear regression and regression coefficient, and we thus conclude that the bands correspond to monomer through to hexamer. These results are similar to those obtained for the rat asialoglycoprotein receptor[26] and chicken hepatic lectin,[27] and suggest that in solution, at least, CD23 is capable of hexamer formation and that this is independent of the stalk region as the 25 000 and 16 000 MW fragments are both cross-linked. We have not examined the biological activities of the cross-linked receptor.

Recent work on the chicken hepatic lectin[28] using hydrodynamic studies and DFDNB cross-linking show that the protein is trimeric on the cell surface and directly after detergent solubilization, but that hexamers form after some time in solution. We have used DFDNB to cross-link CD23 on the surface of cells (Fig. 3) and these results show that the protein has cross-linked to higher molecular weight products, potentially dimer and trimer, with no evidence to suggest any hexamer formation. Similar work on the mouse receptor[14] shows that this is also oligomeric on the cell surface, although the molecular weight of the band produced was not accurately determined and could correspond to either trimer or tetramer. It is possible, however that these higher molecular weights correspond to CD23 cross-linked to another adjacent cell surface molecule, rather than to itself. It has been observed that CD23 is associated with major histocompatibility complex (MHC) class II molecules[29] and such close associations may well bring lysine residues into close enough proximity to cross-link to CD23. However, cross-linking appears consistent with results obtained with pure 45 000 MW and EDC, which gives trimers, and also with the results obtained for the chicken hepatic lectin.

DISCUSSION

These results lead to several conclusions about the oligomeric nature of CD23. First, it is clear that the lectin heads alone can self-associate, and that this is independent of the stalk and possibly the C-terminal tail, shown by the fact that the 25 000 and 16 000 MW fragments cross-link to hexamers with DFDNB. However, it appears that this does not reflect the situation on the cell surface, as the same cross-linker only gives potential trimers of the intact molecule, a situation similar to that found in studies of the chicken hepatic lectin.[28] Cross-linking with EDC gives virtually no cross-linked products for the 16 000 and 25 000 MW fragments which have no stalk, but gives good cross-linking of the 37 000 and 45 000 MW to trimers. This implies that the sites able to cross-link with EDC

are almost solely in the stalk region and that this region is trimeric. The hexamers obtained by DFDNB cross-linking are therefore likely to represent dimers of trimers occurring only in solution, suggesting that they may well be end-on interactions of the lectin heads, a situation which cannot occur on a single cell and would thus explain the lack of hexamer formation on the cell surface.

The cross-linking experiments reported here may reveal that CD23 and its 37 000 MW fragment exist as trimers *in vivo* or possibly as an equilibrium mixture of monomers and trimers that are trapped by cross-linking in the associated state. This question could be resolved by determination of the molecular weight of the uncross-linked protein but insufficient material was available in the present study. This could prove difficult, however, as self-associations with a low affinity exist in another IgE-binding protein εBP[30] and while these are clearly detected by chemical cross-linking, native column chromatography caused disassociation to monomers. Studies on the highly homologous rat asialoglycoprotein receptor however, have ruled out association–disassociation equilibrium.[31] Moreover, cross-linking of mouse CD23 after purification on an antibody column shows that once this complex is disrupted the monomers cannot reassociate or bind to IgE.[14] Further work is in progress to examine the natural state of the human receptor.

The results are consistent with the stalk region forming a trimeric α-helical coiled coil stalk, the formation of which could be either nucleated or stabilized by interactions between the lectin heads and the transmembrane helices and/or binding of ligands such as IgE and CD21. It means that CD23 has three binding sites for IgE and CD21, which could have important functional implications. IgE is thought to have two accessible CD23 binding sites,[8] and would occupy two of the three lectin heads, leaving one free for other interactions, e.g. with CD21 (see Fig. 6a in ref. 1). The stoichiometry of the binding of CD23 to CD21 is unknown, but assuming there is one site per chain, CD23 could cross-link one IgE and one CD21 molecule, or up to three CD21 molecules on the B-cell surface to up-regulate IgE synthesis (see Fig. 6b in ref. 1; ref. 9).

The only established function of CD23 *in vivo* is the feedback regulation of IgE synthesis in the murine system.[32,33] This may take place by antigen cross-linking of the complexes of IgE antibodies with membrane-bound CD23 on IgE-committed B cells.[34,35]

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