CD23 (FcεRII) release from cell membranes is mediated by a membrane-bound metalloprotease

Ariane E. MAROLEWSKI*, Derek R. BUCKLE†, Gary CHRISTIE†, David L. EARNSHAW†, Pearl L. FLAMBERG*, Lisa A. MARSHALL*, David G. SMITH† and Ruth J. MAYER*¹

*SmithKline Beecham Pharmaceuticals, Upper Merion, 709 Swedeland Road, King of Prussia, PA 19406, U.S.A., and †SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, U.K.

CD23 (low-affinity IgE receptor, FcεRII) is expressed as a Type II extracellular protein on a variety of cells such as B-cells, monocytes and macrophages and is cleaved from the cell surface to generate several distinct fragments. The expression of CD23 on the cell surface as well as the generation of soluble fragments of CD23 has been shown to be involved in the regulation of IgE synthesis. Here we report that the release of CD23 from the cell surface is mediated by a metalloprotease. An assay utilizing purified CD23 and an neo-epitope antibody specific for one of the known cleavage products is described and used to demonstrate unambiguously the cleavage of CD23 by a distinct protease. Characterization of the mechanism of CD23 processing shows that the protease exists as an integral membrane protein

with a functional molecular mass of approx. 63 kDa as determined by gel-filtration chromatography. The CD23-cleaving activity found in enriched plasma membranes from RPMI 8866 cells is inhibited by the metalloprotease inhibitors 1,10-phenanthroline and imidazole and by the matrix metalloprotease inhibitor batimastat, but not by inhibitors of cysteine proteases, serine proteases or acid proteases. The same or a similar activity that cleaves CD23 to the known 33 kDa fragment and is inhibited by batimastat is present in diverse cell types such as unstimulated fibroblasts and monocytic cell lines not expressing CD23, as well as in the Epstein–Barr virus-transformed B-cell line, RPMI 8866, which constitutively expresses CD23.

INTRODUCTION

CD23 or FcεRII, the low-affinity IgE receptor, has been shown to have a role in the human immune response, in particular in the regulation of IgE synthesis [1]. Two isoforms of CD23 are known: α-CD23, which is expressed constitutively on B-cells, and β-CD23, whose expression is transiently stimulated by interleukin 4 [2]. When β-CD23 is expressed on the cell surface of human B-cells and monocytes in response to interleukin 4, it is processed concomitantly to give multiple defined soluble fragments (sCD23) [3,4]. These fragments are biologically active as immunostimulatory cytokines and are also involved in the regulation of IgE synthesis [5]. The mechanism by which soluble CD23 is formed has not yet been characterized.

The human CD23 protein is composed of several domains [6], illustrated in Figure 1. The cytoplasmic domain, residues 1–23, is largely hydrophilic and is thought to be involved in signal transduction on engagement of the receptor. The α and β isoforms of CD23 differ only in the seven N-terminal residues within this domain. A single hydrophobic membrane-spanning helix, residues 24–44, follows the cytoplasmic tail. The remainder of the protein is extracellular and consists of two domains, a stalk region and a C-type lectin domain. The stalk region (residues 45–163) contains a leucine zipper motif with multiple heptad repeats, extending from residue 82 to residue 157. This coiled-coil motif implies that CD23 exists as a multimer on the cell surface, which has been verified by cross-linking experiments [7]. The lectin domain displays homology with the asialoglycoprotein receptor and is the site of IgE binding [8,9].

Four major sCD23 fragments (apparent molecular masses 37, 33, 29 and 25 kDa) have been identified, all deriving from cleavage within the stalk region and all containing the complete lectin domain [4]. The sequence of fragment formation and

conditions that alter the proportions of fragments have been explored [4,10,11]; proteolysis has been attributed to autocatalysis by CD23 [3] and to hydrolysis by an undefined cysteine protease [12]. This paper provides evidence that the CD23-processing enzyme from human RPMI 8866 cells that generates the 37 and 33 kDa fragments is a metalloprotease distinct from the substrate CD23.

MATERIALS AND METHODS

Cell culture

RPMI 8866 and BJAB (human B-cell lines), human lung fibroblasts, U937 and THP1 (human monocytic cell lines) and H9 and Jurkat (human T-cell lines) cells were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium containing L-glutamine and 10% (v/v) fetal bovine serum, with the exception of Chinese hamster ovary (CHO) cells [4], which were grown in Dulbecco's minimal essential medium with high glucose and 10% (v/v) fetal bovine serum. The seeding density was typically 10⁵ cells/ml and final densities were (1–2) × 10⁶ cells/ml. Cell culture was performed in T75 flasks incubated at 37 °C in an air/CO₂ (19:1) atmosphere. Cells were harvested by centrifugation, washed once with PBS and stored pelleted at –80 °C.

Membrane extraction

Frozen cells (3 g) were thawed, resuspended in 12 ml of homogenization buffer [20 mM Hepes/150 mM NaCl/1.5 mM MgCl₂/1 mM dithiothreitol (pH 7.5)] and lysed by N₂ cavitation once at 5.8–6.2 MPa in a Parr apparatus. The lysed cells were centrifuged at 10000 g at 4 °C for 35 min; the light layer of the

Abbreviations used: CHO, Chinese hamster ovary; iCD23, 45 kDa CD23; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; sCD23, soluble CD23 fragments; TNF, tumour necrosis factor.

¹ To whom correspondence should be addressed (e-mail Ruth-J.Mayer@sbphrd.com).

Figure 1 Diagram of CD23 monomer

Abbreviation: aa, amino acids.

pellet was resuspended in 4 ml of 0.2 M potassium phosphate, pH 7.4, and the heavier nuclear pellet was discarded. Membrane extraction was performed with a solution of final composition 6.6% (w/v) dextran (water content determined by Karl Fisher titration), 6.6% (w/w) polyethylene glycol and 0.25 M sucrose, to which was added the entire volume of non-extracted membranes [13]. The solution was mixed by inversion 50 times and centrifuged for 5 min at 1000 *g* to separate the layers. The upper layer was removed, diluted 1:3 with 20 mM potassium phosphate, pH 7.4, and centrifuged at 100000 *g* for 45–90 min. The final extracted membrane pellet was resuspended in 1.0 ml of PBS containing protease inhibitors [0.25 μ M *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; Sigma), 1 μ M leupeptin (Sigma) and 10 units/ml aprotinin (Calbiochem)] and stored at -80°C , except for experiments to determine inhibition by protease inhibitors, in which PBS alone was used. The specific activity of the plasma membrane marker enzyme γ -glutamyl transferase (Sigma kit) was increased 7–10-fold during the extraction step. For examining CD23-cleaving activity in a number of different cell types, a membrane extraction was performed with 10^8 cells in a total weight of 8 g for the aqueous extraction step.

Antibody generation

A polyclonal antiserum, BD15, that detected all the soluble fragments of CD23 as well as intact (45 kDa) CD23 (iCD23) was obtained by immunizing rabbits with truncated CD23 [7], consisting of residues 45–321. An antiserum, BD4, selective for the new N-terminus of the 33 kDa fragment was obtained by immunizing rabbits with the peptide $\text{NH}_2\text{LKSQDLELSC}$ coupled to maleimide-activated keyhole limpet haemocyanin

(Pierce Chemical) via the terminal cysteine residue. Antisera were screened for selectivity against all fragments of CD23 as well as the antigenic peptide and related peptides on dot-blots. Peptides were applied to PVDF membrane by using 50 μ l of a 100 μ M solution of peptide in Western transfer buffer, and then detected with standard methods for Western blots as described below. The clone for monoclonal antibody MHM6 [14] was a gift from Dr. John Gordon (Birmingham, U.K.) and the antibody was prepared in mg quantities by standard methods [15]; this antibody recognizes an epitope in the lectin domain of CD23.

Western blots

Proteins separated by SDS/PAGE [12% (w/v) gel] were transferred to PVDF membrane (Bio-Rad) with a Bio-Rad semi-dry transfer apparatus. Non-specific binding was blocked with Superblock buffer (Pierce Chemical). All antibody dilutions and washes were in PBS with 0.1% (v/v) Tween 20. Antibody-positive bands were detected with the Amersham enhanced chemiluminescence detection kit. For quantification, blots were scanned with a Millipore Bioimage digital system and the band intensity was determined with the VISAGE software package.

CD23 ELISA

CD23 or sCD23 concentrations were determined with the CD23 ELISA kit commercially available from The Binding Site (Birmingham, U.K.), except that MHM6 was used as the capture antibody and coated at 5 μ g/ml in 50 mM sodium phosphate/150 mM NaCl/0.02% NaN_3 (pH 7.4) on Nunc Maxisorp plates for 18 h.

Purification of iCD23 and sCD23

Purified iCD23 was prepared via an immunoaffinity resin of MHM6 coupled to Affigel 10 (Bio-Rad). The coupling was performed in accordance with the manufacturer's instructions. Cells from a large-scale RPMI 8866 fermentation were solubilized in 0.5% (v/v) NP40 in PBS buffer and applied overnight to the affinity resin in a volume of 50 ml at 1 ml/min and 4°C . Non-binding proteins were removed by extensive washing in PBS with 0.1% (v/v) NP40 and binding proteins were eluted with 0.1 M glycine, pH 2.3, or 3 M ammonium thiocyanate [16]. The resulting iCD23 was determined as 97–99% pure by Coomassie staining; it contained less than 0.05% 33 kDa CD23. Concentrated cell-free supernatant from a large-scale fermentation of RPMI 8866 cells was similarly applied to an MHM6 affinity column to purify total sCD23 fragments. The composition of this sCD23 was determined by Western blotting with CD23 polyclonal antibody (from The Binding Site, Birmingham) as 35%, 25%, 14% and 25% respectively for the 37, 33, 29 and 25 kDa fragments, reproducing the ratios found in small-scale culture over 72–96 h. The ratio of fragments as detected with BD15 was slightly different (50%, 17%, 15% and 17%) and was representative of the ratios observed by Coomassie staining of these fragments. The different fragment ratios observed with the two antisera are probably due to a different polyclonal composition of these antisera. The 33 kDa fragment from the large-scale culture was subjected to N-terminal sequencing and was verified as being the same as that previously reported [4].

CD23-processing activity assays

Membranes prepared as described above were solubilized by incubation on ice for 1 h in the presence of 0.1% (v/v) NP40 and

centrifuged briefly to pellet the membrane debris. Standard CD23-processing assay conditions, unless otherwise noted, consisted of 1–1.8 μg of RPMI 8866 solubilized membranes, referred to as solubilized membranes, and 100 nM purified iCD23 in a final volume of 20 μl made up with PBS or with 25 mM Hepes, pH 7.5. Assays were incubated at 37 °C for up to 2 h followed by SDS-PAGE and detection by Western blotting. The activities in PBS and in 25 mM Hepes, pH 7.5, were identical, even when membrane pellets were resuspended directly into Hepes buffer and solubilized. To assess the effect of protease inhibitors on activity, inhibitors were added from concentrated solutions to solubilized membranes to the desired final concentration, with no more than 2% (v/v) DMSO in the final assay, a concentration shown not to affect cleavage. CD23-processing activity was also determined in intact RPMI 8866 membranes (6–10 μg of protein), referred to as membranes, by incubating membranes at 37 °C for 1 h, quenching with SDS-containing gel loading buffer, and determining product formation by Western blotting with an antibody recognizing all known fragments of CD23 (BD15 or The Binding Site ELISA kit polyclonal).

Cell-based assays of sCD23 release

Cells were cultured in medium as described above at 2×10^6 cells/ml (RPMI 8866, CHO) or 10^5 cells/ml (BJAB, U937) for 1–72 h in the presence or absence of 50–200 ng/ml recombinant human interleukin 4 as well as various amounts of batimastat. RPMI 8866 cells were incubated for 1 h, CHO cells for 24 h, and BJAB and U937 cells for 72 h in the presence of interleukin 4. The medium was then made 30 μM in batimastat and chilled; cells were removed by centrifugation, and sCD23 in the cell-free supernatant was determined by ELISA. Levels of sCD23 found in the medium at the end of the incubation time in the absence of inhibitor varied between the cell types: typically 40–60 ng/ml for RPMI 8866 and CHO, and 5–10 ng/ml for BJAB and U937.

Data analysis

IC_{50} values were determined from a non-linear fit with the Marquardt algorithm in the program KALEIDAGRAPH from data sets of at least four concentrations of inhibitor in triplicate. K_m values for CD23 and estimates of pK_a of catalysis were determined with the program KINPAC, based on the methods of Cleland [17]. All values of IC_{50} as well as other data are representative of at least two independent determinations. Absolute concentrations of CD23 were determined by ELISA, with the calibration standards provided by The Binding Site. Estimates of product sCD23 concentrations were determined by the relative intensities of bands on scanned Western blots, with sCD23 of known concentration as internal standard. The percentage turnover of substrate was estimated either by directly determining the loss of 45 kDa CD23 band intensity from the primary blot or by reprobing with a suitable antibody; or by calculating product amounts with an sCD23 internal standard and comparing with known substrate concentration. Results from Western blots are presented directly as band intensity from the Visage system; band intensities were compared only within the same blot or blots prepared under identical conditions.

RESULTS

Selectivity of BD4 for the 33 kDa fragment

The selectivity of the antiserum (designated BD4) obtained by immunization with the N-terminal peptide of the 33 kDa frag-

Figure 2 Selectivity of BD4 polyclonal antiserum for 33 kDa sCD23 fragment

Membranes and purified sCD23 fragments were prepared as described in the Materials and methods section. Samples were separated by SDS/PAGE [12%(w/v) gel], transferred to PVDF membrane and Western blotted with combinations of BD4 and antigenic peptide, or with BD15. The positions of molecular mass markers are indicated at the left (in kDa). Lane 1, sCD23 fragments, probed with BD4; lane 2, membranes incubated at 37 °C for 2 h, probed with BD4; lanes 3 and 4, identical samples to those in lanes 1 and 2 respectively, probed with BD4 in the presence of 10 μM antigenic peptide; lane 5, sCD23 as in lane 1, detected with BD15 to show other sCD23 fragments (the intense band corresponds to the 37 kDa fragment).

Figure 3 Production of sCD23 fragments from membranes

Membranes (8 μg of protein per lane) were incubated at 37 °C and aliquots were removed after the times indicated below the lanes. Samples were quenched in loading buffer then subjected to SDS/PAGE and Western blots as described in the Materials and methods section. Upper panel, a blot probed with BD15; lower panel, a blot of identical samples probed with BD4.

ment of CD23 was evaluated against total sCD23 fragments and membranes containing iCD23, and with dot-blots against several peptides. Figure 2 shows the selectivity of BD4 for the 33 kDa fragment compared with other forms of CD23. Lanes 1 and 2 demonstrate that BD4 recognizes only the 33 kDa sCD23 fragment in both purified sCD23 and extracted membranes, and lane 5 shows the overall composition of fragments in lane 1 detected with the BD15 antiserum (detecting all the forms of CD23). Lane 2 also contains 45 kDa CD23 (as would be illustrated by Figure 3A), which is not detected by BD4. Lanes 3 and 4 show that antibody binding can be selectively blocked by addition of the peptide antigen to the primary antibody solution during the Western blot. In addition, the antiserum readily detected the antigenic peptide applied to dot-blots, but did not detect under these conditions any of the following peptides: the acylated antigenic peptide; a peptide differing from the antigenic peptide by substituting Phe for the N-terminal amino acid, and a 20-mer representing the CD23 sequence around the antigenic peptide (residues 92–111). These peptides, which were not recognized in

Figure 4 Time course of production of 37 and 33 kDa sCD23 for RPMI 8866 membranes or solubilized membranes

(A) Data derived from Figure 3; detection was performed with BD15 or BD4 as in Figure 3 and quantified as described in the Materials and methods section. The intensity of the 37 kDa band (■) was obtained from the BD15 blot, whereas the intensity of the 33 kDa band (●) was obtained from the BD4 blot. (B) Solubilized membranes (1.8 μ g per lane) were incubated at 37 °C for various times, quenched and analysed as in (A). A single blot was probed with BD4 for selective detection of 33 kDa product, then reprobed with BD15 without stripping the blot. Intensities the 37 kDa band (■) and the 33 kDa band (●) were obtained from the final blot. The product formation observed in the presence of batimastat confirms that the increase in both fragments is derived by metalloprotease-dependent cleavage. The portion of substrate turned over during the full assay time was approximately 35–40% for membranes and 12–16% for solubilized membranes.

the dot-blots, also did not compete with the antiserum in experiments identical with that shown in Figure 2.

CD23-processing activity in RPMI 8866 membranes or solubilized membranes

RPMI 8866 membranes or the plasma membrane components solubilized from purified plasma membranes with NP40, defined as solubilized membranes, were incubated and cleavage was assessed by Western blotting of the products (Figures 3 and 4). No endogenous iCD23 was added in these experiments. Figure 3 shows the formation of 37 and 33 kDa sCD23 fragments over time from unsolubilized membranes. Detection with BD15 (upper panel) shows the 37 and 33 kDa fragments; detection with BD4 (lower panel) shows the 33 kDa fragment alone but gives a higher-intensity band that allows a more accurate quantification of this product. Figures 4(A) and 4(B) show the quantitative results obtained with membranes and solubilized membranes

Figure 5 Dependence of 33 kDa fragment production on time and concentration of iCD23

(A) Linearity of product formation with time at 100 nM CD23 from 1.8 μ g of solubilized membranes in a total volume of 20 μ l. Samples were treated identically with those in Figure 4 with respect to gel electrophoresis and analysis, with the BD4 antiserum. (B) Solubilized membranes (1.0 μ g) were incubated with various amounts of purified iCD23 for 2 h and product was determined by Western blot as above (■). Substrate turnover was approx. 10% (low concentration) or less. Also shown (●) is the amount of product detected when substrate was incubated in the absence of solubilized membranes. The K_m for CD23 was determined as 215 ± 46 nM.

respectively. Comparison of Figures 4(A) and 4(B) shows that solubilization alters both the time course of production of 37 and 33 kDa and the ratio of 37 to 33 kDa sCD23 fragments. With membranes, the time course was characterized by an initial rapid formation (20 min) of both 37 and 33 kDa products, and then a slower, linear phase of product formation (up to 1 h). With solubilized membranes, 33 kDa CD23 was produced in a linear time course over 3 h. Relatively little of the 37 kDa fragment was produced during the incubation, although some 37 kDa fragment was present at zero time as a result of cleavage during the membrane purification process.

In addition, the assay with solubilized membranes was evaluated with various concentrations of added, purified iCD23. Figure 5(A) shows the linear production of the 33 kDa product from purified iCD23 for up to 3 h with the standard assay conditions of 100 nM added iCD23, as described in the Materials and methods section. Figure 5(B) shows that the addition of increasing amounts of iCD23 increases the amount of 33 kDa product and indicates that the cleavage process is saturable. In the absence of solubilized membranes, iCD23 does not undergo

Figure 6 Inhibition of CD23 processing by protease inhibitors

The extent of CD23 processing to 33 kDa product by solubilized membranes in the presence of protease inhibitors was assayed under the standard assay conditions, and 33 kDa product was detected with BD4 antiserum. Band intensity was quantified; values expressed as fractional inhibition are given below the lanes. Lane 1, no inhibitor, time 0 h; lane 2, no inhibitor, time 2 h; lane 3, 1 mM EDTA (lanes 3–10, 2 h); lane 4, 10 μ M E-64; lane 5, 10 units/ml aprotinin; lane 6, 1 μ M batimastat; lane 7, 10 mM imidazole; lane 8, 10 μ M pepstatin; lane 9, 10 μ M leupeptin; lane 10, 1 mM 1,10-phenanthroline. Lanes were combined from two separate gels run on the same day.

Figure 7 pH profile of CD23-processing activity

Solubilized membranes were diluted 1:6 into Hepes/Mes/Ches [2-(cyclohexylamino)ethanesulphonic acid] buffers (50 mM each) at pH values ranging from 6.0 to 9 (indicated above the gel lanes) and incubated at 37 °C for 2 h. Assays, electrophoresis and Western blots were performed as described in the Materials and methods section, with BD4 antiserum as the primary antibody. Band intensities in lanes from left to right: 0.2, 1.1, 2.3, 5.5, 6.1, 5.5 and 5.5 respectively.

significant cleavage but contributes a small amount of 33 kDa product, which is an impurity in the iCD23 as shown in Figure 5(B).

Inhibition of CD23-processing activity

Inhibitors of the four major protease classes were tested for their effect on CD23 processing by solubilized membranes. Figure 6 shows that inhibitors of serine proteases, aspartate proteases and cysteine proteases as well as EDTA were ineffective at preventing the processing of CD23 in solubilized membranes. In addition, EDTA up to 5 mM and tissue inhibitor of metalloproteases 1 at 10 μ g/ml had no effect on the processing of CD23 (results not shown). Only 1,10-phenanthroline and imidazole, broad-spectrum metalloprotease inhibitors, and the hydroxamic acid batimastat, an inhibitor of the matrix metalloprotease subclass, were capable of inhibiting the cleavage of CD23.

pH profile of CD23-processing activity

The pH dependence of the rate of CD23 processing at pH values from 6 to 9 is depicted in Figure 7. Solubilized membranes were assayed under standard conditions, except that a mixed buffer was substituted for PBS. In comparison the activity in PBS was 30 % higher than with the mixed buffer. Maximum activity in the mixed buffer was observed from pH 7.5 to 9.0; activity decreased rapidly at pH values below 7.0. Exposure to pH 6.0 resulted in an irreversible loss of activity (15–20 % in 2 h) that could not be restored by increasing the pH to 7.4 with or without added Zn^{2+} ;

Table 1 Evaluation of CD23-processing activity in different cell types as assessed by inhibition of total sCD23 release by batimastat in cells expressing CD23 or production of 33 kDa sCD23 fragment by solubilized membranes prepared from resting cells

For IC_{50} values, total sCD23 in the medium was measured in the presence of 0.01–10 μ M batimastat, as described in the Materials and methods section; CHO cells were transfected with human CD23 [4]. Results are given as means $\pm$ S.D. The amount of 33 kDa produced from solubilized membranes was assayed as described in the Materials and methods section and is expressed as a percentage of that produced from an equivalent amount of solubilized RPMI 8866 membranes in the same assay. Abbreviation: n.d., not determined.

Cell type	IC_{50} for batimastat (μ M)	33 kDa fragment produced (% of RPMI 8866)
RPMI 8866	0.17 ± 0.07	100
U937	0.12 ± 0.03	170
THP1	n.d.	140
Fibroblast	n.d.	88
BJAB	0.23 ± 0.05	113
H9	n.d.	< 5
Jurkat	n.d.	< 5
CHO	0.2 ± 0.15	70

activity was lost more rapidly at pH 5.0. This irreversible loss of activity prevented the calculation of a well-defined pK_a .

CD23-processing activity in various cell types

The expression of CD23-processing activity in several cell types was assessed with two separate types of assay (Table 1). First, sCD23 released into the media from cell types expressing both CD23 and processing enzyme could be inhibited by batimastat with comparable IC_{50} values, indicating that a similar class of protease was generating sCD23 in each case. Secondly, solubilized membranes from RPMI 8866, BJAB, CHO, THP1, U937, Jurkat, H9 and fibroblast cells were assayed for CD23-processing activity under standard assay conditions. Western blots with BD4 antiserum showed that solubilized membranes from all of these cell lines cleaved iCD23 to 33 kDa CD23, with the exception of the Jurkat and H9 T-cell lines. For all of the positive cell lines, the intensity of the 33 kDa band was within a factor of 2 of that generated by an identical amount of solubilized membranes from RPMI 8866 cells, on the basis of quantification of the Western blots (Table 1). Use of the selective BD4 antiserum also demonstrated that iCD23 was cleaved at the same site as identified for RPMI 8866 cells, suggesting that a single protease or a closely related family is responsible for cleavage in all of the cell types. No other major fragment products were detected in the blots on reprobing with BD15, with the exception of fibroblasts, where a weak band migrating with a molecular mass of approx. 20 kDa was observed; this could have been derived from non-specific cleavage or secondary cleavage of the 33 kDa band. Of these cells, only RPMI 8866 expressed significant CD23 under the conditions used for cell culture (FACS analysis; results not shown).

Gel-filtration chromatography of CD23-processing enzyme

Solubilized membranes were fractionated on a Superose 12 column and fractions were assayed for CD23-processing activity. CD23-processing activity from RPMI 8866 cells was eluted in fractions corresponding to a molecular mass of approx. 63 kDa (Figure 8), with Bio-Rad gel-filtration standard molecular mass markers. Similar results were obtained with extracts from BJAB and CHO cells.

Figure 8 Elution of CD23-processing activity from Superose 12

Solubilized membranes (0.5 mg) were diluted in 50 mM Hepes/0.1% NP40 (pH 7.5) and applied to a Superose 12 FPLC column (Pharmacia) at 0.3 ml/min. Fractions were collected at 2 min intervals and assayed for CD23-processing activity; product was detected with BD4, quantified as outlined in the Materials and methods section and relative activity given as intensity (■). Molecular mass markers (Bio-Rad gel filtration standards, 158, 44 and 17 kDa positions are plotted) were applied to the same column in a separate run (●) and the elution volume was determined from UV absorption.

DISCUSSION

Proteolytic processing of cell-surface receptors to soluble forms is a phenomenon that has recently been recognized to be widespread in scope [18,19]. As a class the processing mechanisms exhibit considerable similarities, yet the specific cleavage of each substrate also has distinctions. Some differentiation between these various proteases has been observed in regulation characteristics such as constitutive or regulated enzyme activity [20–22] as well as in inhibition by a variety of compounds (results not shown), reinforcing the hypothesis that a family of similar enzymes exists [19,23,24].

Extensive purification of plasma membranes from the several different human cell types investigated shows that the enzyme responsible for CD23 release is membrane-anchored, as is CD23 itself. Interaction between CD23 and its processing enzyme could therefore be facilitated as well as controlled by the relative orientation of the two proteins in the membrane. Correct cleavage to the 33 kDa fragment under conditions where exogenous CD23 is added to solubilized membrane shows that some specificity for the cleavage sequence exists even when the substrate is not presented in the constraint of a membrane. The different ratio of processed fragments obtained before and after solubilization of membrane proteins suggests, however, that the relative conformations are altered on solubilization. One possible interpretation is that release of both proteins from the membrane permits access of the protease to substrate sites normally less accessible in the more rigid membrane conformation. Other reasonable interpretations are that detergent solubilization of the membranes results in loosening of the coiled-coil region or in the release from the membrane of more than one protease with different properties.

Additional characterization of the enzymic activity showed that the rate of cleavage of exogenous CD23 depends on CD23 concentration, with an approximate K_m of 220 nM. The effective concentration of CD23 in the intact membrane would be high compared with the concentration in homogeneous solution, so that normal cleavage of CD23 in membranes should be very efficient. The time course of product formation seen in membranes, consisting of a rapid phase followed by a linear phase,

could suggest that the rate of processing is limited by proximity and diffusion of substrate within the membrane.

Inhibition by class-specific protease inhibitors revealed that the CD23-processing enzyme belongs to the metalloprotease class, as evidenced by inhibition by 1,10-phenanthroline and imidazole, although interestingly not by EDTA at a concentration typically found to inhibit many metalloproteases. The observed pH dependence of activity is also consistent with, although not unique to, that of a neutral Zn^{2+} -dependent metalloprotease [25,26] in which an acidic group is required for catalysis. The possible effect of pH on substrate binding as well as the cause of the irreversible loss of activity at low pH were not determined. The initial proposal that CD23 was autocatalytic [3] was most probably a result of a partial co-purification of a protease with CD23, as iCD23 in our hands did not generate additional sCD23 fragments after purification from RPMI 8866 cells by immunoaffinity column.

Inhibition of CD23 cleavage by batimastat, a broad-spectrum inhibitor of matrix metalloproteases, suggests that the processing enzyme is in the family of enzymes recently discovered to be inhibited by hydroxamic acids, exemplified by tumour necrosis factor (TNF)- α convertase and L-selectin sheddase [18,27]. The enzyme(s) that cleaves TNF is somewhat more susceptible to inhibition by EDTA than the cleavage of either L-selectin or CD23. Cloning of two enzymes as TNF- α convertases indicates that these enzymes are disintegrin-metalloproteases with Zn^{2+} -binding domains high sequence similarity to that of the matrix metalloproteases and are thus susceptible to inhibition by non-selective matrix metalloprotease inhibitors [28,29]. The demonstration that more than one enzyme can cleave pro-TNF- α to mature TNF- α would suggest that even identical cleavages across multiple cell types should be interpreted cautiously and indeed more than one enzyme could be physiologically responsible for the release for CD23 from the surface of differing cell types. A full understanding of the relationship of CD23-processing enzyme to others in this class of metalloproteases will require the identification of additional family members as well as a much more thorough appreciation of the substrate–enzyme relationships across the class.

The regulation of CD23 fragment production seems to be primarily through the regulation of substrate, i.e. CD23, expression. Constitutive expression of active CD23-processing activity seems to be the normal state in all cell types that process CD23. The only cells found so far that do not seem to express an active processing enzyme are T-cell lines; the ability of T-cells to express CD23 is uncertain, but certainly T-cells are not a major source of CD23 [1,30]. The relative level of cleaving activity in the various cells is very similar to that seen in RPMI 8866, in which high levels of CD23 are also present. Expression of CD23 is not constitutive in any of the cell types tested except the Epstein–Barr virus-transformed RPMI 8866, but can be induced in cell types in which processing activity was detected. The regulatory mechanism of broad protease expression coupled with tight substrate regulation seems to be valid for a number of the membrane-bound metalloproteases studied so far [31].

Inhibition of the metalloprotease activity prevents the formation of the 37 and 33 kDa sCD23 fragments, both in membranes [32] and solubilized membranes; the formation of the 29 kDa fragment has not been investigated. Various studies have suggested that the 25 kDa fragment can be generated under some conditions either directly from the cell surface or rapidly from larger fragments by an unrelated mechanism involving an enzyme with characteristics of a cysteine protease [11,12,33,34]. The physiological relevance of this proposed cysteine protease mechanism to initial shedding of CD23 remains to be determined,

although the major form of CD23 that accumulates in serum is the 25 kDa fragment [35]. In related studies [32] we have shown that inhibition of the metalloprotease activity, and thus the release of sCD23, blocks the function of CD23 in enhancing IgE production from B-cells, suggesting a therapeutic utility of the inhibition of CD23 processing via a novel mechanism of IgE regulation.

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