

Oliver Schulz¹,
 Brian J. Sutton²,
 Rebecca L. Beavil²,
 Jianguo Shi²,
 Herb F. Sewell¹,
 Hannah J. Gould²,
 Peter Laing³ and
 Farouk Shakib¹

¹ Division of Molecular & Clinical Immunology, University of Nottingham, Queen's Medical Centre, Nottingham, GB

² The Randall Institute, King's College London, London, GB

³ Peptide Therapeutics plc, Cambridge Science Park, Cambridge, GB

Cleavage of the low-affinity receptor for human IgE (CD23) by a mite cysteine protease: nature of the cleaved fragment in relation to the structure and function of CD23

Der p I, a cysteine protease representing a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, has recently been shown to cleave CD23 from the surface of cultured human B cells (RPMI 8866 B cell line). We have now undertaken a detailed investigation of CD23 cleavage by *Der p* I. We demonstrate that *Der p* I cleaves CD23 at two sites (Ser155-Ser156 and Glu298-Ser299) to produce a 17-kDa fragment containing the lectin domain and only part of the C-terminal tail. No such effect was demonstrable with mouse CD23, a finding which was anticipated based on its lack of the cleavage sites identified on human CD23. Based on the cleavage pattern and the model of CD23, we propose a sequence of events leading to the liberation of the 17-kDa soluble CD23 fragment. The biological significance of such cleavage is underlined by the demonstration that *Der p* I-treated B lymphocytes lose their ability to bind IgE, and that the 17-kDa fragment (amino acids 156–298) contains the minimum structural requirement (amino acids 156–288) for binding to both IgE and CD21.

1 Introduction

The low-affinity receptor for IgE (FcεRII), also known as CD23, is a calcium-dependent type II integral membrane protein which belongs to the lectin family of adhesion molecules. CD23 is a 45-kDa differentiation marker for IgM⁺/IgD⁺ B cells, but is also found on other cells, including eosinophils, follicular dendritic cells, macrophages, and platelets. The extracellular portion of this receptor consists of a lectin homology domain flanked on the C-terminal side by a short tail containing an inverse RGD sequence, and on the N-terminal side by an α-helical coiled-coil stalk that mediates trimer formation [1–3].

On B lymphocytes, CD23 has been shown to be involved in the regulation of IgE synthesis *in vivo* [4, 5]. When CD23 is not occupied by IgE, it undergoes proteolytic cleavage to release soluble CD23 (sCD23) fragments, which have been shown to up-regulate IgE synthesis in an autocrine fashion [6]. The binding of IgE to CD23 apparently not only protects the receptor against proteolytic cleavage [7], but also allows membrane CD23 to deliver a negative IgE regulatory signal to the B lymphocyte [8, 9]. Endogenous or exogenous proteases that cleave CD23 from the surface of B lymphocytes would, therefore,

potentially disrupt the IgE regulatory mechanism, thereby leading to excessive IgE synthesis.

Very little is known about the nature of endogenous proteases that cleave membrane CD23. However, we have recently demonstrated that *Der p* I, a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, cleaves CD23 from the surface of cultured human B cells (RPMI 8866 B cell line) [10]. We have now undertaken a detailed investigation of CD23 cleavage by *Der p* I, and found that *Der p* I cleaves CD23 at two sites (Ser155-Ser156 and Glu298-Ser299) to produce a 17-kDa fragment (amino acids 156–298) containing the minimum structural requirement (amino acids 156–288) for binding to both IgE and CD21.

2 Materials and methods

2.1 Antibodies and proteins

FITC-labeled mouse anti-human CD23 mAb Bu38 was obtained from The Binding Site (Birmingham, GB) and FITC-labeled rat anti-mouse CD23 mAb B3B4 was a kind gift from Dr. D. Conrad (Richmond, VA). Mouse anti-human CD23 mAb MHM6 was a kind gift from Prof. J. Gordon (Birmingham, GB). A recombinant protein representing the extracellular portion of human CD23 (40-kDa, residues Asp48-Ser321, preceded by an isoleucine residue) was generated by the method described for a different soluble CD23 fragment [11] using the forward PCR primer 5'ATCGACACCACACAGAGTCTAAA3' and the reverse primer 5'TCGGATCCTCAAGAGTGGAGAGG-GGC3'. The recombinant soluble CD23 was isolated on an anti-CD23 mAb MHM6 affinity column (Sephacrose, Pharmacia, Uppsala, Sweden), and further purified by gel filtration on a Superdex 75 HR20/30 column (Pharmacia). *Der p* I was purified by antibody affinity chromatography as described [10]. Myeloma IgE (WT) was a kind gift from Prof. D. R. Stanworth (Cambridge, GB).

[I 16477]

Received November 11, 1996; in revised form December 18, 1996; accepted December 18, 1996.

Correspondence: Farouk Shakib, Division of Molecular & Clinical Immunology, School of Medicine & Health Sciences, Queen's Medical Centre, Nottingham NG7 2UH, GB
 Fax: +44-115-9709-125

Abbreviations: rMBP: Rat mannose-binding protein *Der p* I: *Dermatophagoides pteronyssinus* cysteine proteinase I

Key words: CD23 / Cysteine protease / *Der p* I / IgE / *Dermatophagoides pteronyssinus*

2.2 Monitoring CD23 expression on cells

Human RPMI 8866 B cells and CHO-K1 cells transfected with mouse CD23 [12] were kind gifts from Prof. J. Gordon (Birmingham, GB) and Dr. D. Conrad (Richmond, VA), respectively. The RPMI 8866 B cells were grown in RPMI 1640, 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B. The mouse CD23-transfected CHO-K1 cells were grown in glutamine-free DMEM, 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B. FITC-labeled mouse anti-human CD23 mAb Bu38 or rat anti-mouse CD23 mAb B3B4 (both 100 µl, 2 µg/ml mAb) were used to assess CD23 expression on human RPMI 8866 B cells and mouse CD23 transfected CHO-K1 cells, respectively. The reactivity and specificity of both anti-CD23 mAb have been characterized [13, 14]. Cells were analyzed on a FACScan (Becton Dickinson, Oxford, GB) as described [10]. In the IgE-binding assay, 8 µg/ml FITC-labeled IgE was incubated for 1 h at room temperature with 2×10^5 RPMI 8866 B cells.

2.3 CD23 cleavage assay

Der p I was pre-incubated for 5 min at 37°C with 5 mM cysteine and added to 2×10^5 – 3×10^5 CD23-expressing cells in a total volume of 200 µl RPMI 1640, 10 mM Hepes (RPMI 8866 B cells) or glutamine-free DMEM and 10 mM Hepes (mouse CD23-transfected CHO-K1 cells). The mixture was then incubated for 1 h at 37°C and the cells, collected by centrifugation, were washed in fresh medium and incubated for 30 min at room temperature with the appropriate FITC-labeled anti-CD23 mAb. For cleavage of recombinant 40-kDa CD23, 1 µg *Der p* I in 30 µl PBS was incubated for 1 h at 37°C with 20 µg of CD23. The cleavage product was assessed by silver-stained SDS-PAGE on a 15 % polyacrylamide gel.

2.4 Purification of sCD23

The *Der p* I cleavage product of human CD23 was purified from the culture supernatant of 2×10^8 cells by affinity chromatography using mouse anti-CD23 mAb MHM6. Bound sCD23 was then eluted using 0.1 M acetic acid adjusted to pH 2.1 with HCl, and then immediately neutralized with 1 M Tris-HCl pH 9.0. The *Der p* I cleavage product of the recombinant CD23 was purified by reverse-phase HPLC (Applied Biosystems HPLC 140A) using Aquapore butyl Brownlee cartridge (30 mm × 2.1 mm; Applied Biosystems, Warrington, GB). The absorbance was monitored at 220 nm and the solvent system consisted of 0.1 % (vol) trifluoroacetic acid in water (solvent A) and 0.05 % (vol) trifluoroacetic acid in 70 % acetonitrile (solvent B).

2.5 N-terminal sequencing

N-terminal sequencing of sCD23 fragments was determined by automatic Edman degradation on a Biosystems 473A Microsequencer (Applied Biosystems). Samples, purified by either HPLC or affinity chromatography, were loaded onto polybrene-treated and precycled glass fiber

filters. Phenylthiohydantoin-amino acids were identified by chromatography on a C₁₈ column (PTH C-18, 2.1 mm × 200 mm).

2.6 Molecular modeling

A molecular model of trimeric human CD23 was generated based upon the crystal structure of the trimeric rat mannose binding protein (rMBP; protein databank entry 1RTM), using the programme HOMOLGY, within INSIGHT II (Molecular Simulations Inc., Cambridge, GB), running on a Silicon Graphics workstation. rMBP contains a triple stranded α-helical coiled-coil neck region immediately adjacent to the trimer of lectin domains [15], and sequence comparison revealed that residues 133–164 of CD23 contains a hydrophobic heptad repeat pattern similar to that present in the neck region of rMBP (residues 73–104). This strongly suggests that CD23 also has a triple helical coiled-coil neck region. In CD23, this region is contiguous with the previously predicted α-helical coiled-coil stalk region [2], although a change in the phase of the heptad repeat occurs at the junction between the stalk and the neck. At this same position in rMBP, the coiled-coil neck region is connected to the collagen triple-helix stalk region. The neck region of CD23 was therefore modeled upon the structure of rMBP, and then joined to a previously published model of CD23 lectin domain [16]. At the junction between these two sections, there is a loop of six residues in rMBP, while in CD23, there are nine residues. A new loop was therefore modeled in CD23 using the loop search facility within HOMOLGY. This loop contains an additional cysteine residue not present in rMBP, and links the lectin side of the loop (residue 174) to another cysteine (residue 163) in the coiled-coil neck region. Inspection of the mouse and rat CD23 sequences suggest that these sequences are compatible with this model of the CD23 trimer.

3 Results and discussion

3.1 Determination of the CD23 cleavage sites

Cleavage of a recombinant 40-kDa protein, representing the extracellular portion of human CD23 by *Der p* I produced a 17-kDa fragment; no such effect was demonstrable in the presence of the cysteine protease inhibitor E64 [17] (Fig. 1a). The 17-kDa fragment of CD23 obtained in this way was purified by HPLC (Fig. 1b) and its N-terminal sequence determined. Alignment of the sequence obtained (SGFVCN) with the published CD23 sequence [18] placed the *Der p* I cleavage site on the N-terminal side of the lectin domain, between Ser155 and Ser156. Soluble CD23 was also purified from the culture supernatant of *Der p* I-treated RPMI 8866 B cells and its N-terminal sequence (SGFVCN) confirmed the above cleavage site. The molecular mass (*i.e.* 17-kDa) of the *Der p* I-generated soluble CD23 fragment is suggestive of a molecular species lacking at least part of the C-terminal tail. This therefore prompted us to look for a second cleavage site on the C-terminal side of the lectin domain. Indeed, a second CD23 fragment was identifiable by HPLC in the *Der p* I digest of the recombinant 40-kDa CD23 (Fig. 1b). N-terminal sequencing of this peptide revealed a sequence

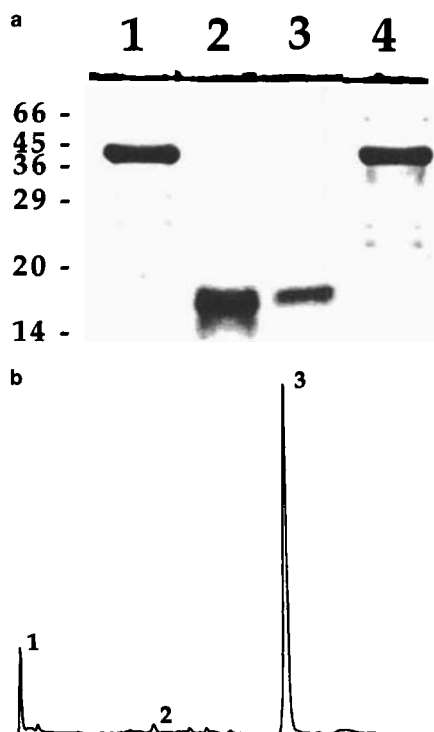


Figure 1. (a) Silver-stained SDS-PAGE analysis of the *Der p* I cleavage product of recombinant human CD23. Lanes 1–4 represent intact 40-kDa CD23, *Der p* I-cleaved 17-kDa CD23, HPLC-purified 17-kDa CD23, and intact 40-kDa CD23 after treatment with *Der p* I in the presence of E64, respectively. Molecular mass standards are indicated in kDa on the left. (b) HPLC elution profile of the *Der p* I cleavage product of recombinant human 40-kDa CD23. Peak 1 is the column flow-through. N-terminal sequencing of peak 2 identified the sequence SMGPDSR, which represents residues 299–305 within the C-terminal tail of CD23, and sequencing of peak 3 identified the sequence SGFVCN which represents residues 156–161 within the stalk region. The ratio between the area under peaks 2 and 3 is 1:40, which is to be expected considering the difference in peptide length (sevenfold) and yield (sixfold), assuming near-total recovery of the starting amount (600 pmol) of the 40-kDa CD23.

(SMGPDSR) spanning residues 299–305 of the C-terminal tail of CD23, thereby placing the second cleavage site between Glu298 and Ser299.

To relate these cleavage sites to the overall three-dimensional structure of the molecule, a model of CD23 showing the serine residues 155 and 156 was generated (Fig. 2). These two residues lie buried at the trimer interface within the coiled-coil neck region, immediately below the lectin domains. Although the C-terminal tail region cannot be modeled, the location of the final residue in the lectin domain can be seen at the end of the β strand immediately above the serine residues in Fig. 2. Thus, the tail structure is expected to cover this region and may well contribute to the stability of the trimer. By analogy with the rMBP structure, and given the additional disulfide bond linking the lectin domain to the neck and the presence of the tail region, little or no flexibility between the lectin domains is to be expected. The cleavage site will therefore only become accessible upon dissociation of the trimer, a process that may well be enhanced by prior cleavage of the tail region. Based on the cleavage pattern and the model

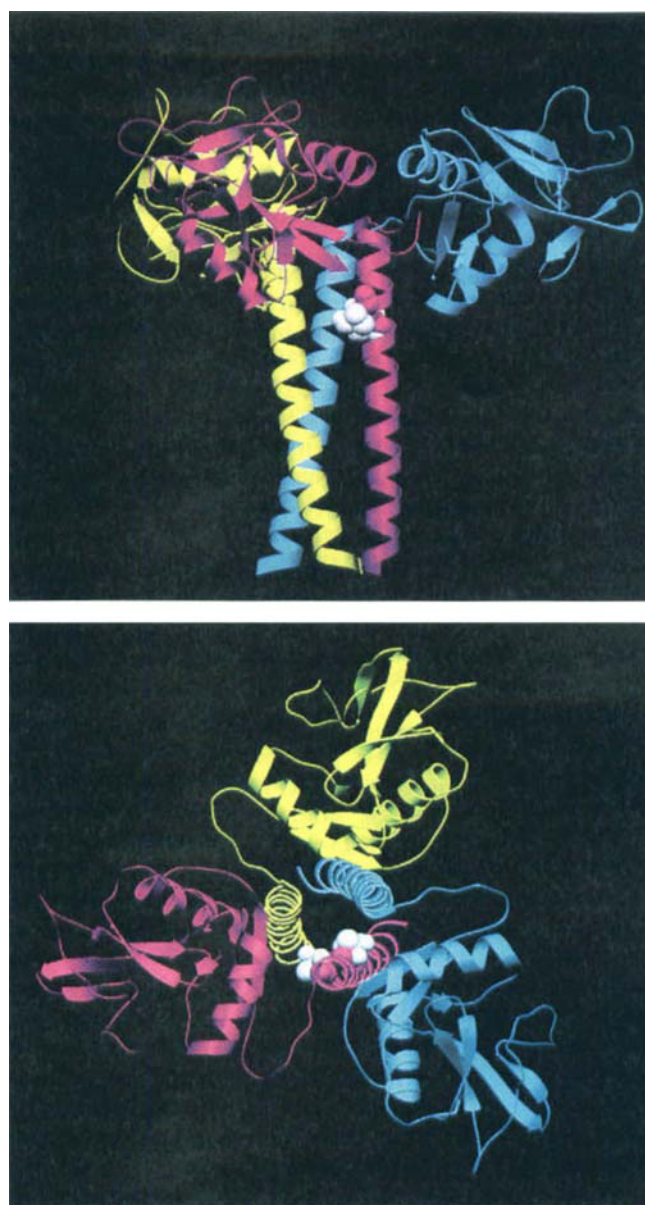


Figure 2. A model for the human CD23 trimer: the trace of the polypeptide chain is shown, and the arrows represent strands of β sheet. (a) Shows the three lectin domains and the triple α -helical coiled-coil stalk and neck region. Residues Ser155 and Ser156 in the neck region, a site of proteolytic cleavage by *Der p* I, are shown in a space-filling representation for the magenta-colored subunit only. The modeled lectin domain (based upon [15]) terminates in β strand in the vicinity of the neck region adjacent to the serine residues. (b) The trimer model with Ser155 and Ser156 represented as in (a), viewed along the threefold axis of symmetry.

of CD23, it is possible to speculate upon the sequence of events leading to the liberation of the 17-kDa sCD23 fragment. We suggest that *Der p* I cleaves CD23 in a sequential manner, starting with the site in the C-terminal tail (Glu298-Ser299). Removal of this segment destabilizes the trimer and allows access to the Ser155-Ser156 cleavage site. Clearly, this could be tested by generating a cell line transfected with a human CD23 construct lacking the Glu298-Ser299 cleavage site.

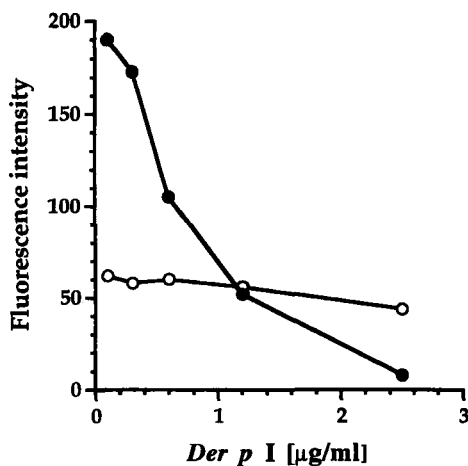


Figure 3. Differential effect of *Der p* I on human and mouse CD23 expression on RPMI 8866 B cells and CHO-K1 cells, respectively. *Der p* I cleaves human CD23 (filled circles, monitored by Bu38 antibody), but not mouse CD23 (open circles, monitored by B3B4 antibody). Data represent the means of triplicate determinations; the SEM were less than 5%.

Given that the Ser155-Ser156 motif (LGVSSGFV) is absent in mouse CD23 (ILISKGTA) [19], we next tested whether *Der p* I can cleave CD23 from the surface of CHO cells transfected with mouse CD23. We found that mouse CD23 is not significantly affected by *Der p* I (Fig. 3) and although this may be due to sequence differences in both the C-terminal tail and the neck region [19], there is an alternative explanation. The C-terminal tail in mouse CD23 is shorter compared to human CD23, whereas the stalk region is longer [20]. These differences may affect oligomerization of the receptor and thus accessibility to a site in the neck region.

3.2 Biological consequences of CD23 cleavage

To assess the impact of membrane CD23 cleavage by *Der p* I on IgE uptake by the RPMI 8866 B cells, FITC-labeled IgE binding and CD23 expression were assessed at different time points. Treatment of the B cells with 3 µg/ml *Der p* I showed that the kinetics of the loss in IgE binding capacity closely followed that of CD23 cleavage (Fig. 4). This loss in the ability of the B cells to bind IgE was expected since, as we have shown earlier, *Der p* I removes the lectin domain which contains the IgE binding site [20].

Der p I induced cleavage of membrane-bound CD23 on B lymphocytes may lead to enhanced IgE synthesis. First, CD23 knockout mice show increased IgE synthesis, suggesting that membrane CD23 provides a negative feedback signal [8]. Therefore, cleavage of CD23 by *Der p* I may up-regulate IgE synthesis by disrupting this negative feedback signal, which is thought to be delivered by IgE binding to membrane CD23. Second, we note that the 17-kDa sCD23 fragment released by *Der p* I (amino acids 156–298) contains the minimum structural requirement (residues 156–288) for binding to both IgE [11, 21] and CD21 (J. Shi and H. J. Gould, unpublished observations). sCD23 has been shown to enhance IgE synthesis by binding to CD21 (also known as CR2) [22], and smaller sCD23 fragments have been reported to be IgE-suppressive [23]. We are

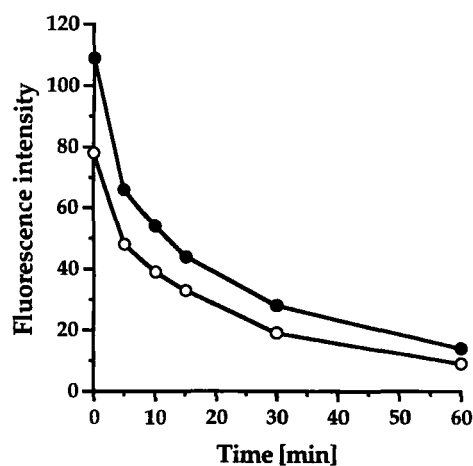


Figure 4. Kinetics of *Der p* I (3 µg/ml)-induced cleavage of CD23 from RPMI 8866 cells (filled circles) and that of the consequent loss in ability to bind FITC-labeled IgE (open circles). Data represent the means of triplicate determinations; the SEM were less than 5%.

currently studying the effect of the 17-kDa sCD23 fragment released by *Der p* I on IgE synthesis *in vitro*.

4 Concluding remarks

We [10] and others [24] have recently demonstrated that *Der p* I, the cysteine protease from the house dust mite, cleaves CD23 from the surface of RPMI 8866 B cells. We have now shown that *Der p* I cleaves human CD23 (membrane-expressed and the soluble recombinant form representing the entire extracellular sequence) on both the N-terminal and the C-terminal sides of the lectin domain, thereby producing a 17-kDa sCD23 fragment. It is interesting to note in this connection that papain, a cysteine protease showing sequence similarities with *Der p* I [25], releases an sCD23 fragment of similar molecular size which binds IgE [26, 27]. While it remains to be seen whether *Der p* I cleavage can up-regulate IgE synthesis directly via the released sCD23, cleavage of this fragment from membrane-bound CD23 on B cells will clearly disrupt the negative feedback regulation of IgE synthesis mediated by this receptor.

The authors wish to thank Prof. J. Gordon and Dr. D. Conrad for their generous gifts of cells and antibodies. The authors are grateful to Alison Galvin for her help with flow cytometry. O.S. is a recipient of a Peptide Therapeutics/University of Nottingham joint PhD studentship. B.J.S. and H.J.G. thank the Medical Research Council and National Asthma Campaign for their support.

5 References

- Sutton, B. J. and Gould, H. J., *Nature* 1993. 366: 421.
- Beavil, A. J., Edmeades, R. L., Gould, H. J. and Sutton, B. J., *Proc. Natl. Acad. Sci. USA* 1992. 89: 753.
- Beavil, R. L., Graber, P., Aubonney, N., Bonnefoy, J.-Y. and Gould, H. J., *Immunology* 1995. 84: 202.
- Flores-Romo, L., Shields, J., Humbert, Y., Graber, P., Aubry, J.-P., Gauchat, J.-F., Ayala, G., Allet, B., Chavez, M., Bazin, H., Capron, M. and Bonnefoy, J.-Y., *Science* 1993. 261: 1038.

- 5 Lamers, M. C. and Yu, P., *Immunol. Rev.* 1995. 148: 71.
- 6 Sarfati, M. and Delespesse, G., *J. Immunol.* 1989. 141: 2195.
- 7 Lee, W. T., Rao, M. and Conrad, D. H., *J. Immunol.* 1987. 139: 1191.
- 8 Yu, P., Kosco-Vilbois, M., Richards, M., Köhler, G. and Lamers, M. C., *Nature* 1994. 369: 753.
- 9 Sherr, E., Macy, E., Kimata, H., Gilly, M. and Saxon, A., *J. Immunol.* 1989. 142: 481.
- 10 Schulz, O., Laing, P., Sewell, H. F. and Shakib, F., *Eur. J. Immunol.* 1995. 25: 3191.
- 11 Shi, J., Ghirlando, R., Beavil, R. L., Beavil, A. J., Keown, M. B., Young, R. J., Owens, R. J., Sutton, B. J. and Gould, H. J., *Biochemistry* 1997, in press.
- 12 Bartlett, W. C., Kelly, A. E., Johnson, C. M. and Conrad, D. H., *J. Immunol.* 1995. 154: 4240.
- 13 Rao, M., Lee, W. T. and Conrad, D. H., *J. Immunol.* 1987. 138: 1845.
- 14 Schwartz-Albiez, B. and Moldenhauer, G., In Knapp, W., Dorken, B. and Gilks, W. R. (Eds.), *Leucocyte Typing IV*, Oxford University Press, Oxford 1989. p. 142.
- 15 Weis, W. I. and Drickamer, K., *Structure* 1994. 2: 1227.
- 16 Padlan, E. A. and Helm, B. A., *Receptor* 1993. 3: 325.
- 17 Barrett, A. J., Kenbhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. and Hanada, K., *Biochem. J.* 1982. 201: 189.
- 18 Ikuta, K., Takami, M., Kim, C. W., Honjo, T., Miyoshi, T., Tagaya, Y., Kawabe, T. and Yodoi, J., *Proc. Natl. Acad. Sci. USA* 1987. 84: 819.
- 19 Bettler, B., Hofstetter, H., Rao, M., Yokoyama, W. M., Kilchherr, F. and Conrad, D. H., *Proc. Natl. Acad. Sci. USA* 1989. 86: 7566.
- 20 Bettler, B., Texido, G., Raggini, S., Ruegg, D. and Hofstetter, H., *J. Biol. Chem.* 1992. 267: 185.
- 21 Mossalayi, M. D., Arock, M., Delespesse, G., Hofstetter, H., Bettler, B., Dalloul, A. H., Kilchherr, E., Quaz, F., Debre, P. and Sarfati, M., *EMBO J.* 1992. 11: 4323.
- 22 Aubry, J.-P., Pochon, S., Graber, P., Jansen, K. U. and Bonnefoy, J.-Y., *Nature* 1992. 358: 505.
- 23 Sarfati, M., Bettler, B., Letellier, M., Fournier, S., Rubio-Trujillo, M. and Hofstetter, H., *Immunology* 1992. 76: 662.
- 24 Hewitt, C. R. A., Brown, A. P., Hart, B. J. and Pritchard, D. I., *J. Exp. Med.* 1995. 182: 1537.
- 25 Chua, K. Y., Stewart, G. A., Thomas, W. R., Simpson, R. J., Dilworth, R. J., Plozza, T. M. and Turner, K. J., *J. Exp. Med.* 1988. 167: 175.
- 26 Peterson, L. H. and Conrad, D. H., *J. Immunol.* 1985. 135: 2654.
- 27 Sarfati, M., Nakajima, T., Frost, H., Kilchherr, E. and Delespesse, G., *Immunology* 1987. 60: 539.